Lipid Nanocarriers in Cancer Diagnosis and Therapy

Edited by

Eliana B. Souto
Cancer is a complex group of pathologies, characterised by abnormal transformation of healthy cells into cancerous cells involving cell proliferation, invasion into surrounding tissues and sometimes metastisation. This uncontrolled growth continues along the cell cycle, producing new abnormal cells that can reach systemic blood circulation. Metastasis leads to formation of newer colonies in vital organs not affected by the primary cancer, giving rise to secondary tumours. Despite great achievements on understanding its aetiology, in parallel with the biotechnological development of new anticancer drugs, cancer remains one of the leading causes of death worldwide. Preclinical and clinical studies demonstrated successful gene transfer and suppression of tumour growth when functional wild-type tumour suppressor gene contained in a viral vector were administered intratumourally. However, this approach holds the risk of immunogenicity by producing antiviral antibodies, thereby limiting repeated treatments. Furthermore, metastatic cancers need to be treated via systemic gene delivery approach, and viral vectors have low therapeutic efficacy when administered intravenously. Nonviral nanotechnologies are therefore being exploited as potential alternatives. When comparing conventional anticancer treatments with novel approaches based on nanotechnologies, it has long been reported that targeted and in situ drug delivery provide a selective killing process of cancer cells, minimising the toxicity on healthy organs and tissues and consequent adverse side effects. On the other hand, even if located in the tumour region, the efficacy of anticancer drugs can be affected by the development of multidrug resistance. This resistance mainly results in unreasonable tumour size reduction or recrudescence after initial therapeutic effect. Nevertheless, even some nanocarriers were able to overcome the problem of multidrug resistance to some extent. This type of less aggressive approach is critically more cost effective and shows greater patients’ compliance. Examples of nanotechnologies include, for example, dendrimers, polymeric micelles, superparamagnetic iron oxide cores, gold nanoparticles, liposomes and other lipid nanocarriers.

Among these, lipid nanocarriers are an emerging field of research holding great promise for cancer diagnosis and treatment, since they have the advantage of multifunctionalisation for simultaneous imaging analysis and delivery of anticancer drugs, genes and vaccines. They may be passively or actively targeted to solid tumors, where the drug is slowly released inside the tumor. Furthermore, lipid nanocarriers can
Preface

act as sustained-release delivery system, and control of properties, such as diameter, surface charge, pharmacokinetic and bioavailability profiles and dosing schedule, can significantly improve the therapeutic outcome of anticancer drugs. These nanocarriers are composed of well-tolerated and physiological lipids, thus nonimmunogenicity and biodegradability are other advantages, along with facilities to be produced in large scale, and possibility to deliver therapeutic genes to treat metastatic cancers.

This book deals with the current state of the art and challenges in the field of lipid nanocarriers, for drug delivery and targeting, on how alteration of some of their properties can impact the therapeutic efficacy and adverse side effects, and discusses the potential use of these multifunctional lipid nanocarriers for image-guided drug delivery. The first part of the book deals with the fundamentals behind the rationale of developing lipid nanocarriers maintained for cancer diagnosis, prevention and therapy, whereas the second part is focused on more specific applications, including lung, breast, brain, skin and ocular cancer targeting, and also appropriate adrenocortical tumours.

For the successful achievements of these goals, a synergistic and interdisciplinary collaboration of several professionals is demanding, where biologists, oncologists, engineers, toxicologists, pharmacologists, physicians and nanotechnologists can bring together solutions towards designing and developing feasible imaging and therapeutic platforms. The editor is grateful to the outstanding group of international researchers, who have contributed with their most valuable expertise bringing a first-hand continuing professional experience. This book draws its contributors from Brazil, Canada, China, France, India, Japan, Portugal, Spain, Taiwan, and USA, who have helped make this work possible. While preparing the book, the editor has received the exceptional support of Fundação Ensino e Cultura Fernando Pessoa and Fundação para a Ciência e Tecnologia, for recognising the virtue of this scientific task. Sincere acknowledgements are addressed to the editing and managing staff at iSmithers for their tireless efforts and assistance.

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Contributors

Mansoor Amiji
Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University, Room 110, Mugar Life Sciences Building, 360 Huntington Avenue, Boston, MA 02115, USA

Joana R. Araújo
Department of Physical Chemistry, Faculty of Pharmacy, University of Barcelona, Avenue Joan XXIII s/n, Barcelona 08028, Spain

Husain Attarwala
Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University, Room 110, Mugar Life Sciences Building, 360 Huntington Avenue, Boston, MA 02115, USA

Jair Bar
Division of Medical Oncology, Department of Medicine, The Ottawa Hospital Cancer Centre, 501 Smith Road, Ottawa, ON KIH 8L6, Canada

Ana Maria Carmona-Ribeiro
Biocolloids Lab, Department of Biochemistry, Institute of Chemistry, University of São Paulo, Avenue Professor Lineu Prestes, 748 - Butantã, São Paulo, SP, CEP 05508-900, Brazil

Yi-Hsuan Chi
Institute of Cellular and Organismic Biology, Academia Sinica, Taipei 115, Taiwan

Padma V. Devarajan
Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Matunga, Mumbai 400019, India
Contributors

Maria A. Egea
Department of Physical Chemistry, Faculty of Pharmacy, University of Barcelona, Avenue Joan XXIII s/n, Barcelona 08028, Spain

Clara B. Fernandes
Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, N.P. Marg, Matunga, Mumbai 400019, India

Srinivas Ganta
Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University, Room 110, Mugar Life Sciences Building, 360 Huntington Avenue, Boston, MA 02115, USA

Thanga M. Geetha
Biocon-Bristol Myers Squibb Research Centre, Syngene International Ltd., Bangalore 560099, India

Dalapathi Guggulothu
Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, N.P. Marg, Matunga, Mumbai 400019, India

Renuka T. Gupta
C.U. Shah College of Pharmacy, S.ND.T Women’s University, Sir Vithaldas Vidya Vihar, Santacruz (W), Mumbai 400049, India

Tatsuhiro Ishida
Subdivision of Biopharmaceutical Sciences, Department of Pharmacokinetics and Biopharmaceutics, Institute of Health Biosciences, The University of Tokushima, 1-78-1, Sho-machi, Tokushima 770-8505, Japan

Jiankang Jin
Department of Thoracic and Cardiovascular Surgery, The University of Texas of M.D. Anderson Cancer Center, Houston, TX 77030, USA

Sajan Jose
Department of Pharmaceutical Sciences, Mahatma Gandhi University, Cheruvandoor Campus, Ettumanoor 686631, Kerala, India
Contributors

Vandita Kakkar
M-Pharmacy, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, India

Mayurkumar Kalariya
Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University, Room 110, Mugar Life Sciences Building, 360 Huntington Avenue, Boston, MA 02115, USA

Indu Pal Kaur
University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, India

Hiroshi Kiwada
Subdivision of Biopharmaceutical Sciences, Department of Pharmacokinetics and Biopharmaceutics, Institute of Health Biosciences, The University of Tokushima, 1-78-1, Sho-machi, Tokushima 770-8505, Japan

Amr S. Abu Lila
Subdivision of Biopharmaceutical Sciences, Department of Pharmacokinetics and Biopharmaceutics, Institute of Health Biosciences, The University of Tokushima, 1-78-1, Sho-machi, Tokushima 770-8505, Japan

Maria L. García López
Department of Physical Chemistry, Faculty of Pharmacy, University of Barcelona, Avenue Joan XXIII s/n, Barcelona 08028, Spain

Véronique Marsaud
UMR CNRS 8612, Pharmacologie Cellulaire et Moléculaire des Anticancéreux, CNRS, UMR 8612, Faculté de Pharmacie, Châtenay-Malabry F-92296, France and Université Paris-Sud, Orsay F-91405, France

Aditya P. Nayak
Department of Studies and Research in Biochemistry, P.G Center, Shivagangothri, Kuvempu University, Davangere, Karnataka 577002, India
Contributors

Elisabet González Mira
Department of Physical Chemistry, Faculty of Pharmacy, University of Barcelona, Avenue Joan XXIII s/n, Barcelona 08028, Spain

Rayasa S.R. Murthy
Department of Pharmaceutics, ISF College of Pharmacy, Ferozepur Road, Ghal Kalan, Moga 142001, Punjab, India

Saša Nikolić
Department of Physical Chemistry, Faculty of Pharmacy, University of Barcelona, Avenue Joan XXIII s/n, Barcelona 08028, Spain

Vandana B. Patravale
Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, N.P. Marg, Matunga, Mumbai 400019, India

Vincent Plassat
UMR CNRS 8612, Pharmacologie Cellulaire et Moléculaire des Anticancéreux, CNRS, UMR 8612, Faculté de Pharmacie, Châtenay-Malabry F-92296, France and Université Paris-Sud, Orsay F-91405, France

Priyanka S. Prabhu
Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, N.P. Marg, Matunga, Mumbai 400019, India

Rajagopal Ramesh
Department of Thoracic and Cardiovascular Surgery, The University of Texas of M.D. Anderson Cancer Center, Houston, TX 77030, USA; Department of Pathology and of Pharmaceutical Sciences, The Peggy and Charles Stephenson Oklahoma University Cancer Institute, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

Yogesh M. Rane
Formulation Development (NDDS) Department, Pharma Research, Wockhardt Research Centre, Aurangabad 431210, India
Contributors

Jack-Michel Renoir
UMR CNRS 8612, Pharmacologie Cellulaire et Moléculaire des Anticancéreux, CNRS, UMR 8612, Faculté de Pharmacie, Châtenay-Malabry F-92296, France; Université Paris-Sud, Orsay F-91405, France, and INSERM U749, Institut Gustave Roussy, Villejuif Cedex, France

Jack A. Roth
Department of Thoracic and Cardiovascular Surgery, The University of Texas of M.D. Anderson Cancer Center, Houston, TX 77030, USA

Manish Shanker
Department of Thoracic and Cardiovascular Surgery, The University of Texas of M.D. Anderson Cancer Center, Houston, TX 77030, USA

Rajshree L. Shinde
Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology (ICT), Matunga, Mumbai 400019, India

Kamalinder K. Singh
C.U. Shah College of Pharmacy, S.ND.T Women’s University, Sir Vithaldas Vidya Vihar, Santacruz (W), Mumbai 400049, India

Eduarda S.F. Souto
Higher School of Nursing, University of Porto, Rua Dr. António Bernardino de Almeida, Porto 4200-072, Portugal

Eliana B. Souto
Faculty of Health Sciences, Fernando Pessoa University, Rua Carlos da Maia, Nr. 296, Office S.1, Porto P-4200-150, Portugal and Institute of Biotechnology and Bioengineering, Centre of Genomics and Biotechnology, University of Trás-os-Montes and Alto Douro (CGB-UTAD/IBB), P.O. Box 1013, P-5001-801, Vila Real, Portugal

Selma B. Souto
Department of Endocrinology, Hospital de São João, University of Porto Medical School, Alameda Prof. Dr. Hernâni Monteiro, 4200-319 Porto, Portugal
Contributors

Giorgia Urbinati
UMR CNRS 8612, Pharmacologie Cellulaire et Moléculaire des Anticancéreux, CNRS, UMR 8612, Faculté de Pharmacie, Châtenay-Malabry F-92296, France and Université Paris-Sud, Orsay F-91405, France

Sarah J. West
Department of Thoracic and Cardiovascular Surgery, The University of Texas of M.D. Anderson Cancer Center, Houston, TX 77030, USA

Paul Wheatley-Price
Division of Medical Oncology, Department of Medicine, The Ottawa Hospital Cancer Centre, 501 Smith Rd, Ottawa, ON K1H 8L6, Canada

Chien-Hsun Wu
Institute of Cellular and Organismic Biology, Academia Sinica, Taipei 115, Taiwan

Han-Chung Wu
Institute of Cellular and Organismic Biology, Academia Sinica, Taipei 115, Taiwan
Reviewing the Gaps in the Safety and Risk Assessment of Nanoparticles and Nanomaterials

Yogesh M. Rane and Eliana B. Souto

1.1 Introduction

During the early 1990s, innovative polymer-based therapeutics were most frequently developed as novel anti-cancer agents, and thus can be considered as the first generation of nanomedicines. More recently, a much broader range of life-threatening and debilitating diseases (e.g., viral infections, arthritis, multiple sclerosis and hormone abnormalities) have been targeted via intravenous, subcutaneous or oral routes. Given the increasing demand for more acceptable materials and sustainable production methods, novel nanocarriers based on lipid materials have been proposed as alternatives to polymeric nanocarriers. The growing debate over the safety of nanomedicines and the need for evolution of an appropriate regulatory framework are at the forefront of the regulatory pipelines [1].

Nanomaterials have different chemical, physical and biological characteristics than their larger counterparts of similar chemical composition. These differences have contributed to both positive and negative aspects of nanotechnology – nanotechnology, on the one hand, may have novel and interesting medical and/or industrial applications but, on the other hand, may also represent increased risk to human and environmental health. In general, the most important challenge of nanotechnology, particularly for nanomedicine, is to understand which nanosized specific characteristics interact with particular biological systems and functions, to optimise the therapeutic potential and reduce the undesired responses. The evaluation of the safety of nanomedicines is of particular relevance for long-term application. At present, it is still too early to predict, on the basis of the characteristics of the nanomaterial, a possible biological response because no reliable database exists. Therefore, a case-by-case approach for hazard identification is still required, thus it is difficult to establish a risk assessment analysis [2].

Despite the widespread concerns about the safety of nanosized particles, preclinical and clinical experience gained during the development of lipid excipients shows that judicious application of lipid nanocarriers for each specific application will ensure development of safe and important materials for biomedical and pharmaceutical
use [3]. With increasing development of technology and instrumentation, more uniformity in the results across various nanotechnologies will help bridge the gap between safety and efficacy of nanomedicines. Likewise, it will urge scientists across the globe to take a cautious approach towards nanomedicine usage and explore research findings. However, with respect to inhaled nanoparticles, extensive know-how is now available regarding toxicological effects. The effects of inhaled nanoparticles are attributed to their direct effects on the central nervous system, their translocation from the lung into the bloodstream and their ability to invoke inflammatory responses in the lung with subsequent systemic effects. Since the evidence for health risks of nanoparticles after inhalation has been increasing over the last decade, Borm and Kreyling [4] tried to translate these findings and principles observed in inhalation toxicology into recommendations and methods for testing nanoparticles for drug delivery. They identified a large gap in research on nanoparticles in inhalation toxicology and in nanoscale drug delivery, and advised a closer interaction between both disciplines, to gain an insight into the role of nanoparticle size and properties, and their mechanisms of acute and chronic interaction with biological systems. In this respect, Oberdorster and co-workers [5] have identified the following aims: (i) the development of viable in vivo nanoparticle detection techniques; (ii) the development and production of inexpensive real-time monitoring instruments and methods for mass concentration (for nanoparticle dispersions of low concentration), surface area concentration and size distribution; (iii) the development of standardised, well-characterised nanoparticles; (iv) the development of radiolabelled samples, and samples that can be tracked and detected through neutron activation; (v) the development of more advanced surface chemistry characterisation techniques, in particular, techniques for detecting and speciating biological molecules on the surface of nanoparticles; and (vi) the development of electron microscopy techniques for biologically relevant nanoscale analysis.

### 1.2 Risk Assessment and Sustainability

Risk assessment is the evaluation of scientific information on the properties of a material, the dose-response relationship and the extent of exposure. The product of risk assessment is a statement about the likelihood of exposed humans being harmed and about the extent of the degree of risk (risk characterisation) [6]. Risk assessment is a complex process, involving integration of information across a range of domains including source characterisation, fate and transport, modelling, exposure assessment and dose-response characteristics [7]. On the other hand, sustainability risk management deals with emerging clinical needs and ethical issues. Risk managers should anticipate these risks and develop appropriate strategies, bearing in mind the need for [6]:

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(i) Physicochemical properties of nanoparticles – The diversity and complexity of nanoparticles make their identification and characterisation more difficult than that of their counterparts.

(ii) Detection and analysis of nanoparticles – The in vivo detection of nanoparticles is challenged by their small size in parallel with their unique physical structure and physicochemical characteristics. The variety of physiochemical properties can significantly affect sample collection and analysis. A range of methods are available for measuring the number, surface area and mass concentration of nanoparticles.

(iii) Biological effects – The toxicity assessment is potentially influenced by a variety of physicochemical properties of engineered nanoparticles.

Balbus and co-workers [8] have reported their findings about nanotoxicology, answering two specific questions: (i) what information is needed to understand the human health impact of engineered nanoparticles, and (ii) how is this information best obtained? In vivo toxicological tests are required because of their familiarity and interpretability. In addition, the dose of nanoparticles needs to be determined depending on the toxicological test results. Nanoparticles should be validated by worldwide laboratories and made available for benchmarking tests of other newly created nanoparticles. Prediction of the potential toxicity of emerging nanoparticles will require hypothesis-driven research that will elucidate how physicochemical parameters influence toxic effects on biological systems.

As a general rule, risk assessment of nanotechnologies requires (i) the definition of nanoparticle technologies and application to facilitate regulatory discussions, prioritisation of research and exchange the study outcomes; (ii) analytical tools for the characterisation of nanoparticles in complex biological matrices; (iii) the establishment of relevant dose metrics for nanoparticles used for interpretation of both scientific studies and regulatory frameworks; and (iv) the assessment of kinetics and dynamics (behaviour) of nanoparticles to estimate human exposure to nanoparticles [9]. The type of tests and biological end points used within standard hazard assessment frameworks are generally appropriate for risk assessment of nanoparticles. These include the way in which the nanoparticle is dosed into and maintained within the test medium, a better understanding and reporting of factors that influence the behaviour of nanoparticles and agreement on how dosimetric data should be reported [10].

1.2.1 Effect of Nanosized Particles

The size of nanoparticles alone may not be the critical factor determining their toxicity, as the overall number and thus the total surface area may also be important.
As a particle decreases in size, the surface area per unit mass increases and the particle number decreases. With the decrease in particle size, a greater proportion of atoms or molecules are found at the surface compared to those inside. Thus, nanoparticles have a much larger surface area per unit mass compared with larger particles. The increase in the surface-to-volume ratio has effects on the particle surface energy, which renders them biologically more reactive. Nanoparticles can be produced with a variety of shapes, structures and surface properties. The effects of these properties on the toxicity of nanomaterials are also unknown. The shape of nanoparticles may have effects on the kinetics of deposition and absorption in the body. The desirable properties of engineered nanoparticles closely depend on the size, shape and structure, both physically and chemically. Biological activity also depends on physicochemical parameters not usually considered in toxicity screening studies. Quantitative data obtained from toxicity studies on engineered nanoparticles are still limited. Raw materials selected for the production of nanoparticles need to be characterised by toxicity screening studies. Requirements for in vitro and in vivo screening studies will differ according to the delivery route and production process. Additionally, understanding human exposures in the context of developing appropriate screening studies will present a further set of characterisation requirements. Oberdorster and co-workers [5] proposed four screening study contexts and characterisation recommendations: (i) human exposure characterisation; (ii) characterisation of material following administration; (iii) characterisation of administered material; and (iv) characterisation of as-produced or as-supplied material. Currently, engineered nanoparticles are predominantly at the research or preproduction stage and there are relatively few environments where exposures are known to occur. However, if commercialisation of products using nanoparticles develops as expected, the potential for clinical exploitation and thereafter for human exposure is likely to increase dramatically over the coming decades. Therefore, estimates of future use and potential human exposures should be considered in the development of toxicity screening.

Upon administration of nanoparticles, their characterisation in a test system or model will provide the highest quality data on dose and material properties that are related to observed responses. However, this is limited by current methodological facilities. Characterisation after administration is particularly advantageous since physicochemical changes in the nanoparticles are likely to occur before and after administration. Potential changes that can occur include changes in aggregation state, adsorption of biomolecules and biochemically induced changes in the surface chemistry of nanoparticles. This approach addresses potential physicochemical changes between the bulk material and the administered nanoparticles (such as agglomeration state) and allows more robust causal associations between the material and observed responses to be developed. Characterisation of nanoparticles as produced or as supplied represents the most direct approach for obtaining physicochemical information and may provide
useful baseline data on the material under test. Most engineered nanoparticles have a functionality based on their physicochemistry. It is therefore likely that information of relevance to toxicity screening studies will be available from suppliers or producers in many cases. However, due to the lack of currently accepted nanomaterial characterisation standards, it is strongly recommended that whenever possible, independent characterisation should be undertaken. Characterisation of supplied nanomaterial may not appropriately represent physicochemical properties of the material during or following administration. For this reason, exclusive reliance on this approach is discouraged, and is recommended where characterisation of material during or after administration is clearly not feasible.

Depending on the particle surface chemistry, reactive groups on the nanoparticle surface will certainly modify the biological effects. Under ambient conditions, some nanoparticles can form aggregates or agglomerates. These agglomerates have several forms, from dendritic structure to chain or spherical structures. To maintain the characteristics of nanoparticles, these are often stabilised with hydrophilic coatings to prevent agglomeration. The properties of nanoparticles can be significantly changed by surface modification, and the distribution of nanoparticles in the body strongly depends on the surface characteristics [11, 12]. Oberdorster and co-workers [5] have recommended that after characterising the physicochemical properties of test materials, in any toxicity screening study, characteristics such as size distribution, agglomeration state, shape, crystal structure, chemical composition (including spatially averaged (bulk) and spatially resolved heterogeneous composition), surface area, chemistry and charge, and porosity should be described. They further advised that careful consideration should be given to the metric used for quantification of dose. Although response may be associated with a wide range of physicochemical characteristics, measuring dose against a physical metric of mass, surface area or particle number for a well-characterised material will enable quantitative interpretation of data. Appropriate selection of the dose metric will depend on the hypothesised parameter most closely associated with anticipated response or the metric that may be most accurately measured. Where nanoparticles are administered in a liquid medium, the nature and amount of material within the suspension should be fully characterised before delivery in terms of number, surface area and mass concentration.

To enable retrospective interpretation of toxicity data and replication of tests, it is strongly recommended that all information on the production and processing of nanoparticles be recorded. It is essential to document fully storage time and conditions (including temperature, humidity, exposure to light and atmosphere composition), because physicochemical changes occur during storage. If possible, the physicochemical stability of samples over time should be demonstrated. Where a test material is a heterogeneous mixture of different components, information
is required on the relative abundance of the different components, and whether associations in the bulk material are maintained in the administered material, or whether different components are preferentially administered with specific delivery mechanisms. The agglomeration state of a nanomaterial during and following administration may have a significant impact on its biological activity. If possible, some insight into the binding forces within agglomerates (e.g., relatively weak van der Waals forces or relatively strong sintered bonds) should be obtained. Material agglomeration or deagglomeration in different liquid media should also be investigated whenever possible. Characterisation of a material as administered is of highest priority, supplemented by characterisation after in vitro or in vivo administration. Table 1.1 depicts the recommendations on material characterisation as suggested by Oberdorster and co-workers [5].

1.2.2 Integrated Platforms for Nanoparticle Toxicity Analysis

Usually, most of the research on the toxicology of nanomaterials has focused on the effects of nanoparticles that enter the body accidentally. However, much less research on the toxicology of nanoparticles that are used for biomedical applications, in which the nanoparticles are deliberately placed in the body, has been carried out. Moreover, there are no harmonised standards for assessing the toxicity of nanoparticles to the immune system (immunotoxicity). Dobrovolskaia and McNeil [13] demonstrated that nanoparticles can stimulate and/or suppress the immune responses and that their surface chemistry largely determines their compatibility with the immune system. Modification of the surface properties of nanoparticles can significantly reduce their immunotoxicity and can make them useful platforms for drug delivery. Hund-Rinke and Simon [14] studied two nanoparticle populations (i.e., 25 and 100 nm size) assuming that the future technical development will reduce particle size further. Studies indicated that the smaller nanoparticles were more toxic in the usual tests with an assumption that the overall toxicological potential of nanoparticles will increase in future. Emphasis for improvements focuses on (i) the cause of the toxicity (whether specific surface area plays a role, e.g., dominance of crystalline form or aggregates formed of the size); (ii) improvement of the test design (e.g., stability of the dispersions); (iii) development of a set of tests suitable for the assessment of nanoparticles; and (iv) assessment of the results on the basis of optimised test systems. For nanoparticles with potential risk, a risk-benefit analysis can be performed and, if necessary, risk-reducing measures can be undertaken.

The potential benefits of nanotechnology are large, but there are also perceived risks, which must be addressed early in time. The precautionary principle should not be used to stop research on nanomedicines. Development of a sound balance between
Reviewing the Gaps in the Safety and Risk Assessment of Nanoparticles

The benefits and the potential hazards of nanotechnology demand creation of a sustainable database for the purpose of risk assessment [15–17].

Another item of relevance is the genotoxic effect of nanoparticles. Genotoxicity describes a deleterious action on a cell’s genetic material affecting its integrity, whereas genotoxic materials are known to be potentially mutagenic or carcinogenic, specifically those capable of causing genetic mutation and of contributing to the development of tumours [18]. Genotoxicity provides evidence that the compound has reached DNA.

### Table 1.1 Recommendations on material characterisation

<table>
<thead>
<tr>
<th>Characterisation</th>
<th>Toxicity screening studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supplied material</td>
</tr>
<tr>
<td>Size distribution and polydispersity</td>
<td>A</td>
</tr>
<tr>
<td>Shape area</td>
<td>A</td>
</tr>
<tr>
<td>Surface area</td>
<td>A</td>
</tr>
<tr>
<td>Composition</td>
<td>A</td>
</tr>
<tr>
<td>Surface chemistry</td>
<td>A</td>
</tr>
<tr>
<td>Surface contamination</td>
<td>D</td>
</tr>
<tr>
<td>Surface charge</td>
<td>A</td>
</tr>
<tr>
<td>Crystal structure</td>
<td>A</td>
</tr>
<tr>
<td>Physicochemical structure of the particle</td>
<td>A</td>
</tr>
<tr>
<td>Agglomeration state</td>
<td>D</td>
</tr>
<tr>
<td>Porosity</td>
<td>B</td>
</tr>
<tr>
<td>Production method</td>
<td>A</td>
</tr>
<tr>
<td>Prior storage of material</td>
<td>A</td>
</tr>
<tr>
<td>Concentration</td>
<td>–</td>
</tr>
</tbody>
</table>

(A) Characterisation is recommended to be essential; (B) characterisation is of added value but is not recommended as essential due to constraints associated with complexity, cost and availability; (C) characterisation is considered to provide added value but nonessential information; and (D) characterisation is not considered of significant value to screening studies.

either directly or through an indirect mechanism, so that DNA damage can occur. The damage could result in death of cell, with or without additional cell divisions before death occurs [19].

*In vitro* studies show that particles generate reactive oxygen species (ROS), deplete endogenous antioxidants, alter mitochondrial function and produce oxidative damage to lipids and DNA [20]. Surface area, reactivity and chemical composition play important roles in the oxidative potential of nanoparticles. Schins [21] reviewed work devoted to genotoxicity and cancer risk assessment and came to the conclusion that particles and fibres form a rather specific group among all toxicants. The physicochemical behaviour of nonparticulate, chemical carcinogens is significantly different from that of fibrous and nonfibrous particles. ROS play a major role in the primary genotoxicity of particles, which may derive from their surface properties, the presence of transition metals, intracellular iron mobilisation and lipid peroxidation. Primary genotoxicity is also relevant to particle size, shape, crystallinity and solubility. It may also depend upon particle uptake, interaction with cell division machinery and the presence of mutagens carried with the particle. Secondary genotoxicity can be attributed to excessive and persistent formation of ROS from inflammatory cells. Schins identified mechanisms of particle genotoxicity by acellular assays, *in vitro* tests, *in vivo* studies (usually in mice or rats) and finally biomarker studies in humans with occupational exposure. *In vitro* studies helped to identify primary genotoxic properties of particles, whereas *in vivo* studies provided further support for the correlation between particle-induced inflammatory reactions and secondary genotoxicity. For proper risk assessment of particles, it is necessary to identify the relative impact of primary and secondary genotoxicity in realistic exposure conditions.

The International Conference on Harmonisation (ICH) defined genotoxicity tests as *in vitro* and *in vivo* tests designed to detect compounds that induce genetic damage directly or indirectly by various mechanisms. These tests enable hazard identification with respect to damage to DNA and its fixation. Fixation of DNA damage in the form of gene mutations, large-scale chromosomal damage, recombination and numerical chromosome changes is generally considered to be essential for heritable effects and in the multistep process of malignancy, a complex process in which genetic changes may play only a part. Materials that are positive in such tests have potential to be human carcinogens and/or mutagens. ICH also designates such tests for identification of carcinogenesis and genotoxicity and limits their use for identification of heritable diseases.

Examples and theories reported for non-nanostructures (molecules and larger particles) provide information on the mechanisms of genotoxic effects, which can be diverse and their elucidation can be more and more demanding. Thus, a pragmatic approach would be the use of a battery of standard genotoxicity testing methods covering a wide
range of mechanisms. Application of standard methods to nanoparticles demands, however, several adaptations, and the interpretation of results from the genotoxicity tests may need additional considerations.

1.3 Conclusions

In recent years, countries with economic boom have shown significant rise in investment and expenditure on research and healthcare services, predominantly those addressing cancer diseases. The clinical trials currently being undertaken for general diseases or for diseases that affect substantial proportions of population have increased. Furthermore, interest in genomic science has also increased owing to its ability to improve the understanding of gene, environment and disease interactions. Therefore, the ethical training of researchers, members of ethics review committees and personnel from governing bodies is of utmost relevance, in parallel with the research devoted to nanotoxicology, nanotherapeutics and nanoregulatory expertise. Evaluation should relate not just only to efficiency and promotion of innovation but also to an acknowledgement that public-funded basic physicochemical research should be accepted to incorporate strong higher order public goods elements from its inception and not merely after product development at the technology transfer stage.

References


Lipid Nanoparticles in Cancer Therapy: Past, Present and Future

Aditya P. Nayak, Eduarda S.F. Souto, Rayasa S.R. Murthy and Eliana B. Souto

2.1 Introduction

Although the ‘confrontation against cancer’ is on since several decades, we are not able to control, conquer and eliminate it. Among the current chemotherapy options, radiotherapy is the most common method of treatment for both localised and metastasised cancers. Here, the success rates are very limited and case dependent. It has long been reported that the efficiency of anti-neoplastic drugs can be increased by targeting and maintaining their concentration at the site of action for a sufficient length of time [1]. Some of the abnormalities shown by cancer cells may contribute to pursue such attempt. In fact, in comparison to healthy tissue, neoplastic tissues show a rapidly expanding tumour vasculature often with a discontinuous endothelium, with gaps between cells that may be several hundred nanometres long following a compromised clearance via lymphatics, the so-called ‘enhanced permeability and retention (EPR) effect’. These abnormalities can be used to deliver anti-cancer drugs specifically to the tumour region using the colloidal drug delivery systems by altering the route of administration, distribution, metabolism and elimination. In the case of intravenous (IV) injection, highest amount of nanoparticles will get accumulated within the cancer cells due to hyperpermeability and hindered lymphatic clearance [2–5]. However, even if located in the neoplastic region, the efficacy of anti-cancer drugs can be affected by the development of multidrug resistance (MDR), which is one of the most common reasons for the diminished efficacy of drugs in almost all diseases in general and cancer in particular. This resistance mainly results in no reasonable tumour size reduction or recrudescence after initial therapeutic effect. Colloidal delivery systems were able to overcome the problem of MDR to some extent [6].

2.2 Concepts and Definitions of Lipid Nanoparticles

Lipid nanoparticles (LNP) are colloidal particles composed of a lipid matrix that is solid at room and body temperature. LNP have been tested for a number of administration routes including parenteral [7–10], oral and peroral [11–14], ocular
Drugs loaded in LNP are also reported to show improved bioavailability, targeting capacity [22–24] and enhanced cytotoxicity against MDR cancer cells [25, 26]. LNP have been proposed as an alternative to other controlled drug delivery systems (CDDS) such as lipid nanoemulsions, liposomes and polymeric nanoparticles as a result of improved advantages. The solid lipids figuring in LNP matrix immobilise drug molecules and thus reduce drug leakage, which is commonly seen in many other CDDS, such as lipid nanoemulsions and liposomes. Furthermore, LNP are generally less toxic in comparison to some polymeric nanoparticles, because physiological and biocompatible lipids are utilised. LNP are also suited for mass production [27–31] and sterilisation [32], and can be produced in an eco-friendly environment by avoiding organic solvents [9]. The aforementioned properties of LNP, in particular the controlled-release kinetics and site-specific drug delivery, make them particularly attractive for the delivery of anti-cancer drugs.

Typically, LNP consist of solid lipid(s), surfactant(s), cosurfactants (if required) and incorporated active ingredients. The lipids used in the preparation of LNP include fatty acids with different lengths of hydrocarbon chain, fatty esters, fatty alcohol, glycerides with different structures and mixtures of glycerol esters. Currently used lipids and their structures are listed in Table 2.1. Waxes such as Cutina CP (cetyl palmitate), solid paraffin and beeswaxes are also sometimes used for LNP preparation. In addition to the conventional lipids, synthetic lipids, such as para-acyl-calix[4]arene, are also developed to prepare LNP [33].

<table>
<thead>
<tr>
<th>Class of lipid</th>
<th>General structure</th>
<th>Examples (n value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids</td>
<td>H₃C-[CH₂]ₙ-COOH</td>
<td>Dodecanoic acid (n = 10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myristic acid (n = 12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Palmitic acid (n = 14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stearic acid (n = 16)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>H₂C-O-CO-[CH₂]ₙ-CH₃</td>
<td>Caprylate triglycerides (n = 6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caprate triglycerides (n = 8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trilaurin (n = 10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tripalmitin (n = 14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tristearin (n = 16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tribehenin (n = 20)</td>
</tr>
</tbody>
</table>
Lipid nanoparticles are critical in cancer therapy, past, present, and future. Lipid composition is vital for preparing LNP with the desired characteristics, including drug-loading capacity, stability, and sustained-release behavior. Loading capacity for the same drug differs depending on its apparent partition coefficients with lipid phases, which vary with the balance of the hydrophobic and hydrophilic functional groups of the lipid molecules. For instance, hydrophobicity of a fatty acid increases with the increase in the length of its hydrocarbon chain. A fatty ester is more hydrophobic than the fatty acid of the same chain length, because the hydrophilic carboxyl group of the fatty acid is replaced by the more hydrophobic ester group. Triglycerides are more hydrophobic than mono- and diglycerides because all three hydrophilic hydroxyl groups are substituted by fatty ester. In addition, polymorphism of lipids also affects the properties of LNP. Lipid polymorphism refers to the existence of multiple crystalline forms of solid lipids with varied crystalline lattice. Polymorphic forms having imperfect crystalline lattice show higher drug-loading capacity as drug molecules are primarily loaded into

### Table 2.1 Continued

<table>
<thead>
<tr>
<th>Class of lipid</th>
<th>General structure</th>
<th>Examples (n value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoglycerides</td>
<td>H₂C-O-CO-[CH₂]ₙ-CH₃</td>
<td>Glycerol monostearate (n = 16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxy monoglycerides</td>
<td>H₂C-O-CO-[CH₂]ₙ-CH-[CH₃₂]ₘ CH₃</td>
<td>Glycerol hydroxystearate (n = 10; m = 5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC-OH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂C-OH</td>
<td></td>
</tr>
<tr>
<td>Diglycerides</td>
<td>H₂C-O-CO-[CH₂]ₙ-CH₃</td>
<td>Glycerol behenate (di-n = 20)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty esters</td>
<td>O</td>
<td>Cetyl palmitate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₃₁C₁₅-C-O-C₁₆H₃₃</td>
<td></td>
</tr>
</tbody>
</table>

Lipid composition is critical to prepare LNP with the desired characteristics including the drug-loading capacity, stability, and sustained-release behavior. Loading capacity for the same drug differs depending on its apparent partition coefficients with lipid phases, which vary with the balance of the hydrophobic and hydrophilic functional groups of the lipid molecules. For instance, hydrophobicity of a fatty acid increases with the increase in the length of its hydrocarbon chain. A fatty ester is more hydrophobic than the fatty acid of the same chain length, because the hydrophilic carboxyl group of the fatty acid is replaced by the more hydrophobic ester group. Triglycerides are more hydrophobic than mono- and diglycerides because all three hydrophilic hydroxyl groups are substituted by fatty ester. In addition, polymorphism of lipids also affects the properties of LNP. Lipid polymorphism refers to the existence of multiple crystalline forms of solid lipids with varied crystalline lattice. Polymorphic forms having imperfect crystalline lattice show higher drug-loading capacity as drug molecules are primarily loaded into
LNP in the defects of the lattices of solid lipids. When the lipid molecules undergo polymorphic transition from the metastable form into the stable form, they become more orderly packed, and some of the defects in the lattices disappear. As a result, drug expulsion from the lipid core to the particle surface may occur, contributing to high initial burst release and drug leakage during storage. To avoid crystallinity and polymorphism problems, lipid oils, for example, Miglyol 812 (caprylic/capric triglycerides) or oleic acid, or a binary mixture of physically incompatible solid lipids has been incorporated into solid lipids to disrupt the crystallinity of the solid lipid matrix. The resulting modified form of LNP is often referred to as nanostructured lipid carrier.

Cationic LNP formulations containing positively charged lipids are reported for gene delivery [34], as the positive surface charge may enhance the in vivo transfection efficiency of genes. Examples of cationic lipids include stearylamine, N,N-di-(β-stearoylethyl)-N,N-dimethylammonium chloride, benzalkonium chloride, cetyltrimethylammonium bromide (CTAB), dimethyldioctadecylammonium bromide (DDAB), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and cetylpyridinium chloride (CPC). From the point of view of cytotoxicity, two-tailed cationic lipids are usually preferable to one-tailed cationic lipids (i.e., CPC and CTAB) and cationic surfactants. Surfactants are used in LNP to disperse the melt lipid into aqueous phase and then to stabilise LNP after cooling. The surfactants used include ionic, nonionic and amphoteric surfactants (Table 2.2). Although ionic surfactants such as sodium dodecyl sulfate form LNP with narrow size distribution and better stability, some of them are associated with undesirable toxicity.

<table>
<thead>
<tr>
<th>Ionic surfactants</th>
<th>Nonionic surfactants</th>
<th>Amphoteric surfactants</th>
<th>Cosurfactants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium cholate, sodium cocoamphoacetate, sodium dodecyl sulfate, sodium glycocholate, sodium oleate, sodium taurocholate, sodium taurodeoxycholate</td>
<td>Brij-78, poloxamer 188, poloxamer 407, poloxamine 908, polyglycerol methylglucose distearate (Tego Care 450), Solutol HS15, Span 85, Tween-80, tyloxapol, Tween-20, trehalose</td>
<td>Egg phosphatidylcholine, egg lecithin (Lipoid E80), soy phosphatidylcholine (SP), (Epikuron 200, 95% SP), (Lipoid S100), (Lipoid S75, 68% SP), (Lipoid S75, 68% SP), (Phospholipon 90G, 90%)</td>
<td>Butanol, butyric acid</td>
</tr>
</tbody>
</table>
Surfactants for LNP preparation are selected based on several factors, namely, hydrophilic-lipophilic balance (HLB) value, route of administration, toxicity, \textit{in vivo} lipid biodegradation and the effect on lipid modification and particle size. Surfactants with HLB values in the range of 8–18 are suitable for the preparation of LNP through oil-in-water dispersion medium. Nonionic surfactants are preferred for oral and parenteral preparations over those with ionic properties because of their lower toxicity and irritancy. In general, the toxicity of surfactants decreases in the order cationic > anionic > nonionic > amphoteric. LNP have the inherent disadvantage of low encapsulation of water-soluble agents because of the lipophilicity of solid lipids. However, to incorporate hydrophilic drugs into LNP, counterions such as polymers and esters (organic salts) are used to neutralise the charge of the drugs (Table 2.3). This may increase the apparent partition coefficient of the selected drug in the lipid phase and enhance its loading.

<table>
<thead>
<tr>
<th>Organic salts</th>
<th>Ionic polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monodecylphosphate</td>
<td>Dextran sulfate sodium salt</td>
</tr>
<tr>
<td>Monohexadecylphosphate</td>
<td>Hydrolysed and polymerised epoxidised soybean oil</td>
</tr>
<tr>
<td>Monoctylphosphate</td>
<td></td>
</tr>
<tr>
<td>Sodium hexadecylphosphate</td>
<td></td>
</tr>
</tbody>
</table>

In addition to lipids and surfactants, other agents are also used particularly in preparing some modified forms of NLP. For example, surface-modified NLP for long circulation are prepared by coating with hydrophilic polymers such as poloxamers, poloxamines or polyethylene glycol (PEG). This technique is valuable to minimise clearance of NLP by phagocytosis [35] due to high surface hydrophobicity of the particles.

2.3 \textit{In Vitro} Properties and \textit{In Vivo} Features of Lipid Nanoparticles

\textit{In vitro} characterisation of anti-cancer drugs, particularly for solid tumours, can only serve as a good starting point to screen out inactive or less active agents. There are several drug resistance mechanisms, sometimes referred as noncellular mechanisms, that exist only in \textit{in vivo} situations. Thus, a compound showing strong \textit{in vitro} anti-tumour activity may fail miserably \textit{in vivo} in tumour-bearing animals. However, information on the \textit{in vivo} efficacy of LNP formulations of anti-tumour drugs remains
limited. Most studies that included animal models focused on drug biodistribution. Serpe and co-workers [36] studied the *in vivo* efficacy of LNP formulation of SN-38 (prodrug of irinotecan) in mice model xenografted with HT-28 (human colon carcinoma cell line) tumour. *In vivo* anti-cancer studies generally include pharmacokinetics and biodistribution analyses and anti-cancer studies on tumour-induced mice models. In principle, unmodified nanocarriers and vesicular systems get rapidly cleared from the systemic circulation by the reticuloendothelial system (RES). LNP can be coated with stealth agents, such as PEG, to minimise the clearance by RES to achieve extended systemic circulation time. Using stearic acid-PEG2000, long-circulating LNP formulations of doxorubicin and paclitaxel have been formulated [23, 37]. However, many papers have reported that unmodified LNP were also able to remain in the bloodstream for long periods of time in the animal studies performed [22, 38–40]. The area-under-the-curve (AUC) values of several anti-cancer drugs obtained in pharmacokinetic studies showed that unmodified LNP were able to increase the AUC values by 3-fold up to over 20-fold in comparison to drug solutions, indicating low RES clearance. However, in the same studies, the AUC values of drugs delivered by the long-circulating LNP were approximately 0.25-fold to over threefold higher than that of unmodified formulations.

The influence of route of administration on the pharmacokinetics and drug biodistribution of the etoposide-loaded LNP was studied by Reddy and co-workers [41]. Improvements in tumoral drug accumulation were observed when the etoposide formulations were intraperitoneally or subcutaneously (SC) injected in comparison to IV injection. The SC administration even resulted in multiple-fold increases in tumour drug concentrations 24 hours postinjection. The authors suggested that the slower and progressive penetration of the nanoparticles (and the loaded drug) from the SC injection site into the tumour may result in more favourable patterns of drug distribution. Tumoral drug accumulation was confirmed in tumour-bearing animals, which served as *in vivo* tumour models [41]; nearly 67% and 30% increases in tumour drug concentrations were measured 1 and 24 hours postinjection, respectively. This is promising in terms of cancer chemotherapy. In the study by Zara and co-workers [42], duodenal administration of idarubicin-loaded LNP also led to a higher AUC than when the NLP were IV administered. These studies indicate the potentialities of LNP for targeted anti-tumour delivery of drugs by modulating the pharmacokinetics and body distribution of drugs. The results of these studies show that the route of administration is an essential factor to consider when designing animal or clinical studies of LNP for anti-tumour drug delivery.

Many studies also report the accumulation of LNP-delivered drugs in the brain. Most cytotoxic drugs do not usually accumulate in the brain tissue because of P-glycoprotein (P-gp) efflux operating in the blood-brain barrier (BBB). This barrier presents a serious challenge to chemotherapeutic treatment of central nervous system tumours. The
ability of LNP to carry drugs across the P-gp-rich BBB is consistent with the previously described findings in P-gp-overexpressing cancer cell lines. This P-gp bypassing feature of LNP may be useful for cancer chemotherapy if properly exploited.

In most of the biodistribution studies so far conducted with anthracyclines loaded in LNP [22, 25, 36, 42–45], the drug concentrations in organs with potentially high toxicological levels (e.g., heart) were significantly low. By selectively reducing the anthracycline concentration in the cardiac tissues, effective drug therapy using higher drug dosing may be recommended with lesser risk of cardiotoxicity. Overall, LNP are useful for the improvement of the pharmacokinetics and biodistribution profiles of anti-cancer drugs. Drugs administered tend to stay longer, penetrate the tumour sites better and avoid some vulnerable organs.

Basically, cytotoxic drug therapy is aimed at destroying as many cancer cells as possible and at preventing their proliferation. Therefore, it is important to ensure that the drug administered as an LNP formulation should be at least as cytotoxic to cancer cells as the same quantity of conventional free drug solution. However, most of the LNP formulations are reported to demonstrate cancer cytotoxicity comparable or even superior to the corresponding free drugs. Miglietta and co-workers [46] demonstrated improved cytotoxicity, which was retained for 72 hours, with LNP incorporating doxorubicin or paclitaxel by performing Trypan blue exclusion assays on the breast cancer cell line MCF-7 and the leukaemia cell line HL60. A similar study carried out with LNP formulations carrying doxorubicin or cholesteryl butyrate on the colorectal cancer cell line HT-29 showed that LNP formulations carrying the drugs were significantly more cytotoxic than the corresponding free drugs [36]. However, paclitaxel-loaded LNP showed cytotoxicity similar to that of the drug solution possibly due to the extremely slow release of paclitaxel from the lipid matrix [47]. Studies on the combination therapy of anti-cancer agents loaded in LNP showed synergistic activity, thus giving scope for overall dose reduction in the course of therapy. The anti-adhesive effect of cholesteryl butyrate LNP was studied by Dianzani and co-workers [48] by coincubating cholesteryl butyrate LNP with human polymorphonuclear (PMN) cells and human umbilical vein endothelial cells (HUVEC) and the results were compared with that of simple sodium butyrate. Adhesion was quantified by computerised microimaging fluorescence analysis. Both cholesteryl butyrate LNP and sodium butyrate displayed anti-adhesive effects on f-Met-Leu-Phe (FMLP)- and interleukin-1β-stimulated cells as determined by a concentration-response curve, but cholesteryl butyrate LNP were in all cases more active. Moreover, these particles inhibited FMLP-induced adhesion of PMN cells to fetal calf serum-coated plastic wells, thus showing a direct effect on PMN cells, while sodium butyrate had little effect. Confocal microscopy showed that fluorescent LNP entered PMN cells and HUVEC after 10 minutes of incubation.
MDR phenotype in cancer cells presents a significant obstacle to anti-tumour therapy at a cellular level. MDR was demonstrated when tumour cells that have been exposed to one cytotoxic agent develop cross-resistance to a broad range of structurally and functionally unrelated compounds [49, 50]. Typically, hydrophobic and amphipathic anti-cancer drugs, for example, vinca alkaloids (vincristine, vinblastine), taxanes (paclitaxel, docetaxel), epipodophyllotoxins (etoposide, teniposide), anthracyclines (doxorubicin, daunorubicin, epirubicin), topotecan and mitomycin C [51, 52], are most frequently associated with MDR, which is mediated by membrane-bound drug efflux transporters such as P-gp [53, 54]. Many approaches are worked out to reduce P-gp expression to ultimately reduce MDR, which would possibly increase the success rate of cancer chemotherapy. One approach is to use chemicals that show higher P-gp expression than the drug, and thus to restore the drug sensitivity of MDR cancer cells. Several agents, for example, verapamil, cyclosporin A, quinidine, tamoxifen, and also calmodulin antagonists, have been identified to reverse MDR resistance [52], and they were often referred as chemosensitisers. Most of these MDR reversal agents show pharmacological activity and thus have not been of practical use in clinical setting. Furthermore, due to their low affinities for P-gp, high doses of these agents are required to achieve the desired chemosensitising effect, which may result in unacceptable toxicity [55]. Many of these chemosensitisers are also substrates for other enzymes and transporters. This further leads to unpredictable pharmacokinetic interactions when used with other anti-tumour agents.

LNP have been shown to be effective in overcoming the efflux process and thus anti-cancer drugs loaded in LNP might be effective in avoiding and/or overcoming MDR. Wong and co-workers [25, 26] reported enhanced doxorubicin uptake and retention in MDR breast cancer cells using polymer-lipid hybrid nanoparticles. These hybrid nanoparticles dispersed using a nonionic block copolymer (Pluronic F68) were also reported to enhance the cytotoxicity of doxorubicin in P-gp-overexpressing breast cancer cell lines (MDA435/LCC6/MXR1). An eightfold increase in the anti-tumour activity of doxorubicin hybrid nanoparticles in comparison to free doxorubicin was shown by clonogenic assays. In addition, increased drug uptake with prolonged drug retention was observed with doxorubicin-loaded hybrid nanoparticles compared to free drug. This was further supported by fluorescence microscopy images. These findings reveal the potential of LNP for drug-resistant cancer treatment and advocate the need of encapsulated anti-cancer drug to achieve benefit in drug-resistant cancer therapy.

Blood vessels in tumours are different from normal blood vessels because they have abnormal architectures and impaired functional regulation. These abnormalities are well known, particularly their greatly enhanced permeability for macromolecules, which are retained in tumours for extended periods. This phenomenon is called the EPR effect [56]. This effect, related to the transport of macromolecular drugs
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composed of liposomes, micelles, proteinaceous or polymer-conjugated macromolecules, lipid particles and nanoparticles into the tumour, is the hallmark of solid tumour vasculature. These macromolecular species are therefore ideal for selective delivery to tumours. The EPR effect has facilitated the development of macromolecular drugs consisting of various polymer-drug conjugates (pendant type), polymeric micelles, liposomes and polymeric nanoparticles, and LNP that exhibit better therapeutic efficacy and fewer side effects than the parent low-molecular-weight compounds. As solid tumours frequently lack anti-oxidative stress enzymes, generating oxidative stress in tumour tissue may be another unique anti-cancer strategy. Most tumour cells have a weak or limited defence system against reactive oxygen species, and the oxygen radical-generating techniques that are developed are primarily endogenous. Consequently, an approach to cancer therapy based on the EPR effect and oxyradical induction in order to produce apoptosis appears promising [56].

2.4 Lipid Nanoparticles for Cancer Chemotherapeutics

Cytotoxic drugs are used in addition to other modalities (e.g., surgery, radiotherapy) to improve their effectiveness and prevent cancer recurrence. Even though these drugs treat cancers by preferentially causing death or arrest in the growth of cancer cells, their cytotoxicity is not highly specific for cancer cells. The healthy cells, particularly those that are rapidly dividing, inevitably suffer from the chemotherapeutic treatment. The major goal in cancer chemotherapy is to achieve reasonably high drug concentration in cancer tissues for effective cancer cell kill without causing damage to the healthy tissues. In addition, clinical resistance to anti-cancer drugs is a hurdle for effective cancer therapy [57, 58]. The major form of drug resistance is related to the expression of ATP-dependent membrane-associated drug transporters in cancer cells. Cancer cells overexpress these drug transporters and thus cause resistance to a broad range of structurally diverse anti-cancer compounds, a phenotype referred as MDR. Under these conditions, anti-cancer drugs are actively transported out of the cancer cells by these transporters, rendering the chemotherapy ineffective even when an adequate dose of drug is administered.

LNP have been evaluated for their anti-tumour activities, stabilities and/or biodistribution in cultured cell lines and animal models. Properties of LNP that are desirable for anti-tumour drug delivery include (i) versatility, which allows encapsulation of cytotoxic agents of diverse physicochemical properties; (ii) improved anti-cancer drug stability; (iii) improved \textit{in vitro} cytotoxicity against cancer cells; (iv) ability to load more than one drug in one carrier system; (v) activity against cancer cells that are normally refractory to chemotherapy; (vi) enhanced drug efficacy in animal models; and (vii) improved pharmacokinetics and \textit{in vivo} drug distribution.
The majority of anti-tumour agents, particularly the cytotoxic compounds, are fairly lipophilic and poorly water-soluble. However, there are several exceptions where there are also water-soluble compounds. In addition, some lipophilic molecules can also be converted into water-soluble ionic salts for their use in clinical settings than their corresponding water-insoluble free bases. The efficiency and extent of drug incorporation into LNP are strongly influenced by the partition behaviour of the drug in a lipid-in-water biphasic system. The lipophilic, uncharged anti-tumour drugs may be encapsulated with relative ease using classical LNP preparation methods, while the hydrophilic and charged compounds require new strategies to enhance their incorporation into LNP. Methods to control the release profile of these compounds from LNP are also necessary because they may rapidly diffuse from the drug carriers into the surrounding aqueous medium.

2.4.1 Delivery of Poorly Water-soluble Compounds

Several LNP have been successfully formulated for encapsulation of poorly water-soluble anti-cancer drugs. In general, these poorly water-soluble compounds partition reasonably well in the lipid phase and can be efficiently encapsulated by the conventional LNP without much modifications. However, these agents carry other problems such as poor stability and risk of precipitation. In fact, each group of drugs has its specific problems that need to be solved before the drug can be properly delivered using LNP.

The poor water solubility of camptothecin has seriously diminished its practical value. This problem was solved by loading the drug in LNP to the extent of encapsulation efficiency of 99.6% [39]. Similarly, to improve the water solubility of SN-38, LNP formulation was prepared [59]. The concentration of SN-38 in the formulation reached 1.0 mg/ml. Paclitaxel-loaded LNP were developed in an attempt to eliminate Cremophor EL (which is known to cause serious hypersensitivity reactions and nephrotoxicity in human subjects) in its present nonaqueous micellar formulation [36, 46, 47]. LNP were prepared using surfactants or stabilisers such as Pluronic F68, Brij-78 and phosphatidylcholine. Etoposide, a poorly water-soluble drug, was successfully encapsulated in LNP composed of tripalmitin to achieve an encapsulation efficiency of 98.96% and a loading capacity of 4% [41].

The anti-neoplastic activity of camptothecin is strongly correlated to the functionality of the lactone ring of the drug molecule. However, in an aqueous environment, particularly at basic pH, this lactone ring is vulnerable to hydrolysis, leading to the formation of therapeutically inactive carboxylate. This was overcome by preparing LNP that provide lipid environment to prevent hydrolytic degradation. Yang and Zhu [39] reported that the drug mostly remained in its active lactone form in the LNP.
formulation until it was released. Similarly, the lactone ring stability of irinotecan and SN-38 was also improved when delivered as LNP.

Conventional paclitaxel formulation faces the problem of precipitation on dilution with saline to bring its concentration to 0.6–1.2 mg/ml for clinical use. This requires filtration of the drug solution prior to administration. Studies have shown that the LNP formulation of paclitaxel did not exhibit this phenomenon even when it was diluted to nanomolar range using cell culture medium for in vitro cytotoxicity evaluation [36]. However, fast release of paclitaxel from a carrier system could cause precipitation due to supersaturation and this problem can be prevented by controlling its release by suitable formulation design [47]. A lipid emulsion-based formulation was also reported to overcome the precipitation problem, even though precipitation on long-term storage of these products was found difficult to avoid [60]. Stevens and co-workers [37] prepared a lipophilic paclitaxel prodrug (paclitaxel-20-carbonyl-cholesterol) to overcome this difficulty. This prodrug was able to demonstrate over 10% of drug release into bovine serum albumin in a relatively linear fashion within 2 hours. Cavalli and co-workers [47], however, showed that their LNP formulation containing the unaltered paclitaxel was autoclavable without a significant reduction in the amount of the incorporated drug and was found stable for over 18 months after lyophilisation, and the lyophilised nanoparticles appeared easily dispersible when cryoprotectant (2% trehalose) was added. Another chemically unstable drug, all-trans retinoic acid (ATRA), which has been reported to show sensitivity to heat, light and oxidation, was chemically stabilised in LNP during storage [61]. More than 90% of the encapsulated ATRA remained intact after 1 month of storage at 4 °C versus less than 60% when the drug was stored in the form of methanol solution or Tween-80 solution under the same conditions.

2.4.2 Delivery of Water-soluble Ionic Salts

Water-soluble drugs generally pose problems of poor loading in LNP due to neutralisation of their ionic charges with a counterion. This problem can simply be avoided by using the free bases of these agents. However, the use of free base compounds in many cases will lead to solubility problems as faced by lipophilic drugs, such as slow release and possibility of insufficient blood concentration levels for efficient biological action in vivo. Thus, it is generally preferred to use the water-soluble salt of an anti-cancer drug for LNP formulation using any of the following strategies:

1. Ion pair approach: Gasco and co-workers used decyl phosphate or hexadecyl phosphate to form ion pairs with water-soluble drugs (e.g., doxorubicin hydrochloride, idarubicin hydrochloride) to enhance their loading into LNP prepared using stearic acid and egg lecithin. The ion pair showed increased
lipophilicity characterised by 1000-fold increase in lipid-to-water apparent partition coefficients in the case of doxorubicin hydrochloride and 300-fold in the case of idarubicin hydrochloride. LNP prepared using these lipophilic ion pairs carried payloads of doxorubicin and idarubicin up to 7% and 8.4%, respectively. However, because of the high lipophilicity, these LNP formulations would release drugs slowly and thus could pose theoretical risk of resistance development due to chronic exposure to sublethal concentrations of cytotoxic drugs in cancer cells [23, 24, 43, 62, 63].

2. Polymeric coating of LNP: In this approach, polymers, such as dextran sulfate, may be used as counterions to prepare LNP of positively charged drugs, for example, doxorubicin hydrochloride. Wong and co-workers [64] reported the encapsulation of doxorubicin hydrochloride up to 70% in LNP in the presence of dextran sulfate versus approximately 40% in its absence. Wong and co-workers [25] used another polymer composed of hydrolysed and polymerised epoxidised soybean oil to formulate doxorubicin hydrochloride LNP. The formulation showed marginal increase in payload (6–20%); the drug release was faster compared to ion pair-based LNP, indicating the possibilities of incomplete neutralisation of charges on the polymer molecules. In addition, these residual charges might help draw the water molecules into the lipid matrix to accelerate its disintegration, leading to faster drug release.

2.4.3 Delivery of Water-soluble Nonionic Drug Molecules

Strategies of designing LNP described above are not applicable for small water-soluble nonionic hydrophilic molecules. For instance, 3′-azido-3′-deoxythymidine is a nonionic anti-viral water-soluble drug with a low molecular weight. This drug was conjugated with palmitate to increase lipophilicity for encapsulating in LNP [65]. 5-Fluorouracil (5-FU) is a similar drug and has been extensively researched for LNP encapsulation. Other drugs of similar category, for example, mitomycin C and cisplatin (also its analogue carboplatin), can also be considered as water-soluble drugs that need modification for LNP encapsulation. Wang and co-workers [38] reduced the water solubility of 5-FU by conjugating two octanoyl groups to the 5-FU molecule to obtain 3′,5′-dioctanoyl-5-fluoro-2′-deoxyuridine. This lipophilic drug derivative was loaded into LNP with an encapsulation efficiency at over 90%. 5-FU can be easily diluted and administered without the problem of drug precipitation as seen for paclitaxel. However, LNP formulations provide additional benefit of controlled release, and they possibly improve the anti-tumour activities, side-effect profiles and biodistribution patterns of the loaded drugs. Successful development of LNP formulations for this class of anti-tumour drugs can still be rewarding.
2.5 Conclusions

Chemotherapy, used either alone or in combination with other therapeutic strategies like surgery, radiation therapy and immunotherapy, is an inevitable treatment option for any type of cancer. In spite of the large number of chemotherapeutic agents available to treat cancer, these drugs may produce toxic manifestations and can cause damage to vital organs and thus require site-specific targeted delivery to tumour sites. Out of many strategies, particulate delivery systems have received wide importance due to their targeting potential, safety and in addition their capability for stabilisation of the loaded drug, long circulation, *in vivo* stability, sustained release and intracellular delivery. Biodegradable polymers and lipids have received maximum attention in designing nanoparticulate delivery systems to deliver wide varieties of cancer chemotherapeutic and diagnostic agents. Being the part of biological system, lecithins and their derivatives used in the preparation of LNP are safe, nontoxic and effective in targeting applications.

References


Lipid Nanoparticles in Cancer Therapy: Past, Present and Future


3 Multifunctional Lipid Nanocarriers for Cancer Prevention and Therapy

Mayurkumar Kalariya, Srinivas Ganta, Husain Attarwala and Mansoor Amiji

3.1 Introduction

Cancer is a group of diseases with complex aetiology, where normal cells transforms into cancerous cells involving uncontrolled growth, invasion into surrounding tissue and sometimes metastasis to other parts of the body. Despite growing understanding of cancer aetiology and advancements in cancer therapy, the disease continues to be one of the leading causes of death worldwide, with modest progress achieved in reducing the morbidity and mortality of this disease. The recent National Cancer Institute (http://www.cancer.gov/) annual report indicates that some progress has been made in reducing the cancer-related deaths in US with early detection, prevention and treatment strategies. However, the World Health Organisation (WHO) predicts cancer deaths worldwide will rise from 7.9 million during 2007 to 12 million by 2030. Many reasons have been cited for the continuous rise of cancer and failure of cancer therapy, such as chronic exposure to carcinogenic factors and diagnosis of cancer at the late stage that exhibit metastasis into body organs, thus yielding an increase in mortality rate.

3.1.1 Cancer Vaccines and Chemoprevention

An important strategy in cancer prevention is the use of prophylactic cancer vaccines, which can prevent the initiation of cancer in healthy people. Conversely, therapeutic vaccines are intended to treat cancers that have already developed. Prophylactic vaccines are designed from antigens of the infectious agents which are responsible for development of cancer [1]. These antigens are capable of inducing humoral responses and establishing cancer-specific immunologic memory in the body [2]. The US Food and Drug Administration (FDA) has approved two groups of prophylactic cancer vaccines: the first against the hepatitis B virus (Recombivax HB®, Engerix-B®, Fendrix®), which can develop liver cancer in chronically infected individuals, and the second vaccines against human papillomavirus (HPV) type 16 and 18 (Gardasil® and Cervarix®). HPV infection accounts for about 70% of all cases of cervical cancer
worldwide (http://www.cancer.gov/). Therapeutic cancer vaccines are used to treat already existing cancer by boosting the body’s natural defence to recognise and attack malignant cells. However, none of the therapeutic cancer vaccines which entered clinical trials have as yet been approved by the FDA or the European Medicines Agency (EMEA) [3]. To realise efficient cancer vaccines with broad clinical use, one of the main challenges will be bringing together the expertise that includes vaccine delivery technologies, immune adjuvants and formulation technologies.

Chemopreventive compounds are known to modify each stage of the carcinogenesis process [4, 5] by preventing carcinogens from striking their cellular targets and damaging DNA (initiation). Several mechanisms have been invoked: enhancement of carcinogen detoxification, scavenging of reactive oxygen species, modification of carcinogen uptake and metabolism and enhancement of DNA repair. Chemopreventive compounds are also seen to inhibit promotion and progression phases after the development of premalignant cells by interfering with cell cycle regulation, signal transduction, transcriptional regulation and apoptosis [6]. Accordingly, chemoprevention is considered as an effective strategy to control cancer disease. Several studies have also shown a positive correlation between consumption of a diet rich in vegetables, fruits and herbal products and lower incidence of cancers [7, 8]. The examples of chemopreventive agents are discussed in subsequent sections with an effort to address their delivery issues using nanotechnology-derived products.

### 3.1.2 Cancer Treatment Strategies

Cancer treatment relies on integration of surgical, radiation and chemotherapy approaches. The requirement of each approach depends on the type of cancer and stage of its development. Chemotherapy is the main method of treatment for only a few cancers, but it is increasingly used as an adjunct to surgery or radiation for many types of cancers [9, 10]. Currently used anti-cancer drugs mainly affect cell division and thus impact all rapidly proliferating normal tissues, such as the bone marrow, gastrointestinal tract (GIT) and hair follicles, resulting in toxic effects [9]. As a result, anticancer drugs are frequently given at suboptimal doses, risking effectiveness of therapy as well as development of multidrug resistance. Many approaches for improving the selectivity of anti-cancer drugs are being pursued at present. Novel anti-cancer drugs are being developed that interfere with specific pathways activated only in cancer cells, but not in normal cells. Towards this end, new drug molecules have been identified to interfere with select signal-transduction pathways, down-regulate proto-oncogenes that are involved in cancer-cell proliferation or suppress tumour angiogenesis. In parallel, efficient delivery of currently used anti-cancer drugs is being explored to ensure that the drug reaches its target tumour site, while sparing normal tissues in vivo.

Another recent advancement in cancer treatment is the use of gene therapy to improve the clinical outcomes. Many studies are under way to treat different types of cancers
using gene therapeutics. Some studies target normal cells to enhance their ability to fight cancer, while other studies target cancer cells, with the aim of destroying them or stopping their growth [11, 12]. Gene delivery into tumour cells or tumour-associated stroma results in stimulation of anti-cancer immune response, induction of tumour cell death, inhibition of angiogenesis and control of tumour cell growth [12]. The most common vectors that are used in gene delivery are viral vectors because of their highly efficient transfecting ability. However, nonviral vectors are gaining momentum in gene delivery, and they are being tailored to be as efficient as viral vectors but without the toxicity and immunogenicity associated with viral vectors. Various nonviral vectors (e.g., lipid- and polymer-based) have been shown to achieve efficient gene delivery and functional expression [13].

3.1.3 Delivery Challenges in Cancer Prevention and Therapy

Chemopreventive agents and small-molecule or macromolecular therapeutics must circumvent a series of physiological barriers before they reach the target site in the body and produce therapeutic outcomes. Many clinically used anti-cancer drugs do not produce enough oral bioavailability due to their poor aqueous solubility as well as affinity to efflux pumps (adenosine triphosphate-binding cassette (ABC) efflux transporters) and susceptibility to cytochrome P450-metabolising enzymes in the GIT. For instance, the potent anti-cancer drug paclitaxel shows an oral bioavailability of less than 6% [14], because of its poor aqueous solubility and metabolic interactions in the GIT [15]. On the other hand, novel anti-cancer agents discovered from high-throughput screening are receptor-specific (hits and leads) but often, molecules derived from these screens have high molecular weight and are poorly water soluble, therefore posing limitations on delivery [16]. It is estimated that around 40% of new drugs, coming directly from drug discovery each year, are poorly soluble in water. As a result, many of them exhibit a poor oral bioavailability and unfavourable pharmacokinetic profiles, and are eliminated rapidly [17]. A drug delivery strategy can be employed in such situations to rescue some of these drug candidates which are providing potential benefits in cancer therapy.

The delivery challenges are particularly important in the case of macromolecular agents, such as peptides, protein, genes, oligonucleotides and small interfering RNA (siRNA) [18]. Since there is a need for the macromolecules to enter into subcellular compartments in an effective manner to produce the required cellular functions [18, 19], it is important to design drug and gene delivery systems that protect the payload from degradation in the physiological environment, while allowing transport of drug molecules through anatomic and physiological barriers to increase their availability at the target site. Besides the delivery challenges, anti-cancer drugs can cause significant toxicity; therefore, the opportunity to preferentially deliver them to the target site will improve both the safety and efficacy of these agents. By incorporating the drug in optimised drug delivery carriers or conjugating the drugs with different polymers, for example,
polyethylene glycol (PEG) or polyethylene oxide (PEO), it is possible to modify the pharmacokinetics and biodistribution of the drugs and improve their efficacy.

3.2 Lipid-based Nanotechnology for Cancer

3.2.1 Nanotechnology Applications in Medicine

Nanotechnology is a multidisciplinary field, which covers a vast and diverse array of devices up to several hundred nanometres in size (generally <100 nm in at least one dimension) and fabricated using atomic and molecular components. Nanocarriers have a large surface to volume ratio and their physicochemical properties, such as friction and interaction with other molecules, are distinct from equivalent materials at a larger scale. Nanocarriers used as drug delivery vehicles consist of different biodegradable materials such as natural or synthetic polymers, lipids or metals and are developed by top-down or bottom-up engineering of components. The most common examples of these nanocarriers include polymeric nanoparticles, dendrimers, nanoshells, liposomes, micelles, nanoemulsions and solid lipid nanoparticles (SLN) [20]. A schematic representation of different types of nanocarriers used for drug delivery is presented in Figure 3.1. These carriers have the potential to improve the

![Figure 3.1 A schematic representation of different types of nanocarriers. Reproduced with permission from G. Orive, E. Anitua, J.L. Pedraz and D.F. Emerich, Nature Reviews Neuroscience, 2009, 10, 9, 682. ©2009, Nature Publishing Group [21](figure)](image)
therapeutic index of currently available drugs by achieving steady-state therapeutic levels over an extended period of time and reducing drug distribution to nontarget compartments. They can improve solubility and stability of potentially effective new chemical entities having poor pharmacokinetic and/or inappropriate biodistribution properties. Additionally, nanocarriers may facilitate the development of multifunctional systems for targeted drug delivery [22, 23], combined therapies [24] or systems for simultaneous therapeutic and diagnostic applications. More recently, the breakthrough potential of the cancer nanotechnology is becoming increasingly recognised with several examples of first generation nanocarriers approved by the FDA for therapy (Abraxane® [25], Doxil® [10], DaunoXome® [26]) and diagnosis (Feridex® [27]).

3.2.2 Cancer Nanomedicine

The drug delivery systems have the potential to overcome the drawbacks often encountered with chemotherapeutic agents, such as lack of selectivity, toxic side effects and a high occurrence of drug resistance [28]. Intravascularly injectable nanocarriers are a major class of systems for drug delivery in the treatment of cancer. They are envisaged for use in the targeted delivery of therapeutic agents, with a concurrent, substantial reduction of deleterious side effects. Synergistic effect of these two approaches may result in interception and containment of lesions before they reach the lethal or even the malignant phenotype with minimal or no concurrent loss of life quality.

3.2.3 Passive and Active Targeting Strategies

Drug targeting is a strategy aiming at the delivery of a compound to a particular tissue of the body. Targeting methods range from covalently linked antibodies [29, 30] to mechanisms based on the size and physical properties of the nanocarriers. Due to the enhanced permeability and retention (EPR) effect, nanocarriers can specifically accumulate anti-cancer drugs at the tumour site. Additionally, their recognition and internalisation by the target tissues can be further enhanced by surface conjugation with targeting moieties such as antibodies, peptides or nucleic acids. In general, nanocarriers consist of at least three elements: a core constituent material, a therapeutic and/or imaging payload and surface modifiers that enhance the biodistribution and delivery of the payload to the site of action [20]. A major clinical advantage sought by the use of targeted nanocarriers over simple immunoconjugated drugs is the specific delivery of large amounts of therapeutic or imaging agents per targeting biorecognition event. Figure 3.2 shows a model of passive and active tumour-targeted delivery using nanocarriers.
Passive targeting by EPR effect refers to selective extravasation and retention of long-circulating nanocarriers at the diseased site due to leaky microvasculature with lack of defining structures and enhanced permeability. Most clinically viable nanocarrier-based targeting strategies utilise the principle of passive targeting [31]. Due to the production of growth factors and cytokines, tumours and inflamed tissues in the body exhibit increased angiogenesis and vasodilatation, which allows for extravasation of passively targeted nanocarriers [32]. Depending on the tumour type, growth rates and microenvironment tumour vasculature organisation may differ; yet most solid tumours exhibit a vascular pore cut-off size between 380 and 780 nm [33]. Nanocarriers larger than 200–400 nm cannot escape through normal vasculature [34], but would extravasate through the leaky vasculature at tumour sites. Consequently, nanocarriers offer the opportunity to increase drug accumulation in the vicinity of tumour cells and significantly reduce drug distribution and associated toxicity to normal tissues. Additionally, impaired lymphatic drainage results in fluid retention within tumour tissues and high interstitial pressure. Collectively leaky microvasculature and the broken lymphatic system result in EPR effect and ‘passive’ cancer targeting due to accumulation of nanocarriers in tumour at higher concentration than in other tissues [35].

For successful passive targeting, extended blood circulation time is desired for nanocarriers to ensure their multiple passages through target site. This can be achieved by particle surface modification using hydrophilic polymers, such as PEG, to render them ‘stealth’ to opsonisation by the reticuloendothelial system (RES). For example, PEG-grafted liposomes in the 70–200 nm size range, comprising 3–7 mol%
methoxy-PEG-2000 grafted to distearoyl phosphatidylethanolamine (DSPE) or dipalmitoyl phosphatidylethanolamine demonstrated extended circulation half-lives of 15–24 hours in rodents and up to 45 hours in humans, whereas the unmodified liposomes showed half-life of only 2 hours. PEG reduces the nanocarrier surface interaction with opsonins due to the flexible hydrophilic polymer chains by steric repulsion [36]. Moreover, the use of physical energy, such as hyperthermia [37] and ultrasound [38], has been reported to increase local microvascular permeability and thereby enhance drug delivery to solid tumours and other targeted tissues. Extreme precaution is ensured while treating patients having coexisting conditions with leaky vasculature because passive distribution of these carriers may result in accumulation of therapeutics at multiple disease sites. Limitations to passive targeting with long-circulating nanocarriers include heterogeneity in tumour vasculature, elevated interstitial fluid pressure and reduced interaction with tumour cell because of PEG modification. Therefore, passive targeting effect may not be achieved in the treatment of all tumours.

Active targeting involves nanocarrier surface functionalisation using ligands that can specifically recognise tumour-specific or tumour-associated receptors overexpressed on tumour cell surface [36], thus delivering the payload exclusively to the disease site. In the nineteenth century, Paul Ehrlich proposed a concept of the ‘magic bullet’, an ideal package that would target and deliver drugs to a specific place in the body even before the concept of rational targeting ligand was introduced [39]. Active targeting relies on specific interactions between the nanocarrier surface ligands (such as antibodies, peptide mimics or nucleic acids) and tumour cell receptors (such as human epidermal growth factor receptor 2 (HER2) [40], transferrin receptors (TfR) [41], folic acid receptor [42] or vasoactive intestinal peptide receptors (VIP-R) [43]) that are overexpressed on tumour cells and would selectively increase rate and extent of drug delivery to the target [36, 44, 45]. Consequently, this approach has potential to lower the systemic toxicity of chemotherapy in the treatment of cancer. Ligand-mediated active targeting and cellular internalisation are predominantly important for therapeutics that are not easily internalised by tumour cells and require facilitation by fusion, endocytosis or other mechanisms to access their subcellular targets [46]. Whole antibodies that expose their constant regions on the liposomal surface are more susceptible to the Fc-receptor-mediated phagocytosis by the cells of the mononuclear phagocytic system (MPS). Immunogenicity is therefore an important consideration and must be addressed during targeting ligand selection [47].

Covalent and noncovalent coupling methods have been employed to conjugate targeting ligands onto the nanocarrier surface. Covalent coupling methods involve interaction between reactive group(s), such as formation of a disulfide bond, crosslinking between two primary amines using a divalent coupling agent (e.g., a dialdehyde), reaction between a carboxylic acid and primary amine or reaction between a primary amine and a free aldehyde [48]. Noncovalent binding involves physical association of
targeting ligands to the nanocarrier surface and avoids the use of rigorous, destructive reaction reagents. However, noncovalent binding may produce potential problems such as weak binding, poor control of the reaction and inappropriate orientation of the coupled ligand. When compared to drug-ligand conjugates, active targeting of nanocarriers offers several advantages, such as capability to deliver large drug payloads at the disease site, noncompromised drug activity since the ligand is attached to the nanocarrier surface, increased binding probability due to the presence of numerous ligands onto the nanocarrier surface and efficient drug distribution into the tumour interstitium. Additionally, due to its optimal size, the nanocarrier-ligand conjugate can extravasate preferentially at the disease site, thereby reducing drug and ligand toxicities to the normal tissue [32].

A number of \textit{in vitro} and \textit{in vivo} studies demonstrated that active targeting of nanocarriers can effectively increase the tumour distribution of encapsulated chemotherapeutic agents when compared to their nontargeted counterparts. For example, trastuzumab (Herceptin®)-conjugated paclitaxel-loaded PEGylated immunoliposome showed substantially higher cellular uptake than the PEGylated liposome \textit{in vitro} in cancer cells overexpressing HER2 [40]. Additionally, a pharmacokinetic study showed prolonged circulation time of paclitaxel encapsulated in PEGylated immunoliposomes as compared to commercial paclitaxel (Taxol®) in rats. Another example involving colloidal gold-labelled liposomes showed accumulation of HER2-targeted immunoliposomes within tumour cells, whereas nontargeted liposomes were predominantly confined to the extracellular matrix [49]. Similarly, the deposition of epidermal growth factor receptor (EGFR)-targeted immunoliposomes in tumour cells was reported sixfold higher than nontargeted liposomes [50]. In a different study, \textit{in vivo} breast cancer imaging using vasoactive intestinal peptide-grafted $^{99m}$Tc-HMPAO encapsulating sterically stabilised liposomes (SSL) showed higher accumulation of targeted SSL in cancer cells than nontargeted SSL [43]. A different study utilising whole-body gamma-scintigraphic imaging demonstrated two- to threefold increased accumulation of nucleosome-targeted immunoliposomes at the tumour site compared to nonspecific IgG-conjugated or plain liposomes in murine carcinoma models [51]. Consequently, these studies suggest that the accumulation achieved by targeted nanocarriers in tumour cells is superior to that of nontargeted nanocarriers following extravasation. Anti-tumour effect of intratumourally injected nontargeted nanoparticles was inferior to corresponding targeted nanoparticles, suggesting that the former cleared out from the tumour sites due to lack of cellular uptake [52]. Therefore, nanocarriers should possess both EPR effect and specific interaction with tumour cell receptors to selectively increase rate and extent of drug delivery to the site of action [31].

Extracellularly activated long-circulating nanocarriers have potential to achieve both passive and active targeting. They exploit distinct tumour cell microenvironment as a molecular signal to trigger drug release or to facilitate their cellular uptake upon
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arrival at disease site. Tumour cells produce more lactic acid due to increased glycolysis and membrane proton pump activity and secrete the acid into extracellular space. Matrix metalloproteinases (MMP) overproduction is also prominent due to its key role in angiogenesis, metastasis and extracellular environment during tumour propagation. Therefore, slightly acidic pH (pH 6.5–7.2) [53] and increased levels of proteases are unique characteristics of the extracellular microenvironment surrounding a tumour [54]. A pH-sensitive drug delivery system was designed by shielding HIV-1 Tat peptide-conjugated liposome surface with PEG using a hydrazone linker. The PEG layer disconnects because of linker hydrolysis at acidic pH (pH 5.0–6.0) rendering actively targeted liposomes as a result of surface-exposed Tat peptide. In vitro incubation of pH-responsive liposomes with NIH-3T3 murine fibroblast or U87 human glioma cells demonstrated increased cellular internalisation [55] via Tat peptide moieties compared to non-pH-responsive liposomes. In another study, PEG-modified liposomes for gene delivery were developed by linking PEG to phospholipid via an MMP-sensitive peptide. In a study involving MMP overexpressing in vitro models, the MMP-sensitive liposomes exhibited considerably superior transfection efficiency when compared to the MMP-insensitive liposomes. Moreover, in vivo administration of MMP-sensitive liposomes in mouse models showed 9–15 times increased value of area under the curve due to prolonged circulation and threefold higher transfection efficiency in the MMP overexpressing tumours than MMP-insensitive liposomes [56].

Over many years, liposomal formulations for anti-cancers continue to be refined and applied to more cancer indications [57, 58]. Liposome formulations of doxorubicin (Doxil) are now approved and used for the treatment of Kaposi’s sarcoma, breast cancer and refractory ovarian cancer. Furthermore, liposomes loaded with daunorubicin (DaunoXome), cytarabine (DepoCyt®) and amphotericin B (AmBisome®) have also been approved by FDA for a variety of cancer indications. Recently, an orphan designation was granted by the EMEA to liposomal paclitaxel for the treatment of pancreatic cancer. Human breast carcinoma cells incubated with nanoshells in vitro revealed photothermally induced cell kill on exposure to near-infrared radiation illustrating the use of remote activation as a therapeutic targeting strategy [59].

Several types of nanocarriers have been exploited for magnetic resonance imaging (MRI) contrast and in research protocols. These include gadolinium-based nanoparticles [60], iron oxide-based nanoparticles [61], multiple-mode imaging contrast agents that combine magnetic resonance with biological targeting [62] and optical detection. Self-assembling synthetic polymer-based dendrimers were used for the MRI of the lymphatic drainage in a mouse model of breast cancer [63]. This indicates that dendrimer-based contrast agents might be useful for noninvasive detection of cancer cells in lymph nodes of patients, to provide early signals of disease or information about patterns of metastatic spread. Low-density lipid nanoparticles have been used to enhance ultrasound tumour imaging [64].
3.2.4 Multifunctional Nanotechnology

Lipid-based nanocarriers can be produced to achieve a variety of useful properties, specifically, increased circulation time to allow, for example, their accumulation at the diseased site with compromised vasculature, the targeting and enhanced cellular penetration via surface-attached ligands, the \textit{in vivo} imaging due to encapsulated image contrast enhancers and/or drug release through stimulation under certain physiological conditions. These multifunctional nanocarriers engineered to combine several of these useful properties in a single system significantly enhance the efficacy of many therapeutic and diagnostic agents [65, 66]. Through a synergistic effect, multifunctional nanocarriers overcome distinct physiological barriers and deliver the therapeutic payload or image contrast enhancers to the target sites in the body. As shown in \textbf{Figure 3.3}, nanocarriers have emerged as ideal platforms to accomplish

\textbf{Figure 3.3} A conceptual model of multifunctional lipid nanocarriers for target-specific systemic delivery of drugs and/or genes and imaging contrast enhancers for image-guided therapy
multifunctionalisation due to the unique properties of nanoscale matter, the diversity of available materials and infinite design schemes; they are also ideal platforms to achieve surface modifications for targeted delivery and cellular internalisation [20]. Selection of the core material is highly dependent on the inherent and dynamic properties of the biologically active agent such as therapeutic index, lipophilicity, charge and size distribution. Similarly, while combining therapeutic agents and/or imaging/diagnostic modalities, one must consider possible interactions such as synergy, quenching (interference), enhanced toxicity or altered release kinetics. Finally, surface ligands, such as antibodies, peptide mimics or nucleic acids and tumour cell receptors, attached to the nanocarrier surface enhance the biodistribution and delivery of the payload to the site of action. These multifunctional nanocarriers encapsulating therapeutic and imaging agents facilitate online monitoring of tumour location, tumour-targeting levels, intratumoural localisation and drug release kinetics during treatment [28]. Thus, a more personalised approach in cancer therapy can be adapted on the basis of the individual patient’s status and treatment response.

3.3 Illustrative Examples in Cancer Prevention

3.3.1 Lipid Nanocarriers for Cancer Vaccines

During the late nineteenth century, William Coley first observed bacterial infection-mediated tumour regression that led to the concept of tumour regulation via innate immune system employed in modern cancer vaccines. Later, in 1982, a study by Van Pel and Boon [67–69] suggested that cancer cells are antigenic; therefore, an immune system activated against tumour epitopes can produce tumour regression. A schematic representation of the immunological response at the tumour microenvironment is shown in Figure 3.4. Additionally, memory development of these antigens can result in prevention of cancer recurrence due to immune system attack on the reencountered antigens [70, 71]. Tumour antigens can be divided into two types: (i) tumour-associated, and (ii) tumour-specific antigens. Tumour-specific antigens are exclusively expressed in tumour cells, whereas tumour-associated antigens, although present in normal cells, are overexpressed in tumour cells [72, 73].

Compared to traditional live and attenuated vaccines; novel peptide and recombinant DNA or DNA-expressed protein antigens elicit a weak immunogenic response due to lack of multiple epitopes and pathogen-specific molecular patterns that can be recognised by the immune system. However, immunogenicity of these antigens can be enhanced by coadministration with a vaccine adjuvant such as mineral salts (aluminium hydroxide and aluminium or calcium phosphate gels, otherwise known as alum), an immune stimulating complex (ISCOM), which is a cagelike structure composed of
cholesterol, phospholipids and saponins, and poly(D,L-lactide-co-glycolide) (PLGA) microparticles. An ideal adjuvant should be potent, safe, biodegradable and biocompatible. Alum is relatively safe and the only vaccine adjuvant approved by FDA; yet it can produce weak adjuvant effects for cell-mediated immunity or for protein subunit antigens. Additionally, alum-containing vaccines may stimulate immunoglobulin E (IgE) production and subsequent allergic reactions. In another approach to enhance immunogenicity, the antigen can be fused with immunostimulatory molecule that is a nonself antigen. For example, tumour-specific antigens conjugated with a toll-like receptor agonist such as CpG unmethylated oligonucleotides activated the innate immune system followed by monocyte- and macrophage-mediated proinflammatory cytokine release. Proinflammatory cytokine-induced T-cell activation by the Th1 pathway is of significant importance in the management of cancer disease progression [72, 74]. Alternatively, immunogenicity of antigens can be enhanced by incorporating them into macromolecular nanocarriers. As foreign bodies, these nanocarriers can be efficiently recognised by antigen presenting cells (APC) and thereby increase direct presentation or cross-presentation of surface-attached or encapsulated antigens followed by cascade activation leading to cellular or humoral immunity [74].

**Figure 3.4** Schematic representation of the immunological response at the tumour microenvironment. Reproduced with permission from B. Goldman and L. DeFrancesco, *Nature Biotechnology*, 2009, 27, 2, 129. ©2009, Nature Publishing Group [70]
Recently, several lipid-based nanocarriers have been developed for efficient antigen delivery to APC followed by immune activation, thereby finding use in cancer prevention and/or treatment. Particulate nanocarriers that can act as vaccine adjuvant include emulsions, liposomes and virosomes.

Encapsulation of an immunostimulatory molecule in a delivery system enhances its immune response and lowers systemic side effects when targeted to the site of action [75]. Liposomes may act as adjuvant and delivery vehicle for cancer vaccines [74]. Lectin-bearing polymerised liposomes demonstrated preferential uptake by the primary target site for oral vaccine delivery, such as M cells of Peyer’s patches and organised lymphoid tissue lining in the small intestine [76]. A liposomal vaccine encapsulating an immuno-adjuvant – monophosphoryl lipid A and an antigen – MUC1 (mucinous carcinoma-associated glycoprotein) overexpressed on non-small cell lung cancer cells was efficacious and prolonged the patient survival time in phase II clinical trials. Additionally, the vaccine was found to be safe over long-term use [77, 78]. Fusogenic liposomes prepared by fusion of conventional liposomes with inactivated Sendai virus can deliver the encapsulated payload directly inside the cell cytoplasm by a fusion mechanism as opposed to endosomal uptake of conventional liposomes. Cytoplasmic delivery of antigen can lead to the development of major histocompatibility complex class I restricted cytotoxic T-cell response [79]. Therefore, the liposomes can serve as efficient nanocarriers for the delivery of cancer antigens. ISCOM are immunostimulatory complexes about 40 nm in diameter containing spherical micellar assemblies [80] made up of saponins, cholesterol, phospholipids and an immunogen (usually protein). They induce a wide range of antigen-mediated immune responses, for example, production of antibodies, CD8+ T cells and cytotoxic T cells, which can be a major concern for the development of effective cancer vaccines [81]. ISCOMs demonstrated successful cancer prevention in animal tumour models of EL4 erythema-expressing ovalbumin (OVA), B16-OVA, B16-NY-ESO-1 and Lewis lung-OVA [82].

3.3.2 Lipid Nanocarriers for Cancer Prevention

Chemoprevention refers to the use of chemical compounds for prophylactic use or applied at an early precancerous stage to prevent tumour formation. The strategy requires prolonged administration of the drug and one of the challenges is to ensure that these chemopreventive drugs do not present overt toxicity. Clinical trials involving prolonged use of hormone inhibitors, for example, tamoxifen, have shown an increased risk of cardiovascular toxicity in a small group of individuals [83]. Chemopreventive drugs encapsulated in nanocarriers can be delivered preferentially at the target site, thereby creating increased drug concentration at the site of action and lowered systemic exposure. Additionally, lipid nanocarriers can promote drug absorption through the GIT. Natural chemopreventive drugs, such as the phenolic natural product curcumin,
exhibited chemoprotective property to prevent generation of azoxymethane-mediated colon tumour and phorbol ester-mediated skin tumour through various mechanisms, for example, suppression of protein kinase C and EGFR and activation of nuclear factor kappa B (NF-κB) [84, 85]. Clinical efficacy of curcumin administered in the free form is limited because of poor absorption through the GIT [86]. Liposomal delivery of curcumin demonstrated a comparable or higher efficacy than oxaliplatin for induction of apoptosis and inhibition of angiogenesis in vitro in human colorectal cell lines and in vivo in animal models of colorectal cancer xenografts [86].

In another example, both stability and intracellular delivery of resveratrol, a phenolic compound found in extract from grapes, were increased by loading into SLN [87]. The cytostatic effect of resveratrol was more pronounced when administered in SLN, as compared to the same dose administered from a conventional drug solution. Therefore, the delivery of resveratrol by SLN contributed to the drug effectiveness on decreasing cell proliferation, with potential benefits for prevention of skin cancer. Diallyl sulfide obtained from garlic extracts possesses chemopreventive property that arrests cell cycle in human colon cancer cells at G2/M phase [88]. Encapsulated diallyl sulfide-loaded pH-sensitive liposomes offered maximum cancer-protective efficacy when compared to the drug in solution due to their ability to deliver the payload inside the cell cytoplasm via a pH-dependent endosomal destabilisation of the delivery vehicle and release of the payload [89]. Tamoxifen is nonsteroidal oestrogen antagonist used in the treatment of breast cancer. Different clinical trials confirmed the preventive role of tamoxifen in oestrogen-positive breast cancers [90–92]. Cardiovascular toxicity attributed to the prolonged clinical use of tamoxifen [83] can be reduced by targeted delivery at the site of action. Nanocarrier-mediated delivery of tamoxifen can increase efficacy and reduce side effects associated with tamoxifen therapy. Additionally, tamoxifen-encapsulated nanoemulsion produced increased inhibition of cell proliferation and induction of cellular apoptosis compared to tamoxifen suspension [93]. In another study, liposomal delivery of tamoxifen demonstrated enhanced efficacy in vivo in tamoxifen-resistant breast cancer mouse model [94].

3.4 Illustrative Examples in Cancer Therapy

3.4.1 Multifunctional Liposomal Formulations

Multifunctional liposomal delivery system, anchored with a cell-penetrating peptide and a pH-sensitive PEG-shield, was prepared to enhance systemic circulation and cellular uptake through site-specific exposure of cell-penetrating peptide (e.g., HIV Tat-1 peptide) [95]. Peptide-conjugated liposome surface was shielded using PEG linked to phosphatidylethanolamine via a hydrazone linker. Due to the EPR effect, PEGylated liposomes accumulate in targets and at the tumour microenvironment, the pH 5.0–6.5 hydrazone linker suffers hydrolysis releasing the PEG chains. Consequently, because of
the surface-exposed Tat peptide, the actively targeted liposomes enhance the cellular uptake. Intratumoural administration of these pH-responsive construct demonstrated enhanced green fluorescent protein (GFP) transfection efficiency compared to the pH nonsensitive counterpart. Another targeted delivery system, quantum dot-conjugated immunoliposomes (QD-IL)-encapsulated doxorubicin was developed to combine imaging and therapeutic modalities in a single macromolecular construct [96]. QD-IL prepared by insertion of anti-HER2 single-chain variable fragment (scFv) exhibited increased receptor-mediated endocytosis in HER2-overexpressing SK-BR-3 and MCF-7/HER2 cells compared to nontargeted quantum dot-conjugated liposomes. Similarly, doxorubicin-loaded QD-IL demonstrated enhanced cytotoxicity when compared to QD-IL without chemotherapeutic payload. In addition, localisation of QD-IL at tumour sites was confirmed by in vivo fluorescence imaging in MCF-7/HER2 xenograft models. In another example, 111In-radiolabelled PEG-liposome surface was modified with the nucleosome-specific monoclonal antibody 2C5 by postinsertion technique [97]. The tumour-targeted liposome biodistribution and accumulation in various mice tumours were evaluated in vivo using gamma scintigraphy. The 2C5 antibody-modified long-circulating tumour-targeted liposomes (2C5-LCL) showed a three- to eightfold increase in specific cell binding, in vitro to various cancer cell lines of diverse origin. In vivo administration of 111In-radiolabelled 2C5-LCL (111In-2C5-LCL) demonstrated prolonged circulation and twofold increase in tumour accumulation. Whole-body imaging of tumour-bearing mice model revealed significantly faster and superior in vivo tumour visualisation with 111In-2C5-LCL than 2C5-free nontargeted liposomes in tested tumour models. Additionally, doxorubicin-loaded 111In-2C5-LCL demonstrated significantly enhanced tumour accumulation, tumour growth inhibition and suppression of metastatic processes in Lewis lung carcinoma [51].

3.4.2 Multifunctional Nanoemulsion Formulations

Nanoemulsions are increasingly investigated for the delivery of hydrophobic drugs to improve their bioavailability or make their administration possible. Cancer therapy can significantly benefit from simultaneous imaging and targeted drug delivery. Therefore, nanoemulsion formulations are being developed as a multimodality platform for tumour targeting and imaging. For example, paclitaxel in a radiocontrast gadolinium ion (Gd³⁺) nanoemulsion was developed for simultaneous MRI and site-specific drug delivery [98]. Pine nut oil containing about 20% γ-linoleic acid (GLA) droplets were stabilised with phosphatidylethanolamine-conjugated diethylenetriaminepentaacetic acid that provides high affinity for specific metal ions and lipophilic core to dissolve the hydrophobic anti-cancer drug. In vitro study demonstrated efficient drug delivery to the tumour cells combined with lowered magnetic relaxation time, thus multifunctional capability of the delivery system. In another example, hydrophobically coated iron oxide particles with surface-attached near-infrared fluorophore were incorporated
in the dispersed phase of oil-in-water (o/w) nanoemulsion [99]. This nanocomposite exhibited simultaneous magnetic resonance and optical properties due to iron oxide and infrared fluorophore respectively; therefore, it has potential to be used as a contrast agent for multimode molecular imaging. Magnetic resonance and fluorescence imaging demonstrated their accumulation in subcutaneous human tumours in nude mice in vivo, and with Perl’s staining of histological tumour sections ex vivo. Moreover, the lipid monolayer-surrounded perfluorocarbon core can be functionalised with targeting ligands and therefore lipophilic drugs contained within nanoemulsions can be delivered to target cells. In addition, liquid perfluorocarbon nanoemulsions enhance the cellular MRI signal due to the high magnetic resonance sensitivity of the $^{19}$F isotope [100]. A similar nanoemulsion system, containing perfluorocarbon to enhance MRI contrast and quantum dot nanoparticles to enable optical imaging, was developed to study cell and small-animal molecular imaging in biology and medicine.

3.5 Illustrative Examples of Lipid Nanocarriers for Nucleic Acid Delivery

Nucleic acid-based therapies such as plasmid DNA, oligonucleotides and siRNA have extensively been investigated for hereditary disorders, cancer, infectious diseases, cardiovascular disease, neurodegenerative diseases, wound healing and tissue engineering [101]. However, free nucleic acid fragments and constructs are swiftly removed from the blood circulation after systemic administration. In addition, the anatomical and physiological barriers restrict the entry of these molecules into the target extracellular or intracellular compartments [102, 103]. Intracellular distribution is particularly important for nucleic acid therapeutics, since their site of action is located in the intracellular compartments [18], but these are again subjected to degradation by the endolysosomal enzymes or nuclease while delivering them into the cytoplasm or the nucleus [104]. Lipid systems have demonstrated transfection ability in both in vitro and in vivo models [105–109]. The mechanism of internalisation of these nanocarriers is either by nonspecific or receptor-mediated cellular uptake [19]. Cationic nanocarriers can destabilise the endosomal membrane after endocytosis by target cells, resulting in safer release of the active content into the cytoplasm [107, 108, 110]. The specific cellular uptake occurs through recognition of the targeting ligand with cell surface receptors triggering endocytosis of the nanocarrier-receptor complex.

Different types of liposomes for gene delivery are explored, such as anionic liposomes, cationic liposomes, pH-sensitive liposomes and redox-responsive liposomes. Cationic lipid-based liposomes are the most promising carriers in gene delivery. They can complex with negatively charged plasmid DNA via electrostatic interactions, resulting in the formation of liposome-DNA complexes (lipoplexes)
that present biocompatibility, low toxicity potential and the option of scale-up for clinical applications [111]. This complex is not firmly formed and it can release DNA from cationic lipid in the cytoplasm, which is critical to the successful delivery of DNA into the cell [112]. Despite few clinical trials underway, the in vivo stability concerns have not yet been fully resolved for these systems. For instance, the cationic liposome-DNA complexes form aggregates with blood components and these structures can block the lung capillaries [113]. It has been shown that PEG modification of cationic liposomes increased the physical stability of formulations and also improved their transfection efficiencies [114]. The transfection ability also depends on the type of lipid used for their production. For instance, 2,3-dioleoyl oxy N-[2-(spermine carboxamido)ethyl]-N,N-dimethyl-1-propanaminium (DOSPA) and dioctadecyl amidoglycil spermine (DOGS) had shown better transfection results than N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) due to their multivalent head groups [115]. In addition, the linkage between the hydrophobic alkyl side chains, the phospholipid head group and the structure of the hydrophobic moieties (saturated, unsaturated or cholesterol anchors) affects the successful transfer of the gene molecules into the cells [116]. The helper lipids also play an important role in a successful transfection. The most commonly used helper lipid in lipofection is dioleoylphosphatidylethanolamine (DOPE), which can form the inverted hexagonal structure in liposome, making free the DNA from lipoplexes and releasing them into the cytoplasm [117].

The pH-sensitive liposomes have shown good potential in gene delivery. These can be prepared to achieve pH sensitivity using pH stimuli-responsive components. pH-responsive liposomes can be endocytosed in intact form and they fuse with the endovascular membrane, where they undergo phase transitions in response to acidic pH, resulting in liposomal destabilisation and release of their content into the cytoplasm [111]. These systems are composed of polymorphic lipids, such as unsaturated phosphatidylethanolamine with amphiphilic head groups, acting as stabilisers at neutral pH [118]. Amphiphilic groups are protonated in acidic environment, causing destabilisation of the liposomal bilayer, thus allowing release of the liposomal contents [119]. These systems have been effectively applied for the in vitro cytoplasmic delivery of anti-tumour drugs, proteins, antigens, antisense oligonucleotides and plasmid DNA [120, 121]. Liposomes made with targeting potential (folate- or transferrin-anchored) and pH responsiveness showed improved cytosolic drug delivery [122].

Another strategy with liposomal gene delivery exploits the redox potential that exists at tumour cells because of 100–1000-fold higher intracellular glutathione levels [18]. In this approach, liposomes fabricated from disulfide (–S–S–) crosslinked lipids can undergo disruption in intracellular reducing environment, causing release of its payload at intracellular target sites [18]. Redox-responsive liposomes are fabricated with
phospholipids, the hydrophobic and hydrophilic parts of a small lipid portion of which are linked through a –S–S– bond. These systems show good stability while in systemic circulation, but upon reaching a reducing environment, the liposomal membrane destabilises through the cleavage of the –S–S– bond, with subsequent release of its contents at target sites [123]. For instance, redox-stimuli liposomes prepared using thio-cholesterol-based cationic lipids showed DNA release in a reducing environment [124].

Cationic lipid-based emulsions composed of natural oil (soybean oil, linseed oil or squalene) and cationic phospholipids have been investigated for gene delivery. Studies have shown that the stability and transfection ability of cationic lipid emulsions were significantly enhanced in vivo, compared to those of cationic liposomes [108]. For instance, a cationic lipid emulsion containing an insulin expressing plasmid DNA was intravenously injected into diabetic mice, resulting in decreased blood glucose levels for 7 days, showing the potential of cationic lipid emulsions as efficient gene carriers for treatment of type 1 diabetes mellitus [125]. SLN have also shown transfection ability both in vitro and in vivo [106]. A recent study showed that the SLN composed of Precirol® ATO 5, DOTAP and Tween-80 and complexed with plasmid DNA led to enhanced GFP in hepatic tissue and spleen [106].

3.6 Conclusions

This chapter has discussed some of the different lipid-based nanomedicine platforms that are being investigated for cancer prevention and therapy. These platforms include liposomes, SLN and nanoemulsions as versatile delivery vehicles. The surface of lipid nanocarriers can be modified for active or passive targeting of the specific tissue or disease site. Additionally, compositions of these systems can be tailored in order to stimulate drug release in the desired physiologic environment. Moreover, multifunctionalisation of nanocarriers provides the opportunity for strategic delivery of drugs, genes and combinations with contrast enhancers for simultaneous in vivo imaging. With illustrative examples from contemporary literature, nanocarriers are used in delivery of vaccines and chemopreventive strategies, as well as therapeutic platforms and nucleic acid constructs in effective clinical cancer management. The results show that multifunctional lipid-based nanocarriers, made with judicious selection of biocompatible materials, can serve an important role in advancing cancer prevention and treatment strategies from bench to bedside.

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4 Targeting Solid Lipid Nanoparticles for Drug Delivery in Cancer Therapy

Kamalinder K. Singh and Renuka T. Gupta

4.1 Introduction

The human body contains an amazing complex of millions of cells that experience several transformation processes daily. Every cell is programmed to undergo a series of successive steps or phases of the cell cycle in order to successfully reproduce into two normal cells. During the cell cycle, each cell is encoded to synthesise proteins and enzymes needed to promote its growth, then replicate and divide its set of chromosomes. On successful completion of the cycle, a cell produces two completely new cells, which further multiply to produce four new cells, these four cells produce eight cells, and so on. For reasons not evident, a cell that spends too much or too little time in a phase might not be successful in completing the job of that phase; for example, it may produce excess proteins or inadequate amounts of enzymes. This abnormal cell continues its journey further along the cell cycle, producing two new abnormal cells at the end. These two cells then replicate to generate four abnormal cells, four cells divide into eight cells, continuing further till a mass of abnormal cells is produced. This marks the beginning of cancer.

Cancer is defined as a disorder wherein malfunctioning at any step in a cell’s life process results in three distinct major changes not associated with normal cells [1]. These three unique traits, namely, abnormal proliferation, uncontrolled differentiation and capability to migrate from their original site to distant organs throughout the body, a process known as ‘metastasis’, is a trademark of all cancers. During metastasis, cancerous cells break through the innumerable barriers within the body and are able to travel through the body’s circulatory system and invade other organs. Metastasis leads to formation of newer colonies in vital organs not primarily affected by the disease, giving rise to secondary tumours and destroying surrounding tissue [1, 2].

4.2 Chemotherapy and Its Limitations

Chemotherapeutic drugs or anti-cancer drugs act on DNA of the cells and alter their ability to reproduce by arresting their growth and multiplication. They are often used alone, or in conjunction with radiation therapy or surgery, and are
administered via intravenous (IV) or peroral route. Since anti-cancer drugs act on dividing cells, all cells in various phases of the cell cycle are vulnerable to chemotherapy. This lack of selectivity towards cancer cells coupled with indiscriminate action, even on normal unaffected cells, is the cause of the very serious toxic side effects observed after administration. As a result, cells that are actively dividing such as cells of the bone marrow, cells lining the mouth and cells of the gastric and intestinal mucosa are greatly affected after a chemotherapy session. In order to withstand the rigors of chemotherapy, these drugs are usually administered in cycles, which are repeated over a specific period of time. A chemotherapy schedule is designed depending upon the type and stage of cancer and the specific action of the drug being administered. Since normal cells are capable of repairing themselves following chemotherapy, sufficient time is accounted for their recovery in between treatments [3].

4.2.1 Need for Targeted Drug Delivery Systems

In spite of the most stringent chemotherapy schedules, the threat of metastasis lingers. Metastasis cannot be treated even by surgery and the process begins even before cancer can be detected clinically [1].

Targeted therapies offer a major significant advantage over chemotherapy by being more selective towards cancerous cells [4, 5]. Selective targeting of potent anti-cancer drugs can lead to a dramatic enhancement of therapeutic efficacy, simultaneously reducing toxic side effects on normal cells in the body. Target-oriented systems can be tailored for the delivery of chemotherapeutic agents, either alone or in combination, to control metastasis while not affecting the physiology of normal cells. Encapsulation of an anti-cancer drug into a suitable carrier system (nanocarrier) can significantly protect sensitive tissues from drug toxicity. Most of the cancer cells have innumerable cell surface receptors that are significantly different from those present on normal cells. The targeted therapies take advantage of these differences in the normal and cancerous cells to preferentially attack cancer cells, thereby significantly reducing the toxicity threshold of these anti-cancer agents to normal tissues.

4.2.2 Particulate Carriers for Anti-cancer Drug Delivery

Targeted drug delivery systems involve the use of a suitable carrier system. The nanocarrier that is devised for drug delivery should possess good stability and should be biodegradable and nontoxic. The drug should be physically associated with the carrier and its release should be by either diffusion or matrix degradation. Limitations for using particulate carrier systems include long circulation times, phagocytosis by macrophages leading to localisation in the liver and spleen and risk of embolism [6–8].
Nanocarriers are increasingly being used as particulate carriers in cancer therapy. These carriers consist of small particulate carriers in the range of 100–400 nm. In order to target these carriers to cancerous cells, it is important to understand tumour cell biology.

As described in Section 4.1, tumour cells possess characteristics very different from the normal cells. As shown in Figure 4.1, the tumour tissue possesses abnormally increased permeability and leaky vasculature (EPR effect). Active cellular targeting can be reached by modification of the SLN surface with cell-specific recognition and binding ligands. The modified SLN attach externally to the tumour cell-specific receptors, are recognised by these cell-specific receptors and undergo phagocytosis. Once inside the cancer cell, SLN release the encapsulated drug moiety into the cell or may release drug extraneously into the tumour tissue leading to high drug concentration within the target cancer cells.

excessive leaky blood vessels unlike the normal cells, which have a tightly bound vasculature. The leaky endothelium serves as a perfect platform for the nanoparticle to extravasate or escape into the tumour vasculature (passive tissue targeting) at a higher rate as compared to the normal tissues [2, 3]. Also, an improperly organised lymphatic drainage system from the tumour tissue into the exterior leads to greater retention of the particulates within the tumour mass and a prolonged retention of the nanoparticles, enhancing the slow, active targeting of the encapsulated drug moiety or moieties into the surrounding cancerous cells. This effect, dubbed as the enhanced permeability and retention (EPR) effect, is largely responsible for the higher rate of targeting to cancerous cells observed with nanoparticulate carriers [4, 5, 7–10].

Solid lipid nanoparticles (SLN) are of particular importance, and will be discussed in this chapter.

### 4.3 Solid Lipid Nanoparticles

SLN are a type of carrier systems that are increasingly being investigated as colloidal particulate carriers since the early 1990s. SLN are colloidal particles having particle size in the nanometre range (below 1000 nm) [5]. These nanoparticles are made from solid lipids that are liquid on heating and solidify at room and body temperature.

Although nanoparticles were investigated as carriers for anti-cancer drugs as early as the 1980s, the use of SLN as effective colloidal carriers for anti-cancer therapy began only in the early 2000. The presence of a solid hydrophobic core as against the liquid core in lipid emulsions offers an increased physical stability and also protects incorporated drugs from degradation. SLN are able to provide controlled drug release by drug diffusion or matrix degradation; they are biocompatible and well tolerated; and their surface properties can be modified to offer site-specific targeting. Although limitations such as insufficient drug-loading capacity, drug expulsion on storage and presence of high water content in the dispersions pose challenges to the formulator, SLN are nontoxic and are easy to prepare and scale-up, and efforts are being directed to overcome some of the formulation challenges.

A number of reviews on SLN detailing their production, characterisation, sterilisation and applications by Muller and co-workers [11–15], Mehnert and co-workers [13–16] and Westesen and co-workers [17–21] are available in the literature. These groups have done considerable work in the area involving the use of SLN as an alternative colloidal carrier system for drug delivery.

In recent years, reviews involving the use of SLN as carrier systems for anti-cancer drugs can be obtained from the literature [22–44]. These reviews cover the potential
use of SLN as possible carriers for anti-cancer drugs in general [22–33], and specifically for the treatment of skin [34, 35], breast and prostate cancer [36]. These reviews highlight the specific use of SLN for oral [37], parenteral, dermal/transdermal and ocular [35] and pulmonary [39] routes of administration of anti-cancer agents. Applications of SLN in gene therapy [40, 41], as drug delivery system for peptides and proteins [42] and in medical imaging [43] and diagnosis [44] for use in cancer treatment are also reviewed.

This chapter summarises the extensive research work that has been done and is currently under way to incorporate anti-cancer drugs, which are hydrophobic and/or hydrophilic in nature. It also discusses modifications that have been undertaken to improve the encapsulation efficiency, release characteristics, multidrug resistance (MDR), cell uptake and targetability of SLN by novel surface modifications with a special mention of modifications undertaken to drive SLN as potential candidates for brain targeting. The chapter concludes by raising concerns about the toxicity issues that may arise as a result of using SLN as drug carrier vehicles.

4.3.1 Encapsulation of Hydrophobic Drugs

Incorporation of hydrophobic drugs into SLN is comparatively simpler than incorporating hydrophilic drug molecules because of the lipid nature of the carrier being used. It is possible to encapsulate a hydrophobic moiety into the hydrophobic core and form a uniformly dispersed melt with the lipid. However, during homogenisation, it is quite possible that the drug gets expelled out of the lipid phase. The chemistry of bonding between the lipid and the drug depends largely on the nature of both the drug and the lipid, which gives rise to differences in the encapsulation efficiency and subsequently drug loading onto the lipid.

SLN have successfully been prepared, characterised and evaluated to incorporate a number of poorly soluble, highly unstable and toxic anti-cancer moieties. The type and concentration of emulsifiers, concentration of drug and homogenisation pressure during the homogenisation process critically affect the particle size and need to be optimised as demonstrated by Yang and Zhu [45] for camptothecin SLN (CA-SLN). The optimised CA-SLN were monodisperse and negatively charged, with an average diameter of about 200 nm. These optimised SLN, freeze dried using 10% of mannitol and 5% of glucose as cryoprotectants, could be reconstituted in distilled water within 5 minutes without change in nanoparticle size. It was found that camptothecin (CA) might exist in an amorphous state in SLN. In vitro release studies showed sustained drug release for up to a week and the released CA was found to quickly change to an open carboxylate form in phosphate buffer at biological pH. The results thus indicated the potential applicability of SLN as vehicles for sustained-release delivery of camptothecin (CA) and similar lipophilic drugs.
Nimesulide is a nonsteroidal anti-inflammatory drug with anti-tumour effect and low solubility in water. Four different samples of SLN of nimesulide using palmitic acid, stearic acid, Compritol 888 ATO and mixture of Compritol 888 ATO and Miglyol 812 (20%) as lipids have been reported to possess colloidal size ranging from 85 to 132 nm, negative zeta potential values, good loading capacity and sustained release for up to 14 hours with the best results obtained for Compritol 888 ATO SLN. *In vitro* anti-tumour activity on HT39 and SW480 cell lines demonstrated comparable activity to that of the free drug, suggesting the applicability of SLN as potential carriers for insoluble anti-cancer drugs such as nimesulide [46].

Podophyllotoxin-loaded SLN were evaluated for their anti-tumour properties on 293T and HeLa cell lines and were found to be less toxic to the cells and more effective in anti-tumour potency compared to unconjugated drug. Thus, incorporation of podophyllotoxin into SLN demonstrated potential application as an alternative carrier for anti-tumour drugs [47]. Etoposide is a poorly water-soluble podophyllotoxin analogue. Tumouricidal effects after single-dose intraperitoneal (IP) administration of three formulations of etoposide SLN in Dalton’s lymphoma showed highest survival time of mice with tripalmitin etoposide SLN followed by glycerol monostearate and glycerol distearate etoposide SLN. Thus, the nature of the lipid was found to influence the apoptotic induction property, resulting in an increase in the survival time of tumour-bearing mice in the case of tripalmitin compared with the other two lipids [48]. It was observed that the route of administration also influences tumour uptake and affects tumour regression. Free etoposide and tripalmitin etoposide SLN were radiolabelled with technetium-99m (Tc-99m) and injected by subcutaneous (SC), IV or IP route. The tumour uptake of drug-loaded SLN was significantly high (59-fold higher than with IV and 8-fold higher than with IP 24 hours post-injection) after SC injection (p < 0.001) as compared with the other two routes of administration. The SC injection of etoposide SLN led to slower and progressive penetration from the site of administration into the tumour, resulting in several-fold enhanced tumour uptake [49].

Stearic acid (StA)-based methotrexate SLN (MTX-SLN) showed prolonged half-life (t\(_{1/2}\)) and mean residence time (MRT) as compared to MTX solution. *In vivo* studies in mice bearing Ehrlich ascites carcinoma showed an increased lifespan when treated with MTX-SLN [50]. MTX-SLN prepared using hot microemulsion congealing technique has been studied for topical treatment of psoriasis. A Taguchi orthogonal experimental design was used to establish optimum combination of lipid, drug:l lipid molar ratio, concentration of surfactant and type of cosurfactant to achieve maximum loading of drug and desired particle size. It was found that the type of lipid, drug: lipid molar ratio and concentration of surfactant were critical variables influencing particle size and drug loading (p < 0.05). However, the concentration of cosurfactant was a noninfluencing parameter. The optimised MTX-loaded SLN were smooth and spherical with an average diameter of about 123 nm and encapsulation efficiency of
52.16% and further incorporated in Carbopol 934P gel base. *In vitro* skin deposition studies on human cadaver skin showed significantly higher (p < 0.05) deposition of MTX from the SLN gel as compared to the conventional gel. Double-blind clinical studies of potential batches conducted on 24 mild-to-moderate psoriasis patients demonstrated improvement in the therapeutic response (p < 0.01) at all the evaluation time points with a subsequent reduction in local side effects, suggesting a significant improvement in the therapeutic index in the treatment of psoriasis by MTX-loaded SLN incorporated in gel base over the plain commercial drug gel [51].

MTX-SLN were prepared in our laboratory using tristearin as the solid lipid and were found to be stable for up to 12 months [52]. An *in vitro* [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) chemosensitivity assay, a colorimetric assay that measures the reduction in the metabolic activity of mitochondria, was conducted on B16F10 melanoma cell lines and cell survival was measured 24 hours after exposure. The half-maximal inhibitory concentration (IC$_{50}$) for commercial MTX injection (MTX-INJ) and MTX-SLN was found to be 0.025 and 0.003125 μg/ml respectively, that is, IC$_{50}$ values of MTX-SLN were eightfold lower than those of the commercial formulation. A similar trend was observed in colony formation assay and wound healing assay in MTX-SLN as compared to MTX-INJ. The nanosized lipid particles containing MTX were effective at a lower concentration than the commercial injection. This suggested improved efficacy of MTX encapsulated in lipid nanoparticles, resulting in a higher uptake of the lipid particulate carrier by melanoma cells.

*In vivo* tissue distribution studies were conducted in Wistar rats by continuous IV infusion over a period of 30 minutes. The area under the drug concentration–time curve AUC$_{0}^{24}$ for MTX-SLN was significantly higher (almost 2 times) than the AUC$_{0}^{24}$ obtained for MTX-INJ, suggesting higher bioavailability of the test formulation as compared to the commercial injection. MTX drug levels in lungs in the case of MTX-SLN were consistently higher than the levels observed with MTX-INJ over the period of 24 hours. AUC$_{0}^{24}$ and mean residence time (MRT) of MTX-SLN in lungs were significantly higher (p < 0.05) than the respective AUC$_{0}^{24}$ and MRT of MTX-INJ. Following MTX-INJ, peak levels of the drug were attained slowly at 2 hours in the brain and the levels declined gradually in approximately 8 hours, while in the case of ovary, the C$_{max}$ was reached immediately with a rapid decline within 1 hour. In the case of MTX-SLN, peak levels of the drug were achieved within 1 and 2 hours in the brain and ovary, respectively, and the levels were maintained for almost 12 hours. Thus, MTX-SLN demonstrated the ability to act as a controlled-release carrier system for drug delivery to brain and ovary.

MTX-SLN on oral administration were found to undergo extensive enterohepatic recycling in plasma and other tissues like lungs, liver, spleen, brain, ovary, heart and kidneys. MTX-SLN were found to concentrate in highest amounts in lungs as
compared to commercial MTX tablets, indicating their potential in the treatment of lung cancer, by both parenteral and peroral routes of administration [52].

Tamoxifen citrate is a nonsteroidal anti-oestrogen used in breast cancer therapy. Tamoxifen citrate-loaded SLN were prepared by emulsification and high-pressure homogenisation technique using tristearin, tripalmitin and glyceryl behenate as the solid lipids. Tristearin SLN showed about 3.5-fold ($p < 0.001$) and 3-fold ($p < 0.001$) higher $t_{1/2}$ and MRT in plasma, respectively, than the free tamoxifen, indicating the potential of SLN as a long-circulating system in blood with feasibility to deliver tamoxifen to cancerous tissues through the EPR effect [53]. Fontana and co-workers [54] have reported the preparation of tamoxifen citrate SLN by microemulsion and precipitation techniques. Preliminary study in vitro on MCF-7 breast cancer cell lines demonstrated anti-tumoural activity comparable to the free drug.

Paclitaxel (PTX)-loaded glyceryl monostearate [55] and glyceryl palmitostearate [56] nanoparticles have been investigated for their anti-proliferative activity on B16F10 cell lines and the anti-proliferative activity of PTX was not found to be affected by encapsulation.

Design and characterisation of SLN containing PTX fabricated by a modified solvent injection technique using StA as lipid and stabilised by a mixture of surfactants, for the oral delivery of PTX, devoid of the side effects of Cremophor® EL, have been reported [57]. The in vitro cytotoxicity assay confirmed that PTX entrapped in SLN showed higher cytotoxicity against cultured hepatocellular carcinoma cells than PTX alone.

In vitro study of two types of long-circulating StA SLN containing PTX showed very slow drug release profile and the in vivo study demonstrated prolonged circulation times [58].

PTX-loaded tristearin SLN (PTX-SLN) were prepared in our laboratory and the in vitro antimetastatic studies were conducted on B16F10 melanoma cell lines [52]. The $IC_{50}$ for commercial PTX injection (PTX-INJ) was 0.008 μg/ml whereas the $IC_{50}$ for PTX-SLN was 0.03125 μg/ml after 24 hours exposure. These results are in contrast to our earlier observation with MTX-SLN where the $IC_{50}$ of MTX-SLN was reduced by 8 times as compared with the $IC_{50}$ of commercial formulation. This could be due to two possible reasons: first, the lipophilic nature of PTX probably makes the drug more difficult to diffuse out of the lipid matrix and be available for action on the melanoma cells; and second, the presence of Cremophor EL in the commercial injection could be responsible for its higher toxicity to melanoma cells.

In vivo pharmacokinetics of PTX-SLN was evaluated by continuous IV infusion in Wistar rats for a period of 45 minutes [52]. Both PTX-INJ and PTX-SLN showed
biphasic decline in plasma concentration at all time points, a characteristic of PTX, but the $C_{\text{max}}$ was markedly different ($p < 0.05$). The $C_{\text{max}}$ in plasma was 7 times higher in the case of PTX-SLN than in the case of PTX-INJ. The $C_{\text{max}}$ and $\text{AUC}_{0}^{24}$ in lungs in the case of PTX-SLN were 10 and 8 times higher, respectively, than that for commercial injection. This indicated better targeting potential with reduced retention time in heart and kidneys, suggesting better therapeutic performance of PTX-SLN than commercial injection.

Quercetin, a polyphenol flavonoid, has recently gained attention for its anti-cancer properties. Quercetin SLN prepared by an emulsification and low-temperature solidification method with size in the range of 50–200 nm allowed efficient uptake in the intestine, particularly in the lymphoid sections of the tissue, as determined by in situ gastrointestinal perfusion studies in rats. Oral administration of quercetin SLN or suspension in rats showed that the $t_{1/2}$, MRT and relative bioavailability of quercetin SLN as compared to that of suspension were 3.1, 2.2 and 5.71 times higher, respectively, demonstrating that SLN are effective carriers for oral delivery to enhance absorption of a poorly water-soluble drug quercetin [59].

Beta-elemene, a volatile oil used for the treatment of cancer, was encapsulated into SLN by a combination of probe sonication and membrane extrusion. Body distribution of these SLN in rats after IV administration showed higher levels of beta-elemene in the liver, spleen and kidney within 5 minutes of injection with a simultaneous 30% decrease in concentrations in the heart and lung, indicating the potential of these SLN for treatment of liver cancer [60].

All-trans retinoic acid (ATRA; also called tretinoin) is an anti-cancer drug with poor water solubility and limited stability to light, heat and oxidants. Freeze-dried tricaprin-based ATRA SLN were found to be stable for 3 months. The anti-proliferative effect of the SLN powder was not significantly different from that of the free drug; however, a significant reduction of the haemolytic potential of ATRA was observed with SLN, suggesting improved chemical stability and biological activity of ATRA in SLN [61].

Thus considerable research suggesting the feasibility of SLN as a better, prolonged and efficacious carrier system for toxic hydrophobic anti-cancer agents has been reported.

### 4.3.2 Encapsulation of Hydrophilic Drugs

Incorporation of hydrophilic drugs into SLN is challenging since the hydrophilic nature of the drug causes its partitioning into the aqueous phase during hot melt homogenisation. It has been reported that this segregation can be minimised by replacing water with oil, or by cold homogenisation [62]. SLN incorporating hydrophilic active
substances have been successfully prepared using alternative formulation techniques and evaluated for their physicochemical, *in vitro* and *in vivo* properties.

Vinorelbine bitartrate, a hydrophilic and temperature-sensitive drug, has been loaded into SLN by cold homogenisation technique resulting in formulations with particle sizes ranging from 150 to 350 nm and zeta potential about +20 mV. Atomic force microscopy images suggested irregular spheres with a smooth surface. Increasing lecithin or oleic acid content led to an improvement in the encapsulation efficiency, which reduced with an increase in the amount of added drug. Sustained drug release up to 48 hours was observed and the release rate was delayed by the addition of lecithin or oleic acid in the formulations. Stability up to 2 months under room temperature was observed. Cellular cytotoxicity of vinorelbine bitartrate-loaded SLN against MCF-7 cells was found to improve significantly due to entrapment in the lipid matrix of SLN [63].

Mitoxantrone is a water-soluble anthracenedione anti-neoplastic agent. SLN of mitoxantrone using glyceryl behenate as the solid lipid were prepared with a mean particle size of 61 nm, drug content of 4.18 ± 0.10% and encapsulation yield of 87.23 ± 2.16%. No toxicity was observed after local injection of SLN, whereas severe liver and lung toxicity was produced by injecting drug solution. Significant reduction in lymph node metastases was observed with the SLN as compared to free drug solution and control placebos when tested in MCF-7 breast cancer in nude mice and animal model of P388 lymph node metastases in Kunming mice. Thus, the study suggested the prospects of delivering mitoxantrone for breast cancer and its lymph node metastases with excellent therapeutic effect and reduced side effects [64].

Cisplatin is a platinum complex and water-soluble anti-cancer compound. Spherical and uniform SLN of cisplatin were obtained by an emulsification dispersion-ultrasonication method. They showed higher drug targeting to the liver and brain due to improved lipophilicity combined with a low concentration in the kidney in rats, suggesting targetability and low toxicity of cisplatin [65].

Another hydrophilic drug that has been extensively studied and incorporated into SLN is doxorubicin (DOX). Serpe and co-workers [66] investigated intracellular accumulation and cytotoxicity of DOX loaded into SLN and PEGylated liposomes (liposomes coated with polyethylene glycol (PEG)) as against the free drug in human colorectal adenocarcinoma HT-29, retinoblastoma Y79 and glioblastoma U373 cell lines. The IC$_{50}$ values for DOX-SLN were significantly lower after 24 hours exposure than those for free DOX in all cell lines and after 48 hours exposure the values were lower than those for liposomal DOX in HT-29 and Y79 cells. The enhanced cytotoxicity of SLN was attributed to the increased drug incorporation into cells, due to the nature of the lipid carrier system to enhance drug action by causing a marked
uptake and accumulation of SLN within the cell. In the case of SLN of DOX and PTX developed by the same group, the IC$_{50}$ value of DOX-SLN was higher than that of conventional DOX formulation on HT-29 colorectal cancer cell lines [67]. In another study, cellular uptake and cytotoxicity of DOX and PTX solid lipid nanospheres investigated on two cell lines, human promyelocytic leukaemia (HL60) and human breast carcinoma (MCF-7), showed higher cytotoxicity of DOX-SLN as compared to the conventional DOX solution, even at lower concentrations [68]. Pharmacokinetics of DOX-SLN after IV administration in male Wistar albino rats showed markedly higher blood concentrations of the drug at each time point as compared to the commercial solution. Higher drug concentrations were obtained in the lung, spleen and brain, while lower drug concentrations were observed in the liver, heart and kidneys, showing alteration in the body distribution profile when DOX was administered in the form of SLN [69]. Thus, SLN act as potential carrier vehicles for hydrophilic drugs by improving efficacy and targetability.

4.4 Modifications to Improve Encapsulation Efficiency and Release Characteristics

The primary challenge faced during drug incorporation into SLN is low encapsulation efficiency. A higher dose loaded onto a lower lipid concentration leads to very less drug being encapsulated inside the lipid core and more drug adsorbed onto the surface, leading to a burst release, which is observed within the first 30 minutes in dissolution profile studies. On the contrary, a low dose incorporated into a higher lipid concentration leads to more drug being present in the core of the lipid, and subsequently a very slow release rate that may not produce the desired therapeutic concentration. These two extremes need to be intricately balanced, considering the nature of the drug molecule being encapsulated. Hydrophobic drugs complement the hydrophobic nature of the lipid core, whereas hydrophilic drugs have a greater chance of being expelled into the external aqueous phase during homogenisation. Considerable research efforts have been directed towards increasing the encapsulation efficiency and improving the release characteristics of both hydrophobic and hydrophilic drugs from SLN either by modifying the method of preparation or by combining various excipients and emulsifiers. Nanostructured lipid carriers (NLC) are a result of these efforts; they contain a liquid lipid combined along with the solid lipid in the core shell. NLC have dramatically improved the encapsulation efficiency of a number of hydrophilic drugs and exhibit a much more precise release characteristic than SLN.

SLN of DOX have been prepared by solvent emulsification-diffusion method using glyceryl caprate as the lipid core and curdlan as the shell material and the formulation
parameters were optimised to obtain nanoparticles with a mean particle size of 199 nm, entrapment efficiency of 67.5 ± 2.4% and drug-loading capacity of 2.8 ± 0.1%. Freeze-dried SLN were stable after 1 year of storage at 4 °C. Drug release and cell viability assay showed that properties of the prepared SLN did not change during freeze drying [70].

SLN and NLC of DOX were prepared using monostearin as the solid lipid. Octadecylamine was added in small amount to aid in the formation of crosslinking by glutaraldehyde in the presence of oleic acid. An improvement in the drug-loading capacity and encapsulation efficiency was observed and a reduction in the burst release was obtained after crosslinking with glutaraldehyde in the case of NLC [71].

CA is a potent anti-cancer drug with limited solubility and stability. It was incorporated into Precirol and Compritol to form SLN, into a combination of Precirol and squalene to form NLC and into squalene to form lipid emulsion. Precirol SLN showed higher cytotoxicity than the free control, sustained release of drug, limited haemolysis and good storage stability, suggesting their feasibility for use as delivery system for parenteral CA administration [72].

Use of prodrug of an active moiety is another approach to improve its incorporation into lipid phase. Cholesteryl butyrate was selected as the lipid matrix as well as the prodrug for delivery of butyric acid to tumour cells since butyric acid is metabolised rapidly and has a short $t_{1/2}$. SLN of different concentrations of cholesteryl butyrate were prepared and evaluated for their anti-proliferative effect on non-small cell lung carcinoma cell line NIH-H460 and compared against sodium butyrate. A complete growth inhibition of the cells was observed at a concentration as low as 0.25 mM. Fluorescence microscopy demonstrated that these SLN completely internalised into the cells after 5 minutes of treatment. Thus, SLN could serve as an alternative approach for butyric acid delivery into the tumour cells [73].

4.5 Modifications to Improve Multidrug Resistance

P-glycoprotein (P-gp) transporter is a multispecific drug efflux transporter that plays a major role in regulating the bioavailability of various anti-cancer drugs. P-gp transporter affects the pharmacological response by obstructing the permeability of anti-cancer drugs. The expression of P-gp on tumour cells results in the development of multidrug-resistant phenotype that greatly affects the outcome of chemotherapy [74].

SLN of anti-cancer anthracyclines idarubicin (IDA) and DOX have been demonstrated to have potential to overcome P-gp-mediated MDR in leukaemia. SLN were prepared from warm microemulsion precursors with emulsifying wax as the oil phase.
Brij 78 and vitamin E d-alpha tocopheryl polyethyleneglycol succinate were used as surfactants and anionic ion-pairing agents. Sodium taurodeoxycholate and sodium tetradecyl sulfate were added to neutralise the charges on the cationic anthracyclines and improve drug entrapment in the SLN. *In vitro* cytotoxicity studies demonstrated a ninefold lower IC$_{50}$ value of DOX-SLN as compared to free DOX solution in resistant P388/ADR cell line. *In vivo* studies in P388/ADR leukaemia mouse model suggested significantly higher median survival time of DOX-SLN than that of free DOX and controls. In the case of IDA-SLN, IC$_{50}$ values were comparable to those of free IDA. *In vitro* and *in vivo* studies in P388/ADR cell lines and P388/ADR mouse tumour models, respectively, showed equal effectiveness for both IDA-SLN and free IDA. Thus the study suggested that although SLN showed promise as effective carriers for anti-cancer drugs for the treatment of P-gp-mediated MDR in leukaemia, selection of proper drug was critical [75].

Wong and co-workers [76] have developed a new polymer-lipid hybrid nanoparticle (PLN) system that can efficiently load and release the hydrophilic DOX hydrochloride and enhance DOX toxicity against MDR cancer cells. In this study, cationic DOX was complexed with a new soybean oil-based anionic polymer and codispersed with a lipid in water to form DOX-loaded SLN. These polymer-lipid hybrid SLN showed a drug encapsulation efficiency of 60–80% with a particle size range of 80–350 nm and had a prolonged release profile up to 2 weeks. *In vitro* cytotoxicity studies in MDR human breast cancer cell line (MDA435/LCC6/MDR1) showed an eightfold increase in cell kill when compared to treatment with DOX solution at equivalent doses. Cellular DOX uptake and retention by the MDR cells were significantly enhanced ($p < 0.05$) when DOX was delivered in DOX-PLN form. *In vivo* evaluation of DOX-PLN conducted by intratumoural injection in a murine solid tumour model created by injecting EMT6 mouse mammary cancer cells intramuscularly into the hind legs of BALB/c mice showed substantially larger central necrotic regions than the untreated tumours. The DOX-PLN residues could be seen extensively distributed among the dead cell debris, pointing that the anti-cancer effect of DOX-PLN was mainly a combined result of intratumoural nanoparticle distribution and sustained drug release. The study demonstrated that DOX-PLN possesses significant *in vivo* cytotoxic activity with a potential to improve the therapeutic index of locoregional solid tumour chemotherapy with minimal systemic toxicity [77].

Another novel anti-cancer synergistic combination of DOX and mitomycin C (MMC) was designed into a PLN conjugate in order to be effective in MDR breast cancer. The DOX-MMC co-loaded PLN were tested on MDA435/LCC6 human breast cancer cells and were found to be effective in killing the MDR cells at 20- to 30-fold lower doses than the free drugs. The combination was found to be more effective than PLN loaded with either drug, demonstrating the importance of simultaneous uptake of the drugs by the cells leading to a synergistic effect. This rational combination was able
to overcome MDR at a significantly lower dose than free drugs, signifying the ability to improve chemotherapy and reduce the limitations arising due to toxicity [78].

In another study, Choi and co-workers [79] developed DOX-loaded SLN by using biocompatible compounds showing no in vitro haemolytic activity in human erythrocytes. In comparison to DOX, DOX-SLN were found to efficiently enhance the apoptotic cell death by causing a greater accumulation of DOX in P-gp-overexpressing MCF-7/ADR cells (a type of DOX-resistant breast cancer cell line). Thus, DOX encapsulated into SLN could be a useful therapeutic application to overcome the MDR of adriamycin-resistant breast cancer.

4.6 Modifications to Improve Cellular Uptake and Targetability

The in vitro cytotoxicity studies of the various anti-cancer drugs incorporated into SLN have been extensively studied in a number of cell lines. Incorporation of drugs into SLN has consistently resulted in higher cellular uptake in cell lines mainly due to the lipophilic nature of the carrier. In an in vitro cytotoxicity study by Muller and co-workers [80], the nature of the lipid was found to have no effect on the viability of HL60 and human granulocyte cells. However, binding to SLN surface led to a marked reduction in the cytotoxic effects of the surfactants. Muller and Olbrich [81] investigated the phagocytic uptake of the SLN by macrophages and the subsequent cytotoxicity at the cellular level. SLN by virtue of their small size and the lipid nature of the surface were identified as foreign particles and subsequently taken up by the mononuclear phagocytic system (MPS) and cleared immediately from the system by phagocytosis. SLN uptake by phagocytosis was required to deliver drugs to treat diseases caused by Mycobacterium tuberculosis or Mycobacterium avium that affect MPS cells. It has also been demonstrated that controlled surface modification of SLN was able to reduce macrophage uptake and it was possible to escape identification by MPS and deliver drugs to other sites of the body [82]. Sterically stabilised SLN using hydrophilic block copolymers poloxamine 908 and poloxamer 407 reduced the phagocytic uptake to approximately 8–15% as compared to phagocytosis of hydrophobic polystyrene particles [83].

Wan and co-workers [84] prepared PEGylated SLN of vinorelbine bitartrate, a hydrophilic anti-cancer drug, by using the conjugate of PEG2000-StA. In vitro cellular uptake studies indicated that phagocytosis by RAW264.7 cells was inhibited effectively and the uptake by MCF-7 and A549 cancer cell lines was significantly improved with PEG-modified SLN than with nonmodified SLN.

A novel docetaxel-loaded SLN system prepared with galactosylated dioleoylphosphatidyl ethanolamine showed a very high encapsulation efficiency (>90%) and low burst
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effect within the first day with sustained drug release for the next 29 days \textit{in vitro}. Cytotoxicity studies in BEL7402 hepatocellular cell lines showed superior cytotoxicity of these surface-modified SLN as compared to free drug and nontargeted SLN. Cellular uptake and biodistribution studies indicated better anti-tumour efficacy of the targeted SLN. Histology of the liver demonstrated no toxicity on both healthy and affected liver cells. The study suggested that these modified targeted SLN of docetaxel showed promise for use in the treatment of advanced and metastatic hepatocellular carcinoma by enhanced \textit{in vivo} anti-tumour effects [85].

Ferritin is a globular protein complex that stores iron intracellularly and is produced by all cells. It is overexpressed on the surface of tumour cells of breast, liver, colon, etc. due to an increase in the number of receptors responsible for ferritin storage. SLN of the hydrophilic drug 5-fluorouracil (5-FU) coupled with ferritin showed 7.7-fold higher \textit{in vitro} cell uptake in MDA-MB-468 breast cancer cells as compared to plain SLN. \textit{In vivo} studies on BALB/c mice bearing MDA-MB-468 tumours resulting in effective reduction in tumour growth as compared to treatment with free 5-FU and plain SLN [86].

Mutations in p53 tumour suppressor gene are a very common genetic abnormality in lung cancer. A novel drug delivery system consisting of cationic SLN-mediated nanoparticles with enhanced p53 gene transfer to lung cancer cells has been reported and may have potential for clinical application as a nonviral vector-mediated lung cancer therapy due to its effective induction of apoptosis and tumour growth inhibition [87].

Cytotoxicity study of PTX-SLN encapsulated in different lipids such as glycerol tristearate, monostearin and StA showed that cellular cytotoxicity of PTX was highly enhanced by encapsulation within the lipid matrix. PEGylated and folate-modified monostearin SLN of PTX were found to further enhance the cellular uptake of SLN and cytotoxicity of PTX due to the presence of PEG chains on the surface and improved endocytosis mediated by folate receptor [88].

PTX-loaded sterically stabilised SLN prepared using trimyristin as the solid lipid and PEGylated phospholipid as stabiliser were found to show comparable cytotoxicity to the commercially available Cremophor EL-based PTX when studied in OVCAR-3 human ovarian and MCF-7 breast cancer cell lines [89]. Nonstealth and stealth SLN of PTX prepared using warm oil-in-water (o/w) microemulsion technique were found to be stable over time without precipitation of PTX and have been proposed for parenteral administration of PTX [90].

Pharmacokinetics of nonstealth and stealth SLN of DOX after IV administration to rats showed the presence of DOX and its metabolite doxorubicinol even 24 hours postinjection, while they were not detectable in the case of commercial DOX solution.
The presence of doxorubicinol in the brain 4 hours postinjection confirmed passage of SLN across the blood-brain barrier (BBB), and the levels continued to remain higher in the brain 24 hours postinjection. The brain showed higher DOX concentration after administration of stealth SLN as compared to nonstealth SLN. A significant decrease in DOX concentration in the heart was observed in the case of both types of SLN [91].

Signal transducer and activator of transcription 3 (STAT3) activation has been shown to impart several oncogenic features in many solid and blood tumours. A polymer-lipid conjugate nanoparticulate system comprising polyethylenimine (PEI) modified with StA has been investigated to deliver siRNA for efficient STAT3 downregulation in B16 melanoma cells. Compared to the PEI complexes, the PEI-StA complexes showed higher potency in STAT3 downregulation in B16 cells, 2.5 times higher caspase 3 activity accompanied by a significant induction of interleukin-6 secretion and reduction in vascular endothelial growth factor levels. Cell viability was reduced to 16% with the PEI-StA complexes as compared to 69% with the PEI complexes after the third daily dose. In vivo results consistently showed a regression in tumour growth and tumour weight with siRNA-PEI-StA complexes as compared to siRNA-PEI treatment [92].

StA-chlorambucil (CLB)-dimethyl dioctadecyl ammonium bromide (DDAB) nanocarriers showed enhanced stability and drug encapsulation over StA-CLB SLN, sterically stabilised SLN with PEGylated phospholipids and NLC with oleic acid as adjunct lipid. The adsorption and complexation of DDAB on StA-CLB nanoparticles also altered the pharmacokinetics and biodistribution of CLB, as indicated by rapid plasma clearance, low tissue toxicity and greater tumour accumulation of nanocarriers as compared to CLB solution [93].

Dexamethasone (DXM) is an anti-inflammatory agent that is preadministered during a chemotherapy session and is known to enhance the anti-tumour efficacy of the chemotherapeutic agent. Incorporation of this anti-inflammatory agent into SLN is expected to promote efficacy of the agent while simultaneously reducing systemic toxicity. Palmitate ester of DXM was synthesised to impart lipophilicity and incorporated into SLN containing a PEGylating agent, PEG 6000 monostearate. This surface modification was found to decrease the uptake of DXM-SLN by murine macrophages, pointing to the possibility of a higher uptake by tumour tissues and an enhancement of the uptake of chemotherapeutic agent to the tumour site [94].

Gadolinium SLN using polyoxyl-2-stearyl ether as the lipid core were prepared by cooling warm microemulsion templates at room temperature. To obtain folate-coated nanoparticles, a folate ligand was added to either the microemulsion templates or nanoparticle suspensions. Cell uptake studies conducted on KB cell lines (human nasopharyngeal epidermal carcinoma cell line) showed that though the method of
addition of folate ligand did not significantly affect nanoparticle cell uptake, the concentration of folate ligand added profoundly influenced the cellular uptake of nanoparticles. Thus, these engineered folate-coated nanoparticles had the potential to serve as nanocarriers for gadolinium neutron capture therapy of tumours [95].

4.7 Modifications to Achieve Brain Targeting

Brain tumours are extremely challenging to treat because of the complex anatomy and physiology of the brain. Currently, surgical resection, a highly invasive technique, is the primary treatment regimen, followed by radiotherapy and chemotherapy. The main hurdle in the brain targeting of anti-cancer agents is low central nervous system (CNS) penetration due to the presence of the blood-brain barrier (BBB). The BBB is an extremely complex, organised network of cells that acts as a doorkeeper to any molecule entering the brain. This physiological barrier is composed of a tight intercellular junction formed principally of capillary endothelial cells that are highly effective in restricting the movement of drugs or solutes in and out of the brain [96, 97]. Drug trafficking is influenced by a number of factors, one of which is the chemical nature of the drug. In general, hydrophobic drugs have better chances of penetrating the brain, although not all of them can easily penetrate the BBB. Although the entry of hydrophilic moieties is generally prohibited, the transport of small molecules such as glucose, galactose, amino acids, nucleosides, nucleotides, lactates, pyruvates, vitamins and hormones is facilitated by a number of specific transporters present in the BBB, depending on the requirement [98].

A number of strategies have been reviewed using SLN for targeting drugs to the brain bypassing the BBB [96–109]. These reviews highlight the various challenges that need to be overcome to target drug molecules to the brain, the significance of the BBB, the possible receptor and transporter mechanisms within the BBB that can be used to advantage to transport drug molecules to the brain and SLN as the rising potential drug delivery carriers to the brain.

One of the first reported studies on brain targeting was conducted by Yang and co-workers [110], who studied the body distribution of CA-SLN when injected IV in mice. CA-loaded StA SLN with an average diameter of 196.8 nm and a zeta potential of −69.3 mV showed in vitro drug release for up to a week in pH 7.4 phosphate-buffered saline at 37 °C. The area under the drug concentration–time curve (AUC)/dose and MRT of CA-SLN in mice after IV administration were much higher than that of the control CA solution, especially in the brain, heart and reticuloendothelial system (RES) organs, with the brain AUC ratio being highest among all tested organs. Furthermore, orally administered CA-SLN also showed the highest increase in drug
availability in terms of AUC in the brain, suggesting the feasibility of use of CA and other anti-tumour drugs by peroral route of administration [111].

Muller and co-workers [112–114] have applied a novel targeting strategy called the Pathfinder® technology to target drugs to the brain. This group used the concept of differential protein adsorption, a phenomenon observed after IV injection of drug loaded onto SLN. After parenteral administration, proteins from the blood adsorb onto the SLN. The adsorption pattern observed depends largely on the physicochemical surface properties of the particles. These surface-adsorbed proteins determine the in vivo fate of the particles and decide upon the organs that the SLN would preferentially target. For example, adsorption of opsonins such as immunoglobulins IgG and IgM would lead to recognition by macrophages of the MPS and subsequent elimination of SLN by the MPS. Adsorption by dysopsonins such as albumin and apolipoprotein A-I, A-IV, C-III and H minimises or avoids phagocytic uptake. Thus after IV administration, the carrier would control the fate of the drug in vivo and not the properties of the drug. Apolipoprotein E (ApoE) has been identified as a protein that plays an important role in transporting lipoproteins across the brain via the low-density lipoprotein receptor. Coating SLN with ApoE by surface modification or pretreatment prior to injecting could potentially aid in the brain targeting of drugs.

A study by Goppert and Muller [115] using different polysorbates as stabilisers on the SLN showed that all the polysorbates had an affinity to adsorb ApoE. SLN stabilised with polysorbate 80 showed the highest adsorption of ApoE and albumin and a reduction in ApoC-II adsorption. They concluded that polysorbate 80-stabilised SLN had the highest potential to deliver drugs to the brain.

IDA-loaded SLN were compared against free drug solution for an improvement in bioavailability by duodenal route over IV route in rats. AUC and elimination $t_{1/2}$ were, respectively, 21 and 30 times higher for SLN than that for drug solution. Tissue concentrations of IDA and idarubicinol, the main metabolite of IDA, were lower in the heart, lung, spleen and kidneys after SLN administration. The drug and its metabolite were detected in the brain, indicating their ability to cross the BBB. AUC after IV administration was lower than after the duodenal administration, suggesting that duodenal administration alters the pharmacokinetics and tissue distribution of IDA [116].

In a study by Huang and co-workers [117], SLN of temozolomide (TMZ), an alkylating agent used in the treatment of intracalvarial spongioblastoma and malignant tumours of the nervous system, were prepared using StA as the lipid by emulsification-solidification method. TMZ-SLN with an average diameter of 65.9 ± 11.8 nm and a zeta potential of $-37.2 \pm 3.6$ mV were obtained. In vitro drug release was in accordance with Higuchi equation and sustained up to 3 days in phosphate-buffered
saline (pH 6.8) at 37 °C. In vivo studies showed that the AUC/dose and MRT of the TMZ-SLN were much higher and prolonged than those observed in the case of TMZ solution, specifically in the brain and RES organs. The AUC ratio was highest in the brain, suggesting the probability of using SLN of TMZ for sustained release and brain targeting.

SLN of cisplatin has been prepared by microemulsification technique using StA, soy lecithin 95% and sodium glycolate. An average diameter of 250–500 nm, zeta potential between $-9.8$ and $-11.2$ mV and maximum entrapment efficiency of 74.53% were observed. The in vivo studies in Wistar rats showed preferential targeting to the liver followed by brain and lungs. Higher drug targeting in the brain pointed to an increased lipophilicity of cisplatin-loaded SLN, which possibly led to an increased brain targeting efficiency [118].

Negatively and positively charged tripalmitin SLN loaded with etoposide were prepared by melt emulsification and high-pressure homogenisation technique. The spray-dried free-flowing powder had a mean diameter of 391 and 362 nm for negatively and positively charged SLN, respectively, with a $>96\%$ entrapment efficiency. These SLN were radiolabelled with Tc-99m and in vivo pharmacokinetics was evaluated by IV injection in rats. Positively charged SLN exhibited higher blood concentrations and prolonged blood residence time. A significantly lower uptake by RES organs such as liver and spleen ($p < 0.001$) for both positively and negatively charged SLN was observed as compared with etoposide. Positively charged SLN showed a 14-fold higher distribution to bone and brain than etoposide and negatively charged SLN at 4 hours post-injection, suggesting their suitability to serve as a delivery system for targeting to brain tumours by EPR effect [119]. As a further development to this study, the IP route was found to be more advantageous for brain targeting of etoposide-loaded positively charged tripalmitin SLN than the SC route [49].

Superparamagnetic iron oxide (Endorem®) was incorporated into StA SLN by two different methods, one from water-in-oil-in-water multiple warm microemulsion and another from o/w microemulsion, and freeze dried. The average diameter of SLN obtained from multiple microemulsion was 233 nm and that from o/w microemulsion was 159 nm. In vitro analysis showed relaxometric properties similar to those of plain superparamagnetic iron. In vivo study was conducted using SLN prepared by o/w microemulsion method and compared with Endorem as the control. It was observed that the superparamagnetic SLN had slower blood clearance than the control Endorem preparation, and magnetic resonance imaging (MRI) data suggested CNS uptake of SLN up to 135 minutes till the completion of the experiment while plain Endorem showed liver uptake and no CNS penetration. It was concluded that SLN loaded with superparamagnetic iron oxide were capable of crossing the BBB, suggesting the possibility to be used as CNS MRI contrast agents [120].
Wang and co-workers [121] synthesised an esterified prodrug 3’,5’-dioctanoyl-5-fluoro-2’-deoxyuridine (DO-FUdR) of 5-fluoro-2’-deoxyuridine (FUdR) in order to reduce the high metabolic rate of FUdR in the body and make it available in the body for brain targeting. The prodrug was incorporated into SLN (DO-FUdR-SLN) by a thin-layer ultrasonication technique and optimised by applying a central composite design. The median particle size of DO-FUdR-SLN was 76 nm with 29.02% drug loading and 96.62% entrapment efficiency. IV administration of SLN showed 5.32- and 10.97-fold higher brain AUC of DO-FUdR and DO-FUdR-SLN, respectively, than that of FUdR, suggesting the suitability of SLN as a carrier system for CNS targeting.

Nonstealth and stealth SLN of DOX were injected via IV route in rabbits and compared against the commercial DOX solution. It was found that DOX AUC increased as a function of the amount of stealth agent present in the nonstealth SLN. DOX could be detected in blood 6 hours post-injection in the case of nonstealth and stealth SLN, while no DOX was present in the case of DOX solution. DOX was detected in the brain only in the case of nonstealth SLN administration. In the case of stealth SLN, DOX could enter the brain only when the highest amount of stealth agent was included. Also, nonstealth and stealth SLN formulations showed significant decrease in heart and liver concentrations of DOX, pointing to a safe and efficacious formulation for brain targeting [122].

4.8 Toxicity Concerns

The use of SLN, NLC and lipid drug conjugate in parenteral drug delivery and the toxicity caused by these kinds of lipid nanoparticles with a glance on the fate of lipid nanoparticles after their parenteral delivery in vivo have been reviewed extensively [123–125]. Most of the research work cited above has concluded that the inclusion of anti-cancer drugs into SLN has considerably reduced toxicity and improved safety of the drug by encapsulating the drug into the lipid carrier when tested in vivo in animal models. SLN have demonstrated lower cytotoxicity as compared to polymeric (polyalkylcyanoacrylate (PACA) and polylactic/glycolic acid (PLGA)) nanocarriers [80]. Also, in vitro cytotoxic studies in human granulocytes showed that surface-modified SLN were 10-fold less cytotoxic than PLGA and 100-fold less cytotoxic than PACA nanoparticles [83].

Due to its nanosize, SLN formulation is able to transcend all biological barriers within the cells and the toxicity associated with this phenomenon has not been investigated in detail. More stringent tests are required to assess the safety and toxicity of these nanocarriers before their commercial use since the health and safety of humans is of primary concern.
Inhalation toxicity is one of the prime concerns and a review by Card and co-workers [126] on pulmonary toxicity of nanoparticles highlights important issues related to possible toxic effects on the respiratory system due to inhalation and systemic toxicity of administered nanoparticles. An interesting review paper on consideration of haemocompatibility of nanosized drug delivery systems highlights the issues of biocompatibility before considering their potential use in therapeutics [127]. Haemocompatibility is very important especially since many of the SLN formulations we have discussed earlier are administered parenterally. Upon systemic administration, the SLN essentially first comes in contact with the blood and then later on is carried by blood to various tissues. Hence any interaction of the formulation with the blood components must be ruled out before administration. Interaction with blood can cause haemolysis, coagulation, platelet adhesion/aggregation, complement activation, etc., which can further lead to embolism or serious complications. Although regulatory guidelines have not yet been framed for stringent analysis of nanoparticles, it is suggested to apply tests intended for parenteral/sterile products for nanoparticles as well.

Detailed acute and repeated dose toxicity studies of MTX-SLN and PTX-SLN formulations developed in our laboratory have been conducted on Wistar rats [52]. The lipid tristearin used in SLN served as an effective barrier protecting organs and blood from potential toxic effects of the anti-cancer agents, thus proving an efficacious and safe carrier system for delivery of drugs to the target sites. A blank SLN formulation was also included in the study to understand the effect of plain lipid in the form of SLN on animals. Blank SLN did not show significant toxic effects on the tissues and other parameters, suggesting that the lipid carrier was equally safe to use in the body.

Research studies involving toxicity and safety issues arising due to the use of SLN are still in their early stages of development. Stringent tests are necessary to screen the potential toxicity of SLN even though physiological lipids are used in their fabrication. Complex issues may arise once SLN of submicrometre size are in the internal milieu of the body for prolonged periods of time. Hence, both short- and long-term toxicity studies need to be undertaken in order to ensure the safety of these newer drug delivery systems.

4.9 Conclusions

Recent years have seen an increase in investigations of the application of SLN as potential carriers for delivery of drugs, especially for anti-cancer drugs. Nanocarriers show good targeting potential to the liver, lungs, spleen and brain after IV administration. Because they tend to have fewer effects on non-cancer cells and therefore tend to be
less toxic, targeted agents are particularly amenable to use in combination with other agents since their combined side-effect profiles would still be acceptable. In fact, it is reasoned that the future of cancer treatment lies in identifying the best combinations of targeted therapies for each individual patient based on the molecular profile of the tumour. This would be a significant advance in cancer treatment.

References

Targeting Solid Lipid Nanoparticles for Drug Delivery in Cancer Therapy


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Kamalinder K. Singh and Renuka T. Gupta


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Targeting Liposomes for Drug Delivery in Cancer Therapy

Han-Chung Wu, Yi-Hsuan Chi and Chien-Hsun Wu

5.1 Introduction

Cancer chemotherapy is often severely limited by the toxicity caused by the anti-cancer agents to normal cells. Doxorubicin, which is usually associated with severe cardiotoxicity and hepatotoxicity, has been found to reduce these side effects when administered using liposomes that specifically localised the nanoparticle formulation to the tumour [1]. It was suggested 35 years ago that liposomes could be used as drug carriers in cancer chemotherapy [2], but initial liposome drug delivery systems failed because they were rapidly cleared from the circulation by the macrophages involved in what is known as the reticuloendothelial system (RES) or mononuclear phagocyte system (MPS). The first generation of liposomes was cleared rapidly from the circulation system because the MPS engulfed their unmodified phospholipid bilayers, which impeded the distribution of drugs to solid tumours.

A major breakthrough in the prolongation of liposome circulation half-life occurred when we became able to decrease MPS recognition by coating liposomes with polyethylene glycol (PEG) [3, 4]. The coupling of PEG to liposomes (PEGylated liposomes) decreased drug clearance of the liposomes by the kidneys and the MPS, giving them a much longer half-life in the blood than the first-generation liposomes [5, 6]. In humans, mean plasma residence times range from several hours for pure lipid systems to several days for PEGylated liposomes [5, 6]. The use of PEGylated liposomal doxorubicin (PLD) was found to significantly improve the therapeutic index of doxorubicin by preclinical trials [6–10] and clinical studies [11–13]. Many of such drug delivery systems are being used to improve the pharmacokinetics and pharmacodynamics of the drugs they deliver [1, 14].

The success of liposome accumulation within the tumour lies in the extensive angiogenesis and leakiness of tumour vessels. Angiogenic tumour vessels have an average pore size of 100–600 nm [15], significantly larger than those in normal endothelium, which are typically <6 nm wide [16]. The best size for a liposome is reported to be 100 nm for drug transfer from blood to tumour and for longer retention [17, 18],
the latter promoted by inadequate lymphatic drainage often found in tumour tissues [19]. The combination of these factors leads to an accumulation of the drug delivery system within the tumour, creating a passive targeting phenomenon called the ‘enhanced permeability and retention (EPR) effect’ [20, 21]. Studies of EPR-mediated passive-targeting liposomes have found drug concentrations to be 5–10 times higher in solid tumours treated this way than in those treated with free drugs alone [22, 23]. Liposome particles have also been used to deliver other chemotherapeutic agents, radionuclides and genes [24–27].

Several approaches have been developed to improve the selective targeting of cytotoxic drugs towards tumours and avoid harming normal organs. A promising new strategy is to encapsulate anti-cancer drugs in liposomes conjugated with moieties such as antibodies or peptides that can target particular types of tumour cells or tumour vasculatures [28, 29]. Once targeting liposomes are localised within tumour tissues, the drug they contain is released and becomes available through receptor-mediated endocytosis. Endocytosis delivers the targeting liposomes to the endosomes, where the pH drops to 5.9–6.0; the liposomes are then broken down to release encapsulated drugs into the intracellular space. The molecules progress to lysosomes, where the pH further reduces from 6.0 to 5.0. These lysosomes contain enzymes that break down the liposomes allowing the cargo to be released into the intracellular space, markedly increasing therapeutic efficacy [29–31]. In this chapter, we discuss liposomal anti-cancer drugs in clinical use, review the progressive development of liposomal drug delivery systems and compare the use of targeting liposomes with nontargeted liposomes and conventional anti-cancer drugs.

5.2 Types of Liposomal Anti-cancer Drugs and Their Clinical Use

Because of their severe toxicity to normal tissues, most small-molecule chemotherapeutic agents have a narrow therapeutic window. By encapsulating drugs in drug delivery particles such as liposomes, the volume of drug distribution is significantly reduced while their concentrations within the tumour can be increased [16]. Remote loading methods such as the ammonium sulfate method [32] and the pH gradient method [33] can encapsulate weak bases such as doxorubicin or vinorelbine into the liposomes with more than 95% efficiency. Therefore, much effort has been devoted to developing new liposomal anti-cancer drugs encapsulating such agents as vinca alkaloids, taxanes, cisplatin, camptothecin analogues, podophyllotoxin derivatives and nitrogen mustard analogues. Several liposomal anti-cancer drugs have been developed. Their characteristics and clinical use are summarised below.
5.2.1 Anthracyclines: Liposomal Doxorubicin and Daunorubicin

5.2.1.1 Liposomal Doxorubicin

The anthracycline anti-neoplastic antibiotics doxorubicin (adriamycin) and daunorubicin (daunomycin) are commonly used to treat a wide range of cancers, including haematological malignancies, many types of carcinoma and soft-tissue sarcomas. Despite their advantages, clinical benefits of anthracyclines are compromised by several adverse reactions including cardiotoxicity, haematotoxicity, gastrointestinal and skin toxicity [34]. Since chronic anthracycline-induced cardiotoxicity is associated with a bad prognosis [35], several strategies have been developed to prevent cardiotoxicity. For example, by enclosing doxorubicin in liposomes, cardiac uptake of toxic agent can be reduced, anti-tumour activity can be preserved in mice, and the circulation lifetime of liposomal doxorubicin can be prolonged [11, 12, 36]. To date, the liposomal doxorubicin has the greatest clinical impact of all liposomal drugs. A wide range of liposomal anthracycline products has been approved and marketed for clinical use (Table 5.1). Doxil is a PLD distributed in the USA by Tibotec Therapeutics [54], and is known as Caelyx in Europe where it is marketed by Schering-Plough [55]. Doxil/ Caelyx is approved for treatment of acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma [56], refractory ovarian cancer [57], metastatic breast cancer [58, 59] and multiple myeloma [60]. Unlike Doxil or Caelyx, Myocet (Medeus Pharma, Stevenage, Herts, UK) is a ‘non-PEGylated’ liposome encapsulating doxorubicin.

<table>
<thead>
<tr>
<th>Table 5.1 Liposomal anthracycline products</th>
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<tbody>
<tr>
<td><strong>Trade name (company)</strong></td>
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<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Liposomal doxorubicin</td>
</tr>
<tr>
<td>Doxil (ALZA Pharm)</td>
</tr>
<tr>
<td>Caelyx (Schering-Plough)</td>
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Table 5.1 Continued

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<th>Indication</th>
<th>Year of approval or current status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocet (Elan Pharm)</td>
<td>Metastatic breast cancer (along with cyclophosphamide)</td>
<td>2000 (Europe)</td>
<td>[45]</td>
</tr>
</tbody>
</table>

### Liposomal daunorubicin

<table>
<thead>
<tr>
<th>DaunoXome (Nexstar Pharm)</th>
<th>First-line Kaposi’s sarcoma</th>
<th>1996</th>
<th>[46]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML (combination therapy)</td>
<td>Phase III</td>
<td></td>
<td>[47–49]</td>
</tr>
<tr>
<td>ALL (combination therapy)</td>
<td>Phase II</td>
<td></td>
<td>[50]</td>
</tr>
<tr>
<td>Malignant pleural mesothelioma</td>
<td>Phase II</td>
<td></td>
<td>[51]</td>
</tr>
<tr>
<td>AML, CMML, MDS (RAEB, RAEB-T) (along with semaxanib)</td>
<td>Phase I/II</td>
<td>[40] (NCT00005942)</td>
<td></td>
</tr>
<tr>
<td>Metastatic breast cancer</td>
<td>Phase I</td>
<td></td>
<td>[52]</td>
</tr>
<tr>
<td>Relapsed or resistant solid tumours</td>
<td>Phase I</td>
<td></td>
<td>[53]</td>
</tr>
</tbody>
</table>

citrate complex. The use of Myocet in combination with cyclophosphamide has been approved for treatment of metastatic breast cancer in Europe, but not in the USA [1].

5.2.1.2 Liposomal Daunorubicin

Daunorubicin is an anthracycline aminoglycoside anti-neoplastic agent commonly used to treat acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL) and chronic myeloblastic leukaemias (CML) [61]. It has also been approved to treat neuroblastoma (NB) and rhabdomyosarcoma [62]. Liposomal daunorubicin markedly increases the area under the curve (AUC) in plasma and in tumours and has much lower AUC in normal tissues compared with the free drug in mice [63]. DaunoXome (DNX), a liposomal daunorubicin citrate product, also received the US Food and Drug Administration (FDA) approval to treat AIDS-related Kaposi’s sarcoma in 1996 [46]. The clinical evaluation of the treatment efficacy of the drug in specific types of leukaemia and non-Hodgkin’s lymphomas is currently under way.

Though inflicting less damage on cardiac tissue through passive targeting mechanism, liposomal doxorubicin and daunorubicin still have some unavoidable side effects. Liposomal doxorubicin and liposomal daunorubicin have similar side-effect profiles [64–66]. Palmar-plantar erythrodysesthesia syndrome (PPES), a painful skin toxicity characterised by a patchy or confluent erythema involving the palmar aspect of the hands and soles of the feet, also known as hand-foot syndrome, is a commonly reported side effect of liposomal doxorubicin [67, 68], and it has been reported for liposomal daunorubicin as well, though not as often and when liposomal daunorubicin is administered in high doses [69].

5.2.2 Pyrimidine Analogues: Liposomal Cytarabine and Gemcitabine

5.2.2.1 Liposomal Cytarabine

Cytarabine, also known as cytosine arabinoside or ara C, is an FDA-approved chemotherapeutic agent used mainly to treat specific types of leukaemia, including AML and CML [70, 71] and ALL [72]. It is also used in the treatment of lymphoma [73], meningeal leukaemia and lymphoma (cancers found in the lining of the brain and spinal cord) [74–76]. The drug can be given by intravenous (IV), intrathecal (IT) or subcutaneous (SC) injection. However, its adverse side effects include severe bone marrow suppression, neurotoxicity and gastrointestinal toxicity.

Liposomal cytarabine, which reduces such side effects and has a longer half-life within the CSF than cytarabine, was approved by FDA in 1999 to be given as IT
therapy for the treatment of lymphomatous meningitis (lymphoma that has spread to the meninges) [77, 78]. It was initially marketed in the USA by SkyePharma under the trade name DepoCyt and is currently being studied clinically in the treatment of other types of cancer (Table 5.2).

Although most of the side effects caused by conventional cytarabine disappeared once it was encapsulated, there is still risk of chemical arachnoiditis, a most common and well-documented syndrome manifested primarily by nausea, vomiting, headache and fever emerged in both neoplastic meningitis and IT chemotherapy patients. However, increasing the number of treatment cycles does not increase the cumulative risks [84]. The incidence and severity of chemical arachnoiditis are significantly reduced with the coadministration of dexamethasone with DepoCyt [85].

<table>
<thead>
<tr>
<th>Trade name (company)</th>
<th>Indication</th>
<th>Year of approval or current status</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DepoCyt, liposomal cytosine arabinoside (SkyePharma)</td>
<td>Lymphomatous meningitis</td>
<td>1999 [77]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neoplastic meningitis</td>
<td>Phase IV [78]</td>
<td></td>
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<tr>
<td></td>
<td>ALL</td>
<td>Phase III [79]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aggressive non-Hodgkin lymphoma</td>
<td>Phase II [79, 80]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Refractory diffuse large B-cell lymphoma</td>
<td>Phase II [40]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metastatic breast cancer with CNS involvement</td>
<td>Phase II [81]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glioblastoma, glioma, astrocytoma, brain tumour</td>
<td>Phase I/II [40, 82]</td>
<td></td>
</tr>
<tr>
<td>CPX-351a (cytarabine:daunorubicin) liposome injection (Celator Pharm)</td>
<td>AML</td>
<td>Phase II [83]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALL</td>
<td>Phase I [40]</td>
<td>(NCT00389428)</td>
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CNS, central nervous system; MDS, myelodysplastic syndrome; CPX-351, a liposomal formulation of a fixed combination of cytarabine and daunorubicin in a 5:1 molar ratio
5.2.2.2 Liposomal Gemcitabine

By conjugating lipid with gemcitabine, problems of leakage during circulation might be circumvented. These approaches have been used to improve gemcitabine metabolic stability, pharmacokinetic behaviour and the \textit{in vivo} anti-tumour activity \cite{86-88}. After incorporation into a target cell, the biodegradable lipid-drug conjugate is cleaved by intracellular enzymes, and the active agent is released. These modifications improve protection from deamination by plasma cytidine deaminase, and the gemcitabine prodrug liposome has a long half-life and high AUC in tumours \cite{88}.

5.2.3 Vinca Alkaloids: Liposomal Vincristine and Vinorelbine

Vinca alkaloids are potent anti-cancer agents from the \textit{Catharanthus roseus} (Madagascar periwinkle) that act by binding to tubulin and blocking metaphase in actively dividing cells \cite{89}. While vincristine is a dimeric Catharanthus alkaloid, vinorelbine (5′-nor-anhydro-vinblastine) is a semisynthetic vinca alkaloid which is better tolerated.

Vincristine is one of the most widely used chemotherapeutic agents in adult and paediatric cancer. In 1963, vincristine was first approved by the FDA for the treatment of acute leukaemia in children \cite{90}. Vincristine is almost always used in combination chemotherapy regimens. Vincristine is currently approved as part of the CHOP for the first-line treatment of non-Hodgkin’s lymphomas \cite{91}, MOPP, COPP, BEACOPP for Hodgkin’s lymphoma \cite{92}, Stanford V chemotherapy regimen in acute lymphoblastic leukaemia and Wilms’ tumour \cite{93}. It is also being used in the treatment of rhabdomyosarcoma, breast cancer and small cell lung cancer \cite{94}.

The most frequent and clinically important side effect of vincristine is dose-limiting neurotoxicity, manifested mainly as peripheral neuropathy. It has also been associated with Charcot-Marie-Tooth disease (CMT) \cite{95, 96}. Nonneural side effects of vincristine are less common but can include alopecia (hair loss), myelosuppression, hyponatraemia and constipation \cite{97}.

Vinorelbine is a clinically approved drug used to treat some types of cancers, including metastatic breast cancer \cite{98} and non-small cell lung cancer (NSCLC) \cite{99}. Unlike other older vinca alkaloids, due to its reduced affinity for axonal microtubules \cite{100}, vinorelbine is less neurotoxic \cite{101}, with only numbness or tingling in hands or feet reported. It does have other side effects, including bone marrow suppression, alopecia, diarrhoea, nausea, hyponatraemia and constipation.

Liposome-based delivery further improved the therapeutic index of vincristine and vinorelbine through optimising pharmacokinetics profile and minimising toxicity.
While several liposomal vincristines have been formulated, less success has been found with vinorelbine, which is considerably more difficult to stabilise in liposomes [104, 105]. The first-generation liposomal vincristine sulfate product, Onco TCS, using a DSPC/cholesterol liposome formulation, submitted New Drug Application (NDA) in 2004, but it has not yet been approved by FDA for marketing (Table 5.3). This product may cause dose-limiting toxicities such as pain and constipation, and other symptoms such as fever, rigors, fatigue, myalgia and peripheral neuropathy [109].

For further improvement, sphingomyelin/cholesterol liposomal vincristine (Marqibo™), a novel formulation of liposomal vincristine encapsulated in the aqueous interior of liposomes composed of sphingomyelin and cholesterol, has been introduced [110]. Marqibo was studied by Sarris and co-workers [91, 107] in relapsed non-Hodgkin’s lymphoma and ALL in a phase II trial (Table 5.3), and has been found to have a favourable side-effect profile and haematological improvement [110, 111]. Unavoidably, side-effect analysis was reported that Marqibo (vincristine sulfate liposome infusion, VSLI) caused VSLI-associated fever, constipation, muscle cramps, anxiety/psychiatric disorders and paraesthesia [112]. INEX Pharmaceuticals (Burnaby, BC, Canada) authorised Hana Bioscience (South San Francisco, CA, USA) to perform further development, clinical trials and commercialisation of Marqibo in 2006.

A number of tumours have been found to be resistant to vincristine mediated by multidrug resistance. Liang and co-workers [113] demonstrated that using the stealthy liposomal encapsulation of vincristine plus quinacrine could be used reliably to treat resistant human leukaemia. In addition, a cationic liposomal vincristine formulation was developed to improve vincristine retention, circulation half-life and to increase anti-tumour activity [114]. The use of the monoacyl cationic lipids stearylamine and sphingosine has significantly improved vincristine retention in the circulation of mice and has increased the anti-tumour activity against leukaemia in the murine model [114].

Of the three vinca alkaloids used in liposomal formulations [104], vinorelbine has been considerably more difficult to stabilise in liposomes, because of the leakages of drug before reaching the tumour [105]. However, it has been stably encapsulated in liposomes using triethylammonium sucrose octasulfate and has been found to display high encapsulation efficiency and in vivo drug retention [115]. It has been found to be highly active against human breast and lung tumour xenografts and the efficacy was considerably improved in both a human colon carcinoma and a murine colon carcinoma model [115]. Two promising liposomal vinorelbine products have undergone early clinical trials for certain cancer types (Table 5.3).
Table 5.3 Liposomal vinca alkaloid products

<table>
<thead>
<tr>
<th>Trade name (company)</th>
<th>Indication</th>
<th>Year of approval or current status</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liposomal vincristine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onco-TCS (Inex Pharm)</td>
<td>Relapsed non-Hodgkin’s lymphoma</td>
<td>Not approved by NDA submission in 2004</td>
<td>[1, 91]</td>
</tr>
<tr>
<td>Marqibo®, VSLI (Inex Pharm; Hana Biosciences, Inc.)</td>
<td>Relapsed non-Hodgkin’s lymphoma</td>
<td>Phase II</td>
<td>[40, 106] (NCT00006383; NCT00144963)</td>
</tr>
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<td></td>
<td>Acute lymphoblastic leukaemia</td>
<td>Phase II</td>
<td>[40, 107] (NCT00495079)</td>
</tr>
<tr>
<td></td>
<td>Metastatic malignant uveal melanoma</td>
<td>Phase II</td>
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<td>Malignant melanoma</td>
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<td>[40] (NCT00145041)</td>
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<td><strong>Liposomal vinorelbine products</strong></td>
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<tr>
<td>INX-0125, VLI (Inex Pharm; Hana Biosciences, Inc.)</td>
<td>Advanced solid tumours</td>
<td>Phase I</td>
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</tr>
<tr>
<td></td>
<td>Hodgkin’s lymphoma</td>
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<tr>
<td></td>
<td>Non-Hodgkin’s lymphoma</td>
<td></td>
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<tr>
<td>NanoVNB® (Taiwan Liposome Company, TLC)</td>
<td>NSCLC</td>
<td>Phase I/II (Taiwan)</td>
<td>[108] (<a href="http://www.tlcbio.com">http://www.tlcbio.com</a>)</td>
</tr>
<tr>
<td></td>
<td>Metastatic breast cancer</td>
<td></td>
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<tr>
<td></td>
<td>Metastatic colorectal cancer</td>
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<tr>
<td></td>
<td>Head and neck cancer</td>
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VLI: Vinorelbine liposome injection
VSLI: Vincristine sulfate liposome infusion
5.2.4 Taxanes: Liposomal Paclitaxel and Docetaxel

Paclitaxel and docetaxel are members of the taxane family, which are tubulin-stabilising chemotherapeutics widely used for a variety of solid tumours. Paclitaxel is approved by FDA to treat ovarian cancer, breast cancer and AIDS-related Kaposi’s sarcoma [116–118]. It is also approved to be used together with cisplatin to treat advanced ovarian cancer and NSCLC [119, 120]. Docetaxel has a set of clinical uses similar to that of paclitaxel for the treatment of breast, ovarian and non-small cell lung cancers, and even locally advanced, metastatic or relapsed types of such cancer types [121, 122].

These drugs are hydrophobic, so paclitaxel is dissolved in dehydrated ethanol and polyethoxylated castor oil (Cremophor EL), whereas docetaxel is dissolved in Tween-80 (polysorbate ethanol). Cremophor EL is dose limited as it has been found to cause severe hypersensitivity reactions [123], though they can minimised by premedication with glucocorticoids and antihistamines. An albumin-bound paclitaxel nanoparticle (Abraxane, Abraxis Bioscience; Costa Mesa, CA, USA) was approved by FDA in 2005 as an alternative to Cremophor EL for the treatment of breast cancer [124–126]. However, its use has still been associated with such side effects as neurotoxicity (peripheral neuropathy), bone marrow suppression (neutropenia, thrombocytopenia and anaemia), alopecia, mucositis, arthralgias and myalgias. These dose-limited toxicities often require a reduction of dosage but impair drug efficacy. Premedication with dexamethasone is used to mitigate some of the side effects [127].

Liposome-entrapped paclitaxel easy-to-use (LEP-ETU) is a liposomal formulation of paclitaxel designed to reduce toxicities associated with Cremophor EL or paclitaxel itself while maintaining or enhancing efficacy [128]. This formulation is also called PNU-93914, liposomal paclitaxel, LipoTaxen and paclitaxel liposome (Table 5.4). A phase I study of LEP-ETU reported the maximum tolerated dose (MTD) of 325 mg/m² q3w

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<th>Table 5.4 Liposomal taxane products</th>
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<tr>
<td><strong>Trade name (company)</strong></td>
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<tr>
<td>Liposomal paclitaxel</td>
</tr>
<tr>
<td>PNU-93914, LEP-ETU (Neopharm)</td>
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</table>
Targeting Liposomes for Drug Delivery in Cancer Therapy

To provide acceptable neutropenic events compared with 175 mg/m² paclitaxel q3w. A 275 mg/m² dose may offer an improved therapeutic index [129]. The most frequently reported adverse events were fatigue, alopecia and myalgia [132]. Other liposomal products of paclitaxel that are undergoing clinical trials are listed in Table 5.4. Kunstfeld and colleagues demonstrated paclitaxel encapsulated in cationic liposomes has a remarkable in vivo antiangiogenic activity and prevents tumour growth and invasiveness, and improves survival of mice [133]. Another cationic liposomal formulation of paclitaxel, EndoTAG-1, acts by targeting activated negatively charged endothelial cells of tumour blood vessels (2009 ESMO World Congress on Gastrointestinal Cancer).

While most of the toxicities associated with docetaxel partially overlap with those associated with paclitaxel, the delayed-onset pleural effusions and oedema that have been associated with some cases treated with docetaxel may be due to the nonionic surfactant polysorbate-80 vehicle [134, 135]. To eliminate the need for these vehicles and improve the drug’s anti-tumour efficacy, docetaxel has been incorporated in liposomes [136–138]. Yoshida and co-workers [139] developed a multifunctional liposome that combines chemotherapy and hyperthermia therapy. Both magnetite and docetaxel were loaded together in liposomes, and when exposed to alternating current magnetic fields, effective tumour regression could be achieved at a low-grade

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<tr>
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<tr>
<td>Liposomal paclitaxel (Nanjing Sike Pharm; Shandong Luye Pharm)</td>
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<td></td>
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<tr>
<td>EndoTAG-1 (MediGene)</td>
</tr>
<tr>
<td><strong>Polymeric micelle formulation of paclitaxel</strong></td>
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<tr>
<td>Genexol-PM® (SamYang Genex)</td>
</tr>
<tr>
<td><strong>Liposomal docetaxel</strong></td>
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<tr>
<td>ATI-1123 (Azaya Therapeutics)</td>
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temperature and the anti-tumour effect of docetaxel could be enhanced. ATI-1123, a liposomal formulation of docetaxel, is currently undergoing phase I clinical trials (Table 5.4).

5.2.5 Platinum Compound: Liposomal Cisplatin and Oxaliplatin

Three platinum-based chemotherapy drugs, cisplatin, carboplatin and oxaliplatin, have been approved by the FDA in the treatment of patients with some malignancies such as testicular, ovarian and colorectal cancer [140]. The first member of this class of compounds, cisplatin or cis-diamminedichloro-platinum (II) (CDDP), is recommended by the FDA for metastatic testicular [141], metastatic ovarian, transitional cell bladder cancer, NSCLC (in combination with gemcitabine) and cervical cancer (in combination with radiation) [142].

Clinical use of cisplatin has been limited by its severe side effects, including nephrotoxicity [143], gastrointestinal toxicity, peripheral neuropathy [144], ototoxicity [145], asthenia and haematological toxicity. Nephrotoxicity is the most significant cumulative dose-related symptom (at cumulative dose in the range 300–600 mg/m²). Therefore, the other platinum compounds such as carboplatin as well as oxaliplatin that have been developed and tested over the last two decades to improve the efficacy and to reduce the toxicity of cisplatin [146, 147]. Carboplatin has been found to be as efficacious as cisplatin but less nephrotoxic.

A liposomal formulation of cisplatin has been developed and has a prolonged circulation time and an increased tumour platinum uptake. In addition, its anti-tumour effect is significantly better than that of free cisplatin in vivo [148]. However, this formulation of liposomes containing cisplatin was not found to be efficacious in the phase I and phase II clinical studies [149], mostly due to its low bioavailability and slow release kinetics, which reduces drug concentration to suboptimal therapeutic level.

Lipoplatin™ (Regulon, Inc., Mountain View, CA, USA) is a PEGylated liposomal cisplatin product with an average diameter of 110 nm [150] (Table 5.5). Encasement in liposomes improves cisplatin’s tumour bioavailability and toxicity profile [155]. However, Lipoplatin must be administered by IV infusion at slow rate (~25 mg/m²/hour) because rapid infusion (1–2 hours) results in higher nephrotoxicity and accentuates other side effects [142].

ALZA Pharmaceuticals developed another STEALTH (sterically stabilised) liposomal formulation of cisplatin (SPI-77), which has different formulation from Lipoplatin [156]. SPI-77 showed promise in preclinical models, but such results were not replicated in clinical trials [157–159]. Until now, more clinical trials of SPI-77 are
ongoing. In subsequent phase I studies, the dose was increased to 320 mg/m² but resulted in severe proximal myopathy [157].

Improvements in the delivery vehicles for platinum drugs are currently being sought. For example, the diaminocyclohexane (DACH)-based AP-5346 and aroplatin/liposomal cis-bis-neodecanoato-trans-(R,R)-1,2-diaminocyclohexane platinum (II)

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<th>Table 5.5 Liposomal platinum products</th>
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</tr>
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<td><strong>Liposomal cisplatin</strong></td>
</tr>
<tr>
<td>Lipoplatin™ (Regulon) &amp; Pancreatic cancer (along with 5-FU, gemcitabine) &amp; Phase III &amp; [40] (NCT00416507)</td>
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<tr>
<td>NSCLC (along with gemcitabine) &amp; Phase III &amp; [151]</td>
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<tr>
<td>Gastric cancer &amp; Phase I/II &amp; [152]</td>
</tr>
<tr>
<td>SPI-77 (ALZA Pharm) &amp; Recurrent ovarian epithelial cancer &amp; Phase II &amp; [40] (NCT00004083)</td>
</tr>
<tr>
<td>Advanced NSCLC &amp; Phase II &amp; [153]</td>
</tr>
<tr>
<td>Head and neck cancer &amp; Phase I/II &amp; [149]</td>
</tr>
<tr>
<td><strong>Liposomal oxaliplatin</strong></td>
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<tr>
<td>Aroplatin™, L-NDDP (Aronex Pharm) &amp; Malignant pleural mesothelioma &amp; Phase II &amp; [40, 154] (NCT00004033)</td>
</tr>
<tr>
<td>Advanced colorectal cancer &amp; Phase II &amp; [40] (NCT00081536; NCT00043199)</td>
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<tr>
<td>Advanced pancreatic cancer &amp; Phase I/II &amp; [40] (NCT00081549)</td>
</tr>
<tr>
<td>Oesophageal, ovarian cancer, hepatocellular carcinoma &amp; Phase I/II &amp; [40] (NCT00057395)</td>
</tr>
<tr>
<td>MBP-426 (Mebiopharm Co.) &amp; Advanced/metastatic solid tumours &amp; Phase I &amp; [40] (NCT00355888)</td>
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</table>

5-FU, 5-Fluorouracil; L-NDDP, liposomal cis-bis-neodecanoato-trans-(R,R)-1,2-diaminocyclohexane platinum
(L-NDDP) are in early clinical development (Table 5.5). Júnior and co-workers [160] have developed a new formulation of stealth pH-sensitive liposomes containing cisplatin. These liposomes are composed of unsaturated phosphatidylethanolamine and take advantage of its fusogenic properties to ensure the release of cisplatin into the cytosol. These liposomes have proved to be effective against human lung cancer cell and have been able to circumvent the resistance through the use of the cisplatin-resistant GLC4 cell line [160]. The work of Schroeder and co-workers [161] presents a strategy for the release of cisplatin from liposomes in vivo using low-frequency ultrasound. They demonstrated that short exposure to low-frequency ultrasound effectively releases the anti-cancer chemotherapeutic agent cisplatin from liposomes, thereby significantly improving its therapeutic efficacy.

Other liposomal formulations of platinum-based drugs have been developed, including carboplatin [162, 163] and oxaliplatin [164–166]. The liposomal carboplatin developed by Hamelers and co-workers [163] is 1000-fold more cytotoxic than conventional carboplatin. Studies by Abu Lila and co-workers [166] suggested that oxaliplatin encapsulated in PEG-coated cationic liposomes achieve selective and efficient drug delivery to tumour-associated angiogenic vessels, and show a remarkable in vivo antiangiogenic activity and suppressed tumour growth.

5.2.6 Liposomal Camptothecin Analogues

Camptothecin derivatives have become integral to the management of lung and colon cancer. They continue to be the subject of intense investigation. A number of analogues of camptothecin were subsequently synthesised including topotecan, irinotecan and lurtotecan. These derivatives were designed to be more water soluble while retaining anti-tumour activity. Both topotecan (Hycamtin, GlaxoSmithKline, Research Triangle Park, NC) and irinotecan (Camptosar, Pharmacia and Upjohn Company, Kalamazoo, MI, USA) are now registered in the USA and elsewhere. In an effort to enhance drug delivery to tumours and minimise exposure to normal tissues, camptothecin analogues have been encapsulated in liposomes.

5.2.6.1 Liposomal Topotecan

Topotecan, a semisynthetic water-soluble derivative of camptothecin, is a topoisomerase I inhibitor. An earlier study that developed topotecan with multilamellar vesicles found that it had an acidic interior and that topotecan was stabilised as the lactone form [167]. Unfortunately, this formulation is limited by its rapid elimination from blood circulation. To overcome the problem, stealth liposomal
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topotecan was developed [168, 169]. Compared to free drug and conventional liposomes, PEGylated liposomes improved cytotoxic effect of topotecan against cancer cell lines [168, 169]. The pharmacokinetic studies in animal model showed that PEGylated liposomal formulations increased the concentration of total topotecan in plasma [169]. Drummond and colleagues encapsulated topotecan in liposomes using transmembrane gradients of triethylammonium salts of polyphosphate or sucrose octasulfate [170]. They improved its stability, pharmacokinetic profile and anti-tumour activity.

5.2.6.2 Liposomal Irinotecan

Irinotecan is a water-soluble prodrug that can be converted to SN-38, a topoisomerase I inhibitor. Irinotecan was discovered to have serious side effects including myelosuppression and gastrointestinal disorders. Chou and co-workers [171], preparing a liposomal irinotecan by pH gradient loading, reported a slight increase in the retention of irinotecan within liposomes. Furthermore, a variety of preparation methods have been used to improve the loading efficiency and in vivo drug retention. Liposomes can entrap irinotecan at high drug-to-lipid ratios using a modified gradient loading method featuring a sterically hindered amine [172]. This liposomal irinotecan showed markedly superior efficacy compared with the free drug in human breast and colon cancer xenograft models [172].

A method for encapsulating irinotecan into liposomes containing copper gluconate with triethanolamine has been developed [173]. Another method uses divalent cation ionophore A23187 and copper-containing liposomes [174]. Both methods aim to improve encapsulation and retention of drug and increase its therapeutic activity.

5.2.6.3 Liposomal Lurtotecan

Lurtotecan (also known as GI147211 and GG211), a semisynthetic, water-soluble analogue of camptothecin, has been encapsulated in small liposomes composed of hydrogenated phosphatidylcholine and cholesterol. Relative to free lurtotecan, liposome-encapsulated lurtotecan (NX 211, Gilead Sciences) has a prolonged plasma half-life, a 1500-fold increase in the plasma area under the drug concentration curve, a 40-fold increase in distribution to implanted tumours in mice and, in single-dose efficacy studies in xenograft models, a threefold or greater increase in therapeutic index [175]. This product is undergoing phase II clinical trials in patients with refractory ovarian cancer, advanced head and neck cancer and recurrent small cell lung cancer (Table 5.6). Further clinical toxicity analysis found neutropenia and thrombocytopenia occurred in patients treated with NX 211 [184–187]. The MTD
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<th>Trade or code name (company)</th>
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<td><strong>Liposomal 9-nitrocamptothecin</strong></td>
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<tr>
<td>L9NC (S.P. Pharm)</td>
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<td>Corpus uteri, endometrial cancer</td>
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<td></td>
<td>Ewing’s sarcoma (along with temozolomide)</td>
<td>Phase I/II</td>
<td>[40] (NCT00492141)</td>
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<tr>
<td><strong>Liposomal irinotecan, irinotecan metabolite (SN38)</strong></td>
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<td>Nanoliposomal CPT-11, NL CPT-11 (University of California, San Francisco, CA, USA)</td>
<td>Recurrent high-grade gliomas</td>
<td>Phase I</td>
<td>[40] (NCT00734682)</td>
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<tr>
<td>Liposomal SN-38, LE-SN38 (Neopharm)</td>
<td>Advanced solid tumours</td>
<td>Phase I/II</td>
<td>[40, 177] (NCT00046540)</td>
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<tr>
<td></td>
<td>Metastatic colorectal cancer</td>
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<tr>
<td>PEP02 (PharmaEngine)</td>
<td>Gastric/gastro-oesophageal junction adenocarcinoma</td>
<td>Phase II</td>
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<td></td>
<td>Metastatic colorectal cancer</td>
<td>Phase I</td>
<td>[40] (NCT00940758)</td>
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<tr>
<td>IHL-305, irinotecan liposome injection (Yakult Honsha)</td>
<td>Advanced solid tumours</td>
<td>Phase I</td>
<td>[40] (NCT00364143)</td>
</tr>
<tr>
<td>CPX-1, irinotecan HCl: fluorouridine liposome injection (Celator Pharm)</td>
<td>Advanced solid tumours</td>
<td>Phase I</td>
<td>[40, 178]</td>
</tr>
<tr>
<td></td>
<td>Advanced colorectal cancer</td>
<td>Phase II</td>
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of 4.3 mg/m² has been limited by an effort to reduce neutropenia and thrombocytopenia. Therefore, the recommended phase II dose is 3.8 mg/m² [184].

5.2.7 Podophyllotoxin Derivatives: Liposomal Etoposide

Etoposide is an anti-neoplastic agent that acts by forming a complex with topoisomerase II and DNA, causing DNA breaks and cell death. However, the administration of etoposide is limited by its lipophilicity. Liposomal etoposide was developed to improve the delivery of the drug. The encapsulation of etoposide in PEG-coated PE gave the liposomal drug a better pharmacokinetic profile than its conventional counterpart [188]. Another strategy is to encapsulate etoposide in cationic liposomes. This cationic liposomal etoposide enhances the drug release and improves anti-tumour activity (Table 5.7) [204–206].
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<tbody>
<tr>
<td><strong>Liposomal mitoxantrone</strong></td>
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<tr>
<td>LEM (Neopharm)</td>
<td>Advanced solid cancer</td>
<td>Phase II</td>
<td>[40, 189, 190] (NCT00024492)</td>
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<tr>
<td><strong>Liposomal all-trans-retinoic acid</strong></td>
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<td>ATRA-IV (Antigenics Inc.)</td>
<td>T cell non-Hodgkin’s lymphoma</td>
<td>Phase II</td>
<td>[191]</td>
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<td></td>
<td>Advanced solid tumour malignancies (along with Depakote®)</td>
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<td>[192]</td>
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<tr>
<td></td>
<td>Advanced renal cell carcinoma (along with interferon alpha2b)</td>
<td>Phase I/II</td>
<td>[193, 194]</td>
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<td>ATRAGEN, liposomal tretinoin (Aronex Pharm)</td>
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<td>Phase II</td>
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<td>Advanced renal cell carcinoma (along with interferon alpha2b)</td>
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<td><strong>Liposomal interleukin 2</strong></td>
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<td>Oncolipin (Biomira USA)</td>
<td>Non-small-cell lung cancer (immune stimulant along with liposomal vaccine)</td>
<td>Phase II</td>
<td>[195]</td>
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<td>Stage III malignant melanoma (polyvalent melanoma vaccine along with recombinant interferon alpha2b)</td>
<td>Phase II</td>
<td>[40] (NCT00004104)</td>
</tr>
<tr>
<td>hII-2 plasmid liposomes (Valentis, Lithuania)</td>
<td>Recurrent/refractory SCC of the head and neck (along with methotrexate)</td>
<td>Phase II</td>
<td>[40] (NCT00006033)</td>
</tr>
<tr>
<td>Oncoquest-L² vaccine (Biomira USA)</td>
<td>Stage III, stage IV, or recurrent follicular lymphomas</td>
<td>Phase I</td>
<td>[40, 196] (NCT00020462)</td>
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### Table 5.7 Continued

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<td><strong>Liposomal thymidylate synthase inhibitor</strong></td>
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<td>OSI-7904L (OSI Pharm)</td>
<td>Advanced or metastatic gastric/ gastro-oesophageal adenocarcinoma</td>
<td>Phase II</td>
<td>[40, 197] (NCT00073502)</td>
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<tr>
<td></td>
<td>Refractory/recurrent advanced colorectal cancer (along with oxaliplatin)</td>
<td>Phase I</td>
<td>[40, 198] (NCT00081237)</td>
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<td>Refractory solid tumours</td>
<td>Phase I</td>
<td>[199]</td>
</tr>
<tr>
<td></td>
<td>Advanced solid tumours (along with cisplatin)</td>
<td>Phase I</td>
<td>[40, 200] (NCT00116896)</td>
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<td>Locally advanced or metastatic biliary tract adenocarcinoma (along with 5-fluorouracil/leucovorin)</td>
<td>Phase II</td>
<td>[40, 201] (NCT00088270)</td>
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<td>Locally advanced or metastatic SCC of the head and neck</td>
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<td>[40] (NCT00116909)</td>
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<td><strong>Liposome ether lipid</strong></td>
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<tr>
<td>TLC ELL12 (Elan Pharm)</td>
<td>Refractory solid tumours</td>
<td>Phase I</td>
<td>[202]</td>
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<td></td>
<td>Lung, prostate, skin cancer</td>
<td>Phase I</td>
<td>[203]</td>
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*Liposomes containing tumour-derived antigen and IL-2*

LEM: Liposome-encapsulated mitoxantrone

SCC: Squamous cell carcinoma

### 5.2.8 Nitrogen Mustard Analogues: Liposomal Chlorambucil

Chlorambucil is an orally active anti-neoplastic aromatic nitrogen mustard alkylating agent that has been mainly used in the treatment of chronic lymphocytic leukaemia [207]. It is orally administered but is rapidly metabolised, and thus its stability in aqueous environments is low and quickly eliminated. Ganta and co-workers [208] developed a long-circulating liposomal formulation of chlorambucil to improve its pharmacokinetics and tissue distribution, which they found
to improve its therapeutic efficacy. They reported it to significantly enhance its therapeutic effect on colon adenocarcinoma tumour in mice with no apparent increase in toxicity [208].

Another strategy developed by Pedersen and co-workers [209] is to insert the lipid-chlorambucil prodrug conjugates to liposomes. This would solve the problems of drug leakage during circulation because the lipids protect the active compound. The drugs are contained in the liposome membrane until activated by the enzyme, secretory phospholipase A2, which overexpressed in tumours.

5.3 Ligand-mediated Targeting Liposomes

Although PEGylated liposomes have many advantages, passively targeted liposomes have some disadvantages. Normal organ uptake of liposomes leads to the accumulation of the encapsulated drug in MPS cells in the liver, spleen and bone marrow [149], which may cause toxicities to these tissues. With the increased circulation time and confinement of the particulate drug delivery system comes an increased possibility of haematological toxicities, including neutropenia, thrombocytopenia and leucopenia [210, 211]. In addition, the use of Doxil has introduced new side effects, including hand-foot syndrome and mucositis [212, 213], which may result from the slow release of the drug [214]. Another disadvantage can be seen with the use of the liposomal cisplatin formulation (SPI-077), which has been found to accumulate substantially in tumour tissues but to have no anti-tumour effect [149, 215]. Cisplatin, which cannot diffuse through the intact liposomal membrane, stays inside the PEGylated liposome and thus displays little anti-tumour activity [216].

The rate of release of drug from the nanoparticles can be controlled to match the pharmacodynamic profile of the drug. This can be achieved by two approaches: (i) conjugating a targeting ligand to increase the rate of intracellular delivery and bioavailability of the drug and (ii) incorporating a pH-sensitive component in the formulation to facilitate the escape of the drug from the endosomal compartment, or physically triggering drug release locally at the target tissue (Figure 5.1). Ongoing research aims to develop what is known as active or ligand-mediated targeting liposomes in order to improve the tumour site-specific action of the delivery system [30, 31, 217–219]. Liposomes with ligands that target internalising antigens have been found to reach tumour cells more specifically, increase the bioavailability of the anti-cancer agent in solid tumours and have greater cytotoxic effects [23, 30, 31]. Therefore, using ligands that specifically bind to cancer cells (including cancer stem cells) and tumour blood vessels with
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Figure 5.1 Multifunctional targeting liposomes. A single lipid bilayer membrane separates an internal aqueous compartment from the external medium. Combination of anti-cancer drugs or imaging agents was encapsulated in the internal compartment. The incorporation of surface charge lipids allows for the binding with DNA or RNA interference (RNAi). Tumour-targeting ligands like antibodies, single-chain variable fragments (scFv), peptides or other recognition ligands were coupled to PEG-DSPE (polyethylene glycol-derived distearoylphosphatidylethanolamine). These targeting liposomes are capable of detecting and killing malignant cells, and monitoring the treatment effect. The targeting liposomes using drug or ligand combinations target the same liposomal drug against different tumour-associated molecules, target different drugs against the same tumour-associated molecule, or target different drugs against different tumour-associated molecules. After IV administration, targeting liposomes can extravasate and on arrival in the tumour tissues, they are bound and internalised by cancer cells through receptor-mediated endocytosis and encapsulated drugs are released into the intracellular space of the cancer cells. Combinations of liposomal drugs may help to lower drug dosages and increase responses, which can help reduce drug toxicities.

liposomal drug delivery system enhances the ability of that system to attack cancer cells and minimises side effects.

Differences in plasma membrane molecules of cancer cells or tumour blood vessels can be used to guide therapeutic or imaging agents to tumour tissues [23, 218, 220]. Such molecular markers in tumour tissues can serve as targets for anti-tumour treatment. Moieties that target cancer cells include monoclonal antibodies or scFv (single-chain variable fragments) antibody fragments, peptides, growth factors, carbohydrates,
glycoproteins and receptor ligands that are overexpressed or selectively expressed on cancer cell surfaces [28, 30, 218, 221].

5.3.1 Antibody-mediated Targeting Liposomes

Antibodies or antibody fragments are frequently used as targeting moieties for targeting liposomes. A significant advantage in using this class of targeting agents is phage display that can be used to select antibody fragments with high selectivity and high affinity for the target cells. Although various monoclonal antibodies have been shown to deliver liposomes to many targets, the optimisation of properties of immunoliposomes is an ongoing concern.

HER2 is a receptor tyrosine kinase, a product of the HER2 (c-erbB2) proto-oncogene, which has been shown to play an important role in the development and progression of breast, ovarian and other cancers. An interesting concept was developed to target HER2-overexpressing tumours using anti-HER2 liposomes [222]. The binding of anti-HER2 targeting liposomes, immunoliposomes (liposomes coated with a targeting antibodies or antibody fragments), is followed by rapid receptor-mediated endocytosis and subsequent intracellular drug release in HER2-overexpressing cancer cells [223, 224]. Uptake of anti-HER2 targeting liposomes in HER2-overexpressing cancer cells can be as high as 23,000 liposomes per cell [224]. Anti-HER2 targeting Lipo-Dox has been reported to display efficient intracellular uptake in tumour cells throughout tumour tissue, while nontargeted liposomes were found to be restricted to tumour stroma, either in the extracellular space or within tissue macrophages [225]. Immunoliposome delivery is also highly specific, with total uptake more than 700-fold higher in HER2-overexpressing than in nonoverexpressing cells [222]. Anti-HER2 targeting liposomal doxorubicin (Lipo-Dox) administered via IV in three weekly doses was found to have highly potent anti-cancer activity associated with tumour inhibition, regressions of large established tumours and cures [218]. Recently, it was found that anti-HER2 scFv antibody fragment did not cause any significant change in the clearance rate of liposomal doxorubicin [226]. Optimal results were reportedly obtained with anti-HER2 targeting liposomal doxorubicin formulated with 15 ligands per liposome [226]. Anti-HER2 targeting liposomal doxorubicin retains most of its original binding capacity to HER2-expressing cells after in vivo passage, indicating that the ligand is stably maintained in vivo in association with the doxorubicin liposomal carrier [226].

Randomised clinical trials have associated overexpressed HER2 with clinical improvements achieved from anthracycline-based chemotherapy [227–229]. Recombinant mAb HER2 (RhuMAb, also known as trastuzumab; Herceptin®), a humanised
mAb directed against HER2, is approved for the treatment of women with HER2-overexpressing metastatic breast cancer. The clinical efficacy of Herceptin is improved when it is used in combination with doxorubicin, but this combination increases the cardiac toxicity [230, 231]. Anti-HER2 targeting Lipo-Dox may represent ideal therapy for HER2-overexpressing cancer cells, resulting in less cardiotoxicity than doxorubicin or the combination of doxorubicin plus trastuzumab. Anti-HER-linked Lipo-Dox clinical trials, NSC 701315, have been established for good manufacturing practice manufacture of anti-HER2 targeting Lipo-Dox, and further development is ongoing in collaboration with Alza Corp (Mountain View, CA, USA) and Centocor, Inc. (Malvern, PA, USA) [85].

A chimeric mAb to epidermal growth factor receptor (EGFR), IMC-C225 (Erbitux®), has received FDA approval for the treatment of irinotecan refractory or intolerant metastatic colorectal cancer. Targeting liposomes were constructed using either C225-Fab′ or novel scFv C10 derived from a phage-displayed antibody library. EGFR-targeted immunoliposomes were specifically bound to and were internalised into EGFR-expressing cell lines in vitro, and EGFR-targeted immunoliposomes containing the anti-cancer drugs doxorubicin, vinorelbine or methotrexate were significantly more cytotoxic than the corresponding nontargeted liposomal drugs [232]. Pharmacokinetic and biodistribution studies confirmed long circulation times (t1/2 = 21 hours) and efficient accumulation in tumours (up to 15% ID/g) irrespective of the presence of the targeting ligand. Although total accumulations of anti-EGFR immunoliposomes and nontargeted liposomes in EGFR-overexpressing tumours were comparable, only immunoliposomes internalised extensively within tumour cells (92% of analysed cells versus <5% for nontargeted liposomes), indicating different mechanisms of delivery at the cellular level [233].

B- and T-cell malignancies are present in blood or lymph nodes and should be more accessible to targeting liposomes than solid tumours. Some studies have developed the targeting of immunoliposomal anti-cancer drugs against the B-cell antigens CD19 and CD20 in xenograft models of human B-cell lymphoma [30, 234]. CD19 or CD20 are B lineage-restricted antigens that have little or no expression on the stem cells. Normal B cells express these antigens and may be killed during therapy, but bone marrow stem cells are expected to reconstitute the normal lymphocyte population. In vitro anti-CD19- or anti-CD2-targeted liposomal formulations of doxorubicin or vinorelbine had increased cytotoxicity against B-cell lymphoma relative to nontargeted liposomes. In vivo, a significant improvement in therapeutic response was associated with either doxorubicin- or vinorelbine-loaded targeting liposomes against the CD19 compared with nontargeted liposomal drug or free drug. When targeted against the noninternalising antigen CD20, immunoliposomal vinorelbine, but not immunoliposomal doxorubicin, had greater therapeutic responses than the corresponding nontargeted liposomal drug or free drug. Allen and co-workers [235] showed that
anti-CD19-targeted liposomal doxorubicin improved the therapeutic efficacy in murine B-cell lymphoma and the toxicities of liposomal doxorubicin could be reduced by targeting with anti-CD19 antibodies. In a comparison of circulation half-life of whole monoclonal antibody-, Fab’- and scFv-conjugated anti-CD19 liposomal doxorubicin, it was found that liposomal doxorubicin targeted via whole monoclonal antibody was cleared most rapidly from the circulation due to Fc-mediated uptake in the liver and spleen [236]. Liposomal doxorubicin targeting via Fab’ fragments had the longest circulation half-life. All three formulations (whole monoclonal antibody, Fab’ and scFv) of immunoliposomes extended the mean survival time of Raji-bearing mice compared to liposomal doxorubicin or free doxorubicin [236]. Immunoliposomal doxorubicin, targeted via an anti-idiotype antibody to murine B-cell lymphoma, has also been found to prolong survival in mice compared to nontargeted liposomes or free doxorubicin [237].

Another current clinical target for B-cell lymphoma is CD22, a B-cell specific member of the sialic acid-binding Ig-like lectin (siglec) family that recognises α2-6-linked sialylated glycans as ligands [238–240]. The internalising mAb (LL2) against CD22 antigen has also been used to increase the cytotoxicity of liposomal doxorubicin against lymphoma cells [241]. Recently, the targeting liposomes conjugated with high-affinity glycan ligands of CD22 have been found to actively to be bound to and endocytosed by CD22 on B cells, significantly extending life in a murine model of human B-cell lymphoma. Moreover, the ligand-conjugated liposomes have been found able to recognise and kill malignant B cells from peripheral blood samples obtained from patients with hairy cell leukaemia, marginal zone lymphoma and chronic lymphocytic leukaemia [242].

Human NB cells express abundant amounts of the disialoganglioside GD2 at their cell surface and the presence of this antigen on normal tissues, such as the cerebellum and peripheral nerves, is limited. Pastorino and co-workers [243] demonstrated that treatment of nude mice xenografts of the human NB cells with doxorubicin-loaded liposomes conjugated to Fab’ fragments of anti-GD2 resulted in long-term survival. In another study, anti-GD2 immunoliposomes containing fenretinide-induced apoptosis in NB lines and demonstrated strong anti-NB activity in vivo [244]. Since NB-derived tumours, such as melanoma, express abundant amounts of the GD2 antigen, this may be a good targeting molecule for immunoliposomes in the treatment of other GD2-positive tumours. NB tumour-bearing mice injected with immunoliposomes conjugated with anti-GD2 antibodies and entrapping a c-myb antisense oligonucleotide containing CpG motifs (anti-GD2-Lipo-CpG-myb-AS) have been found to improve anti-tumour activity and increase survival time (i.e., more than 120 days) [245]. Asparagine-glycine-arginine (NGR) peptide-conjugated liposomal doxorubicin (NGR-Lipo-Dox) reduced the angiogenic potential of various NB xenografts, with synergistic inhibition observed for the combination of NGR-Lipo-Dox with anti-GD2
antibody-conjugated liposomal doxorubicin (anti-GD2-Lipo-Dox). A significant improvement in anti-tumour effects has been seen in NB-bearing animal models treated with the NGR-Lipo-Dox and anti-GD2-Lipo-Dox formulations [246]. Long-term survival was achieved only in animals treated with the combined tumour- and vascular-targeted formulations, confirming the pivotal role of combination therapies in treating aggressive metastatic NB [246].

In a solid tumour model, Sugano and co-workers [219] have demonstrated that treatment of severe combined immunodeficient (SCID) mice bearing established lung tumour xenografts, with immunoliposomal doxorubicin targeted via internalising anti-β1 integrin Fab’ fragments significantly suppressed tumour growth as well as prevented the metastasis of tumour cells to liver and adrenal glands. Nucleosome-specific antibodies capable of recognising various tumour cells through tumour-cell-surface-bound nucleosomes improved Doxil targeting to tumour cells and increased its cytotoxicity [247].

Novel mAb fragments can now be rapidly derived from a high-diversity repertoire, such as fully human scFv from a phage-displayed antibody library. The development of rapid and efficient methods to conjugate scFv to liposomes now makes this strategy highly feasible. This strategy will accelerate the progress of immunoliposomes in the future.

5.3.2 Peptide-mediated Targeting Liposomes

Although monoclonal antibodies have shown clinical potential as tumour-targeting agents, they are limited by immunogenicity associated with immunoliposomes, toxicity to liver and bone marrow from nonspecific antibody uptake, large molecular size and poor tumour penetration [248, 249]. These limitations can be overcome by using peptide ligands, which are smaller, less immunogenic molecules and easier to produce and manipulate. Furthermore, peptide ligands have moderate affinity to antigens, which is beneficial because extremely high affinity of antibody binding can impair tumour penetration [250]. Compared with antibody ligands, peptide ligands can improve tumour penetration and decrease MPS clearance of conjugated liposomes [217, 249, 251, 252]. The increasing use of peptides as targeting ligands has been aided by the method of phage display to identify novel ligands. Ongoing research aims to enhance the tumour site-specific action of the liposomes by attaching them to peptide ligands that target tumour cell [23, 217, 251] and tumour vasculature surface molecules [28, 31, 253].

In one study, investigators have identified the three-amino acid peptide motif, NGR that can target angiogenic blood vessels [220]. The tumour-homing property of the
NGR motif relies on recognition of an isoform of the CD13/aminopeptidase N (CD13/APN) selectively expressed within tumour blood vessels [27, 253, 254]. CD13/APN transcription has been shown to be a potent regulator of angiogenesis and it has been shown to be induced by angiogenic growth factors via the RAS/MAPK pathway [255, 256]. In one study, an NGR-containing peptide was coupled to the surface of liposomal doxorubicin and was used to treat orthotopic NB xenografts in SCID mice [243]. That study reported apoptosis of the tumour endothelial cells and destruction of the tumour vasculature. Indirect tumour cell kill occurred when tumour endothelium was destroyed by NGR-targeted liposomes, which led in turn to direct tumour cell kill via localisation of the NGR-targeted liposomal doxorubicin to the tumour interstitial space with release and uptake of the liposomal drug by the tumour cells [243].

Laakkonen and co-workers [257, 258] identified a cyclic nine-amino acid peptide LyP-1 (Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys) that could selectively recognise lymphatics and tumour cells in certain tumour types and subsequently inhibit tumour growth. Fluorescein-labelled LyP-1 peptide was detected in tumour structures that were positive for three lymphatic endothelial markers and negative for three blood vessel markers [257]. Recently, it was found that the p32 or gC1qR receptor, whose expression is elevated on the surface of tumour-associated cells undergoing stress, is the target molecule for the LyP-1 peptide [259]. Mice containing MDA-MB-435 xenograft tumours that are treated with the LyP-1-targeted cooperative nanomaterial system have been found to have significant reductions in tumour volume compared to those containing individual nanoparticles or untargeted cooperative system [260].

A novel 5-mer peptide Ala-Pro-Arg-Pro-Gly (APRPG) was isolated from a phage display peptide library from angiogenic vessels formed by a dorsal air sac method [261]. APRPG-modified liposomes DSPE-PEG-APRPG encapsulating doxorubicin, were able to suppress tumour neovascularure in colon carcinoma growing in mice [261]. Another angiogenic homing peptide Gly-Pro-Leu-Pro-Leu-Arg (GPLPLR) against membrane type-1 matrix metalloproteinase (MT1-MMP) has also been identified from a phage-displayed peptide library [262]. Positron emission tomography showed fourfold more tumour accumulation in methylcholanthrene-induced sarcoma-bearing mice of the targeted liposomes over nontargeted liposomes and a significant suppression of tumour growth, associated with damage to angiogenic endothelial cells [262]. Liposome targeting via vasoactive intestinal peptide (VIP), a 28-amino acid mammalian neuropeptide, improved imaging of breast cancer in a rat model [263]. VIP was covalently attached to the surface of stealth liposomes containing a radionuclide, Tc-99m hexamethylpropylene amine oxime (Tc99-m HMPAO) and proved useful for tumour imaging [263].

A new peptide that binds specifically to nasopharyngeal cells and translocates across cell membranes, known as l-peptide, was identified by Lee and co-workers [217] from a phage-displayed random peptide library. l-peptide-conjugated liposomes
containing doxorubicin produced marked cytotoxicity of nasopharyngeal cells and their xenografts in SCID mice [217]. High-affinity cyclic arginine-glycine-aspartic acid (RGD) peptides have been isolated from phage display RGD motif libraries of endothelial or melanoma cells [264]. A cyclic RGD peptide coupled to liposomal doxorubicin has been shown to have improved efficacy in a C26 colon carcinoma mouse model compared to free doxorubicin or untargeted liposomes [264]. In a study using a doxorubicin-insensitive mouse model of C26 colon carcinoma, RGD-coupled long-circulating liposomal doxorubicin showed superior therapeutic efficacy compared to nontargeted liposomes [265].

Lo and co-workers [251] identified a novel targeting peptide by using phage-displayed random peptide library screening, which specifically binds to the cell surface of hepatocellular carcinoma (HCC) cells both in vitro and in vivo. With conjugation of the targeting peptide and liposomes containing doxorubicin, an improvement of therapeutic efficacy in HCC xenografts was observed without systemic side effects. Recently, Du and co-workers [266] also identified the HCC-specific peptide, AGKGTPSLETTP, by in vivo phage display. Reduction in tumour size and prolongation of long-term survival were also found in xenograft-bearing models compared with free doxorubicin-treated group. Chang and co-workers [23] isolated several novel peptides capable of binding to NSCLC cell lines. Targeting peptides were both able to recognise the surgical specimens of NSCLC. When the targeting peptide was coupled to liposomes carrying vinorelbine or doxorubicin, therapeutic index and survival rate markedly increased. Furthermore, the increase in drug accumulation in tumour tissues brought about by the use of targeting liposomes was 5.7 times higher than that brought about by the use of free drugs and the higher concentration of bioavailable doxorubicin delivered by these liposomes markedly increased cancer cell apoptosis [23].

The use of liposomes with peptide ligands that target tumour blood vessels may combine blood vessel and tumour cell destruction with conventional anti-cancer activities of the encapsulated drug. In mice bearing human cancer xenograft, low dose of SP5-52-conjugated liposomal doxorubicin markedly inhibited tumour vascularisation and tumour growth [28]. The severe damage to tumour vasculature caused by SP5-52-conjugated liposomal doxorubicin throughout the tumours suggested an improvement in chemotherapeutic efficacy [28]. This potential for dual action of the targeted liposomal doxorubicin to tumour cells and blood vessels may result in a higher and more durable anti-cancer effect than a simple anti-angiogenic approach. The increased level of tumoural accumulation of the targeting liposomes was correlated with its anti-tumour efficacy, which was superior to that of the free drug as well as the drug formulated in the nontargeting liposomes [31]. Tumours treated with targeting liposomes demonstrated a marked decrease in vessel density, high level of apoptotic death and delayed tumour growth compared to other control treatments [28, 31]. Proteolytic degradation of the
basement membrane by the MMP-2 and MMP-9 is an essential step in tumour angiogenesis. Recently, Mueller and co-workers [267] found a phage-displayed peptide sequence TLTYTWS that specifically binds to collagen IV modified by MMP-2. TLTYTWS peptide inhibits angiogenesis in an in vivo assay in a concentration-dependent manner.

Peptide-mediated targeting liposomes have been found to circulate for longer periods in the blood maximising delivery to the high interstitial fluid pressure (IFP) of the tumour site [23, 28, 31] and to be less toxic to blood WBC [23, 251]. This enhanced the therapeutic index of drug in its treatment of several human cancer xenografts [28, 31]. Peptide-mediated liposomes that target tumour tissues represent a new generation of chemotherapy delivery systems that offer superior efficacy and safety profiles. For future clinical development, peptide-mediated targeting liposome components including targeting ligands, conjugation methodologies, liposome designs and chemistry, manufacturing and control (CMC) management can be further optimised and refined.

### 5.3.3 Folate-mediated Targeting Liposomes

Targeting tumours with folate-modified liposomes represents a popular approach, because folate receptors (FR) are frequently overexpressed in a range of tumour cells. After early studies established the possibility of delivering macromolecules [268] and then liposomes [269] into living cells using FR endocytosis, research interest in folate-targeted drug delivery by liposomes grew rapidly [221, 270]. Liposomal daunorubicin [271] as well as doxorubicin [272] have been delivered into various tumour cells through FR increasing cytotoxicity. The application of folate-modified doxorubicin-loaded liposomes to the treatment of acute myelogenous leukaemia was combined with the induction of FR using all-trans retinoic acid [272]. It has been proposed that folate-targeted liposomes can be used as delivery vehicles for boron neutron capture therapy [273] and also used for targeting tumours with haptens for tumour immunotherapy [270]. Within the field of gene therapy, folate-targeted liposomes have been used for both gene targeting to tumour cells [274] and for targeting tumours with antisense oligonucleotides [275].

### 5.3.4 Transferrin-mediated Targeting Liposomes

Transferrin receptors (TfR) are overexpressed on the surface of many tumour cells. Therefore, antibodies against TfR, as well as transferrin (Tf) itself, are popular ligands for liposome targeting to tumours and inside tumour cells [276]. The studies involve the coupling of Tf to PEG on PEGylated liposomes to combine longevity and target ability for drug delivery into solid tumours [277]. A similar
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approach was applied to the intracellular delivery of cisplatin into gastric cancer [278]. Tf-coupled doxorubicin-loaded liposomes demonstrate increased binding and toxicity against C6 glioma cells [279]. Tf [280] as well as anti-TfR antibodies [281, 282] have also been used to facilitate gene delivery into cells by cationic liposomes.

CALAA-01 (Calando Pharmaceuticals-01) is a TfR-targeted anti-cancer drug designed to inhibit tumour growth. The active ingredient in CALAA-01 is a small interfering RNA (siRNA). This siRNA inhibits tumour growth via RNA interference to reduce expression of the M2 subunit of ribonucleotide reductase (RRM2). The CALAA-01 siRNA is protected from nuclease degradation within a stabilised polymer delivery system targeted to tumour cells [283, 284]. Upon administration, TfR-targeted anti-RRM2 siRNA CALAA-01 binds to TfR, releases anti-RRM2 siRNA after endocytosis and silences the expression of RRM2 via the RNAi pathway, thus inhibiting the tumour growth of TfR-overexpressed cancer cells [283, 285]. Phase I safety study of IV CALAA-01 in adults with solid tumours was started on 29 May 2008 (clinicaltrials.gov/ct2/show/NCT00689065).

5.4 Future Directions of Liposome-based Anti-cancer Drugs

The advances in liposomal drug delivery systems suggest the potential for the development of multifunctional ‘smart drug delivery systems’ which can be decorated with different types of targeting ligands, diagnostic or imaging reagents and the therapeutic anti-cancer drugs (Figure 5.1). The availability of such multifunctional drug delivery systems in the near future would allow us to detect, target, modulate delivery and track the progression of therapy. Future directions for liposomal delivery systems include new drug candidates; direct intracellular delivery to overcome pharmacologic limitations of free drugs, including inadequate selectivity or poor cellular uptake; and biological agents, including protein-based and RNAi-based constructs (Figure 5.1).

In the near future, multifunctional liposomes may be capable of detecting malignant cells in the body, killing the cancer cells with minimal side effects and monitoring treatment effects remotely and noninvasively. Some highly complex nanoparticles have reached the clinic. For example, CALAA-01 is a four-component system that assembles into a highly multifunctional, targeted nanoparticle that contains siRNA. This multicomponent system is now in clinical studies and this example shows that complex nanoparticles can be manufactured at cGMP and satisfy regulatory requirements.
Currently, there are several nanoparticle-based formulations in clinical trials showing decreased side effects or improved efficacy, but none of them replace the market of the free drug. The unsolved issues regarding the limited tissue targeting and drug release and the high cost of the nanoparticle-based formulation restricts its widespread use. It is anticipated that the emergence of new nanotechnologies will facilitate the development of new delivery systems that not only specifically deliver drugs to the target tissue but also release them locally and efficiently. In addition, inexpensive but safe materials have to be explored to reduce the cost of the nanotechnology-based therapy so that a greater patient population can benefit.

There is no doubt that nanoparticle therapeutics with increasing multifunctionality will exist in the future. As newer and more complex nanoparticle systems appear, better methodologies to define biocompatibility will need to be created, especially those that can assess intracellular biocompatibility. While the details of issues regarding scale-up and cGMP production are not often discussed, sophisticated nanoparticles, denoted as CALAA-01 show that efforts towards overcoming cGMP and regulatory hurdles is progressing. Although many challenges exist for the translation of nanoparticles into approved products for patients, their potential advantages should drive their successful development as well as fuel the continuing emergence of a new class of anti-cancer therapies.

5.5 Conclusions

The poor pharmacokinetic profiles, narrow therapeutic windows and the indiscriminate harm to normal tissues without preference for tumour sites of all current cytotoxic chemotherapy drugs limits their use. Liposomal systems, such as RES-avoiding and long-circulating systems, can provide stable formulation, improved pharmacokinetics and ‘passive’ targeting to tumour tissue. More recently, many liposomal drugs including clinical or preclinical products for gene delivery and cancer therapy stills being the principal areas of interest. The newest generation of drug carriers under development, which represent multicapability nanotechnology-oriented strategies, features direct molecular targeting of cancer cells via ligand-mediated interactions for greater specificity and efficiency.

Nanoparticles provide opportunities for designing and tuning properties that are not possible with other types of therapeutics, and as more clinical data become available, the nanoparticle approach should improve further as the optimal properties are elucidated. Liposome-based drug delivery systems are evolving, and newer, more sophisticated multifunctional nanoparticles are reaching the clinic. Results from these trials are already fuelling enthusiasm for this type of
therapeutic modality. Some of the important features of liposomal drug delivery systems, such as extended pharmacokinetic data, observed in preclinical studies have been confirmed in humans; pump-mediated multidrug resistance (MDR) might be overcome; and side effects significantly reduced while providing improved efficacy. These combined features may allow for new therapeutic strategies such as maintenance therapy.

The development of targeting liposomes has been made possible by advances in liposomal systems. Targeting liposomes consisting of novel ligands conjugated to liposomal doxorubicin, currently in development, should make possible selectively binding to and internalising in tumour cells. The modular organisation of targeting liposomes technology enables a combinatorial approach in which a repertoire of peptide or scFv libraries can be used in conjunction with a series of liposomal drugs to yield a new generation of molecularly targeted agents. In addition to targeting ligands, development of novel liposomes includes additional classes of anti-cancer drugs formulated into one of these existing platforms. For example, future directions for liposome delivery drugs include new drug candidates, novel biological agents and oligonucleotide-based constructs.

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Han-Chung Wu, Yi-Hsuan Chi and Chien-Hsun Wu


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6 Cationic Liposomes and Tumour Vasculature Targeting: A Therapeutic Approach that has Potential for Solid Tumours

Amr S. Abu Lila, Tatsuhiro Ishida and Hiroshi Kiwada

6.1 Introduction

One of the primary goals of cancer treatment is to deliver sufficient amounts of the drug to targeted tumours, while minimising delivery to normal tissues. However, the efficacy of most chemotherapeutic agents is compromised, at least in part, by the dose-limiting side effects resulting from the indiscriminate distribution of the agents to normal tissues. In addition, the dose that reaches the tumour may be as little as 5–10% of the dose that accumulates in normal organs [1, 2]. This is due to the high interstitial fluid pressure of solid tumours, compared to that of normal tissue, which leads to decreased transcapillary transport of anti-cancer drugs into tumour tissues [3–5]. Consequently, tumour cells are exposed to a lower effective concentration of the drug than normal cells. This phenomenon is usually accompanied by the development of drug resistance, metastasis and eventual therapeutic failure [6, 7].

One avenue towards the development of more selective anti-cancer drugs consists of the targeted delivery of anti-cancer drugs to the tumour by means of nanocarrier delivery systems [8]. Among the commonly used nanocarrier delivery systems, liposomes have received the most widespread clinical acceptance as versatile carriers of cytotoxic drugs [9–12]. However, earlier clinical trials were potentially limited by rapid elimination of the liposomes from systemic circulation, mostly because of uptake by the cells of the mononuclear phagocyte system (MPS). Coating liposomal surface with a water-soluble and flexible polymer, such as polyethylene glycol (PEG), prevented liposomes from interacting with serum opsonins and prevented direct recognition by the MPS cells and thus prolonged their blood circulation time [12–15]. In addition, PEGylated liposomes 100–200 nm in size were confirmed to accumulate selectively in tissues with increased vascular permeability, such as those found at tumour sites or caused by infection or inflammation [16, 17], via a phenomenon known as the ‘enhanced permeability and retention (EPR) effect’ [18, 19]. The advantages of these liposomes led to their being marketed worldwide, starting
in 1995, as doxorubicin-encapsulating PEG-coated liposomes (PEGylated liposomal doxorubicin; Doxil) [20, 21].

The growth of solid tumours is dependent on their capacity to induce the growth of blood vessels to supply them with oxygen and nutrients. The blood vessels in tumours present specific characteristics not observed in normal tissues, including extensive angiogenesis, leaky vascular architecture and impaired lymphatic drainage [22]. These unique physiological features of tumour vasculature have been exploited extensively for the passive accumulation of PEG-coated liposomes (100–200 nm) in solid tumours via the EPR effect [18, 19], which results in selective tumour targeting of anti-cancer drugs associated with the liposomes. However, the use of such liposomes to achieve efficient passive tumour targeting is somewhat limited. The high interstitial pressure of tumour tissue poses a potential barrier against the efficient delivery and distribution of the anti-cancer drug, either in liposome-associated or free form, in the tumour interstitium. Enhanced uptake of liposomes by the liver and spleen sometimes causes cytotoxicity in these tissues, as a result of enhanced accumulation of the encapsulated drug [23].

6.2 Tumour Cell Targeting versus Tumour Vasculature Targeting

In addition to the barriers described above, many physiological barriers are known to hinder the effective delivery of drugs associated with long-circulating liposomes, such as PEG-coated liposomes, to tumours [24, 25]. The relatively high interstitial fluid pressure, disorganised arrangement of tumour vessels and long interstitial drug transport distances pose the most serious threats to the success of interstitial targeting strategies [5, 26]. In addition, in certain solid tumours, overexpression of the P-glycoprotein gene provokes drug resistance to certain therapeutic agents such as doxorubicin and cisplatin [27]. Furthermore, to achieve the targeting of cells in a solid tumour, the liposome must first extravasate from the vasculature into the interstitium, and then travel through the interstitium into the tumour tissue [28]. This process hinders the effective delivery of anti-cancer drugs associated with liposomes to solid tumours.

The targeting of vascular endothelium presents several advantages compared with tumour targeting. First, normal endothelial cells are quiescent and, therefore, side effects on the nontargeted endothelium are expected to be minimal [29]. Secondly, vascular endothelial cells are genetically stable, nontransformed cells that are presumably less adaptive than tumour cells and less likely to acquire mutations leading to drug resistance [30]. Thirdly, endothelial cells proliferating in solid tumours share similar phenotypes among different tumours. This makes vascular targeting applicable to a wide variety of tumour types [31]. Fourthly, effective and uniform delivery of
anti-cancer drugs to solid tumours has proved challenging: the drugs do not penetrate beyond the perivascular region, due to physical barriers presented by fibrous tumour tissue and to elevated interstitial pressure, which reduces fluid convection, as discussed earlier [32]. In contrast, endothelial cells lining the vessels are easily accessible to the bloodstream, and consequently the liposomes efficiently reach and bind to the cells. Finally, many cancer cells depend upon a few endothelial cells for their growth and survival, and, therefore, the death of a single endothelial cell may result in the death of more than 100 tumour cells [33, 34].

Many recent studies have shown that endothelial cells lining tumour vessels overexpress specific cell surface antigens, which are absent or barely detectable in established or quiescent normal blood vessels. These unique characteristics of tumour endothelial cells can be exploited to achieve active vascular targeting of liposomes. This is accomplished by coupling specific molecules, such as antibodies, specific peptides, growth factors or a cationic charge, to the surface of liposomes [35–38]. This chapter, however, focuses on cationic liposomes as promising carriers for the delivery of small molecular therapeutics (anti-cancer drugs) to tumour vasculature.

### 6.3 Tumour Angiogenesis

A pioneering study at the National Cancer Institute by Glenn Algire in 1945 [39] led to the conclusion that ‘the rapid growth of tumour explants is dependent on the development of a rich vascular supply’. Later, much evidence supported the concept that most tumours are dependent on angiogenesis for the provision of oxygen and nutrients to the tumour cells, to sustain their progressive growth. In 1971, Folkman [40] proposed the innovative idea that tumour growth and metastasis are angiogenesis-dependent. Thus, angiogenesis inhibitors could be used to treat cancer. In 2004, the U.S. Food and Drug Administration (FDA) approved the first anti-angiogenic drug for the treatment of cancer – a humanised monoclonal antibody against vascular endothelial growth factor-A (VEGF-A) called Avastin (bevacizumab) [41].

### 6.3.1 Formation of Tumour Vessels

Tumour angiogenesis, the formation of neovessels from preexisting ones, is not a singular process; at least two types of angiogenesis are believed to contribute to vessel growth in tumours [42]. One involves the stimulation of new blood vessel capillaries that sprout in the vasculature of the neighbouring mature host [43]. The other involves the recruitment of circulating endothelial precursor cells, shed from the vessel wall or mobilised from the bone marrow, to promote neovascularisation [44, 45]. Various molecular regulators are involved in these different mechanisms.
of vascular growth. Among these, members of the families of vascular endothelial growth factor (VEGF) and angiopoietin (Ang) have a predominant role [46, 47]. In addition, several molecules, including a number of angiogenesis inhibitors, seem to be involved in tumour angiogenesis. The temporal and spatial expression of these regulators is not as well coordinated in tumours as in physiological angiogenesis, and their mechanism of action is poorly understood [48].

Tumour vessels also lack protective mechanisms that normal vessels attain during growth. They may lack functional perivascular cells that provide necessary vasoactive control to accommodate metabolic needs, in response to changes in oxygen or hormonal balance, and to induce vascular quiescence. Finally, the vessel wall is not always formed by a homogenous layer of endothelial cells [49]. Instead, it may be lined with only cancer cells or a mosaic of cancer and endothelial cells [50].

### 6.3.2 Structure and Function of Tumour Vessels

#### 6.3.2.1 Chaotic Architecture and Blood Flow

With the increasing promise of vascular targeting in solid tumours, a thorough understanding of the cellular structure and function of tumour vessels has become even more important. In comparison with blood vessels in normal tissues, tumour vessels are recognised as dynamic, both in terms of the formation of new vessels by angiogenesis and the remodelling of existing vessels [51, 52]. Tumour vessels are tortuous and tend to be arranged in irregular arrays and to have dilated lumens [53]. In addition, arteriovenous shunts and trifurcations, not normally observed in normal tissue, are found in tumour vasculature [54]. These aberrant features of tumour vasculature lead to turbulent, uneven blood flow with frequent stasis and sudden changes in flow direction [55]. In addition, hypoxic and acidic regions may be created within tumour tissue [56]. These conditions lower therapeutic effectiveness, modulate the production of angiogenic stimulators and inhibitors and create the opportunity for tumour cells to be more malignant and metastatic.

#### 6.3.2.2 High Vascular Permeability

Tumour blood vessels are also abnormal in terms of their ultrastructure. Compared with the walls of normal blood vessels, the walls of tumour blood vessels have discontinuous or absent basement membranes, fewer pericytes and a lack of perivascular smooth muscle [57]. In addition, tumour blood vessels exhibit endothelial cell gaps, with an average size of ~100–600 nm [22]. These pores are significantly larger than the gaps found in normal endothelium, which are typically <6 nm wide [12]. These characteristics make tumour vessels leakier than normal vessels, and enable the
preferrential accumulation of macromolecules and polymeric drugs in tumour tissue via a passive targeting phenomenon, known as the EPR effect [18, 19].

6.3.2.3 Nonuniform Surface Markers

Tumour blood vessels are characterised by unique biochemical features that distinguish them from resting blood vessels. These features include the expression of a number of angiogenesis-related molecules, such as certain integrins, endothelial cell growth factor receptors, proteases and negatively charged macromolecules on the cell surface, such as glycoproteins, anionic phospholipids and proteoglycans, which are absent or barely detectable in established or quiescent normal mature blood vessels [58–60]. Such surface markers, designated as ‘vascular zip codes’, can serve as exploitable selective targets for the achievement of active vascular targeting of chemotherapeutic agents by means of nanocarrier systems, including liposomes.

6.3.3 Lack of Functional Lymphatics

The lymphatic vessels in tumours are also structurally and functionally abnormal in that functional lymphatics are absent within the tumour and enlarged at the periphery. One explanation may be that tumour cells grown in a confined space generate mechanical stress, which may compress the newly formed lymphatic channels inside the tumour [61]. On the other hand, at the tumour margin, where mechanical stress is predicted to be lower, functional lymphatic vessels as well as a greater fraction of open lymphatics are found [62]. These enlarged lymphatics may collect interstitial fluid and metastatic tumour cells, and thus facilitate lymphatic metastasis.

6.3.4 Methods for Evaluating Angiogenesis

As mentioned above, angiogenesis requires the coordinated function of a variety of molecules including proteases, growth factors, cell adhesion molecules and extracellular matrix components. The establishment of models to study angiogenesis is thus considered crucial for basic research in this area. A number of in vivo and in vitro models have been developed to test the efficacy of both pro- and anti-angiogenic agents. Angiogenesis involves cellular processes such as endothelial cell proliferation, migration and invasion [63]. However, recapitulation of all these processes in one comprehensive in vitro model has not been accomplished. Instead, a variety of in vitro models have been established, which may imitate certain cellular or biochemical events during the angiogenic cascade [64]. These
models include cell proliferation assay, cell migration assay and tube formation assay [65–67]. However, the cellular processes involved in angiogenesis do not occur in isolation, but are regulated instead. Therefore, these in vitro models are best viewed as providing initial information, which is subject to confirmation by in vivo assays.

To gain a more detailed understanding of angiogenesis as it occurs in tissues, and to discover and evaluate the potency of new anti-angiogenic compounds, a number of in vivo models that mimic the angiogenic cascade in the context of a true physiological microenvironment have been developed. Some of the in vivo models include the chick chorioallantoic membrane assay, rabbit cornea assay, Matrigel plugs model and mouse dorsal air sac (DAS) model [68–71]. Among various widely accepted in vivo models used to evaluate angiogenesis and the potency of new anti-angiogenic compounds, the DAS model is considered the most reliable screening method for nanocarrier systems that target tumour-induced neovascularity, and for evaluation of the anti-angiogenic efficacy of anti-cancer agents encapsulated in such nanocarrier systems [72]. In the mouse DAS model, a chamber is prepared by covering both sides of a Millipore ring (10 mm diameter, 3 mm thickness) with Millipore filters (0.45 μm pore size), and by filling it with a suspension of tumour cells. The chamber is then implanted into a subcutaneous dorsal air sac created by the subcutaneous injection of air (10 ml) in anaesthetised mice. At differing intervals after chamber implantation, the animals are euthanised and the skin is removed. The implanted chambers are removed from the subcutaneous fascia of the animals, and a black ring with the same inner diameter as that of the Millipore ring is placed at the same site. The angiogenic response, indicated by the formation of zigzag-shaped blood vessels, can be observed under a dissecting microscope, and the vascular response can be quantified by computer image analysis software.

6.4 Tumour Vascular Targeting with Cationic Liposomes

Tumour growth, progression and metastasis are critically dependent on blood supply, which has received increased attention as a potential target of new anti-cancer therapies. Anti-angiogenic therapy to limit and even reverse the growth of tumours is under investigation and shows promise. Moreover, as mentioned above, tumour endothelial cells express specific surface antigens, not present in the resting blood vessels of normal tissues, which are suitable for targeting purposes. These specific ‘vascular zip codes’ can be exploited to achieve active vascular targeting by means of nanocarrier drug delivery systems such as liposomes. Many approaches have been applied to enhance the efficiency of targeting liposomes to tumour endothelial cells. These include the coupling of specific molecules, such as antibodies, peptides or growth factors, or incorporation of cationic charges on the surface of the liposomes [73–75]. This chapter, however, is focused exclusively
on cationic liposomes as one of the most promising carriers for targeting tumour vasculature.

As early as 1987, a cationic liposome containing the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium chloride was utilised as a synthetic carrier to deliver genes into cells [76]. This pioneering work opened a new field of research into cationic liposomes, which serve as carriers for the delivery of genetic materials. In comparison with other gene delivery modes, such as viral vectors, cationic liposomes are technically simple and quick to formulate, not as biologically hazardous as viral vectors, effective in transferring genetic material of unlimited size and may be relatively easily adapted for specific applications [77]. There are, however, some drawbacks with lipid vectors, including lower efficiency than viral vectors in gene transfer and transient gene expression [78–80]. Such drawbacks have provoked a reevaluation of their use as gene vectors.

Recently, there has been renewed interest in cationic liposomes, mainly due to their inherent, yet unexplained, ability to selectively target tumour vasculature. This selective affinity of cationic liposomes to tumour vasculature provides an opportunity for the development of many anti-angiogenic and/or anti-cancer formulations based on cationic liposomes. In addition, a wide area of research has focused on manipulation of the structural features of cationic liposomes to improve their vascular targeting efficiency and reduce toxicity-related reactions.

### 6.4.1 Development of Cationic Liposomes Homing to Tumour Vasculature

We recently tried to develop PEG-coated cationic liposomes that have in vivo long-term circulation properties, as well as selective binding properties, to achieve tumour vascular targeting [72]. The DAS model was employed to evaluate whether designed PEG-coated cationic liposomes have the properties of long circulation and selective targeting to tumour angiogenic vessels. As described above, the DAS model is technically simple and provides a natural environment in which blood vessels and their tumour-induced formation can be studied. In addition, the model takes only about 5 days to develop; thus, it is less time-consuming than the tumour-bearing mouse model, which takes more than 10 days to develop.

Upon using the DAS model, we changed the lipid composition of the liposomes so that we could select a PEG-coated cationic liposome that accumulated preferentially and selectively in angiogenic vessels induced in the skin. A typical structure of PEG-coated cationic liposomes is described in Figure 6.1.

The selected PEG-coated cationic liposome was composed of the following in a molar ratio of 2:1:0.2:0.2, respectively: hydrogenated soy phosphatidylcholine...
Amr S. Abu Lila, Tatsuhiko Ishida and Hiroshi Kiwada

(HSPC), cholesterol (CHOL), O,O′-ditetradecanoyl-N-(α-trimethylammonioacetyl) diethanolamine chloride (DC-6-14), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-\(n\)-[methoxy (polyethylene glycol)-2000] (mPEG\(_{2000}\)-DSPE). The cationic lipid DC-6-14 was used generously to prepare a nonviral vector (cationic liposome) for the delivery of nucleic acids [81]. The size of the liposome was approximately 250 nm (homogenous size), and the zeta potential was relatively positive (+11.2 ± 0.7). In addition, the PEG-coated cationic liposome showed no selective accumulation/binding to preexisting mature blood vessels in the skin (Figure 6.2). This reveals an important difference between normal tissues and tumour tissues in the distribution of cationic liposomes in blood vessels, which may be exploited in attempts to achieve successful anti-angiogenic therapy. Kalra and co-workers [82] previously demonstrated that PEG-coated cationic liposomes, composed of 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl]-3-trimethylammonium propane (DOTAP), 1,2-dioleoyl-sn-glycerol phosphatidyl choline, CHOL and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-PEG5000 (PEG\(_{5000}\)-DOPE) in a molar ratio of (10:7:2:1), associate with approximately 27% and 5% of vessel areas in tumours and normal tissues, respectively, in human and murine tumour mouse models.

Figure 6.1 Typical structure of a PEG-coated cationic liposome. A vesicle capable of targeting tumour vasculature consists of two compartments: a hydrophilic core and a hydrophobic, sandwiched bilayer.
6.4.2 Cationic Liposomes and Encapsulated Drug Behaviour

It was then assumed that by encapsulating cytotoxic drugs within the PEG-coated cationic liposomes that were developed, potent anti-angiogenic efficacy might be obtained. This concept was examined by encapsulating oxaliplatin (l-OHP) in the liposomes [72]. Although many conventional cytotoxic agents, such as vinblastine and paclitaxel, have recently been confirmed to exert anti-angiogenic activity against tumour vasculature [83–85], free l-OHP has not been reported to suppress tumour-related angiogenesis. This might be attributed to low l-OHP accumulation in tumour tissue as a result of its high partitioning to erythrocytes [86, 87]. Screening of the anti-angiogenic potential of l-OHP encapsulated in the PEG-coated cationic liposomes was performed, using the mouse DAS model. The results of this study emphasised that encapsulation of l-OHP in PEG-coated cationic liposomes dose not only prolongs its circulation time by protecting the drug from partitioning to erythrocytes, which constitutes a major limitation of the in vivo therapeutic efficacy of the drug, but allows the selective delivery of l-OHP to tumour vasculature. In addition, l-OHP delivered to the tumour vasculature succeeded in suppressing tumour-induced angiogenesis completely, compared with either l-OHP-containing PEG-coated neutral liposomes (lacking cationic lipid) or free l-OHP. A quantitative evaluation of the anti-angiogenic effect was obtained by determining the capillary network area of angiogenic vessels.
on the micrographs, using an Angiogenesis Image Analyzer (version 2, Kurabo) (Figure 6.3). This anti-angiogenic response of l-OHP-containing PEG-coated cationic liposomes was attributed to the selective delivery of l-OHP to the angiogenic vessels and subsequent uptake by tumour endothelial cells. By contrast, the other l-OHP formulations are likely to suffer from high partitioning to erythrocytes (especially free l-OHP formulation) or massive distribution to the skin interstitium (especially l-OHP-containing PEG-coated neutral liposomes), leading to a local drug concentration that is not high enough to exert a therapeutic effect.

Figure 6.3 Specificity of the in vivo anti-angiogenic effect of l-OHP-containing PEG-coated cationic liposomes. The anti-angiogenic effect of l-OHP-containing PEG-coated cationic liposomes was compared with that of other formulations, that is, free l-OHP, l-OHP encapsulated in PEG-coated neutral liposomes and ‘empty’ (no drug containing) PEG-coated cationic liposomes. Mice with an implanted chamber received the formulations (5 mg l-OHP/kg, 45 mg total lipid/mice) on day 3 after chamber implantation. On day 5, the skin area attached to the chamber was examined microscopically. The capillary network area of newly formed vessels in the photographs taken was quantitatively determined. The values (n = 5) represent the mean ± SD, ***p < 0.005 versus positive control; NS, not significant. Reproduced with permission from A.S. Abu-Lila, T. Suzuki, Y. Doi, T. Ishida and H. Kiwada, Journal of Controlled Release, 2009, 134, 1, 18.©2009, Elsevier [72]
6.4.3 Effect of Dosing Schedule on the Anti-tumour Efficacy of Cationic Liposomal Formulations

Despite the fact that the anti-tumour efficacy of anti-cancer drugs encapsulated in neutral or anionic liposomes is dependent on the dosing schedule, little is known about the impact of the dosing schedule on the anti-angiogenic efficacy of cationic liposomal formulations. Eichhorn and co-workers [88] were the first to investigate the impact of the dosing schedule on the anti-angiogenic activity of EndoTAG™-1 (paclitaxel-containing cationic liposomes). They demonstrated that a single weekly dose was less efficient, compared to metronomic dosing with three to five intravenous applications per week at a lower dose. They ascribed these results to the endothelial cell turnover time in solid tumours. The minimal doubling time of the tumour endothelium is approximately 2.5 days in mouse solid tumours [89]. Such rapid endothelial cell turnover was assumed to compensate for the anti-vascular effect of EndoTAG™-1 when it was administered only once a week. In fact, on this dosing schedule, tumour microvessel density was unchanged. An improved therapeutic effect with EndoTAG™-1 was achieved by drug application every 2–4 days, which is in accordance with the endothelial turnover time (~2.5 days).

Recently, we addressed the effect of the dosing schedule on the anti-tumour activity of l-OHP-containing PEG-coated cationic liposomes [90]. We emphasised that the intratumour accumulation of l-OHP-containing PEG-coated cationic liposomes is dependent on the dosing schedule. Administration of liposomal l-OHP every 4 days significantly enhanced the intratumour accumulation of PEG-coated cationic liposome that was subsequently injected, and the therapeutic efficacy was increased. By contrast, administration of liposomal l-OHP once a week resulted in lower anti-tumour activity, compared to the 4-day administration schedule. This difference in therapeutic efficacy between the two dosing regimens may be correlated with the degree of tumour angiogenic vessel maturation. As shown earlier, the cationic liposomes could selectively bind to the newly formed (immature) tumour angiogenic vessels, but not to the preexisting mature blood vessels [57, 82]. A 1-week interval between injections might be enough for the maturation of tumour angiogenic vessels, and the cationic liposomes might, therefore, lose their binding sites in the solid tumour. Consequently, the therapeutic efficacy of l-OHP-containing PEG-coated cationic liposomes was lower when administered once a week than when administered every 4 days.

6.4.4 Pharmacokinetics of Cationic Liposomes

In spite of numerous publications about the in vivo fate of neutral and negatively charged liposomes, surprisingly little is known about the in vivo fate of cationic liposomes. Only a few reports about the pharmacokinetics of cationic liposomes
exist, and their results are either ambiguous or conflicting [91]. In general, cationic liposomes have been viewed as incompatible in vivo, and they are quickly cleared from the bloodstream when injected intravenously [92, 93]. Although their distribution among organs can be modulated by varying either their lipid content or their size, cationic liposomes accumulate mostly in the lungs, the liver and to a lesser extent in the spleen [94]. Reportedly, the accumulation of cationic liposomes in lungs can be explained by a first-pass effect. In the presence of serum proteins, cationic liposomes could form aggregates, which would be captured in the first capillary bed encountered – namely, in lungs.

Some studies have highlighted the effect on the pharmacokinetics and biodistribution of cationic liposomes of the molar percentage of cationic lipid, as well as PEGylated lipid, incorporated into the liposomes [95]. Generally, PEG-coated cationic liposomes have shown an enhanced pharmacokinetic profile, with a longer circulation time than non-PEG-coated cationic liposomes. In addition, incorporation of a high mol% of cationic lipid in the composition of liposomes was found to hinder the in vivo targetability of cationic liposomes, as the result of an increase in the blood clearance of the liposomes. Stuart and co-workers [95] have demonstrated that in the absence of PEG (mPEG2000-DSPE), cationic liposomes consisting of 5–50 mol% cationic lipid (DOTAP) were rapidly cleared from circulation, resulting in <5% of liposomes present in blood at 24 hours postinjection. Additionally, the inclusion of mPEG2000-DSPE did not increase the blood levels of

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Half-life (h)</th>
<th>Clearance (ml/h)</th>
<th>AUC_{\alpha=0→\alpha} (%dose·hour/ml)</th>
<th>V_d (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-coated cationic liposomes</td>
<td>10.4 ± 0.7ns</td>
<td>0.133 ± 0.007*</td>
<td>750.6 ± 35.2*</td>
<td>1.99 ± 0.10**</td>
</tr>
<tr>
<td>PEG-coated neutral liposomes</td>
<td>9.4 ± 0.5</td>
<td>0.113 ± 0.005</td>
<td>884.9 ± 41.7</td>
<td>1.53 ± 0.07</td>
</tr>
</tbody>
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Cationic Liposomes and Tumour Vasculature Targeting

liposomes containing 50 mol% cationic lipid (DOTAP/HSPC in a molar ratio of 1:1). However, when the cationic lipid was reduced to 20 mol%, the inclusion of 5 mol% mPEG₂₀₀₀-DSPE significantly increased the blood levels of liposomes. At ≤10 mol% cationic lipid, mPEG₂₀₀₀-DSPE had its maximum protective effect on the liposome circulation times.

Results from our laboratory have shown that, in tumour-bearing mice, the circulation half-life of PEG-coated cationic liposomes consisting of HSPC/CHOL/DC-6-14/mPEG₂₀₀₀-DSPE (2:1:0.2:0.2 molar ratio) was similar to that of PEG-coated neutral liposomes consisting of HSPC/CHOL/mPEG₂₀₀₀-DSPE (2:1:0.2 molar ratio), at 10.4 ± 0.7 and 9.4 ± 0.5 hours, respectively [90]. However, the PEG-coated cationic liposomes showed a lower area under the blood concentration versus time curve, compared with PEG-coated neutral liposomes. Apparently, the presence of a surface-positive charge enhances both blood clearance (Clearance) and tissue distribution (V_d) of PEG-coated cationic liposomes (Table 6.1). In addition, the PEG-coated cationic liposomes and PEG-coated neutral liposomes accumulated to a similar extent in the liver and spleen. This confirms that PEGylation of cationic liposomes effectively prevents the rapid clearance of cationic liposomes from blood circulation, as observed for non-PEGylated cationic liposomes (Figure 6.4).

Figure 6.4 Organ distribution of PEG-coated liposomes. On day 12 after tumour inoculation, LLCC tumour-bearing mice (n = 4) were injected intravenously with either radio-labelled PEG-coated cationic (closed columns) or neutral liposomes (open columns). At 24 hours postinjection, the animals were euthanised and the radioactivity in each organ was determined. Data are presented as mean ± SD. (In the case of the spleen, the value was per 500 mg instead of per gram). Reproduced with permission from A.S. Abu Lila, Y. Doi, K. Nakamura, T. Ishida and H. Kiwada, Journal of Controlled Release, 2010, 142, 2, 167. ©2010, Elsevier [90]
6.5 Dual Targeting Approach of Both Tumour Endothelial Cells and Tumour Cells

The chemotherapeutic agents used currently to treat cancer are highly toxic, and this places a limit on the dose that a patient can tolerate [96]. Encapsulation of these agents in delivery systems such as liposomes, which selectively target drugs to tumour tissue, might alleviate this problem, because high drug concentrations could be attained within the tumour without affecting normal tissue [8–10]. Moreover, targeting of therapeutic agents to the vasculature of tumours, as opposed to the tumour cells themselves, may offer some additional advantages. Tumours are critically dependent on a blood supply; disrupting this supply can severely suppress tumour growth [97]. Tumour blood vessels are easily accessible to intravenously administered therapeutics, and they are composed of normal cells that do not readily acquire mutations leading to drug resistance [98]. Accordingly, it is easy to imagine that a strategy that targets both the tumour vasculature and the tumour cells using targeted liposomes may be more effective than a strategy that targets only the tumour vasculature, which can leave a cuff of unaffected tumour cells at the tumour periphery that can subsequently regrow and kill the animals or patients.

Pastorino and co-workers [99] provided the proof-of-principle study for the hypothesis that the combined administration of liposomal anti-cancer drugs, which target tumour cells and tumour vasculature, improves therapeutic efficacy relative to each therapy used individually. To target tumour vasculature, doxorubicin-loaded liposomes were modified with asparagine-glycine-arginine (NGR) peptides that target the angiogenic endothelial cell marker aminopeptidase N [100]. To target tumour cells, they used anti-GD2 monoclonal antibody against the disialoganglioside receptor GD2, which is widely expressed in cancer cells of neuronal origin [101]. In an orthotopic neuroblastoma xenograft model, the combined formulations showed superior anti-tumour efficiency over each liposomal formulation administered separately. This enhanced anti-tumour activity was attributed to the complementary modes of action of the two therapeutic approaches: doxorubicin-loaded liposomes modified with NGR peptides act primarily on the tumour vasculature and doxorubicin -loaded liposomes modified with anti-GD2 monoclonal antibody mainly affect tumour cells. In this way, an effective ‘two-compartment’ tumour therapy was realised, which affected both the tumour cell and the vascular compartment within the tumour.

A PEG-coated cationic liposome composed of HSPC:CHOL:DC-6-14:mPEG2000-DSPE was recently developed, and it was confirmed that this type of cationic liposome is a promising carrier for the delivery of encapsulated chemotherapeutic agents to tumour vasculature [72]. In that study, it was also shown that l-OHP encapsulated in such a cationic liposome can exert a potent anti-angiogenic activity in a DAS model.
In addition, the authors recently demonstrated that, in a murine solid tumour model, l-OHP-containing PEG-coated cationic liposomes showed superior anti-tumour activity in terms of suppression of tumour growth and prolongation of mouse life span compared to either free l-OHP or l-OHP-containing PEG-coated neutral liposomes (Figure 6.5a and b) [102]. This superior anti-tumour activity was confirmed to be due

**Figure 6.5** Anti-tumour efficacy of various l-OHP formulations on LLCC-tumour bearing mice. On day 4, 8 or 12 after tumour inoculation (indicated by arrows), either free l-OHP (■), l-OHP containing PEG-coated cationic liposomes (●), l-OHP containing PEG-coated neutral liposomes (▲), empty PEG-coated cationic liposomes (x) or 5% dextrose solution (control) (●) was administered via the tail vein at a 1-OHP dose of 5 mg/kg. Each treatment group contained 6 mice. (a) Anti-tumour activity, as assessed by tumour size, and (b) survival of LLCC-bearing mice. Data in (a) represent mean ± SD (n = 6). ***p < 0.01 against other formulations. Reproduced with permission from A.S. Abu Lila, S. Kizuki, Y. Doi, T. Suzuki, T. Ishida and H. Kiwada, *Journal of Controlled Release*, 2009, 137, 1, 8. ©2009, Elsevier [102]
to the delivery of l-OHP to dual targets, tumour endothelium and tumour cells, by means of PEG-coated cationic liposomes. Such dual targeting approach, that is, vascular targeting and tumour targeting with a single liposomal anti-cancer drug formulation, has the potential to overcome some of the major limitations of conventional strategies.

Dai and co-workers [103] have also recently addressed the utilisation of a single liposomal formulation to achieve the dual targeting of tumour vasculature and tumour cells. For this purpose, they coupled a derivative of ATN-161 (N-acetyl-proline-histidine-serine-cysteine-asparagine-amide, PHSCN), a ligand for integrin αvβ1, which is the receptor overexpressed on tumour neovasculature and some tumour cells, to the surface of PEG-coated doxorubicin liposomes to obtain PHSCNK-modified doxorubicin-loaded PEG-coated liposomes (PHSCNK-PL-DXR). The coupling of PHSCNK to the liposomal surface was confirmed to enhance the cellular uptake and cytotoxicity of doxorubicin on both human umbilical vein endothelial cells and breast cancer cells, due to integrin-mediated endocytosis. The authors therefore concluded that PHSCNK-PL-doxorubicin, which can actively deliver the drug into both tumour neovasculature and tumour cells, may be a promising targeted delivery system for anti-cancer drugs.

6.6 Effect of Sequential Administration on the Anti-tumour Efficacy of Cationic Liposomal Formulations

Many liposome-based drug delivery systems encapsulating chemotherapeutic agents have exerted efficient anti-tumour activity in preclinical studies after successive injections of the liposomal formulation, but not after a single injection [104–106]. It is believed that such efficient anti-tumour activity can be attributed to the selective delivery and preferential accumulation of liposomal anti-cancer drugs in tumour tissue via the EPR effect [18, 19, 107]. However, an important, yet underappreciated, factor that might be involved in the anti-tumour efficiency of liposomal anti-cancer drugs is the enhancement effect of the primary dose on the accumulation of subsequently injected doses. Therefore, we followed the intratumour accumulation of PEG-coated cationic liposomes after either a single or two subsequent injection(s) of l-OHP-containing PEG-coated cationic liposomes [90]. The results of this study showed that the tumour accumulation levels of test-PEG-coated cationic liposomes in mice pretreated with a single liposomal l-OHP injection were similar to those in nontreated (control) mice. On the other hand, in mice pretreated with two successive injections of liposomal l-OHP, the tumour accumulation of cationic test liposomes was significantly higher than that in nontreated (control) mice or in mice pretreated with a single injection (Figures 6.6 and 6.7). The enhanced intratumour accumulation of test PEG-coated cationic liposomes after two successive injections of liposomal l-OHP was attributed to the cumulative cytotoxic effect of the preinjected liposomal l-OHP.
Figure 6.6 Intratumoural accumulation of PEG-coated liposomes after sequential liposomal l-OHP administration. LLCC tumour-bearing mice received either a single or two sequential injection(s) of liposomal l-OHP every 4 days. Four days later, the treated mice received radio-labelled PEG-coated cationic liposomes. Mice receiving only the radio-labelled PEG-coated cationic liposomes served as controls. At 6, 24 and 48 hours post injection, the radioactivity in tumours was determined. Data are presented as the percentage of the injected dose per gram of tumour tissue and SD (n = 4). Reproduced with permission from A.S. Abu Lila, Y. Doi, K. Nakamura, T. Ishida and H. Kiwada, *Journal of Controlled Release*, 2010, 142, 2, 167. ©2010, Elsevier [90]

formulation on both endothelial cells and tumour cells. Because of their prolonged circulation time in the blood, l-OHP-containing PEG-coated cationic liposomes are likely to have easy access to tumour endothelial cells and to readily extravasate from the blood stream into the tumour interstitial space due to the EPR effect, thus gaining access to the tumour cells. Once in the tumour tissue, l-OHP-containing liposomes can be internalised by both tumour endothelial cells and tumour cells, following interaction with those cells, as demonstrated earlier [102]. Thus, liposomal l-OHP will be allowed to exert its cytotoxic effect [108, 109] and may bring about a decrease in the number of tumour cells and, consequently, a decrease in tumour interstitial pressure, thus allowing deeper penetration of the test-cationic liposomes (Figures 6.6 and 6.7).

On the other hand, the failure of a single pretreatment with liposomal l-OHP to enhance the intratumour accumulation of the subsequently injected test dose was related to
the insufficient delivery of l-OHP to tumour tissue or/and a suboptimal time interval between doses. After a single injection, the fraction of the dose of l-OHP-containing PEG-coated cationic liposomes that reached the tumour tissue seemed to become bound almost entirely to tumour endothelial cells, while only a small proportion remained available for extravasation into the tumour interstitium. This relatively small fraction of liposomal l-OHP extravasating into the interstitial space of tumour tissue might be insufficient to exert a potent cytotoxic effect against the tumour cells, and thus fail to enhance the intratumour accumulation of the subsequently injected second dose. In addition, the time interval (4 days) between the doses might not be sufficient for l-OHP to be released from the liposomes in a sufficiently high concentration to exert a cytotoxic effect against viable tumour cells, as demonstrated in Section 6.4.3.

6.7 Clinical Applications of Cationic Liposomes: Promise and Obstacles

6.7.1 Safety of Cationic Liposomes in Preclinical and Clinical Use

As so far addressed, cationic liposome has proved to be an effective tool for drug delivery in many preclinical models. In addition, several clinical trials for cancer therapy using
anti-cancer drug-loaded cationic liposomes are ongoing. Whilst the in vitro toxicity of cationic liposomes is well addressed in many studies (the reader is referred to an interesting review on in vitro toxicity of cationic liposomes for further detailed description of this area [110]), data about their in vivo toxicity are scarce. General concerns about their in vivo safety have recently been intensified by a few harsh setbacks.

6.7.1.1 Interaction of Cationic Liposomes with Blood Components

It has been reported that cationic liposomes are able to interact with various types of biological components (e.g., cells and plasma proteins) because of their positive surface charge [53, 110, 111]. The presence of plasma proteins is also thought to be a limiting factor for in vivo availability of cationic liposomes. Therefore, an emphasis on the interaction of cationic liposomes with blood components is crucial for the successful development of an effective liposomal drug delivery system. Chonn and co-workers [112] previously demonstrated a direct correlation between the amount of plasma protein bound to cationic liposomes (non-PEGylation) and their rate of elimination from systemic circulation. Such studies led to the realisation that a reduction in the binding of opsonising plasma proteins to liposomes could improve their biodistribution. This was accomplished by the PEGylation of liposomes. PEGylation, the inclusion of high-molecular-weight PEG to the liposome surface, significantly increases liposome stability in blood by limiting the interaction of conventional liposomes with circulating blood proteins and MPS cells, as described earlier, and thus prolongs their plasma circulation time [113, 114]. Moreover, since many blood components are negatively charged, intravascular administration of cationic liposomes might result in aggregation with serum components and/or microemboli formation. Consequently, tissue ischemia may be problematic [115].

6.7.1.2 Activation of Immune Responses

Immune responses to the cationic lipids that are frequently used to formulate cationic liposomes are much less understood. However, Zelphati and co-workers [116] have shown that the complement system can be activated by cationic lipids. Complement activation could result in complement components binding to the liposomes (non-PEGylation) and targeting them to receptors for complement components that are found in lungs [117] or in Kupffer cells in the liver; this could also explain the rapid clearance of non-PEGylated cationic liposomes from the blood and uptake into lungs and subsequently into the liver. Chonn and co-workers [112] have demonstrated that the activation of the complement system by liposomes was affected by the surface charge density, as well as by the dose of liposomes incubated with the serum. Moreover, the nature of the surface charge, whether negative or positive, is important
in determining which complement pathway is activated by the liposomes. In contrast to anionic liposomes, which activated the classical pathway of complement activation, cationic liposomes, under conditions where the classical pathway of complement activation was effectively blocked, were able to reduce the C3-C9 complement levels in a dose-dependent manner, indicating that cationic liposomes activated the alternative pathway of complement activation.

Filion and co-workers [118] have addressed the immunomodulatory activity of cationic liposomes composed of DOPE/DOTAP (1:1 molar ratio) toward immune effector cells, specifically macrophages. They demonstrated that cationic liposomes, via their cationic lipid component, can down-regulate at least two immunomodulators, NO and TNF-α, produced by activated macrophages. They attributed such down-regulation of NO and TNF-α synthesis to the inhibition of protein kinase C activity by cationic lipids incorporated in the composition of cationic liposomes [119]. Moreover, the addition of dipalmitoyl phosphatidylethanol amine-polyethylene glycol 2000 (PEG<sub>2000</sub>-DPPE) to these cationic liposomes successfully restored NO and TNF-α synthesis by activated macrophages, by inhibiting the uptake of these liposomes by macrophages [120].

### 6.7.1.3 Induction of Inflammatory Reactions

Inflammatory reactions have been observed upon in vivo use of cationic liposomes. However, the mechanism by which cationic liposomes elicit such inflammation is still not completely understood. Freimark and co-workers [121] reported that intratracheal instillation of cationic liposomes induced cytokine production and cellular influx in the lung airways, leading to severe lung inflammation. Dokka and co-workers [122] demonstrated that instillation of cationic liposomes elicited dose-dependent toxicity and pulmonary inflammation. They found that the inflammatory reaction was correlated with an oxidative burst that resulted from a dose-dependent increase in the generation of reactive oxygen intermediates (ROI), induced by cationic liposome instillation.

### 6.7.2 Clinical Applications of Cationic Liposomes in Cancer Therapy

The success achieved in the preclinical models provides a strong rationale for the use of cationic liposomal cytotoxic therapeutic agents for the treatment of human cancer. To date, cationic liposomal paclitaxel has been the most extensively evaluated in the clinical setting [123]. The first clinical trial [124] was performed to evaluate the safety of EndoTAG<sup>TM</sup>-1 in patients suffering from advanced metastatic colorectal cancer. Approximately 13% of the patients under study showed stable disease, and the
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treatment was well tolerated. A phase 1b clinical trial [125] was conducted to evaluate the safety and anti-tumour efficacy of EndoTAG™-1 in patients with metastatic breast cancer and tumour progression after anthracycline-based chemotherapy. The overall assessment of tumour response showed 6% partial response and 28% stable disease in patients receiving a dose of 0.55 mg/kg on days 1, 3 and 5 of a 3-week cycle. Nausea and vomiting were the major side effects associated with treatment. A clinical phase II study was conducted to investigate the safety and efficacy of EndoTAG™-1 in combination with standard gemcitabine treatment in patients with locally advanced and/or metastatic pancreatic cancer [126]. Two hundred patients have been enrolled in this phase II study and preliminary data confirm a favourable safety profile for EndoTAG™-1 in combination with gemcitabine treatment. Moreover, the study has shown promising preliminary therapeutic results, as the median overall survival was increased by EndoTAG™-1 combination therapy compared to gemcitabine standard monotherapy [127]. Such data extracted from the preclinical and clinical studies could potentially serve as a basis for the future development of cationic liposomal drug delivery systems for cancer treatment. The achievements, and any limitations, of these clinical trials should encourage researchers to invest their efforts in the development of cationic liposomal formulations amenable to clinical applications.

6.8 Conclusions

While the majority of anti-cancer drugs are designed to directly kill tumour cells, recent strategies are focusing on drugs that kill cancer cells indirectly by interfering with tumour blood supply. There are at least two general approaches to depriving tumours of their blood supply. One is to inhibit tumour-induced neovascularisation from both the sprouting neighbouring vessels and the recruitment of circulating endothelial cell precursors from the bone marrow [128–131]. This has been achieved by the use of angiogenesis inhibitors. Several angiogenesis inhibitors have shown efficacy in the reduction of tumour neovascularisation and tumour growth in preclinical models [132–134]. This approach gained a significant boost with the FDA approval of the angiogenesis inhibitor, bevacizumab, for the treatment of a variety of solid tumours. A second approach to reducing the tumour blood supply is mediated by direct targeting tumour vasculature, taking advantage of the exclusive overexpression of specific surface antigens (‘vascular zip codes’) on the surface of tumour endothelial cells [135, 136]. This unique characteristic of tumour endothelial cells has been exploited to achieve active vascular targeting by means of nanocarrier drug delivery systems. Among the commonly used nanocarrier systems used to deliver cytotoxic therapeutic drugs to tumour vasculature, cationic liposomes have been considered a promising candidate for the delivery of anti-angiogenic agents to tumour vasculature, based on their inherent ability to bind selectively to tumour endothelial cells. This
novel therapeutic strategy was first realised by the synthesis of EndoTAG\textsuperscript{TM}-1, comprised of paclitaxel encapsulated in cationic liposomes [83]. Recently, we succeeded in developing a PEG-coated cationic liposome that targets to tumour vasculature. In addition, when the cytotoxic agent, l-OHP, was encapsulated in such liposomes, a potent anti-angiogenic activity was achieved [72].

Although anti-angiogenic inhibitors and vascular targeting agents can regress primary tumour growth and inhibit tumour metastasis in experimental tumour models, anti-vascular tumour therapy as monotherapy has failed to provide convincing results in clinical trials [137, 138]. To date, anti-angiogenic drugs and vascular targeting agents cannot eradicate tumours completely, and remarkable anti-tumour effects can be achieved only by combining anti-vascular tumour therapy with conventional radio- and/or chemotherapy directly targeting the tumour cell compartment. These clinical data support the earlier predictions of Teicher [139], who postulated that the combined administration of anti-angiogenic and cytotoxic radio- and/or chemotherapy would yield maximal benefits, because such combinations would destroy two separate compartments: tumour cells and endothelial cells. Cytotoxic agents would kill cancer cells directly, and anti-angiogenic agents would kill cancer cells indirectly by depriving them of nutrients and oxygen. The strategy of using PEG-coated cationic liposome, which is capable of delivering anti-cancer drugs to both tumour vasculature and tumour cells, may meet the criteria of current chemotherapy. Accordingly, the proposed dual targeting approach, that is, vascular targeting and tumour targeting with a single liposomal anti-cancer formulation, may have the potential to overcome some of the major shortcomings of conventional strategies.

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Cationic Liposomes and Tumour Vasculature Targeting


Lipid-based Nanocarriers for Cancer Gene Therapy

Rajagopal Ramesh, Manish Shanker, Jiankang Jin, Sarah J. West and Jack A. Roth

7.1 Introduction

Cancer is the second most common cause of death in the US. The American Cancer Society (http://www.acs.org/) estimated that about 1,479,350 new cases of cancer, excluding noninvasive cancer and basal and squamous cell skin cancers, were diagnosed in 2009 [1]. About 562,340 of the patients diagnosed with cancer will die of the disease. Additionally, in the US, the lifetime risk of developing cancer for men is less than one in two, while for women the risk is more than one in three [1]. Although the incidence of cancer is very high, the occurrence of certain types of cancer could be greatly reduced by minimising the exposure to external factors (tobacco, chemicals, radiation, alcohol, infectious organisms). For example, the incidence of alcohol- and tobacco-related cancers could be reduced by discontinuing their use. Behavioural changes could also significantly reduce the development of certain types of cancer, such as cancer of the liver and cervix. Reducing exposure to the sun and to indoor tanning devices could similarly decrease the incidence of skin cancer.

By utilising modern screening methods for early detection and dissemination of cancer awareness, a reduction in the incidence of certain cancers has been observed. For example, stopping the use of cigarettes has reduced the incidence of lung cancer in men. However, in recent years, an increasing trend of cigarette smoking with corresponding higher incidence of lung cancer has been observed in women, indicating the need for the development of more robust methods for preventing lung cancer incidence in women.

Development of new therapies has also contributed to improvements in the 5-year survival rates of cancer patients. The 5-year survival rate for all cancers diagnosed from 1996 to 2004 was 66% compared to the survival rate of 50% for the period 1975–1977 [1]. Despite the advances made in cancer prevention and therapy, the overall 5-year survival rates for certain types of cancer, especially those of epithelial origin, are less than 15% [2]. These results suggest that, in addition to improving cancer screening and diagnostic methods, new and effective treatments are warranted.
A novel and attractive form of cancer treatment is gene therapy, which relies on transferring therapeutic genes to the tumour. Several gene therapy-based clinical trials have been conducted for treatment of numerous human diseases including cancer [3]. The most widely tested gene delivery vehicle has been adenovirus (Ad) due to its ability to transfect both dividing and nondividing cells with high efficiency and with a low probability of nonhomologous recombination with the host genome. Ad-based gene transfer strategies with the $p53$ tumour suppressor gene (TSG) have shown clinical promise in phase I/II trials [4–7]. However, the drawbacks of Ad-based gene therapy are the ability of the virus to induce host immune response and toxicity and the potential to produce infectious virus [8–11]. As a result, Ad-based cancer therapy has been limited to locoregional treatment and it is ineffective as systemic delivery vehicle for the treatment of metastatic cancers [10, 11]. Therefore, there is still a need to overcome the inability to treat metastatic disease. An alternative to adenoviral vectors is the nonviral nanocarrier-based gene delivery system, which was shown effective in delivering therapeutic genes to metastatic sites when administered systemically [3, 12]. The advantage of using nonviral nanocarrier systems is that they are easy to manufacture and they help avoid the problems often encountered with Ad [12, 13].

The ideal strategy envisioned for cancer treatment is a nanocarrier that systemically delivers a therapeutic gene to the primary tumour site and to distant metastatic sites without undue toxicity to normal tissues. Development of such effective systemic therapies will have broad applications in cancer treatment.

In the following sections, we will discuss some of the lipid-based nanocarriers that have been developed and tested in preclinical studies followed by a discussion on nanocarriers that are being tested in the clinic for cancer gene therapy.

### 7.2 Preclinical Studies Testing Lipid-based Nanocarriers for Cancer Gene Therapy

Lipid-based nanocarriers are small particles of 5–500 nm in size and can be produced from various lipids or lipid mixtures that can vary in charge (positive, negative and neutral), fluidity and packing geometry. A majority of the nanocarriers currently being developed are used in diagnosis and detection of a disease [14–19]. However, nanocarriers are also being tested to deliver therapeutic genes to treat diseases such as cancer [20–22].

Numerous studies using cationic, neutral and anionic lipids have documented the potential use of lipid-based nanocarriers for gene transfer [23–29]. However, the results
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from a majority of these studies have established cationic lipids to be the preferred lipid component for nucleic acid delivery, owing to the high efficiency of nucleic acid transfer associated with these lipid formulations. Often, cationic lipids are mixed with neutral lipids to increase the rigidity and stability of the nanocarrier. However, the usefulness of these cationic lipid-based formulations for in vivo gene delivery has been limited by inflammation-associated toxicity induced by the nanocarrier and their poor stability in vivo [30–34]. The interaction between the lipid component of the nanocarrier and serum proteins has a dramatic negative impact on the stability of the nanocarrier [35, 36].

Cationic lipid-based nanocarriers attract and bind negatively charged serum proteins, leading to their rapid removal from circulation by macrophages and the reticuloendothelial system (RES). As a result, the majority of the in vivo nanocarrier delivery systems use subcutaneous (SC), intradermal, intratumoural (IT) or intraperitoneal (IP) injection to avoid the inflammation-associated toxicity and stability problems associated with cationic nanocarriers in circulation [37]. The interaction between lipid nanocarriers and plasma proteins is responsible for the disparity between the efficiency of in vitro and in vivo gene transfer [38–45].

To improve the in vivo stability of cationic lipid nanocarriers and increase their accumulation in the tumour, PEGylating the outer surface of the nanocarrier using polyethylene glycol (PEG) has been investigated. Studies have shown that PEGylation does improve the stability and half-life of cationic lipid nanocarriers in vivo and evades the host immune system resulting in increased accumulation of the drug in the tumour and enhanced therapeutic efficacy [46, 47]. Another approach taken to minimise the interaction between positively charged lipid nanocarrier and serum proteins is to use neutral or anionic lipids in formulating the nanocarrier rather than cationic lipids [47]. Several reports using neutral and anionic lipid formulations have demonstrated increased gene delivery to the tumour and enhanced therapeutic effect and have attributed these observations to the ability of the nanocarriers to escape serum protein interaction and RES clearance [48–50]. Although neutral and anionic lipids have shown effective gene delivery, these lipid formulations are usually used to deliver siRNA, antisense oligonucleotides and chemotherapeutic drugs for cancer treatment [51–54]. Several chemotherapeutic drugs formulated in these neutral or anionic lipid formulations are routinely used in the clinic. Despite the advances made with neutral and anionic lipid-based nanocarriers, they have not been developed and tested as gene delivery vehicles for clinical cancer gene therapy.

One cationic lipid nanocarrier that has been tested as gene transfer vehicle and shown to be stable in vivo is the N-[1-(2, 3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP):cholesterol (DOTAP:Chol) lipid formulation. Early studies in murine models using DOTAP:Chol nanocarrier at an equimolar ratio demonstrated
that these nanocarriers are efficient *in vivo* nucleic acid transfer vehicles [24–26]. Furthermore, intravenous (IV) administration of a plasmid DNA carrying the chloramphenicol acetyltransferase (CAT) reporter gene and encapsulated in the DOTAP:Chol nanocarrier (DOTAP:Chol-CAT) resulted in preferential transgene expression in the mouse lung with lower expression levels in a variety of other tissues [24]. Systemic administration of DOTAP:Chol nanocarrier containing the luciferase (Luc) marker gene showed preferential accumulation of the particles in the lung as noted by higher expression of Luc [55]. Luc expression was initially observed in both the lung and liver. However, Luc expression increased in the lung over a period of 24 hours, while it was lost in the liver, demonstrating that retention and expression of transgene continues in the lung over an extended period. Loss of Luc expression in the liver is likely due to rapid RES-mediated clearance of the nanocarrier.

Size fractionation studies suggested that nanocarriers in the range of 200–450 nm in size were optimal for nucleic acid transfer. However, more recent studies suggest that nanocarriers that are 10–100 nm are better for IV delivery and they escape the RES, thereby improving gene delivery and transfer [56, 57]. Nanocarriers that are less than 100 nm in size appear to be more efficiently taken up by tumour cells, while nanocarriers that are greater than 400 nm in size are efficiently taken up by the host macrophages and cleared rapidly. Nanocarriers whose size is in the range of 100–400 nm appear to strike a balance between tumour uptake and macrophage clearance. Although the size of the nanocarrier is critical and important for efficient gene delivery, it should be noted that for different cancers and diseases, the particle size will likely need to be modified. Furthermore, studies comparing nanocarriers of different sizes and formulations in a broad range of tumour models are warranted prior to making a conclusion on the size of the nanocarriers needed for efficient gene transfer in humans diagnosed with cancer.

Crook and co-workers [58] reported that DOTAP when mixed with cholesterol increased the *in vitro* delivery of nucleic acids in the presence of serum. The amount of serum proteins associating with the DOTAP:Chol nanocarrier was determined to be equivalent in DOTAP:Chol and DOTAP nanocarriers. The increase in nucleic acid transfer was attributed to the increased binding of the cholesterol-containing nanocarrier. The presence of cholesterol in the formulation stabilises the nanocarrier against serum protein disruption and supports its use as an *in vivo* nucleic acid delivery vehicle. Thus, the DOTAP:Chol nanocarrier achieves a balance between toxicity and *in vivo* nucleic acid transfer efficiencies compared to other cationic nanocarriers [24–26].

Based on these reports, we have tested the use of DOTAP:Chol nanocarrier as a local and systemic gene delivery vector in experimental mouse models. Initial studies were focused on IT treatments with a marker gene to determine the efficiency of gene
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Figure 7.1 Human H1299 lung tumour established SC in nude mice was injected IT with DOTAP:Chol nanocarrier containing the β-gal gene. Immunohistochemical analysis showed β-gal expression in the tumour at 24 hours after treatment. Tumour tissue from untreated mice served as control.

transfer. IT administration of β-galactosidase (β-gal) marker gene into SC H1299 lung tumour xenograft showed about 15–20% transduction, as determined by the transgene expression in the tumour tissue (Figure 7.1) [26]. This degree of transgene expression was unexpected as nonviral gene transfer and expression in vivo has been reported to be very low and in the range of 5–10%.

It is unclear how the DOTAP:Chol nanocarrier formulation provides such high-efficiency gene transfer and expression, but nevertheless our findings were encouraging to conduct additional in vivo studies. Subsequent therapeutic studies from our laboratory showed that daily IT treatment of p53 gene encapsulated in the DOTAP:Chol nanocarrier for a total of six doses resulted in suppression of SC lung tumour growth [26]. Tumour suppression was accompanied by tumour cell apoptosis. Additionally, the DOTAP:Chol-p53 nanocarrier treatment was shown to be effective in lung tumours irrespective of the endogenous p53 status (wild type, mutant or null).

To determine whether the IT treatment approach was restricted to p53 therapy, we conducted studies testing additional TSG such as Fhit, Fus1 and the melanoma differentiation-associated gene-7 (mda-7)/interleukin (IL)-24 in lung cancer [26, 59, 60]. In these studies, a significant delay in tumour growth was observed in mice whose tumours were treated with the nanocarrier containing the appropriate therapeutic gene (Figure 7.2).

The effective tumour suppression produced by the individual TSG varied, with complete tumour regression observed in a few mice that were treated with mda-7/IL-24. These results provide support for applying DOTAP:Chol nanocarrier-based gene therapy for treatment of localised tumours that are unresectable and have failed to respond to conventional chemotherapy or radiotherapy.
Next we investigated whether DOTAP:Chol nanocarrier was effective in systemically delivering TSG to experimental lung metastasis established in a nude mouse model. Prior to the start of the efficacy studies, we conducted marker gene studies. Human A549 lung tumour-bearing nude mice were injected with DOTAP:Chol nanocarrier containing β-gal gene IV via the tail vein. At 24 hours after injection of the nanocarrier, the mice were euthanised and tumour-bearing lungs were isolated and stained for β-gal expression. As shown in Figure 7.3, tumours in the lung stained for β-gal as evidenced by the blue staining of pulmonary tumour nodules.

This study showed successful gene transfer and transgene expression in the lung tumours after systemic administration of DOTAP:Chol nanocarrier.

Subsequent studies focused on delivering therapeutic TSG for treatment of experimental lung metastasis. IV administration of DOTAP:Chol-p53 nanocarrier to nude mice bearing experimental human lung tumour metastasis resulted in marked reduction in the number of tumour nodules in the lung and increased animal survival [26]. The therapeutic effect on lung tumour suppression was also observed when other TSG such as the Fhit gene were used (Figure 7.4), indicating that DOTAP:Chol nanocarrier can be used for delivering several therapeutic genes.
Figure 7.3 Lung tumour nodules from mice treated IV with DOTAP:Chol nanocarrier containing the β-gal gene stain positive for β-gal.

Figure 7.4 Gross and histopathological photographs showing that IV delivery of DOTAP:Chol nanocarrier containing Fhit TSG suppresses lung tumour growth as evidenced by the reduction in the number of tumour nodules compared to the tumour nodules in the lungs of mice that were untreated or treated with a nanocarrier containing control plasmid DNA.
Furthermore, repeated treatments in mice showed an increase in the transgene expression level suggesting additive effects. One argument frequently made is that when repeated treatments are spanned across less than a 3-day interval, transgene expression is reduced or shut off due to induction of host inflammatory response [61]. The findings from these studies are in contrast to our findings, and the discrepancy in the study results was argued to be due to the use of immunodeficient mice in our studies, which likely produced reduced inflammatory response against the nanocarrier, and as a consequence transgene expression was unaffected after repeated treatments. The majority of the studies conducted by other laboratories used immunocompetent mice to demonstrate the inhibitory effects of successive repeated treatments on transgene expression.

To investigate if difference in mouse strain contributed to different outcomes regarding the effect of repeated treatments on transgene expression, we conducted studies in an immunocompetent C3H mouse model bearing syngeneic tumours. Experimental lung tumours were established in C3H mice by injecting UV2237 fibrosarcoma. Treatment of these tumour-bearing mice showed a dose-dependent increase in p53 transgene expression with further increases in transgene expression after each treatment. These results showed that repeated treatments with a therapeutic gene contained in DOTAP:Chol nanocarrier are feasible, and the transgene expression additively increases with multiple treatments irrespective of the mouse strain [26]. However, one major observation was that in all of our studies we have used mice bearing experimental lung metastasis while studies from other laboratories often used non-tumour-bearing mice. This led us to speculate that the host pathology could contribute in regulating the inflammatory response.

We hypothesised that the immune status of mice bearing in situ tumours is likely altered and less functional than the immune status of mice that do not have tumours. We also hypothesised that in tumour-bearing mice inflammatory response is reduced compared to mice that do not have tumours. To test our hypothesis, we conducted studies comparing tumour-bearing (TB) and non-tumour-bearing (NTB) immunocompetent mice and measured the inflammatory response to the DOTAP:Chol nanocarrier carrying a plasmid DNA. The inflammatory response as determined by the cytokine profile and alveolar macrophage activity was markedly reduced in TB mice compared to NTB mice [62]. Furthermore, the plasmid DNA dose required to induce inflammatory response in TB mice equivalent to the level of inflammatory response produced in NTB mice was much higher. For the first time, our results demonstrated that the host pathology status plays a role in regulating the inflammatory response induced by lipid-based nanocarriers.

On the basis of our findings, the therapeutic effects and efficacy of systemic delivery of DOTAP:Chol nanocarrier carrying TSG (p53, Fhit, Fus1, mda-7/IL-24) were
investigated in the laboratory [26, 59, 60]. IV administration of DOTAP:Chol nanocarrier containing p53 into experimental lung tumour-bearing mice for a total of six doses resulted in effective gene delivery to the tumours and in a therapeutic effect as determined by a marked reduction in the number of lung tumour nodules and increased animal survival [26]. We showed that the nanocarrier was selectively and increasingly taken up by the tumours when compared to the surrounding normal lung tissues, resulting in increased transgene expression in the tumours and enhanced therapeutic effect [63]. The tumour size in the lung dictated the amount of nanocarrier uptake, with larger tumours showing increased uptake of the nanocarrier compared to smaller tumours. We also showed that these DNA-containing nanocarriers induced no significant toxicity in TB mice at the plasmid DNA doses used. Our results demonstrated that, in addition to size, shape and charge of the nanocarrier, other factors such as host immune status and pathology, and tumour size also contribute to overall uptake of the nanocarrier, transgene expression and therapeutic efficacy.

Based on our preclinical findings demonstrating the systemic use of DOTAP:Chol nanocarrier-based gene delivery for cancer therapy, we at the M.D. Anderson Cancer Center have recently initiated a phase I clinical trial for the systemic treatment of non-small cell lung cancer (NSCLC), which is discussed in Section 7.3.

Although studies from our laboratory have shown that DOTAP:Chol nanocarrier is useful for cancer gene therapy and is safe with no major toxicity at the plasmid DNA doses tested, DNA concentrations above 50 μg showed inflammation-associated toxicity in vivo (unpublished data). A dose-dependent increase in toxicity with acute toxicity and death of mice occurring at 100 μg DNA concentration was observed. Our findings concurred with reports from other laboratories [31, 32]. The molecular phenomenon underlying lipid nanocarrier-induced toxicity has been attributed to the presence of CpG motifs in the plasmid backbone [64–66]. Studies have shown that reducing the CpG motifs or modifying the CpG motifs with synthetic sequences reduced the inflammatory response [67, 68]. However, despite the modifications made to the CpG sequences and the plasmid backbone, the inflammatory response is not completely abolished [69]. There are reports that lipid nanocarrier-mediated induction of acute inflammatory response can occur in the absence of any CpG motif, suggesting that CpG is not the sole contributor for inducing inflammation [70, 71]. To date, the underlying molecular mechanism by which lipid nanocarrier containing plasmid DNA elicits inflammatory response remains unclear and is an active area of research in several laboratories.

While laboratories developing nanocarrier-based gene therapy for cancer are studying the role of CpG in inflammation, studies in our laboratory have focused on alternative methods to overcome nanocarrier-induced inflammatory response, even in the presence of CpG sequences in the plasmid DNA. The rationale is that by developing
new approaches to overcome CpG-induced inflammatory response one may be able to translate and apply the lipid nanocarrier-based gene delivery in the clinic. Treatment of normal lung fibroblast cells with DOTAP:Chol nanocarrier containing a plasmid DNA induced inflammation-associated signalling pathways resulting in the release of proinflammatory cytokines. Induction of the inflammatory response occurred as early as 2 hours after treatment. However, in the presence of anti-inflammatory agents, such as naproxen, the nanocarrier-mediated inflammatory response was abrogated [30]. Similar observations were made when additional anti-inflammatory agents such as ibuprofen, sulindac sulfate and celecoxib were used to determine their inhibitory effects on nanocarrier-induced inflammation (unpublished data). To further test if naproxen could suppress the inflammatory response in vivo, immunocompetent C3H mice were either pretreated or not pretreated with naproxen followed by IV administration of DOTAP:Chol nanocarrier containing a lethal dose (100 μg) of plasmid DNA. The group of mice not pretreated with naproxen was susceptible to the acute inflammatory response and showed signs of morbidity and eventually mortality, which often occurred within 24 hours of nanocarrier injection. However, the group of mice that was pretreated with naproxen tolerated the lethal dose with no signs of toxicity even after 2 weeks (unpublished data). These results demonstrated that use of anti-inflammatory agents could overcome lipid nanocarrier-mediated inflammatory response. By using plasmid DNA with reduced CpG motifs and combining with the anti-inflammatory agent, it is expected that the inflammatory response will be completely abrogated and the therapy will be safer for clinical use.

7.3 Clinical Studies Testing Lipid-based Nanocarriers for Cancer Gene Therapy

Numerous targeted and untargeted lipid-based nanocarriers, as described in Section 7.2, have been developed and tested as gene delivery vehicles and have been shown to effectively deliver therapeutic genes and produce anti-cancer effects [26–30, 72–74]. However, of the many tested and promising nanocarriers in preclinical studies, only a few have successfully been translated to the clinic. The inability to test many of the nanocarriers in the clinic could be attributed to numerous reasons, among which the prime factors are toxicity, stability and inability to be produced in large scale for clinical testing. Additionally, despite the development of methods such as PEGylation of the vector to reduce toxicity and increase in vivo stability, complete elimination of toxicity has not been achieved and has hampered the testing of these nonviral vectors in the clinic.

All of the nanocarriers that have entered phase I clinical trial to date are cationic lipid-based formulations and have been tested against solid tumours and via IP or
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IV routes. The first lipid-based nanocarrier to be tested in the clinic was formulated with cationic 3\(\beta\)[N-(N’N’-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) in combination with dioleoylphosphatidylethanolamine (DOPE) for delivering E1A gene for the treatment of human ovarian and breast cancers [75, 76]. In this phase I clinical trial, E1A gene encapsulated in DC-Chol nanocarrier (DCC-E1A) was administered to patients diagnosed with ovarian cancer \((n = 12)\) or breast cancer \((n = 6)\) whose tumours either overexpressed HER-2/neu or expressed low HER-2/neu and had failed to respond to conventional therapies. DCC-E1A was administered to ovarian and breast cancer patients once a week via IP and intrapleural routes, respectively. The objective of this trial was to determine the maximum tolerated dose (MTD) of DCC-E1A and the ability to deliver E1A gene and observe any E1A-mediated HER-2/neu repression in tumour cells. Patients were treated in cohorts with initial starting DNA dose at 1.8 mg/m\(^2\) and subsequent doses increasing 100% to 3.6 and 7.2 mg/m\(^2\). The results from this clinical trial showed the MTD to be 3.6 mg/m\(^2\). The most common treatment-related toxicities observed with the highest DNA dose was nausea, fever, vomiting and discomfort at the injection site. Molecular analysis demonstrated that DCC-E1A treatment reduced HER-2/neu expression in tumour cells and induced tumour cell apoptosis [76].

Successful completion of the DCC-E1A phase I trial was followed by a multicentre phase I clinical trial testing DCC-E1A in patients with recurrent epithelial ovarian cancer that overexpressed HER-2/neu [77]. In this trial, 15 patients were recruited and treated with increasing doses (1.8, 3.6 and 7.2 mg DNA/m\(^2\)). The MTD was 3.6 mg DNA/m\(^2\) and concurred with the report by Hortobagyi and co-workers [76]. However, in this trial, no correlation between dose and biological activity was observed. The authors concluded that DCC-E1A therapy is safe and feasible but for DCC-E1A therapy to be effective it has to be combined with other conventional or novel therapies [77].

DCC nanocarrier-based E1A therapy has also been investigated as a treatment for recurrent head and neck cancer [78]. In this trial, nine patients were treated with DCC-E1A via IT administration. The DNA dose tested in this trial was 15, 30, 60 and 120 \(\mu\)g DNA/cm of tumour. Although treatment was well tolerated, most of the patients reported pain and bleeding at the injection site. No MTD was achieved in this trial. In some patients, minor tumour response and/or stabilisation of disease was observed. In a follow-up phase II trial, DCC-E1A nanotherapy was conducted in 24 patients with recurrent, unresectable head and neck cancer [79]. Patients were treated with DCC-E1A (30 \(\mu\)g/cm\(^3\)) by IT injection for a total of 10 doses over a period of 8 weeks. The results showed that DCC-E1A therapy was safe and well tolerated. Additionally, DCC-E1A therapy produced clinical responses in a few patients, which ranged from complete response \((n = 1)\) to partial response \((n = 2)\) to disease stabilisation \((n = 7)\). Although some degree of therapeutic benefits was observed, combination
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of DCC-E1A therapy with other treatment modalities was warranted to produce an enhanced tumour response.

Despite the encouraging results obtained from the initial phase I/II clinical trials, there were no advances in the testing of DCC-E1A in phase II/III trials and in making DCC-E1A a clinical product.

Using the cationic 1,2-dimyristoylpropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE):DOPE formulation, a phase I clinical trial for IT delivery of IL-2 gene was performed for advanced head and neck cancer [80]. In this dose-escalating phase I clinical trial, 11 patients diagnosed with head and neck cancer were treated with the cationic lipid-based nanocarrier containing a plasmid DNA expressing IL-2 from 0.06 to 0.6 mg of plasmid DNA. The results from this trial showed that the IL-2 nanocarrier treatment was safe and well tolerated with no adverse toxicity observed. However, clinical responses were observed in only one patient. These studies have not continued or progressed to phase II clinical trial for further testing.

Another cationic lipid-based nanocarrier that is currently being tested in a phase I clinical trial is the DOTAP:Chol formulation [81]. In this clinical trial, DOTAP:Chol nanocarrier is being tested as systemic gene delivery vehicle for systemic treatment of NSCLC. This is the first systemic lipid nanocarrier-based gene therapy clinical trial in the world. The objective of this trial is to IV deliver the Fus1 TSG encapsulated in the DOTAP:Chol nanocarrier and determine the MTD. To date, patients diagnosed with metastatic stage IV NSCLC who have failed chemotherapy have been recruited in the trial and have received DOTAP:Chol-Fus1 treatment. Preliminary results show that treatment is well tolerated with no signs of treatment-related toxicity [81]. When completed, the results from this clinical trial will identify the MTD and allow conducting subsequent phase II trials with a defined Fus1 DNA dose and focus on both toxicity and therapeutic efficacy.

Based on the preclinical studies and the phase I trial testing systemic DOTAP:Chol nanocarrier delivery of Fus1 TSG in NSCLC, two additional phase I clinical trials for the treatment of pancreatic cancer, ovarian cancer and breast cancer have been approved by the Food and Drug Administration (FDA) and initiated in our institution. For treatment of pancreatic cancer, a plasmid DNA containing a mutant form of Bcl2-interacting killer (Bik) gene called BikDD that is under the control of cholecystokinin type A receptor promoter and regulated by VP16-GAL4-WPRE integrated systemic amplifier (VISA) will be used. The CVISA-BikDD plasmid encapsulated in DOTAP:Chol nanocarrier will be administered IV to determine the MTD and optimal biologically active dose and compared with the clinical response. This trial is currently open and recruiting patients. The results of this trial are expected to be known in 2012.
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For the breast cancer clinical trial, patients will be treated IV with DOTAP:Chol nanocarrier containing the E1A plasmid DNA. Expression of E1A is controlled by the widely used cytomegalovirus (CMV) promoter. Use of CMV promoter entails constitutive and high gene expression levels.

Another DOTAP:Chol nanocarrier-based cancer gene therapy trial that is currently being discussed is for the treatment of metastatic melanoma. Although the phase I trial has yet to receive approval from the FDA, plans are under way to deliver the tumour suppressor/cytokine gene, *IL-24*, encapsulated in the DOTAP:Chol nanocarrier for systemic treatment of metastatic melanoma. This phase I trial, when approved by the FDA, will also be conducted at the M.D. Anderson Cancer Center, Houston, TX, USA.

More recently, a phase I clinical trial for systemic treatment of solid tumours with a tumour-targeted cationic lipid nanocarrier carrying the *p53* TSG (SGT-53) has been initiated. This nanocarrier is formulated using DOTAP:DOPE to carry the *p53* TSG and coated on the outside with a targeting moiety to achieve tumour-targeted gene delivery. Tumour-specific *p53* gene delivery will be achieved via the anti-transferrin receptor single-chain antibody fragment, which is attached to the outside of the nanocarrier and targets the transferrin receptor which is overexpressed in cancer cells. The aim of this dose-escalating study trial is to determine the safety and the MTD. Patients recruited in this trial will be treated with plasmid DNA concentrations from 0.6 mg DNA per IV infusion escalating to 7.2 mg DNA per infusion. According to the treatment schedule planned patients will receive two treatments per week for a total of 5 weeks (10 treatments). Additionally, measurement of dose-related tumour response and toxicity is envisaged ([http://www.clinicaltrials.gov/](http://www.clinicaltrials.gov/)).

### 7.4 Conclusions

Cancer gene therapy has come a long way since the inception of gene therapy to treat genetic diseases two decades ago. During this period, our understanding of the biology of viral vectors has improved, and both their application and limitation in cancer therapy have led to the development and testing of lipid-based nanocarriers for cancer gene therapy. Although nanocarriers have several advantages over viral vectors, they also have limitations, including nanocarrier-induced inflammatory response [82]. Despite these limitations, it is exciting that a few of the lipid-based nanocarriers are currently being tested as gene delivery vehicles in clinical trials for treatment of cancer. It is also evident that cationic lipid-based nanocarriers are still the preferred vector of choice for systemic gene delivery, as evidenced by the number of cancer gene therapy clinical trials that are currently testing these nanocarriers.
When completed, the results from the phase I clinical trials will provide information leading to improvements in nanocarrier formulation, and strategies to overcome potential toxicity will be developed and their subsequent testing in phase II/III clinical trials will be undertaken. Given the rapid growth and interest in the field of nanotechnology and nanomaterials, it is anticipated that in the next few years, new and novel nanocarrier gene delivery systems will be developed and made available for testing in the clinic for cancer treatment. Some of the nanocarriers will be multifunctional in their properties and will be used for diagnosis, molecular imaging and therapy of cancer. Studies testing the utility of lipid-based nanocarriers for magnetic resonance imaging and molecular imaging of tumours have been reported [83, 84]. The use of gold nanoparticles coated on the outside with DNA and encapsulated in a cationic lipid was shown to be efficient in gene transduction and expression [85]. Using a similar concept, it is possible to develop hybrid gold nanoparticles containing an inner magnetic iron oxide core and coated on the outside with DNA, which, in turn, are encapsulated within a lipid nanocarrier that is decorated on the outside with a tumour-targeted ligand, making them multifunctional in their properties. Additionally, in the next few years, significant advances would have been made in the field of nanotechnology and nanomedicine and novel methods devised to regulate and control cationic lipid nanocarrier-mediated toxicity, thereby making more lipid-based multifunctional nanocarriers available for clinical cancer gene therapy.

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8 Lipid-based Biomimetics: General Perspectives in Drug and Vaccine Delivery

Ana Maria Carmona-Ribeiro

8.1 Introduction

The World Health Organization (WHO) has reported over 10 million new cases of cancer each year and over 6 million deaths annually caused by the disease [1]. Statistics predicts that the number of new cancer cases will increase to more than 15 million in 2020. Late diagnosis, metastasis and development of multidrug resistance (MDR) are major problems that are faced in clinics [2]. Nanobiotechnology has the potential to uncover the structure and function of biosystems at the nanoscale level, providing effective tools to treat diseases at a molecular scale, and to rationalise delivery of drugs and genes to solid tumours following systemic administration [3]. Vehicles applied in pharmaceutical product development include polymer-based nanoparticles, lipid-based nanoparticles (liposomes, nanoemulsions, solid lipid nanoparticles (SLN) and biomimetic nanoparticles), self-assembling nanostructures such as micelles and dendrimers-based nanostructures among others. In the recent years, much research has gone into the characterisation of novel carriers and their biological effects and potential applications. These include bottom-up and molecular self-assembly, biological effects of naked nanoparticles and nano-safety, drug encapsulation and nanotherapeutics, and novel nanoparticles for use in microscopy, imaging and diagnostics [4]. To be successful, a cancer treatment approach needs to overcome physiological barriers such as vascular endothelial pores, heterogeneous blood supply and heterogeneous architecture, to name just a few [5]. Optimal methods and devices of delivery are strongly required. In the past, many anti-cancer drugs had only limited success and had major adverse side effects [6]. Nanoparticles have attracted considerable attention worldwide because of their unique functional characters such as small and controllable particle size, high stability, lower toxicity, tuneable hydrophilic-hydrophobic balance and the ability to bear surface features for target specific localisation, and so on. Thus, polymeric nanoparticles constitute a versatile drug delivery system [7], which can potentially overcome physiological barriers and carry the drug to specific cells or intracellular compartments by passive or ligand-mediated targeting approaches [8]. The use of some polymers also allows, at least in principle, to achieve controlled release and sustained drug levels for longer periods of time. Numerous biodegradable polymeric nanoparticles made of natural
polymers such as proteins or polysaccharides have been tried for drug delivery and controlled drug release. More recently, the focus has also been on synthetic polymers, for instance, polycationic nanoparticles for encapsulation and controlled release of amphotericin B [9] and encapsulation of curcumin [10] or doxorubicin (DOX) for human cancer therapy [11]. Stable particles of insoluble drugs such as amphotericin B with very high drug content can be easily and reproducibly prepared through the application of the layer-by-layer (LbL) technology based on alternate adsorption of oppositely charged polyelectrolytes on the surface of drug particles (crystals) [9, 12]. Such polymeric coating prevents drug nanoparticle aggregation and creates a firm polymeric shell on their surface. Drug release rate from such nanocarriers can be easily controlled by assembling multilayer shells with variable shell density and thickness. Various additional functions, such as specific targeting ligands, can be easily attached to the surface of nanocolloidal particles of poorly soluble drugs by using a polymer with free reactive groups for the ‘outer’ coating. This may represent a novel approach to preparing convenient dosage forms of poorly soluble drugs. By incubating drug and poly-2-hydroxyethyl methacrylate (PHEMA) particles, DOX adsorption took place so that in vitro, there was a controlled drug release from the particles [11]. Methods of preparation of polymeric nanoparticles (PNP) fall into two major classes: one deals with the polymerisation of monomers (e.g., emulsion and dispersion polymerisation), whereas the other essentially involves dispersion of polymers (e.g., salting out, emulsification-diffusion and nanoprecipitation) [13]. Reports show that higher entrapment efficiency in PNP can be achieved by incorporation of drug during their preparation rather than adsorption on preformed nanoparticles [14]. Drug release takes place through their simultaneous biodegradation, followed by desorption, diffusion or erosion [15]. PNP exhibit different structures, for example, water-containing nanocapsules, oil-containing nanocapsules, nanospheres, PEGylated (PEG, poly(ethylene glycol)) stealth nanoparticles, PEGylated and targeted nanoparticles and polysaccharide-coated nonstealth nanoparticles [16].

One of the major challenges for cancer therapy is the selective killing of cancer cells without damaging normal cells. Small-molecule inhibition of proteins with an enzymatic activity has been a simple, successful and popular approach in drug discovery. For example, there is inhibition of cell cycle progression when certain proteins are unfolded [17]. Chaperones are an evolutionarily conserved class of proteins that guide the normal folding of many of the key regulators of cell growth, differentiation and survival. This essential guardian function is subverted during oncogenesis to allow malignant transformation and to facilitate rapid somatic evolution. In cancer cells, the newly synthesised oncogenic client proteins bind to chaperones. Upon adenosine triphosphate binding to certain chaperones, client proteins and chaperones form a mature complex. This mature superchaperone complex catalyses the conformational maturation of client proteins [18]. These oncogenic client proteins stimulate cancer
cell proliferation and survival. There are several chaperone inhibitors [19] and many of these [20] have entered preclinical or phase I/II clinical studies [21–24].

Most pharmaceutical agents have primary targets within cells. Therefore, effective formulations of these agents should reach sites of action within the cell. Selective subcellular delivery is likely to have greater therapeutic benefits [25]. Cytosolic delivery, for instance, is desirable for drugs that undergo extensive exportation from the cell via efflux transporters such as multidrug-resistant proteins and P-glycoproteins (P-gp). These efflux mechanisms continuously reduce therapeutic intracellular drug concentrations. MDR, which is related to cancer chemotherapy, tumour stem cells and tumour metastasis, is a huge obstacle to effective cancer therapy. One of the underlying mechanisms of MDR is the increased efflux of anti-cancer drugs by overexpressed P-gp of multidrug-resistant cells. Carriers loaded with drugs have been functionalised with the antibody against P-gp (anti-P-gp) and successfully killed cancer cells. An intracellular nanoparticle, consequently, may act as a drug depot within the cell. Nanotechnology may be used to achieve therapeutic dosing via targeted therapies, establish sustained-release drug profiles, and protect therapeutic compounds intracellularly avoiding their efflux or degradation. For example, biomacromolecules such as proteins have been entrapped and protected in carboxylethyl- or aminopropyl-functionalised mesoporous silica with rigid, uniform open-pore geometry [26]. The combination of high protein loading, high immobilisation efficiency and stability was attributed to the large and uniform pore structure, and to the optimum environment introduced by the functional groups [26]. Protein molecules were sequestered in or excluded from the porous material, depending on electrostatic interaction with the charged functional groups. In order to protect the contents inside the mesoporous silica, additional polyelectrolyte layers have been built on the particles [27]. Multilayered polyelectrolyte (PE) (poly(diallyldimethylammonium chloride), PDDA/poly(sodium 4-styrenesulfonate), PSS) or PE/nanoparticle (PDDA/silica nanoparticles, SiNP) shells were deposited onto the enzyme-loaded spheres [27]. The activity of the encapsulated enzyme was retained, even after exposure to proteases [27]. By using C12-trimethylammonium bromide versus C16-trimethylammonium bromide in the self-assembly of mesoporous nanoparticles, the distribution of pore sizes - a parameter that determines the release kinetics of the drug payload - was tuned from a centre at 1.8–2.5 nm [28]. As a further extension of this work, ibuprofen was introduced into the pores of these particles and placed in a simulated body fluid to determine their potential viability as drug delivery systems. These mesoporous silica materials could be used to deliver relatively large doses of drug in a controlled manner [29].

The complexation of charged macroions by oppositely charged polyelectrolytes is a fundamental process in biological systems and many technical applications. Particular examples are the complexation of histone proteins by DNA in nucleosomal core particles [30] as well as complexes of polyelectrolytes with charged colloids and micelles.
Industrial applications are not limited to drug formulation and stabilisation in the pharmaceutical industry being as diverse as stabilisation of colloidal suspensions, water treatment and paper making [32, 33]. For example, polysaccharide nanoparticles formed by complexation of cationic chitosan and anionic alginate have been employed to carry therapeutic peptides and proteins [34]. They are biocompatible, biodegradable, exhibit low toxicity and adhere to mucosae and other biological structures. Nanoparticles intended for use in the transmucosal delivery of molecules were prepared by the ionic gelation of chitosan hydrochloride with pentasodium tripolyphosphate and concomitant complexation with sodium alginate and they clearly enhanced the systemic absorption of insulin after nasal administration to rabbits [34]. Chitosan alone has also been useful for mucosal and transmucosal delivery of biomolecules [35].

In nanoemulsions of oil-in-water, a dispersed oil phase is stabilised in a water phase by an emulsifying layer of surfactant. Similarly, nanoparticles composed of solid lipids, such as SLN [36], have been stabilised with surfactants such as poloxamer 188, polysorbate 80, lecithin, polyglyceryl 3-methylgluco distearate, sodium cocoamphoacetate or saccharose fatty acid esters and used to carry drugs, peptides or proteins [37]. Advantages of SLN are their composition (biocompatible compounds), the fast and effective production process, including the possibility of large-scale production, the avoidance of organic solvents in the production procedures and the possibility to produce concentrated lipid suspensions. However, the drug-loading capacity of conventional SLN is limited because of the formation of a perfect lipid crystal matrix and other colloidal structures such as micelles, liposomes, mixed micelles and drug nanocrystals might be also present in the aqueous dispersion [38]. The preparation of SLN involves a first step of emulsification in hot water by stirring 10% of melted solid lipid such as stearic acid, 15% of surfactant and up to 10% of cosurfactant via microemulsions. Thereafter, the warm microemulsion is dispersed under stirring in excess cold water. Finally, ultrafiltration or lyophilisation removes the excess of water and increases the SLN concentration [39–42].

Liposomes or vesicles composed of natural lipids are biocompatible, biodegradable and nonimmunogenic [43]. These properties should make phospholipid liposomes suitable candidates for drug delivery. A low in vivo stability, however, was a major challenge solved with the introduction of the sterically stabilised liposomes formed by covalent attachment of PEG polymer chains to the phospholipid polar heads [44]. Also liposomes have found applications as immunoadjuvants in vaccines [45, 46], enhancers or carriers in medical diagnostics [47–49] and transfection vectors in genetic engineering [50–52]. In the cosmetics industry, liposomes represent an improvement offering an aqueous and biocompatible matrix to hydrophobic substances in flagrant contrast to gels and creams that employ skin-irritating oils, alcohols and tensioactives [53–55]. Methods to prepare and characterise bilayer vesicles or liposomes were
comprehensively discussed in the book edited by Roger New in 1989 [56]. Liposome physics and applications were compiled by Danilo Lasic [43] and the book entitled Liposome Letters was edited by Bangham, the father of liposomes, himself who described this book as a *pot pourri* of letters, pseudo-articles, articles and poems [57]. In 1983, the introduction of liposomes to the scientific world that took place in 1965 was wonderfully and enthusiastically documented in the book edited by Bangham [57]. Contributors to this last book are among the leading authorities in the field of liposomes. Professor Bangham, who retired in 1982, was seen in the December 2009 meeting of the International Liposome Society that took place in London, UK.

Amphiphiles form supramolecular assemblies of the bilayer type if they have an approximately cylindrical molecular shape resulting in a geometric parameter between 0.5 and 1.0 [58]. Natural phospholipids are not the only materials that form bilayers. Structural and functional aspects of biological membranes have also been reproduced in a variety of biomimetic systems [59–61]. Vesicle formation is not restricted to lipids but also is the preferential supramolecular assembly for synthetic amphiphiles such as dialkyl(dimethyl)ammonium bromide or chloride [62, 63], sodium dihexadecylphosphate (DHP) [64] and many others molecules [65–67]. The chemical stability of liposomes is low because acid/base catalysed hydrolysis may pinch off one or both hydrocarbon chains from the backbone of the lipid [68, 69] or oxidation may form cyclic peroxides at adjacent double bonds of the hydrocarbon chains resulting ultimately in the breakage of chains via lipoperoxidation [70]. Hydrolysis rate of soybean lecithin in liposomes is a function of pH and temperature, being at highest at extreme pH values where acid-base catalysis is enhanced and/or at the highest temperatures tested [71]. Oxidation may be prevented by using saturated lipids or by adding antioxidants such as vitamin E or butylated hydroxytoluene [72]. Synthetic amphiphiles that form bilayers certainly can be made chemically more stable than natural lipids. However, in contrast to natural lipids, which form colloidal stable bilayer membranes under physiological conditions (150 mM monovalent salt and pH 7.4), their colloid stability is low and their biological stability, that is, their stability in the biological milieu has been poorly investigated. Furthermore, cytotoxicity for some synthetic amphiphiles such as the cationic lipids in general and the dioctadecyl(dimethyl)ammonium bromide (DODAB) lipid in particular has been reported as high. This isolated fact may find useful applications in the design of liposomal antimicrobials where the liposomal carriers are active anti-microbial agents [73–77]. In spite of its toxicity, the capability of DODAB to induce retarded hypersensibility and cellular immune responses led to its usefulness as an efficient immunoadjuvant [78–82].

Other approaches have successfully combined different carriers to produce hybrid nanostructures or biomimetic particles. The combination of particles or flat surfaces with lipids in different assemblies produced interesting particulates and devices with an outer lipid coating [83–91]. Other hybrid nanoparticles combined polysaccharides
mechanically immobilised in matrixes of synthetic polymeric particles during particle synthesis [92–94]. These were obtained from traditional synthesis of polymeric particles by emulsion polymerisation in the presence of natural polysaccharides such as carboxymethylcellulose [92] or chitosan [93] and were excellent for enzyme adsorption [94]. Particles covered by lipids are also finding many interesting applications in drug and vaccine delivery since they profit from all basic knowledge acquired over the last decades for liposomes, vesicles and other lipid assemblies. The importance of the hybrid lipid-particle approach has been realised over the last 5 years with an exponential growth of research in this area. Not only have polymeric particles been used as supports for lipids [95–107] but also inorganic particles such as silica [108–134] or biological particles such as bacteria [73–77], fungi [9, 78], mammalian cells [135] or viruses [136, 137] have been coated by lipids. Drug solubilisation by hydrophobic domains of lipid bilayers followed by encapsulation with polyelectrolytes was another of such hybrid approaches [9]. Drug previously solubilised at hydrophobic borders of bilayer fragments [138] was surrounded by polyelectrolytes layers so that excellent anti-fungal activity against Candida albicans was obtained at low doses of amphotericin B [9] or other anti-fungal drugs such as miconazole [139, 140]. A third strategy stabilised hydrophobic drug particles in water dispersion by adsorption of lipid layers so that effective particulates at high drug-to-lipid molar ratios could be obtained [141, 142].

Carriers have to be tailored for targeting the site of drug action and a multidisciplinary approach is required to achieve this major task. All carriers quoted above such as liposomes, SLN, polymeric ‘micelles’, organic, inorganic, metallic, biological or biomimetic particles have been used in drug delivery to cells as well as organelle targets. They have exploited biological pathways to achieve payload delivery to cellular and intracellular targets and they can sometimes overcome the blood-brain barrier (BBB) [143–145]. Therefore they are potentially useful in the treatment of cancers and diseases of the central nervous system, such as glioblastoma multiforme, neurovascular disorders and neurodegenerative diseases. Drugs that are normally unable to cross the BBB following intravenous injection can be transported across this barrier by binding to poly(butyl cyanoacrylate) nanoparticles and coating with polysorbate 80 [143, 144]. Only drug-loaded nanoparticles coated with polysorbate 80 and/or withapolipoprotein B or E were effective. Polysorbate 80-coated nanoparticles apparently adsorbed these apolipoproteins from the blood after injection and thus mimicked lipoprotein particles that could be taken up by the brain capillary endothelial cells via receptor-mediated endocytosis. Bound drugs may then be further transported into the brain by diffusion following release within the endothelial cells or, alternatively, by transcytosis [143, 144]. A recent review on colloidal nanocarriers by Mishra and co-workers [146] emphasised formulation technology, carrier types and applications. Major carriers were classified among the following carrier types: the polymeric particles [15, 147–153], SLN [154–161], magnetic particles [162–164],
metal and inorganic particles [165–168], quantum dots [169–171], polymer ‘micelles’ [172–175], phospholipid-based micelles [176–178] and liposomes [179–182]. In this chapter, we add to this list the hybrid nanostructures called biomimetic particles [83–142], which combine particles and lipids or polymers and polysaccharides or polymers, lipids and other biomolecules of interest.

A decisive factor to formulate excellent drug carriers relies on our understanding of intermolecular interactions. Therefore, principles of self-assembly and fundamental intermolecular interactions are overviewed and discussed in the next section.

8.2 Intermolecular Interactions

Intermolecular interactions are important for the rational design of novel supramolecular assemblies, including biomimetic materials [183]. For example, during interphase, chromosomes in nuclei become organised by nonspecific, entropic forces acting alone [184]. Nonspecific interactions explain the position of heterochromatic chromocentres and nucleoli in interphase nuclei [185]. In the crowded environment of a cell, DNA and RNA polymerases have been modelled as beads spaced along a string. Aggregation of the large polymerising complexes increases the entropy of the system through an increase in entropy of the many small crowding molecules; this occurs despite the entropic costs of looping the intervening DNA [184]. In fact, the second law of thermodynamics acts through nonspecific entropic forces between engaged polymerases to drive the self-organisation of genomes into loops containing several thousands (and sometimes millions) of base pairs. Nonspecific interactions give rise to entropic effects. The cell nucleus contains up to 0.4 g/ml of macromolecules [186]. These biomacromolecules can be regarded as a mixture of large and small particles in a dense solution [187]. Around each particle a zone of excluded volume exists which is inaccessible to the centres of mass of other particles. Depletion attraction occurs when the translational and rotational degrees of freedom of each particle are limited by all other particles in a crowded environment. When less numerous large particles (such as nucleoli and heterochromatic regions) coexist with more numerous small particles, the total entropy gain of the small particles may outweigh the entropy loss of the large particles when the latter aggregate, thus minimising their excluded volumes [187]. This leads to an apparent force between the large particles, the depletion attraction. In the nucleus, depletion attraction has been implicated to be responsible for grouping DNA polymerases together into replication factories, but can be expected to affect many other structures or functional compartments as well.

Biomolecules in general have multiple sites for intra- and intermolecular weak interactions. The energy needed to break a carbon-carbon covalent bond is 348.6 kJ/mol
whereas only 4–29 kJ/mol are required to break a H-bond, depending on the atoms involved [188]. Wisely, Mother Nature kept together the two strands of double-stranded DNA by weak but cooperative hydrogen bonding so that replication and transcription can occur and life characteristics can be preserved and expressed [189]. The repulsion between nonpolar groups and water drives the hydrophobic effect which is the main factor leading to protein folding or to formation of supramolecular lipid assemblies such as the bilayer membranes [190].

The main weak interactions are the electrostatic interaction, the van der Waals interaction (ion-dipole, dipole-dipole, dipole-induced dipole interactions, induced-dipole induced-dipole), the hydrogen bonds, the hydrophobic interactions and the steric repulsion [188, 191]. The energy involved in the cohesive van der Waals intermolecular interaction between molecules in a liquid fairly corresponds to the vaporisation heat. The vaporisation heat is the energy required to increase the separation distance between the molecules in the liquid and change the physical state from liquid to gas. This energy amounts to about 10 kJ/mol [188]. Other interactions such as orientation-dependent dipolar and H-bonding interactions may occur simultaneously so that the cohesive force that holds molecules together in a liquid cannot be obtained in a simple way. Nevertheless, the relative strengths of different types of interaction are reflected in the boiling points of some compounds. For example, the nonpolar ethane, CH$_3$–CH$_3$, which does not have a dipole moment, has a boiling point of –89 °C whereas methanol, CH$_3$–OH, which has a dipole moment, boils at 64 °C. Upon increasing the molecular size both for a polar or a nonpolar molecule, the importance of the dispersion forces increases. For example, ethane and $n$-hexane boil at –89 and 69 °C, respectively.

Intermolecular interaction energies ($E$) are classified based on their nature and intensity of decay, as a function of the separation distance ($d$) between pairs of atoms or molecules interacting in vacuum. Charge-charge interactions are purely coulombic, electrostatic with the free energy of interaction ($E$) decaying with the separation distance ($d$) as $1/d$. For charge-dipole interactions, $E$ is proportional to $1/d^2$ (fixed dipole) and to $1/d^4$ (freely rotating dipole). For dipole-dipole interactions, $E$ is proportional to $1/d^3$ (two fixed dipoles) or to $1/d^6$ (two freely rotating dipoles). For nonpolar-charge interactions, $E \propto 1/d^4$. For nondipolar-dipole or nonpolar-nonpolar interactions, $E \propto 1/d^6$. The covalent interaction is complicated and short ranged in its quantum mechanical nature and the hydrogen bond is also complicated, short-ranged, with $E$ roughly proportional to $1/d^2$.

The electrostatic interaction may be reduced by the intervening medium. The dielectric constant of water at 25 °C is 78.5 whereas the dielectric constant of dodecane is 2.0. Thus, water is very effective in reducing electrostatic interactions between charged atoms or molecules, whereas intervening media such as dodecane and other
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hydrocarbons act as insulators. The interaction between water and a small ion corresponds to the ion-dipole interaction. The ion causes a strong aligning effect on the surrounding dipolar water molecules and becomes 'hydrated' forming the 'hydrated ions'. The first shell of water molecules around a strongly solvated ion is usually referred to as the primary hydration shell. This shell of oriented dipoles causes a second shell of less strongly oriented dipoles and the second causes a third and so on, so that a solvation zone is formed around the ion that is different from the bulk. When solvation zones of two solvated molecules or ions or surfaces overlap, solvation or structural forces arise that are repulsive and short-ranged; these forces are known as hydration forces if the intervening medium is water.

All atoms and molecules are polarisable. For a nonpolar molecule, the polarisability arises from the displacement of its negatively charged electron cloud relative to its positively charged nucleus under the effect of an external electric field, whereas for a polar molecule the total polarisability includes an additional term due to its permanent dipole moment. For neutral atoms and molecules, transient polarisabilities cause the induced-dipole induced-dipole forces also known as dispersion forces, London forces or electrodynamic forces [192, 193]. The dispersion forces are always present, playing a significant role in adhesion, surface tension, physical adsorption and so on. They are the most important contribution to the total van der Waals force. For the interaction between two methane molecules, which are completely neutral and do not bear any permanent dipole moment, the theoretical dispersion energy contribution to the total van der Waals interaction is 100% [191]. In contrast, for two water molecules that do bear permanent dipole moments, the dispersion energy contribution to the total is 24% with the major contribution being due to the orientation force. Water, however, is not the general case. In general, dispersion forces constitute the dominant term in the equation that describes the van der Waals interaction force between molecules.

Dipolar forces are generally small. For the hydrogen halides: from HCl to HI the strength of the total van der Waals interaction increases even as the dipole moments decrease. Generally, the dispersion term dominates the interaction exceeding the dipole-dependent induction and orientation forces. A serious shortcoming of the London theory of dispersion forces is its neglect of the solvent effect mediating the intermolecular interactions since interactions took place in vacuum. The medium was considered by the generalised theory of van der Waals forces by McLachlan [194]. Overall, the total polarisibility of a molecule is a function of its absorption frequencies and the McLachlan approach computed the dispersion forces between molecules that have a number of different absorption frequencies or ionisation potentials, for which the simple London expression breaks down. The McLachlan approach can be applied to molecules or small particles interacting in a given medium. The dielectric constant of the medium is also a function of the absorption frequency of the medium. The solvent medium may significantly reduce the van der Waals force for two nonpolar
molecules relative to their interaction in vacuum. The attractive or repulsive coulombic interaction has a longer range than most attractive van der Waals contributions because the interaction energy varies with $1/d$ whereas interactions between freely rotating dipoles or nonpolar atoms or molecules decay as a function of $1/d^6$. This will be of importance for determining the total interaction energy also for macroscopic particles or surfaces.

The interactions of macroscopic particles or surfaces may be computed by summing all the pair potentials between the molecules in each particle or surface. The additivity of forces between interacting pairs yields a resulting interaction force between macroscopic bodies that is much larger and decays much more slowly with the separation distance between the particles or the surfaces than the intermolecular forces between a single pair of molecules or atoms do. Together with electrostatic, steric-polymer and short-ranged solvation forces, the van der Waals forces are among the most important interaction forces acting between macroscopic particles and surfaces in liquids. The net interaction energy becomes proportional to the size of the particles, so that the energy can be very much larger than $kT$ even at separations of 100 nm or more [195].

Typical values for the Hamaker constants of condensed phases (solids or liquids) are about $10^{-19}$ J for interactions across vacuum. The atomic density $\rho$ is proportional to $1/v$, where $v$ is the volume of one atom, and the coefficient $C$ in the interatomic pair potential is roughly proportional to the square of the polarisibility $\alpha$ which in turn is roughly proportional to the volume $v$ of an atom. Thus, the Hamaker constant $A$ will be proportional to $C\rho^2$, that is, to $\alpha^2 \rho^2$, that is, to $v^2/\nu^2$, that is, to a constant [195]. This reasoning justifies the fact that Hamaker constants of most condensed phases are inside the $0.4 \times 10^{-19}$ to $4.0 \times 10^{-19}$ J range. For example, the three different media as are hydrocarbon, CCl$_4$ and water, composed of molecules differing greatly in size and polarisibility, have very similar Hamaker constants: $0.5 \times 10^{-19}$, $0.5 \times 10^{-19}$ and $1.5 \times 10^{-19}$ J, respectively [191].

The problem of the pairwise additivity approach is the neglect of the influence of neighbouring atoms on the interaction between any pair of atoms. Also, this approach cannot be extended to bodies interacting in a medium. The Lifshitz theory ignored the atomic structure and treated the large bodies as continuous media with well-defined bulk properties such as their dielectric constants and refractive indices [196, 197]. Theoretical techniques using a modified additivity approach permit calculations of Hamaker constants as a function of static dielectric constants of the media involved and of dielectric constants at imaginary absorption frequencies [193, 198–200]. The dielectric permittivity $\varepsilon (i\nu)$ of a medium varies with frequency $\nu$ in the same way as does the atomic polarisability of an atom. A high Hamaker constant $A$ means a high polarisibility, which, in turn, means a high dielectric constant and refractive index. Hamaker constants of metals and metal oxides can be up to an order of magnitude
higher than those of nonconducting media. Some efficient conducting metals have static dielectric constants that are infinite. Hamaker constants for metals such as silver, copper or gold, are in the range $25 \times 10^{-20}$ to $40 \times 10^{-20}$ J, that is, about 10 times higher than those computed for water, as expected for a much more polarisable material. For hydrocarbons (e.g., $n$-pentane, $n$-octane, $n$-dodecane) interacting across vacuum or air, $A$ varies between $3.8 \times 10^{-20}$ and $5.1 \times 10^{-20}$ J in excellent agreement with exact solutions ($3.7 \times 10^{-20}$ to $5.2 \times 10^{-20}$ J). The Lifshitz theory holds when the separation distances between interacting bodies, $d$, is much larger than molecular dimensions, $\sigma$, because it is a continuum theory. For two dodecane phases interacting across vacuum or air, $A$ is $5.0 \times 10^{-20}$ J, that is, approximately 10 times larger than $A$ for two dodecane phases interacting across water [201–203]. In general, there is a good agreement between experimentally measured van der Waals forces and theory [204–211]. However, in liquids, unlike air or vacuum, other forces are usually present: long-range electric double-layer forces, solvation and steric forces. Similar particles suspended in water or in any liquid of high dielectric constant are usually charged and this prevents their coalescence (coagulation). Because the van der Waals interaction between similar particles is always attractive, they would aggregate if the interaction force between them were only of the van der Waals type. Other repulsive forces such as the electrostatic, the steric (as for polymers or adsorbed biomolecules on surfaces) and the solvation forces do come into play to generate the colloid stability so often observed for particles in liquids.

The origin of the charges on a charged surface can be either dissociation of ionisable surface groups, for example, $\text{–COOH} \rightarrow \text{–COO}^- + \text{H}^+$; or specific binding of ions from solution onto a previously uncharged surface. In the case of an ionic crystalline solid, like silver iodide in water, its charge can be controlled by the activity (concentration) of Ag$^+$ or I$^-$ ions in solution. These ions are potential-determining ions because their activity controls the sign and magnitude of the electrostatic potential on the particle surface. Other examples of potential-determining ions are H$^+$ and OH$^-$ for surfaces of metal oxides (e.g., silica particles), polymer latex (e.g., carboxylate polystyrene microspheres) or charged bilayer vesicles (e.g., dihexadecylphosphate synthetic vesicles).

The final surface charge is balanced by an equal but oppositely charged region of counter-ions, some of which are bound, usually transiently, to the surface within the Stern or Helmholtz layer, while others form an atmosphere of ions in rapid thermal motion close to the surface, the so-called diffuse electric double layer. There is accumulation of counter-ions (oppositely charged with respect to the surface) at the surface and the depletion of co-ions for a planar negatively charged surface in the presence of a water solution of a monovalent salt. The counter-ion number density along the distance from the surfaces can be obtained from the Boltzmann distribution of ions $i$ at a given distance $x$ and from the Poisson equation that is a differential equation relating the net excess charge density at $x$ with the electrostatic potential at $x$. The
solution of the Poisson–Boltzmann equation gives the potential $\Psi$ and thereby the counter-ion number density $\rho$ at any distance from the surface.

The model for the diffuse double layer initially proposed by Stern in 1924 [212] and refined by Grahame in 1947 [213] assumes that all adsorbed counter-ions are confined to the Stern layer which defines the plane where the diffuse double layer begins: the so-called outer Helmholtz plane. The Stern potential at the outer Helmholtz plane is lower than the surface potential at the physical surface where surface charges are located. A third surface potential definition also useful is the $\zeta$-potential defined as the potential at the plane of shear for a moving charged particle. The surface potential at the shear plane is a good approximation of the Stern potential [214]. The accumulation of oppositely charged ions is given by the Boltzmann equation at any distance $x$ from the charged surface.

The scheme available for solving the Poisson–Boltzmann equation for the case of a double layer around a spheric surface involves the Debye–Hueckel approximation at small potentials and a number of analytical approximations at higher potentials as reviewed by Hunter in 1981 [215]. There is a semiempirical equation, as proposed by Loeb and co-workers [216] in 1961, and extensive tables of the potential and charge relation computed by these same authors. Some good approximate analytical expressions were developed by Lee White [217] and by Ohshima and co-workers [218].

During interactions of two similar colloidal particles or charged planar surfaces, their electrical double layers overlap so that a repulsive force develops. If the approach is slow and equilibrium can be established between the ions on the surface and in the bulk, the surface potential would remain constant during the approach. This would be the case for interacting silver iodide particles [219]. If the particle charge is caused by crystal defects, as in some clay minerals, the surface charge can be assumed as constant during the interaction. For interacting oxide surfaces, the interaction itself affects the degree of dissociation of the surface groups so that neither the surface potential nor the surface charge is constant during the approach. In this last case, the charge regulation model may be more appropriate one [220]. Regarding the oxide surfaces, its surface charge is caused by the dissociation of amphoteric surface hydroxyl groups and this is determined by the pH. The surface potential $\Psi_0$ is a function of both pH and electrolyte concentration so that the high surface charges on the oxides can be reconciled with the low measured $\zeta$-potentials, that is, although the amount of bound counter-ions on the surface is low, the binding is quite strong, causing low $\zeta$-potential values [214, 215, 221–225].

The analytical procedures for finding the profile of the electrostatic interaction energy $V$ are rather lengthy and complicated [214, 226, 227]. The problem can be solved numerically by using the computer facilities available nowadays. For example, we
calculated numerically the electrostatic interaction energy as a function of the separation distance between charged spherical vesicles or bilayer-covered polystyrene microspheres interacting in water solutions of a monovalent electrolyte [89, 228, 229]. Chan and co-workers [230] and Healy and co-workers [231], in 1980, have also used direct numerical procedures to evaluate $V$ as a function of the separation distance $D$ for flat double layers interacting under conditions of constant charge, constant surface potential or charge regulation (surface groups undergoing dissociation with the degree of dissociation being influenced by the interaction). The regulation must always lie between the limits of constant charge and potential [232, 233]. Under charge regulation, values for the surface dissociation parameters such as site density and dissociation or binding constants should be independently obtained.

The calculation of the total interaction energy between interacting flat surfaces or particles, taking this energy as the sum of the repulsive double-layer energy and the attractive van der Waals energy, is the basis of the DLVO theory [219, 226]. This theory is successful for many interacting systems but fails for systems where other interaction forces are present such as the solvation forces resulting in structural repulsion at short distances (<1 nm) between surfaces not included in the DLVO theory. Also the hydrophobic effect may cause additional attractive forces for which formal treatments are not available [233]. In a single interacting system, several different interaction forces may be contributing to the total interaction force directly measured using a surface force apparatus [206, 210] or the osmotic stress technique by Parsegian, Rand and co-workers [234]. This is the case for charged phospholipid bilayers interacting across water solutions for which the osmotic stress technique resulted in measurements in fair agreement with the DLVO theory for $D > 1$ nm, and extra-repulsive forces due to hydration of the phospholipids polar heads for $D < 1$ nm [235]. Another good example of the multiplicity of forces acting in a single system is the case of two dihexadecyl phosphate monolayers adsorbed on hydrophobised mica, interacting across water solutions at different pH and monovalent salt concentrations [236]. At low pH values, an extra-attractive force of unknown origin appears in addition to DLVO forces. At high pH values, adsorption of hydrated sodium ions is the more probable explanation for the extrarepulsive force appearing at short ranges. Direct measurement of forces for the dihexadecylphosphate system as a function of the separation distance was possible because the negatively charged mica sheets were first hydrophobised with a cationic DODAB monolayer which interacted hydrophobically with a DHP monolayer forming a bilayer of mixed composition [236]. Figure 8.1 illustrates the bilayer assembly of DODAB/DHP adsorbed on mica so that the interaction forces between outer DHP monolayers could be directly measured using the surface force apparatus.

Early evidences for non-DLVO interactions were the absence of coagulation for some colloids such as silica even at very high electrolyte concentrations [237], redispersion
of some flocculated particles by dilution of the electrolyte (repeptisation) [238] and an additional short-range repulsion for two mica sheets interacting across electrolyte solutions, at KCl concentrations higher than $10^{-4}$ M [239, 240]. At close distances, the van der Waals attraction should pull the mica sheets into adhesive contact in the primary minimum, but adsorption of the hydrated potassium ions at the mica surface caused the so-called hydration-repulsive force which is short-ranged ($<1$ nm) and decays exponentially with a decay length of approximately 1 nm, dominating the attractive van der Waals force [239, 240]. The spontaneous swelling of clays [241] and uncharged lipid bilayers in water [235, 242, 243], repeptisation and the stability of many colloidal dispersions in very high salt [244] could also be alternatively explained by thermal agitation or steric interactions preventing close approach in the case of lipids and other amphiphilic structures. The finite thickness of the Stern layer in the case of interparticle forces would also explain the repulsive, short-ranged interaction forces [233]. The DLVO theory indeed neglects the finite ion sizes that

Figure 8.1 Schematic representation of supported lipid bilayer on mica. Mica, a negatively charged aluminium silicate, supported composite bilayers of DODAB/DHP by deposition of a cationic DODAB monolayer followed by deposition of an anionic DHP monolayer using the Langmuir–Blodgett technique. These supported bilayers were prepared as such in order to determine interaction forces between the two supported bilayers by means of the surface force apparatus. Adapted from P.M. Claesson, A.M. Carmona-Ribeiro and K. Kurihara, Journal of Physical Chemistry, 1989, 93, 2, 917. ©1989, ACS [236]
generate a hard-wall cut-off at a finite separation equal to twice the diameter of the adsorbed hydrated ion (one layer of adsorbed ion per each of the two interacting surfaces). Claesson, Horn and Pashley, in 1984, directly measured forces between two mica surfaces in concentrated aqueous solutions (0.7 M) of tetraalkyl ammonium bromide salts [245]. In this case, the net repulsion steeply rises as the intersurface separation decreases up to contact, with contact being in very good agreement with twice the ion diameter. The thickness of the Stern layer can therefore induce stability in a real system in the sense that a physical ionic barrier exists that prevents adhesion at the primary minimum of the interaction energy curve as a function of the separation distance between the surfaces. Only direct measurements of the ‘hydration’ or, more generally, ‘solvation’ forces using the surface force apparatus could define decay lengths, range and force-distance profiles, which would be important to ascribe to these forces a real dependence on solvent structure [233]. For uncharged amphiphilic structures such as bilayers of lecithins, phosphatidylethanolamines, DODAB, DHP and the oxyethylene surfactants, direct measurements of interaction forces were performed either using the surface force apparatus for layers adsorbed on mica [211, 236, 243, 245–248] or using the osmotic stress/X-ray diffraction method developed by Rand, Parsegian and co-workers [235, 249–254]. The principles behind direct surface force measurements performed using these techniques are as follows. Whereas the surface force apparatus measures interactions between amphiphiles adsorbed or deposited on two bare or hydrophobised mica sheets, the Rand–Parsegian method measures interactions between free lipid multibilayers. The first method measures forces from the elastic deflection of a spring, which is depicted from interferometric patterns formed by the light crossing a sequence of thin, optically transparent films. The second method measures forces from the osmotic pressure applied externally to the interlamellar spacings after this pressure equilibrates the interbilayer repulsive forces. Distances are determined from X-ray diffraction patterns and repeating lamellar distances at each imposed osmotic stress. Both methods yield similar results at least for interaction forces between lipidic layers [233]. Uncharged bilayers composed of lecithins, phosphatidylethanolamines, sugar head-group glycolipids and the oxyethylene surfactants exhibit limited swelling in water with some short-range hydration repulsion arising from their hydrophilic head groups. Also the force-distance profiles are expected to include an attractive van der Waals component. Furthermore, the fluid nature of these structures causes local fluctuations of thickness and head-group area [254] so that this thermal motion of relatively lengthy head groups could generate a kind of ‘steric’ force, which would exhibit a force-distance profile [242, 254] very similar in shape to the profile obtained for polymeric polyethylene oxides [255, 256]. The difference between the profiles is solely with regard to the range: polymers, which are macromolecules of very high molecular weight, display their force curve up to 80–120 nm for molecular weights 160,000. For phospholipids, the range goes only up to 2–3 nm. Shapes for the curves obtained with neutral phospholipids are very similar [191]. For more fluid bilayers in the liquid-crystalline state the range for the
repulsive steric force is slightly larger than the range displayed by more rigid bilayers in the gel state. Whether the ‘hydration’ repulsion measured for amphiphiles such as lipids is due to thermal motion of polar heads or to solvent (water) structuring around the polar heads or to both is still an open question.

The hydrophobic effect is entropic in nature and comes from the immiscibility of inert substances with water [190]. Water droplets on hydrophobic surfaces display large contact angles and reorientation of water around nonpolar solutes or surfaces is entropically very unfavourable. The existing water structure is disrupted by the hydrophobic surface and a more ordered, entropically unfavourable water structure is generated on the surrounding water. Experiments for silica and mica surfaces rendered hydrophobic by chemical methylation or plasma etching revealed that the force is long ranged, decaying exponentially for the first 10 nm and then more gradually [257–259]. For adsorbed, uncharged phospholipid bilayers on mica, the van der Waals component is sufficient to describe the interbilayer interaction as long as the polar, hydrophilic head groups shield the hydrocarbon chains from water [211, 243, 246]. By stressing the elastic and fluid bilayers to expose more of their hydrocarbon moieties to water, the attraction acquires characteristics of the hydrophobic interaction: longer ranges and larger attractive forces [260]. Indeed it is not unusual for lipid bilayers to become defective, exposing hydrophobic regions to water and thereby fusing due to the hydrophobic interaction. Some cationic amphiphile bilayer vesicles composed of dioctadecyldimethylammonium halides may undergo fusion simply by increasing monovalent salt concentration in their medium under isotonic conditions [261–263].

8.3 Carriers in the Biological Milieu

The inadequate understanding of physicochemical interactions between a carrier and host immune system seems responsible for technical difficulties in constructing a multicomponent long-circulating system with optimal performance in vivo [264]. Since the 1970s, when the feasibility of using liposomes with enzymes, anti-cancer and anti-microbial drugs was established [265, 266] as was their ability to potentiate immunoresponses to antigens [267], liposomes have found many uses as vehicles of choice for drug [268–270] and vaccine delivery and targeting [271–273]. Even the most stable conventional carriers such as the liposomes are rather unstable in a biological milieu. In addition to interactions with various plasma components and enzymes, they are actively taken up by the immune system. Their clearance from blood takes place via three major routes: (i) opsonisation or preparation for phagocytosis, that is, chemi- or physisorption of immune and nonimmune components in blood such as immunoglobulins, albumin, complement and fibronec tin; (ii) phagocytosis or macrophages uptake by the fixed macrophages of the reticuloendothelial (RES) system.
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(liver and spleen); and (iii) remotion of the phospholipid molecules from the vesicles by high-density lipoproteins (HDL) in the plasma leading to vesicle disintegration [43, 44, 274–276]. The problem of HDL-induced destabilisation of bilayers has been resolved by the addition of excess cholesterol and/or by using phospholipids with a high gel-to-liquid-crystalline transition temperature [277–279]. Targeting drugs carried by liposomes to cells in the RES requires that they retain their load while they are circulating [273]. Surface-grafted polymers can prolong blood circulation times even for fluid liposomes [44] since the repulsive forces between particles can be greatly increased by coating the surface of the particle with natural and synthetic polymers [32]. Nonionic, water-compatible, flexible and well-hydrated polymers have been preferred for attachment to the surface either by adsorption, hydrophobic insertion, electrostatic binding or by grafting through covalent bonding. The repulsion between surfaces with attached grafted polymers was shown to be dependent on the density of grafting and degree of polymerisation [280]. At high densities, the steric repulsion is determined by competition between an elastic force associated with the conformational entropy of the chains and an osmotic force related to the monomer-monomer interaction [281]. The polymers repel opsonins, allowing liposomes to circulate in the blood for longer, in a manner that is apparently dose-independent [282–286]. This prolonged circulation enhances the contact with areas of inflammation or in tumours, where vessels are inherently leaky [287, 288] but the uptake of liposomes in the tumour mass by tumour cells is size-dependent and unlikely to be extensive, especially in solid tumours with large avascularised areas [289]. Using polyclonal antibodies attached to liposomes, the idea of using cell-specific ligands to target liposomes to cells was put forward in the seventies by Gregoriadis and co-workers [289–291]. A variety of tumour cell lines were tested in vitro and a solid tumour was tested in vivo regarding cell uptake of liposomes to which polyclonal antibodies were previously attached [289, 290]. Also, a galactose-terminating glycoprotein was inserted in liposomes to direct them to the galactose receptor of the liver [290, 291]. These approaches substantially increased target cell recognition, liposome binding to the target cells and cell penetration. In the 1980s, conditions for obtaining optimal targeting were described upon varying structural characteristics of vesicles incorporating anti-target monoclonal antibodies (immunoliposomes) or other cell-specific ligands on their surface [292–295]. Major limitations with the immunoliposomes approach are related to the accelerated interception of the vesicles by the RES system so that premature clearance of the liposomes occurred, to their operationally useful minimum size of 40–100 nm in diameter which is too large for extravasation from the capillaries so that their use to cellular or molecular targets that only can be attained by extravasation is hampered, and to the foreign immunoglobulin that is bound to them leading to a liposome-mediated promotion of immunogenicity with production of antibodies in the host that can neutralise and prematurely remove injected immunoliposomes [273, 296, 297]. In contrast to electrostatic stabilisation, which was effective in many in vitro applications, steric stabilisation is a suitable way of
stabilising particles in biological systems and in media with high or variable ionic strength. Antibodies were coupled to liposomes that incorporate GM1 ganglioside or phosphatidylinositol (PI) at between 5 and 10 mol% into the bilayer [298, 299] or PEG polymer chains covalently coupled to lipids [300]. This approach resulted in efficient localisation of drug-containing immunoliposomes in lung tumours with substantial reduction in tumour size [299, 301]. Liposome targeting using human desialylated glycoproteins or neoglycoproteins made by conjugation of receptor-specific sugars to human albumin were considered safe [273]. After binding to cells, the fate of the liposomes involves endocytosis with bilayer disruption inside the lysosomes, cell organelles rich in phospholipases. This disruption releases liposomal contents allowing them to act inside the cell. For labile contents at acidic pH, evasion from lysosomal localisation was achieved by using pH-sensitive liposomes for which the acidic environment induced liposome fusion with the lysosomal membrane and release of liposomal contents in the cytoplasm [302].

Coupling strategies for improving biological stability of liposomes via steric stabilisation involved grafting oligo(ethylene oxide) to different lipids [44, 303]. Phosphatidylethanolamine has been often used for the attachment of longer PEG chains [44]. In the case of attachment of PEG chains to cholesterol, no prolonged blood circulation times were obtained because these cholesterol derivatives presented very high critical micelle concentration so that they can be quickly lost from the bilayer upon dilution [303]. Electrophoretic mobility and ζ-potential measurements for charged PEG-liposomes showed decreased mobility of PEG-coated liposomes relative to the conventional sample with the same surface charge, this result being ascribed to the hydrodynamic drag by the polymer [304]. Flexibility of the surface-attached polymer chains reduces the adsorption of blood components on biocompatible surfaces generating the so-called mobile steric hindrance [305–307]. The spontaneous uptake of liposomes and other carriers by the liver and the spleen favours applications in the treatment of intracellular microbial infections or for vaccination or on the extended retention of small liposomes in the circulatory system for intravascular or extravascular access to cancer or infected cells [273]. Liposomal amphotericin B has been advantageously used for the treatment of systemic candidiasis in cancer [308], neutropenic [309] and transplant patients [310], being more effective and less nephrotoxic than the conventional formulation. The same advantages were obtained for anthracycline antibiotics as daunorubicin and DOX, cytostatic drugs widely used in the treatment of a variety of human cancers that had their cardiotoxicity reduced upon their incorporation in liposomes [311–317]. The first liposome-based vaccine licensed for use in humans was against hepatitis A virus (HAV) [318, 319]. The protocol for producing the vaccine involved several steps: (i) purification of the capsid of the HAV; (ii) purification of the influenza virus and preparation of a DNA-free extract containing envelope glycoproteins and phospholipids to be mixed with the HAV capsid; and (iii) enrichment of the above mixture in with egg-yolk phosphatidylcholine (PC) and
phosphatidylethanolamine [318]. In this vaccine, a key feature is that the envelope of the influenza virus contains haemagglutinin (HA) which, in the vaccine, mediates fusion with the target cell membranes accounting for the good results obtained with the hepatitis A liposomal vaccine [319, 320]. The viral envelope is reconstituted in absence of the genetic material of the native viruses retaining, however, the cell entry and membrane-fusion characteristics of the virus. Thus, influenza-derived virosomes are taken up by cells through a process of receptor-mediated endocytosis, which directs the vaccine to the endosomal cell compartment. Subsequently, the virosomal membrane fuses with the endosomal membrane from within the lumen of the endosomes. This fusion process requires low pH and is mediated by HA establishing continuity between the lumen of the virosole and the cell cytosol. Therefore, antigen-containing virosomes interacting with antigen-presenting cells are expected to introduce part of the antigen into the cell cytosol and, thus, into the major histocompatibility complex class I presentation pathway.

Especially where vaccines are either ineffective or unavailable, the de novo production of a vaccine antigen by the host's cells in vivo defines an interesting research area called genetic immunisation [52]. The uptake by cells of antigen-encoding plasmid DNA somehow causes transfection and antigen production in a manner similar to that found by antigens of internalised viruses leading to protective humoral and cell-mediated immunity. The problem with this type of vaccine is DNA degradation by interstitial deoxyribonucleases so that DNA needs protection from the extracellular milieu. Liposomes have been used to protect DNA and simultaneously deliver the antigen to the antigen-presenting cells (the macrophages) which are eager to swallow the liposomes [321–323].

Another important aspect of modern vaccination is the design of subunit vaccines using lipid-based carriers as immunoadjuvants. The problem of presenting peptides, which typically have a low immunogenicity, in suitable adjuvants to mimic epitopes of pathogens was classically solved by coupling the peptides to carrier proteins. However, more interesting from a vaccination standpoint, is the development of some powerful peptide carriers as are the hydrophobic anchors that can be inserted in liposomes. In a first generation of liposomal constructs, short peptide antigens were associated to liposomes, surface-bound, encapsulated or membrane-associated [324–328]. To reproduce the presentation by a pathogen of antigens to the immune system and to trigger a functional immune response, a second generation of liposome constructs has been exploited by chemically coupling two different peptides to the same preformed liposome on two different hydrophobic anchors [329]. The association at the surface of the same vesicle of two independent epitopes that remain independent from a molecular point of view and thus may have different fates with regard to the immunocompetent cells (e.g., B lymphocyte or T helper cells targeting) generated highly immunogenic diepitope constructs [329]. The two epitopes were
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coujugated respectively to functionalised phosphatidylethanolamine (one hydrophobic anchor) and to a lipopeptide (the other hydrophobic anchor) and inserted in the liposome bilayer generating a particularly potent and long lasting (1 year) immune immunoglobulin G response in Balb/c mice; importantly these immunoglobulins cross-reacted with the parent protein [329].

In Alzheimer’s disease, the brain accumulates the β-amyloid peptide in the form of insoluble amyloid plaques. Amyloid filaments, similar to those found in amyloid plaques can be assembled in vitro from chemically synthesised β-amyloid peptides. Antibodies raised against the N-terminal region (1–28) of the β-amyloid peptide bind to the in vitro formed β-amyloid assemblies disaggregating the fibrils and restoring the peptide solubility. Also the antibody was found to prevent in vitro fibrillar aggregation of the β-amyloid peptide [330]. In order to anchor the β-amyloid peptide in liposomes, two palmitoylated lysines were appended at each of the two termini of the peptide. The palmitoylated peptide was then inserted in the bilayer of liposomes [331]. Mice immunised with the liposomal palmitoylated β-amyloid peptide developed very high antibody titres and the antisera thus, obtained was shown to solubilise, in vitro, β-amyloid fibres [331].

Folic acid, attached to PEG-derivatised distearoyl-phosphatidyl ethanolamine (DSPE) has been used to target in vitro liposomes to folate receptor (FR)-overexpressing tumour cells [332–334]. Binding and subsequent internalisation of dipalmitoyl-phosphatidyl ethanolamine (DPE)-rhodamine-labelled liposomes by a high FR-expressing murine lung carcinoma line with inhibition by free folic acid was demonstrated by confocal fluorescence microscopy [334]. The in vitro cytotoxicity obtained with DOX-loaded folate-targeted liposomes was 10-fold greater than that of the nontargeted liposome formulation, but remained generally inferior to that of free drug against both drug-sensitive and drug-resistant tumour cells [334]. Probably, the biological activity of liposomal DOX released inside the cell is reduced in vitro due to the aggregated state of the drug, and requires a long time and/or an in vivo environment for full expression [334]. The approach is an effective route of intracellular drug delivery to multidrug-resistant tumour cells.

The need for controlled drug delivery indicates the essential role of lipid-based biomimetics to build effective drug delivery systems. The monoclonal antibodies carried by liposomes were not always effective in targeting anti-cancer drugs, particularly when bulky liposomes were used. Viruses have learned to smuggle DNA into the nucleus incorporating their own DNA in the genome of the host cell. This is precisely what is required in gene therapy but is not always feasible because target cells are not always accessible. The question is whether especially designed carriers could compete with viruses. In fact, only a very modest liposome-mediated gene expression has been obtained via a mechanism that is not completely known and difficult to
control. Liposome vectors formulated with cationic lipids and a helper lipid readily form complexes with DNA known as lipoplexes. In vitro cationic lipids are being widely used for cell transfection. However, several drawbacks arise in clinical trials for gene therapy [335]. There is aggregation and inactivation by serum, which limit the stability and activity of the cationic carriers. By condensing DNA with the polymer or changing the surface charge with anionic molecules, protecting the surface with PEG and increasing the target specificity by attaching the ligand, the efficiency and stability of the vector were improved [336]. However, there is still the problem of an inflammatory toxicity coming from certain motifs in the plasmid DNA that should be reduced [336]. Nonviral vectors including nanoparticles and liposomes can be engineered for reduced immunogenicity, infection or mutagenesis, and can transport large nucleic acid payloads [337–340]. These vectors typically employ cationic liposomes or cationic polymers such as chitosan, polylsine or polyethylenimine (PEI) complexed with anionic nucleic acids [337, 341]. Among the cationic polymers, PEI has demonstrated the highest gene delivery efficiency in vitro because of its large cationic charge that facilitates a high degree of DNA complexation and an effective ability to escape the cellular endosome upon internalisation via the ‘proton sponge’ effect [342]. PEI displays, however, inherent toxicity and lack of cell specificity for selective transfection, a problem partially circumvented by covalent modification of the cationic polymer with PEG [337, 341]. PEI was conjugated to antibodies [343], peptides such as RGD (arginine-glycine-aspartic acid) for integrin receptor targeting of endothelial cells of tumour metastases [344] or small organic molecules such as folic acid for FR targeting in colon adenocarcinoma and oral epidermoid cancer cells [345] or the peptide chlorotoxin (CTX) [346] which binds with specificity and affinity for the vast majority of brain tumours (74 out of 79) [347], prostate cancer, sarcoma and intestinal cancer [346]. CTX peptide has been providing excellent targeting in previous in vitro and in vivo nanoparticle studies [348, 349] and has recently been coupled to a branched PEI polymer functionalised with grafted PEG and the DNA loading optimised, providing an interesting example of hybrid nanoparticles highly effective against cancer cells in culture [350]. This approach could potentially lead to further use of this carrier system in vitro and in vivo for benchside and bedside applications as a selective, stealth, nonviral targeting agent. Similarly, cationic lipids in the form of liposomes or biomimetic particles have been evolving as the basis of systems with targeting and steric stabilisation as additional functionalities, improving biocompatibility and specificity. Codeelivery in one liposomal drug delivery system of DOX simultaneously with small-interfering RNA (siRNA) targeted to MDR1/ MRP1 and BCL2 mRNA enhanced cell-death induction by increasing intracellular DOX concentration and suppressing cellular antiapoptotic defence [351]. The carrier consisted of cationic liposomes (carrier, 100–140 nm), DOX (anti-cancer drug) and siRNA targeted to molecules representing pump and nonpump cellular resistance so that the complex was approximately 500 nm in mean diameter and presented a ζ-potential of +4 mV [351].
Minko and co-workers [352] have been developing very interesting hybrid strategies to be applied in cancer chemotherapy. Enhancement of the efficacy of chemotherapeutic drugs is achieved by the suppression of antiapoptotic cellular defence. DOX, with a suppressor of antiapoptotic cellular defence synthetic peptide corresponding to the minimal sequence of BCL-2 homology 3 (BH3) domain, the BH3 peptide, was delivered into cells by fusion with a fusogenic peptide sequence [353]. The cytotoxicity of DOX, Ant-BH3 and Ant-BH3 mixed in with DOX, mitochondrial transmembrane potential, expression of genes encoding pro- and antiapoptotic members of BCL-2 protein family and caspases, caspases activity and apoptosis induction were assessed in human ovarian carcinoma cells. It was found that the combination in one drug formulation of DOX and Ant-BH3 enhanced the apoptosis induction by an anti-cancer drug, and prevented the development of antiapoptotic cellular drug resistance. The results confirmed that anti-cancer drug-BH3 combination might form the basis for a new advanced anti-cancer proapoptotic drug delivery system. Considerable efforts have been made recently to suppress MDR and/or antiapoptotic cellular defence [353–355]. These included synthetic analogues of the BCL2 homology 3 (BH3) domain of the proapoptotic members of the BCL2 protein family, including BAK, BAX and BAD [352, 356–360]; antisense oligonucleotides and siRNA targeted to BCL2, MDR1 and MRP mRNA [361–363], c-Jun NH2-terminal kinase [364]; traditional ribozyme drugs [365]; several drug groups from a traditional mitomycin C [366]; and the exotic plant stress hormones family of jasmonates [367]. However, these attempts have not demonstrated a high efficiency in terms of their anti-cancer effect. The inhibition of only one contributor to cellular resistance is not sufficient for overcoming all mechanisms of cancer-cell resistance to chemotherapy. Optimal efficacy of the treatment required delivery of all cell-death inducer(s) and suppressor(s) of both types of cellular-drug resistance simultaneously inside the cancer cell [351, 368].

New technologies particularly RNA interference (RNAi) mediated by siRNA have become more and more interesting. They provide effective therapeutic entities to silence pathogenic gene products associated with disease, including cancer, viral infections and autoimmune disorders. The Nobel Prize in Physiology or Medicine has been awarded to Andrew Fire and Craig Mello for their discovery of RNAi, that is, the suppression of gene activity by double-stranded RNA [369]. siRNA molecules, notably the antisense strand, recognise and inhibit the corresponding mRNA, thereby silencing the appropriate gene. RNAi works at a posttranscriptional level by targeting mRNA as a mean for inhibiting the synthesis of the encoded protein [370]. Salient features of RNAi were identified by Fire and Mello in their 1998 paper. These are (i) the ability of a few molecules of double-stranded RNA to direct destruction of a much larger amount of the corresponding mRNA, suggesting a catalytic mechanism; (ii) the transmission of RNAi across generations; (iii) the power of RNAi to bring genetics to any organism whose genome sequence is
known; and (iv) the near universality of RNAi among eukaryotes. Main strategies for delivering RNAi to cells were recently reviewed by Castanotto and Rossi [371]. The discovery that gene expression could be controlled by base-pairing of small RNA with messenger RNA containing complementary sequence, a process known as RNAi, has markedly advanced our understanding of eukaryotic gene regulation and function [371]. The modulation of oncogenes expression by siRNA is set to revolutionise the treatment of cancer. Remarkably, despite being just one decade from its discovery, the phenomenon is already being used therapeutically in human clinical trials [371]. Cationic carriers will certainly be useful in novel RNAi-based therapeutics.

To assess the stability of siRNA under conditions relevant to clinical use with particular emphasis on topical delivery considerations, a study of three different unmodified siRNA was performed [372]. The results indicated that neither repeated freeze/thaw cycles, extended incubations (over 1 year at 21 °C), nor shorter incubations at high temperatures (up to 95 °C) have any effect on siRNA integrity as measured by nondenaturing polyacrylamide gel electrophoresis and functional activity assays. Degradation was also not observed following exposure to hair or skin at 37 °C. However, incubation in foetal bovine or human sera at 37 °C led to degradation and loss of activity. Therefore, siRNA in the bloodstream is likely inactivated, thereby limiting systemic exposure. The design of lipid-based formulations of siRNA for systemic use aims at protecting the RNA from degradation in the circulation [373]. Cationic lipids have been essential components of such formulations because they are required to achieve efficient loading of anionic polymers such as siRNA and they facilitate intracellular delivery of polynucleotides such as siRNA by destabilising biological membranes following uptake by endocytosis.

Cationic polymeric nanoparticles have also been designed aiming at siRNA protection [374]. In this last approach, nanoparticles were prepared by emulsion polymerisation of isobutylcyanoacrylate and isohexylcyanoacrylate with chitosan. Thereafter, loading of the nanoparticles with siRNA was achieved by adsorption. The biological activity of the siRNA-loaded nanoparticles was assessed on mice bearing a papillary thyroid carcinoma after intratumoural and intravenous administration. The adsorbed antisense siRNA in vivo lead to a strong anti-tumoural activity so that tumour growth was almost stopped after intravenous injection of the antisense siRNA-loaded nanoparticles, while in all control experiments, the tumour size was increased by at least 10 times. In vivo studies have also confirmed the relevance of using chitosan-coated poly(isobutylcyanoacrylate) nanoparticles as a delivery system to inhibit the expression of ret/PTC1 oncogene as a treatment of the papillary thyroid carcinoma [375]. The siRNA approach was also used for specific targeting and downregulation of fusion oncogenes in chronic myeloid leukaemia and in a bone cancer, the Ewing sarcoma [376].
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Osteosarcoma is the most common malignant primary bone tumour. Its growth is strongly supported by bone resorption. Antibodies or recombinant proteins have inhibited the cytokine receptor activator of nuclear factor-κB ligand. Recently, its expression was inhibited using genetic approaches based on siRNA [377]. The delivery of siRNA to its cellular target and demonstration of its efficiency in vivo relied on two new osteosarcoma models expressing firefly luciferase. These luciferase-expressing osteosarcomas showed conserved osteolytic and osteogenic activities in mice and were detectable by in vivo bioluminescence imaging. Bioluminescence analysis revealed extensive cell death in response to ifosfamide treatment. Additionally, a protocol for in vivo administration of siRNA combined with cationic liposome was established.

8.4 Lipid Coating on Particles and Its Applications

The self-assembly of amphiphiles into spherical micelles, cylinders, bilayers and other structures in water solution is fairly well understood [191]. However, the effect a solid surface may have on amphiphile assemblies deposited on the surface is not at all clear. Advanced microscopy techniques such as atomic force microscopy (AFM) provided direct visualisation of assembled amphiphiles at atomically flat substrates where surface corrugation was much smaller than the dimensions of the surfactant assemblies [378], but many important surfaces that interact with surfactants, lipids or biomolecules are neither flat nor smooth as, for example, the surface of biological cells. Adhesion of vesicles formed from Escherichia coli lipids, a lipid mixture rich in lipopolysaccharides with bulky and strongly hydrated polar head groups, took place on glass surfaces [379], similarly to the formation of an adhered vesicle layer for cationic vesicles made of DODAB onto the rough and highly hydrated surface of bacteria [73]. The electrostatic attraction between these cationic vesicles and the negatively charged cell surfaces drove the adsorption of the nondisrupted cationic vesicles onto bacteria [73]. Lipidic vesicles assembled on silica and titanium dioxide particles delivered the oil-soluble bactericide Triclosan™ to oral bacteria and inhibited their growth [380]. The cationic DODAB vesicles themselves efficiently interacted with several pathogenic bacteria revealing their potency as bactericides [73–76, 381]. Quantification of the amount of DODAB adsorbed from vesicles onto E. coli cells was performed from determination of adsorption isotherms. The limiting adsorption was consistent with deposition of a layer of adjacent vesicles on the cell. Absence of vesicle disruption upon interaction with the bacteria was depicted from absence of [C14]-sucrose leakage in experiments where this marker was used to label the inner water compartment of the vesicles [75]. The physical and chemical factors that determine the deposition of bilayer vesicles from aqueous media onto flat solid surfaces or onto particles are still poorly understood. Vesicle deposition onto a solid surface would be determined initially by the classical combination of the
repulsive force arising from the interaction of the electrical double layers associated with the vesicle and the solid surface and the attractive dispersion force between the vesicle and the solid. Vesicles are not, however, permanently rigid structures, and depending on their size and chemical composition and that of the aqueous medium they can distort, aggregate, disrupt and fuse with each other. Deposition of vesicles onto a solid surface could give rise to any particular one or a combination of these processes. Unilamellar PC vesicles were reported to break open and adhere to a mica surface to form a bilayer coating, in spite of the indirect evidence obtained from the measured separation between two surfaces when pushed together [382]. Further compression of the closely apposed bilayers can result in fusion into a single bilayer. Dipalmitoylphosphatidylcholine (DPPC) and PI adsorb from vesicles onto negatively charged and hydrophobic Ballotini glass beads as a monolayer with their head groups outermost [383]. Phospholipid monolayers with lipid hapten inserted are supported by hydrophobic glass and are useful for specific adhesion of macrophages and cell surface recognition studies, but cannot serve as hosts for transmembrane proteins [384]. Supported phospholipid bilayers on hydrophilic surfaces such as glass or silicon wafers have been obtained from direct fusion of small unilamellar vesicles in the liquid-crystalline state [385–388]. Phospholipid vesicle fusion at the hydrophilic surface was induced by traces of divalent cations. A second method method for preparing supported membranes was the controlled transfer of monolayers to the surface using the Langmuir trough [236, 389–393].

Charged synthetic vesicles of dioctadecyldimethylammonium chloride (DODAC), DODAB or DHP have found a myriad of new uses [67, 84, 86, 381, 394–401]. They are highly charged in water, in the gel state at room temperature and have a much smoother surface charge distribution than other colloids [399]. Because of these properties, they have been used to test theories for colloids [67, 228–263, 396–400] despite their polydispersity. Homodisperse polystyrene latices, on the other hand, may have hairy, rough or conducting surfaces. In 1992, bilayer-covered polystyrene microspheres were produced [84]. Small and large vesicles prepared from DHP and DODAC or DODAB interact with oppositely charged polystyrene microspheres forming bilayer-covered latices [84]. Small unilamellar phospholipid vesicles and polystyrene microspheres also interact in aqueous solution to form homodisperse and stable phospholipid covered latices [85]. In a first step, the vesicle breaks open and the bilayer attaches to the latex; in a second step the hydrophobic attraction between the phospholipid bilayer and the hydrophobic polystyrene surface induces coverage with one phospholipid monolayer with polar heads outermost. Thereafter, the van der Waals attraction between the phospholipid layer and free vesicles in the dispersion drives the deposition of one or more phospholipid bilayers onto the monolayer-covered latex [85]. Also for DPPC and distearoylphosphatidylcholine bilayers, this time adsorbed at the hydrophilic silicon/water interface, single and double bilayers have been prepared with neutron reflectivity measurements allowing
precise and nondestructive characterisation of the structure, hydration and roughness of the layers: 2–3 nm as distance between two deposited bilayers, the second one being highly hydrated [402].

The interaction between lipid bilayer vesicles and polymeric surfaces was evaluated from contact angles and ellipsometric determination of thickness of the deposited molecular films in air. Polymeric films of polystyrene sulfate (PS) or polystyrene amidine (PSA) latices on silicon wafers were prepared by spin-coating from toluene solution and dried under vacuum [403]. Small unilamellar vesicles composed of PC, DODAB or sDHP over a range of concentrations (0–1.2 mM) interacted in pure water in the time of approximately 16 hours with the polymeric films. After rinsing and drying, the lipidic films on polymer coated-silicon wafers had thickness values in air equivalent to one monolayer of each lipid (about 2.2–2.5 nm), with the exception of PC films over a certain range of PC concentrations (0–0.5 mM), which were 5.5–6.5 nm thick. In all cases, the molecular lipid films in air yielded hydrophobic coverage with advancing contact angles equal to 75, 82 and 89° for PC, DODAB and DHP, respectively. The data in air fairly agreed with previously reported adsorption isotherms for these lipids onto latex particles in pure water [67].

Self-assembly of a bilayer-forming cationic lipid at oppositely charged polymeric films led to different assemblies depending on concentration of NaCl added to the vesicles. Salt concentrations previously shown to induce intervesicle fusion favoured also vesicle fusion to the air-water and to the flat polymer-water interface [89]. However, in absence of salt (pure water), electrostatics was not enough to produce bilayer-assembly of DODAB on the PSS films [403]. This situation contrasted singularly with the one previously described for charged vesicles and oppositely charged polystyrene microspheres for which bilayer deposition took place in pure water with adsorption driven by ion-pairing between charged polar heads of the lipid and oppositely charged functional groups on the microspheres [84]. For more hydrophilic spherical supports, such as silica, DODAB vesicles interacted with the particles to produce bilayer-covered particles both in pure water and at 10 mM ionic strength [108, 111, 113]. The same occurred for flat mica sheets, which bear a large negative charge density [404]. It is possible that our PS film spin-coated on the wafer did not present a charge density large enough to induce flattening and deposition of the cationic bilayer. Also, the surface charge density on the DODAB bilayer vesicle is reduced by bromide counter-ion binding [405] and the adsorption was shown to be controlled by vesicle diffusion, another clear indication that the electrostatic attraction between the PSS film and DODAB vesicle was not driving adsorption at very low salt concentration such as 0.1 mM NaCl. On the other hand, inducing hydrophobic defects on the vesicle bilayer upon addition of 50 mM NaCl [263] accelerated DODAB lipid adsorption to rates above those controlled by vesicle diffusion [89]. Another advantage became apparent from the vesicle deposition induced by hydrophobic defects:
adjacent vesicles adhering at the polymer surface are prone to fuse with each other because they still bear hydrophobic defects after attachment to the surface. The \textit{in situ} kinetics for DODAB adsorption upon addition of 50 mM NaCl onto the PSS film clearly demonstrated the efficiency of the ‘hydrophobic defects’ method to produce a flat and stable bilayer coverage on the polymer surface [89, 403].

The quartz crystal microbalance with dissipation monitoring (QCM-D) technique offered a possibility to investigate, in real time, changes in the viscoelastic properties of adsorbed biomolecular layers, which could be directly related to the conformation of the layer [406]. This surface-sensitive method with high time resolution QCM-D [407], and an imaging method with high spatial resolution AFM [408], were used to investigate the behaviour of vesicles with varying net charges, from positively charged pure dioleoyltrimethylammonium-propane (DOTAP) to mixtures of neutral dioleoylphosphatidylcholine (DOPC) and negatively charged dioleoylphosphatidylserine. QCM-D, provided information about the mass and the conformational changes of adsorbed material: it was a useful tool to study the formation of supported lipid bilayers [404, 407–409]. The decomposition of adsorbed vesicles into bilayer patches took place at an elevated critical vesicular coverage [408]. AFM allowed investigating the morphology of single vesicles and isolated bilayer patches [408, 410–412] and following the formation of supported lipid bilayers (SLB), provided structural details of the SLB formation process [413].

In certain cases, the presence of proteins in the liposomes prevented formation of supported bilayers on planar SiO$_2$ surfaces [414]. The study was performed with two types of transmembrane proteins reconstituted into liposomes: proton translocating nicotinamide nucleotide transhydrogenase (TH) and gramicidin A. TH is a redox-driven proton-pumping protein present in energy-transducing membranes in animal mitochondria and in certain bacteria [415, 416]. TH contains one membrane-spanning domain, domain II (30 kDa), which is composed of 13 $\alpha$-helices, and two hydrophilic domains, domain I (43 kDa) and domain III (30 kDa), both exposed to the solution on the same side of the membrane [415, 417]. Trypsin treatment of TH removed domain I, which made it possible to study the influence of this domain on the SLB formation process. The water-exposed domains of TH, incorporated in the bilayer membrane of liposomes, prevented SPB formation on SiO$_2$ [414].

Tethered polymer-SPB have been designed to separate the lipid bilayers from the supporting solid substrate thus allowing reconstitution of integral membrane proteins [418–420]. The tether polymer would be attached at its two ends to the substrate and to some of the membrane lipids [418]. For example, the channel activity of a phytotoxin was probed in tethered membranes on gold surfaces by means of impedance spectroscopy so that characterisation of water-soluble spontaneously inserting bacterial toxins was possible [420].
Immobilising liposomes onto solid chromatographic phases for drug partitioning has been developing recently as a major applied field in bioanalytical chemistry [421–425]. Drug partitioning into lipid bilayers was studied by chromatography on liposomes and biomembranes immobilised in gel beads by freeze-thawing. The drug retention volume was expressed as a capacity factor, $K_s$, normalised with respect to the amount of immobilised phospholipid. Log $K_s$ values for positively charged drugs on brain phosphatidylserine (Ps): egg PC liposomes decreased as the ionic strength was increased, increased as the Ps: PC ratio or the pH was increased and varied linearly with the temperature. Log $K_s$ values for beta-blockers, phenothiazines and benzodiazepines on egg phospholipid (EPL) liposomes correlated well with corresponding values on red cell membrane lipid liposomes, and on human red cell membrane vesicles containing transmembrane proteins. A fair correlation was observed between the values on EPL liposomes and those on native membranes of adsorbed red cells. Compared to the data obtained with liposomes, the retentions of hydrophilic drugs became larger and the range of log $K_s$ values narrower on the vesicles and the membranes that exposed hydrophilic protein surfaces and oligosaccharides. Lower correlations were observed between drug retention on EPL liposomes and egg PC liposomes; and between retention on liposomes (or vesicles) and immobilised artificial membrane (IAM) monolayers of PC analogues. Absorption of orally administered drugs in humans (literature data) was nearly complete for drugs of log $K_s$ values in the interval 1.2–2.5 on vesicles. Both vesicles and liposomes can thus be used for chromatographic analysis of drug-membrane interaction and prediction of drug absorption. The correlation between the absorption of drugs in humans after oral administration (literature data) versus log $K_s$ values on the vesicles showed low absorption at the outskirts of the range 0.6–3.0 found for the analysed substances, whereas drug absorption was nearly complete at intermediate log $K_s$ values. At low log $K_s$ values the drug is too hydrophilic for partitioning into the membrane, whereas at higher log $K_s$ values the absorption is probably hampered by the high partitioning into the membranes and the slow diffusion out from the membrane.

Coating of capillaries in electrophoresis with liposomes of 1-palmitoyl-2-oleyl-sn-glycero-3-phosphatidylcholine (POPC) or POPC with different proportions of bovine brain Ps and cholesterol formed a bilayer structure on the silica surface enabling the separation of neutral compounds [422]. The effectiveness of the coating in separation was evaluated with use of uncharged steroids as model compounds. Taking into consideration both separation and stability, best results were achieved with anionic 80:20 mol% POPC/PS liposomes and [N-(2-hydroxyethyl) piperazine-N9-(2-ethanesulfonic acid)] (HEPES) as the buffer. Successful separation of steroids was achieved only when HEPES buffer was used in the coating procedure and in the background electrolyte solution for the separation. With all other buffers, the peaks of the model compounds overlapped. Divalent cations also affected the stability and
structure of phospholipid vesicles and the binding and immobilisation of proteins into phospholipid membranes [425]. The effect of calcium, magnesium and zinc on coating fused silica capillaries for electrophoresis with zwitterionic PC was determined. A molar ratio of 1:3 PC/Ca^{2+} or PC/Mg^{2+} gave the best coating stability owing to the increased rigidity of the phospholipid membrane furnished by the divalent metal ions [425]. The effect of temperature on the coating stability was investigated by coating 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) liposomes at temperatures above and below the gel- to fluid-state transition. Better results were obtained with DPPC in the more rigid gel state than in the fluid state: the electroosmotic flow was much suppressed and the PC coating was stabilised. Coating the fused silica capillary with PC liposome-metal ion buffer solutions resulted in good electrophoretic separation of basic model proteins (pI-values 7.8–11.0). The electrophoretic results demonstrate the importance of stabilising the phospholipid coating on fused silica capillaries, either by the addition of divalent metal ions (Ca^{2+}, Mg^{2+} or Zn^{2+}) or by working in the gel-state region of the phospholipid.

On particles, Bayerl and co-workers [426] showed the formation of supported phospholipid bilayers on silica, Esumi and co-workers [427] deposited a synthetic cationic bilayer of DODAB and reported phospholipid adsorption on silica [428] and Carmona-Ribeiro and co-workers [84] first demonstrated deposition of a synthetic lipid bilayer onto oppositely charged latex via electrostatic attraction or deposition of a neutral phospholipid monolayer on amidine latex via hydrophobic interaction between hydrocarbon chains of the phospholipid and the hydrophobic latex surface [85].

A series of monodisperse sulfate polystyrene latex dispersions were covered with DODAB bilayers from small vesicles for particle sizes ranging from 76 to 412 nm [86]. The ζ-potential of these bilayer-covered particles in water remained constant (and positive) over the entire range of sizes tested. The kinetics of NaCl-induced flocculation for the bilayer-covered microspheres was obtained and the results used to construct curves of the logarithm of the stability ratio against the log of electrolyte concentration. At a given salt concentration, stability increases, reaches a maximum, and then decreases as a function of size. Slopes of the stability curves were calculated theoretically and compared with those obtained experimentally [86]. The DLVO approximation by Reerink and Overbeek for an ideal colloid predicted an increase of slope with particle size which was not observed experimentally but DLVO models which include aggregation at the secondary minimum turned out to be qualitatively consistent with the experimental dependence of colloidal stability on particle size [86]. Dioctadecyldimethylammonium (DODA) bromide (DODAB), chloride (DODAC), and acetate (DODAAC) bilayer-covered microspheres were more stable than vesicles of similar sizes under identical medium composition. Possibly, the vesicle colloidal instability was due to asymmetry of charge distribution that
occurs when salt is added to the vesicle outside, that is, counter-ions bind at the outer vesicle surface but cannot bind at the inner vesicle surface because salt does not penetrate into the vesicle interior [429]. With acetate as the counter-ion at pH 5.1–5.3, specific acetate binding at the inner vesicle surface would be possible due to permeation of the neutral acetic acid through the vesicle membrane, thereby resulting in a high colloid stability. This stability was the highest ever observed for DODA vesicles. As counter-ion size and hydration increased (from bromide to acetate) so did colloid stability of vesicles and covered microspheres [429]. The effect of increasing particle size was to decrease colloid stability due to reversible aggregation at a secondary minimum [429].

Contact angle measurements on polystyrene or poly(styrene/methacrylate) surfaces with aqueous DODAB, DODAC or DODAAc dispersions over a range of lipid concentrations (10⁻⁶ to 10⁻⁴ M) were reported [430]. For the polystyrene surface without charge, angles decreased as a function of lipid concentration for the three lipids, an indication that lipid molecules would be lying on the hydrophobic homopolymer, whereas for the charged copolymers angles first increased and then attained a plateau value as a function of lipid concentration. Counter-ion effect on the nature of the deposited lipid layer was to increase hydrophilicity according to: acetate > chloride > acetate, whereas the effect of increasing the copolymer surface charge was to promote a higher degree of vertical orientation of the hydrocarbon chains facilitating bilayer deposition [430]. The most hydrophobic surface under air was the one obtained from the interaction between DODAB and the most charged copolymer [430].

Whereas polystyrene microspheres represent well a hydrophobic surface, silica particles are good models for a hydrophilic surface. The mechanism of the interaction between bilayer membranes and silica was temptatively evaluated by determining adsorption isotherms of four different bilayer-forming amphiphiles on hydrophilic silica over a range of experimental conditions [108]. The separate use of synthetic charged membranes with phosphate or tetraalkylammonium groups as polar heads such as are DODAB and DHP bilayer vesicles, to obtain adsorption isotherms on silica established the relative importance of phosphate or tetraalkylammonium on the mechanism of phospholipid deposition onto hydrophillic silica particles at 10 mM buffer and pH 7.4 [108]. Formation of ion pairs between the quaternary ammonium in the choline moiety of the phospholipid and the deprotonated silanol drove vesicle adhesion to the particle but vesicle rupture and bilayer deposition was determined by the cooperative occurrence of several hydrogen bridges between silanol and the phosphate moiety on the phospholipid [108]. A low affinity between neutral phospholipids and the silica surface and a high affinity for the cationic amphiphile over a range of pH values was obtained [111]. Tris-hydroxymethyl aminomethane (Tris) used as buffer increased affinity between PC and silica at pH” 7.4 [111]. This result
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was interpreted as due to Tris adsorption on silica with an increase in the surface density of hydroxyls on the surface available to hydrogen bridging with phosphate phospholipid groups [111]. Bilayer deposition, however, was unambiguously confirmed by the three techniques only for the interaction DPPC vesicles/silica over 1 hour at 65 °C and for the interaction DODAB vesicles/silica over the all range of experimental conditions tested [111]. A simple spectrophotometric method for identifying entire bilayer deposition onto solid particles was developed from incorporation of the optical probe Merocyanine 540 onto the outer bilayer vesicle surface. Upon bilayer deposition on the particle, sandwiching the marker between bilayer and solid particle reduced light absorption, this reduction being quantitatively related to bilayer deposition [111].

For the interaction between cationic DODAB/DPPC and anionic PI/DPPC vesicles with zinc citrate dispersions, liposome adsorption took place without liposome disruption [431]. Also, for several types of liposomes interacting with hydrophilic solid surfaces containing ionisable groups such as citrate [431, 432] or silanol [108, 111], the pH affected the extent of adsorption, adsorption increasing with decreasing pH for anionic liposomes and increasing with increasing pH for cationic liposomes. The fusion and spreading of phospholipid bilayers on negatively charged glass surfaces is dependent on pH and ionic strength with membrane fusion of negatively charged membranes being favoured by low pH and high ionic strength as driven by the van der Waals attraction and membrane fusion of positively charged membranes onto the surface taking place under all conditions tested [433].

There seems to be important differences between the interactions that involve liposomes with flat or curved solid surfaces, though these differences were not systematically studied except for a few observations on the unwillingness of bilayers to curve on rough surfaces and the difficulty of covering surface defects with bilayers [433] or on differences in affinity between DPPC and PC bilayers and curved or flat SiO₂ surfaces [108, 110]. It seems reasonable to expect that differences in molecular geometry as predicted from the self-assembly model do lead to differences in packing of these molecules also at the solid surface. Therefore, the molecule having a geometry that is closer to a cylinder will prefer to deposit as a flat aggregate on a flat surface instead of depositing on highly curved particles. Similarly, molecular shapes departing from the cylindrical geometry would not be comfortable depositing on flat surfaces. For example, CTAB, a micelle-forming amphiphile, adsorbs onto hydrophobic surfaces as hemicylinders of liquid-crystalline aggregates of amphiphilic molecules formed at the interface [378]. The bilayer bending that is needed for deposition on curved surfaces requires energy that can be offered by electrostatic attraction between oppositely charged groups on the bilayer or on the surface and/or highly cooperative hydrogen bonding between phospholipid polar heads and certain groups on the surface, for example, silanols. From the comparison between Langmuirian
adsorption isotherms for PC, DPPC or DODAB deposition on silica particles with 50 nm mean diameter taken from references [108, 111], affinity constants between the lipid and the surface were calculated which increased in the following order PC < DPPC < DODAB. From the molecular shape for PC and DPPC in the bilayer, which is more cylindrical for PC than for DPPC, the geometric parameter for PC results larger than the geometric parameter for DPPC and it becomes difficult for the PC bilayer to bend and deposit on a curved silica particle but bending is easier for a DPPC bilayer. The contrary should be expected for PC and DPPC deposition on a silicon wafer [110]. For DODAB, the electrostatic interaction between vesicle and surface yielded the highest affinity.

Contact angle measurements are simple to perform and can be used to indicate the increase in surface hydrophobicity under air that results from depositing a bilayer on a given flat surface under water [109–111, 410]. For charged poly(styrene/methacrylate) copolymers advancing and receding contact angles increase as a function of DODA bromide, DODA chloride or DODA acetate concentration in solution attaining a maximal plateau value [430]. Also the highest surface hydrophobicity was obtained for the copolymer with the highest charge density demonstrating that bilayer deposition is dependent on a large electrostatic attraction between surface and bilayer [430]. The contact angle approach is useful to identify whether bilayer deposition took place on a surface in solution at a given set of experimental conditions [430].

Essentially, biomimetic particles composed of a particle core coated with lipids associated the advantages of particles and lipids. The solid core conferred mechanical stability to the lipid layers and, for biodegradable cores, particles could eventually be used in vivo as a carrier for bioactive compounds. The main advantages of the lipid envelope were biocompatibility, a biomimetic behaviour of cell membranes and the ability to interact with a wide variety of molecules, either within the membrane or on the surface, depending on the physicochemical properties of the carried species. Over the last two decades, the use of membrane coatings on colloidal particles offered an extensive repertoire of chemical functionality [434]. The concept that PC bilayer-covered silica particles represented a valuable asset to isolation and reconstitution of a model receptor-ligand pair was proved for the cholera toxin receptor and cholera toxin which were successfully reconstituted in the biomimetic particles [119]. The reconstitution was highly dependent on optimisation of PC bilayer deposition on the silica particles as achieved from systematic studies of PC adsorption over a range of pH and ionic strength [116].

De Cuyper and co-workers [435] deposited a phospholipid wrapping on nanometre-sized magnetisable iron oxide grains to obtain magnetoliposomes for biomedical applications, such as allergy diagnosis from in vitro detection of IgE, contrast agents.
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in magnetic resonance imaging [436], recovery and separation of proteins from protein mixtures [437] and other in vivo applications [438]. Another stabilising procedure for iron oxide grains was encapsulation of an aqueous magnetic fluid composed of several iron oxide grains in giant liposomes [439–444].

Glass microspheres coated with phospholipid layers evaluated the binding specificity of enzymes on cell surface [445] or allowed quantitative analysis of lipid-antibodies binding [446, 447]. Hydrogels exhibited a pH-dependent volume which enabled the control of the storage or release of encapsulated drugs [448–450]. Polymer hollow capsules were produced by Mohwald and co-workers [451–458] from the polyelectrolyte LbL technique on biological cells as templating cores and thereafter dissolving the template. Permeability control of ions and small neutral molecules was achieved by analogy with the barrier function of biological membranes from the lipid coating on these capsules [459]. Formation of multilayered coatings on particles [459] and particle stabilisation/flocculation [460–462] have been the subject of much recent effort devoted to the understanding of interactions between model mixtures of oppositely charged pairs such as charged particles and oppositely charged polymers. The competitive adsorption of positively charged chitosan and cationic DODAB bilayers onto latex particles was systematically studied, aiming at latex sterilisation and preservation due to the biocidal properties of DODAB cationic bilayer and chitosan as the cationic polyelectrolyte [463]. The results evidenced the competition between the DODAB cationic bilayer and chitosan for negatively charged sites on latex. The competitive effect could be advantageously used, at minute lipid and polyelectrolyte amounts, to keep the most effective bactericide, DODAB, in the bulk solution thereby sterilising the particulate dispersion against bacteria [463].

The swelling mechanism and structural properties of 4 to 4 alternating polyelectrolyte layers of alternating charges composed of four negatively charged PSSand four positively charged PDDA polyelectrolyte layers were reported [464, 465] and the assembly used to adsorb phospholipid vesicles [466]. There was an equilibrium swollen thickness for the eight layers in the aqueous environment of 17 nm obtained from neutron reflectivity measurements [466]. The thickness of the lipid film formed via fusion of anionic dimyristoyl-\(\alpha\)-phosphatidylcholine (DMPC)/dimyristoyl-\(\alpha\)-phosphatidylglycerole (DMPG) (10:1) vesicles onto the support (4 PSS/4 PDDA) was estimated after 5–7 hours interaction time and found to be equal to 14.5 ± 1.5 nm [466]. This was significantly larger than the thickness of a DMPC bilayer reported in the literature as 4.3 nm [465], indicating that probably nonfused vesicles were left on top of or within the polyelectrolyte layers [466]. In order to circumvent the vesicle disruption step required for bilayer deposition onto the particle, the use of bilayer fragments was proposed [113, 138, 139, 467]. Thereby, flat bilayer patches could be adsorbed onto particles.
Cryo-TEM provided beautiful images of adsorbed liposomes and of supported lipid bilayers on silica [117] which confirmed previous information obtained by quantitative physicochemical methods, such as determination of lipid adsorption isotherms [108, 111, 113, 117, 119, 123]. For silica particles, irregularities in the silica surface did not prevent the spreading of small unilamellar vesicles or the formation of supported lipid bilayers. The functionalisation of colloidal particles with a new class of lipids, organoalkoxysilane-based lipids (Si-lipids) [468] pointed out the higher mechanic stability of the siloxane network at the particle-water interface [469]. The supported lipid bilayers assembly was called ‘robust’ by Katagiri and Caruso [469] due to the outer syloxane network. Removal of the core from Si lipid-terminated PE-coated particles proceeded without significant delamination of the Si-lipid, provided a viable approach to the preparation of lipid-functionalised capsules [469]. The high stability exhibited by the Si-lipids was considered crucial in areas where subsequent processing of these colloidal materials would be required, for example, the insertion of membrane-bound proteins and ion channels and for the attachment of surface receptors. In addition, the silanol group would have an affinity for bone possibly promoting hydroxyapatite (i.e., the main inorganic component of bone) formation in simulated body fluid [470–472] potentially making these particles covered with Si-lipid membranes biocompatible. Further, capsules coated with Si-lipids would be potentially excellent candidates as delivery systems, especially for controlled release [469]. Thus, the use of an organoalkoxysilane-type lipid (Si-lipid) with a polymerisable moiety in the head group yielded robust lipid coatings on polyelectrolyte-coated particles [469] or on silica particles [114]. These particle-supported Si-lipid membranes were highly stable upon exposure to surfactant and ethanolic solutions [469].

The predominant lipid assemblies in DODAB or DHP dispersions obtained by sonication above the bilayer phase transition temperature are the bilayer fragments or lipid bilayer discs [67, 91, 97]. At low ionic strength, they remain stable in dispersion and provide sites for solubilisation of hydrophobic drugs such as amphotericin B or miconazole [138, 139, 473] with in vivo therapeutic activity in animal models [473]. They efficiently solubilised the drugs at low drug-to-lipid molar ratios [138, 139]. Conversely, at high drug-to-lipid molar ratios, insoluble and hydrophobic drug particles in water solution can be conveniently coated and stabilised by DODAB or DHP bilayer fragments [139, 141]. In addition, some drug-DODAB lipid combinations act in combination to provide very potent anti-microbial pairs as was the case for miconazole coated by DODAB [141].

Bilayer-coating for the encapsulation of cisplatin circumvented the limited solubility of cisplatin in water and produced cisplatin nanocapsules, bean-shaped nanoprecipitates of cisplatin coated by a lipid bilayer [142]. The nanocapsules
represented a novel lipid formulation of cisplatin at high cisplatin-to-lipid molar ratio and exhibiting strongly improved cytotoxicity against tumour cells in vitro as compared to the free drug. The formation of the nanocapsules critically depended on the presence of negatively charged phospholipids and positively charged aqua-species of cisplatin [474, 475]. Coating of the surface of liposomes with PEG is a popular strategy to increase the stability and circulation time of liposomes [474]. Following that strategy, the effect of PEG on the stability of the cisplatin nanocapsules was studied by incorporating PEG conjugated to phosphatidylethanolamine (DSPE-PEG2000). It was found that the release of contents of cisplatin nanocapsules depended on the temperature, the surrounding medium and the lipid composition of the bilayer coat. Sterically stabilised cisplatin nanocapsules containing 6 mol% DSPE-PEG served as the starting formulation for in vivo studies addressing the anti-tumour efficacy of cisplatin nanocapsules in tumour-bearing mice; there was a requirement of anionic phospholipid for successful nanoencapsulation of the cationic aqua-cisplatin [476]. Recently, the route of uptake and the intracellular fate of the nanocapsules were investigated and found to depend on uptake by caveolae-mediated endocytosis so that downregulation of caveolin-1 with siRNA inhibited the uptake and cytotoxic effect of nanocapsules in IGROV-1 cells (human ovarian carcinoma cells) [476]. Considering that reduced drug uptake is a major cause of cisplatin resistance in cancer cell lines and tumours, the cisplatin nanocapsules may circumvent the mechanisms leading to the reduced drug accumulation and thus kill platinum-resistant cells.

A recent and interesting approach has been the synthesis of ether phospholipid conjugates by Pedersen and co-workers [477]. The lipids are prepared from the anti-cancer drug chlorambucil and have C16 and C18 ether chains with PC or phosphatidylglycerol head groups. Thereby the prodrug has the ability to form unilamellar liposomes (86–125 nm) which can be hydrolysed by phospholipase A2, resulting in chlorambucil release. These formulations displayed cytotoxicity toward several cancer cell lines in the presence of phospholipase A(2), with IC(50) values in the 8–36 micromolar range.

The success of PEG-lipid stabilised liposomes in drug delivery is one of the key factors for the interest in these polymer/lipid systems. PEG-lipid effects on physical-chemical properties of the lipid bilayer were evaluated by Edwards and co-workers [478, 479]. The transition from a dispersed lamellar phase (liposomes) to a micellar phase consisting of small spherical micelles occurred via the formation of small discoidal micelles. The onset of disc formation already took place at low PEG-lipid concentrations (<5 mol%) and the size of the discs decreased as more PEG-lipid was added to the lipid mixture [478]. Stable dispersions dominated by flat bilayer discs were also prepared from a carefully optimised mixture
of 1,2-distearoyl-sn-glycero-3-phosphocholine, cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-5000] [PEG-DSPE(5000)]. By varying the content of the latter component, the average diameter of the discs could be changed in the interval from about 15 to 60 nm. The utility of the discs as artificial model membranes was confirmed and compared to unilamellar and multilamellar liposomes in a series of drug partition studies; the discs served also as an alternative to study drug-membrane interactions by immobilisation in glass capillaries for drug-partitioning studies [479]. Discs compared favourably with unilamellar and multilamellar liposomes for hydrophilic drug partitioning employing immobilised discs in glass capillaries [479]. Bilayer discs are an attractive and sometimes superior alternative to liposomes [480, 481]. The major repulsive interactions preventing fusion of bilayer fragments and discs in dispersion are electrostatic, steric and/or eletrosteric [482].

Supramolecular assemblies of DODAB bilayer fragments by themselves or after interaction with supporting particles were recently combined with three different model antigens separately and tested as immunoadjuvants [105]. Thus, the cationic immunoadjuvant was either reduced to a single-component, nanosized system, the DODAB bilayer fragments, or was a dispersion of cationic particles with controllable nature and size as obtained after covering silica or PS latex with a cationic DODAB bilayer [98, 105, 129]. DODAB bilayer fragments interacted with proteins both via the hydrophobic effect and the electrostatic attraction at low ionic strength. DODAB-based adjuvants exhibited good colloid stability while complexed with the antigens, complete absence of toxicity in mice (i.e., local or general reactions) and a remarkable induction of Th 1 immune response at reduced doses of cationic and toxic DODAB lipid. DODAB vesicle disruption by probe sonication was performed at low ionic strength (0.1–5.0 mM monovalent salt) so that the DODAB bilayer fragments were stable and could be driven to interact with silica or PSS latex to produce the cationic particulates. The final DODAB concentration required to cover all particles with a bilayer can be easily calculated from the total surface area for particles and bilayers and from the mean molecular area for the lipid at the air-water interface [84, 96–98, 113, 129]. DODAB-based immunoadjuvants carrying antigens at reduced DODAB dose (0.01–0.1 mM) induced superior delayed-type hypersensitivity responses in mice in comparison to alum [105]. Figure 8.2 illustrates the superior cellular response (delayed hypersensitivity type) elicited by the cationic biomimetic particles such as DODAB-covered PS or silica particles or DODAB bilayer fragments while carrying antigen.

The interaction between lipids and particles has been reviewed over the last two decades in a few review articles and book chapters [67, 83, 87, 88, 91, 97, 482–485] and lately, other excellent reviews have appeared in the literature [99, 121].

**Figure 8.2** illustrates the superior cellular response (delayed hypersensitivity type) elicited by the cationic biomimetic particles such as DODAB-covered PS or silica particles or DODAB bilayer fragments while carrying antigen.
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8.5 Conclusions

The problem of delivering drugs, antigens or biomolecules to their targets in vivo is central and multidisciplinary and biomimetic assemblies are a major asset to improved and less toxic drug and vaccine delivery. Lipids provide adequate matrixes for supporting important biomolecules such as proteins, DNA, siRNA, oligonucleotides and polysaccharides on model surfaces such as latex, silica, silicon wafers, metals, polymers, insoluble drugs, biological cells and viruses. Biomolecular recognition between receptor and ligand can
be isolated and reconstituted by means of receptor immobilisation into supported lipid bilayers on particles. In this chapter, especial emphasis was placed on cationic assemblies since they conveniently adsorb or become adsorbed onto negatively charged biomolecules or prokaryotic or eukaryotic cells with high affinity. Changing the cell surface charge from negative to positive, they differentially reduced cell viability against bacteria, fungus and cultured mammalian cells revealing its high anti-microbial activity and differential cytotoxicity in vitro. The use of bilayer fragments combined with drugs, biomolecules or particles produced novel lipid-based biomimetics to deliver difficult drugs or design vaccines. Hydrophobic drug granules or aggregated recombinant antigens became well dispersed in water solution via lipid adsorption on drug particles as nanocapsules or protein adsorption onto supported cationic bilayers. In other instances, hydrophobic drug molecules were attached as monomers to borders of lipid bilayer fragments yielding drug formulations effective in vivo at low drug-to-lipid-molar ratio. Cationic biomimetic particles from silica or latex covered with one cationic lipid bilayer proved effective for adsorption, presentation and targeting of biomolecules in vivo. Thereby antigens were effectively presented to the immune system by particles at defined and controllable sizes.

Acknowledgements

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Ana Maria Carmona-Ribeiro


9 Advances in Liposomal Formulations for Targeting Lung Cancer

Jair Bar and Paul Wheatley-Price

9.1 Introduction

Lung cancer is the leading cause for cancer-related mortality in the western world [1]. Approximately 85% of lung cancer patients have non-small cell lung carcinoma (NSCLC), and about a half of these are candidates for surgical resection of the tumour or to its eradication by radiotherapy. The other half of NSCLC patients are in advanced stage of the disease and are ineligible for curative treatments [2]. Chemotherapy is the current treatment for these patients and in parallel with palliative care can maintain quality of life and prolong survival. In addition, chemotherapy has a role in patients with surgically resected NSCLC, being routinely offered to patients with resected stage Ib, II or IIIa disease as adjuvant treatment [3]. Chemotherapy carries a risk of significant toxicities, and while many patients may not suffer any adverse side effects, others may suffer unpleasant and sometimes life-threatening complications. Besides chemotherapy, novel treatments are making their appearance on the stage of lung cancer treatments. Epidermal growth factor receptor (EGFR) inhibitors are now becoming the preferred treatments for patients with a mutated EGFR gene [4], although testing for these mutations is only starting to become routine practice in many countries. EGFR inhibitors may lack some of the more serious toxicities of chemotherapy, but these drugs can also cause significant morbidity. Newer targeted treatments, such as anaplastic lymphoma kinase inhibitors, may offer significant benefit for specific subsets of NSCLC patients [5]. All these treatments are given systemically, either intravenously, as most chemotherapeutic agents are administered, or orally, as most targeted therapies are given. An ideal anti-cancer treatment would be one that only affects the tumour cells without any adverse effect on the host. Targeting these treatments specifically to lung cancer tumour cells could theoretically improve the clinical benefits and reduce the levels of toxicity currently pointed out for many anti-cancer drugs.
9.2 Targeting Lung Cancer Cells

Cancer cells are built similarly to the normal cells of the body in which they arise. Therefore, treatments targeted to kill or stop their proliferation can also harm normal cells. This is one of the major limitations of all systemically administered anti-cancer drugs. Several types of chemotherapies exist, designed to target cells that proliferate at a high rate, assuming this is a common phenotype of cancer cells. This targeting aims at DNA replication, nucleic acid metabolism and cytoskeleton remodelling, since these cellular events are active in proliferating cells. However, not all cancer cells proliferate faster than normal cells. In some cases, it is the lack of apoptosis, programmed cell death, which discriminates between cancer and normal cells and allows cancer cells to form a malignant tumour mass. Additional mechanisms might be at play in the unchecked progression of cancer cells, besides differences in proliferation rates [6]. Targeting cancer-specific signalling pathways is the basis of novel anti-cancer agents. Accordingly, the term ‘targeted therapy’ in the realm of cancer treatment usually refers to pathway-specific inhibitory treatments. Several examples exist where cancer cells become dependent on overactivation of a specific signalling pathway, and the blockage of this pathway brings about rapid cell death. In other cases, blocking a signal transduction pathway causes a transient growth arrest, while other signalling pathways are activated in a compensatory way. The physical delivery of therapeutic molecules to cancer cells, or to sites of tumour deposits, is an approach potentially applicable to all these forms of targeted agents.

9.2.1 Physical Targeting

Physical targeting of lung cancer is very commonly performed by surgeons and radiotherapists in clinical practice. While significant improvements have occurred in recent years, at the time of diagnosis, many lung cancers have already metastasised. Thus, for many patients, locally targeted methods cannot encompass the disease, whether disseminated in the lungs, invading adjacent tissues or metastasised to distant organs. Several alternative methods for targeting the tumours exist and will be discussed in this section. Not all of these methods have been applied in the treatment of lung cancer, but are applicable to most solid tumours.

9.2.1.1 Intravascular Targeting

Localised tumours can be accessed by intravascular catheters. Chemoembolisation involves selective perfusion of a chemotherapeutic agent to the tumour vasculature, followed by embolisation and blocking further blood flow in the relevant blood vessels. Chemoembolisation reduces the systemic exposure of the perfused drug while
Advances in Liposomal Formulations for Targeting Lung Cancer

enhancing local exposure. Methods for embolisation of blood vessels include microspheres that block small vessels [7] and gelfoam blockage of larger vessels, sometimes followed by iodised oil (Lipiodol) administration. This method is used most often for tumours residing in the liver, but has also been applied clinically for lung metastases from various primaries with good local control and little toxicity [8].

9.2.1.2 Aerosol Targeting

Lung cancers that are limited to the airways, but too extensive for surgical resection or radiotherapy, might be targeted through the airways themselves. Aerosol treatment is being assessed in this context, involving airborne microscopic droplets that are inhaled through the airways to the lung alveoli. Aerosol droplets can deliver water-soluble chemotherapy, liposomes, DNA for potential gene therapy or other therapeutic agents [9]. The feasibility of chemotherapy administration using this approach has been demonstrated clinically in a small patient cohort using 5-fluorouracil [10] and doxorubicin [11]. Detailed analysis of resected tissues after inhalation of 5-fluorouracil using this approach revealed high levels of the drug in tumour tissues and regional lymph nodes, and low levels in normal lungs and the serum [10]. Toxicity was minimal and primarily pulmonary [11], and several responses were noted [10, 11]. Promising additional chemotherapeutic agents when administered as aerosol in animal models include gemcitabine [12], paclitaxel and docetaxel [13]. Cytokines, such as granulocyte-macrophage colony-stimulating factor, can also be effectively delivered directly to the lungs by inhalation and might have site-specific effects on the local immune system with unexpected tumour responses [14]. Aerosol-mediated gene therapy in mice models demonstrated a longer lung half-life and less distribution to other organs compared to intravenous delivery [15].

Delivery of the aerosol can be achieved by several methods, including metered-dose inhalers, dry powder inhalers and nebulisers. The most commonly used nebulisers are jet nebulisers; however, as with the inhalers, adequate drug delivery is dependent on the patients’ respiratory function. There may be significant deposition of droplets within the delivery apparatus and in the upper airways. Ultrasonic nebulisers might solve some of these problems, allowing a better controlled size of the droplets and less dependence on the respiratory function. Surface acoustic waves-based ultrasonic nebulisers are the latest development in this field and might also be relevant for the delivery of lipid-based nanocarriers [16].

Targeting aerosol treatment to specific areas of the lungs has been demonstrated in a mouse preclinical model by the use of magnetic nanoparticles [17]. Droplets that contain a magnetised component together with the targeted agent allow their cotargeting by a magnetic field, without requirement for conjugation of the therapeutic agent to the magnetic particles.
9.2.1.3 Magnetic Targeting

In the 1990s, epirubicin conjugated to magnetic particles (ferrofluids) was administered intravenously to patients with metastatic solid tumours in a phase I trial, targeted by an externally applied magnetic field [18]. The treatment was well tolerated, and some responses were noted. However, improvements are required since the ferrofluids reached the tumour only in 50% of patients. Targeting adriamycin to primary liver cancer using an external magnetic field has failed in a recent clinical trial [19, 20].

9.2.2 Biological Targeting

The methods for physical targeting of agents to cancer cells discussed above depend on the ability to localise the tumour spatially. In addition, the efficacy of such an approach depends on the tumour being localised, as distant spread of micrometastasis would render any local treatment a merely transient palliation. In order to treat disseminated disease, including micrometastatic foci that are not detectable by any imaging modality, biological properties of cancer cells and their microenvironment must be harnessed.

9.2.2.1 Enhanced Permeability and Retention Effect

Tumours commonly demonstrate active angiogenesis and vasculogenesis, and have a high vascular density. The blood vessels of tumours are leakier than normal blood vessels, in part due to tumour expression of permeability factors such as vascular endothelial growth factor (VEGF) and nitric oxide. In addition, the lymphatic outflow from tumour interstitium is impaired. These factors are the basis of the enhanced permeability and retention (EPR) phenomena seen in cancers, which allows macromolecules to accumulate preferentially in tumour interstitial tissues [21]. The EPR effect is seen whenever molecules larger than 40 kDa are circulating in the blood of tumour-bearing experimental animals. For example, polymeric biocompatible compounds were shown to reach tumour concentrations more than 10 times the concentrations observed in noncancerous tissue [22]. To take advantage of the EPR effect for enhanced tumour drug delivery, drugs need to have a longer half-life in the circulation, which can be achieved by conjugation of small therapeutic agents to large polymers. In addition, a negative or neutral charge of molecules would increase their circulation half-life by reducing the binding to endothelium, as the luminal surface of blood vessels is mostly negatively charged [22, 23]. The selective accumulation of macromolecules in tumours might be enhanced by modulating blood flow and blood pressures. Angiotensin II infusion causes increased systemic...
Blood pressure and reduced accumulation of large molecules in the bone marrow and small intestine, due to vasoconstriction in these organs. In contrast, blood flow and drug accumulation increased in tumours, where homoeostatic autoregulation of blood flow is abnormal [24]. The principles of EPR also govern the accumulation of nanocarriers in tumours.

9.2.2.2 Ligand-targeted Therapy

Ligand-targeted therapy (LTT) requires an antigen that is specific for the tumour tissue. Ideally, it is a membrane epitope, accessible to the targeting moiety. An antigen specifically existing on the endothelial cells of the tumour presents an attractive target for directing therapies to the tumour site. The targeted antigen has to be expressed in sufficient amount on the cell membrane so that the targeting can be effective [25]. For successful drug delivery into the cell, the targeted antigen should undergo internalisation [26]. However, this is not essential for therapeutic agents that can kill the target cells without internalisation (e.g., radionucleotide-antibody conjugates), or through activation of the immune system. The identification of a tumour-specific antigen is the first hurdle in designing LTT.

Antibodies, or fragments of monoclonal antibodies, are common targeting tools for cancer-specific antigens. Complexes including antigen-binding fragments (Fab’) have a longer circulation half-life compared to those with whole monoclonal antibodies, possibly due to the lack of the immunogenic Fc domain [27]. Antibodies targeted against tumour ligands, such as rituximab (targeting CD20), or those targeting membrane receptors, such as trastuzumab (targeting Her2/neu) and cetuximab (targeting EGFR), are currently in standard clinical practice. Ligands of cancer-enriched receptors (e.g., folic acid, glucose) are also means of targeting cancer cells. Relatively novel targeting molecules are aptamers, short nucleic acid chains designed to bind to a predetermined molecule. Aptamers were shown in a preclinical investigation to be useful for targeting therapeutic molecules to prostate tumours [28].

9.2.2.3 Targeted Release

Various characteristics of tumour microenvironments can be used to activate release of therapeutic molecules from the carrying moiety. One such characteristic is the acidic pH typical of tumours. Targeting of molecules for intracellular release can be achieved by using molecular bonds that are disrupted in a reducing environment, such as disulfide crosslinks. Exogenously applied light or heat can be utilised to disrupt light- or heat-sensitive bonds and release molecules in an organ or volume that can be exposed to these stimuli.
9.3 Lipid-based Targeted Therapies

Lipid molecules have a polar moiety (hydrophilic), and a hydrophobic or lipophilic moiety. They are utilised in therapeutic compounds most commonly as liposomes, which are lipid vesicles. Liposomes may enhance targeting of the loaded chemotherapeutic agent to the cancer cells, mostly through the EPR effect.

Liposomes can be produced to be unilayered, including a single monolayer (a micelle), as a bilayered phospholipid membrane or multilayered, structured as an onion. A specific type of liposome-like structures are micelles composed of block copolymers, meaning two types of polymers linked together. One of the polymers is hydrophilic and the other hydrophobic, forming a monolayered micelle at a certain range of concentration. Polymeric micelles have the delivery advantages of liposomes and design flexibility of polymers. The following discussion of liposomes relates also to polymeric micelles.

Liposomes that have a simple phospholipid external membrane are rapidly cleared from the circulation by macrophage phagocytosis. These liposomes are called ‘non-stealth liposomes’, and can be used when the reticuloendothelial system (RES) is the target. Modulation of the hydrophobic nature, charge and size of liposomes can reduce their opsonisation and thus their uptake by the RES [29]. Polyethylene glycol (PEG) chains coupled with the external membrane create ‘stealth liposomes’, masked from immune system uptake and allowing for prolonged circulation and eventual delivery to tumours. Compared to free doxorubicin, stealth liposomes formulation of this drug was shown to accumulate to higher levels in malignant exudates [30]. Liposomal daunorubicin was found equivalent to a combination of several chemotherapies in the treatment of AIDS-related Kaposi’s sarcoma [31].

9.3.1 Ligand-targeted Liposomes

Liposome targeting to cancer can be achieved by their attachment to tumour-specific antibodies, forming immunoliposomes, a type of LTT. Another method for ligand-targeting of liposomes is by receptor targeting. The folate receptor, which is overexpressed by many tumours, is another potential tumour-specific target. Folate can be attached to a liposome, for example, through PEG molecules. This was shown to increase drug delivery to tumour cells expressing the folate receptor [32].

Incorporation of polymers including arginine-glycine-aspartic acid (RGD) tripeptides can target liposomes to cells expressing RGD receptors [33]. This approach might be more specific to tumour endothelial cells than to cancer cells.
Various integrins, as well as other endothelial cell adhesion molecules, are expressed on endothelial cells in an organ-specific manner. Heterotypic interactions, between adhesion molecules expressed by cancer cells and the adhesion molecules expressed by the various endothelial cell beds, are a critical step in the process of adhesion and extravasation of tumour cells during the formation of metastasis. This same mechanism can be exploited to target a therapeutic agent to the relevant vascular bed. Targeting specific integrins that are relatively specific for cancer endothelial cells has been attempted by using RGD-based peptide ligands [33–35]. An example is a peptide designed to bind human lung endothelial structures specifically in lung cancer, while not binding endothelium of normal lung tissue [36]. When linked to an adriamycin-carrying liposome, this peptide improved the efficacy of treatment of lung and oral human cancer xenografts in nude mice compared to the naked liposome. Targeting tumour vasculature by specific integrin-binding peptides could be an important route of anti-cancer treatment. Importantly, the physical properties of some liposomes by themselves (i.e., their size and charge), and the fenestrated structure of tumour microvasculature, seem to promote targeting of liposomes to tumour endothelial cells [37].

9.3.2 Drug Release from Liposomes

Besides active targeting of liposomes by LTT and passive targeting by EPR, liposomal content can be designed to be released in specific microenvironments, thus enhancing tumour targeting. Nontargeted liposomes contents are released in the extracellular compartment or inside cells by fusion of the liposome membrane with the cells’ plasma membrane [38]. pH-dependent release can be designed to target mildly acidic (pH \( \sim 6.5 \)) microenvironments, which would be more specific to the extracellular cancer microenvironment. Such targeted release can be used for therapeutic agents that are active without entering the cell (such as radioactive isotopes or toxins). Liposomes targeted to tumours by LTT usually undergo endocytosis followed by lysosomal processing. Enhanced intracellular targeting of liposome contents can be achieved by designing it to be released in lower pH of 4–5, typical of endosomes or lysosomes. Alternatively, lipids structures in which disulfide bonds anchor the carried drug can be used. These liposomes are stable in the vascular system and the extracellular environment and they release their contents preferably in the reducing intracellular environment, characterised by higher glutathione levels [39]. Comprehensive reviews of pH or reducing environment-triggered release of liposome contents have been published recently [40, 41].

The intracellular uptake of liposome contents by endocytosis can be enhanced by utilising specific fusogenic lipids as external components of liposomes [37]. For example, Lipoplatin® (Regulon, Mountain View, CA, USA) is a liposomal formulation of
cисплатин, который включает диципалмитил фосфатидил глицерол (DPPG) в его мембрану. Возможно, это происходит из-за отрицательного заряда Lipoplatin, что приводит к улучшению внутриклеточного поглощения путем фузии с клеточными мембранами. Примечательно, в этом случае, лечение липосомами может обойти один из механизмов сопротивления cisplatin, который развивают раковые клетки, что связано с пониженной экспрессией Ctr1, белка, необходимого для поглощения свободного cisplatin [37].

9.4 Therapeutic Agents Delivered by Liposomes

Липосомы могут быть физически направлены на дыхательные пути и легкие при ингаляции. Большинство методов ингаляции могут быть использованы для доставки липосом, включая производство сухих порошковых липосом и использование сухо-пылевого ингалятора [42].

Целенаправленное доставление терапевтических агентов с помощью липосом можно использовать для доставки большой выбора терапевтических агентов. Солевые или липофильные молекулы могут быть заключены в гидрофильную ядро билемарочной липосомы, или они могут быть включены в липидную оболочку, соответственно. Комплексы полонуклеотидов ДНК или РНК называются полиплексами, или липоплексами, если полькуатив это липиды [43]. В модели мышей с метастазами в легких, катионический пептид, заключенный в липосому, позволил целенаправленную доставку смеси siRNAs, проектированных на тиление Mdm2, Myc и VEGF [44], и продлил жизнь животным. Генетическая терапия доставки через липосомы в настоящее время испытывается в фазе I клинических испытаний (NCT00059605).

Другой класс терапевтических агентов, который может быть доставлен через липосомы — это токсины. Дифтерийный токсин A был доставлен к раковым клеткам в модели мышей, доказав возможность этого подхода [45]. Радиотоксины — это специфичные виды токсинов с интенсивным канцерогенным эффектом. Эти агенты, включающие радиоизотопы, что могут убить клетки, используя ионизирующее излучение, вызывая нарушение ДНК. Йод-125 был направлен к раковым клеткам с помощью липосомы более эффективно, чем свободный йод-125 [46].

9.5 Clinical Use of Liposomes for Lung Cancer

Большинство техник, описанных выше, только были протестированы в предклинических моделях и в ранних фазах клинических испытаний. Несмотря на их кажущиеся преимущества, липосомальные терапии до сих пор не являются стандартной частью ухода за многими заболеваниями. В отношении рака легких, несколько публикаций были опубликованы о применении липосомальной терапии в клиническом контексте, которые сведены в таблицу 9.1.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Liposomal formulation</th>
<th>Company</th>
<th>Clinical scenario</th>
<th>Phase of development</th>
<th>Number of patients</th>
<th>References/NIH clinical trials registry identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>PEGylated liposomal adriamycin (PLA)</td>
<td>Several</td>
<td>Stage III NSCLC, concurrent with radiotherapy</td>
<td>Phase I/II</td>
<td>25, 14</td>
<td>[47, 48]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stage IV NSCLC</td>
<td>Phase I/II</td>
<td>20, 17, planned 48 on phase II trial</td>
<td>[49, 50], NCT01051362</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SCLC, second line</td>
<td>Phase I/II</td>
<td>9, 32</td>
<td>[51, 52]</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Liposomal PEGylated cisplatin (SPI-77)</td>
<td>ALZA Pharmaceuticals®, CA, USA</td>
<td>Stage IV NSCLC, first line</td>
<td>Phase II</td>
<td>26</td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td>Liposomal-cisplatin analogue</td>
<td></td>
<td>Malignant pleural mesothelioma (intrapleural administration)</td>
<td>Phase II</td>
<td>Estimated 30</td>
<td>NCT00004033</td>
</tr>
<tr>
<td>Drug</td>
<td>Liposomal formulation</td>
<td>Company</td>
<td>Clinical scenario</td>
<td>Phase of development</td>
<td>Number of patients&lt;sup&gt;a&lt;/sup&gt;</td>
<td>References/NIH clinical trials registry identifier</td>
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<tr>
<td>Lipoplatin&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Regulon, CA, USA</td>
<td>Advanced NSCLC, resistant tumours</td>
<td>Phase I</td>
<td>13</td>
<td>[54]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced NSCLC, first line, with gemcitabine</td>
<td>Phase II, randomised</td>
<td>47</td>
<td>[55]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stage IV NSCLC, second line, single agent</td>
<td>Phase II, randomised</td>
<td>19</td>
<td>[56]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced NSCLC, first line, with paclitaxel or with gemcitabine</td>
<td>Phase III</td>
<td>114, 52</td>
<td>[56–59]</td>
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<tr>
<td>Oxaliplatin</td>
<td>Liposomal oxaliplatin bound to transferrin (MBP-426)</td>
<td>Mebiopharm Co., Japan</td>
<td>Solid tumours</td>
<td>Phase I</td>
<td>Estimated 30</td>
<td>NCT00355888</td>
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<td>Liposomal SN38 (LE-SN38)</td>
<td>NeoPharm, IL, USA</td>
<td>SCLC</td>
<td>Phase II</td>
<td>Target 73, currently suspended</td>
<td>NCT00104754</td>
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<td>Topotecan</td>
<td>Liposomal topotecan (Brakiva™, OPTISOME)</td>
<td>Hana Biosciences, Inc., CA, USA</td>
<td>SCLC, ovarian cancer and other solid cancers</td>
<td>Phase I</td>
<td>Estimated 50</td>
<td>NCT00765973</td>
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<tr>
<td>Lurtotecan</td>
<td>Liposomal lurtotecan (NX 211, OSI-211)</td>
<td>OSI Pharmaceuticals, NY, USA</td>
<td>Solid tumours</td>
<td>Phase I</td>
<td>6, 2 NSCLC patients</td>
<td>[60, 61]</td>
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<td>Estimated 47</td>
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<td>ATI-1123 (Liposomal, protein-stabilised nanoparticledocetaxel)</td>
<td>Azaya Therapeutics, Inc., TX, USA</td>
<td>Solid tumours</td>
<td>Phase I</td>
<td>Estimated 36</td>
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<td>BLP25 liposome vaccine (Stimuvax®)</td>
<td>EMD Serono, MA, USA</td>
<td>Maintenance treatment for NSCLC</td>
<td>Phase IIb</td>
<td>88</td>
<td>[62, 63]</td>
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<td>Drug</td>
<td>Liposomal formulation</td>
<td>Company</td>
<td>Clinical scenario</td>
<td>Phase of development</td>
<td>Number of patients&lt;sup&gt;a&lt;/sup&gt;</td>
<td>References/NIH clinical trials registry identifier</td>
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<td>Phase I</td>
<td>Estimated 51</td>
<td>NCT00059605</td>
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</table>

<sup>a</sup>Number of patients on trials reflects the estimated recruitment as indicated in the NIH clinical trials registry. On the randomised trials, numbers of the patients on the liposome-treated study arm are noted.

DOTAP: Dioleoyl trimethylammonium propane

NIH: National Institutes of Health

NSCLC: Non-small-cell lung carcinoma

SCLC: Small cell lung carcinoma
9.5.1 Liposomal Formulations of Anthracyclines

The anthracycline class of cytotoxic drugs, most commonly doxorubicin (also known as adriamycin) or epirubicin, have been in clinical use for many decades. A liposomal formulation of doxorubicin has established utility in the treatment of breast cancer and ovarian cancer, but has not been validated in NSCLC. Encapsulating these drugs in a liposome is an effective way of drug delivery. The liposomes are more likely to exit the bloodstream in leaky vessels, as found commonly in tumours, therefore increasing drug delivery specifically to the site of action. Regarding anthracyclines, this means in particular less drug delivery to the heart, a site of known toxicity for conventional formulations of these drugs.

PEGylated liposomal doxorubicin (PLD, also called stealth liposomal doxorubicin) has been tested in a number of small phase I and phase II lung cancer studies, but not in phase III clinical trials. In one such study, the stealth formulation was administered with docetaxel, concurrently with radiation in 25 stage III NSCLC patients, accompanied by subcutaneous amifostine to reduce toxicity. An encouraging 40% radiological complete response rate was reported [47]. A similar stealth formulation was given with vinorelbine to 14 NSCLC patients receiving radiation for stage III disease, demonstrating that this combination was also feasible [48].

There are also a few small studies regarding liposomal doxorubicin in patients with advanced or metastatic NSCLC. In 20 patients with advanced NSCLC, treated in the first-line setting with docetaxel, gemcitabine and liposomal doxorubicin, a 33% response rate and median survival of 11 months was observed, with reduced toxicity compared to historical data [49]. However, this combination has not been further tested. When PEGylated liposomal doxorubicin was given as a single agent in pretreated patients, a response was observed only in 1 out of 17 patients, and the common toxicities reported were stomatitis and palmar-plantar erythrodysesthesia (PPE, also called hand-foot syndrome) [50]. These side effects are well recognised from studies using liposomal doxorubicin, probably indicating accumulation of the drug in mucosal membranes and in certain skin areas. Accumulation of liposomal doxorubicin in eccrine sweat glands had been suggested as an explanation for its unique pattern of skin toxicity [65]. PEGylated liposomal doxorubicin is being currently investigated in a phase II trial carried out in China, as first-line treatment in combination with carboplatin for advanced NSCLC patients (NCT01051362).

PEGylated liposomal doxorubicin has also been tested in previously treated small cell lung carcinoma (SCLC) patients. Nine patients received liposomal doxorubicin and sandostatin LAR, but no responses were observed despite time to progression being relatively long at 9.1 months [51]. Another study of 14 patients with pretreated
SCLC also showed no objective responses to treatment with PEGylated liposomal doxorubicin [66]. Standard second-line therapy in SCLC involves a chemotherapy regimen of cyclophosphamide, doxorubicin (adriamycin) and vincristine (CAV). A phase II study of 32 relapsed SCLC patients treated in the second-line setting with cyclophosphamide, PEGylated liposomal doxorubicin and vincristine again showed only modest activity with a response rate of 10%, less than the reported 15–25% for standard CAV regimens [52].

9.5.2 Liposomal Formulations of Topoisomerase I Inhibitors and Taxanes

Lurtotecan (also called GI147211) is a novel water-soluble topoisomerase I inhibitor that has demonstrated interesting activity in SCLC patients [67], with dose-limiting myelosuppression. Preclinical data suggests that improved efficacy depends on prolonged exposure to the drug. Liposomal lurtotecan (NX 211, OSI Pharmaceuticals, NY, USA) has a prolonged systemic half-life, a 40-fold increase in tumour concentration and a threefold higher therapeutic index in mouse models, when compared to nonencapsulated lurtotecan [68]. NX 211 has been tested in phase I studies that included NSCLC patients, some of whom had stable disease or partial response to therapy [60, 61]. NX 211 was tested in a phase II study in patients with recurrent SCLC (NCT00046787) that was completed; however, the results have not been reported yet to the best of our knowledge.

LE-SN38 (NeoPharm, IL, USA) is a liposomal formulation of SN38, the active metabolite of irinotecan, another topoisomerase I inhibitor. Irinotecan is a prodrug that is converted to the active SN38 by enzymatic activity in the patients’ tissues. Irinotecan is used in the clinic instead of SN38 itself, due to the insolubility of SN38. LE-SN38 allows administration of the active metabolite to patients, and was shown in a phase I study to have acceptable toxicity even with a significant drug exposure [69]. It has been tested in a phase II trial in SCLC (NCT00104754). Brakiva™ (Hana Biosciences, Inc., CA, USA), a liposomal formulation of the topoisomerase I inhibitor, topotecan, is also being tested in SCLC in an ongoing phase I clinical trial (NCT00765973). Another water-insoluble drug, docetaxel, is being administered to patients in an aqueous surfactant solution of Tween-80, causing significant toxicity. A formulation that would solve the solubility problem could be very useful clinically. A liposomal formulation of docetaxel, ATI-1123 (Azaya Therapeutics, Inc., TX, USA), has been created, using a proprietary technique called protein stabilised nanoparticle (PSN™). ATI-1123 is being tested in a phase I study with solid cancer patients, including NSCLC (NCT01041235).

As previously discussed, liposomes can be delivered as aerosols, through the airways, to lung tumours. An aerosolised liposomal formulation of another topoisomerase I
inhibitor, camptothecin, was administered to patients with lung metastasis or primary lung cancer in a phase II study. Chemical pharyngitis and fatigue were the observed toxicities, depending on the administered dose. Partial remissions were observed in two patients with uterine cancer, and stabilisation of disease seen in three patients with primary lung cancer [64].

9.5.3 Liposomal Formulations of Cancer Vaccines and Gene Therapy Agents

A phase IIb study of BLP25 liposome vaccine Stimuvax® (EMD Serono, MA, USA) was carried out in patients with advanced lung cancer without progression of disease after first-line palliative chemotherapy [62]. The BLP25 vaccine targets MUC1, which is overexpressed in NSCLC and is therefore an attractive target. Treatment with BLP25 was associated with a trend towards longer survival, and no significant toxicity even with treatment duration of more than 2 years [63]. Phase III trials have been initiated with Stimuvax (NCT00409188, NCT01015443); however, these trials have recently been suspended as a precautionary measure due to unexpected serious adverse reaction in another Stimuvax clinical trial (phase II multiple-myeloma trial EMR63325-008).

The cationic liposome, dioleoyl trimethylammonium propane/cholesterol packaging a plasmid DNA encoding the FUS1 gene (a putative lung cancer tumour suppressor gene), was shown to inhibit growth of lung cancer cell lines in vitro and in mice models [70]. A phase I clinical trial is currently recruiting advanced NSCLC patients for this treatment (NCT00059605).

9.5.4 Liposomal Formulations of Cisplatin

Cisplatin is one of the most active drugs in lung cancer, but also potentially one of the most toxic, causing significant gastrointestinal, neurologic, haematologic and renal toxicity in some patients. SPI-77 (ALZA Pharmaceuticals®, CA, USA; formerly SEQUUS Pharmaceuticals) is a liposomal PEGylated formulation of cisplatin that was developed to reduce systemic toxicity and to improve delivery of cisplatin to tumours. SPI-77 has been tested in a 26 patient phase II study in advanced NSCLC; however, only modest activity was demonstrated [53]. Liposomal cisplatin analogue is being tested in a phase II trial where it is injected into the intrapleural space in patients with malignant pleural mesothelioma (NCT00004033). MBP-426 (Mebiopharm Co., Ltd., Japan) is a liposomal oxaliplatin formulation, another platinum compound, bound to human transferin, currently being tested in solid tumours in phase I studies (NCT00006036).
Lipoplatin is another liposomal formulation of cisplatin that is in clinical development, and has been tested in NSCLC [54–58]. Some technical differences between SPI-77 and Lipoplatin exist, which might confer the latter with improved characteristics and possibly better clinical results. An important distinction among the two might be the use of anionic lipids; DPPG is used in Lipoplatin, while only neutral lipids are used for SPI-77. The DPPG is proposed to improve the cellular uptake by fusion of the Lipoplatin with the cell membrane, and thus might enhance its cytotoxic activity [37]. In the reported clinical trials, Lipoplatin was administered as an 8-hour infusion. A randomised phase II trial of 88 patients, comparing Lipoplatin and gemcitabine versus cisplatin and gemcitabine, demonstrated similar efficacy between the groups, with lower rates of nephrotoxicity and leucopoenia in the Lipoplatin-treated cohort [55]. A phase III trial compared a Lipoplatin-paclitaxel regimen to cisplatin-paclitaxel in 236 advanced NSCLC patients [57, 58]. Response rates and clinical outcome were similar, but the Lipoplatin-treated patients had significantly less nephrotoxicity, neurotoxicity, gastrointestinal toxicity and myelotoxicity. A noninferiority phase III trial investigating Lipoplatin/gemcitabine combination in comparison to cisplatin/gemcitabine is ongoing [59]. Preliminary results are encouraging, with evidence of equivalence or superiority of the Lipoplatin formulation, with reduced nephrotoxicity, nausea and neurotoxicity.

9.6 Conclusions

Liposomes are versatile tools that have the potential of revolutionising the field of anti-cancer treatments. Liposomes could allow the delivery of a variety of therapeutic agents into cancer cells or to the blood vessels. The enhanced retention and permeability effect dictates their targeting to tumour tissues. In addition, liposomes could be designed to target cancer-specific molecules, thus enhancing their specificity and tumour targeting.

Early results from clinical trials in lung cancer are intriguing, and indicate potential for an improvement in the therapeutic index of cancer chemotherapy. To date, Lipoplatin, the liposomal formulation of cisplatin, is the only agent that has phase III data in NSCLC. Efficacy seems comparable to that of cisplatin, in platinum-based doublets, and reduced toxicity has been observed. Further studies are required with this agent before final recommendations about its utility can be given. Other interesting studies that are ongoing include a liposomal anti-cancer vaccine and gene therapy in a liposomal package. Such novel approaches are encouraged in the face of the poor prognosis of advanced lung cancer treated with conventional chemotherapy.
Advances in Liposomal Formulations for Targeting Lung Cancer

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Advances in Liposomal Formulations for Targeting Lung Cancer


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Jair Bar and Paul Wheatley-Price


10 Nanocarriers Targeting Breast Cancers to Deliver Modulators of Oestrogen Receptor

Giorgia Urbinati, Véronique Marsaud, Vincent Plassat and Jack-Michel Renoir

10.1 Introduction

Depending on the tumour location, the first parameter to consider before developing a drug delivery system is whether the delivery needs to be systemic or local. In case of breast cancers (BC), which are often deeply buried in tissues, a systemic intravenous (IV) administration has the great advantage of a rapid action and biodistribution. However, delivery systems need to possess specific properties in order to circumvent unfavourable biodistribution upon IV administration and rapid clearance from the circulation. Responding to these criteria, nanoparticles which emerged as a class of promising nanomedicines (nanocarriers), are small devices (size in the range of 30–300 nm) in which the therapeutic agent is encapsulated. Liposomes have been the frontrunners among these nanocarriers [1]. The major gain of function brought by a nanocarrier is its capacity to alter the pharmacokinetic properties of the drug molecule. Today, a tremendous amount of nanocarriers have been developed, in many cases without precise objective in the type of cancer to be treated.

BC is one of the leading forms of cancer in the Western world. Approximately 70% of all BC are oestradiol-dependent. The binding of oestradiol (E₂) to functional oestradiol/oestrogen receptors (ER) accelerates cell cycle progression and inhibits apoptosis [2, 3]. In normal mammary gland cells, E₂ binds two types of ER – ERα and ERβ – which control cell proliferation and differentiation. ER-positive BC are usually treated with anti-oestrogens (AE) that block the action of ERα or aromatase inhibitors (AI), which block E₂ synthesis [4–6]. Tamoxifen, a selective oestrogen receptor modulator (SERM), is the most commonly used AE, and about half of all ER-positive breast tumours respond to treatment based on this drug [7]. Recent studies have suggested that ERβ acts as a dominant negative on ERα-mediated transcription [8–11], but breast tumours with high ERβ levels are rare since ERα exerts very often its prevalent activity upon BC cells proliferation. Moreover, ERβ levels gradually decrease as the tumour progresses from preinvasive to invasive state [12, 13].
Oestrogen receptor-negative BC represents about 30% of BC and does not respond to hormone therapy. They are more aggressive than E2-sensitive BC and their actual treatment requires the association of several chemotherapeutic agents followed by immunotherapy and radiotherapy exerted after surgery. Moreover, chemical therapeutic agents have a plethora of side effects, a feature that requires improvements in the nature of the drugs, as well as in their dose and route of administration. Up to now, several ‘molecular targeted therapeutics’ like targeted drug delivery nanocarriers are designed to improve BC treatments and to target more precisely the tumour cells, but only a few have been accepted in the clinic. However, treatments used in BC often lead to resistance and evolution of cancer conducts to development of metastasis which constitutes an increased aggressiveness of the disease. Thus, other therapeutic strategies are required to combat resistance and to reach metastatic cells.

This chapter reviews some of the therapies actually used, describing a series of molecular pathways within BC cells known to participate in the evolution of the disease, and envisaging new strategies some of which are actually in clinical trials. The choice of the drug to be incorporated in nanocarriers is now dictated by improved knowledge of the pathways leading to malignant transformation, particularly through understanding of the mechanism(s) by which ER impact BC cell behaviour. The design of the nanocarriers suitable for the encapsulation of such molecule is, in part, driven by its physicochemical characteristics and requires an ‘intelligent design’ [14]. Indeed, a large number of molecules which alter cell viability and survival possess weak stability in vivo, and are insoluble and/or highly toxic in living animals and patients. In addition, they often inhibit different targets. Most of these targets are present in the tumour and healthy tissues; as a consequence, the efficiency of such anti-cancer agents requires the development of innovative tumour-targeted strategies.

10.2 Oestrogen Receptor in the Mammary Gland

10.2.1 Oestrogen Receptors in the Normal Breast

In the normal breast, oestrogen receptors ERα and ERβ are present in small but similar amounts. The classical 65 kDa ERα [15] and the 55 kDa ERβ [16] are transcription factors 55% identical in their ligand-binding domains (LBD) and approximately 97% similar in their DNA-binding domains (DBD) (Figure 10.1) [19]. Both ER bind to E2 with high affinity, but they differ in their ability to bind other natural and synthetic ligands. In particular, ERβ has higher affinity for phyto-oestrogens like genistein, quercetin or coumestrol [20, 21]. The type of response elicited by ligand binding also depends on the cell type and promoters, for both ER. ERα and ERβ ‘communicate’ most of the mitogenic and survival stimuli of oestrogens through direct effects on
Nanocarriers Targeting Breast Cancers

In the normal breast, ERα-containing epithelial cells do not proliferate in response to oestrogen [22]. The reasons for this striking observation were obtained from studies of mice with an inactivated ERα (ER–/– mice). Surprisingly, despite its clear effect on ductal cell proliferation [23], ERα is not colocalised with proliferation markers Ki67, cyclin A and proliferating cell nuclear antigen in cells [24, 25]. Indeed, Gustafsson’s laboratory has shown that ERα is the receptor isoform in epithelial cells that receives the proliferation signal from E2 very early in the G1 phase of the cell cycle, this signal triggering the loss of ERα from the nucleus [25]. By contrast, ERβ is the most abundant ER isoform in normal breast and studies in ERβ–/– mice have indicated that this isoform is not essential for proliferation [24]. Moreover, ERβ modulates ERα levels in the uterus [26]. It is therefore thought that

Figure 10.1 Schematic representation of human ER. (a) The six domains of ER, with the activation functions 1 and 2 (AF-1 and AF-2), are indicated (AB, immunological, variable domain; C, DNA binding domain; D, hinge domain; E, ligand binding domain; F, C-terminal domain) as well as serine and tyrosine phosphorylated sites (in black) and acetylated lysines (in blue) which have been identified in ERα. Kinases responsible for phosphorylation are arrowed above these sites. Arginine R260 in ERα is methylatable by PRMT1 [18]. ERα46, a truncated form of ERα lacking AF1 and identified in endothelial cells is also shown, and (b) The two other ERβ isoforms differ from ERβ by the lack of F domain and a particular localisation. Reproduced with permission from G.P. Skliris, E. Leygue, P.H. Watson and L.C. Murphy, Journal of Steroid Biochemistry and Molecular Biology, 2008, 109, 1. ©2008, Elsevier [17]
E$_2$ stimulates normal mammary epithelial cells in a paracrine manner, causing these cells to secrete growth factors that stimulate neighbouring ER-negative epithelial cells, resulting in proliferation.

**10.2.2 Oestrogen Receptor in Breast Cancers**

In breast tumours, unlike normal breast, ER$\alpha$ tends to be more largely expressed than ER$\beta$, which is very often undetectable, and E$_2$ increases the transcription of genes involved in cell cycle progression (such as those encoding c-myc, cyclins D, A and E) and decreases that of other genes, such as those encoding the cyclin-dependent kinase (CDK) inhibitor p21$^{\text{Waf1/Cip1}}$. Therefore, the growth of ER$\alpha$-expressing cells from these BC is E$_2$-dependent and the removal of E$_2$ leads to regression [28]. Moreover, ER$\alpha$ gene amplification is frequently observed in BC cells [29]. Thus, ER$\alpha$ is a well-established predictive marker of hormone sensitivity and a positive prognostic marker in BC identifying tumours for which endocrine treatment is likely to be efficient. The presence of ER$\beta$ in BC lesions is thought to be associated with more benign breast tumours [30]. As indicated above, ER$\beta$ inhibits both ER$\alpha$-mediated transcription in various cancer cells, including human BC MCF-7 cells [9, 11, 31–33], and E$_2$-induced proliferation of the BC T47-D cell line [34]. In addition, ER$\alpha$ and ER$\beta$ differentially regulate both the proliferation and apoptosis of normal murine mammary epithelial cells [3, 26]. The ER$\alpha$/ER$\beta$ ratio is now believed to represent the key element in the regulation of E$_2$ activity in BC cells.

**10.3 Oestrogen Receptor-mediated Signalling**

ER-mediated transcription is a highly complex process involving a multitude of coregulatory factors and ‘crosstalk’ between different signalling pathways (**Figure 10.2**). There have been many reviews which focused on the various pathways involved, which will therefore be summarised only briefly here (for precise details see [36–38] and references therein). Among several different types of ER activation mechanisms identified so far, the majority lead to genomic action, others displaying nongenomic characteristics.

**10.3.1 Oestradiol-dependent Transcription**

ER are traditionally defined as ligand-dependent transcription factors shuttling between the cytoplasm and nucleus. According to the ‘classical model’, following E$_2$-binding, ER dimers bind specific DNA sequences. Genes regulated by oestrogens are important for proliferation, differentiation, survival, and, particularly in cancer for the stimulation of invasion, metastasis and angiogenesis (i.e., prolactin [39],
progesterone receptor [40] or c-fos [41]). In response to ligand binding, ERα and ERβ undergo conformational changes which control their association/dissociation with coactivators, which facilitate the recruitment of regulatory complexes for chromatin remodelling, leading to the recruitment of the transcriptional machinery and gene transcription [42–46] (Figure 10.2).

Figure 10.2 Schematic representation of the various ER-mediated pathways. The classical transcription model implies the binding of estradiol (E₂) to unliganded ER leading to dissociation of the receptor from a after molecular chaperone complex composed of Hsp90, immunophilins and the p23 protein (see [35] for a review), then direct (a) or indirect (b) binding of the ER-E₂ dimer complexes to DNA at ERE or SRE sites. In case of (b), AP-1 (c-Fos/c-Jun) or Sp-1 transcription factors are recruited with several coactivators (CoAc, like CBP/P300 and HATs). Binding of growth factors (EGF, IGF, heregulin) and prolactin (PRL) (f) to their membrane receptors triggers a cascade of phosphorylations (through activation of the MAP kinase pathway) which phosphorylate ER or other transcription factors (TF) (d, e), which is sufficient to activate transcription. A small fraction of ER can localise at the membrane (c) where it interacts with several other membrane proteins (MNAR, Src) leading to activation of the PI3K/Akt pathway which in turn can phosphorylate TF and/or phosphorylate factors (HAT, CoAc) implied in the (c) and (d) pathways. All these pathways lead to enhanced ER-mediated transcription.
The effects of this ligand-dependent transcriptional activation requires from hours to days to manifest [42]. The involvement of coregulators in steroid receptor action was initially suggested for the glucocorticoid receptor [47], and was later extended to other steroid receptors. These factors can be broadly divided into coactivators (CoAc), which enhance the transcriptional activity of receptors, and corepressors, which enhance the repressive activity of receptors [48]. All the mechanisms involved in full ER-mediated transcriptional activation include changes to chromatin structure mediated by adenosine triphosphate (ATP)-dependent chromatin-remodelling enzymes, together with factors with histone acetyltransferase (HAT) activity. Several ER coactivators, including CBP/p300, p/CAF and TAFII250, have intrinsic HAT activity [49–51]. The other coactivators of the p160 coactivator family (steroid hormone receptor coactivator -1 (SRC-1), -2 (SRC-2) and -3 (SRC-3, or AIB1)) serve as platforms for the recruitment of HATs and methyltransferases to facilitate ER transcription. These molecules are all structurally similar [52], and they all interact with ERα via their C-terminal LBD region, which binds to other factors, such as CBP (CREB-binding protein), p300 and arginine methyltransferase 1 (CARM-1) [53].

Ligand activation of ER may also imply indirect binding of ER to DNA by protein-protein interactions with transcription factors, such as AP-1 or Sp-1 [54]. In both direct and indirect ER association with DNA, recruitment of coactivators modulates gene activation and subsequent protein expression (Figure 10.2).

10.3.2 Oestrogen Receptor Activation through Phosphorylations

Several phosphorylatable sites (Figure 10.1) have been identified in both ER isotypes in response to oestrogen binding or to various growth factors or other kinases. Serine 118 is the most studied since it is phosphorylated by MAPKs in response to E2, EGF and IGF1 [55] or prolactin [56]. Phosphorylated ER can bind directly or indirectly to DNA, recruits coactivators and triggers transcription (Figure 10.2). Most of the phosphorylated sites on ERα are involved in transcription and also in ER dimerisation (Ser 236) [57], nuclear import (Thr 311) [58] or E2 fixation (Tyr 537 [59]). All these posttranslational modifications converge towards the regulation of ERα transcription. It is important to underline that ER-mediated transactivation can be maximally achieved only by its phosphorylation without E2 binding.

The influence of phosphorylation on ERβ has been less studied but Tremblay and co-workers [60, 61] have highlighted the prominent role of phosphorylation in ligand-independent activation of ERβ. For example, ERβ has a role in regulating cyclooxygenase-2 (COX-2) in foeto-placental endothelial cells in the absence of E2 [62]. Data have also been accumulated in favour of the implication of ERβ activation in tumours in which therapeutic resistance ensues [60, 63]. Phosphorylation of ERβ
by the p38 pathway [64] and by ErbB2/ErbB3 at Ser-255, a site consensus for Akt [61], as well as by PKA, plays obviously an important role in the regulation of the usage of cofactors such as SRC-1 and CBP/p300, whose function in receptor-mediated transcription is essential.

10.3.3 Nongenomic Action of Oestrogen Receptor

In addition to its well-documented effects on classical transcription, E$_2$ has been shown a long time ago to induce rapid effects emanating from the membrane [65]. Since then, various E$_2$-induced signalling cascades in the extranuclear compartment have been identified (nongenomic mechanism), through direct interactions of ER with various proteins, including growth factor-dependent kinases and adaptor proteins (Figure 10.2). The formation of multiprotein complexes leads to the activation of many downstream signalling molecules which, depending on the cell type and cellular context, includes C-SRC, caveolin-1, the regulatory subunit of PI3K (p85), MAPK, Akt, p21ras and protein kinase C [66–68]. Importantly, the rapid membrane-initiated effects of oestrogen can be triggered by either ER$_{\alpha}$ or ER$_{\beta}$ which can also both localise in endothelial cells and vascular muscles [69, 70]. ER$_{\alpha}$46, an abundant N-terminus (A/B or AF-1)-deleted ER$_{\alpha}$ splice variant (Figure 10.1), is an efficient transducer of membrane-initiated response in endothelial cells, participating in the rapid stimulation of the vascular endothelial nitric oxide synthase (eNOS) leading to E$_2$/ER-mediated vasodilatation [71]. These effects are of primary importance for explaining the AE inhibitory activity on tumour vasculature [72].

10.3.4 The Intriguing Role of Oestrogen Receptor $\beta$

Despite the fact that ER$_{\alpha}$ and ER$_{\beta}$ shared overlapping properties, studies from knockout mice revealed that these two ER have distinct and unique roles *in vivo* [73]. ER$_{\beta}$ has been shown to inhibit human BC cell proliferation by repressing transcription of the *c-myc*, *cyclin D$_1$* and *cyclin A* genes and increasing the expression of the cyclin-dependent kinase inhibitors p21$^{\text{Waf1/Cip1}}$ and p27$^{\text{kip1}}$, leading to cell cycle arrest in the G$_2$ phase [33, 74, 75]. Consistent with the reported inhibition of growth by ER$_{\beta}$ expression in various mouse models, in which ER$_{\beta}$ opposes the proliferative effects of ER$_{\alpha}$ [34, 76, 77], ER$_{\beta}$ was proposed to act as a tumour suppressor [33, 78, 79]. To sustain this hypothesis, it was shown that ER$_{\beta}$ inhibits angiogenesis and tumour growth in a T47-D xenograft model [77], and the small interfering RNA (siRNA)-mediated knockdown of ER$_{\beta}$ increases the expression of genes relevant to tumour cell proliferation [80]. ER$_{\beta}$ expression is linked to less aggressive tumours in BC [13], and its expression in other cancers such as those affecting ovaries [78], lung [81], prostate [82] and colon [83], suggests some benefits in ligand-targeting ER$_{\beta}$. 
10.4 Classical Therapeutics used in Breast Cancer

10.4.1 Chemotherapy and Radiotherapy

A variety of molecules targeting numerous pathways involved in cell proliferation, cell division and skeleton maintenance have been developed. Despite not being the scope of this chapter, one should keep in mind that among them, the anti-metabolites gemcitabine and 5-fluorouracile are most frequently used in therapy. Adjuvant chemotherapy (after surgery) generally uses the association anthracyclin/cyclophosmanid (3–4 cycles) followed by docetaxel for another period of 3–4 cycles. Neo-adjuvant chemotherapy is often used to reduce the size of the tumour before surgery or to reduce the size of exeresis.

Radiotherapy has an important place in the treatment of infiltrating BC, in association with pre- or postsurgery or even alone, and it is demanding after conservative surgery treatment.

10.4.2 Hormone Therapy

10.4.2.1 Anti-oestrogen

The pharmaceutical industry has synthesised an enormous number of different types of AE, ER ligands able to compete with E₂ for binding to ER [84, 85]; the structures of some are shown in Figure 10.3. For almost 30 years, the E₂-induced growth of BC was overcome by treatment with the SERM tamoxifen, which remains the most widely used AE in the treatment of ER-positive BC [86], decreasing the likelihood of recurrence by 40–50% and reducing mortality rates. Its success is due to the balance between its agonistic and antagonistic activities. Indeed, SERM, like tamoxifen or raloxifen, act as AE in selected target tissues but as oestrogens in other tissues [87]. For example, tamoxifen behaves as a pure antagonist in the breast but not in the uterus, bones and the cardiovascular system [88]. On the contrary, raloxifen remains an antagonist in the uterus. The involvement of different coregulators as mediators of these variable effects has been proposed: (i) tamoxifen may inhibit activation of the ER AF-2 domain but not AF-1 activity, allowing AF-1 to recruit coactivators, such as SRC-1 [89–91]; and (ii) the antagonistic activity of tamoxifen may be accounted for by the capacity of the tamoxifen-ER complex to recruit corepressors, such as SMRT and N-CoR [92]. Such corepressors may facilitate the corecruitment of histone deacetylase (HDAC) to the ER-ERE complex [45], thereby catalysing the removal of acetyl groups, generating a balance in the steady-state level of acetylated histones and blocking transcription [44, 93].

Besides being a powerful chemoprophylactic agent in BC [94], tamoxifen has been demonstrating useful against osteoporosis [95]. However, because of its agonistic activity on the endometrium, it also induces uterine carcinomas in 1–2% of treated women [96, 97].
Pure AE, such as Faslodex® and RU58668 (RU), act by inducing the delocalisation of ERα in the nuclear matrix of target cells, thereby promoting the rapid proteasome-mediated degradation of this ER isotype [98–102] and thus inhibiting ERα-mediated transcription. Faslodex (ICI 182,780 or fulvestrant), the sole selective E₂ receptor downregulator (SERD), Food and Drug Administration (FDA) approved, is effective for the treatment of advanced and tamoxifen-resistant BC
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[103], acting after one deep intramuscular injection of 250 mg in an oily solution once every 5 weeks [104].

High cellular ERβ levels are associated with a favourable prognosis. It has been suggested that increasing ERβ transactivation would be beneficial in the treatment of BC [105]. However, only a small number of ligands with high affinity for ERβ have been identified, only one of which (PHTPP) is an ERβ antagonist, highlighting the need for more intensive searches for ERβ-specific ligands. Our group and others have previously shown that levels of ERβ, unlike ERα do not decrease and instead remain high when this receptor is bound to SERD like RU [106] and Faslodex [79, 107, 108], two potent pure AE with similar anti-tumour characteristics [109, 110]. This observation, led us to suggest that the use of pure AE in cells expressing both ER isotypes might be beneficial in first-line treatment of BC [72, 102, 111].

10.4.2.2 Aromatase Inhibitors

The abolition of E2 production remains an option for premenopausal women with ER-positive tumours. Indeed, drug-based approaches are more widely used than surgical approaches, such as oophorectomy. Selective AI and luteinising hormone-releasing hormone (LHRH) agonists have been designed to reduce circulating oestrogen levels. Menopausal status is considered an important determinant factor in hormone therapy; in premenopausal women, a number of different trials suggested that similar results are obtained with AE, such as tamoxifen and oophorectomy. In postmenopausal women, the ovary is no longer the primary source of oestrogen. Instead, E2 is produced by the conversion of androstenedione to androgen, then to oestrone and E2 in the peripheral tissues, including breast. AI blocking the final steps in the conversion of androgen to E2 has been widely used in the treatment of postmenopausal women with advanced BC [112, 113]. Indeed, anastrozole and letrozole but not exemestane (Figure 10.3) have stronger anti-proliferative activity than tamoxifen in patients with ER-positive tumours, and this treatment can also be used to reduce the side effects of the drug [114–117].

10.5 Nanocarriers Targeting Oestrogen Receptor-dependent Breast Cancer

10.5.1 General Considerations

Despite the development of new molecules with anti-proliferative properties, the clinical use and efficacy of chemotherapeutics is hampered by serious limitations: (i) drug resistance at the tumour level due to physiological barriers; (ii) drug resistance at the cellular level; (iii) distribution, metabolism and clearance of anti-cancer
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drugs in the body; and (iv) administration hurdles due to poor molecule solubility. At the level of solid tumours, the high interstitial pressure may lead to an outward convective interstitial fluid flow, opposing the diffusion of the drug from the vascular space to the tumour tissue [118]. The poor vascularity of some tumour regions is also of concern. Generally, since the body distribution of an anti-cancer molecule is essentially based on its physicochemical properties, which, very often, do not fit the characteristics of the disease area, large amounts of drug are given, rising to toxicity and massive distribution into healthy organs.

As a consequence, the accumulation of toxic drugs, such as anthracyclins, chemical inhibitors, nucleosides and paclitaxel, in the desired tissue is still hard to predict and their delivery at the desired rate to the site of action remains a challenge. In addition, various drugs are rapidly metabolised or other interesting molecules highly active in vitro, cannot reach clinical trials because of their low solubility. Therefore, new systems are required for their controlled release or site-specific delivery, especially for molecules that modify the hormonal equilibrium in the body. Particularly, in the case of BC treatment, there is a crucial need to avoid secondary side effects of some AE in healthy tissue. Nanotechnology approaches, in which a constant dose of anti-tumour compound is delivered directly to cancer cells, may offer new therapeutic options for hormone-dependent BC at early stages. The major challenge in this approach lies in the design of devices taken up specifically by targeted cells, and their ability to release their content over an extended period to achieve a clinical response [119–121]. Conventional hormone therapy has proven very effective for the treatment of oestrogen-sensitive BC, unfortunately with sometimes deleterious side effects, such as bone thinning or risk of osteoporosis [122], and enhanced endometrial cancer. The strategies to circumvent these severe snags are the same as those currently followed for any chemotherapeutic treatment of solid tumours. They aim at enhancing the therapeutic index of the anti-cancer agents by increasing their concentration in the malignant cells while decreasing the exposure in normal host tissues. In this field, the use of long-circulating nanocarriers has given great hope in nanomedicine over the past decades, and offers a significant therapeutic potential by adequately exploiting the so-called ‘enhanced permeability and retention effect’ (EPR effect). This arises from the dysregulated nature of tumour angiogenesis leading to leaky vasculature so that submicronic particulate systems with prolonged circulation in blood will preferentially extravasate from the abnormal vessels and accumulate in tumour tissue [123]. At present, emerging technology for cancer diagnosis and therapy focuses on active nanoparticle targeting to deliver the drugs to the tumour, and specifically at the intracellular level with increasing accuracy, in order to spare the healthy tissues. The most suitable method for active targeting involves biochemical approaches by attaching onto nanoparticle surface, antibodies, integrin-specific ligands or folate residues [124]. The characteristics of an ideal tumour-targeted nanocarrier include the possibility of decreasing drug accumulation in nontarget tissues, increasing tumour
cell-specific drug internalisation through active targeting, having biocompatible and biodegradable potential, having the capacity to protect the encapsulated drug from degradation and from premature clearance, having strong capacity to ensure minimal drug leakage during transit to target as well as the ability to retain the drug at the target site for the desired period of time and, finally, having the capacity to facilitate cellular uptake and intracellular trafficking.

### 10.5.2 Conventional Nanoparticles

In solid tumours such as BC, the vascular endothelium is discontinuous [125], and can be crossed by nanoparticles by extravasation. Various types of nanocarriers (Figure 10.4), with reduced toxicity, have been designed over the last three decades for the delivery of a number of anti-cancer drugs triggering different targets. The first-generation systems were based on liposomes, capable of incorporating hydrophobic compounds which were subsequently replaced by nanospheres (NS) and nanocapsules (NC), the latter having the capacity of encapsulating hydrophilic large molecules, such as DNA. Unfortunately, all these first-generation colloidal polymeric systems were susceptible to capture by the mononuclear phagocyte system (MPS) after injection into the bloodstream, resulting in their rapid destruction in the detoxification organs (liver, spleen, bone marrow).

For hormone-dependent BC, antagonists with high ERα/ß affinity ($K_d$ between 0.1 and 1 nM) were used. Our group and others have incorporated tamoxifen (or its active 4-hydroxy metabolite, 4HT) into poly(ε-caprolactone) (PCL) [126, 127] and poly(δ-l-lactic acid) (PLA), poly (δ,δ-lactic acid/co-glycolic acid) (PLGA) [127] but due to rapid clearance, these formulations have not been further evaluated in vivo.

### 10.5.3 Stealth® Nanoparticles

New nanocarriers have been designed to resist the opsonisation process and the macrophage capture. To do so, efforts have been made to circumvent the MPS process by attaching hydrophilic chains, for example, polyethylene glycol (PEG) to the surface of such so-called sterically stabilised nanoparticles. This resulted in local concentrations of highly hydrated groups on the surface that sterically hindered both hydrophobic and electrostatic interactions with various blood components (immunoglobulins, C3 complement protein) onto the surface of the carrier [128–130]. Such sterically stabilised colloidal formulations circulate for a longer time in the bloodstream resulting in higher rates of capture of the encapsulated drug by the cancer cells [131]. However, this concept has to be viewed cautiously because some reports established that absorption of opsonins may not be encumbered by PEG chains, suggesting that the prolonged half-life of PEG-grafted nanoparticles could be due to properties other than repulsion of blood proteins [120, 132].
One of the first nanocarriers modified with PEG chains were liposomes (Figure 10.4). Different types of AE were incorporated in PEG liposomes. The pure AE RU58668 (RU) was incorporated in stealth liposomes made of Egg-PC/CHOL/DSPE-PEG\textsuperscript{2000} (64:30:6). The incorporation of cholesterol reduced significantly
the release of RU, likely by increasing rigidity of the nanocarrier [133]. Moreover, this formulation revealed a strong capacity not only to block the growth of human MCF-7 BC cells xenografted in mice [111, 134, 135] but also to arrest tumour progression in multiple myeloma, another pathology suspected to be sensitive to AE which induce apoptosis in tumour cells [133, 136–139]. Recently, we have incorporated 4HT at a concentration close to 1 mM in a pH-gradient liposome formulation [140]. The alternative polymeric nanoparticle formulation displayed low tamoxifen loading, and thus the drug was placed onto the surface resulting in its rapid release. Other works have described the loading of tamoxifen or 4-HT in lipid vesicles, however, at a lower encapsulation yield [141–144]. In general, PH-gradient liposome formulations reduce drug diffusion from the vesicles. This type of liposomes was first described more than 10 years ago for the incorporation of amino-containing anti-cancer drugs such as doxorubicin [129, 145, 146], gemcitabine [147, 148], topotecan [149], vincristine [150] and irinotecan [151, 152]. The concept takes advantage of the different behaviour that molecules (weak acids and bases) have once they are protonated [147]. Because of its amine group (Figure 10.3), 4HT is charged at acidic pH, but uncharged when the pH is basic. If the external pH of the system is alkaline, the molecule will tend to be uncharged and because of its lipophylic properties, it will be able to cross the lipid barrier of liposomes. After encapsulation of 4HT inside the core of pH-gradient liposomes (DSPC/CHOL/DSPE-PEG2000) where the pH is 5.1, the AE becomes protonated, thus unable to escape from the aqueous core of the vesicle. This transmembrane pH-gradient method promotes 4HT encapsulation and retains the molecule longer than conventional liposomes [140], revealing a strong anti-tumour capacity after IV injection in multiple myeloma xenografts at a low concentration (3 mg/kg/week). The main advantages of the PEG-coated liposomes lie in their relative biological inertia, weak immunogenicity and low intrinsic toxicity added to the double capability of encapsulating hydrophilic substances within their internal aqueous volume and hydrophobic ones within their lipid bilayer shell.

Various formulations other than liposomes have been developed for the delivery of highly cytotoxic anti-cancer drugs, mainly encapsulating doxorubicin. In fact, when encapsulated in poly-lactic (PLA) nanoparticles, drug dosing was much lower than that of free doxorubicin [153]. Doxorubicin is one of the most commonly used chemotherapeutic drugs being tested in various formulations based on the use of biodegradable nanoparticles [154, 155]. Poly(Me-PEG-cyanoacrylate-co-hexadecylcyanoacrylate; PEG-PHDCA) were used to encapsulate 4HT [156]. However, its release profile was found to be too fast and drug loading too low for expecting significant effects in vivo; this is attributed to the adsorption of the drug onto the particle surface. Moreover, a detrimental cytotoxicity of the polymer was established [156], although weaker than that of
Nanocarriers Targeting Breast Cancers

poly(alkylcyanoacrylates) [126]. By contrast, RU, the highly promising pure AE, was successfully incorporated into both polyester nanocarriers at high loading rates [134]. PEGylation of these nanocarriers had no effect on the drug loading but significantly enhanced the half-life in blood [127]. Importantly, the RU release rate from both PEGylated nanocarriers was very slow (half-life between 25 and 30 hours in BC cell culture, depending on the composition of the nanocarrier) [127, 157]. All these RU-loaded PEGylated nanocarriers arrest the E2-induced tumour growth of MCF-7 cells xenografted in nude mice with strongly enhancing apoptosis at a very low drug concentration (4 HT nanospheres, 0.2 mg 4HT/kg/week). The accumulation of these nanocarriers with encapsulated AE at tumour sites (i.e., $^3$H-4HT trapped in $^{14}$C-radiolabeled polymer) has been demonstrated [111]. By contrast to free AE, in all the AE-loaded nanocarriers, high levels of drug-mediated apoptosis were observed in xenografts bearing solid tumours, together with a large decrease in host vasculature [111, 135]. These effects are thought to be the consequence of the slow RU release from the nanocarrier and a prolonged circulation time due to PEG chains favouring the time-dependent drug accumulation. Whether released outside or within tumour cells following endocytosis of the nanocarriers, AE inhibit the E2-induced activation of ER from epithelial and endothelial cells. In epithelial cells, they strongly induce apoptosis, by activating the caspase cascade and inhibiting vascular endothelial growth factor (VEGF) secretion. In endothelial cells, they block VEGF synthesis by inhibiting E2-dependent activation of the VEGF promoter [158] and then angiogenesis [159, 160].

More recently, ferrocifen derivatives of tamoxifen have been introduced in various PEGylated polymeric nanocarriers [161] as well as those based on lipids [162], at a high rate and with promising preclinical data for BC and glioblastoma treatment.

10.6 Active Targeted Nanoparticles

The primary role of a ligand-targeted nanocarrier (lipid-based [163] or polymer-based [164]) is to enhance cellular uptake into cancer cells and to minimise the accumulation in healthy tissues. PEGylated nanocarriers are considered to deliver the encapsulated agent in a passive way without specifically selecting tumour cells rather than tumour interstitial space. Stealth technology has been considerably improved by conjugation with tumour cell recognition molecules. The potential of this approach has been demonstrated in a number of studies both in vitro and in vivo [165–167]. Several types of ligands have been used for this purpose, for example, vitamins, glycoproteins, peptides, aptamers and mainly antibody fragments [168]. This concept is more than 25 years old, but has gone through only small improvements due to several difficulties encountered. The main problems, other than those inherent to the chemistry, are
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the dilemma of maintaining the stealthy characteristics of the formulation by leaving a certain number of free PEG chains grafted on the surface and attaching sufficient tumour recognition molecules to PEG to ensure that the particle reaches the tumour sites (Figure 10.4).

**10.6.1 Small Molecule-targeted Liposomes**

Several attempts have been made to graft specific tumour target recognition factors: some have used small chemical molecules binding specifically to surface receptors, whereas others have used antibodies.

Among molecules already proposed for use in the targeting of liposomes and nanoparticles to BC cells, folic acid (FA), a high affinity ligand (Kd ≤ 1 nM) for its membrane folate receptor (FR) has gained major interest. However, it should be borne in mind that FA and analogue ligands activate pathways involved in cell proliferation, adhesion or dissemination. Thus, rather than using such activators, it may be preferential to functionalise the carriers with FA-inhibitory analogues. Many innovative strategies for targeting FR for the delivery of diverse therapeutic agents (antisense oligonucleotides, genes and cytotoxic agents) have been proposed [169]. Interestingly, both FR expression and the FR-α promoter are negatively regulated by ER, and this repression is diminished by AE such as tamoxifen and Faslodex [170]. It may therefore be of therapeutic interest to improve the growth-inhibitory activity of AE by encapsulating them in nanocarriers targeting BC tumours linked to folate or folate inhibitors. Unfortunately, FR seems to be weakly expressed in ERα-positive cells such as the MCF-7 cell line [171, 172], calling into question the probable efficiency of such carriers in E2-dependent BC tumour targeting.

Neurotensin receptor-1 (NR-1) has been characterised at the surface of BC cells, in which neurotensin (NTS) has trophic and antiapoptotic effects [173]. NTS is expressed and up regulated by E2 in normal epithelial BC cells and high expression of NR-1 correlated with the size of the tumour and the number of metastatic lymph nodes [174]. NTS-polyplex [175], described for efficient thymidine kinase gene delivery, could also be useful for the delivery of E2-induced downregulated antiapoptotic genes such as Bcl-2 [176] or tumour suppressor genes like p53, PTEN and ERβ. However, rather than using the NTS peptide itself (13 amino acids long), it may be easier to graft a small (5–6 amino acids long) NTS-inhibitory analogue, with the preserved minimal binding sequence to NTS, onto the surface of the particles. Such a commercially available smaller peptide should still recognise NR-1 if correctly attached to the PEG chain, and trigger endocytosis, but should block its receptor activation and, therefore, the induction of cell growth, tumour progression and differentiation. It should also enhance apoptosis through the delivery of the encapsulated AE or other anti-cancer agents to cancer cells.
Since the growth of solid tumours is dependent on their capacity to form new blood vessels to supply them with oxygen and nutrients, a strategy aiming at blocking the angiogenesis pathways has been described. Angiogenesis is a very sophisticated and complex mechanism involving a number of growth factors and surface receptors. Phage-displayed peptide libraries have been used to identify cancer cell epitopes and tumour vasculature-specific peptides [177]. Recently, the efficacy of doxorubicin on solid tumours from nude mice transplanted with BC cells was shown to be enhanced by liposomes to which peptides specific for binding to endothelium cells were grafted [178]. Indeed, targeting the vasculature to combat BC progression is actually a research field that has lead to the development of a number of chemical inhibitors and anti-VEGF antibodies, such as bevacizumab (Avastin®).

### 10.6.2 Magneto-liposomes

An alternative physical approach that consists in magnetic targeting liposomes has revealed high efficiency in the accumulation of anti-cancer drugs in selected tissues or cells with the application of an external magnetic field gradient [179–181].

Magnetic-fluid-loaded liposomes (MFL) were produced based on 200 nm unilamellar PEG-stabilised phospholipid vesicles which encapsulated a physiologically compatible suspension of nanocrystals of maghemite (γ-Fe₂O₃) [182]. Such hybrid nanocarriers have superparamagnetic properties loaded with an excellent contrast agent for magnetic resonance imaging. When injected intravenously, MFL display long-circulation behaviour in blood as intact vesicles [183]. They can be magnetically driven by an external magnet towards solid tumours subcutaneously implanted in mice, without damaging the lipid bilayer and without suffering leakage from their internal aqueous content [184]. Indeed, a magnetic field gradient significantly enhances MFL uptake by human cancer cells [185], but it remains to be demonstrated to what extent magnetic targeting can provide effective therapeutic benefits by loading MFL with an anti-cancer drug.

### 10.6.3 Immuno-targeted Liposomes

Nanoparticles can be rendered selective by coupling specific antibodies (or Fab fragments or single-chain Fv) to the ends of PEG chains decorating the nanocarriers. Tumour-targeting liposomes incorporating the antitransferrin receptor single-chain fragment have been developed for gene therapy and siRNA [186–188]. The density of the cell-surface receptor to be reached is also a crucial parameter for efficient targeting.

Trastuzumab (Herceptin®), a monoclonal antibody directed against one member of the epidermal growth factor receptor (EGFR) family, HER2 (or ErbB-2/NEU),...
received FDA approval for clinical use for BC treatment in 1998, and it is widely used actually to treat hormone-resistant BC tumours [189]. Trastuzumab is currently the leading monoclonal antibody used to treat advanced BC, and there is an inverse correlation between the expression levels of ERα and those of ErbB-2 and SRC3/AIB1 [190]. Using anti-HER2 as bait for immuno-targeting is actually the most employed method for rendering active various nanocarriers with encapsulated anti-cancer drugs. Importantly, HER2-targeting of lipid nanoparticles with a long half-life in the blood does not result in higher rates of tumour localisation than that obtained with PEGylated nanoparticles [191, 192]. Rather it does result in higher rates of internalisation within tumour cells, instead of accumulating only in the interstitial space of the tumour tissue [166, 193]. This enhancement is due to an antibody-mediated endocytosis phenomenon [163]. Trastuzumab has been shown to potentiate the activity of several anti-cancer drugs, such as doxorubicin [194] in metastatic BC and lipid-conjugated telomerase antagonists sensitise Erb-B2-positive BC to trastuzumab [195]. These examples illustrate the benefits of using the HER2 antibody for targeting nanocarriers to BC cells.

It was recently shown that human BC cells injected into nude mice rapidly infiltrate lymph nodes far from the injection site, which, in a manner similar to stem cells, express the CD-44 surface antigen [196]. The hyaluronic acid receptor CD44 is involved in cell migration of BC cells [197] and metastasis [198]. Indeed, breast tumours, especially those with high level of Brca1, contain distinct CD44+/CD24-BC cells with cancer stem cell characteristics [199]. Thus, the targeting of CD-44 might decrease the risk of metastasis and may represent a novel approach to overcome chemotherapy resistance [197]. This strategy is also supported by recent data showing that targeting CD44 with the P245 monoclonal antibody in human BC xenografts significantly reduces tumour growth. This effect is associated with inhibition of growth-inhibiting factors like proinflamatory human cytokines (IL-1β, TGFβ1), oncostatin M and TNF-7α. In addition, treatment of xenografts with P245 antibody prevents tumour relapse after adriamycin/cyclophosphamide chemotherapy [200]. Our group has demonstrated that hyaluronic acid complexation to lipoplexes containing a DNA pCMV-luciferase plasmid enhance the cellular lipoplex uptake [201].

Pseudomonas exotoxin A and other toxins such as ricin have been linked to antibodies and this strategy represents a new hope for the treatment of cancers [202], despite severe nonspecific toxicity. Such immunotoxins have been modified by coupling to PEG and PEG-loaded PLGA nanoparticles having incorporated p38KDEL, a 38 kDa mutant form of pseudomonas exotoxin A. Another alternative linking p38KDEL to the Fab' fragment of herceptin (PE-NP-HER) could be used [203, 204]. This approach allows inhibiting human breast tumour progression from nude mice twice as much as pseudomonas exotoxin A-HER and may allow further preclinical evaluation.
Cetuximab is a chimeric IgG1 monoclonal antibody which competes for the binding of EGF to EGFR resulting in an efficient blockage of downstream pathways promoting tumour growth. Despite some promising therapeutic success in trials associating cetuximab with paclitaxel [205], this strategy was abandoned due to prohibitive dermatological toxicity. Nevertheless, the use of cetuximab as an antibody linked to liposome/nanoparticles may allow further trials for targeting BC cells expressing high EGFR levels.

10.7 Novel Molecular-targeted Approaches for Liposomal Delivery

10.7.1 Silencing Ribonucleic Acids and Antisense Oligonucleotides

RNA interference is of potential interest as a future treatment for BC [206]. ERα controls E2-induced proliferation and ERβ is involved in differentiation. Thus, ERα elimination from tumour cells is therefore likely to be beneficial. A number of factors involved in the E2-induced proliferation of BC cells other than ERα could be targeted with antisense oligonucleotides or siRNAs. Potential targets include coactivators, such as SRC1 and SRC3/AIB1, which are particularly strongly expressed in aggressive tumours [207]. Another potential target in BC cells is the PI3K downstream kinase Akt. This serine/threonine kinase is part of one of the key signalling pathways downstream from E2, as it activates signalling through anti-apoptotic pathways [208]. It remains unclear how E2 activates Akt in ER-negative BC cells [209], but it is evident that resistance to AE therapy may in some cases involve activation of the PI3K/Akt pathway [210] or other phosphorylation pathways [211–213]. The use of siRNA or short hairpin RNA (shRNA) targeting Akt and enabling the incorporation of RNA inhibitors in the cytoplasm of BC cells is a promising approach in efforts to combat this resistance. For example, the inhibition of Akt by a short inhibitory RNA blocks Ser167 phosphorylation in ERα, restoring sensitivity to tamoxifen [214].

Similarly, antisense oligonucleotides against type I insulin-like growth factor receptor mRNA inactivate Erb-B2, PI3K/Akt and p42/p44 MAPK signalling in the C4HD tumour model, a model of carcinogenesis in which the synthetic progestin medroxyprogesterone acetate (MPA) induces mammary adenocarcinoma in mice. BC growth is inhibited with no modulation of progesterone receptor activity [215]. By analogy with ER-positive cells, in which these pathways are rapidly activated following the exposure of BC cells to E2, and with AE-resistant tumours, it is tempting to speculate that antisense oligonucleotides or siRNA targeting growth factor receptors (IGFR, EGFR) and kinases (MAPK, Akt, PKA) may be potentially interesting molecules for use in the treatment of BC in patients.
10.7.2 Heat Shock Protein 90 Inhibitors

The molecular chaperone Hsp90 is essential for the correct folding and full biological activity of an elevated number of client proteins including all the steroid hormone receptors [35]. Hsp90 interacts with various co-chaperones and partner proteins, in concert with immunophilins providing cis/trans peptidylprolyl isomerase activity to form a large heterocomplex that binds ATP. More than 20 partner proteins assisting Hsp90 in the protein-folding process have been identified [216] and more than 150 client proteins are known (for a complete list of Hsp90 client proteins and references see http://www.picard.ch/). These client proteins include transcription factors, such as the tumour suppressor p53, Stat3, steroid receptors – particularly ERα and PR [217, 218] and ERβ [106] – a number of kinases, such as Erb-B2, Akt, Bcr-Abl, several CDK, Raf-1 MEK, v-Src and other proteins associated with oncogenesis and/or angiogenesis, such as the proteasome, telomerase, Mdm2, Hif-1α and SV40 large T-antigen. Hsp90 associates with ERα in an ATP-dependent manner, maintaining the receptors in an inactive conformation unable to bind DNA. This leads to apoptosis enhancement. Thus, the inhibition of Hsp90 may be considered a promising approach for cancer treatment as it results in the simultaneous degradation of multiple targets [216, 219–225]. Many drugs inhibiting ATP binding to Hsp90 have been developed. Some, such as geldanamycin, purines and radicicol interact with an N-terminal site [220], whereas others, such as novobiocin and its analogues [226, 227], interact with a putative C-terminal site [228]. These inhibitors cause dissociation of the protein client from Hsp90, leading to their ubiquitinylation and proteasome-mediated degradation [229]. However, the ubiquitination of Hsp90 in both healthy tissues and tumours constitutes a hurdle which is necessary to overcome to avoid strong detrimental side effects. The incorporation of Hsp90 inhibitors into efficient delivery nanocarriers is a challenge for the medical success of these drugs. Our group has incorporated 4TCNA, a new coumarin analogue inhibitor of Hsp90 and strong inducer of the proteasome-mediated degradation of ERα and of Erb-B2 [230] in PEGylated liposomes (EggPC/CHOL/DSPE-PEG2000; ratio 64:30:6; 2.2 mg/kg/week) and the tumour growth of ER-negative BC cells xenografted in nude mice was completely abolished (Urbinati, Marsaud and Renoir, unpublished work).

10.7.3 Histone Deacetylase Inhibitors

Among enzymes controlling the ER-mediated transactivation process, one group – the histone deacetylases (HDAC), which catalyse the removal of acetyl residues, maintaining the steady-state balance in acetylated histone levels – plays a critical role [44]. HDAC have been classified in four different classes. The most widely cell-distributed ones are class I and II HDAC. Sirtuins form a group of highly conserved nicotinamide (NAD⁺)-dependent deacetylases classified as class III HDAC [231]. SIRT1 is known to deacetylate more than ten substrates, including nuclear ERα and its coactivators PGC1α, p300, thereby affecting nuclear hormone receptor-mediated
transcriptional regulation [232]. The regulation of tumour cell growth by sirtuins suggests that inhibitors of these molecules could constitute a potent treatment for BC. HDAC induce angiogenesis by negative regulation of tumour suppressor genes [233]. In recent years, histone deacetylase inhibitors (HDACi) (Table 10.1) have emerged as promising anti-cancer agents [251] able to reverse changes in the pattern of expression of many genes involved in cell death and proliferation [252–254]. Various inhibitors [255] have been identified and classified on the basis of structure:

<p>| Table 10.1 Structure of inhibitors suitable for encapsulation in targeted nanosystems |
|---------------------------------|---------------------------------|
| Structure | Clinical trials | References |
| HDAC inhibitors | | |
| TSA | None; highly toxic in cells; warrants its delivery through liposome | [233, 241, 266, 299] |
| SAHA (Vorinostat) | Class I and II HDAC inhibitor; phase III; solid tumours, lymphoma, MM | [234, 251, 252, 258] |
| MS275 | HDAC1,2,3; phase I; lymphoma | [239, 251, 252] |
| CG1521 | HDAC2 inhibitor; solid tumors, lymphoma, MM Phase II in combination with chemotherapeutics | [242] |
| PXD101 (belinostat) | Phase I; Solid tumours (colon, renal, melanoma, breast, prostate, ovary) | [301] |</p>
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<th>Structure</th>
<th>Clinical trials</th>
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<td><strong>Proteasome inhibitors</strong></td>
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<tr>
<td>MG132</td>
<td>Its strong inhibitory capacity warrants its use through nanosystems (work in progress in our lab)</td>
<td>[246]</td>
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<tr>
<td>MG 341 Velcade (bortezomid)</td>
<td>Phase II, MM, lymphomas, breast, prostate</td>
<td>[293, 301]</td>
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<td><strong>Hsp90 inhibitors</strong></td>
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<tr>
<td>Geldanamycin</td>
<td>Phase II with its analogue 17-AAG</td>
<td>[247, 299]</td>
</tr>
<tr>
<td>Radicicol</td>
<td>Unstable in vivo, warrants incorporation in stealth NS</td>
<td>[248]</td>
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<tr>
<td>Novobiocin</td>
<td>Coumarin analogues are better inhibitors than novobiocin</td>
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short-chain fatty acids (valproic acid [256] or sodium butyrate [237]), cyclic and noncyclic hydroxamic acids (suberoyl anilide hydroxamic acid (SAHA) [234], trichostatin A (TSA) [241] or 7-phenyl-2, 4, 6, hepta-trienoic hydroxamic acid (CG-1521) [242]), peptides (depsipeptide [257] or apicidin [238]) and benzamides, such as MS-275 [239]. The promising results obtained in preclinical studies with certain HDACi, such as SAHA, MS-275, depsipeptide and valproic acid, have led to clinical trials for haematological cancers, such as cutaneous T-cell lymphoma, multiple myeloma and leukaemia, and for some solid tumours (breast and colorectal carcinomas) [258] (Table 10.1). HDACi also affect nonhistone substrates, including steroid receptors [259], such as ER\textsubscript{α} in particular [260], and transcriptional coactivators [261]. The transactivation capacity and stability of ER\textsubscript{α} seem to be regulated by the p300-dependent acetylation of lysines 266/268 and 302/303 [262, 263]. The accumulation of ER\textsubscript{α} in BC cells, by contrast to what is observed for ER\textsubscript{β}, decreases following exposure to HDACi, such as TSA, and HDACi increase the E\textsubscript{2}-dependent transactivation of ER [79].

By contrast, exposure of ER-negative BC cells to HDACi, allows the reexpression of ER\textsubscript{α}, implicating histone deacetylation as a critical component of ER gene silencing in BC cells [264]. TSA is one of the most active HDACi \textit{in vitro}; unfortunately, free TSA is rapidly metabolised in nude mice [265, 266], but apparently not in rats with DMBA-induced BC tumours [267]. We recently showed that TSA-loaded PEGylated liposomes of Egg PC/CHOL/DSPE-PEG\textsuperscript{2000} are strong inducers of tumour-growth inhibition both in hormone-dependent and hormone-independent BC cell xenografts. Moreover, since the ER\textsubscript{α} promoter possesses a methylated region in hormone-resistant disease, and since the methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-DC) enhanced ER\textsubscript{α} reexpression [268], it has been suggested that reexpression of ER\textsubscript{α} by TSA and 5-aza-DC restore

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<td><strong>Farnesyl transferase inhibitors</strong></td>
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| **HDAC: Histone deacetylase** |
| **SAHA: Suberoyl anilide hydroxamic acid** |
| **TSA: Trichostatin** |
tamoxifen sensitivity in ER-negative BC cells [269]. Thus, HDACi use could help accelerating the degradation of ERα in ER-positive BC, while restoring ERα in ER-negative BC cells, thus favouring de novo AE therapy. This situation would enhance the anti-tumour activity of some of the inhibitors already in phase I and II clinical trials [270].

Since HDACi enhance the anti-proliferative action of AE on BC cells, the combination of HDACi with hormone therapy may be beneficial [235]. ERβ expression leads to a dramatic increase in the anti-proliferative activity of HDACi, correlated with changes in the transcription of genes involved in cell cycle control, such as p21\textsuperscript{WAF1/Cip1} and cyclin D1 and cyclin E [79]. Moreover, the ER molecular chaperone, Hsp90, is also a HDAC target, and studies have shown that the hyperacetylation of Hsp90 induced by HDAC6 inhibition increases the proteasomal degradation of Hsp90 client proteins [271].

Importantly, HDACi and AE therapy interfere with oestrogen signalling at various points [272]. Thus, preclinical therapeutic approaches checking for the anti-tumour activity of nanocarriers charged with an HDACi and an AE were undertaken.

### 10.7.4 PI3K/Akt Inhibitors

PI3K plays an important role in survival, proliferation, mortality and neoangiogenesis. It is generally disregulated in cancer cells or overexpressed leading to enhanced Akt activity which has antiapoptotic influence. Also Akt-dependent cell growth is enhanced through Erb-B2-mediated activation of PI3K. Inhibition of Akt by LY294002 associated with docetaxel protects cells from fibronectin-induced apoptosis. Then, PI3K/Akt pathway may represent a promising strategy for enhancing sensitivity to docetaxel [273]. In this prospect, the combination of wortmannin (an inhibitor of PI3K/Akt) with tamoxifen or fulvestrant inhibits tumour growth of long-time E\textsubscript{2}-deprived aromatase-transfected human ER-positive BC cells better than either drug alone. Thus, blocking ER and PI3K/Akt pathway or even upstream growth factor-activated pathways is a promising therapeutic approach. This can be performed through encapsulation of chemical EGFR inhibitors in various types of stealth nanocarriers (see below) or through the binding of EGFR antibody to the nanocarriers having encapsulated a chemical inhibitor or a siRNA against Akt. In addition, as shown from data revealing that loss of PTEN (a negative regulator of PI3K) is strongly associated with metastasis and poor survival [274], inhibitors such as rapamycin and temsirolimus (CCI-779) are interesting drugs for being incorporated in stealth nanocarriers. Combined with Erb-B2 antibodies, or better chemically coupled with a Fab fragment or a single-chain Fv fragment of herceptin, they constitute a promising device as already showed in preclinical studies, giving promising results with the use of free CCI-779 and trastuzumab [275] (Table 10.2).
<table>
<thead>
<tr>
<th>Table 10.2 Novel therapeutic approaches for metastatic breast cancer</th>
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<tbody>
<tr>
<td><strong>Agent</strong></td>
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<tr>
<td><strong>Antibodies</strong></td>
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<tr>
<td><em>Extracellular inhibitors</em></td>
</tr>
<tr>
<td>Trastuzumab (Herceptin®)</td>
</tr>
<tr>
<td>Pertuzumab</td>
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<tr>
<td>Cetuximab</td>
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<tr>
<td><em>Anti-VEGF</em></td>
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<td>Bevacizumab (Avastin®)</td>
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<td><em>IGF-Receptor</em></td>
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<td>IMC-A12</td>
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<td><em>TRAIL receptors</em></td>
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<td>Mapatumumab</td>
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<td>Lexatumumab</td>
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<tr>
<td><strong>Chemical inhibitors</strong></td>
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<tr>
<td><em>Intracellular TK inhibitors</em></td>
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<tr>
<td>Gefitinib (Iressa®)</td>
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<tr>
<td>Erlotinib (Tarceva®)</td>
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<td>Neratinib</td>
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<tr>
<td>Lapatinib (Tykerb®)</td>
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<td>Sunitinib (Sutent®)</td>
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<td>Sorafenib (Nexavar®)</td>
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<tr>
<td><em>Other kinase inhibitors</em></td>
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<tr>
<td><em>Src family kinases</em></td>
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<tr>
<td>Dasatinib (Sprycel®)</td>
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Table 10.2 Continued

<table>
<thead>
<tr>
<th>Agent</th>
<th>Specific target</th>
<th>Beneficial association</th>
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<tr>
<td><strong>Hsp90 inhibitors</strong></td>
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</tr>
<tr>
<td>Tanespimycin (17 AAG)</td>
<td>Multiple (Erb-B2, Akt, Erb-B1, etc.)</td>
<td>After AE failure, in multiple resistance with HDACi</td>
</tr>
<tr>
<td>IPI-504 (Restapimycin)</td>
<td>Multiple</td>
<td>Trastuzumab</td>
</tr>
<tr>
<td><strong>HDAC inhibitors</strong></td>
<td></td>
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</tr>
<tr>
<td>Vorinostat</td>
<td>Multiple</td>
<td>After AE and AI failure, plus paclitaxel, bevasuzimab</td>
</tr>
<tr>
<td><strong>Proteasome-inhibitors</strong></td>
<td></td>
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</tr>
<tr>
<td>Bortezomib (Velcade®)</td>
<td>Multiple</td>
<td>Trastuzumab, gemcitabine, doxorubicin, cetuximab</td>
</tr>
<tr>
<td><strong>mTOR inhibitors</strong></td>
<td></td>
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<tr>
<td>Temsirolimus (Torisel®)</td>
<td>Multiple</td>
<td>AE, IMC-A12</td>
</tr>
<tr>
<td>Everolimus (Afitinor®)</td>
<td>Multiple</td>
<td>Letrozole</td>
</tr>
<tr>
<td><strong>Marine-derived inhibitors</strong></td>
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<tr>
<td>Trabectedin (Yondelis®)</td>
<td>Multiple</td>
<td>Anthracyclins, taxanes</td>
</tr>
<tr>
<td><strong>Folate antimetabolites</strong></td>
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<tr>
<td>Premetexed (Alimta®)</td>
<td>Folate receptor</td>
<td>AE</td>
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</tbody>
</table>

**AE**: Antioestrogen

**HDACi**: Histone deacetylase inhibitors

**VEGF**: Vascular endothelial growth factor

Very importantly, understanding the role of the growth hormone –IGF, IGF-binding protein- axis and its potent involvement in resistance together with the balance between IGF and the IGFBP has led to the development of more than 25 IGFR inhibitory molecules [276]. Some of them lead to the inhibition of Akt activation and may be useful in the therapy of various cancers.

### 10.7.5 Tyrosine Kinase Inhibitors

Many reports have highlighted the evidence for crosstalk between E$_2$ and growth factor-induced cytoplasmic signalling [55, 277–282]. Besides the PI3K and Akt pathways, particularly promising targets for the treatment of BC include members of the EGFR family, which are often overexpressed, dysregulated or mutated in such diseases. These transmembrane receptor kinases are activated by the binding of various ligands, such as EGF, TGF-β, amphiregulin, epiregulin and neuregulin. EGFR activation involves
homo- or heterodimerisation of the receptor, followed by the autophosphorylation of tyrosine residues at the C-terminal end of the receptor in the cytoplasm, initiating a cascade of signalling pathways [283]. The receptor tyrosine kinase cascade ends with endocytosis of the phosphorylated receptor-ligand complex [284]. EGFR activation can be blocked by two types of inhibitors – monoclonal antibodies recognising the extracellular domain and low-molecular-weight tyrosine kinase inhibitors (TKI), which interact with the tyrosine kinase intracellular domain.

In preclinical studies, gefitinib (Iressa, ZD1839) (Table 10.2) has been shown to inhibit the proliferation of ductal carcinoma in xenografts [285], and to reverse endocrine resistance in BC cell lines when given in combination with tamoxifen [286]. Therefore, targeting EGFR in BC is a promising strategy worthy of investigation, particularly in case of tamoxifen resistance since such a phenotype is accompanied by an enhanced motile and invasive characteristic of BC cells [287]. In combination with other agents, such as paclitaxel and doxorubicin, gefitinib has shown to inhibit the growth of BC cells resistant to tamoxifen [288].

An enormous number of TKI are actually in development by the pharmaceutical industry (Table 10.2). Several are actually in clinical trials but resistance has been observed; indeed, blocking the intracellular domain responsible for their kinase activity does not prevent activation of the kinase through its extracellular domain, which triggers the growth factor-induced proliferation of cancer cells. The use of a nanodevice containing a TKI able to block the intracellular domain and a monoclonal antibody grafted on its surface able to block the extracellular domain, could form a two-shot gun to overcome this kind of resistance.

10.7.6 Inhibitors of the Ras Family

Another crosstalk involves low-molecular weight GTP-ases, such as Ras, which is activated by prenylation. Ras activity is controlled by the GDP/GTP cycle. Ras and the other members of this superfamily of proteins (Rho, Rab) are posttranslationally modified by isoprenoid lipids. This modification is catalysed by farnesyltransferase and geranylgeranyltransferase, which catalyse the covalent attachment of farnesyl (C15) and geranylgeranyl (C20) groups, respectively, to the carboxy-terminal cysteine of prenylated proteins. The overproduction of Ras family proteins in BC is generally associated with a more aggressive type of BC [289]. Farnesyl transferase inhibitors (FTI), despite negative clinical trials in tamoxifen-resistant BC with tipifarnib [290], constitute a new class of anti-cancer drugs currently under evaluation because FTI-277 has been shown to have additive effects with tamoxifen in the inhibition of cell cycle progression in MCF-7 cells [249]. Farnesylated proteins generally maintain high levels of ERα expression throughout the cell and FTI decrease both the number of receptors and their transcriptional activity [291]. Other data suggest that inhibition
of the geranylgeranylation of RhoA and RhoC may be useful for treatment purposes, to prevent invasion by BC cells and the metastasis tendency of these cells [292] (Table 10.1). Due to their hydrophobic structure and their potent anti-cancer activity in vitro, the encapsulation of these FT inhibitors in BC-targeted nanocarriers could be of therapeutic interest. Their encapsulation into appropriate nanocarriers would improve their anti-proliferative and proapoptotic activities.

10.7.7 Ubiquitin-proteasome Inhibitors

Cell survival requires precise control over protein turnover and most protein degradation (including that of ERα in particular) in eukaryotes occurs in the proteasome after ubiquitin conjugation. This pathway regulates cell cycle, p53 and CDK, as well as proteins of the Bcl family and steroid and membrane receptors. It has therefore been suggested that proteasome inhibitors, such as bortezomib (MG-341, or Velcade®), may be useful for treating BC. Indeed, inhibitors of the proteasome provoke accumulation of proapoptotic proteins (such as BAX) [301]. However, proteasome inhibitors seem to be most effective against solid tumours when used in combination with other agents, like trastuzumab actually in phase II clinical trials [293]. Several other trials associating bortezomib with other drugs are currently under investigation for metastatic BC treatment, and promising results have also been obtained for multiple myeloma [294]. Interestingly, inhibition of NFκB by proteasome inhibitors, like MG132, potentiates the apoptotic activity of HDACi [295].

10.7.8 Gene Therapy

Several lines of evidence suggest that ERβ should be the receptor subtype to be kept active when trying to stop E2-induced tumour progression (see above). This ER subtype, unlike ERα, is absent or weakly expressed in many BC tumours, particularly those developing from undifferentiated cells. Nevertheless, the reexpression of ERβ in ERα-positive MCF-7 and T47-D BC cells has been shown to increase the efficacy of AEs for inducing apoptosis and cell cycle arrest [235] and to result in lower levels of tumourigenicity in nude mice [34], respectively. Thus, the delivery of the ERβ gene to breast tumours by the incorporation of plasmids containing this gene into lipoplexes may be particularly useful for the treatment of BC [201]. Other gene therapies can be considered for BC, like the reexpression of p53 and RhoB tumour suppressors or expression of shRNA inducing silencing of gene implicated in tumour growth such as Akt, AIB-1, Bcl-2, VEGF or ERα. Indeed, recent data from our laboratory showed that stealth nanocarriers loaded with a mix of two specific ERα siRNAs decreased the tumour growth by 50% in an MCF-7 BC xenograft model [296], with antiangiogenic activity which can be enhanced by small amount of RU incorporated
in stealth nanocarriers [72]. Importantly, it was observed that even in xenografts of MDA-MB-231 cells, the coadministration of two nanocarriers loaded with RU and siRNA-targeting ERα were able to induce a profound normalisation of the tumour vasculature with a decrease of CD34 expression [72]. Such data strengthen the use of nanocarriers loaded with drugs targeting ERα for its destruction in endothelial cells in coadministration with other drugs, such as TSA to increase tumour cell death.

Inactivation of the \( p53 \) gene appears to lead to cancer, and more than 35% of BC has mutated p53 which occur mainly in ER-negative, Erb-B2 amplified BC. Cationic lipoplexes loaded with antisense mutated p53 were designed and 48 hours after transfection \( \textit{in vitro} \) in BC cells, a decrease in mutated p53 expression was noticed [297], suggesting that this strategy has a promising future mainly considering that cells with p53 mutations are more sensitive to cisplatin and melphalan. It could also be of interest to envisage the association of the wild-type p53 plasmid with HER2-targeted lipoplexes since a viral transfection strategy with Ad5CMV-p53 combined with chemotherapy gave promising results. These effects were obtained following intratumoural administration [298] and due to physical difficulties of this approach, it can be expected that an IV systemic administration with a stable nonviral vector complexing wild-type p53 could have enhanced activity.

\[ 10.8 \text{ Conclusions} \]

Despite extraordinary advances in our fundamental understanding of the molecular mechanisms underlying carcinogenesis in BC cells and the establishment of molecular targeted therapy, BC remains the second leading cause of deaths among women in Western countries. Both intrinsic and acquired resistances, probably due to multifaceted deregulated pathways, may account for the failure of current treatments. Targeted drug delivery strategies with controlled release are one of the major areas of interest in cancer nanotechnology, as the probable next generation of cancer treatments. Systemically administered foreign substances (such as injected chemicals, biomolecules and nanocarriers) encounter multiple ‘biological barriers’ blocking their passage to their intended destinations, thereby reducing the drug availability. Some of the most notable biobarriers are physiological (e.g., the reticuloendothelial system, epithelial/endothelial membranes and cellular drug extrusion mechanisms) or biophysical (e.g., interstitial pressure gradients, transport across extracellular matrices, and specificity and density of tumour-specific surface receptors). The IV administration of stealth nanocarriers in cancer disease may overcome some of these natural barriers.

PEGylated nanoparticles and liposomes have been demonstrated to cross the discontinuous endothelium and to accumulate in solid tumours, which are often richly
vascularised. However, this process is still unclear and the fate of PEGylated nanocarriers has sometimes been found not to respond to this behaviour [120]. Work is still required not only to find explanations for this fate, but also to establish the appropriate conditions to avoid the macrophage-initiated capture of long-circulating PEGylated liposomes still present \textit{in vivo}. Many reports have shown that stealth nanocarriers deliver high concentrations of small encapsulated anti-cancer molecules to tumours. Nevertheless, they are unlikely to do the same for larger molecules, such as proteins, shRNA and DNA. In these cases, specific methods of targeting tumour cells are required. Immuno-targeting is probably the most promising approach, because the design of nanoparticles, liposomes or lipoplex linked to an antibody, such as trastuzumab, and containing a powerful anti-cancer agent may constitute a two-shot gun. In such cases, the antibody may block the cell-proliferative and/or cell-disseminative effects of the recognised antigen receptor, favouring endocytosis of the nanocarriers and the delivery of the encapsulated anti-cancer agent within the cell. It is also likely, as suggested by works dealing with various receptor-targeted nanocarriers, that active targeting minimises the influence of the architecture, composition, size and molecular mass on the treatment efficacy [299].

At present, despite the use of the drug delivery approach, the tendency is to associate various drugs targeting pathways involved in cancer cell progression. A number of clinical trials are actually ongoing, some being summarised in Table 10.2. A promising therapeutic activity has been reported for the combination of AE with HDACi which enhance \textit{in vitro} the anti-proliferative effects of AE in BC cells and block tamoxifen-induced proliferation of uterine cells [235]. Similarly, \(\text{ER}\beta\) increases the efficacy of AE like tamoxifen by blocking cell cycle in G2 [108] and RU58668 blocks cell cycle in G1, and enhances apoptosis. Thus, a promising delivery approach for treatment of BC and resistance could be the IV injection of devices immuno-targeting Erb-B2 and/or CD44 and loaded with AE, \(\text{ER}\beta\) and an HDACi in a tripartite combination.

Unfortunately, there is a major limitation for the development of nanocarriers in the translation from the bench to industrial production. Indeed, production of nanocarriers in large amounts encounters serious engineering problems which need to be solved before nanocarriers can have access to the clinic. In addition, the cost of immuno-liposome therapy has to be taken into consideration and this also constitutes a serious limitation to their development.

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11 Lipid Nanocarriers Targeting Adrenocortical Tumour Cells: Genetics, Clinical Implications, Treatment Strategies and Molecular Targets

Sajan Jose and Selma B. Souto

11.1 Introduction

Adrenocortical carcinoma (ACC) is a rare endocrine malignancy characterised by a limited understanding of its development and pathophysiology, dismal clinical prognosis and lack of efficient therapeutic regimens. The annual incidence of ACC ranges from 0.5 to 2 cases per million [1], and it accounts for up to 0.2% of all cancer deaths [2]. ACC is rare in childhood [3]. In the US, only about 25 new cases occur each year; however, in southern Brazil, cases occur 10 times more often. Whereas complete operative resection remains the only potentially curative option for ACC, approximately half of all patients have associated metastatic disease [4], resulting in a 5-year survival rate of less than 10% [5]. A better understanding of the aetiology and pathogenesis of this devastating disease could lead to more effective drug designs and the development of molecularly targeted treatments. These tumours are 1.5-fold more common in females than males [6]. The incidence of these malignancies increases during chronic gonadotropin overload, for example, in pregnancy [7] or after menopause [8], resulting in adrenocorticotropic hormone (ACTH)-independent macronodular adrenal hyperplasia (AIMAH) and Cushingoid symptoms maintained by ectopic adrenal Lhcgr expression [9]. The disease is most commonly detected in the fifth decade of life, although there is a secondary peak in children less than 10 years old. The clinical presentation of patients in these two groups is somewhat different. About 40% of adult patients with ACC present with a nonsecretory mass detected incidentally or during evaluation for abdominal or flank pain. Of the approximately 60% of tumours bearing a secretory syndrome, a mixed Cushing’s syndrome and virilisation caused by cosecretion of cortisol and adrenal androgens is most common (35%), followed by pure Cushing’s syndrome (30%) and pure virilisation (20%). Feminising (oestrogen-secreting) tumours are rare (10%), and aldosterone-secreting ACC are even less common (2%) [10]. By contrast, 90% of childhood ACC is secretory, and the large majority of the tumours secrete androgens, either as the sole hormone (55%) or in combination with cortisol (30%). Pure Cushing’s syndrome
is seen in less than 5% of paediatric ACC cases, as are other types of hormonal profiles [3]. At the time of presentation, the median tumour size in adults is approximately 10 cm, and 30–40% of patients have clear evidence for metastatic disease [10]. With the current ease of computed tomography and magnetic resonance imaging, the detection of these cancers at earlier stages is becoming more common, and consideration for an ACC is a strong indicator to remove an incidentally detected adrenal mass. Imaging characteristics strongly suggestive of a benign adrenal nodule include a low unenhanced computed tomography scan density or a rapid loss of signal enhancement after intravenous contrast injection [11]. Current guidelines recommend surgical removal of lesions greater than 6 cm. Lesions less than 4 cm and with low risk by imaging criteria are unlikely to have malignant potential and are generally not resected. More than 60% of incidentalomas less than 4 cm are benign adenomas, while less than 2% represent primary adrenal carcinomas. In contrast, the risk of adrenal carcinoma increases up to 25% in lesions that are greater than 6 cm, while benign adrenal adenomas account for less than 15%. For lesions between 4 and 6 cm, either close follow-up or adrenalectomy is considered a reasonable approach. Adrenalectomy should be strongly considered if the imaging findings, including rapid growth rate, decreased lipid content and other features described previously, suggest that the lesion is not an adenoma. Most of these tumours are unilateral, benign, adrenocortical adenomas and bear a very poor prognosis. Bilateral adrenocortical tumours are observed in two rare situations, either of which can induce steroid oversecretion and AIMAH.

11.2 Aetiology of Adrenocortical Carcinoma

At present, most of the genetic alterations known in adrenocortical tumours have been discovered by analysis of mutations associated with familial syndromes [12].

11.2.1 Li-Fraumeni Syndrome, the TP53 Gene and the 17p13 Locus

Li-Fraumeni syndrome (LFS) is a dominantly inherited disorder that confers susceptibility to cancers, mostly soft-tissue sarcoma, brain tumours, breast cancer, leukaemia and adrenocortical cancer [12]. Adrenocortical cancer occurs in up to 3% of patients with LFS, usually in children [12, 13]. The majority of families with LFS have a heterozygous germline inactivating mutation of the tumour-suppressor gene TP53 [12, 13]. Such germline mutations are present in 50–80% of children with apparently isolated, sporadic adrenocortical cancer in North America and Europe [14]. In southern Brazil, the incidence of paediatric adrenocortical cancer is 10 times higher than that in the rest of the world. Almost all patients bear the same germline mutation
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(p53 Arg337His), which corresponds to a location in exon 10 of the TP53 gene [15], and have apparently sporadic adrenocortical cancer. This tissue-restricted pattern could result from a pH-dependent alteration of the mutant Arg337His in p53 [16]. A second variant is caused by a heterozygous germline mutation in the hCHK2 gene, while a 4 cM region on 1q23 has been implicated in a third variant [12]. The prevalence of loss of heterozygosity at 17p13 in adrenocortical tumours increases in parallel with the Weiss score, a classical pathological index of malignancy in such tumours (this score is usually below 3 in adrenocortical adenomas and above 3 in adrenocortical cancer) [17]. A large prospective study has shown that loss of heterozygosity in 17p13 is an independent variable that can predict recurrence after complete surgical removal of localised adrenocortical tumours [18].

11.2.2 Beckwith-Wiedemann Syndrome, the IGF2 Gene and the 11p15 Locus

Beckwith-Wiedemann syndrome (BWS) is a phenotypically variable paediatric developmental disease [19, 20]. Its main features are omphalocoele, macroglossia and macrosomia or hemihypertrophy [20, 21]. In 10% of patients, a tumour is detected during the first years of life, most often Wilms’ tumour (5%) or adrenocortical tumours (3%) [21]. BWS is a complex genetic disease caused by variable genetic and epigenetic defects involving several genes of the imprinted (i.e., only the paternal or maternal allele is expressed) 11p15 locus [21, 22]. The gene for IGF2 is also located on chromosome 11p15, and is subject to imprinting. Unipaternal disomy of 11p15 (i.e., loss of the maternal allele and duplication of the paternal allele) can be found in patients with BWS [21]. The common consequence of the various 11p15 alterations found in BWS is the dysregulation of IGF2 imprinting and the overexpression of IGF2. Receptors for insulin-like growth factors IGF1 and IGF2 are present in adrenal tissues [23]. The mitogenic effect of IGF2 is dependent on the IGF1 receptor (IGF1R) in adrenocortical cancer cells [16].

Interestingly, a dramatic overexpression of IGF2 can be observed in sporadic adrenocortical tumours in adults [16]. In the past 3 years, gene-profiling studies reported that IGF2 was the most dramatically overexpressed gene in adrenocortical cancer, compared with its expression levels in adrenocortical adenomas and in normal adrenal tissue [24]. The prevalence of IGF2 overexpression increases in parallel with the Weiss score. As a consequence, IGF2 overexpression is almost always found in adrenocortical cancers, being most often but not always associated with unipaternal disomy [18]. Loss of heterozygosity at the 11p15 locus is frequent in most adrenocortical cancers (80%), and it is linked to unipaternal disomy. Taken together, these observations suggest that activation of the IGF pathway is a common pathological mechanism used by tumour cells during adrenocortical tumorigenesis.
11.2.3 Multiple Endocrine Neoplasia Type 1, MEN1 Gene and 11q13 Locus

Multiple endocrine neoplasia type 1 (MEN1) is a dominantly inherited syndrome characterised by the presence of parathyroid (95%), endocrine pancreas (45%) and pituitary (45%) tumours. Adrenocortical tumours and/or adrenocortical hyperplasia are present in 25–40% of patients [19]. Somatic mutation of the MEN1 gene is extremely rare in sporadic adrenocortical tumours [25]. By contrast, loss of heterozygosity at 11q13 is almost always found in sporadic adrenocortical cancer, and it is found in 10–14% of sporadic adrenocortical adenomas [25]. The discrepancy between the very low prevalence of MEN1 mutation and the high prevalence of loss of heterozygosity at 11q13 suggests that another tumour-suppressor gene, at the same locus, could be involved in these sporadic adrenocortical tumours.

11.2.4 Carney’s Complex, Protein Kinase A R1A and 17q22–24 Locus

Carney’s complex (CNC) is a dominantly inherited disorder with a variety of clinical and pathological manifestations [26]. The main characteristics of CNC are spotty skin pigmentation (lentiginosis), endocrine overactivity and cardiac myxomas [26]. ACTH-independent Cushing’s syndrome due to primary pigmented nodular adrenocortical disease (PPNAD) is the main endocrine manifestation of CNC. CNC seems to be a genetically heterogeneous disease, and gene linkage analysis has shown that at least two loci are involved, namely, 2p16 and 17q22–24 [27]. The gene located on 17q22–24 has been identified as Protein Kinase A R1A [28]. In sporadic adrenocortical tumours, somatic Protein Kinase A R1A mutations have been found in a subset of hormone-secreting adrenocortical adenomas [29]. These tumours show clinical, biological and pathological features similar to those in PPNAD. Loss of heterozygosity on 17q22–24 has also been observed in sporadic adrenocortical tumours.

11.3 Clinical Implications

11.3.1 Recognising Familial or Syndromic Adrenocortical Tumours

Patients with adrenal Cushing’s syndrome and bilateral adrenal involvement are often a diagnostic challenge. It is crucial, however, not to misdiagnose a patient bearing an adrenocortical tumour within the context of a familial or syndromic disorder, and to recognise the genetic origin of the disease. Children with a unilateral adrenocortical tumour might have germline TP53 or 11p15 alterations. The identification of the genetic origin of an adrenocortical tumour can also help to classify it and to determine its prognosis [30].
The clinical presentation of Cushing’s syndrome in patients with CNC can be misleading, particularly in patients with isolated, sporadic adrenal disease. Patients’ diagnosis should be based on the young age of the patient, the paradoxical cortisol response to the dexamethasone suppression test [31] and detailed radiologic evaluation of both adrenal glands. Ultimately, a genotype study for Protein Kinase A R1A germline mutations should be undertaken. The obvious implication is to conduct family-wide screening, to search for other features of CNC (particularly for life-threatening heart myxomas) in affected individuals and to monitor affected patients indefinitely.

It is now recognised that adrenocortical cancer can feature in syndromic disorders, particularly in children. In these young patients, discussion should take place over whether germline genotyping of TP53 or analysis of the 11p15 locus should systematically be performed in search of LFS or BWS, respectively. Analysis of tumour DNA might provide some initial clues.

11.3.2 Evaluating the Prognosis of Patients with Adrenocortical Tumours

As is the case with many types of endocrine tumours, it is often difficult to distinguish with certainty between benign and malignant adrenocortical tumours. Currently, this distinction is based on various pathological criteria, among which the Weiss score (based on nine different histopathological items) is probably the best known [17]. The Weiss score up to 3 indicates benign tumour and score greater than 3 indicates malignant tumour.

The search for molecular markers of malignant adrenocortical tumours has long been a problem. As indicated above (in the discussion of LFS and BWS), the high prevalence of abnormalities on the 11p15 and 17p13 loci in genuinely malignant (i.e., recurrent or metastasising) adrenocortical tumour provides potential molecular markers of malignancy. As mentioned previously, loss of heterozygosity on 17p13 in adrenocortical tumours has been proven as an independent predictor of recurrence after complete tumour resection. Such molecular markers, which could be routinely examined at the DNA level, should provide highly reproducible information, especially when pathological examination cannot firmly establish a diagnosis of malignancy [24].

11.4 Current Therapies for Adrenocortical Carcinoma

Radical surgery is the standard therapy for patients with localised and regional ACC (stages I–III) [4]. Due to a high risk of recurrence, most centres recommend adjuvant therapy even after complete resection. The best available data derive from a large
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retrospective multicentre study demonstrating the efficacy of mitotane – a synthetic derivative of the insecticide dichlorodiphenyltrichloroethane – to reduce significantly the risk of recurrence and death [32]. The high recurrence rate of up to 85% in ACC after seemingly complete tumour resection [33] prompted researchers to use mitotane as adjuvant therapy. The first evidence of a benefit of such an approach derived from two retrospective reports, describing 10 patients who received mitotane after complete surgery with a survival time above historical controls [34].

11.4.1 Mechanism and Biotransformation of Mitotane

Mitotane exerts a specific, direct, cytotoxic effect on adrenocortical cells producing focal degeneration of the fascicular and, particularly, the reticular zone, whereas changes in the zona glomerulosa are relatively slight [35, 36]. Ultrastructural changes after mitotane administration include severe mitochondrial damage in normal and neoplastic adrenocortical cells, followed by pyknosis and cellular destruction. Metabolic activation and covalent binding of a reactive metabolite to adrenal macromolecules are essential for the adrenolytic activity of mitotane [37, 38]. Mitotane is hydroxylated and transformed into an acyl chloride. The reactive acyl chloride either covalently binds to macromolecules, predominantly mitochondrial proteins, thereby mediating the biological activity of mitotane, or is transformed to the acetic acid derivative o,p′-dichlorodiphenyl acetic acid (o,p′-DDA), the main metabolite of mitotane [39]. o,p′-DDA levels were 3- to 10-fold higher than the parent compound levels in plasma [40]. Both metabolic transformation and covalent binding occur primarily in adrenal mitochondria, whereas biotransformation in the adrenal microsomal fraction is negligible [38].

The most favourable results have been seen with the so-called ‘Italian’ protocol, consisting of etoposide, doxorubicin and cisplatin, with concurrent mitotane administration [41]. In the initial 28 patients receiving this regimen, the overall response rate was 53.5%, although the large majority (13 out of 15) was partial responses. In this study, careful attention was paid to maintain mitotane serum levels between 14 and 20 g/dl, which provided clinical benefit with minimal toxicity. A second active regimen is the combination of streptozotocin and mitotane (SO therapy) [42].

Salvage chemotherapy with vincristine, cisplatin, teniposide and cyclophosphamide has been reported to show activity in patients who had failed the SO treatment [43]. Others have suggested using a regimen of taxanes and gemcitabine as therapy for refractory ACC. Although this regimen has shown some activity in advanced solid tumours unresponsive to other treatments [44], insufficient clinical trial data preclude making a recommendation regarding its use for ACC.
11.4.2 Emerging Technologies in the Treatment of Adrenocortical Carcinoma

11.4.2.1 Overcoming Drug Resistance in Adrenocortical Carcinoma

It has long been recognised that ACC is resistant to standard cytotoxic chemotherapy [45]. At the molecular level, ACC expresses high levels of the multidrug resistance protein MDR1 (also known as P-glycoprotein). This protein, encoded by the ABCB1 gene, is an approximately 170–180,000 kDa membrane glycoprotein that functions as an ATP-dependent drug efflux pump, transporting out of the cell hydrophobic cytotoxic agents, such as doxorubicin, vinblastine and taxol. Normal adrenocortical tissue produces high levels of MDR1 [46, 47], and this expression is retained in most ACC [48, 49]. To overcome this drug resistance, competitive inhibitors of MDR1-mediated drug transport have been tested as a means to increase the effectiveness of chemotherapy. Early trials included compounds such as d-verapamil (which, unlike l-verapamil, is not a calcium channel blocker) and mitotane itself [50]. These studies yielded low rates of response, as did a trial using a second-generation competitor known as PSC833 (valspodar) [51]. Despite these failures, the search for more potent MDR1 inhibitors has continued, and a current phase II study is evaluating the effect of chemotherapy plus tariquidar (XR9576), a third-generation noncompetitive inhibitor of the MDR1 efflux pump [52, 53].

11.4.2.2 Vascular-targeted Therapies in Adrenocortical Carcinoma

Vascular-targeted therapies can be divided into two distinct classes: those that prevent new blood vessel growth (antiangiogenic agents) and those that disrupt established tumour vasculature [54]. Vascular endothelial growth factor (VEGF) is the predominant signal for both endothelial proliferation and migration into sites of neovascularisation, and blockade of this signal has been a major goal of research in this field. Treatment strategies with VEGF include antibodies (e.g., bevacizumab) aimed at blocking the effect at the prereceptor level, as well as a variety of small-molecule inhibitors of the VEGF receptor kinases. In addition to therapies aimed at inhibiting the development of new blood vessels, it may also be possible to develop agents that specifically target tumour vasculature. Tumour blood vessels tend to be poorly organised, with regions of hypoxia and significant acidosis due to the accumulation of the products of anaerobic glycolysis. This environment leads to a relatively ‘immature’ phenotype of the cells [55], and also causes expression of specific markers on the endothelial lining of tumour vasculature. Such targets include roundabout-4 and the fibronectin extra domain B [56]. Additionally, serial analysis of gene expression of tumour endothelium led to the identification of anonymous genes known as tumour endothelial markers (TEM), of which TEM1 (endosalin), TEM5 and TEM8 have
further been shown to be specific for tumour vasculature [57]. These proteins, all of which are cell surface antigens, provide potential targets for the development of agents that target them directly or use them as homing signals to direct other therapeutic molecules, such as via monoclonal antibodies [56].

11.5 Emerging Molecular Targets in Adrenocortical Carcinoma

Elucidating the numerous molecular factors and signalling pathways involved in various adrenocortical tumours has provided new potential targets for treatment. In AIMAH, illegitimate expression of a given receptor might address a specific, unconventional, pharmacologic approach to therapy. Examples include the use of somatostatin analogues in tumours that express the gastric inhibitory polypeptide receptor, gonadotropin-releasing hormone agonists in tumours that express luteinising hormone receptors or adrenergic antagonists in tumours that express catecholamine receptors [58].

In adrenocortical cancer, overexpression of \( IGF2 \) is thought to act in a paracrine fashion through the \( IGF1R \). Various molecules are presently being tested that antagonise this signalling pathway, and which might be useful in adrenocortical cancer. Similarly, molecules that antagonise the cAMP or Wnt signalling pathways might prove beneficial in some specific adrenocortical tumours. A recent study showed that the majority of ACC cell lines tested display constitutive IGF ligand production and activation of downstream effector pathways [59]. Both \( IGF1R \) antagonists, Section 11.2.2, cause significant dose-dependent growth inhibition in ACC cell lines. Furthermore, the authors observed that mitotane, the first-line adrenolytic drug used in patients with ACC, results in enhanced growth inhibition when used in combination with the \( IGF1R \) antagonists. The activity of \( IGF1R \) antagonists was evaluated against ACC xenografts in athymic nude mice. IGF inhibition markedly reduced tumour growth, which was greater than that observed with mitotane treatment, and combination therapy with mitotane significantly enhanced tumour growth suppression [59]. Finally, in adrenocortical cancer, the frequent activation of angiogenic pathways (via VEGF) and/or growth factor pathways (via fibroblast growth factor and its receptors) might indicate future directions for new therapies [60].

11.6 Challenges in Lipid-based Drug Delivery Systems for Adrenocortical Carcinoma

As previously noted, because ACC is a rare tumour, gathering a sufficient number of patients for study in clinical trial is a difficult task. Furthermore, the pharmaceutical
industry may be reluctant in undertaking the development of chemotherapeutics, where the size of the potential market for the agent is relatively small. Progress in the development of novel therapeutic strategies would be greatly enhanced in parallel with research on experimental models of adrenocortical cancer. With respect to \textit{in vitro} testing, there are only three widely available cell lines, SW13, H295 and H295R (a derivative of H295 selected for adherent growth). These cell lines do not adequately represent the heterogeneity of ACC. ACC is also rare in mice; however, there are mouse models in which ACC has been observed. Most ACC has been detected either in gonadectomised animals or in the context of specific targeted mutations such as PTEN, p53 and CDNK1C, but the disease has not been characterised in these models [61]. These cell lines are suitable for testing lipid-based drug delivery systems for ACC. Examples would be the encapsulation of common chemotherapeutics, such as vinblastine, vincristine, cisplatin, teniposide and cyclophosphamide.

PEGylated liposomes (liposomes coated with polyethylene glycol (PEG)) were used to encapsulate the hydrophobic vinorelbine (5′-nor-anhydro-vinblastine) with a wide range of drug release patterns and half-life circulation times [62]. The results depended on the stabilising agent (e.g., sulfate, poly(phosphate) or sucrose octasulfate) and on the drug-to-lipid ratio. Liposomes prepared using sucrose octasulfate displayed the longest half-life in circulation (9.4 hours) and \textit{in vivo} retention in the nanoparticle (27.2 hours). The authors demonstrated that efficacy could be improved in HT-29 and C-26 cell lines, when vinorelbine was encapsulated in sucrose octasulfate-stabilised liposomes. This drug has also been loaded in PEG2000-stearic acid-based solid lipid nanoparticles (SLN) [63]. The particle size and zeta potential of resulted SLN were in the range of 180–250 nm and 0–10 mV, respectively. The encapsulation efficiency of vinorelbine in SLN slightly decreased after PEGylation (approximately 60%). On the other hand, PEGylation contributed for a more sustained release of vinorelbine, in comparison to SLN without PEG modification. The cellular uptake studies showed that phagocytosis by RAW264.7 cells was inhibited by PEGylated SLN, while the uptake by cancer cells (MCF-7 and A549) was enhanced, followed by a significant enhancement of the \textit{in vivo} anti-cancer activity. Vinorelbine-loaded SLN were prepared by a cold homogenisation technique, varying the lipid composition, drug content and homogenising times [64]. The mean particle size of SLN ranged from 150 to 350 nm. The enhancement of lecithin content in lipid matrix resulted in a smaller mean size. The encapsulation efficiency was improved with increasing lecithin or oleic acid content in lipid matrix (approximately 80%); however, the loading capacity decreased (up to 6.6%). The pharmacokinetic studies showed that vinorelbine release could last for 48 hours, and the rate was delayed by the addition of lecithin or oleic acid to the SLN formulation. Cellular cytotoxicity testing against MCF-7 cells could be improved by loading the drug in SLN.
Stable lipid nanoparticles, similar to conventional liposomes, were produced by Johnston and co-workers [65]. Vincristine retention and circulation lifetime properties of liposomal nanoparticles were compared with dihydrosphingomyelin (DHSM)-loaded liposomes. The authors reported that replacement of egg sphingomyelin (ESM) by DHSM in sphingomyelin/cholesterol (Chol) (55/45; mol/mol) resulted in substantially improved vincristine retention properties both in vitro and in vivo. In the case of vincristine-loaded nanoparticles, the drug release half-time was approximately threefold longer for DHSM/Chol as compared to ESM/Chol, both in vitro and in vivo. Further increases in half-time were observed when the drug-to-lipid ratio of vincristine-loaded formulations was increased. In addition, DHSM/Chol liposomes also exhibit improved circulation lifetimes in vivo as compared to those based on ESM/Chol.

Cisplatin has been encapsulated into lipid nanoparticles [66–68]. This drug was loaded into SLN to obtain a targeted and less toxic drug delivery system [66]. Particles were spherical and uniform, with a mean particle size and zeta potential of approximately 120 nm and –46 mV, respectively. The encapsulation efficiency reached 82.3%. The in vitro release profile revealed that cisplatin was released from SLN efficiently and completely in normal saline compared with other release media. A tissue distribution study was conducted in male rats after intravenous administration of 8 mg/ml cisplatin-loaded SLN in comparison to a drug solution in normal saline. The authors reported that a targeted effect to the liver as well as a low concentration in the kidney in rats was observed for SLN formulations. Other studies support these findings where the in vivo result of SLN formulations of cisplatin revealed that the drug is preferentially targeted to the liver followed by brain and lungs [68]. Teniposide is a well-known model drug for micelles and liposomes [69, 70]. This drug was loaded in liposomes [69], and checked for the cytotoxic interaction of two ether lipids, octadecylphosphocholine and ET-18-OCH3, on cancer cells in vitro. Enhanced cytotoxic activity was found with combinations of the ether lipids against leukaemic cells. However, the advantage of loading the chemotherapeutic in liposomes was the decrease of haemolytic activity, in comparison to the free drug.

11.7 Conclusions

Although treatment of ACC is still rather difficult, scientific research is focused on the development of novel strategies to improve the efficiency of traditional diagnosis and therapy. The literature suggests lipid nanoparticles as excellent carrier candidates for encapsulating anti-cancer drugs for tumour chemotherapy. These platforms offer unique properties for accurate targeted drug delivery and a much enhanced efficiency of drug to overcome problems such as stability and toxicity.
References


Lipid Nanocarriers Targeting Adrenocortical Tumour Cells


57. A. Nanda and B. St Croix, *Current Opinion in Oncology*, 2004, 16, 44.


Brain cancer is the third worst cancer following cancer of the pancreas and lungs. The incidence of brain cancer increases with the increase in age and brain cancer represents one of the major cancers particularly in young people. Statistics of brain cancer patients show a grim picture, with the incidence of afflicted population slowly increasing and survival rates of less than 25% after 5 years [1].

The feature of brain cancer that complicates treatment is the natural blood-brain barrier (BBB), a strong fort, protecting the brain. The BBB while permitting supply of nutrients to the brain prevents transport of toxins and other exogenous substances including drugs to the brain. Strategies that allow the transport of substances across the formidable BBB need to be addressed, to enable delivery of adequate drug for improved therapy in brain cancer. This chapter discusses brain cancer, the BBB and strategies for overcoming BBB, followed by a detailed discussion on the design of microemulsions (ME) and nanoemulsions (NE) for improved therapy in brain cancer.

Brain cancer is associated with tumours that are aggressive and invasive in nature and are characterised as primary brain tumours, which originate in the brain cells, and metastatic or secondary brain tumours, which result due to migration of cancerous cells from different primary cancers in the body to the brain [2–4]. The latter process is termed metastasis.

In the case of primary brain tumours, it can take up to 2 years for the symptoms to appear and for manifestation of the disease. They are normally characterised based on the cells from which they originate. Nine categories of primary brain tumours
have been identified by the World Health Organization [5, 6]. Of the nine categories, infiltrative astrocytoma (IAC), the most common type of brain tumour in children, originates in the brainstem, cerebellum, white matter of the cerebrum or spinal cord, and glioblastoma multiforme (GBM), the most common cancer in adults, originates in glial cells in the cerebrum. IAC and GBM account for nearly 85% of the brain tumours [7]. Average survival rates in patients of GBM are stated to be only 14 months, despite aggressive treatments including combinations of surgical resection, radiation and chemotherapy. The others include oligodendroglioma originating in the oligodendrocytes, mixed oligoastrocytoma, which originates in the oligodendrocytes and astrocytes, ependymoma found in the ependymocytes, medulloblastoma, which originates in the primitive neural cells, menigioma found in the meninges and others. All gliomas range from low-grade to high-grade tumours. The high-grade tumours are chaotic and undifferentiated, more aggressive and associated with lower survival rates. The primary brain tumours rarely spread beyond the central nervous system (CNS). Surgical removal of the tumour is often feasible in primary brain tumours. Nevertheless, the prognosis for patients with brain tumour is poor [8].

12.2.2 Metastatic Brain Tumours

Metastatic brain tumours are most often those that spread from the lung, breast and kidney and from malignant melanomas, and are much more common than primary brain tumours. Incidences of brain metastases are 16% in lung cancer patients and lower in breast cancer and colon cancer patients. However, it could be as high as 55% in patients with melanoma [2, 3].

Metastatic brain cancer indicates advanced disease and is associated with low survival rates. Metastatic tumours are usually named after the type of tissue from which the original cancer cells arise (e.g., metastatic lung or breast cancer). Surgical removal in such cases is precluded. Treatment resorted to is therefore radiotherapy and chemotherapy, singly or in combination [9]. Noninvasive chemotherapy options for brain cancer however are severely limited by the impermeable nature of the BBB. Overcoming the BBB, therefore, is the first step to be addressed in the design of improved and noninvasive chemotherapeutic drug delivery systems for brain cancer. This necessitates a thorough understanding of the BBB.

12.3 Blood-brain Barrier

The BBB provides a unique, selective physical and electrical barrier [10, 11]. High-density endothelial cells line the cerebral capillaries, together with astrocytic end-feet processes. At the interface between blood and the brain, the endothelial cells
and associated astrocytes are stitched together to form tight junctions, which are composed of transmembrane proteins such as occludins, claudins and junctional adhesion molecule [12–21]. Each of these transmembrane proteins is anchored to the endothelial cells by another protein complex that includes zonula occludens protein 1 and associated proteins. Formation of tight junctions effectively blocks the paracellular pathway [22]. The membranes of the endothelial cells have two distinct sides, the luminal (blood side) and abluminal (brain side). The abluminal side of the endothelium has pericytes attached at irregular intervals. The pericytes are covered by the basal lamina, a membrane about 30–40 nm in thickness comprising type IV collagen, heparin sulfate, proteoglycans, laminin, fibronectin and other extracellular matrix proteins. The basal lamina is intimately associated with the astrocyte end feet. The space between the endothelium, pericyte and the astrocyte foot process forms the interface between blood and brain. For a molecule to cross from the luminal side into the abluminal side and into the brain interstitial space, it must cross the perivascular space bordered by the plasma membrane of the capillary endothelial cells, pericytes and astrocyte foot processes. Astrocyte end feet, which cover almost the entire endothelial surface (>90%), control the permeability of the BBB. Besides the physical barriers the high electrical resistance of brain capillaries, stated to be 1000–2000 Ω cm², prevents polar and ionic substances from entering the brain [23]. This high electrical resistance is attributed to high expression of occludin [16]. The blood cerebrospinal fluid (CSF) barrier selectively excludes exogenous substances including drugs from reaching the CSF. The choroid plexus, which is the source of CSF production, functions as a highly selective barrier to drugs [24, 25]. However, the surface area of the blood-CSF barrier is significantly lower than that of the BBB. The implications of this barrier in drug delivery could be considered insignificant in comparison with the BBB [26].

12.3.1 Transport across the Blood-brain Barrier

Dual transport mechanisms are possible across the BBB. While focusing on drug delivery across the BBB, it is important to consider methods that allow a molecule to cross from the luminal side into the brain interstitial spaces. It is also pertinent to understand the natural mechanism in the BBB that effluxes out foreign substances including drugs. Transport mechanisms into the brain across the BBB can be broadly categorised as passive diffusion and endogenous carrier-mediated transport.

12.3.1.1 Passive Diffusion

Passive diffusion is a process whereby drugs or endogenous substances travel across the BBB and is dependent on the concentration gradient from blood to brain, and the physicochemical properties of the drug. Drugs that are generally lipophilic, as
predicted by their octanol/water partition coefficient, and have a molecular weight (MW) of <400–500 Da can exhibit transport by passive diffusion. Numerous quantitative relationships have been cast to correlate BBB penetration to lipophilicity and molecular mass as well as other chemical structural features [27–29]. Drugs with MW >400 Da and/or drugs that form eight or more hydrogen bonds with the surrounding water represent poor transport across the BBB [11, 30–32]. Additionally, the presence of quaternary ammonium group or more than one carboxyl group can also negatively influence diffusion through the BBB [33]. CNS drugs that show enhanced permeability through the BBB were found to have fewer hydrogen bond donors, fewer positive charges, greater lipophilicity and reduced flexibility [34]. Thus, transcellular passive diffusion through the BBB is favoured for small (<400 Da), nonpolar and lipophilic compounds. The size, flexibility, conformation, ionisation (nonionised form penetrates BBB), lipophilicity of the drug molecule and its cellular enzyme stability also influence transport across the brain.

12.3.1.2 Endogenous Carrier-mediated Transport

Although lipid-soluble drugs are expected to readily diffuse across the BBB, many of these drugs have shown lower permeability than predicted by their lipid solubility. These drugs, including chemotherapeutic agents, are substrates for drug efflux transporters [35], which are present in the BBB and blood-CSF barrier [36–39]. These transporters actively efflux drugs from the CNS, thus limiting brain uptake. Endogenous transport systems at the BBB include carrier-mediated facilitated transport [40] and receptor-mediated endocytosis (RME) [34], which favour transport from the blood side to the brain. However, adenosine triphosphate (ATP)-dependent active transport mechanisms limit transport by effluxing drug transported across the BBB [24]. Transport by carrier-mediated transport system therefore would depend on which of the systems predominate.

12.3.1.2.1 Carrier-mediated Facilitated Transport

Solute carrier (SLC) transporters play a vital role in various cellular physiological processes, including transport of nutrients, neurotransmitters and metabolites across the BBB. They belong to the SLC superfamily of transporters comprising 43 families with members responsible for transport of specific substrates, including sugars, amino acids, oligopeptides, organic anions and organic cations [41]. The transport of endogenous substrates or anti-cancer drugs can be into or out of the brain, and depends on the subcellular localisation of these transporters on the BBB. Among the SLC superfamily, the organic cation transporter (OCT) system (SLC21) and organic anion/cation transport system (SLC22) play a major role in transporting anti-cancer drugs across the BBB. The more recently discovered organic anion transporter (OAT) and OCT families in the brain endothelium can also be considered as constituting the BBB. Organic anion and cation transporters can favour exchange of anions and
cations from the blood to the brain or vice versa depending on the ionic or drug gradients. In humans, OAT3 is found mainly expressed in the basolateral membrane [42]. Organic anion-transporting polypeptide-A (OATP-A) is also expressed in brain endothelia, and also forms part of the BBB [43]. In the mouse model, Oatp-3 has been shown to play a significant role in the BBB [44].

Other influx transporters at the BBB include glucose transporter type 1 (GLUT-1) for glucose [45], monocarboxylic acid transporter 1 for monocarboxylic acids, large neutral amino acid transporter 1 for large neutral amino acids [46], cationic amino acid transporter 1 for cationic amino acids, equilibrative nucleoside transporter 1 and 2 and concentrative nucleoside transporter 1 and 2 for nucleosides and transporters for choline [47]. These carriers facilitate the entry of polar drugs like melphalan into the brain although their role in transport is considered limited [48, 49].

12.3.1.2.2 Receptor-mediated Endocytosis

RME is facilitated when a drug or drug delivery system expresses affinity for a receptor. RME triggered by receptor-ligand interaction involves uptake at the luminal (blood) side followed by intracellular movement and exocytosis at the abluminal (brain) side of brain endothelial cell. The BBB expresses a number of receptors. A number of these receptors are overexpressed in brain cancer. Tagging the drug with ligand molecules that are specific for these receptors enables selective and improved delivery of drugs, which is generally independent of the size of the ligand [50]. Many receptor-mediated endocytotic pathways involve the formation of clathrin-coated pits, which can envelop not only drug-ligand conjugates but also nanosize particles, form vesicles, detach from the cell surface and carry the particles into the cytosolic compartment [51]. This process facilitates transport of drug-loaded nanocarriers through the BBB, bypassing the drug efflux transporters. Receptors that have significance in drug delivery include transferrin receptors, insulin receptors, lipoprotein-related protein-1 and lipoprotein-related protein-2 receptors, diphtheria toxin receptors [52], leptin receptors [53], oestrogen receptor beta [54], folate receptors [55, 56], interleukin (IL)-4 [57] and IL-13 [58, 59]. RME enables transport of large molecules and nanosize particles, and hence represents an important pathway to deliver anti-cancer drugs into the brain.

12.3.1.2.3 Efflux Transporters or Adenosine Triphosphate-dependent Active Transport

Several membrane transporters, mainly belonging to the superfamily of ATP-binding cassette (ABC) membrane transporters [60], are located in the BBB. Their role is to mediate the efflux of exogenous substances, including drugs, from the brain to the blood. These systems limit accumulation of drugs in the brain. The major efflux transporters [24] include P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP) family.
Padma V. Devarajan and Rajshree L. Shinde

- **P-glycoprotein**

P-gp, a member of the ABC transporter superfamily, also known as multidrug resistance-1 protein, ABCB1 or CD243 (cluster of differentiation 243), is a 170 kDa efflux pump expressed at the apical side of the BBB [61, 62]. P-gp is known to actively pump a variety of anti-cancer drugs, including paclitaxel, topotecan and anthracyclines, back into systemic circulation [63]. The presence of P-gp has been confirmed in resistant glioblastomas [64]. Hence despite the leaky nature of glioma vasculature, anti-cancer drug penetration into brain tumours is limited. Data suggest that the delivery of anti-cancer drugs to brain tumour, despite the compromised integrity of the BBB, is severely hampered by P-gp.

- **Breast cancer resistance protein**

BCRP is also expressed at the apical side of the BBB, and can efflux cytotoxic compounds like mitoxantrone, topotecan, flavopiridol, methotrexate (MTX), sulfated conjugates of therapeutic drugs and hormones like oestrogen sulfate [65]. In comparison with P-gp, the overlapping substrate profile and similar localisation at the BBB suggest that BCRP may also limit transport across the BBB. The effects of BCRP are suggested to be similar to those of P-gp [66].

- **Multidrug resistance-associated protein family**

The MRP family efflux pump comprises nine members (MRP-1–9), which can transport structurally diverse lipophilic anions. MRP-1, MRP-2, MRP-4 and MRP-5, which are expressed at the apical side of the BBB, play an important role in chemoresistance at the BBB [67, 68]. MRP-1 functions as a glutathione and glucuronate conjugate pump and it also confers resistance to anthracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins and MTX. MRP-4 and MRP-5, responsible for transporting cyclic adenosine monophosphate and cyclic guanosine monophosphate, are suggested as resistance factors for nucleotide analogue drugs [67, 69].

**12.3.1.3 Adsorptive-mediated Endocytosis**

One of the newer methods proposed for transport of drugs across the BBB is adsorptive-mediated endocytosis using peptide vectors [70]. Transcytosis of these peptides through the BBB is slated to occur as follows: adsorption or binding on the endothelial cell membrane followed by internalisation, diffusion through the cytoplasm and finally release on the abluminal side. These peptides are the pegelin (SynB1 and SynB3) and penetratin peptides, which are amphipathic and positively charged. Following conjugation to drugs, these peptides serve as vectors to transport the peptide vector...
drug conjugate through the BBB and also overcome P-gp efflux [71]. This has been confirmed with doxorubicin (DOX) [72, 73].

12.4 Blood-brain Barrier in Brain Cancer

Compromise in the BBB has been reported with both primary and metastatic cancerous tumours [74]. The proliferation and invasion of tumoral cells generally cause local disruption of the BBB [75]. The normal and disrupted BBB is schematically depicted in Figure 12.1.

Alterations observed in the endothelial cells include a compromised tight junction and increase in the perivascular space. Mediators such as arachidonic acid (ARA),

![Figure 12.1 The permeability junctions in normal and disrupted BBB. (a) The normal BBB is composed of an intricate network of astrocytes, pericytes, endothelial cells and neurons that form tight, impermeable junctions, which exclude large cells, macromolecules and excess fluid from CNS. (b) In the setting of brain tumour, the tumour astrocytes are more densely packed and irregular, the basement membrane is disrupted and thickened and tight junctions are widened, allowing passage of macromolecules and fluid. Reproduced with permission from E.R. Gerstner, D.G. Duda, E. di Tomaso, P.A. Ryg, J.S. Loeffler, A.G. Sorensen, P. Ivy, R.K. Jain and T.T. Batchelor, Nature Reviews Clinical Oncology, 2009, 6, 229. ©2009, Macmillan Publishers Ltd][76]
leukotrienes, prostaglandin E and thromboxane B2 increase the permeability of the capillary endothelium. Moreover, formation of new blood vessels in the tumour by the proangiogenic factors, including a basic fibroblast growth factor and a vascular endothelial growth factor, improves blood circulation in the tumours, while fenestrations in the tumour blood vessels result in increase in permeability [76]. This could favour permeation of drug into the tumour. Disruption of the BBB does not occur in the healthy tissue. The BBB is therefore characterised by localised regions of enhanced permeability surrounding the tumour. Increase in pinocytic vacuoles is suggestive of possible transport by pinocytosis [77].

Furthermore, significant decrease of transporter P-gp in the endothelial cells is also reported [64, 78]. Reports on the level of P-gp in tumour vasculature and normal tissue are however conflicting. While no change was seen in MRP-2 in malignant glioma cells, increased expression of MRP-1 and MRP-3 was observed [79]. In summary, although the BBB is compromised in the presence of tumours and exhibits increased level of permeability, this increase is not adequate to permit therapeutic levels of anti-cancer drugs in the parenchyma. There exists an urgent need to have appropriate strategies to enable adequate transport of anti-cancer drugs to the brain.

12.5 Drug Delivery Strategies

Two major issues in drug delivery to the brain include poor transport of drugs across the BBB and efflux of drugs from the brain back to the blood. Chemotherapy approaches often fail due to insufficient drug concentration of anti-cancer agents in the brain parenchyma. Hence effective strategies to enhance drug uptake in the brain must address, on the one hand, traversing the BBB and, on the other hand, limiting efflux of drugs by efflux transporters. Drug delivery approaches would include strategies for local delivery or systemic delivery.

12.5.1 Local Delivery

Local delivery would include invasive approaches that would bulldoze through the BBB and hence necessitates surgical intervention. Drugs are delivered to the brain by first drilling a hole in the head. Three basic delivery methods, namely, intracerebroventricular (ICV) injection, intracerebral (IC) implantation and convection-enhanced diffusion (CED), have been investigated [33, 80]. All these methods enable direct delivery at the tumour site. ICV drug delivery to the brain results in high drug exposure at the ependymal surface of the brain, which can cause a subependymal astrogliotic reaction [81, 82]. Intra-arterial delivery of hypertonic solutions of mannitol or urea...
is reported to facilitate transient opening of tight junctions in the endothelial cells [83]. This temporary disruption of the BBB enables delivery of drugs, large molecules and nanocarrier systems [84–86]. This method however carries a high risk. In some cases, localised BBB disruption by ultrasound has been well tolerated and without any evident tissue damages [87–90].

IC implantations of anti-cancer drugs in polymeric matrices are established. Such implants are commercially available. Gliadel® (Guilford Pharmaceuticals, Hertfordshire, UK), a polyanhydride biodegradable polymer wafer containing BCNU (carmustine), is commercially available since 1996 [91]. Other implants containing paclitaxel, cisplatin or Gliadel in combination have been investigated clinically. Implanted ‘osmotic minipumps’ that allow local and continuous drug delivery for a significant period have been used successfully for the treatment of experimental tumours [92]. CED as an alternative to drug infusion is reported to enable improved drug distribution in the brain. CED involves infusing the anti-cancer drugs in and around the tumour under hydrostatic pressure through an implanted catheter [93]. CED has been evaluated for drug solutions [94] and liposomal formulations with positive results [95].

In summary, although local delivery to the brain can be achieved using anti-cancer drug solutions, anti-cancer drugs in nanocarriers and/or implants, local delivery is possible only through surgical intervention. Systemic delivery that is nonsurgical and could enable administration by intravenous (IV) or oral route would definitely provide quantum advantage to the clinician and the patient.

12.5.2 Systemic Delivery

Success in the systemic delivery of drugs to treat brain cancer would provide major advantages in the therapy of brain cancer. Systemic delivery in brain cancer would include delivery by two routes: IV and oral. Although delivery of drugs to the brain by the nasal route has been suggested, the cytotoxic nature of the anti-cancer molecules would preclude administration by this route, due to local toxicity to the nasal tissue.

Important requirements of drug candidates to be delivered by systemic routes include high lipophilicity coupled with low MW. Furthermore, the drug must not be a substrate for efflux transporters. The relevant properties of anti-cancer drugs reportedly used for brain cancer therapy or in investigational research for brain cancer therapy are tabulated in Table 12.1. The table reflects that while many drugs are lipophilic as indicated by their log P values, except for tamoxifen, all the drugs are of high MW (>500 Da). Moreover, the majority of the drugs are substrates for P-gp and other efflux transporters.
Table 12.1 Properties of drugs used in the treatment of brain cancer

<table>
<thead>
<tr>
<th>Drug (substrate)</th>
<th>Log P</th>
<th>MW (Da)</th>
<th>Transporter</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen</td>
<td>6.589</td>
<td>371.515</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>5.829</td>
<td>778.932</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Photofrin</td>
<td>5.127</td>
<td>1179.36</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>5.029</td>
<td>853.906</td>
<td>P-gp</td>
<td>[267]</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>4.918</td>
<td>824.958</td>
<td>P-gp, MRP-1,2,3</td>
<td>[268]</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>4.258</td>
<td>807.879</td>
<td>P-gp</td>
<td>[269]</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>3.46</td>
<td>446.902</td>
<td>BCRP</td>
<td>[10, 24]</td>
</tr>
<tr>
<td>Imatinib</td>
<td>3.218</td>
<td>493.603</td>
<td>BCRP</td>
<td>[270]</td>
</tr>
<tr>
<td>Teniposide</td>
<td>1.963</td>
<td>656.655</td>
<td>P-gp, MRP-1,2,3,6</td>
<td>[10]</td>
</tr>
<tr>
<td>Topotecan</td>
<td>1.21</td>
<td>421.446</td>
<td>BCRP</td>
<td>[271, 272]</td>
</tr>
<tr>
<td>Etoposide</td>
<td>1.197</td>
<td>588.557</td>
<td>P-gp, MRP-1,2,3,6</td>
<td>[273]</td>
</tr>
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<td>Idarubicin</td>
<td>0.83</td>
<td>497.494</td>
<td>P-gp</td>
<td>[10, 24]</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>0.766</td>
<td>527.52</td>
<td>P-gp, MRP-1,2,3,6</td>
<td>[271]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.52</td>
<td>543.52</td>
<td>P-gp, MRP-1,2,3,6</td>
<td>[271]</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.517</td>
<td>1255.42</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Epirubicin</td>
<td>0.445</td>
<td>543.519</td>
<td>P-gp, MRP-1,2,3</td>
<td>[2, 11]</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>0.335</td>
<td>152–177</td>
<td>MRP-4,5</td>
<td>[2, 11]</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>0.086</td>
<td>167.193</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Melphalan</td>
<td>-0.432</td>
<td>305.2</td>
<td>MRP-1,2,3</td>
<td>[2]</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>-0.924</td>
<td>444.481</td>
<td>P-gp, BCRP</td>
<td>[271]</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>-1.08</td>
<td>454.44</td>
<td>P-gp, MRP-3,4, BCRP</td>
<td>[2]</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>-2.19</td>
<td>300.05</td>
<td>MRP-2,6</td>
<td>[2]</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>–</td>
<td>393.436</td>
<td>BCRP</td>
<td>[2]</td>
</tr>
</tbody>
</table>

Oral delivery would be the preferred route; however, most anti-cancer drugs exhibit severe limitations in oral drug absorption. Oral drug delivery efforts are today limited to research findings in animal models. Even drugs like tamoxifen that are commercially available for oral administration pose bioavailability issues. Moreover, insufficient drug concentrations achieved even after IV delivery of anti-cancer drug solutions necessitate strategies in formulation design to overcome the BBB [96].
Formulation strategies to overcome the BBB must focus on increasing lipophilicity, rely on endogenous transporters in the BBB and explore the possibility of RME. Coadministration of drugs with inhibitors of efflux transporters is yet another strategy [97, 98]. These strategies may be evaluated singly or in combination to design an efficient drug delivery system for improved therapy in brain cancer [99].

Limitations of administration of drugs in solution even with inhibitors of efflux transporter and the promise shown by nanocarriers to overcome efflux transporters [100] and/or facilitate RME have triggered the exploration of nanocarriers for systemic delivery of anti-cancer drugs to the brain. A number of nanocarriers are under evaluation, with less than a handful in commercial use. The design of newer nanocarriers for improved therapeutic efficacy is therefore a need of the hour.

12.5.3 Nano Drug Delivery Approaches for Brain Cancer

Nano drug delivery carriers present a promising approach for the improved delivery of therapeutic agents to the brain [101–104]. These systems permit administration by IV or oral route. The important advantages of nano drug delivery systems include the possibility to modulate their size, composition, lipophilicity, shape and surface charge. Following IV administration, nanocarriers of >200 nm are primarily sequestered by the reticuloendothelial system (RES) [103]. Conferring stealth property to nanocarriers by coating them with hydrophilic polymers such as polyethylene glycol (PEG), polyethylene oxide (PEO) or poloxamers makes them highly hydrophilic and long circulating, as they can bypass the RES [105–107]. Decreasing particle size to <100 nm could also enable significant RES bypass and long circulation times. Long-circulating carriers could accumulate over a period of time in the brain due to the compromised BBB and leaky vasculature in the tumour by the enhanced permeability and retention effect [108–111]. Nevertheless, PEG-coated carriers are not easily transported across the BBB as a result of their low affinity for brain tissue [27, 112]. Recent evidence suggests that the physiological upper limit of pore size in the BBB of malignant glioma microvasculature is 12 nm [27]. It follows therefore that those long-circulating nanocarriers <12 nm may effectively cross the BBB of malignant glioma microvasculature.

Nanosystems explored for delivery to the brain include polymeric nanoparticles (NP), lipid-based systems such as liposomes, immunoliposomes, polymeric and solid lipid NP, polymeric micelles and dendrimers, peptide vectors and others.

12.5.3.1 Liposomes

Liposomes are phospholipid-based vesicular systems with an inner aqueous layer surrounded completely by a phospholipid membrane bilayer. Liposomes are nanosystems
that exhibit high biocompatibility, low toxicity, ease of preparation and commercial availability. Liposomes are therefore among the most extensively evaluated nanosystems for the delivery of anti-cancer drugs to the brain [113–117]. However, the efficacy of liposomes in targeting brain tumours has been limited. PEGylation of liposomes resulted in prolonged half-life due to long circulation times with enhanced accumulation in tumours [118–122]. Long-circulating stealth liposomes (Caelyx®) have shown 13–19 times higher concentration of DOX in glioblastomas and 7–13 times higher concentration in metastatic lesions in human patients [123]. This suggests that stealth liposomes can bypass the BBB in brain tumour. Mice receiving weekly intraperitoneal injection of DOX in IL-13-conjugated liposomes showed a fivefold reduction in the intracranial tumour volume [124]. Furthermore, there was no evidence of toxicity to the endothelial cells in animals receiving the DOX-containing liposomes.

Immunoliposomes may be designed by tagging or attaching liposome-containing drugs with an antibody that recognises receptors along the endothelium [102, 125–127]. Endogenous large-molecule peptides such as transferrin, insulin and leptin cross the BBB via receptor-mediated transport [127]. Monoclonal antibodies for these receptors, when attached to liposomes containing drugs, are recognised as ligands by these receptors to be endocytosed. Immunoliposomes for delivery of antisense ribonucleic acid (RNA) to the CNS are reported [128]. Significantly higher brain concentrations of daunorubicin in an in vivo animal model with the OX26 immunoliposome have been reported [129]. Monoclonal antibodies directed against insulin receptors have also been developed for delivery of daunorubicin [10]. Transferrin-conjugated liposomes were prepared by coupling the -NH₂ groups present on the surface of stearylamine-containing liposomes with the -COOH groups of transferrin. Following a single IV injection in rats, a 17-fold enhancement in the brain uptake of 5-fluorouracil from transferrin-coupled liposomes compared to a 10-fold increment in the absence of transferrin-coupled liposomes was observed, suggesting transferrin receptor-mediated endocytosis [130, 131].

12.5.3.2 Polymeric Nanoparticles

NP include both nanocapsules, a core-shell structure (a reservoir system), and nanospheres (a matrix system) of size ranging from 1 to 1000 nm. The drug can be entrapped or encapsulated within the polymeric matrix [132]. In addition, NP provide the advantage of high drug-loading capacity and protection against chemical and enzymatic degradation. The major drug evaluated for brain delivery in polymeric NP is DOX. The biodistribution of NP can be altered, for uptake by endothelial cells of the brain, by coating their surface with hydrophilic surfactants. PEGylated NP have provided significant advantage by enabling high drug concentration at the BBB for uptake [133–137]. More specifically, it has been shown that poly(butylcyanoacrylate) (PBCA) coated with Tween-80 exhibited significantly higher brain uptake than
uncoated NP of DOX [26, 99, 138–143] and MTX [140]. A twofold increase in paclitaxel concentration in the brain was seen following IV administration of polysorbate-coated cetyl alcohol NP (<100 nm) [144]. It has been suggested that apolipoproteins could be involved in the enhanced brain penetration of NP coated with Tween-80 [138]. PBCA NP of DOX evaluated in rats with glioblastoma showed 20% long-term survivors [143], while PEG-hexadecylcyano NP of DOX showed no improvement in efficacy in 9L glioma cell line-induced brain tumour model. The combined delivery of a vaccine and an antisense nucleotide to brain tumours using PBCA NP coated with polysorbate 80 is reported [145]. Furthermore, NP for endogenous transporter-mediated absorption have been developed [146]. NP with receptor-specific ligands can promote brain delivery through RME. Coadministration with P-gp inhibitors is an additional strategy exploited to improve delivery to the brain [19, 26, 97, 147, 148].

Magnetic NP, composed of a magnetic (e.g., iron oxide/magnetite) core and a biocompatible polymeric shell (e.g., dextran, starch), have also been proposed to target brain tumours [149–152].

12.5.3.3 Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

The inherent nature of lipidic molecules to cross the BBB can be exploited to design lipid-based nanocarriers [153]. This could bring in a synergy of the lipidic nature of the nanocarrier with the additional possibility of nanocarrier-based uptake mechanisms.

Solid lipid nanoparticles (SLN) consist of solid lipids stabilised with emulsifiers. They can be designed at sizes <100 nm. The commonly used emulsifiers include poloxamers, polysorbates and bile salts. The potential use of SLN specifically for delivery of anti-tumour drugs to the brain has been evaluated for camptothecin [154, 155], DOX [156, 157], paclitaxel [158, 159], vinblastine [11] and etoposide [11]. Stealth SLN have also been studied [156, 157, 160]. Tween-80-coated SLN showed improved potential in enhancing brain uptake [107, 161].

Nanostructured lipid carriers, containing both solid lipid and liquid lipid, are a new type of lipid NP that offer the advantage of improved drug-loading capacity and release properties [162]. Their application in the delivery of anti-cancer drugs is slated to be promising [163].

12.5.3.4 Polymeric Micelles

Amphiphilic block polymers self-assemble in aqueous polymeric solutions to form micelles above their critical micellar concentration [164]. The size of polymeric
micelles generally ranges from 10 to 100 nm. Amphiphilic polymers evaluated include poly(propylene glycol) (PPG), poly(\(dl\)-lactide), poly(caprolactone) and PEG. Of particular importance are the Pluronic block copolymers that contain two hydrophilic (PEG) and one hydrophobic (PPG) blocks (PEG-PPG-PEG), which are also known to inhibit P-gp. Increase in drug permeability was seen with a number of drugs including the anti-cancer drugs paclitaxel and vinblastine using bovine brain microvessel endothelial cell monolayer model [165, 166]. Polyethylene glycol-phosphatidylethanolamine (PEG-PE) micellar system of paclitaxel has been reported [167]. Surface-modified PEG-PE micellar systems showed improved cytotoxicity in the human glioblastoma cell line LN 18 [168].

### 12.5.3.5 Dendrimers

Dendrimers or star polymers have a core to which branches are attached. The surface properties of dendrimers can be tailored by modifying the terminal ends of the branches. Their special advantage is size, which can be <12 nm. Polyether-co-polyester (PEPE) dendrimers loaded with MTX and conjugated to \(d\)-glucosamine have been synthesised [169]. Glucose conjugation to the dendrimers enabled enhanced delivery across the BBB by carrier-mediated transport. Glucosylated dendrimers showed higher cell uptake than nonglucosylated dendrimers suggestive of endocytosis in glioma cell lines and in avascular human glioma tumour spheroids. Tumour-targeting specificity was also achieved through facilitative glucose metabolism by the GLUT in the tumours. The lower IC-50 (inhibitory concentration-50) of MTX in dendrimers compared to free MTX suggested that PEPE dendrimers had increased potency.

IV administered functionalised dendrimers less than approximately 11.7–11.9 nm in diameter were able to traverse pores of the blood-brain tumour barrier of RG-2 malignant gliomas, as the pore size reported in malignant glioma vasculature is 12 nm. Of the permeable functionalised dendrimer generations, those that exhibited long circulation times showed higher accumulation within the glioma cells [170].

### 12.5.3.6 Peptide Vectors

Linking compounds with peptide vectors that enable passage across the BBB is another potential strategy to enhance transport across the BBB [171]. Paclitaxel coupled to peptide vectors exhibited increased solubility and enhanced brain uptake [71]. Peptide vector-conjugated DOX showed increased penetration across biological membranes [72].

Bionanocapsules (BNC) are composed of the surface antigen of hepatitis B virus and various components, such as chemical compounds, protein, genes and small interference RNA. To target brain tumours selectively, BNC were conjugated with anti-human
epidermal growth factor receptor (EGFR) antibody that recognises EGFR\textsubscript{vIII} known to overexpress in a variety of human malignancies of epithelial origin, particularly in gliomas. The BNC were both efficiently and selectively delivered to glioma cells in Gli36 glioma cell lines (expressing EGFR\textsubscript{vIII} but not wild-type EGFR) and Gli35 tumour-bearing rats, indicating another promising brain tumour-targeting drug delivery system [172]. Trans-activating transcription-conjugated NP have also shown enhanced delivery to the brain [70].

In summary, a common feature of all the nanocarriers discussed above is their recognition by the mononuclear phagocyte system (MPS) of the RES. Nanocarriers that can successfully evade MPS recognition would provide a major advantage of increased concentration of the carrier at the BBB. Particulate carriers in particular are more easily recognised by the MPS than carriers of liquid droplets. Designing lipophilic liquid nanocarriers in an attempt to bypass the RES, coupled with lipophilicity of carrier, presents an attractive approach for improved delivery to the brain. ME and NE, emulsion-based lipid nanocarrier system, represent such carriers.

This part of the chapter discusses advances in ME and NE as drug delivery vehicles, with a focus on strategies to design these systems for improved delivery in brain cancer.

### 12.6 Microemulsions and Nanoemulsions

ME and NE are lipid-based nanocarrier systems wherein small oil droplets constitute the dispersed phase and could be as small as 20 nm. More recently, the potential of ME and NE for brain delivery has been reviewed [173, 174]. Moreover, their ability to deliver improved concentration of drugs to the brain has been attributed to the choice of their components [174, 175].

ME are clear, thermodynamically stable, isotropic mixtures of oil, water and surfactant, frequently used in combination with cosurfactants [176, 177]. They are essentially stable, single-phase swollen micellar solutions that offer the advantage of spontaneous formation and ease of manufacture. NE are kinetically stable mixtures of oil, water and surfactant. Cosurfactants are not essential in NE. NE do not form spontaneously. The essential similarity between ME and NE is the size of the oil droplets, which normally ranges between 20 and 200 nm. NE are also referred to as miniemulsions, ultrafine emulsions and submicron emulsions [178]. A schematic representation of ME and NE is shown in Figure 12.2.

ME exhibit thermodynamic stability, which ensures a long shelf life. Although NE are thermodynamically unstable, due to their small droplet size they possess good stability.
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against sedimentation or creaming. Ostwald ripening is the main mechanism of NE breakdown, which can be prevented [178, 179]. Both ME and NE exist as water-in-oil (w/o) and oil-in-water (o/w) forms; however, ME exhibit one more structure - the bicontinuous phase (Figure 12.3).

ME are thermodynamically stable systems that form spontaneously on mixing and are dependent on the composition. The properties of NE, however, depend on the composition and the method of preparation. ME are potentially excellent carriers for bioactive molecules and have attracted much interest in drug delivery [180–183].

Figure 12.2 Schematic representation of ME and NE

Figure 12.3 Schematic representation of ME microstructures: (a) oil-in-water ME, (b) bicontinuous ME and (c) water-in-oil ME. Reproduced with permission from M.J. Lawrence and G.D. Rees, Advanced Drug Delivery Reviews, 2000, 45, 89. ©2000, Elsevier Publishers Ltd [181]
Both o/w and w/o ME and NE have the potential to enhance the oral and parenteral bioavailability of drugs, including various peptides. However, for therapy in brain cancer, by oral and parenteral routes, o/w ME and NE are preferred.

Self-microemulsifying drug delivery systems (SMEDDS) are ideally isotropic mixtures of oils and surfactants (sometimes cosurfactants are also added) that on dilution with an aqueous phase emulsify under conditions of gentle agitation to form ME [180, 181]. These could be considered as pro-ME and find advantage in the design of microemulsion systems’. Selection of excipients for SMEDDS would essentially follow the same principles as that for ME.

12.6.1 Preparation of Microemulsions and Nanoemulsions

12.6.1.1 Microemulsions

A unique feature of ME is their spontaneous formation. ME are therefore prepared by simple mixing of the oil, surfactant, cosurfactant and aqueous phase. The drug is normally incorporated by dissolving in either the oil or the formed ME. Selection of the components is normally carried out in two stages.

Equilibrium solubility: The first stage includes assessing the solubility of the drug in a range of possible ME components. Based on the data obtained, the general criterion is to select the oil, surfactant and cosurfactant in which the drug shows maximum solubility. However, if the intent is to retain the drug preferably in the oil phase, a judicious selection of other components is important.

Phase studies: The second stage is the construction of pseudoternary phase diagrams. Pseudoternary phase diagrams plot three variables each represented on one side of the triangle [184]. The ratio of the surfactant to cosurfactant is generally kept constant so that all the four variables can be represented. The pseudoternary phase diagrams of oil, surfactant, cosurfactant and water may be constructed using the oil titration or water titration method.

For o/w ME, the oil titration method is the more appropriate method. Mixtures of different concentrations of oil, surfactant and cosurfactant prepared by simple mixing techniques are titrated with oil to quantify the amount of oil microemulsified, as evidenced by transparency in the system. Phase studies enable identification of the concentration range of the oil, surfactant and cosurfactant for the formation of the ME existence region [181]. A schematic three-phase pseudoternary diagram depicting the ME region is illustrated in Figure 12.4. Each corner of the triangle represents 100% of the component. The ME area in the phase diagram is directly related to the extent of microemulsification of the oil. Selection of the components is often an act
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of balance between the components exhibiting maximum ME existence region and maximum solubilisation of the drug.

12.6.1.2 Nanoemulsions

NE are thermodynamically unstable systems. Methods to form NE therefore are based on input of energy. Both high-energy and low-energy methods may be used [185].

High-energy methods: The equipment used for high-energy emulsification include the microfluidiser and the high-pressure homogeniser. Ultrasound methods, although suggested, are not popular [186].

Microfluidiser: Microfluidisers comprise an interaction chamber, consisting of a series of microchannels, through which the emulsion is forced under pressure, using a positive displacement pump operating at very high pressures (up to 20,000 psi). The emulsion flows through the microchannels on to an impingement area, resulting in very fine emulsion droplets [187, 188]. The particle size of the fine emulsion

Figure 12.4 Schematic three-phase pseudoternary diagram depicting the ME region
is inversely related to the operating pressure and the number of passes of the coarse emulsion through the interaction chamber. The resulting NE can then be filtered through a 0.2 μm filter under nitrogen to remove any large particles present, resulting in a uniform NE. Chlorambucil NE of size around 150 nm have been prepared by microfluidisation [189].

High-pressure homogenisers: In high-pressure homogenisers, coarse dispersions of oil, surfactant and aqueous phase are passed through a small orifice at high pressure (500–5000 psi) wherein the emulsion is subjected to high shear and turbulence. This results in the generation of emulsions with very small droplet size [185].

Low-energy methods: The most commonly investigated low-energy method is the phase inversion temperature (PIT) method. Although solvent diffusion has been suggested as a low-energy method, it does not find application in the design of NE for drug delivery. The method however is useful when NE are used as templates to prepare NP.

PIT method: The PIT concept was first introduced by Shinoda [190] and Shinoda and Saito [191]. The affinity of nonionic surfactants, mainly polyethoxylated surfactants, for water and oil changes as a function of the temperature, and exhibits phase inversion [192]. The temperature at which such phase inversion occurs is called the PIT or hydrophilic-lipophilic balance (HLB) temperature. At PIT nonionic surfactants exhibit a similar affinity for the two immiscible phases, exhibiting very low interfacial tension [177], of the order of 10^{-2}–10^{-5} (mN/m), to form NE (bicontinuous) or other nanoscaled systems. At the PIT, rapid cooling or sudden dilution with water or oil results in immediate generation of NE with small size and narrow size distribution. To prevent coalescence and enable formation of kinetically stable NE, the cooling rate or dilution must be rapid. The PIT process is relatively simple and facilitates easy industrial scale-up.

12.6.2 Components of Microemulsions and Nanoemulsions

The primary components of ME and NE are the surfactant, oil phase and the aqueous phase [184, 193].Cosurfactants are often considered as essential components of ME. ME and NE may optionally include standard formulation excipients like preservatives, stabilisers, buffers, antioxidants, polymers and other excipients, which would be dictated primarily by the drug or the chemical nature of the oil phase. A detailed discussion of the optional components is out of the scope of this chapter. For components for different routes of administration, readers are directed to the chapter by Patravle and Date [194]. The primary components of ME and NE include the following:

Oil phase: Two important aspects need to be considered during selection of the oily phase of ME. The oil must be good solvent for the drug to enable high drug loading in
ME and the oil must facilitate ready microemulsification to enable maximum loading of oil in ME. Phase studies enable selection of oils based on the region of ME. The ME existence region is influenced by the chain length and the molecular volume of the oil. Vegetable oils containing triglycerides of long-chain fatty acids yield small ME existence region and are difficult to microemulsify [195, 196]. While ME existence region increases with the decrease in the hydrophobicity of the oil phase, solubilisation of hydrophobic drugs increases with the increase in the hydrophobicity of oil. As a general rule, solubilising capacity of oils would decrease in the order vegetable oils > medium-chain triglycerides > medium-chain mono- and diglycerides [197]. The choice of the oil phase is finally a compromise between its ability to solubilise the drug and its microemulsification property. Mixtures of oils may satisfy both requirements [198]. Certain oils like vitamin E (d-α-tocopherol) show high solubilising potential for very poorly soluble drugs like itraconazole, saquinavir and paclitaxel, which are not readily solubilised [199].

The selection of oil for NE, however, is primarily dictated by its solubilisation capacity for the drug. Other features could include viscosity of the oil as an important property that would affect the choice of the method of preparation, necessitating high-energy methods. The same oils may be used in ME and NE, and include vegetable oils, long- and medium-chain triglycerides and medium-chain mono- and diglycerides. There are detailed reports on oil phases normally included in ME [193, 200, 201].

Surfactants: Surfactants are an integral component of all emulsion systems. ME and NE, wherein the globule size is in the nanometre range, rely heavily on the surfactant for their stability. The stability of ME and NE is dictated by the choice of surfactant. Furthermore, the spontaneous formation of ME is facilitated only at high surfactant concentrations. O/w ME and NE would in general necessitate high-HLB surfactants. Combinations of surfactants may also be used. In the case of NE however, those prepared by the low-energy PIT method would necessitate an appropriate combination of low- and high-HLB surfactants to enable spontaneous formation by phase inversion.

The surfactants for ME should exhibit good microemulsification property to enable high oil incorporation [181]. High solubility of the drug in the surfactant can enable high drug loading in ME and NE. Nevertheless, this needs to be looked at with caution for labile drugs wherein it may be important to preferentially entrap the drug in the oil phase for stability. Furthermore, where it is expected that the oil droplet would be preferentially absorbed due to its nanosize, maintaining high drug concentration in the oil phase would be prudent.

Surfactants may be used singly or in combination to obtain the desired HLB to ensure adequate stability. The preferred surfactants for human applications include
lecithins, phospholipid derivatives, sorbitan monoesters, polysorbates, poloxamers, polyethoxylated castor oil derivatives and Solutol® HS 15 (PEG-660-12-hydroxystearate) [201].

A major issue with surfactants is their potential toxicity. Natural lecithin and phospholipid derivatives therefore represent the surfactants of choice based on biocompatibility [184]. Nevertheless, lecithins used alone exhibit the tendency to form lamellar liquid crystalline phases, which need to be inhibited. Inclusion of auxiliary surfactants like polysorbates, which can inhibit this formation, therefore becomes unavoidable [202].

In general, formulations with lower surfactant concentration are therefore preferred. An important advantage of NE over ME in this context is their ability to form at lower surfactant concentrations. Nevertheless, this needs to be weighed against the high thermodynamic stability and the ease of preparation and large-scale manufacture of ME.

Cosurfactants: Cosurfactants in ME play an important role. In the case of ME, surfactants that tend to form liquid crystalline phases, resulting in rigid surfactant films, cannot lower the oil-water interfacial tension sufficiently to yield ME. Cosurfactants when added penetrate the surfactant layer, disrupting the interfacial film to provide fluidity, lower the interfacial tension and thereby enable an increase in the ME region. Cosurfactants may also enable solubilisation of the oil. A small area of ME existence is reported in the absence of cosurfactant, in phospholipid ME [203]. Furthermore, an increase in ME area was observed with the increase in the chain length of alcohol, while an increase in hydroxyl groups in the cosurfactant decreased the ME region. Cosurfactants that can be used in ME include short-chain alcohols such as ethanol and benzyl alcohol, propylene glycol, glycerol, PEG 400 and glycofurol (tetrahydrofurfuryl alcohol PEG ether or tetruglycol). Further details on cosurfactants are available in the literature [181, 197, 204, 205]. NE generally does not require a cosurfactant for formation and stability; however, cosurfactants may be used as solubilisers or viscosity modifiers in ME.

12.7 Microemulsions and Nanoemulsions for Cancer Therapy

Interest in ME and NE as delivery systems for cancer therapy is relatively recent. Paclitaxel is one of the most extensively evaluated drugs in ME and NE. Cremophor-free ME of paclitaxel have been developed to overcome vehicle-induced toxicity [206]. Controlled-release ME of paclitaxel containing poly(lactic-co-glycolic acid) (PLGA) showed significantly slower release compared to an SMEDDS of paclitaxel without PLGA [207]. Moreover, improved activity was observed in an SKOV-3 human
ovarian cancer cell-bearing nude mice model [173]. ME of MTX suppressed tumour cell growth on multiple tumour lines. A cholesterol-rich NE of paclitaxel was seen to concentrate in cancer tissues after IV injection [208]. Similar results were observed with etoposide [209]. NE preparations of the anti-cancer drug dacarbazine significantly increased its efficacy in a xenograft mouse melanoma model [210]. Daunorubicin lipidic NE showed decreased toxicity [211]. Gadolinium lipid NE has been studied for IV delivery to tumours in neutron-capture therapy [212]. An NE of antioxidant synergy formulation reduced tumour growth rate in neuroblastoma-bearing nude mice [213]. High uptake of a lipidic NE by breast carcinoma cells was observed after intrallesional injection in patients [214]. Acoustically active perfluorocarbon NE as drug delivery carriers for camptothecin showed enhanced cytotoxicity against cancer cells [215]. Self-nanoemulsifying drug delivery systems of tamoxifen showed superior release, suggesting improved oral efficacy [216], while an NE of tamoxifen inhibited cell proliferation (20-fold) and increased cell apoptosis (4-fold) in the HTB-20 breast cancer cell line [217]. NE containing encapsulated antigens has been shown to be a useful vehicle for enhancing anti-tumour immune response and was found suitable for different administration routes [218].

12.8 Functional Microemulsions and Nanoemulsions to Cross the Blood-brain Barrier

Functional ME and NE essentially comprise oils and surfactants (and cosurfactants in the case of ME), which in addition to their role as vehicle or carrier could modulate brain uptake through one or more of the strategies illustrated earlier in Section 12.5. In such ME and NE, one or more of the primary components would be functional and hence contribute to enhanced transport across the BBB. Functional ME and NE would therefore comprise functional oils, functional surfactants and functional cosurfactants either singly or in combination.

12.8.1 Functional Oils

Although ME are reported with a number of oils including vegetable oils, long- and medium-chain triglycerides and medium-chain mono- and diglycerides, recent reports suggest the role of polyunsaturated fatty acids (PUFA) in ME as playing an important role in enhancing brain delivery [174, 175]. Oils could also function as P-gp inhibitors [219, 220], absorption enhancers [221], and so on.

**PUFA oils**: PUFA include \( \alpha \)-linolenic acid (ALA), \( \gamma \)-linolenic acid, linoleic acid (LA), ARA, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). ALA acid is the parent omega (\( \omega \))-3 essential fatty acid, while LA is the parent \( \omega \)-6 essential fatty acid.
Biologically, ALA, at low conversion rate, can be converted to EPA and DHA. DHA, an \( \omega-3 \) fatty acid, is an important constituent of human brain and retina [222–224].

Sources of PUFA oils: PUFA are normally present in nut and plant oils and in marine oils. LA (\( \omega-6 \)) is essentially present in corn oil, soybean oil and sunflower oil. However, the more important PUFA are the \( \omega-3 \) acids. ALA (\( \omega-3 \)) is mainly found in soybean oil, canola oil, walnut oil, olive oil and flaxseed oil [225]. Marine oils, particularly from fish such as herring, menhaden, pilchard, sardine, anchovy, tuna, salmon and mackerel, as well as seal oil, krill and marine algae are rich sources of \( \omega-3 \) PUFA, especially EPA and DHA [226, 227]. A list of oils and the PUFA present in them is included in Table 12.2 for reference.

**Table 12.2 Omega-3 (\( \omega-3 \)) fatty acids from plant and marine sources**

<table>
<thead>
<tr>
<th>Plant (seed oils)</th>
<th>% ALA</th>
<th>Marine (fish) [274]</th>
<th>( \omega-3 ) Fatty acids (%) (DHA/EPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chia</td>
<td>64</td>
<td>Pollock</td>
<td>1.1–1.9</td>
</tr>
<tr>
<td>Kiwifruit (chinese gooseberry)</td>
<td>62</td>
<td>Tuna</td>
<td>0.21–1.1</td>
</tr>
<tr>
<td>Perilla</td>
<td>58</td>
<td>Halibut</td>
<td>0.60–1.12</td>
</tr>
<tr>
<td>Flax (linseed)</td>
<td>55</td>
<td>Swordfish</td>
<td>0.97</td>
</tr>
<tr>
<td>Lingonberry</td>
<td>49</td>
<td>Tilefish</td>
<td>0.90</td>
</tr>
<tr>
<td>Camelina</td>
<td>36</td>
<td>Flounder</td>
<td>0.48</td>
</tr>
<tr>
<td>Purslane</td>
<td>35</td>
<td>Salmon</td>
<td>0.45</td>
</tr>
<tr>
<td>Black raspberry</td>
<td>33</td>
<td>King mackerel</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catfish</td>
<td>0.22–0.3</td>
</tr>
</tbody>
</table>

PUFA and cancer: Long-chain PUFA such as EPA and DHA, present in fish and in oils derived thereof, inhibit carcinogenesis and show potential anti-tumour activity [228–230]. *In vitro* studies with human tumour cell lines have shown convincingly that the \( \omega-3 \) PUFA, principally DHA, reduce the growth of tumour cells in various types of cancer, including breast, bowel and pancreas, and in chronic myeloid leukaemia and melanoma [231]. Ingestion of DHA and EPA reduces the growth of tumours in rodents, including tumours of the mammary gland, colon, prostate, liver and pancreas. \( \omega-3 \) fatty acids affect growth, differentiation, cellular apoptosis, angiogenesis and metastasis [232, 233]. PUFA are also beneficial in a number of other diseases [234]. Additional advantages of PUFA include the finding that DHA-enriched cells of tumours are more susceptible to oxidative damage [231]. Moreover, \( \omega-3 \) PUFA can exhibit synergistic effect with several anti-neoplastic agents and radiotherapy [235].
PUFA in microemulsions and nanoemulsions: PUFA are very labile and prone to oxidation. Furthermore, ω-3 fish oils exhibit high sensitivity to heat, light and oxygen. Formulation of such oils as the internal phase in emulsions is one approach to enable protection and enhance their stability. Other stabilisers may be optionally included. PUFA exhibit binding to serum albumin, which suppresses their tumour cytotoxicity. Emulsion formulations could help limit this binding, thereby maximising the benefit of PUFA [236, 237].

PUFA and brain absorption: Following oral administration, PUFA are incorporated into the chylomicrons in the form of triglycerides. This can facilitate transport of the PUFA through the lymphatic system to the brain. The high concentration of DHA in the brain capillary endothelium suggests that DHA is taken up from the diet via blood plasma, by DHA transporters including specific fatty acid-binding lipoprotein carriers [224]. DHA presents the possibility of RME to overcome BBB [238]. ME and NE containing oils rich in DHA could provide the added advantage of enhanced drug delivery to brain. PUFA therefore are an important functional excipient in the ME and NE for brain delivery. The synergistic anti-cancer effects of PUFA can be exploited through design of ME and NE of anti-cancer drugs for enhanced anti-cancer activity [189, 239, 240].

Oils as P-gp inhibitors: Inhibiting the efflux transporter P-gp is an important strategy to enhance brain delivery. The oils Peceol, miglyol, 1-monoolein and 1-monostearin are reported to be P-gp inhibitors [219, 220]. Most anti-cancer drugs are P-gp substrates (Table 12.1). ME and NE of these drugs formulated using such oils could provide improved brain uptake.

Oils as absorption enhancers: PUFA-containing oils have exhibited significant enhancement in brain uptake [174, 175]. A number of low-MW fatty acids, namely, capric acid, caprylic acid and the unsaturated oleic acid, are reported as absorption enhancers. While the safety of capric and caprylic acid could be an issue, oleic acid constituting the oil phase as absorption enhancer may play an important role as a functional excipient [221].

Oils and long-circulation property: The inherently small globule size normally confers stealth property to the ME and NE. Medium-chain glycerides however are extensively metabolised and rapidly eliminated from circulation, limiting their residence time. Long-chain triglycerides on the other hand are metabolised slowly, and hence as functional excipients they provide the advantage of long circulation [241, 242].

12.8.2 Functional Surfactants and Cosurfactants

The primary role of surfactants and cosurfactants in ME and NE is to decrease interfacial tension between the oil and aqueous phase to enable formation of small oil
droplets. In addition, surfactants can play additional and multiple roles as functional excipients.

Stealth agents: ME and NE normally exhibit inherent stealth property due to the small globule size. However, hydrophilic cosurfactants and surfactants like PEG, Tween-80 and poloxamer as stealth agents can enable higher drug concentration at the BBB. The most common stealth agent is PEG [243]. Nevertheless, PEG is to be used with caution for delivery to the brain. While PEGylated liposomal DOX has shown promising results in clinical studies, it has been suggested by others that PEG has low affinity for brain tissue [27, 112]. PEG-modified distearoylphosphatidyl ethanolamine (PEG-DSPE) may be used as a functional surfactant in ME and NE for stealth effect.

Permeation enhancers: Surfactants are reported to enhance permeation by altering biomembrane fluidity [244, 245]. Surfactants can dissolve lipids and proteins, extract lipids and proteins or simply disrupt the lipid membranes to alter fluidity. Surfactants therefore may enhance paracellular permeability by transient opening of tight junctions or increase transcellular permeability. Increased absorption of hydrophobic paclitaxel in the presence of \( \alpha \)-\( \alpha \)-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS) has been reported [246]. The major surfactants evaluated for brain delivery are Tween-80 and poloxamer. The mechanism of enhanced transport with these surfactants is attributed not only to their surface-active properties but also to their ability to mediate RME, and is discussed later.

P-gp inhibitors: A number of surfactants have shown promise as P-gp inhibitors [247–253]. The P-gp inhibitory effect is seen to be related to the concentration of surfactant. In many cases, the effect is seen to increase up to the critical micelle concentration followed by a decrease [97]. It is therefore probable that at concentrations required to form ME, surfactants may not function as P-gp inhibitors.

Receptor-mediated endocytosis: Two surfactants, Tween-80 and poloxamer 188, have been extensively reported to enhance transport across the BBB by facilitating RME by the low-density lipoprotein (LDL) receptors, known to be present in the BBB. Tween-80- and poloxamer-coated nanocarriers are reported to favour opsonisation by apolipoprotein E, thereby enabling adsorption of apolipoprotein E on the surface of nanocarriers [11, 26, 99, 103, 138, 139]. The apolipoprotein E-enriched NP are recognised as LDL and internalised by the LDL uptake system at the brain endothelial cells.

The general list of surfactants and cosurfactants is available in a number of reviews [193, 194, 200, 201]. Functional surfactants and cosurfactants with specific advantage in ME and NE for brain delivery are listed in Table 12.3.
Multifunctional Microemulsions and Nanoemulsions

Functional ME and NE, which play a dual role of vehicle cum functional agent, may be designed using the standard components, namely, oil, surfactant and cosurfactant (in ME). Multifunctional ME and NE are functional ME and NE engineered by inclusion of additional components to confer on them features for improved brain delivery of anti-cancer drugs. Typically, the additional components could include.

Stealth agents: Hydrophilic polymers, which are not primary components of ME and NE, could impart stealth properties. Stealth agents like poloxamers and PEO or other hydrophilic polymers like dextran and pullulan may be included in the aqueous phase.

Efflux transporter inhibitors: P-gp inhibition as a viable strategy to enhance drug delivery in cancer is reviewed recently [10, 24, 97, 98, 254–257]. Efflux inhibitors
could be included in ME and NE to enhance brain uptake. Moreover, they could exhibit potential synergy to enhance brain uptake when used in combination with functional excipients of ME and NE. A number of anti-cancer drugs are substrates for other efflux transporters (Table 12.1). Inhibitors of P-gp and other efflux transporters are compiled in Table 12.4.

<table>
<thead>
<tr>
<th>Transporters</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>Verapamil, cyclosporin A, quinidine, valsplodar, elacridar, biricodar, zosuquidar, tariquidar, dofequidar fumarate (MS209), OC144093, R101933</td>
</tr>
<tr>
<td>MRP-1</td>
<td>Probenecid, sulfinpyrazone, MK-571, cyclosporin A, verapamil, valsplodar</td>
</tr>
<tr>
<td>MRP-2</td>
<td>Probenecid, MK-571, leukotriene C4</td>
</tr>
<tr>
<td>MRP-3</td>
<td>Sulfinpyrazone, indomethacin, probenecid</td>
</tr>
<tr>
<td>MRP-4</td>
<td>Probenecid</td>
</tr>
<tr>
<td>MRP-5</td>
<td>Probenecid, sildenafil</td>
</tr>
<tr>
<td>MRP-6</td>
<td>Probenecid, indomethacin</td>
</tr>
<tr>
<td>BCRP</td>
<td>Elacridar, fumitremorgin C</td>
</tr>
</tbody>
</table>

Ligands for RME: Appropriate ligands for receptors on brain cancer cells may be included in ME and NE. These could be in addition to possibly Tween-80, which is known to facilitate RME. Other ligands, such as transferrin, insulin, leptin and folate, with specificity for the other receptors in the BBB may be incorporated in the systems. The monoclonal antibody OX26 shows affinity for the transferrin receptor [258] and could enable RME on inclusion in the ME and NE. Transferrin and folic acid are other ligands that can be investigated.

Cholesterol-mediated RME: Cholesterol-rich ME and NE have been proposed for targeting to the brain. Cholesterol binds to LDL receptors enabling uptake by RME. Such ME and NE have been evaluated with paclitaxel and etoposide [259, 260].

Polymers in ME and NE: Polymers dissolved in the oil phase of ME and NE could provide controlled drug release. Long-circulating functional ME and NE with controlled-release features could provide added advantage of prolonged effect, following brain uptake. Controlled-release ME of paclitaxel have shown improved efficacy [207]. A schematic representation of multifunctional ME and NE is shown in Figure 12.5.
ME and NE are characterised by performing various physical and chemical tests usually related to liquid oral dosage forms. These tests include assay and drug content, physicochemical tests and stability tests [261–265]. Table 12.5 is a ready reference of the tests and analytical methods useful in the evaluation of ME/NE.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Evaluation parameter</th>
<th>Analytical method/instrument used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Drug content and stability</td>
<td>High-performance liquid chromatography, liquid chromatography-mass spectrometry, gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>2</td>
<td>Globule size analysis</td>
<td>Photon correlation spectroscopy, static light scattering, dynamic light scattering, small-angle neutron scattering</td>
</tr>
<tr>
<td>3</td>
<td>Dilution potential</td>
<td>Visual evaluation following dilution at various ratios</td>
</tr>
<tr>
<td>4</td>
<td>Morphology/structural features of ME</td>
<td>Self-diffusion nuclear magnetic resonance, small-angle X-ray scattering, transmission electron microscopy, scanning electron microscopy</td>
</tr>
</tbody>
</table>

Figure 12.5 Schematic representation of multifunctional ME and NE

12.10 Characterisation of Microemulsion and Nanoemulsion

ME and NE are characterised by performing various physical and chemical tests usually related to liquid oral dosage forms. These tests include assay and drug content, physicochemical tests and stability tests [261–265]. Table 12.5 is a ready reference of the tests and analytical methods useful in the evaluation of ME/NE.
### 12.11 Evaluation *In Vitro* and *In Vivo*

Evaluation of anti-cancer formulations for anti-cancer activity is generally carried out in cell lines, or in small animal tumour models induced using cell lines. A list of cell lines commonly selected for evaluation of efficacy in brain cancer is provided in **Table 12.6**. Additional details on brain cancer cell lines are provided by Al-osman [266].

*In vivo* evaluation would include monitoring tumour regression volume in cancer models, and in normal animals, the ability of the drug or nanocarrier to cross the

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**Table 12.6 Brain cancer cell lines for *in vitro/in vivo* evaluation**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Cell line</th>
<th>Origin</th>
<th>Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>U343 MG</td>
<td>Human astrocytoma</td>
<td>U-373 MG</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>U87</td>
<td>Human glioblastoma</td>
<td>U118</td>
</tr>
<tr>
<td>Glioma</td>
<td>8-MG-BA</td>
<td>Human GBM</td>
<td>T98G</td>
</tr>
<tr>
<td>Glioma</td>
<td>42-MG-BA</td>
<td>Human glioma</td>
<td>U183</td>
</tr>
<tr>
<td>Glioma</td>
<td>U343</td>
<td>Human neuroblastoma</td>
<td>SK-N-MC</td>
</tr>
<tr>
<td>Glioma</td>
<td>C6</td>
<td>Medulloblastoma</td>
<td>D283 MED</td>
</tr>
<tr>
<td>Glioma</td>
<td>F 98</td>
<td>Medulloblastoma</td>
<td>D341 MED</td>
</tr>
<tr>
<td>Glioma</td>
<td>9 L</td>
<td>Medulloblastoma</td>
<td>Daoy</td>
</tr>
<tr>
<td>Glioma</td>
<td>RG-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Table 12.5 Continued**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Evaluation parameter</th>
<th>Analytical method/instrument used</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Partitioning behaviour of drug</td>
<td>Electrokinetic chromatography, gel permeation chromatography</td>
</tr>
<tr>
<td>6</td>
<td>Drug-release studies</td>
<td>Franz diffusion cell</td>
</tr>
<tr>
<td>7</td>
<td>Isotropicity</td>
<td>Cross-polarisers, optical birefringence</td>
</tr>
<tr>
<td>8</td>
<td>Interaction of microemulsion components</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>9</td>
<td>Type of microemulsion</td>
<td>Conductivity</td>
</tr>
<tr>
<td>10</td>
<td>Viscosity</td>
<td>Brookefield viscometer</td>
</tr>
<tr>
<td>11</td>
<td>Centrifugation</td>
<td>Moderate/high-speed centrifuges</td>
</tr>
<tr>
<td>12</td>
<td>Refractive index</td>
<td>Abbe’s refractometer</td>
</tr>
<tr>
<td>13</td>
<td>Freeze-thaw cycling</td>
<td>Freezer and incubator</td>
</tr>
</tbody>
</table>
BBB, which is determined by noninvasive scintigraphic or fluorescent techniques, or by actual analysis of the drug in the brain/tumour following sacrifice of the animal. A detailed discussion is beyond the scope of this chapter.

12.12 Advantages of Microemulsions and Nanoemulsions as Drug Delivery Systems

Among the nano drug delivery carriers being aggressively explored for treatment of brain cancer, ME and NE are among the more recent ones. Specific features of these systems present them as attractive drug delivery systems with good potential for delivery to the brain.

Biological advantage of ME and NE: The small size of the oil droplets in ME and NE, generally <100 nm, imparts inherent stealth features. The design of functional and multifunctional ME and NE could, through synergistic effects of different components, enable enhanced transport of drug across the BBB.

Technological advantage: ME and NE are simple systems and readily amenable to scale-up. ME and NE of size <100 nm are relatively simple to obtain. Particulate nanocarriers of such low size are difficult to obtain and their isolation and stabilisation pose an even greater risk. Moreover, ME can be scaled up by simple agitation methods and is a highly stable system. Many anti-cancer drugs are highly hydrophobic and these are readily incorporated in ME and NE. In general, ME and NE are versatile nanocarriers and can permit loading of both hydrophobic and hydrophilic drugs. A combination of anti-cancer drugs may also be readily incorporated in ME and NE. Furthermore, ME and NE can be tailored to incorporate functional and multifunctional features to maximise brain uptake. Sterilisation by filtration is readily possible for ME and NE, a major advantage for parenteral ME and NE. By and large, in comparison with other nanocarriers, ME and NE show a significant advantage in translation from bench to clinic.

12.13 Conclusions

The versatility of ME and NE to be made functional and multifunctional opens up newer dimensions in the design of such carriers for improved therapy in brain cancer. Research in newer functional excipients, including safer surfactants, synergised with advances in understanding the biology of brain cancer and newer insights into the BBB could one day make noninvasive delivery methods to treat brain cancer, with ME and NE, a reality.
Acknowledgements

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References


Padma V. Devarajan and Rajshree L. Shinde


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274. Fish, Levels of Mercury and Omega-3 Fatty Acids [http://www.americanheart.org/](http://www.americanheart.org/)
13.1 Introduction

Curcumin is a hydrophobic polyphenol derived from turmeric, the rhizome of the herb *Curcuma longa* L. (Figure 13.1). Commercial curcumin is a mixture of curcuminoids, containing approximately 77% diferuloylmethane, 18% demethoxycurcumin and 5% bisdemethoxycurcumin. Curcumin is a highly pleiotropic molecule that modulates numerous targets. It binds to as many as 33 different proteins including thioredoxin reductase, cyclooxygenase-2 (COX-2), protein kinase C (PKC), 5-lipoxygenase and tubulin. The various molecular targets modulated by this agent include transcription factors, growth factors and their receptors, cytokines, enzymes and genes regulating cell proliferation and apoptosis. It has been shown to have antioxidant, anti-inflammatory and anti-mutagenic properties [1–3]. This multitargeting ability of curcumin may be the key to its therapeutic anti-cancer potential. Curcumin has been shown to protect against different cancers, including leukaemia and lymphoma, gastrointestinal cancers, genitourinary cancers, breast cancer, ovarian cancer, head and neck squamous cell carcinoma, lung cancer, melanoma, neurological cancers and sarcoma, in *in vitro* studies including cancerous cell lines.

![Curcumin molecule](image)

**Figure 13.1** Curcumin (bis-α,β-unsaturated β-diketone), a hydrophobic polyphenol derived from turmeric, which is the rhizome of the herb *Curcuma longa* L.
However, there is little or no evidence of its \textit{in vivo} anti-cancer potential for organs like brain. However, there are a few reports on \textit{in vitro} studies demonstrating curcumin-evoked apoptosis in brain tumour cells [4, 5]. A major hurdle for curcumin utility and testing as a therapeutic agent is its poor bioavailability [6]. The main reasons (Figure 13.2) for its reduced bioavailability include low intrinsic activity, poor absorption, high rate of metabolism, inactivity of metabolic products and/or its rapid elimination and clearance from the body. Possible ways adopted or suggested by various researchers all round the globe to overcome these problems are discussed at length in this chapter.

\textbf{Figure 13.2} Problems associated with the multitargeted molecule curcumin

Adjuvants, which can block metabolic pathways of curcumin, are one of the major means that are being used to improve its bioavailability. Nanoparticles, liposomes, micelles and phospholipid complexes are promising carrier systems, which appear to provide longer circulation, better permeability and resistance to metabolic processes. Among the nanoparticulate systems, solid lipid nanoparticles (SLN) have recently found reappraisal as potential drug delivery system (DDS) for brain delivery [7]. SLN are attractive colloidal drug nanocarriers consisting of spherical solid lipid particles in the nanometre range, dispersed in water or in an aqueous surfactant solution. SLN loaded with curcuminoids for topical application were developed and characterised [8]. The \textit{in vivo} study with healthy volunteers revealed the improved efficiency of a topical application cream containing curcuminoid-loaded SLN over that containing...
free curcuminoids [8]. A very recent study by Gota and co-workers [9], in healthy volunteers, demonstrated that plasma level of curcumin after dosing of a solid lipid curcumin particle (SLCP) formulation at 650 mg of SLCP was 22.43 ng/ml, while dosing an equal quantity of unformulated 95% curcuminoids extract did not produce detectable levels. Furthermore, they also investigated three different doses (2, 3 and 4 g) of SLCP in 11 patients with osteosarcoma and reported a high interindividual variability in pharmacokinetics and nonlinear dose dependency, suggesting potentially complex absorption kinetics of this molecule [9].

Overall, nanoparticle-based system for curcumin delivery is still in its infancy and much progress is warranted in this area. Furthermore, the limited literature data reveal that the curcumin bioavailability enhancement has not gained significant attention. Thus, envisaging low bioavailability as one of the main limitations of curcumin-based therapies, the present study was planned, wherein curcumin-loaded solid lipid nanoparticles (C-SLN) were developed so as to achieve high plasma and brain concentrations after peroral administration of low doses of curcumin.

13.2 Chemistry of Curcumin

Chemically, curcumin is a 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E) or diferuloylmethane, which exhibits keto-enol tautomerism having a predominant keto (diketonic) form in acidic and neutral solutions and stable enol form in alkaline medium. It is a yellow-orange powder insoluble in water and ether but soluble in ethanol, methanol, dimethylsulfoxide and acetone [10]. Curcumin has a melting point of 183 °C, a molecular formula of C_{21}H_{20}O_{6} and a molecular weight of 368.37 g [11]. Curcumin gives brilliant yellow hue at pH 2.5–7 and red at pH >7. The fact that curcumin in solution exists primarily in its enolic form [12] has an important bearing on the radical-scavenging ability of curcumin. The stability of curcumin in aqueous media improves at high pH (>11.7) [13, 14]. However, it is stable at acidic pH but unstable at neutral and basic pH [10, 13, 15]. Most curcumin (>90%) is rapidly degraded within 30 minutes of placement in phosphate buffer systems of pH 7.2 [10]. The ability of antioxidants such as ascorbic acid, N-acetyl-l-cysteine (NAC) and glutathione (GSH) to prevent this degradation suggests that an oxidative mechanism is at work.

In contrast, one of curcumin’s major metabolites (tetrahydrocurcumin (THC)) is quite stable at neutral or basic pH [16] and still possesses antioxidant activity [17–19]. Curcumin is soluble in 0.1 M sodium hydroxide, although it remains stable for only 1–2 hours in alkaline solutions. In comparison, curcumin is more stable in cell culture medium containing 10% fetal calf serum and in human blood, with <20% of curcumin
being decomposed within 1 hour, and after incubation for 8 hours, about 50% of curcumin still remaining [14]. Based on mass and spectrophotometrical analysis, trans-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexenal was tentatively identified as a major degradation product, while vanillin, ferulic acid and feruloylmethane were identified as minor degradation products [14].

Since curcumin decomposes rapidly in serum-free medium, precautions must be taken during cell culture experiments. In addition, the biological effects caused by the degradation products of curcumin, especially vanillin, must also be taken into consideration. Vanillin, a naturally occurring flavouring agent, has been reported to inhibit mutagenesis in bacterial and mammalian cells. It may act as an anti-mutagen by modifying DNA replication and DNA repair systems after cellular DNA damage caused by mutagens occurs. Vanillin is also a powerful scavenger of superoxide and hydroxyl radicals. Degradation of curcumin is extremely slow at pH 1–6 [14], as normally encountered in the stomach where pH varies from 2 to 4. This combined with the fact that the major part of curcumin is unabsorbed indicates that sufficient amount is available for local action in the gastrointestinal tract. This probably explains its suitability for the treatment of gastric cancers as indicated by several studies including clinical trials.

13.3 Brain Tumour

Primary malignant brain tumours represent a heterogeneous group of diseases. They probably develop through accumulation of genetic alterations that permit cells to evade normal regulatory mechanisms and escape destruction by the immune system. In addition to inherited alterations in crucial genes that control the cell cycle, such as TP53, the chemical, physical and biological agents that damage DNA are suspected potential neurocarcinogens [20]. However, detailed investigations may be required before a more comprehensive picture of the natural history and pathogenesis of brain tumours is clear. There is intensifying interest in understanding the cause of brain tumours because the prognosis for patients with glioblastoma and other tumour types remains grim. A clinical report from the Mass General Hospital [21] states that a large number of brain tumours are caused by metastatic invasion of cancer cells, including melanoma, from other parts of the body.

Direct targeting of specific cellular and molecular alterations is a strategy in the development of cancer therapeutics [4]. Development of diet-derived chemopreventive agents is an emerging area of cancer research. Many diet-derived compounds show promising anti-cancer activities in epidemiologic as well as experimental studies [5]. The therapeutic efficacy of curcumin in various human malignant glioblastoma cells has been established [22], and curcumin was found to inhibit the nuclear factor-κB
(NF-κB) signalling pathways in these cell lines [4, 23, 24]. Numerous other mechanisms, like the induction of heat shock proteins (HSP) [25], the inhibition of matrix metalloproteinase (MMP) transcriptions [26, 27], tumour necrosis factor (TNF)-related apoptosis-inducing ligand-induced apoptosis [28], inhibition of glucose-6-phosphate transporter gene expression [29], the activation of both receptor-mediated and mitochondria-mediated proteolytic pathways [30], the induction of histone hypoacetylation leading to apoptosis in a poly (ADP-ribose) polymerase and caspase 3-mediated manner [31], the inhibition of the Inhibitor of growth protein 4 signalling pathway [32] and the induction of nonapoptotic autophagic cell death [33, 34], have also been established. Furthermore, curcumin was found to sensitize glioma cells to several chemotherapeutic agents and to radiation therapy [4]. Apart from the above-mentioned mechanisms, other mechanisms of action by which solubilised curcumin acts in vitro have been elaborated by a group of researchers [35]. They observed that the solubilised curcumin causes activation of proapoptotic enzymes caspase 3/7 in human oligodendroglioma (HOG) and lung carcinoma (A549) cells, and in mouse tumour cells N18 (neuroblastoma), GL261 (glioma) and B16F10. A simultaneous decrease in cell viability was also revealed by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assays. Further examination of the B16F10 cells showed that curcumin effectively suppresses cyclin D1, P-NF-κB, Bcl-xL, P-Akt and vascular endothelial growth factor (VEGF), which explains its efficacy in blocking proliferation, survival and invasion of the B16F10 cells in the brain. Taken together, solubilised curcumin effectively blocks brain tumour formation and also eliminates brain tumour cells.

Curcumin recently entered phase I clinical trials for the treatment of high-risk cancers, and recent emerging literature suggests multiple beneficial effects of curcumin in glioma cells, including inhibition of cellular growth, invasion and angiogenesis [4]. The usefulness of solubilised curcumin as a prophylactic against brain cancer via intravenous (IV) or intracranial routes has been reported by a team of scientists [35]. They demonstrated the nontoxic nature of their formulation against normal brain cells. Furthermore, they demonstrated that tail-vein injection or, more effectively, intracerebral injection of curcumin through a cannula blocks brain tumour formation in mice that had already received an intracerebral bolus of mouse melanoma cells (B16F10). Although the above results are conclusive of the fact that the solubilised curcumin when utilised judiciously could prove to be highly efficacious for treatment of brain tumours, the invasive nature of the treatment (intracerebral injection) can be highly discouraging for long-term or frequent use [35].

In another in vivo study, curcumin significantly decreased the incidence of radiation-induced pituitary tumours in rats [25]. In the subcutaneous xenograft model of glioblastoma cells, curcumin inhibited tumour growth significantly and induced autophagy. In another study, when the brain tumours reached 50–70 mm³ in volume, intratumoral
injections of curcumin (100 mg/kg in dimethyl sulfoxide (DMSO)/phosphate-buffered saline) were administered every 24 hours for 7 days. Evaluation of the effect was performed on day 16 of the initial curcumin treatment. An approximately threefold decrease in mean tumour volume was observed in the curcumin-treated group compared to the controls [35].

13.4 Blood-brain Barrier

The blood-brain barrier (BBB) is a specialised system of capillary endothelial cells that protects the brain from harmful substances in the bloodstream, while supplying the brain with the required nutrients for proper function. Unlike peripheral capillaries that allow relatively free exchange of substances across/between cells, the BBB strictly limits transport into the brain through both physical (tight junctions) and metabolic (enzymes) barriers. The cell association, sometimes called the neurovascular unit, constitutes the BBB and is now appreciated to be a complex group of interacting cells, which in combination induce the formation of BBB [36]. Thus, passage across the BBB is often the rate-limiting factor in determining permeation of therapeutic drugs into the brain. The purpose of the BBB is twofold. First, the composition of the internal environment of the brain, the brain interstitial fluid and the cerebrospinal fluid (CSF) has to be controlled within extremely fine limits, far more so than the somatic extracellular fluid, so that the neurons can perform their complex integrative functions [37, 38]. Potent excitatory neurotransmitters in the central nervous system (CNS) and their levels in brain extracellular fluid must be very precisely controlled. This integrative neural function of the CNS relies almost solely on accurate synaptic transmission and spatial and temporal summation. Some amino acids in the blood that are present in high concentrations, such as glycine, glutamic acid and aspartic acid, provide a stable background for the function of central neuronal synapses [39]. Second, a major function of the BBB is neuroprotection. Over a lifetime, the CNS will be exposed to a wide range of neurotoxic metabolites and acquired xenobiotics, which may cause cell damage and death. As neuronal replacement is virtually absent in the CNS of mammals, a phylogenetic class of animals, where individuals may have a relatively long lifespan, any enhancement of neuronal death will result in accelerating degenerative pathologies and advance natural debilitation with age.

However, a direct consequence of the existence of the BBB is the clinical difficulty in delivering therapeutic compounds to the CNS. Polar drugs cannot diffuse freely into the brain [40], as the paracellular pathway is absent; thus, unlike capillaries in the peripheral circulation, the diffusion of aqueous solutes from the blood to the brain extracellular fluid or in the reverse direction cannot occur and a large number of
more lipophilic drugs are subject to the activity of efflux transporters. As a result, a significant number of CNS diseases have poorly met therapy largely due to the difficulty of delivering drugs across the BBB [37].

Various colloidal delivery systems have been tried upon by different researchers to overcome the poor bioavailability associated with a compromised permeability across BBB. These systems include liposomes, microspheres, lipid microspheres, niosomes, nanoparticles and SLNs [41–46]. A high drug loading, physical and chemical stability and a low incidence of toxicity of the carrier used are a few important factors for a delivery system to be effective. Furthermore, the in vivo fate of the carrier, the chances of scaling up the process and the overall cost are other important considerations to be kept in mind before deciding on the suitability of the system [47–50].

The cerebral distribution parameters of drugs are further governed by a number of factors like plasma protein binding, cerebral blood flow rate, influx and efflux rates at the BBB, blood-CSF barrier and the brain parenchyma, rate of drug metabolism in the brain and drug-tissue interactions/binding in the brain.

In brain tumours, permeability is a more complex phenomenon. There are at least two major variables involved. The first variable concerns the tumour microvessel population, that is, the blood-tumour barrier. It has been shown that there may be three distinct microvessel populations in brain tumours [51]. The first consists of continuous, nonfenestrated capillaries like those of normal brain. The second microvessel population consists of continuous, fenestrated capillaries. Tumours with these microvessels exhibit increased permeability to small but not to large molecules. The third capillary population contains interendothelial gaps that may measure as large as 1 μm, for example, RG-2 rat glioma [51]. The second major variable with regard to capillary permeability involves the spatial distribution of the target capillaries. Although brain tumour capillaries may have increased permeability, like those of the RG-2 and D-54 MG tumours, permeability in brain surrounding the tumours rapidly returns to normal brain values within a few millimetres of the tumour margin. If, as shown by Burger (1987) [52], individual tumour cells may reside centimetres away from the edge of a tumour, spatial variability in capillary function will affect drug delivery to all brain tumours [53]. Once injected intravascularly, a drug mixes with the total body volume of blood or, in the case of water-soluble compounds that are not protein bound, with total body plasma. Because a 70 kg human contains 3 l of plasma, to achieve a starting plasma concentration of 1 unit of ‘ideal’ drug per millilitre, 3000 units of drug must be administered [53].

The human body acts as an enormous sink into which a majority of intravascularly administered drug will be distributed, not to the brain tumour, but to other body tissues. Once mixed with total body plasma, the drug distributes throughout body
tissues and is then eliminated. The fraction of drug entering tumour, compared with that circulating through the entire body, is miniscule in every case: <1% of circulating drug will reach the tumour, regardless of the drug’s permeability and regardless of changes in capillary permeability. Therefore, any method that is used to increase brain tumour permeability will still have to deal with the reality that most of the administered drug is distributed to the rest of the body, and as far as treatment of the brain tumour is concerned it is wasted. In most instances, the limit on the total dose of drug that can be given is imposed by the tolerance of normal tissues, which is systemic toxicity. Because the maximum dose that can be given to a patient is determined by factors external to treatment of the tumour (i.e., systemic toxicity), we must accept as a starting principle that the administered drug dose cannot be increased, and we must search for alternative methods to increase the fraction of drug that reaches the tumour [53].

Thus, it is imperative to consider and optimise these parameters while selecting a suitable delivery system for brain delivery. Successful delivery across the BBB has only been achieved in some cases. To reach therapeutic drug level in the brain, nanoparticulate systems as drug carriers with sufficiently high loading capacity and small particle size, which can bypass the reticuloendothelial system (picks up particulate system from blood for elimination), are being looked into as suitable delivery systems [54–57]. Considering the success of these nanoparticles in passing through the BBB and their limitation(s) especially with respect to toxicity and stability, another suitable option for drug delivery into the brain is SLN. They are generally made up of solid hydrophobic core having a monolayer of phospholipid coating. The solid core may contain the drug dissolved or dispersed in the solid high-melting fat matrix with the hydrophobic end of the phospholipid chains embedded in the fat matrix. Thus, they have a potential to carry lipophilic or hydrophilic drug(s) or diagnostics [58–60].

13.5 Role of Curcumin in Cancer

Cancer is well recognised as a disease of old age. It is estimated that the process of tumorigenesis starts at around the age of 20 and detection of cancer is normally around the age of 50 or later; thus most cancers have an estimated incubation time of around 20–30 years. Recent studies indicate that in any given type of cancer 300–500 normal genes have been modified somehow to result in the cancerous phenotype. Studies have estimated that genetic factors cause only 5–10% of all human cancers, while the remaining percentage is caused by lifestyle. In spite of an extensive search for safe and efficacious treatments for cancer, it has involved the use of harmful substances, such as poisonous mustargen introduced in 1941; chemotherapy, introduced in 1971; and the targeted therapies, introduced in 1991.
Progress in cancer research can be followed by the number of approvals from the US Food and Drug Administration (FDA) [1], as indicated by very few in 1970, 7 in 1987, 16 in 1996, 21 in 1998 and 28 in 2006 [61]. More than 70% of the FDA-approved anti-cancer drugs can be traced back to their origin in plant-derived natural products, which were traditionally used as ancient remedies for various ailments.

Although cancers are characterised by the dysregulation of cell signalling pathways at multiple steps, most current anti-cancer therapies involve the modulation of a single target. The ineffectiveness, lack of safety and high cost of monotargeted therapies have led to a lack of faith in these approaches. As a result, many pharmaceutical companies are increasingly interested in developing multitargeted molecules for cancer control. Many plant-based products, however, accomplish multitargeting naturally and, in addition, are inexpensive and safe compared to synthetic agents.

The current paradigm for most of the treatments is to either combine several smart drugs or design drugs that modulate multiple targets (multitargeted therapies), formally referred to as dirty drugs (Figure 13.3). The recent success of a number of promiscuous agents has led many researchers to investigate the rationale and potential of openly unspecific agents. Some authors advocate the development of ‘magic shotguns’ rather than ‘magic bullets’ as a more realistic and potentially successful approach to tackle a disease of such complexity as cancer [62]. This line of thinking is also starting to prevail in other medical disciplines. In a review by Roth and co-workers [62], the authors discussed the use of selective versus nonselective drugs for CNS disorders. Since in most cases multiple molecular lesions or signalling pathways are involved in the pathogenesis of CNS disorders, the authors conclude that attempts to develop more effective treatments for diseases such as schizophrenia and depression by discovering drugs selective for single molecular target, the ‘magic

![Figure 13.3 Dirty drugs with their multiple targeting potential are gaining interest](image)
Indu Pal Kaur and Vandita Kakkar

bullets’ concept, have been largely unsuccessful. They hypothesise that selectively designing nonselective drugs that interact with several molecular targets will lead to new and more effective medications for a variety of CNS disorders. However, because pharmaceutical companies are not usually able to secure intellectual property rights to plant-based products, the development of plant-based anti-cancer therapies has not been prioritised.

Intensive studies on the action mechanisms of curcumin in various biological systems have indicated that it employs multiple anti-tumour-promoting pathways [63]. It is conceivable that the molecular mechanism of action of curcumin is quite complicated and dispersed. The locations of targets of its action vary from genome (DNA) level, to the messenger (RNA) level, to the enzyme (protein) [64]. The action of curcumin may proceed simultaneously or sequentially through these different levels. It appears that when any essential component of a signal transduction pathway is rendered hyperactive or autonomous, it may acquire the ability to drive the cell into unchecked proliferation leading to tumour promotion. Curcumin may attenuate or suppress the hyperactivity of these components of signal transduction and maintain simultaneously the normal cell function.

The various molecular targets (Figure 13.4) modulated by this agent include transcription factors, growth factors and their receptors, cytokines, enzymes and genes regulating cell proliferation and apoptosis [64, 65]. The diversity of the biological

![Modulation of various molecular targets by curcumin](image)

**Figure 13.4** Modulation of various molecular targets by curcumin
actions of curcumin in mammalian species was recently emphasised by a noteworthy study demonstrating its beneficial effects in mice homozygous for a complete knockout of a gene linked with cystic fibrosis.

13.5.1 Contribution of Curcumin in the Induction of Apoptotic Mechanism

The ability of curcumin to induce apoptosis in cancer cells without cytotoxic effects on healthy cells contributes to the understanding of the anti-cancer potential of curcumin. This spice is described to efficiently induce apoptosis in various cell lines including HL-60, K562, MCF-7 and HeLa [65, 66]. Curcumin also leads to apoptosis in scleroderma lung fibroblasts without affecting normal lung fibroblasts [67]. Woo and co-workers [68] suggested that the induction of Caki (human kidney carcinoma cells) programmed cell death is activated by Akt dephosphorylation, Bcl-2, Bcl-xL and inhibition of apoptosis protein inhibitor, as well as cytochrome c release and caspase 3 activation. Later, this was confirmed by the results of Bush and co-workers [69], Anto and co-workers [70] and Pan and co-workers [71] studying caspase 3 activation in melanoma and HL-60 cells. Bush and co-workers [69] described that curcumin induces caspases 8 and 9, although p53 remains unchanged. Nevertheless, the death receptor pathway is activated through Fas in a Fas-ligand-independent way. Anto and co-workers [70] confirmed the role of Bcl-2 and Bcl-XL inhibition by preventing curcumin-induced apoptosis after overexpressing these two key proteins.

However, apoptosis in Jurkat cells is described as independent of caspase 3, its activation being blocked by an increase in GSH levels [72]. Caspase activation by curcumin was described to be blocked by HSP, which do not influence cytochrome c release [73]. Jana and co-workers [74] demonstrated that curcumin inhibits proteasome activity in mice, potentially leading to induction of apoptosis through caspase 9 activation. In another study, Jiang and co-workers [75] demonstrated that induction of apoptosis by curcumin (30 μM) is highly dependent on the origin and malignancy of cell lines. It appears that the typical apoptosis can only be induced in immortalised mouse embryo fibroblast NIH 3T3, erbB2 oncogene-transformed NIH 3T3, mouse sarcoma 180, human colon cancer cell HT29, human kidney cancer cell 293 and human hepatocellular carcinoma HepG2 cells but not in primary cultures of mouse embryonic fibroblast C3H 10T1/2, rat embryonic fibroblast or human foreskin fibroblast cells [75]. Treatment of NIH 3T3 cells with the PKC inhibitor staurosporine, the tyrosine kinase inhibitor herbimycin A or arachidonic acid metabolism inhibitor quinacrine induces typical apoptosis. These findings suggest that blocking the cellular signal transduction in immortalised or transformed cells might trigger the induction of apoptosis by curcumin. They also demonstrated that curcumin (3.5 μg/ml) induces apoptosis in human promyelocytic HL-60 cells.
The apoptosis-inducing activity of curcumin occurred in a dose- and time-dependent manner. Flow cytometric analysis showed that the hypodiploid DNA peak of propidium iodide-stained nuclei appeared 4 hours after treatment with 7 μg/ml curcumin. The apoptotic effect of curcumin was not affected by cycloheximide, actinomycin D, ethylene glycol tetraacetic acid, W7 (calmodulin inhibitor), sodium orthovanadate or genistein whereas an endonuclease inhibitor, ZnSO₄, and a proteinase inhibitor, N-tosyl-1-lysine chloromethyl ketone, could markedly abrogate curcumin-induced apoptosis. The antioxidants NAC, L-ascorbic acid, α-tocopherol, catalase and superoxide dismutase effectively prevented curcumin-induced apoptosis [76].

Zheng and co-workers [77] explored the apoptosis-inducing effects of curcumin in human ovarian tumour A2780 cells. They found that curcumin could significantly inhibit the growth of ovarian cancer cells by inducing apoptosis through upregulation of caspase 3 and downregulation of expression of NF-κB.

### 13.5.2 In Vitro Anti-cancer Effects of Curcumin

Curcumin has been shown to exert a fascinating array of pharmacological effects in cells *in vitro* at physiologically attainable and at supraphysiological concentrations. It has been shown to inhibit the proliferation of a wide variety of tumour cells, including B-cell and T-cell leukaemia [76, 78–80], colon cancer and epidermal carcinoma [81]. Furthermore, it also suppresses the proliferation of various breast tumour cell lines such as BT20 and SKBR3. A few studies on the anti-cancer activity of curcumin against uterine cancer have also been reported. Curcumin exhibits anti-cancer effects in various lung cancer cells through a variety of molecular targets.

At the cellular level, curcumin derivatives inhibit farnesyl protein transferase (FPTase), in A549 cells. The anti-cancer effect of curcumin in murine thymoma cells was found to be due to the blocking of interleukin (IL)-1 signalling by the inhibition of the recruitment of the IL-1 receptor-associated kinase. A study showed that curcumin could prevent tumour-induced thymic atrophy in thymic T cells, leading to the neutralisation of tumour-induced oxidative stress and the restoration of NF-κB activity and the re-education of the TNF-α signalling pathway, resulting in thymic protection [82]. Furthermore, curcumin was described as an effective agent [83], explaining its chemopreventive effect at the level of tumour promotion. This phenomenon could be explained by VEGF and angiopoietin 1 and 2 inhibition in EAT cells, by VEGF and angiopoietin 1 inhibition in NIH 3T3 cells and by inhibition of the tyrosine kinase Flk-1/KDR (VEGF receptor-2) in human umbilical vein endothelial cells [84]. It also displays an inhibiting effect on human telomerase reverse transcriptase expression, reducing telomerase activity in MCF-7 cells [85].
In vitro cellular experiments have shown that short-term treatment with curcumin inhibits epidermal growth factor receptor (EGFR) kinase activity and epidermal growth factor-induced tyrosine phosphorylation of EGFR in A431 cells and depletes cells of Her2/neu protein. Similar to geldanamycin, curcumin is extremely potent at degrading intracellular HER2 and disrupting its tyrosine kinase activity [86]. Curcumin has also been shown to induce apoptosis in acute T-cell leukaemias by inhibiting the phosphatidylinositol 3 kinase/Akt pathway and to induce G2/M arrest and nonapoptotic autophagic cell death in malignant glioma cells by abrogating Akt and extracellular-regulated kinase (ERK) signalling pathways [34].

Effects of curcumin are also apparently mediated through its inhibition of various other serine/threonine protein kinases. Curcumin completely inhibits the activity of several protein kinases including phosphorylase kinase, PKC, cytosolic protamine kinase, autophosphorylation-activated protein kinase and pp60src tyrosine kinase. Other investigators have shown similar suppression of phorbol-12-myristate-13-acetate-induced activation of cellular PKC by curcumin [87, 88].

Most inflammatory stimuli typically activate one of the three independent mitogen-activated protein kinase (MAPK) pathways leading to activation of the p44/42 MAPK (also called ERK1/ERK2), jun N-terminal kinase or p38 MAPK pathway, respectively. Curcumin can apparently inhibit all of these pathways directly or indirectly, thus providing evidence of its potent anti-inflammatory and anti-carcinogenic effects [87, 88].

13.5.3 Preclinical Pharmacodynamic Studies

Following oral administration, curcumin prevented cancer in the colon, skin, stomach, liver, lung, duodenum, soft palate and breasts of rodents [2]. In particular, the effects of dietary curcumin (0.05–2.0%) on colorectal carcinogenesis have been demonstrated in both carcinogen-induced and genetic rodent models. Curcumin inhibited carcinogenic initiation, as reflected by decreased levels of adducts induced by benzo[a]pyrene or by aflatoxin b1 [89, 90]. In the azoxymethane-induced rat colon cancer model, dietary curcumin (0.8%) reduced the number of aberrant crypt foci to one-half compared with control [91]. In intestinal cancer induced in mice by azoxymethane, oral curcumin (2000 ppm) treatment for 14 weeks produced a significant increase in the apoptotic histological index when compared to controls [92]. Genetic models, such as the multiple intestinal neoplasia (e.g., ApcMin) mouse, permit the study of the inhibition of promotion phase of carcinogenesis. Curcumin interfered with adenoma formation in the ApcMin mouse, which harbours an adenomatous polyposis coli gene mutation and is a model of the human disease familial adenomatous polyposis [93]. When administered in the diet at 0.1% and 0.2% for the animals’ lifetime, a significant decrease in adenoma number was observed compared to control animals [94, 95].
This was accompanied by downregulation of the expression of the enzyme COX-2 and attenuation of tissue oxidative status, as reflected by the levels of the oxidative DNA adduct pyrimido-[1,2]purin-10(3H)-one-2-deoxyguanosine.

Chemopreventive effects of curcumin along with its effects on the initiation or post-initiation phase of N-nitrosomethylbenzylamine-induced oesophageal carcinogenesis in male F344 rats are also reported [96]. In another similar study in rodents, curcumin was able to inhibit the development of N-methyl-N-nitro-N-nitrosoguanidine-induced stomach cancer [97], an effect that may be mediated in part by an ability to suppress the proliferation of Helicobacter pylori (the major pathogen in human gastric cancer) [98].

Effects of curcumin (daily dose of 100 mg/kg) in an animal (Wistar rat) model of N-nitrosodiethyamine (DENA)-initiated and phenobarbital (PB)-induced hepatocarcinogenesis were investigated by Sreepriya and Bali [99]. The investigators reported that curcumin prevented the reduction of defensive hepatic GSH antioxidant activity, decreased lipid peroxidation and minimised the histological alterations induced by DENA/PB [100]. In another study, investigators found that the administration of curcumin and a synthetic analogue to nicotine-treated Wistar rats over a period of 22 weeks enhanced biochemical marker enzyme and lipid profiles [101]. Other in vivo studies have investigated the effects of curcumin on tumour angiogenesis and on COX-2 and VEGF biomarkers in hepatocellular carcinoma cells implanted in nude mice [102]. A group of researchers demonstrated that systemic administration of curcumin for 6 consecutive days to rats bearing the highly cachectic Yoshida AH-130 ascites hepatoma significantly inhibited tumour growth [103]. Anti-tumoral and inhibitory effects of curcumin were reported for melanoma cells [104] and melanoma lung metastasis in mice [105].

However, the effect of curcumin is not limited to the above cancers; a study in human breast cancer xenograft model of nude mice bearing the human-derived MDA-MB-435 breast tumour showed a decrease in the load of breast cancer metastases and concomitant suppression of NF-κB, COX-2 and MMP-9.

Curcumin, a multitargeting molecule, when applied topically at a concentration of 3–10 mol, 5 minutes prior to the application of carcinogen, inhibits chemical carcinogenesis of the skin [106, 107]. In this series of studies, tumour initiation was induced by benzo[a]pyrene or 7,12-dimethylbenz[a]anthracene (DMBA) and tumour promotion was induced by 12-O-tetradecanoylphorbol-13-acetate. Potential mechanisms of these effects were considered to involve inhibition of arachidonic acid-induced inflammation, inhibition of hydrogen peroxide formation and inhibition of ornithine decarboxylase activity/transcription, the last of which is a rate-limiting step in polyamine biosynthesis [106, 107].
It should also be noted that studies have also demonstrated no attenuation of chemically induced carcinogenesis by curcumin. For example, dietary curcumin (500 ppm) did not affect prostate carcinogenesis in rats exposed to 3,2-dimethyl-4-aminobiphenol or 2-amino-1-methylimidazo[4,5-b]pyridine [108].

**13.5.4 Combinatorial Chemoprevention by Curcumin**

Topical application of curcumin (10 mmol) 3 times weekly to the buccal pouch of Syrian golden hamsters has demonstrated inhibition of DMBA-induced oral carcinogenesis [109]. In this study of ‘combinatorial chemoprevention’, the effect of topical curcumin appeared to be enhanced by the concomitant consumption of green tea (6 mg tea solids/ml) for 18 weeks [109]. Subsequent studies combining curcumin with other chemopreventive agents have also shown augmented growth inhibitory effects. Although there are numerous reports in the published literature to suggest that curcumin augments the cytotoxicity of anti-cancer drugs such as paclitaxel in cells _in vitro_ [110, 111], observations that confirm this notion _in vivo_ are lacking at present in the lung metastases [110]. Interestingly, certain rodent studies have suggested a potential for curcumin to confound unwanted detrimental effects of cytotoxic anti-cancer drugs. For example, curcumin administered to rats by gavage (100 or 200 mg/kg daily for 7 days) ameliorated chromosomal mutations induced by cyclophosphamide in the bone marrow [112].

More recent studies have evaluated curcumin’s chemosensitising and radiosensitising effects. Aggarwal and co-workers [110] evaluated the chemosensitising effect of curcumin in combination with paclitaxel on breast cancer metastases to the lung. Other researchers have examined the effects of curcumin on human breast cancer (MDA-MB-231) cells in an immunodeficient mouse model of metastasis and observed that the number of lung metastases significantly decreased after intercardiac injection of curcumin, a clear demonstration of curcumin as a promising agent for dietary chemoprevention of metastases [113]. They investigated the effect of curcumin alone and in combination against several cancers and found that (i) the combination of curcumin and gemcitabine inhibits pancreatic cancer growth in nude mice by inhibiting NF-κB-regulated gene expression, cell proliferation and angiogenesis [114]; (ii) the combination of curcumin and docetaxel is effective against human ovarian cancer in nude mice [115]; (iii) curcumin can suppress the growth of human glioblastoma in rodents [34]; and (iv) curcumin sensitises colon cancers in nude mice to oxaliplatin [116]. In addition, other recent studies have shown that curcumin sensitises prostate cancers to chemotherapeutics and radiation by downregulating expression of the MDM2 oncogene [117]. Together, these _in vivo_ animal studies clearly suggest curcumin’s anti-cancer potential when administered either alone or in combination with currently employed chemotherapeutic agents or radiation.
13.5.5 Pharmacokinetic Studies in Animals

Wahlstrom and Blennow [118] were the first to examine the uptake, distribution and excretion of curcumin in Sprague-Dawley rats. Negligible amounts of curcumin in blood plasma of rats after oral administration of 1 g/kg of curcumin showed that curcumin was poorly absorbed from the gut. Curcumin has been found to be safe at doses up to 8 and 12 g. Pan and co-workers [119] investigated the pharmacokinetic properties of curcumin in mice. They found that after intraperitoneal administration of curcumin (0.1 g/kg) to mice, about 2.25 μg/ml of curcumin appeared in the plasma during the first 15 minutes. One hour after administration, the levels of curcumin in the intestine, spleen, liver and kidneys were 177, 26, 27 and 7.5 μg/g, respectively. Only traces (0.41 μg/g) were observed in the brain at 1 hour. Perkins and co-workers [95] examined the pharmacokinetics of curcumin in a Min/+ mouse model of familial adenomatous polyposis (FAP) using either dietary curcumin or a single dose of radiolabelled curcumin given via intraperitoneal route and showed that irrespective of the dose, traces of curcumin were present in the plasma, which were at levels near the limit of detection (5 pmol/ml). A very recent study by Yang and co-workers [120] showed that 10 mg/kg of curcumin given IV in rats gave a maximum serum curcumin level of 0.36 ± 0.05 μg/ml, whereas a 50-fold higher curcumin dose administered orally gave only 0.06 ± 0.01 μg/ml maximum serum level in rat. An oral curcumin dose of 1 g/kg in rats produced a maximum serum curcumin level of 0.5 μg/ml after 45 minutes of curcumin dosing [121]. Similarly, Marczylo and co-workers [122] also showed that a maximum serum curcumin concentration of 6.5 ± 4.5 nM was reached 0.5 hours after oral dosing of curcumin. More recently, curcumin (0.1 g/kg) administered to mice was found to undergo metabolic reduction to dihydrocurcumin and THC, which were further converted to monoglucuronide conjugates [3]. These studies clearly suggest the effect of route of administration on achievable serum levels of curcumin.

Another study evaluated the tissue distribution of curcumin using tritium-labelled drug. The study found that radioactivity was detectable in blood, liver and kidney following doses of 400, 80 or 10 mg of [3H]curcumin. With 400 mg, considerable amounts of radiolabelled products were present in tissues 12 days after dosing. The percentage of curcumin absorbed (60–66% of the given dose) remained constant regardless of the dose, indicating that administration of more curcumin does not result in higher absorption [123]. That is, in rats, there is a dose-dependent limitation to bioavailability.

13.5.6 Pharmacokinetic Studies in Humans

Pharmacokinetic studies in humans have generally produced similar data as for the animal studies, although some variations were observed. The pilot studies suggest that
concentrations of curcumin achieved in plasma and target tissues are low, probably due to its extensive metabolism [6, 16, 88]. In a phase I trial, plasma and urine concentrations of curcumin in patients who had ingested 3600 mg curcumin orally were 11.1 nmol/l and 1.3 μmol/l, respectively [17]. Curcumin concentrations in colorectal tissues of patients on this dose were 7.7–12.7 nmol/g, while levels in the liver were below the limits of detection [4, 5]. In another study, peak plasma concentrations of curcumin 1–2 hours after oral dosing reached 0.41–1.75 μM in patients receiving 4–8 g curcumin [1].

A similar study in healthy human volunteers reported $C_{\text{max}}$ values of 2.30 ± 0.26 and 1.73 ± 0.19 μg/ml after 0.25–72 hours with 10 and 12 g doses, respectively [124]. However, healthy volunteers who ingested 2 g pure curcumin powder after fasting showed less than 10 ng/ml curcumin in their plasma 1 hour after intake [125]. In the same study, coingestion of curcumin with 20 mg of piperine appeared to increase the bioavailability of curcumin by 2000%.

In a study of oral curcumin, patients with preinvasive malignant or high-risk premalignant conditions of the bladder, skin, cervix, stomach or oral mucosa received 0.5–8.0 g curcumin by mouth daily for 3 months [126]. Plasma curcumin concentrations were found to peak 1–2 hours after intake and gradually declined within 12 hours. The 8 g/day dose resulted in a peak serum concentration of 1.75 ± 0.80 μM. When administered orally in micronised form with orange juice at doses of 50–200 mg to 18 healthy volunteers, no curcumin was found in the plasma at or above the limit of quantification (>0.63 ng/ml) [127].

In a clinical phase I dose escalation study using a standardised oral Curcuma extract comprising mainly of curcumin, doses up to 180 mg of curcumin per day were administered to patients with advanced colorectal cancer for up to 4 months without toxicity or detectable systemic bioavailability [128]. In a follow-up study in 15 patients with advanced colorectal cancer refractory to standard chemotherapy, curcumin in the form of ‘curcuminoids C3’ (Sabinsa Corp., East Windsor, NJ, USA, 90% curcumin) was consumed orally for up to 4 months at doses between 0.45 and 3.6 g daily [129]. Oral consumption of 3.6 g of curcumin per day resulted in levels of drug and glucuronide/sulfate conjugates in plasma near the limit of detection (5 pmol/ml).

Curcumin and its conjugates were also detected in 24 hours urine collections. In the six patients who had consumed 3.6 g curcumin, urinary levels (in μM) varied between 0.1 and 1.3 for curcumin, between 0.019 and 0.045 for curcumin sulfate and between 0.21 and 0.51 for curcumin glucuronide. The presence of curcumin and its conjugates in the urine of patients taking 3.6 g of curcumin daily suggests that urinary curcumin/curcumin metabolites might serve as measures of compliance with treatment.
Exploratory studies have also been performed in patients undergoing operations for colorectal cancer who consented to have their tissues analysed for research purposes [130, 131]. Twelve patients with confirmed colorectal cancer received oral curcumin at 0.45, 1.8 or 3.6 g/day for 7 days prior to surgery. The levels of agent-derived species were determined in blood and colorectal tissue obtained at the time of surgical resection. The mean concentrations of curcumin in normal and malignant colorectal tissue of patients who had ingested 3.6 g curcumin daily were 12.7 and 7.7 nmol/g tissue, respectively [130]. The studies further explored the pharmacology of curcumin administered in capsules at daily doses ranging from 0.45 to 3.6 g for up to 4 months. This time, the effect of curcumin on leucocytes was measured in terms of three potential biomarkers: Glutathione S-transferase GST activity, malondialdehyde deoxyguanosine adduct levels and prostaglandin E$_2$ (PGE$_2$) production ex vivo. In a comparison of inducible PGE$_2$ production immediately before and 1 hour after dosing on days 1 and 29, the highest dose (3.6 g) elicited significant decreases (62% and 57%, respectively). Consequently, the investigators chose the 3.6 g dose for further evaluation in a phase II trial in cancers outside the gastrointestinal tract. Curcumin sulfate and curcumin glucuronide were also found in the intestinal tissue taken from these patients; trace levels of curcumin were detected in peripheral blood samples. Compatible with the preclinical data presented earlier, these preliminary results in humans suggest that a daily dose of 3.6 g curcumin achieves measurable levels in colorectal tissue with negligible distribution of the parent drug outside of the gut. When 12 patients with liver metastases from colorectal cancer received oral curcumin (0–3.6 g) daily for 7 days prior to hepatic surgery, curcumin was not found in liver tissue resected 6–7 hours after the last dose of curcumin, whereas trace levels of products of its metabolic reduction were detected [131]. Levels of curcumin and glucuronide and sulfate conjugates in the low-nanomolar range were found in blood samples taken 1 hour after the last dose. The results of this pilot study suggest that doses of oral curcumin required to produce hepatic levels sufficient to exert pharmacological activity are probably not feasible in humans.

Curcumin has potential as palliative therapy for cancerous skin lesions. In a study by Kuttan and co-workers [132], curcumin efficacy was evaluated in 62 patients when it was applied as either an ethanol extract of turmeric or an ointment to external cancerous skin lesions. Regardless of the application, curcumin provided remarkable symptomatic relief that was in many cases relatively durable (lasting several months) and in all cases (except for a single adverse reaction in one subject) extremely safe. In a recent study reported by us, curcumin entrapped in elastic vesicles (curcumin-EV) was significantly effective in controlling UV-induced lesions even at a dose as low as 1–3 μmol. At a dose of 10 μmol there was a complete alleviation of symptoms, when curcumin-EV were applied before and after UV exposure [133].
Apparently, curcumin can also safely exert chemopreventive effects on premalignant lesions. In a prospective phase I dose escalation study, Cheng and co-workers [126] examined the safety, efficacy and pharmacokinetics of curcumin in 25 patients with a variety of high-risk precancerous lesions (recently resected urinary bladder cancer \((n = 2)\), arsenic Bowen’s disease of the skin \((n = 6)\), uterine cervical intraepithelial neoplasm (CIN) \((n = 4)\), oral leukoplakia \((n = 7)\) and intestinal metaplasia of the stomach \((n = 6)\)). Curcumin was administered to the first three patients at a starting dose of 500 mg/day for 3 months and, if no grade 2 or higher toxicities were observed, the dose was increased to 1000, 2000, 4000, 8000 and finally 12,000 mg/day. Curcumin was not toxic at doses of 8000 mg/day or lower, reaching peak serum concentrations at 1–2 hours \((0.51 \pm 0.11 \mu M \text{ at } 4000 \text{ mg}, 0.63 \pm 0.06 \mu M \text{ at } 6000 \text{ mg and } 1.77 \pm 1.87 \mu M \text{ at } 8000 \text{ mg})\) and being gradually eliminated (principally through nonurinary routes) within 12 hours. Although frank malignancies occurred despite curcumin treatment in one patient each with CIN and oral leukoplakia, a remarkable number of patients (i.e., one patient with recently resected bladder cancer, two with oral leukoplakia, one with intestinal metaplasia of the stomach, one with CIN and two with Bowen’s disease) showed histological improvement in precancerous lesions.

At least 12 active clinical trials of curcumin are ongoing in the USA, Israel and Hong Kong. Curcumin is being used alone in most of these trials and in combination with quercetin or sulindac in one. Meanwhile, chemoprevention trials of curcumin in hepatocellular carcinoma, gastric cancer and colon cancer are ongoing in Japan. In the USA, several randomised and nonrandomised phase I/II trials (http://www.Clinical-Trials.gov) are investigating its effects on a range of human malignancies (e.g., colorectal cancer, aberrant crypt foci, FAP, pancreatic cancer, multiple myeloma, Alzheimer’s disease, myelodysplastic syndrome (MDS) and psoriasis) when given alone or in conjunction with other natural substances or nonsteroidal anti-inflammatory drugs.

In a phase II study using unformulated curcumin, free curcumin was barely detectable in 19 patients who were administered 8 g of curcumin daily. However, a trend towards changes in blood markers was observed, suggesting that circulating plasma curcumin levels may not reflect tumour tissue curcumin levels [134]. Two phase II trials are interrogating the effects of curcumin in advanced pancreatic cancers. An Israeli trial is investigating the combined effects of curcumin and gemcitabine in patients with advanced or metastatic adenocarcinomas of the pancreas, while an exploratory clinical trial in the USA is testing the efficacy of curcumin alone in patients with unresectable or metastatic pancreatic cancers.

Another Israeli clinical trial is investigating the clinical efficacy of curcumin alone or in combination with coenzyme Q\(_{10}\) in patients with MDS. At M.D. Anderson
Cancer Center, a pilot trial of curcumin alone or in combination with bioperine (a black pepper extract) is under way in patients with asymptomatic multiple myeloma. In a very recent study in healthy volunteers, the SLCP demonstrated increased bioavailability of curcumin as compared to an unformulated 95% curcuminoids extract at 650 mg dose. To what degree the enhanced bioavailability is a result of increased absorption or is due to reduced conversion of free curcumin to conjugates is still not clear, because in this study the samples were not pretreated with glucuronidase. However, various researchers report only slight (two- to threefold) increases in curcumin absorption by simply dissolving or mixing curcumin in different types of lipids [121, 122, 124, 135]. Yet formulations containing lipids and emulsifiers have shown positive effects in an in vivo colitis model [136].

To summarise the data from pilot and phase I clinical studies performed with curcumin, it appears that low systemic bioavailability following oral dosing is consistent with the findings in preclinical models presented earlier. Efficient first-pass and some degree of intestinal metabolism of curcumin, particularly glucuronidation and sulfation, might explain its poor systemic availability when administered via the oral route. A daily oral dose of 3.6 g of curcumin results in detectable levels in colorectal tissue, which might be sufficient to exert pharmacological activity, with negligible distribution of the parent drug in hepatic tissue or other tissues beyond the gastrointestinal tract.

### 13.5.7 Alleviation of Chemotherapy-induced Symptoms

Patients with cancer suffer from various treatment-related symptoms, including neuropathic pain, depression, fatigue, decreased appetite and sleep disturbance. Many of these symptoms may cause treatment delays and prevent the delivery of full-dose therapy in the scheduled time. In the course of targeting cancer, most chemotherapeutic agents activate NF-κB and induce TNF-α release. Consequentially, many of the symptoms related to cytokine dysregulation are affected by both the disease and the treatment. For example, chemotherapy commonly causes neuropathic pain, depression, fatigue, decreased appetite and sleep disturbance, all of which have been linked to proinflammatory pathways that include NF-κB and TNF-α, as well as other key factors, such as IL-1 and IL-6 [137, 138].

Animal models of ‘sickness behaviour’ support that the symptoms of sickness (e.g., anorexia, disturbed sleep, hyperalgesia and disrupted learning) are related to the inflammatory cytokines, primarily IL-1, IL-6 and TNF-α [139, 140]. Since curcumin can suppress the activation of NF-κB and NF-κB-regulated TNF, IL-1 and IL-6 expression, it could have potential effects against these symptoms.
13.6 Therapeutic Strategies for Bioavailability Enhancement of Curcumin

Unfortunately, the poor absorption, rapid metabolism and elimination of curcumin result in a poor bioavailability of this interesting polyphenolic compound. Possible ways explored by researchers to overcome these problems are discussed below (Figure 13.5). Adjuvants, which can block metabolic pathways of curcumin, are one of the major means that have been explored to improve the bioavailability of curcumin. However, traditionally, turmeric was delivered orally as an emulsion in oil or milk, perhaps because of the hydrophobic nature of its bioactive constituents such as curcumin and turmeric oil. Taking this as a cue, nanoparticles, liposomes, micelles and phospholipid complexes of curcumin have been developed and found to provide longer circulation, better permeability and resistance to metabolic processes.

Figure 13.5 Therapeutic strategies to enhance the bioavailability of curcumin

13.6.1 Adjuvants

Piperine, a well-known inhibitor of hepatic and intestinal glucuronidation, has been shown to enhance the oral bioavailability of curcumin in rats as well as humans. In rats, 2 g/kg of curcumin alone produced a maximum serum curcumin level of 1.35 ± 0.23 μg/ml at 0.83 hours, whereas concomitant administration of piperine (20 mg/kg)
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led to 154% enhancement in the bioavailability of curcumin. In contrast, in humans receiving a dose of 2 g curcumin alone, serum curcumin levels were either undetectable or very low. Concomitant administration of piperine, however, produced 2000% increase in bioavailability [125]. Thus, the effect of piperine on the bioavailability of curcumin has been shown to be much greater in humans than in rats. Brain uptake of curcumin after 2 minutes was increased by 48% due to coadministration of piperine relative to that without piperine. However, the uptake in other organs was not found to be significantly improved by piperine in this study, and this can be explained by the poor solubility of piperine in 10% ethanolic saline (injection medium) [141]. The glucuronidation-inhibiting effect of piperine [125] and the established lesser activity of curcumin glucuronides [142] indicate that inhibition of glucuronidation by piperine may be the major mechanism by which piperine increases the bioavailability of curcumin. The synergistic inhibitory effect of curcumin and genistein against pesticide-induced cell growth of oestrogen-dependent breast carcinoma cell lines (MCF-7) has also been reported [143].

Curcumin uptake by rat skin after topical application of a curcumin hydrogel demonstrated that eugenol and terpineol could enhance curcumin absorption by 2.2- and 2.5-fold, respectively, at 8 hours after topical application on rat skin. The above studies are suggestive of the fact that these adjuvants have the ability to enhance the bioavailability of curcumin [144]. Another study indicates that epigallocatechin-3-gallate, a component of green tea, could counteract certain undesirable activities assigned to curcumin [145]. Thus, the studies indicate that the activity of curcumin can be modulated when different agents are used simultaneously with curcumin.

13.6.2 Self-microemulsifying Drug Delivery System

Self-microemulsifying drug delivery system (SMEDDS) is emerging as one of the most interesting approaches to improve the solubility, dissolution and oral absorption of poorly water-soluble drugs [146]. SMEDDS is an isotropic mixture of oil, surfactant, cosurfactant and drug substance, which can form a microemulsion under the conditions of gastrointestinal fluid and gastrointestinal motility after oral administration. The resultant microemulsion with a particle size less than 100 nm and an improved solubility of hydrophobic drug(s) can enhance absorption across the gastrointestinal tract [147]. Cui and co-workers [148] reported that formation of curcumin-loaded SMEDDS enhances the absorption of curcumin not only due to spontaneous formation of a microemulsion in the gastrointestinal tract but also because the surfactants present therein can reduce the interfacial surface tension and enhance penetration of curcumin into the epithelial cells. Furthermore, as there are a lot of lymphatic tissues such as Peyer’s patches and microfold cells in the rat intestine and because the microemulsion droplets produced from SMEDDS in the perfusion can be uptaken
via the lymphatic tissues [149], absorption via these lymphatic tissues could be an important route for curcumin as well.

### 13.6.3 Liposomes, Micelles and Phospholipid Complexes

Liposomes are excellent DDS since they can carry both hydrophilic and hydrophobic molecules. Li and co-workers [150] investigated the *in vitro* and *in vivo* anti-tumour activity of liposomal curcumin against human pancreatic carcinoma cells and demonstrated that liposomal curcumin inhibits pancreatic carcinoma growth and in addition exhibits antiangiogenic effects.

In a study on preclinical anti-cancer activity of a liposomal curcumin formulation in colorectal cancer, the efficacy of liposomal curcumin was compared with that of oxaliplatin, a standard chemotherapeutic agent for colorectal cancer. A synergism was established between liposomal curcumin and oxaliplatin at a ratio of 4:1 in LoVo cells *in vitro*. Significant tumour growth inhibition was observed in Colo205 and LoVo xenografts, *in vivo*, and the growth inhibition by liposomal curcumin was greater than that for oxaliplatin in Colo 205 cells. The study established the comparable or greater growth inhibitory and apoptotic effects of liposomal curcumin in relation to oxaliplatin both *in vitro* and *in vivo* in colorectal cancer [116].

Ruby and co-workers [151] also reported the anti-tumour and antioxidant activities of neutral unilamellar liposomal curcuminoids in mice. Nevertheless, *in vivo* preclinical studies are warranted to show the increased bioavailability of liposomal curcumin over free curcumin. Kunwar and co-workers [152] evaluated the *in vitro* cellular uptake of liposomal curcumin and albumin-loaded curcumin. They found that liposomal vehicle is capable of loading more curcumin into cells than either human serum albumin or aqueous DMSO, and lymphoma cells showed a preferential uptake of curcumin compared to lymphocytes.

Micelles and phospholipid complexes can improve the gastrointestinal absorption of natural drugs, thereby contributing to higher plasma levels and lower kinetic elimination resulting in improved bioavailability. The intestinal absorption of curcumin and micellar curcumin formulation with phospholipid and bile salt evaluated using an *in vitro* model consisting of everted rat intestinal sacs suggested its biological transformation during absorption. Furthermore, the *in vitro* intestinal absorption of curcumin was found to increase from 47% to 56% when the same was present in micelles [153]. Pharmacokinetic studies by Ma and co-workers [154] also demonstrated that a polymeric micellar curcumin gave a 60-fold higher biological half-life for curcumin in rats compared to curcumin solubilised in a mixture of dimethylacetamide, polyethylene glycol (PEG) and dextrose. Phospholipid complex formulations
of several natural drugs, such as silymarin [155] and dolichol [156], have been found to show improved bioavailability. Liu and co-workers [135], for example, showed a significant improvement in curcumin bioavailability due to curcumin-phospholipid complex formation. In this study, curcumin (100 mg/kg) and curcumin-phospholipid complexes (corresponding to 100 mg/kg of curcumin) were administered orally to Sprague-Dawley male rats. The curcumin-phospholipid complex showed a maximum plasma curcumin level of 600 ng/ml 2.33 hours after oral administration as opposed to that of free curcumin having maximum plasma concentration of 267 ng/ml after 1.62 hours of oral dosing. About a 1.5-fold increase in curcumin half-life in rats was found in this study for the curcumin-phospholipid complex over free curcumin. The above studies indicate that the curcumin-phospholipid complex can significantly increase the circulating levels of presumably active curcumin in rats [135]. Another study conducted by Maiti and co-workers [121] showed a threefold increase in aqueous solubility and a better hepatoprotective effect of a curcumin-phospholipid complex compared to free curcumin. In an attempt to increase the aqueous solubility of hydrophobic drugs, Letchford and co-workers [157] showed a $13 \times 10^5$-fold increase in curcumin solubility in a polymeric micellar formulation containing methoxy poly(ethylene glycol)-block-polycaprolactone diblock copolymers. The enormous increase in the solubility of curcumin in the above-said micelle makes it a promising formulation to be explored further.

13.6.4 Nanoparticulate Delivery System

Nanoparticle-based delivery systems will probably be suitable for highly hydrophobic agents like curcumin, circumventing the pitfalls of poor aqueous solubility. However, very few studies have been published citing curcumin nanoparticles. This approach has been used to deliver natural products such as coenzyme Q$_{10}$ [28], estradiol [158] and ellagic acid [30] and chemotherapeutic agents such as paclitaxel [31] and doxorubicin [32]. In fact, a nanoparticle formulation of paclitaxel in which serum albumin is included as a carrier (Abraxane) has been approved for the treatment of breast cancer [33]. Furthermore, silk fibroin-derived curcumin nanoparticles have been reported to exhibit higher efficacy against breast cancer cells [35]. Same authors also describe a biodegradable curcumin nanoparticulate formulation based on poly(lactide-co-glycolide) (PLGA) and a stabiliser PEG-5000 that exhibits enhanced cellular uptake and increased bioactivity in vitro and superior bioavailability in vivo in comparison to free curcumin.

A study by Bisht and co-workers [159] reported the synthesis, physicochemical characterisation and cancer-related application of a polymer-based nanoparticle of curcumin called ‘nanocurcumin’ with less than 100 nm size. Nanocurcumin was found to have similar in vitro activity as that of free curcumin in pancreatic cell lines. Free curcumin and nanocurcumin also inhibited the activation of the transcription factor NF-κB,
and reduced the steady-state levels of proinflammatory cytokines like IL and TNF-α. However, the authors neither determined the in vivo effect of nanocurcumin in mice nor its biodistribution to show any potential increase in the efficacy of nanocurcumin over free curcumin in vivo. In another study reported by Shaikh and co-workers [160], curcumin-loaded PLGA nanoparticles were prepared by emulsion-diffusion-evaporation method. The authors found a significant increase ($p < 0.001$) in the AUC (area under the curve) value of the nanoparticulate formulation in comparison to that of curcumin suspension and curcumin with piperine, indicating the improved bioavailability with the nanoparticulate systems. A comparison of relative bioavailability exemplifies 26 times enhancement in the bioavailability of nanoparticulate formulation in comparison to free drug. In furtherance with the above, they also report $T_{\text{max}}$ to be 2 hours with a sustained release attained up to 48 hours in plasma in comparison to free curcumin, where there were no detectable levels beyond 6 hours [160].

### 13.6.5 Solid Lipid Nanoparticles

SLN loaded with curcuminoids for topical application were developed and characterised by Tiyaboonchai and co-workers [8]. However there was no improvement in the in vitro release profile of curcumin from SLN in comparison to free curcumin (70% release in 12 hours by SLN versus 90% release in 8 hours by pure curcumin). However, the light and oxygen sensitivity of curcuminoids was markedly reduced by incorporating curcuminoids into this unique type of formulation. An in vivo study with healthy volunteers revealed improved efficiency of a topical application cream containing curcuminoid-loaded SLN over that containing free curcuminoids [8]. Wang and co-workers reported the synthesis of 3′,5′-dioctanoyl-5-fluoro-2′-deoxyuridine (DO-FUdR) to overcome the limited access of the drug 5′-fluoro-2′-deoxyuridine (F UdR) and its incorporation into SLN. The brain area under the concentration/time curve of DO-FUdR-SLN and DO-FUdR was 10.97- and 5.32-fold higher than that of FUdR, respectively. These results indicated that DO-FUdR-SLN had a good brain targeting efficiency in vivo. These authors report that SLN can improve the ability of the drug to penetrate across the BBB and is a promising drug targeting system for the treatment of CNS disorders [54]. Indeed no studies have been tried to show the therapeutic efficacy of drug-loaded solid lipid particles administered orally.

### 13.7 Curcumin-loaded Solid Lipid Nanoparticles for Oral Administration

SLN consist of spherical solid lipid particles in the nanometre range dispersed in water or in aqueous surfactant solution. They are generally made up of solid hydrophobic core having a monolayer of phospholipid coating. The solid core may contain the drug
dissolved or dispersed in the solid high-melting fat matrix with the hydrophobic end of the phospholipid chains embedded in the fat matrix. Thus, SLN have the potential to carry lipophilic or hydrophilic drug(s) or diagnostics [58–60].

Various mechanisms for nanoparticle-mediated drug uptake by the brain have been discussed by Chen and co-workers [138]. These include (i) enhanced retention in the brain-blood capillaries, with an adsorption on to the capillary walls, resulting in a high concentration gradient across the BBB; (ii) opening of tight junctions due to the presence of nanoparticles; and (iii) transcytosis of nanoparticles through the endothelium. Maintenance of few peculiar properties of SLN, prove them to be an apt system for targeting across the BBB. For example, SLN with particle size below 200 nm have an increased blood circulation, thereby leading to an increase in the time for which the drug remains in contact with BBB and for the drug to be taken up by the brain [138, 161, 162]. The chemical nature of the overcoating surfactant is of importance, as only polysorbate-coated particles were found to show a pharmacological effect in the CNS while a coating with poloxamers (184, 188, 388, 407), poloxamine 908, cremophors (EZ or RH40) or polyoxyethylene (23)-laurylether was not effective [163]. The reported mechanism of action was the transport of polysorbate-coated nanoparticles across the BBB via endocytosis by the brain capillary endothelial cells. Among all the surfactants being investigated, polysorbates have the highest potential to deliver the SLN to the brain [164]. In view of the above, we prepared SLN of curcumin (C-SLN) using microemulsification method. To explain in brief, polysorbate 80, 4% soy lecithin solution and water (as per preoptimised formulae) were placed together in a beaker and heated to the lipid melt temperature. Lipid was also melted (82–85 °C). Curcumin was added to the aqueous phase containing polysorbate 80, following which the hot aqueous emulsifier mix was dropped at once into the lipid melt under magnetic stirring to obtain a clear microemulsion. The hot microemulsion thus formed was transferred into an equivalent amount of cold water (º2 °C) under continuous mechanical stirring (5000 rpm) for 1.5 hours. In the aqueous medium, SLN are formed by crystallisation of the oil droplets present in the microemulsions [165]. The prepared SLN were stored in a refrigerator until further analysis. The prepared SLN were characterised for particle size (by transmission electron microscopy (TEM)), encapsulation efficiency and in vitro release. The pharmacokinetics of the prepared SLN after oral administration were examined using a validated liquid chromatography-tandem mass spectrometry (LC/MS/MS) method.

Drug encapsulation efficiency of the developed SLN of curcumin was found to be 81.92 ± 2.91% (n = 6). Glyceryl behenate as a lipid for the preparation of SLN has been reported to show high loading capacities of up to 86.5% [166, 167]. High encapsulation efficiency confirms the suitability of the formula and the method of preparation of SLN.
The average particle size of C-SLN was found to be $134.6 \pm 15.4$ nm when measured using laser diffraction (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). When observed under TEM, SLN were found to be spherical in shape. The size of the nanoparticles observed under TEM however was much smaller in the range of 40–120 nm than the results obtained using photon correlation spectroscopy. This however may be attributed to the fact that Mastersizer is based on the principle of laser diffraction, which unfortunately may not detect the particles below 100 nm, due to the Brownian movement of the particles (Figures 13.6 and 13.7).

The release of curcumin from C-SLN was fitted to a first-order kinetics model and was found to occur by diffusion. The release was prolonged up to 7 days with $85.90 \pm 2.47$%
being released within this duration (Figure 13.8). Almost 47.16 ± 5.97% of the drug was released in the initial 2 days; however, the release was delayed in the later stage. The initial release may be by diffusion from the shell of the SLN, while the subsequent phase of prolonged release may be attributed to the fact that the curcumin dispersed within the core will be released slowly from the solid matrices of lipid through diffusion and dissolution. Recently, Shaikh and co-workers [159] reported a prolonged (22 days) release of curcumin from polymeric nanoparticles; however, only 43% of the total drug entrapped within SLN was released in 22 days.

![Figure 13.8 In vitro release profile of curcumin, from C-SLN, by dialysis method. A 50% ethanol solution was used as the release medium. Data represent the mean ± standard deviation of three tests.](image)

**Figure 13.8 In vitro release profile of curcumin, from C-SLN, by dialysis method. A 50% ethanol solution was used as the release medium. Data represent the mean ± standard deviation of three tests**

### 13.7.1 Pharmacokinetics of Curcumin-loaded Solid Lipid Nanoparticles

Low serum and tissue levels are observed for curcumin, irrespective of the route of administration. This is attributed to poor absorption, extensive intestinal and hepatic metabolism and rapid elimination, all of which restrain the bioavailability of curcumin [6, 16, 168]. Numerous pharmacokinetic studies in humans and rats as discussed above have reported very low serum and plasma concentrations. We report here a pharmacokinetic study conducted by us, using a low concentration of 50 mg/kg of prepared C-SLN versus free curcumin.

Plasma levels of curcumin after oral administration of C-SLN (50 mg/kg) were compared with the levels achieved with the same concentration of free curcumin (using Tween-80 as solubilising agent; C-S). The mean curcumin concentrations in the plasma after oral administration of a single dose of C-SLN and C-S in Wistar rats determined using a highly validated and a sensitive LC/MS/MS method are illustrated
Figure 13.9 Mean plasma concentration-time plot of curcumin in rat after a single oral dose of C-SLN at very high dose (VH-50 mg/kg) and solubilised curcumin; C-S/Cmn (50 mg/kg) (n = 3). Values for C-SLN are significantly different from those of C-S at all the corresponding time points

in Figure 13.9. Oral administration of C-S resulted in a sharp $C_{\text{max}}$ (0.292 $\mu$g/ml) within 15 minutes, after which the plasma concentration declined rapidly, indicating a rapid metabolism of curcumin. In contrast, a relatively slow increase and sustained plasma concentration of curcumin for a longer time was observed after administration of C-SLN. A very low volume of distribution (7.72 ± 0.43 l/kg) (53 times lower than C-S) and a significantly ($p < 0.05$) high $C_{\text{max}}$ of 14.293 $\mu$g/ml at 0.5 hours, which was still detectable at 24 hours (0.012 $\mu$g/ml), suggest a sustained effect of C-SLN. There was a marked difference in the AUC$_{0-\infty}$ between C-S and C-SLN at all doses. The AUC$_{0-\infty}$ for C-SLN was appreciably higher (39 times) at 50 mg/kg dose of C-SLN versus C-S when administered orally to rats. Liu and co-workers [135] reported similar $C_{\text{max}}$ values (0.266 $\mu$g/ml) for free curcumin; however, these levels were achieved at 100 mg/kg dose, which is double the dose used by us (50 mg/kg: $C_{\text{max}}$ 0.292 $\mu$g/ml). Yang and co-workers [120] used a 10 times higher dose (500 mg/kg: $C_{\text{max}}$ 0.060 $\mu$g/ml) and Pan and co-workers [16] used a 20 times higher dose (1 g/kg: $C_{\text{max}}$ 0.220 $\mu$g/ml) and the $C_{\text{max}}$ values recorded were either the same or lower. It may be concluded that either the method used in the present study is more accurate and sensitive, or curcumin does not follow dose-dependent kinetics. Furthermore, the solubilisation of curcumin with Tween-80 may enhance its absorption/permeation and hence bioavailability.

Liu and co-workers [135] achieved a $C_{\text{max}}$ of 0.6 $\mu$g/ml with the prepared phospholipid complexes of curcumin. Yang and co-workers [120] showed that 10 mg/kg of curcumin given IV in rats gave a maximum serum curcumin level of 0.36 ± 0.05 $\mu$g/ml. Multiplying the dose with a factor of 5 (to have an arbitrary value for a 50 mg/kg dose) would mathematically result in a $C_{\text{max}}$ of 1.8 $\mu$g/ml. Envisaging the above, our results are highly appreciable, as oral administration of C-SLN at 50 mg/kg could achieve a $C_{\text{max}}$ of
14.20 μg/ml, which has not been achieved even with a similar dose (as discussed above) when given by IV route. Furthermore, even a 40 times higher oral dose of 2 g could achieve a $C_{\text{max}}$ of only 1.35 ± 0.23 μg/ml in an earlier study [125]. This enhanced bioavailability of C-SLN might be attributed to enhanced permeability and decreased clearance. As the average particle size of nanoparticles is maintained below 200 nm, it helps bypassing the first-pass metabolism in the liver, which has been reported to be the major site of curcumin degradation. In addition, the surfactants, such as Tween-80 and lecithin, have contributed to an increase in the permeability of the intestinal membrane and/or improve the affinity between lipid particles and intestinal membrane, and may also exhibit bioadheson to the gastrointestinal tract wall [159]. Also, embedding curcumin into a solid lipid matrix not only reduces the enzymatic degradation during the exposure of SLNs but may also enhance the process of absorption across cellular membrane and also the BBB. Lastly, C-SLN could provide curcumin with long circulation times and a reduced clearance from systemic circulation, resulting in better bioavailability.

### 13.7.2 Apoptotic Effect of Curcumin-loaded Solid Lipid Nanoparticles on Human Neuroblastoma Cell Lines

Studies on human neuroblastoma cells (obtained from National Cancer Institute, Frederick, MD, USA) clearly indicate the advantage of incorporation of curcumin into SLN. The extent of cell death induced by C-SLN was significantly higher ($p < 0.05$) at all the tested doses. Possible reasons for the better effect could be that (i) curcumin is being presented in a soluble form for interaction with the cancerous cells and (ii) permeability of C-SLN into the neuroblastoma cells is better. The results
reveal enhanced (4 times) apoptotic effects of C-SLN (10 μM) over free curcumin (10 μM), indicating the superiority of the developed SLN (Figure 13.10).

13.7.3 Curcumin-loaded Solid Lipid Nanoparticles across Blood-brain Barrier

To evaluate the ability of C-SLN to cross the BBB, an in vivo experiment was designed. Animals were divided into two groups: one group served as control (n = 3) while the other group was the treated group. The treated group was administered 1 ml of C-SLN, while the control was administered 1 ml of C-S (50 mg/kg). One hour after peroral administration, blood was withdrawn, following which the animals were sacrificed and their brains were harvested. The brain samples were frozen at –80 °C for 4 days, after which cryosections were obtained. Plasma samples and brain cryosections were then observed under a fluorescent microscope (Nikon, Eclipse 80i).

**Figure 13.11** Fluorescent micrograph of rat plasma 1 hour after peroral administration of (a) C-SLN, (b) C-S and (c) C-SLN, dispersion as such

**Figure 13.12** Fluorescent micrograph of rat brain cryosection 1 hour after peroral administration of (a) C-SLN and (b) C-S (solubilised curcumin)
Yellow fluorescent C-SLN are clearly observable in plasma (Figure 13.11) as well as in brain sections of rats administered C-SLN in comparison to animals administered C-S (Figure 13.12).

The studies give a direct evidence of effective delivery of intact C-SLN to the brain and confirm that they maintain their integrity while passing across the gastric mucosa (upon peroral administration) and in plasma.

13.8 Conclusions

The usefulness of natural molecules like curcumin is limited in therapy because of their poor physicochemical characteristics. Curcumin is a multitargeted molecule showing pluripharmacology such that it manifests several anti-cancer mechanisms complementing the overall effects. In spite of wondrous in vitro success, data on curcumin and its clinical effectiveness are still lacking. The ambiguity in translating in vitro and preclinical effectiveness to humans and clinics points towards a need for pharmaceutical couturing of curcumin. The significance of this statement can be understood from a comprehensive review (presented in the chapter) of anti-cancer effects of curcumin, wherein all the pharmacological and clinical studies show its effectiveness for gut-related cancers only. This is as expected, considering that curcumin is a very poorly absorbed drug (less than 1%) and is excreted unchanged in the faeces. Hence its local concentration in the gut is sufficient to show this effect. Similarly, it has also shown its clinical effectiveness upon local application to skin.

In view of the above, we present various strategies that have been tried for bioavailability enhancement of curcumin. Most of these studies however lack pharmacokinetic proof of achieving improved absorption. Further to this, delivering any molecule across the BBB has its own limitations such that developing curcumin for brain tumours can be a marathon task. Moreover, no in vivo study and only a few in vitro studies report on the use of curcumin for brain tumours. In fact, the effectiveness of this molecule for neurodegenerative diseases is well established in animal studies. This may also be attributed to its poor bioavailability such that systemically available curcumin and the amount of drug reaching CNS is sufficient to protect (as required for neurodegeneration) but insufficient to elicit apoptotic effect desired for killing cancerous cells.

In the end, we present an SLN system developed by us and also give evidence for improved plasma kinetics and delivery of curcumin to brain; significantly better apoptotic effect of the developed system against human neuroblastoma cell lines also confirms the potential of the developed system.
Bioavailability Enhancement and Brain Targeting of Curcumin

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Bioavailability Enhancement and Brain Targeting of Curcumin


Bioavailability Enhancement and Brain Targeting of Curcumin


14

Lipid Nanomedicines for Eye Cancer Treatment

Maria L. García López, Saša Nikolić, Joana R. Araújo, Elisabet González Mira and Maria A. Egea

14.1 Anatomy and Physiology of the Eye

The eye is the primary organ of vision and is embryologically an extension of the central nervous system, sharing many common anatomical and physiological properties with the brain and being affected by similar disease processes.

The human eye has a spherical shape with an average diameter of 23 mm. The structural components of the eyeball are divided into three layers or coats and three compartments. The outermost coat comprises the clear transparent cornea and the white, opaque sclera; the middle layer comprises the iris in the anterior, the choroids in the posterior and the ciliary body; and the inner layer is the retina – composed of pigment epithelium, photoreceptors and neurons. The three compartments of the eye are as follows: anterior chamber – the space between the cornea and the iris diaphragm; posterior chamber – the triangular space between the iris in the anterior, the lens and zonule in the posterior and the ciliary body; vitreous chamber – the space behind the lens and zonule. Finally, the intraocular fluids are the aqueous humour – an optically clear solution of water, electrolytes and low protein content the vitreous humour – a transparent gel consisting of a three-dimensional network collagen fibres with the interspaces filled with polymerised hyaluronic acid molecules and water.

The role of the ocular surface, which is dependent on adjacent structures such as the anterior lamellae of the lids, the lashes and the lachrymal system, is to maintain optical clarity of the cornea by regulating the hydration and to protect the globe from mechanical, toxic and infectious trauma [1].

The eyelids are the first line of defence of the eye and provide a movable mucosal lining that covers the entire ocular surface. Structurally, the eyelid is composed of seven layers, being the region in opposition to the globe the most commonly involved in ocular surface pathology.
The conjunctiva is an ectodermally derived mucosal and nonkeratinised epithelium that extends from the mucocutaneous junction of the eyelid margins to the corneoscleral limbus. The thickness varies regionally from 2 to 3 cell layers in the tarsal and fornical conjunctiva to 6–9 layers in the bulbar conjunctiva, containing 5–10% globet cells that produce mucin – a carbohydrate-rich substance important for the maintenance of the tear film. The conjunctiva is the conduit for topically applied drugs to reach the posterior segment of the eye [2].

The cornea is the portal through which visual information from the environment enters the eye. As such, it is a highly specialised optical tissue that must maintain transparency to visible light and resist adverse external forces. Structurally, cornea is an avascular tissue consisting of five layers, the outermost being a nonkeratinised, stratified squamous epithelium, approximately 5–7 cells thick. The basement membrane of the corneal epithelium is attached to Bowman’s layer, which is a thick, fibrous, largely acellular tissue, composed of collagen. The middle layer is the stroma that constitutes a major part of the cornea. It also consists of collagenous lamellae and is considered the hydrophilic component. Descemet’s membrane, the thin layer between the posterior stroma and endothelium, regulates the hydration status of the stroma. Finally, the corneal endothelium, a monolayer of squamous epithelium, plays a critical role in corneal hydration and transparency. These last two layers are both not found to act as transport barriers to drug absorption.

The limbus is the anatomical transition of the sclera and conjunctival epithelium into the cornea and is believed to be the location of the epithelial stem cells of the cornea. At the limbus, the stratified columnar conjunctival epithelium moves to the stratified squamous epithelium of the cornea and the vascular substantia propria of the conjunctival epithelium ends in a rich vascular limbal plexus, important in providing nutrients and oxygen to the mitotically active limbal stem cells. It is clear that healthy stem cells are required to maintain a normal corneal epithelium and to prevent overgrowth of the cornea by conjunctival epithelium [3].

The lens, like the cornea, is transparent and avascular. It is comprised of 65% water and 35% protein and has a thick elastic capsule that prevents molecules from moving into or out of it. The lens is suspended from the ciliary body by the zonule. The ciliary body consists of longitudinal and circular smooth muscle fibres. Accommodation is the process by which the relaxation of zonular fibres allows the lens to become more globular, thereby increasing its refractive power. On the contrary, when the ciliary muscles relax, the zonular fibres become taut and flatten the lens, reducing its refractive power. This is associated with constriction of the pupil and increased depth of focus.

The lachrymal gland is a specialised sebaceous gland located superotemporal to the globe that secretes tears in response to parasympathetic stimulation. Drainage
of these tears involves a number of different mechanisms and physical factors, such as gravity and evaporation. A decisive role is played by capillary attraction, aided by contraction of the lachrymal portion of the orbicularis muscle with blinking, as well as distension of the lachrymal sac by the action of the orbicularis muscle [4].

The roles of the tear film are to provide a smooth uniform refractive surface, to lubricate the ocular surface and thus to facilitate comfortable movement of the lids while minimising mechanical or environmental trauma and to defend against microbial invasion. It is formed by a trilaminar structure. Mucin forms the layer most proximal to the corneal surface and provides a hydrophilic film upon which aqueous tears – secreted by lachrymal glands – layer. The aqueous surface is then covered by lipids – secreted from meibomian glands – essential to retard evaporation and prevent early break-up of the tear film [5]. Stability of the tear film requires not only the maintenance of the precise ratio of the three components (mucin, water and lipids) but also normal blinking, which redistributes the tear film in a uniform layer, promotes secretion and pumps excess tears into the lachrymal sac [6].

The retina is the ‘photographic film’ of the eye that converts light into electrical energy (transduction) for transmission to the brain. It consists of the neuroretina and retinal pigmented epithelium (RPE). The retinal photoreceptors are located on the outer aspect of the neuroretina, an arrangement that arises from inversion of the optic cup and allows close proximity between the photosensitive portion of the receptor cells and the opaque RPE cells, which reduce light scattering. The RPE also plays an important role in regeneration/recycling of photopigments of the eye and during light-dark adaptation. There are two main types of photoreceptors in the retina – the rods and the cones. The rods are responsible for vision in poor light and for the wide field vision and the cones are responsible for acute and colour vision.

The blood supply of the globe is derived from three sources: the central retinal artery, the anterior and posterior ciliary arteries. All of these are derived from the ophthalmic artery, which is a branch of the internal carotid.

Normal function of the retina requires normal retinal and choroidal circulation. The development of blood supply is intimately linked with progress of intraocular structures, developing a complex network in unison with oxygen demands and local tissue gradients of vasoactive growth factors [7].

An important normal physiological function of the retinal vasculature is maintenance of the inner blood-retinal barrier, which prevents nonspecific permeation of the retinal neuropile by macromolecules yet facilitates exchange of respiratory gases,
amino acids, salts, sugars and some peptides. Furthermore, retinal capillaries possess an array of pumps that assist the removal of excess fluid and waste products from the extracellular space into the retinal circulation. The endothelial cells of the retinal vessels form a continuous, nonfenestrated monolayer, with each cell being fused to juxtaposed neighbours by zonulae occludens, tight junctions that maintain barrier function [8].

The optic nerve meets the posterior part of the globe at a point seen inside the eye as the optic disc. It contains about one million nerve fibres, each of which has a cell body in the ganglion cell layer of the retina. Apart from the optic nerve, the posterior pole of the globe is also perforated by several long and short ciliary nerves that contain parasympathetic, sympathetic and sensory fibres, which mainly supply muscles of the iris (dilator and sphincter) and ciliary body (ciliary muscles).

14.2 Neoplastic Diseases of the Eye

Cancer can affect all the three parts of the eye: the eyeball, the orbit and the adnexal structures. An eye neoplasm can be classified as primary or metastatic.

The primary eye cancers comprise eyeball, orbital, eyelid and lachrymal gland tumours. Among the ocular cancers in adults, melanoma is the most common, followed by lymphoma. Melanomas develop from melanocytes. Primary ocular melanoma can involve the uveal tract, conjunctiva, eyelid or orbit. Uveal melanoma is the most common ocular melanoma. Conjunctival melanoma manifests on the surface of the eye and has been increasing in incidence. Eyelid and primary orbital melanoma are the least common variants [9]. Lymphoma is a type of cancer that starts in immune system cells called lymphocytes. There are two main types of lymphoma – Hodgkin’s disease and non-Hodgkin’s lymphoma. Primary intraocular lymphoma is always a non-Hodgkin’s lymphoma. The most common sites of ocular involvement are the vitreous, retina, sub-RPE, the optic nerve head or any combination of these sites [10]. In children, retinoblastoma, arising from cells in the retina, is the most common primary intraocular cancer [11].

The improved survival of patients with common cancers (breast cancer, skin cancer or lung cancer) together with better vigilant surveillance and advances in diagnostic techniques have led to increased detection of eye metastases. Breast carcinoma accounts for the majority of metastatic lesions to the orbit and ocular adnexa [11–15]. Cutaneous malignancies include basal cell carcinoma, squamous cell carcinoma, sebaceous cell carcinoma and malignant melanoma, but the majority of cases that result in ocular metastasis, ocular morbidity and mortality are from sebaceous cell
carcinoma and malignant skin melanoma [16]. Regarding lung cancer, metastases most frequently originate from adenocarcinomas and the choroid is the most common site of metastases [17–19].

Detailed classification and description of ophthalmic cancers is out of the scope of this book. For more information, specialised texts are recommended [20, 21].

### 14.3 Treatment of Ophthalmic Cancers

Since ocular cancers were first recognised, their treatment has gradually evolved. In the early years, enucleation, evisceration, exenteration, choroidectomy and iridocycllectomy were often performed [22–25]. Since that time, radiation therapy alone, systemic chemotherapy alone or a combination of the two has been used extensively [26–28].

Although radiation and surgery remain the main treatments of ophthalmic malignancies, chemotherapy has come to the forefront of ophthalmology with recent advances in the treatment of retinoblastoma [29]. Often, chemotherapy is employed to reduce morbidity and improve the efficacy of radiation and surgery [30, 31].

It has been reported that epithelial neoplasia of the eyelid, conjunctiva and cornea are responsive to chemotherapy. 5-FU has been successfully used to treat conjunctival squamous carcinoma, leading to a complete resolution of the disease [32–34].

Intensive chemotherapy, including thiotepa, melphan or cyclophosphamide, was found potentially curative for some patients with trilateral retinoblastoma, an ocular neoplasm lethal in virtually all cases reported earlier [35].

Due to inefficient drug delivery to the eye, enucleation still remains the definitive curative treatment for retinoblastoma in most cases, although progress has been made in tumour control by intraarterial chemotherapy [36].

Subsequent malignant neoplasms are a major cause of premature death in survivors of hereditary retinoblastoma. Radiotherapy may further increase the risk of death [37, 38]. It has been reported that secondary malignancies developed within the radiation field, where the osteosarcoma was the most frequently occurring secondary neoplasm [39].

In 1996, there were four reports regarding chemotherapy for retinoblastoma, termed chemoreduction [40]. It was shown that intravenous delivery of a combination of three
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to four chemotherapeutics for more than several months could provide control of retinoblastoma without any additional treatment, such as external beam radiotherapy.

Recently, bilateral intraarterial chemotherapy for bilateral retinoblastoma based on melphan alone or in combination with topotecan, has been proven to be both efficient and safe for the patient; it can avoid the use of multiagent systemic chemotherapy, enucleation and/or radiation [41–43]. This therapeutic approach is still invasive for the patient and has to be performed under anaesthesia, while it would be early to make any definitive conclusions regarding the long-term efficacy or risks.

It has been reported that subconjunctival injections of carboplatin may be potentially effective in controlling retinoblastoma in animal models [44]. The same study suggests that subconjunctival carboplatin injections may control tumours in a dose-dependent manner. Tumour control was observed in 50% of treated eyes, in the animal model. Therefore, periocular chemotherapy of retinoblastoma is promising, but its role is still to be defined.

Topotecan is a promising drug with activity against retinoblastoma; however, attaining therapeutic concentrations in the vitreous humour is still a challenge for the treatment of vitreous seeds in retinoblastoma. In a recent study, intravitreal topotecan administration was found to be a possible alternative route for enhanced drug exposure in the vitreous humour, while avoiding systemic toxicities [45].

Uveal melanoma is the most common primary intraocular malignant tumour in adults. To date, there is no convincing evidence that a consensual and effective chemotherapy can have a major role in the treatment of primary choroidal uveal melanoma [46] that often results not only in vision loss but also in metastatic death. Regardless of the primary treatment modality, radiotherapy or enucleation of the eye, risk of metastasis is increased and prognosis is poor in patients of more advanced age, with large tumours or with tumours that involve the ciliary body or exhibit extrascleral extension. Currently, there is no effective treatment for metastatic uveal melanoma [47], although there are first results of preliminary studies using innovative systems, such as liposomes, to deliver already known drugs [48]. Despite some promising preclinical data, imatinib mesylate resulted inactive as a single agent in the treatment of metastatic uveal melanoma [49].

Leukaemic infiltrations of intraocular structures occur in approximately 3% of affected patients, where myeloid leukaemias, more than lymphocytic leukaemias, are more likely to affect the eye. The involvement of the iris, vitreous, choroid, retina, optic nerve head and RPE has been described [50–56]. Nevertheless, most of the chemotherapy protocols for leukaemia treatment do not currently address the eye, because of the low incidence of ocular involvement. There are few reports on the use
of traditional chemotherapy for ocular leukaemia, suggesting that external radiation therapy remains the treatment of choice [57, 58].

Among intraocular lymphomas, comprising vitreoretinal, primary choroidal, primary iridal and secondary uveal lymphoma, the vitreoretinal lymphoma is the most common and the most aggressive one. Since intraocular lymphoma was first recognised, its treatment has evolved from enucleation, through radiation and/or systemic chemotherapy to intravitreal chemotherapy and biological therapy. The aim of this therapy is not only the eradication of the intraocular disease but also the prevention of central nervous system and/or systemic relapse. The most common drug in use for chemotherapy of vitreoretinal lymphoma is methotrexate. When given systemically, methotrexate concentration in the vitreous was found to be 100-fold lower than in the serum, explaining the only partial response [59]. Other drugs, such as ifosfamide, trofosfamide, etoposide, cytarabine, bisulfan and cyclophosphamide, given systemically, led to a partial response or relapse in most of the patients [60–64]. Although an optimal approach in the treatment of intraocular lymphomas has not been identified yet, promising results have been reported for intravitreal chemotherapy using injections of methotrexate [65–67]. In order to overcome the problem of intraocular lymphoma that is resistant to methotrexate therapy, a biological therapy using rituximab has been proposed [68, 69]. Although the preliminary results look promising, more studies will be needed in order to establish a consolidated treatment protocol that would use rituximab in the therapy of vitreoretinal lymphoma.

14.4 Ocular Pharmacokinetics: Challenges and Obstacles

Pharmacology, the study of agents and their actions, can be divided into two branches. Pharmacodynamics is concerned with the effects of a drug on the body and, therefore, encompasses dose-response relationships as well as the molecular mechanisms of drug activity. Pharmacokinetics, on the other hand, is concerned with the effect of the body on the drug. Drug metabolism, transport, absorption and elimination are components of pharmacokinetic analysis.

A drug is not effective unless it is present at its site of action for an adequate period of time. The development of pharmacokinetic principles has enabled the rationalisation of changes in drug concentration that occur in various tissues in the body after administration and the ability to design dosage schedules that maximise drug effectiveness (and minimise drug toxicity) have allowed the use of novel molecular medicines. With new delivery technologies that are designed for particular agents and tissue sites, one can avoid undesirable pharmacokinetic properties, the design of delivery system and drug being intimately related.
Drug diffusion is an essential mechanism for drug dispersion throughout biological systems and can be used as a reliable mechanism for drug delivery. Rates of diffuse transport of molecules vary among biological tissues within an organism, even though their content may be similar (predominantly water). In general, the diffusion coefficient in tissues is significantly slower than the diffusion coefficient for the same solute in water.

The goal of pharmacokinetics is synthesis of isolated basic mechanisms into a functional unit, most often achieved by development of a mathematical model that incorporates descriptions of the uptake, distribution and elimination of a drug in humans or animals. This model can then be used to predict the outcome of different dosage regimens on the time course of drug concentrations in tissues.

For most drugs, pharmacokinetic parameters are estimated in plasma. Drug concentrations in plasma samples can be easily quantified and pharmacokinetic properties routinely calculated. However, for ophthalmic drugs following topical instillation, intravitreal or subconjunctival injection, pharmacokinetic properties are usually evaluated in different ocular tissues and fluids. It is technically more difficult to quantify drug concentrations. In addition, mechanisms of drug delivery to and clearance from ocular tissues following ocular dosing are not well understood.

In multicellular organisms, thin lipid membranes serve as semipermeable barriers between aqueous compartments. The plasma membrane of the cell separates the cytoplasm from the extracellular space and endothelial cell membranes separate the blood within the vascular space from the rest of the tissue. Properties of the lipid membrane are critically important in regulating the movement of molecules between aqueous spaces. While certain barrier properties of membranes can be attributed to the lipid components, accessory molecules within the cell membrane – particularly transport proteins and ion channels – control the rate of permeation of many solutes. Transport proteins permit the cell to regulate the composition of its intracellular environment in response to extracellular conditions. If the solute does not enter the cell readily, it diffuses primarily within the extracellular space.

Drug delivery to the eye can be broadly classified as delivery into anterior and posterior segments. The anterior segment represents a smaller part of the eye and includes the cornea, conjunctiva, iris-ciliary body, lens and anterior/posterior chambers filled with aqueous humour. In contrast, the posterior segment is the major ocular structure, consisting of three layers, the sclera, choroids and retina, surrounding the vitreous body.

The cornea is often the tissue through which drugs in ophthalmic preparations reach the inside of the eye. Because the structure of the cornea consists of epithelium-stroma-epithelium, which is equivalent to a fat-water-fat structure, the penetration of nonpolar compounds through the cornea depends on their oil/water partition coefficients. The epithelium is generally the rate limiting barrier to transcorneal transport, once the most apical corneal epithelial cells form tight junctions that limit the paracellular
permeation [70]. Therefore, lipophilic drugs have typically at least an order of magnitude higher permeability in the cornea than the hydrophilic drugs. The following are the corneal factors contributing to the efficiency of corneal penetration of topically applied ophthalmic drugs: corneal structure and its integrity, the physical-chemical properties of the applied drug and the formulation in which the drug is prepared.

The fluid systems in the eye – the aqueous humour and the vitreous humour – also play an important role in ocular pharmacokinetics. The aqueous humour has a normal volume of 0.3 ml and is formed by active secretion and ultrafiltration from the ciliary processes in the posterior chamber. The fluid enters the anterior chamber through the pupil, circulates in the anterior chamber and drains through the trabecular meshwork into the canal of Schlemm, the aqueous veins and the conjunctival episcleral veins. The vitreous humour has the same origin as the aqueous, but diffuses through the vitreous body and escapes from the eye through the uveoscleral route.

Once a drug has penetrated the cornea, there are several factors that need to be considered in the ultimate pharmacokinetic description of that drug’s fate: the volume or spaces (tissues) into which the drug distributes; binding of drug in both aqueous humour and tissues; partitioning behaviour of drug between aqueous humour and the various ocular tissues, such as iris, lens and vitreous humour; possible differences in equilibrium time between aqueous humour and the various ocular tissues; possibility of drug metabolism in the eye fluid or tissues; and drug effects to either stimulate or inhibit aqueous humour production and turnover. Following eye drop administration, the peak concentration in the anterior chamber is reached after 20–30 minutes. From the aqueous humour, the drug has an easy access to the iris and ciliary body, where it can bind to melanin and form a reservoir that is released gradually to the surrounding cells, prolonging the activity. Distribution to the tightly packed lens is much slower than the distribution to the porous uvea. The first mechanism has a rate of about 3 μl/min and is independent of the drug. Elimination by the second one, on the other hand, depends on the drug’s ability to penetrate across the endothelial walls of the vessels, which is faster for lipophilic drugs.

The eye is protected from the xenobiotics in the bloodstream by two blood-ocular barriers. The anterior, blood-aqueous barrier is composed of the endothelial cells in the uvea and prevents the access of plasma albumin and hydrophilic drugs into the aqueous humour. Inflammation may disrupt the integrity of this barrier. The posterior, blood-retinal barrier is composed of RPE and the tight walls of retinal capillaries [71]. Although drugs easily gain access to the choroidal extravascular space through its leaky walls, distribution into the retina is limited thereafter. Without specific targeting systems, only a minute fraction of an intravenous or oral drug dose gains access to the retina and choroids.

Scleral anatomy is similar to that of corneal stroma and permeability does not apparently depend on lipophilicity but depends strongly on molecular size. On its external
anterior surface, sclera is covered by conjunctiva which is expected to show preference for lipophilic molecules because it is a cellular tissue. Conjunctival penetration and uptake of topically applied drugs is typically an order of magnitude higher than corneal uptake.

An ophthalmic drug applied to the eye may undergo metabolism when the drug penetrates across ocular-blood barriers into the site of action. Studies involving ocular tissues have demonstrated the expression of diverse enzymes that are involved in various stages of drug metabolism and detoxification, such as oxireductases, hydrolases and conjugating enzymes [72], mainly expressed in the iris ciliary body and retina-choroid, primary entry sites from blood into the eye.

Two specific transporters of the antioxidant vitamin C were identified in ocular tissues [73]. Amino acid transporters were confirmed by gene expression in the cornea; these are important in transferring amino acids from blood for protein synthesis and maintenance of structural and functional integrity [74]. Ocheltree and co-workers [75] demonstrated the expression of peptide transporter, a proton-coupled transporter that helps in the translocation of di- and tripeptides, in human RPE cells. There are also glucose transporters that meet the energy need of oxidative metabolism in retina, but although these are more efficient and have more capacity than any other nutrient transporter, their high specificity for glucose renders them inefficient for the purpose of drug delivery [76].

Efflux of various sterols, lipids, endogenous metabolic products and xenobiotics across plasma membrane and cytoplasm into extracellular fluid is mainly governed by efflux transporters. This (ATP-binding cassette) superfamily of proteins is actively involved in detoxification and consequent development of chemoresistence [77].

Epithelial cells express various nutrient transporters and receptors on their surface, which aid in the movement of various substrates across the cell membrane. Although there is a growing recognition of the significance of the ocular drug-metabolising enzymes in governing the rate and extent of drug delivery to various ocular tissues, from the pharmacokinetic perspective, further research is needed in terms of drug transporter and metabolic enzyme expression for the nature of the barriers to be understood. On the other hand, one can easily conclude that the attachment of various transporters/receptors targeted ligands to the drug delivery system, or the design of drugs which efficiently evade efflux pumps, are strategies that can improve ocular drug bioavailability significantly.

14.5 New Frontiers in Drug Delivery to the Eye: Lipid Nanomedicines

Ocular drug delivery remains a challenge, despite numerous scientific efforts. Traditionally, ocular diseases are treated by topical application of solutions,
suspensions or ointments. For chronic diseases involving the posterior segment, different applications routes are used, such as oral, parenteral, subconjunctival, intracameral, sub-Tenon’s, intrascleral and subretinal routes. Nevertheless, both topical and systemic applications often fail to deliver the drugs in therapeutic concentrations to the interior of the eye, posing many limitations to the treatment of diseases of the posterior segment. It has been determined that both anterior and vitreous levels of drugs can be established from subconjunctival injection [78–83]. Intravitreal administration is the most common approach used to deliver to the posterior segment. The duration of delivery in both cases varies a lot upon type of formulation injected. To date, most of the developed products require frequent injections, jeopardising the therapy outcomes due to poor patient compliance, potential side effects and high costs.

A defective and leaky vascular architecture of the tumour tissue and a usually inadequate interstitial fluid drainage by lymphatic system are two main characteristic of the tumour tissue that offer a rationale of using submicron-sized drug delivery systems for the treatment thereof. Properly engineered carriers may achieve passive tumour targeting, solving the problem of poor tissue specificity of the traditional antineoplastics, at least partly. The biodistribution of these carriers can be manipulated by modifying their surface physicochemical properties, further decreasing systemic drug toxicity.

In order to favourably change and control drug pharmacokinetics, and also, in case of anti-neoplastic agents, to protect the normal tissue against nonspecific toxic action of a drug, novel drug delivery systems are necessary. Lipid-based delivery systems, such as plain and multifunctional liposomes, niosomes, nanoemulsions, lipid-core micelles, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) and lipoplexes are the most advanced and the most promising ones. Anti-cancer therapeutics are known for their heterogeneity in terms of molecular structure and physicochemical properties. In addition to the obvious ability of lipid nanocarriers to incorporate lipophylic drugs, liposomes, lipoplexes and the more recently developed variations of SLN (e.g., polymer-lipid hybrid nanoparticles and lipid-drug conjugates) further expand the role of lipid nanomedicines.

One of the first lipid-based drug nanocarriers are liposomes. Liposomes are artificial phospholipid mono- or multilamellar vesicles capable of incorporating drug molecules. They were first described in 1965 [84]. Depending on its characteristics, the drug will be encapsulated in the core or in the lipid bilayer of the liposome. The size of the liposomes depends on their composition and production method [85]. Small and large unilamellar liposomes are around 100–800 nm in size, respectively. Multilamellar liposomes range in size from 500 to 5000 nm. The size, along with their surface charge, steric stabilisation, dose and route of administration determine the pharmacokinetics and biodistribution of liposomes.
Liposomes can act as carriers for a wide variety of drug molecules, proteins, nucleotides and plasmids endowing them with a great potential for their application in ophthalmics [86]. Being completely biodegradable, liposomes offer advantages over most ophthalmic delivery systems.

One of the drawbacks of using conventional liposomes, that is, with no surface modification, is their fast elimination from the blood by the cells of reticuloendothelial system [87]. PEGylation of the liposomes has been suggested in order to avoid this fast clearance of the particles from the blood [88].

Some anti-cancer formulations based on liposomes have already reached the market, while others are now entering clinical trials. Some examples are Myocet® (doxorubicin), DaunoXome® (daunorubicin), LEP-ETU™ (paclitaxel), LE-SN38™ (irinotecan), DepoCyt® (cytarabine), DOXYL®/Caelyx® (doxorubicin) and Lipoplatin® (cisplatin). A novel liposomal vincristine formulation has been shown to be able to offer complete resolution in case of uveal melanoma [48, 89]. Lipoplatin (cisplatin) is a long-circulating PEGylated liposomal formulation with almost negligible nephrotoxicity, ototoxicity and neurotoxicity, as demonstrated in preclinical and phase I human studies [90]. The potential of liposomes to be used in photodynamic therapy of pigmented choroidal melanomas has been assessed in a study using a liposomal preparation of verteporfin (benzoporphyrin derivative monoacid). The results showed superiority of liposomal formulations in increasing concentrations of the dye in the tumour tissue [91].

When the phospholipids are substituted by blends of nonionic surfactants and cholesterol, structures analogues of liposomes are obtained. They are called niosomes. Niosomes are unilamellar or multilamellar vesicles that can encapsulate both lipophilic and hydrophilic drugs. Niosomes were first reported in the seventies of the last century as a future of the cosmetic industry [92] and have since been studied as drug targeting agents. It has been demonstrated that niosomes, just like liposomes, are able to prolong the circulation of encapsulated drug and to increase the contact time of the drug with the tissue in topical preparations [93, 94]. Chemical stability and lower production costs are some of the advantages of niosomes over liposomes [95], as also in topical ocular delivery. Nevertheless, physical instability during storage, resulting in vesicle aggregation or fusion and leaking of the drug are the major drawbacks of these carriers [96]. Vesicle aggregation may be prevented by inclusion of molecules that stabilise the system against the formation of aggregates by repulsive steric or electrostatic effects. Such approach has been reported in the preparation of doxorubicin sorbitan monostearate niosomes stabilised by the inclusion of cholesterol poly-24-oxyethylene ether [97] and by the inclusion of dicetyl-phosphate in 5(6)-carboxyfluorescein-loaded sorbitan monostearate niosomes [98].
Other relatively new lipid-based nanocarriers are SLN and NLC [99–101]. They are prepared from biocompatible lipids and surfactants and stay solid at room and body temperature. This makes the SLN a potentially optimal generic delivery platform for many actives, including anti-cancer cytotoxic agents. Since the early 1990s, a number of cytotoxic agents were tested for incorporation into SLN. These include doxorubicin [102–105], etoposide [106], idarubicin [107], paclitaxel [108] and others. Nevertheless, the history of SLN and NLC is relatively short, compared to that of other drug delivery systems, for example, liposomes. Therefore, there is still a lack of clinical studies describing in vivo behaviour of SLN or NLC in cancer management. The findings of preclinical studies that use cell cultures and animal models have been promising so far [109]. Nevertheless, the main focus is still far from ocular tumour therapy.

14.6 Conclusions

In the last decade, modern chemotherapy has changed the way of cancer management. However, in the field of ocular cancers, surgery combined with radiotherapy is still predominant over pharmacological treatment, if not the only therapeutic approach in many eye neoplastic diseases. This is because of the incapacity of conventional drug delivery systems to provide therapeutic concentrations of cytotoxic agents inside the eye, due to the anatomical and pathophysiological barriers. Therefore, new drug delivery systems are needed to improve drug targeting to the eye. Nanotechnology can help overcome the anatomical barriers and deliver the active ingredient to the desired site, minimising systemic exposure and severe side effects. Among many nanostructures, those composed of lipids seem the most promising ones. They can be formulated using biocompatible ingredients that have already been approved for intravenous application, therefore saving time and eliminating health concerns intrinsic to the carrier itself. Until now, different cytotoxic anti-cancer agents have been incorporated into lipid-based submicron carriers, but few of them have been tested for ocular cancer management. Nevertheless, based on the preclinical studies, the lipid nanomedicines are likely to offer multiple applications in ocular cancer therapy in the future.

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Lipid Nanomedicines for Eye Cancer Treatment


Lipid Nanomedicines for Eye Cancer Treatment


Lipid Nanomedicines for Eye Cancer Treatment


15 Lipid Nanocarriers for Topical Anti-cancer Therapy: An Update

Clara B. Fernandes, Priyanka S. Prabhu, Dalapathi Guggulothu and Vandana B. Patravale

15.1 Introduction

Cancer drugs can cause enormous toxicity; therefore, the opportunity to deliver them locally creates the possibility of improving both the safety and efficacy of cancer chemotherapy. By using suitable approaches, the drug itself can become more effective when delivered directly to its targeted tissue and much higher local drug concentrations can be achieved compared to traditional approaches [1]. Few technologies have impacted cancer as broadly as drug delivery systems. Novel delivery systems have made possible the clinical use of new therapeutics, have allowed cancer treatments that have significantly reduced side effects and have enabled new and better chemotherapeutic regimens using existing pharmaceuticals [2].

Transdermal drug delivery systems encompass a wide array of noninvasive or minimally invasive technologies for delivering drugs and vaccines across the skin without needles [3]. Key advantages of transdermal delivery include easy accessibility of skin, which aids in high patient compliance, avoidance of the gastrointestinal tract and the ability to achieve sustained release. The transdermal route has distinct advantages over traditional routes of drug administration, namely, the oral route, which has poor macromolecular bioavailability, or injections, which are limited by pain and possible side effects due to transiently high plasma drug concentration.

Technologies used by transdermal devices can be divided into passive or active methods based on whether an external source of energy is used for skin permeation enhancement. Passive methods include use of chemical enhancers, emulsions and lipid assemblies as well as biological methods such as peptides. Chemical methods are relatively easy to incorporate into transdermal patches and can be used to deliver varying dosage amounts by changing the application area. However, these methods may have a lag time up to hours and thus cannot be easily adapted for rapid onset or time-varying delivery that may be needed for drugs such as insulin [4]. This review focuses on the lipid-based passive methods used to deliver anti-cancer compounds both for local as well as systemic absorption.
15.2 Introduction to Skin Anatomy and Physiology

The skin or cutis is one of the best biological barriers known to man. It is also the largest organ of the human body, with a total weight of more than 3 kg and a total area of 1.5–2 m² [5]. Human skin consists of three main layers, namely, the epidermis (divided into the stratum corneum (SC) and the viable epidermis), the dermis and the hypodermis [6].

15.2.1 The Epidermis

The epidermis is between 50 and 200 μm thick and in permanent contact with the environment [5]. Human skin is one of the best biological barriers owing to the outermost layer of epidermis, namely, the SC (horny layer) [7]. The thickness of SC is about 10–20 μm [6]. It consists of a few dozen flat and partly overlapping, largely dead cells, so-called corneocytes, which are organised in columnar clusters comprising groups of 3–10 corneocyte stacks [5]. The average number of corneocytes per unit area of SC is $2 \times 10^6$ per cm². Corneocytes in each cluster are very tightly packed and attached to each other through desmosomes, covering 15% of the intercellular space length. The intercellular spaces contain specialised multilamellar lipid sheets with variable ultrastructures that are covalently attached to the corneocyte (envelope) membranes. Cells in each cluster stack tend to overlap at their edges with the cells in adjacent stacks. Such tilelike organisation contributes to the tortuosity of the intercellular space in the SC and improves the quality of the skin permeability barrier [5]. Intercellular lipids in the horny layer mainly encompass the relatively nonpolar substances, such as the free fatty acids, cholesterol and cholesteryl esters, in addition to more than a dozen ceramides. Owing to the fairly long aliphatic chains of the latter, and due to the low overall lipid polarity in the skin, the intercorneocyte lipids are tightly packed and at least locally appear as the lipid multilamellae. The latter, moreover, in many places adhere strongly to the corneocyte (envelope) membranes. All this contributes to the tightness and impermeability of the intact skin: it is hence very difficult to bring molecules with a molecular mass greater than 200–350 Da efficiently across the intact skin; achieving the same task for the molecules greater than 750 Da is practically impossible, even when these molecules have an ideal solubility in the skin [8]. The basic features of the SC resemble the barrier of the endothelial lining of blood vessels. However, in the horny layer, the basic motif is repeated a number of times, which makes the skin a much tighter barrier than the blood vessel wall. The horny layer structure was pictorially described in the ‘brick and mortar’ model, in which the corneocytes represent the ‘bricks’ and the intercellular lipids are the ‘mortar’ [5]. The viable epidermis consists of multiple layers of keratinocytes at various stages of differentiation [6].
15.2.2 The Dermis

The dermis is about 3–5 mm thick and consists of a matrix of connective tissue woven from fibrous proteins (collagen, elastin and reticulin) that are embedded in an amorphous ground substance of mucopolysaccharide. Nerves, blood vessels and lymphatics traverse the matrix and skin appendages (sweat glands and pilosebaceous units) pierce it. The dermis needs an efficient blood supply to convey nutrients, remove waste products, regulate pressure and temperature, mobilise defence forces and contribute to skin colour. Branches from the arterial plexus deliver blood to sweat glands, hair follicles, subcutaneous fat and the dermis itself. This supply reaches to within 0.2 mm of the skin surface, so that it quickly absorbs and systemically dilutes most compounds passing the epidermis. The generous blood volume in the skin usually acts as a ‘sink’ for diffusing molecules reaching the capillaries, keeping penetrant concentrations in the dermis very low, maximising epidermal concentration gradients, and thus promoting percutaneous absorption [9].

15.2.3 The Skin Appendages

The eccrine sweat glands, numbering about 2–5 million, produce sweat (pH 4.0–6.8) and may also secrete drugs, proteins, antibodies and antigens. Their principal function is to aid heat control.

The apocrine sweat glands develop at the pilosebaceous follicle to provide the characteristic adult distribution in the armpit, the breast areola and the perianal region. The milky or oily secretion may be coloured and contains protein, lipids, lipoproteins and saccharides. Surface bacteria metabolise the odourless liquid to produce the characteristic body smell.

Hair follicles develop all over the skin except the red part of the lips, the palms and soles, and parts of the sex organs.

Sebaceous glands are most numerous and largest on the face, forehead, in the ear, on the midline of the back and on anogenital surfaces; the palms and soles usually lack them. These holocrine glands produce sebum from cell disintegration; its principal components are glycerides, free fatty acids, cholesterol, cholesterol esters, wax esters and squalene.

The nails, like hair, consist of ‘hard’ keratin with relatively high sulphur content, mainly cysteine. Unlike the SC, the nail behaves like a hydrophilic matrix with respect to permeability.

The SC has around $5 \times 10^7$ natural intercellular hydrophilic pores per $1 \, \text{cm}^2$ and a ‘mild’ widening or opening of new hydrophilic pores may increase the number of
accessible pathways to about $10^9$ per cm$^2$ (or more broadly to $10^6$–$10^{10}$ per cm$^2$). Normal skin contains no transcellular ‘pores’, but it is possible to puncture such holes into the organ with a sufficiently strong local perturbation, as would be described further. The prevailing, naturally occurring hydrophilic conduits through the SC are merely 0.5–10 nm wide. This is 30–4 times below the expanded intercellular route width of 15–40 nm. The calculated skin porosity is therefore between 0.004% and up to 0.3%. Relative occupancy of total skin surface by the artificially created trans-epidermal hydrophilic pores, including transcellular perforations, is between 0.02% and 25% (typically around 0.25%) in case of electroporation, approximately 0.2% after skin poration with an radiofrequency field, up to 35% when a particle jet is employed, around 5% following sonoporation and ≤20% (typically 0.5–2%) when microneedles are used [10].

15.2.4 Skin Pathogens, Exotoxins and Cutaneous Cellular Defence

Skin surface nurtures many and some potentially pathogenic microbiota. Their density is normally lowest at the sites with lowest surface pH and hydration. The prevailing epicutaneous bacterial species are Staphylococci, especially the reasonably tolerable Staphylococcus epidermidis, Staphylococcus hominis and Staphylococcus capitis (all ~0.5–1.0 μm in size). Micrococci (~0.1–0.5 μm, with Micrococcus luteus as the prevalent species), Streptococcus epidermis and Streptococcus pyogenes (both ~0.6–1.0 μm in size), Propionibacterium acnes (Corynebacterium parvum), Propionibacterium granulosum and Propionibacterium avidum (all 0.3–0.5 μm by ~2 μm) are less abundant. Normal skin microbiota help prevent growth of more pathogenic species. This normally keeps at bay Staphylococcus aureus or Staphylococcus haemolyticus (both ~0.5–1.0 μm), Escherichia coli (~0.5 μm by 2.0 μm) and Pseudomonas aeruginosa (1–5 μm by 0.5–1.0 μm), to name but a few. Skin surface can harbour much tinier pathogens as well, such as herpes virus (~100 nm), papilloma virus (~60 nm) or hepatitis virus (~50 nm). Occasionally, exotoxins are detectable too. The latter range from the relatively large bacterial toxins (tetanus toxin: molecular weight ~135 kDa) to small poisonous chemicals and chemical allergens (nickel, molecular weight = 58.7 Da). Skin protection relies primarily on the very high permeability barrier and surface dryness. This combats infections in combination with the mildly microbicidal superficial acidity. The most important secondary line of defence is the presence of immunoactive cells, especially dendritic cells (DC or Langerhans cells) that form a cutaneous network. Such cells reside nearly exclusively in the epidermis, in contrast to cutaneous macrophages that largely populate the dermal skin region. Creating wider pores across skin barrier can harm health, as skin typically harbours a large number ($10^1$–$10^7$ per cm$^2$) of microbiota including pathogens (viruses, bacteria and fungi), with sizes between 60 nm and greater than 100 μm. The skin barrier should therefore be kept as intact as possible at all times [10].
15.3 Percutaneous Absorption

Percutaneous absorption involves passive diffusion of substances through the skin. Percutaneous absorption may occur via three pathways:

15.3.1 Transepidermal Absorption

The main resistance encountered along this pathway arises in the SC. The belief is that most substances diffuse across the SC via the intercellular lipoidal route. This is a tortuous pathway of limited fractional volume and even more limited productive fractional area in the plane of diffusion. However, there appears to be another microscopic path through the SC for extremely polar compounds and ions [11]. Small, polar, water-soluble molecules gain access to the protein fraction of the cell membrane and cross the lipid barrier presumably through small pores in between the protein subunits [12]. When a permeating drug exits at the SC, it enters the wet cell mass of the epidermis and since the epidermis has no direct blood supply, the drug is forced to diffuse across it to reach the vasculature immediately beneath. The viable epidermis functions as a viscid watery regime to most penetrants. It appears that only ions and polar nonelectrolytes found at the hydrophilic extreme and lipophilic nonelectrolytes at the hydrophobic extreme have any real difficulty in passing through the viable field. The epidermal cell membranes are tightly joined and there is little or no intercellular space for ions and polar nonelectrolyte molecules to squeeze through by diffusion. Extremely lipophilic molecules, on the other hand, are thermodynamically constrained from dissolving in the watery regime of the cell (the cytoplasm). Thus the viable epidermis is rate determining when nonpolar compounds are involved [11].

Passage through the dermal region represents a final hurdle to the systemic entry. This is regardless of whether permeation is by transepidermal or by a shunt route. Permeation through the dermis is through the interlocking channels of the ground substance. Diffusion through the dermis is facile and without molecular selectivity since gaps between the collagen fibres are far too wide to filter large molecules [11].

15.3.2 Transfollicular (Shunt Pathway) Absorption

The skin’s appendages offer only secondary avenues for permeation. Sebaceous and eccrine glands are the only appendages that are seriously considered as shunts bypassing the SC since these are distributed over the entire body. Though eccrine glands are numerous, their orifices are tiny and add up to a miniscule fraction of the body’s surface. Moreover, they are either evacuated or so profusely active that molecules cannot diffuse inwardly against the gland’s output. For these reasons, they are not considered as a serious route for percutaneous absorption. However, the follicular route remains
an important avenue for percutaneous absorption since the opening of the follicular pore, where the hair shaft exits the skin, is relatively large and sebum aids in diffusion of penetrants. Partitioning into sebum, followed by diffusion through the sebum to the depths of the epidermis, is the envisioned mechanism of permeation by this route. Vasculature subserving the hair follicle located in the dermis is the likely point of systemic entry [11]. Shunt diffusion may be of importance in the percutaneous absorption of molecules with very low permeability constants, such as the more polar steroids, and of other large molecules. The relative unimportance of the shunt diffusion pathway is suggested by the observations that diffusion is not impaired in patients with congenital absence of sweat glands; that palmar skin, with its high density of sweat glands, is highly impermeable to most diffusing molecules; and that penetration through hair-bearing rodent skin is quite similar to that through non-hair-bearing areas [12].

15.3.3 Intercellular Pathway

This pathway may be involved in transport of electrolytes through the skin, since these are extremely insoluble in lipid membranes. Except for electrolyte transport, the intercellular route appears to have no major role in percutaneous absorption [12].

15.3.4 Factors Influencing Percutaneous Absorption

15.3.4.1 Physicochemical Factors

15.3.4.1.1 Partition Coefficient of Penetrant

Partition coefficient ($K_{o/w}$) of unity favours percutaneous absorption. Compounds which have a log $P$ of less than 1 will have a difficulty in distributing from the device into the SC. For compounds with log $P$ greater than 2, there are potential problems in achieving steady plasma concentration in a reasonable time span due to the drug being held up in the SC where a reservoir can be established.

15.3.4.1.2 Concentration of Penetrant

The lipophilic nature of the SC (rich in phospholipids, ceramides, cholesterol and cholesterol esters) influences the penetration of active compounds and percutaneous drug absorption is evaluated by means of Fick’s law as follows:

$$J = \frac{dQ}{dt} = \left[ \frac{(K_s \times D)}{h} \right] \times C \times A$$

where $dQ/dt$ is the amount of drug diffused per unit of time or drug flux ($J$), $K_s$ is the partition coefficient, $D$ is the diffusion coefficient, $h$ is the thickness of the SC, $C$ is
the concentration of the active compound and \( A \) is the skin surface area utilised for drug administration [6]. Thus, increasing the concentration of dissolved drug causes a proportional increase in flux. This continues until the saturation concentration (solubility limit) is reached, at which point there is no further change in flux. At concentrations higher than the solubility, excess solid drug functions as a reservoir and helps maintain a constant drug concentration for a prolonged period of time [13].

**15.3.4.1.3 Molecular Weight**

The diffusion coefficient of a drug is a function of the molecular weight of the drug. Larger molecules would have a smaller diffusion coefficient and vice versa since the diffusion coefficient is inversely proportional to the square root or cube root of molecular weight [13].

**15.3.4.1.4 Thermodynamic Activity of Penetrant**

The drug must have balanced affinity for the vehicle and deeper skin tissues. If it has more affinity for deeper skin tissues, there is better percutaneous absorption and if it has greater affinity for the vehicle, there is poor percutaneous absorption. But the drug must have adequate affinity for vehicle so that it does not crystallise out.

**15.3.4.1.5 pH Values**

Application of solutions having very high or very low pH can be destructive to the skin. With moderate pH values, the flux of ionisable drugs can be affected by changes in pH that alter the ratio of charged and uncharged species. Many investigations have shown that the unchanged form has better penetration characteristics. However, for a number of drugs, there may also be significant skin absorption at pH values at which the ionised form is predominant [13].

**15.3.4.1.6 Temperature**

Increase in skin temperature results in an increase in rate of skin permeation. Clinically, skin temperature increases under occlusive dressings. Under occlusion, sweat cannot evaporate nor can heat radiate as readily and the skin surface temperature rises by a few degrees. The increase in permeability may be attributed to the solubility of the drug in skin tissues. Rise in temperature may also increase vasodilatation of skin blood vessels leading to increase in percutaneous absorption.

**15.3.4.1.7 Hydration**

The amount of moisture held by the SC is a function of the environmental relative humidity [13]. The normal water content of SC is 5–15\%, a value that can be increased up to 50\% by occlusion. Occlusion is defined as the complete impairment of passive transepidermal water loss at the application site. Occlusion is achieved by
covering or occluding the skin with plastic sheeting or vehicle containing oily material such as lanolin, isopropyl myristate and paraffin. A predominant effect of occlusion is to increase hydration of the SC, thereby swelling the corneocytes, and promoting the uptake of water into intercellular lipid domains. The magnitude of increased SC hydration is related to the degree of occlusion exerted and is dependent upon the physicochemical nature of the dressing. Occlusion hydrates the keratin in corneocytes and increases the water content between adjacent intercellular lipid lamellae. A penetrant diffusing through the intercellular lipid domains will distribute between the hydrophobic bilayer interiors and the aqueous regions separating the head groups of adjacent bilayers. SC hydration magnifies the latter environment and increases the hydrophilic character of the SC to some extent [14]. Urea moisturises the skin and is a mild keratolytic. Both effects promote skin penetration [9].

15.3.4.1.8 Drug-skin Binding

An ideal topical drug should penetrate into the skin in an active form to reach the target structure. The SC, especially the deeper layers, sometimes acts as a deposit or reservoir and modifies transdermal penetration of some drugs. If the drug binds to any of the layers of skin, then a portion of the drug is dissolved in the membrane and is free to diffuse. The remainder is held by binding sites and though binding is reversible, bound drug molecules are not able to diffuse through the membrane. Binding of this type prolongs the time required to reach steady state penetration. Thus there is a delay in the absorption of substantial quantities [13].

15.3.4.1.9 Effect of Vehicle

Vehicles influence the penetration of drug through the skin in several ways. If the vehicle has high affinity for the drug then the skin/vehicle partition coefficient of the drug is lowered and hence the flux of drug through skin tends to be small. Extraction of membrane components by the vehicle opens additional penetration pathways and increases penetration flux [13]. Occlusive vehicles such as fats and oils reduce water loss and increase moisture content of the skin, thus promoting drug penetration. Humectants such as glycerol, propylene glycol or polyethylene glycol draw moisture from the skin and hence reduce percutaneous absorption [9].

15.3.4.1.10 Penetration Enhancers

Penetration enhancers are substances that temporarily diminish the impermeability of the skin [9]. Dimethyl sulfoxide, dimethylformamide, dimethylacetamide, oleic acid, isopropyl myristate, ethylene glycol and azone are few examples of penetration enhancers. Penetration enhancers promote the delivery across the skin by increasing the drug solubility/partitioning in the skin lipids and by facilitating drug diffusion in and from the barrier. When mixed with the skin lipid membranes, permeation
enhancers partition into the lipid layers and either expand the polar-apolar interface or else form enhancer-rich, partly disordered domains; (partial) lipid extraction from the skin is also possible. This occurs preferentially in the least well structured skin lipid regions. Enhancers thus enlarge the width and the number of hydrophobic pores in the SC by creating excess lipid area in the lipid matrix. This facilitates the diffusive flow of sufficiently hydrophobic and some amphiphatic drugs across the skin [5].

15.3.4.2 Biological Factors

15.3.4.2.1 Skin Age

The skins of the foetus, the young and the elderly are more permeable than adult tissue. Children are more susceptible to the toxic effects of drugs and chemicals, partly because of their greater surface area per unit body weight [9].

15.3.4.2.2 Skin Condition

The intact skin is a tough barrier but many agents damage it. Vesicants such as acids and alkalis injure barrier cells and thereby promote penetration as do cuts, abrasions and dermatitis. In heavy industry, workers' skins may lose their reactivity or 'harden' because of frequent contact with irritant chemicals. Many solvents open up the complex dense structure of the horny layer. Mixtures of polar and nonpolar solvents such as methanol and chloroform remove the lipid fraction, forming artificial shunts through which molecules pass more easily. Disease commonly alters skin condition. Inflammation of skin, loss of SC and altered keratinisation increases permeability. If the skin is thickened with corns and calluses, then drug permeation decreases. After injury or removal of the SC, within 3 days, the skin builds a temporary barrier that persists until the regenerating epidermis can form normal keratinising cells. Even the first complete layer of new SC cells formed over a healing layer can markedly reduce permeation [9].

15.3.4.2.3 Regional Skin Sites

Variations in cutaneous permeability depend on the thickness and the nature of the SC and the density of skin appendages. However, the absorption site varies widely for a specific substance passing through identical skin sites in different healthy volunteers; the most permeable regions in some individuals compare with the least permeable sites in others. The diffusivity of different skin sites decreases in the order: plantar, palmar and dorsum of hand, scrotal and postauricular, axillary and scalp, arm, leg and trunk. The application of transdermal hyoscine to postauricular skin is attributed to the relatively high permeability and ease of access of the latter. The layers of SC in postauricular skin are thinner and less dense, also there are more sweat and sebaceous
glands per unit area and many capillaries reach closer to the surface, increasing the temperature by 4–6 °C relative to the thigh [9].

**15.3.4.2.4 Peripheral Circulation**

Theoretically, changes in the peripheral circulation could affect percutaneous absorption; an increased blood flow could reduce the time a penetrant remains in the dermis and also raise the concentration gradient across the skin [9].

**15.3.4.2.5 Species Differences**

Mammalian skins differ widely in characteristics such as horny-layer thickness, sweat gland and hair follicle densities and pelt condition. The capillary blood supply and the sweating ability differ between humans and common laboratory animals. Such factors affect the routes of penetration and the resistance to permeation. Frequently, mice, rats and rabbits are used to assess percutaneous absorption, but their skins have more hair follicles than human skin and they lack sweat glands. Comparative studies on skin penetration indicate that, in general, monkey and pig skins are most like that of man; hairless mouse skin has some similar characteristics. Rabbit, rat and guinea pig skins are highly permeable and the skin of a Mexican hairless dog has different characteristics to those of man [9].

**15.3.4.2.6 Percutaneous Metabolism**

The skin has been shown to contain the major enzymes found in other tissues of the body. Topically applied compounds may be metabolised in skin resulting in altered pharmacologic or toxicologic activity. The effect of skin metabolism on the biological response to topically applied chemicals is only beginning to be investigated. The task is complicated since skin metabolism is difficult to measure *in vivo* without interference from systemic enzymes. In addition, certain metabolic systems in skin, such as cytochrome P-450, have relatively low activity when compared with liver. *In vitro* studies indicate that significant metabolism can occur during the percutaneous absorption process [15].

**15.4 Lipid-based Particulate Carriers**

Copland and co-workers [16] have classified lipid-based systems according to the behaviour of the lipid formulation components upon interaction with water, both at the air-liquid interface and in the bulk aqueous phase, using the following categories of polar lipids proposed by Small [17]:

**Class I**: Insoluble nonswelling amphiphiles. These lipids are insoluble in the bulk aqueous phase but will spread at the air-liquid interface to form a stable monolayer.
Class II: Insoluble swelling amphiphiles. These lipids have a very low real aqueous solubility but swell to form well-defined liquid crystalline phases; they will also form stable monolayers.

Class III: Soluble amphiphiles. Soluble amphiphiles form micellar solutions when added to an aqueous bulk phase. Addition to an aqueous solvent gives the following subcategories:

Class IIIa: Lipids form micelles when the specific critical micelle-forming concentration of the amphiphile has been reached. At high concentrations of class IIIa amphiphiles, liquid crystal phases are formed.

Class IIIb: Lipids do not exhibit this lyotropic mesomorphism and form micelles in a stepwise fashion upon dissolution in the aqueous bulk.

15.4.1 Class I – Based Delivery Systems

15.4.1.1 Microemulsions

In recent years, there has been a considerable amount of research into the development of microemulsions for cutaneous drug delivery due to their improved drug solubilisation, long shelf life and ease of preparation [18]. Microemulsions are thermodynamically stable colloidal dispersions of water and oil stabilised by a surfactant and, in many cases, also a cosurfactant [19]. They are optically clear with particle size smaller than ~300 nm. Droplets in simple microemulsions resemble (mixed) micelles, except that they contain an extra oily component [20]. A well-balanced microemulsion lacks spontaneous curvature and has a very low interfacial tension [21]. Owing to the properties of these self-assembled dispersions, such as low viscosity, ultralow interfacial tension, enormous interfacial area, good shelf life (stability with time), high solubilising capacity, macroscopic homogeneity and microscopic heterogeneity (microdomains), they are being considered as an attractive formulation strategy for topical delivery [22, 23].

Feng and co-workers [24] demonstrated that topical application of microemulsion of combination of celecoxib (5-lipoxygenase inhibitor) and zileuton (cyclooxygenase inhibitor) had an additive effect in inhibition of the incidence and development of human skin squamous cell carcinoma growth in a nude mouse model. Chen and co-workers [25] investigated the microemulsions for transdermal delivery of triptolide. The triptolide-loaded microemulsions showed an enhanced in vitro permeation through mouse skin compared to an aqueous solution of 20% propylene glycol containing 0.025% triptolide with minimal skin irritation. Subramanian and co-workers [26] proposed topical celecoxib microemulsion for the treatment of UV-B-induced skin cancer since the developed microemulsion showed higher permeation rate and significant anti-inflammatory...
activity studied using the arachidonic acid-induced ear oedema model. In vitro studies showed the composition of the microemulsion greatly influenced the skin flux of the permeation of the anti-neoplastic agent 5-fluorouracil (5-FU) from water-oil (w/o) microemulsion of IPM/AOT/water. The attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) indicated that the microemulsion interacted with a component of the SC and perturbed the architectural structure. The extent of perturbation depended on the concentration of water and AOT in the microemulsion [27].

15.4.1.2 Nanoemulsions

Nanoemulsions are nanometric-sized emulsions, typically with a diameter of up to 600 nm; but unlike microemulsions, nanoemulsions are thermodynamically unstable systems despite the fact that the long-term physical stability of nanoemulsions usually makes them unique [28, 29]. These novel formulations enhance drug delivery into and across skin primarily by increasing concentration gradient across skin. Nanoemulsions also perturb the skin barrier function by virtue of the presence of surfactants, which are well-known penetration enhancers [18]. Therefore, a combination strategy involving penetration enhancement per se using lipid bilayer alteration along with enhanced concentration gradient promotes improved drug partitioning into skin. Their long-term stability, ease of preparation (spontaneous emulsification) and high solubilisation of drug molecules make them promising as a drug delivery tool [30].

This formulation strategy was successfully investigated to localise paclitaxel (PCL) in the deeper layers of the skin with minimal systemic absorption. PCL nanoemulsion of size 21.58 nm was found to penetrate into the deeper skin layers up to subcutaneous tissue within 4 hours and maintain its high local concentration in respective skin layers up to 48 hours, with minimal redistribution [31]. Tagne and co-workers [32] have reported that, relative to suspensions of dacarbazine (DAC) with a mean particle size of 5470 nm, nanoemulsions of DAC having mean particle sizes of 131 nm were more efficacious. They observed that in a mouse xenograft model using a human melanoma cell line, a topical application of nanoemulsions of DAC compared to the suspension preparation of DAC produced up to 10-fold greater percent reductions of tumour size. Recently, w/o nanoemulsion of caffeine was developed for transdermal application in skin cancer. The formulation showed good permeation and was found to be safe for topical administration [33].

15.4.1.3 Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLN, also referred to as lipospheres or solid lipid nanoparticles) are a relatively new class of drug carrier. They are particles of submicron size (50–1000 nm) made from lipids that remain in a solid state at room temperature
and body temperature. SLN can be conveniently prepared using wide variety of lipids including lipid acids, mono-, di- or triglycerides, glyceride mixtures or waxes and stabilised by the biocompatible surfactant(s) of choice (nonionic or ionic) [34].

Chen and co-workers [35] compared podophyllotoxin-loaded SLN with podophyllotoxin tincture with regard to skin permeation, skin penetration and epidermal targeting effect. For one SLN formulation, an increased penetration into porcine skin up to 3.48 times over tincture was reported. Furthermore, it was found, that podophyllotoxin was located in the epidermis and hair follicles when applied as SLN formulation. No drug was found in the dermis after SLN application, while podophyllotoxin after tincture application was distributed in each layer of the skin. Therefore, a localisation effect in the epidermis was suggested and a reduction in systemic side effects was expected after application of podophyllotoxin using a formulation containing SLN.

Triptolide, an anti-neoplastic, immunosuppressive, anti-fertility agent, was encapsulated in SLN consisting of tristearin glyceride, soybean lecithin and polyethylene glycol 400 MS having particle size of 123 ± 0.9 nm, polydispersity index of 0.19 and zeta potential −45 mV. The cumulative transdermal absorption rate in 12 hours was 73.5%, whereas with the conventional triptolide hydrogel, it was 45.3%. The results indicated that SLN could serve as an efficient tool to promote transdermal absorption and sustained release of triptolide [36].

15.4.2 Class II – Based Delivery Systems

15.4.2.1 Liposomes

Liposomes are the most popular nanosized drug carrier aggregates. They are always vesicular, that is, comprise one or several lipid bilayer(s) without surface tension enclosing an aqueous core. They are nearly spherical due to bilayer elastic energy far exceeding the thermal activation threshold. Broadly, the conventional fluid bilayer liposomes have size approximately in the range of 30–200 nm [10]. Phospholipids, the major component of liposomal systems, can easily get integrated with the skin lipids and maintain the desired hydration conditions thereby improve drug penetration and localisation in the skin layers.

Liposome gels consisting of an anti-neoplastic agent, 5-fluorouracil, intended for topical application have been prepared and drug release properties in vitro evaluated. Different formulations of liposomes were prepared by the film hydration method by varying the lipid phase composition (PL 90H/cholesterol mass ratio) and hydration conditions of dry lipid film (drug/aqueous phase mass ratio). Topical liposome gels were prepared by incorporation of lyophilised liposomes into a structured chitosan gel base. The rate of drug release from liposome gels was found to be dependent on
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the bilayer composition and the dry lipid film hydration conditions. Also, liposomes embedded into a structured vehicle of chitosan showed significantly slower release than hydrogels. The drug release obeyed the Higuchi diffusion model, while liposomes acted as reservoir systems for continuous delivery of the encapsulated drug [37].

Interestingly, the incorporation of tamoxifen (TAM) into lipid bilayers has been viewed to bring additional benefit of imparting stability to the liposomes. The latter is related to the cholesterol-like structure of TAM, which on its incorporation in liposomes reduces the flux of molecules. TAM also inhibits the lipid peroxidation, besides its ability to influence the fluidity of liposomal bilayers in a dose-dependent manner [38, 39]. Significantly higher skin permeation of TAM from liposomal formulations (flux values 63.67 μg/cm²/hour and 59.87 μg/cm²/hour for liposomal suspension and liposomal gel) has been achieved, as compared to solution (21.65 μg/cm²/hour) and carbopol gel (24.55 μg/cm²/hour) containing TAM [40].

R. Kumar and co-workers investigated multilamellar liposome for topical delivery of finasteride (FNS), a drug of choice for benign prostate hypertrophy and prostate cancer. Multilamellar drug-loaded liposomes consisting of saturated phospholipid (100 mg), cholesterol (50 mg) and FNS (5 mg) showing highest drug payload (2.9 mg/100 mg of total lipids) and drug entrapment efficiency (88.6%) were prepared by thin-film hydration with sonication method. Significantly higher skin permeation and fivefold higher deposition of drug in skin of FNS through excised abdominal mice skin was achieved from the liposomal formulations (3.66 ± 1.6 μm) than the corresponding plain drug solution and conventional gel [41]. Tea polyphenols, including (+)-catechin, (–)-epicatechin and (–)-epigallocatechin-3-gallate (EGCG), have been shown to possess potent antioxidant and anti-cancer activities. Liposomes containing egg phosphatidylcholine, cholesterol or anionic species were prepared by a solvent evaporation method and then were subjected to a probe sonicator. The release rate study showed that inclusion of an anionic species, such as deoxycholic acid (DA) or dicetyl phosphate (DP), increased the permeability of the lipid bilayers, leading to the rapid release of these formulations, a greater amount of catechins were delivered into the solid tumour by liposomes than by the aqueous solution. The drug release rate and vesicle size of liposomes were found to influence drug deposition in tumour tissues. Furthermore, the isomers, (+)-catechin and (–)-epicatechin, showed different physicochemical properties in liposomes and for local deposition in the skin and tumour. Finally, the presence of gallic acid ester in the structure of EGCG was found to significantly increase the tissue uptake of catechins [42].

15.4.2.2 Transfersomes

Transfersomes are self-optimised aggregates with the ultraflexible membrane capable of delivery of the drug reproducibly either into or through the skin, depending on the
choice of administration or application, with high efficiency. These vesicular transfersomes are several orders of magnitudes more elastic than the standard liposomes and thus well suited for skin penetration. Transfersomes overcome the difficulty of skin penetration by squeezing themselves along the intracellular sealing lipid of the SC [43]. Flexibility of transfersome membranes is achieved by mixing suitable surface-active components in the proper ratios [44]. The resulting flexibility of transfersome membranes minimises the risk of complete vesicle rupture in the skin and allows transfersomes to follow the natural water gradient across the epidermis, when applied under nonocclusive conditions [45].

TAM, anti-breast cancer agent is carried through the skin most efficiently by means of transfersomes and accelerates the growth of murine uteri, where it acts as an antioestrogen, even at doses as low as 0.1–0.2 mg/kg/day. Tenfold greater amounts of TAM in soy oil must be injected subcutaneously in mice to achieve a comparable biological effect. TAM in soy oil given orally also is significantly less efficient than the epicutaneously used transfersomal drug formulation [8].

A combination method of using microneedle pretreatment and elastic liposomes was developed to increase skin permeation of the anti-cancer drug docetaxel (DTX, MW = 807.9). The effect of DTX liposomal systems with and without elastic properties as well as in combination with the microneedle on the permeation of DTX across both rat and porcine skin was investigated in vitro. It was found elastic liposomes loaded with DTX can enhance transdermal delivery of DTX without microneedle treatment. However, an enhanced transdermal flux (1.3–1.4 μg/cm²/hour) for DTX from all liposomal formulations was observed after microneedle treatment. Importantly, the lag time obtained following the application of elastic liposomes through microneedle-treated skin was decreased by nearly 70% compared with that obtained from conventional liposomes [46].

15.4.2.3 Ethosomes

Ethosomes are noninvasive delivery carriers that enable drugs to reach the deep skin layers and/or the systemic circulation. These are soft, malleable vesicles tailored for enhanced delivery of active agents. They are composed mainly of phospholipids, (phosphatidylcholine, phosphatidylserine, phosphatidic acid), high concentration of ethanol and water [47]. The high concentration of ethanol makes the ethosomes unique, as ethanol is known for its disturbance of skin lipid bilayer organisation; therefore, when integrated into a vesicle membrane, it gives that vesicle the ability to penetrate the SC. Also, because of their high ethanol concentration, the lipid membrane is packed less tightly than conventional vesicles but has equivalent stability, allowing a more malleable structure and improves drug distribution ability in SC lipids [48].
Topical photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA) is an alternative therapy for many nonmelanoma skin cancers. The major limitation of this therapy, however, is the low permeability of ALA through the SC of the skin. The objective of the present work was to characterise ethosomes containing ALA and to enhance the skin production of protoporphyrin IX (PpIX), compared to traditional liposomes. Results showed that the average particle sizes of the ethosomes were less than those of liposomes. Moreover, the entrapment efficiency of ALA in the ethosome formulations was 8–66% depending on the surfactant added. The particle size of the ethosomes was still approximately <200 nm after 32 days of storage. An in vivo animal study observed the presence of PpIX in the skin by confocal laser scanning microscopy (CLSM). The results indicated that the penetration ability of ethosomes was greater than that of liposomes. The enhancements of all the formulations were ranging from 11- to 15-fold in contrast to that of control (ALA in an aqueous solution) in terms of PpIX intensity. In addition, colorimetry detected no erythema in the irradiated skin. The results demonstrated that the enhancement ratio of ethosome formulations did not significantly differ between the nonirradiated and irradiated groups except for PE/CH/SS, which may have been due to a photobleaching effect of the PDT-irradiation process [49].

15.4.2.4 Niosomes

Nonionic surfactant based vesicles (niosomes) are formed from the self-assembly of nonionic amphiphiles in aqueous media resulting in closed bilayer structures. These structures are analogous to phospholipid vesicles (liposomes) and are able to encapsulate aqueous solutes and serve as drug carriers. The low cost, greater stability and resultant ease of storage of nonionic surfactants has lead to the exploitation of these compounds as alternatives to phospholipids [50].

An innovative niosomal system made up of α,ω-hexadecyl-bis-(1-aza-18-crown-6) (Bola), Span 80® and cholesterol (2:5:2 molar ratio) was proposed as a topical delivery system for 5-FU, largely used in the treatment of different forms of skin cancers. Bola-niosomes showed a mean size of ~400 nm, which were reduced to ~200 nm by a sonication procedure with a polydispersion index value of 0.1. Bola-niosomes showed a loading capacity of ~40% with respect to the amount of 5-FU added during the preparation. 5-FU-loaded bola-niosomes were tested on SKMEL-28 (human melanoma) and HaCaT (nonmelanoma skin cancer with a specific mutations in the p53 tumour suppressor gene) to assess the cytotoxic activity with respect to the free drug. 5-FU-loaded bola-niosomes showed an improvement of the cytotoxic effect with respect to the free drug. Confocal laser scanning microscopy studies were carried out to evaluate both the extent and the time-dependent bola-niosome-cell interaction. The percutaneous permeation of 5-FU-loaded niosomes was evaluated by using human SC
and epidermis membranes. Bola-niosomes provided an eight- and fourfold increase in the drug penetration with respect to an aqueous drug solution and to a mixture of empty bola-niosomes with a drug aqueous solution [51].

15.4.3 Class III – Based Delivery Systems

15.4.3.1 Micelles

Micelles form spontaneously near and above the amphipathic solubility limit. Polar surfactants in water or relatively apolar surfactants in oil thus take a variety of shapes (cylindrical or thread-like, disc-like, spheroidal and spherical micelle) but exhibit sizes 3–20 nm in the nanorange at least in one direction. Micelles are always smaller than liposomes and far shorter lived, the more so the smaller their interfacial tension. A micelle has either a fatty core separated from an aqueous solvent by the polar heads (normal micelle) or else an aqueous core separated from a fatty solvent by the polar heads (inverse micelle). Micelles of constant size are therefore practically incompressible but flexible if they are long enough. Spherical micelles have aggregation numbers that are normally in the \(10^2\) range, but may contain just 10–20 molecules in the extreme, depending on their specific composition and molecular concentration [10]. It is well known that micellar solutions are able to influence the solubility and stability of lipophilic compounds in water. For example, lipophilic drugs can be solubilised by the hydrophobic environment within the micelles (direct micelles), allowing for improvements in the level of bioavailability [52].

Delocalised lipophilic cations such as dequalinium (DQA), shown to enhance the efficacy of existing anti-cancer modalities such as radiation and photodynamic therapies, have limited solubility in aqueous solutions. To overcome this obstacle, DQA was incorporated into liposomes and micelles. Due to inability to incorporate in liposomes at physiological salt concentrations, micelles made of polyethylene glycol derivatives of phosphatidylethanolamine (PEG-PE), which stably bind up to 30 mol% DQA, were found to be more useful [53].

15.4.3.2 Lipid Cubic Phases

The cubic phase is an isotropic, thermodynamically stable and highly viscous phase. The internal structure of the cubic phase consists of one congruent lipid bilayer, the lipid channel, extending in three dimensions, surrounded on both sides by water. The thickness of the glyceryl monooleate bilayer is approximately 3.5 nm and the water channel diameter is roughly 5 nm [54]. Therefore, the cubic phase can be regarded as a soft nanostructured material. Since the cubic phase is bicontinuous, it can dissolve both hydrophilic, amphiphilic and hydrophobic substances [55]. With
decreasing water or increasing oil content, the originally (quasi)globular structures of microemulsion transform into bicontinuous microemulsions [56].

In vivo topical administration of lipid (monoolein or phytantriol) cubic phases of δ-aminolevulinic acid and its methyl ester (m-ALA) on nude mice skin induced the production of the photosensitiser protoporphyrin IX (PpIX) in living tissue. The formulations were applied for 1 hour, the monoolein cubic systems and the three-component phytantriol sample showed higher fluorescence compared to the standard ointment during the 10 hours of measurement. Both ALA and m-ALA yielded similar results, although the differences between the investigated vehicles were more pronounced when using m-ALA. For the 24 hours applications, the monoolein cubic systems with m-ALA showed faster PpIX formation than the standard ointment, implying higher PpIX levels at short application times (less than 4 hours) [55].

15.5 Lipid-based Nanocarriers for Herbal Anti-cancers

Recently, there has been a surge in research exploring the potential of phytopharmaceuticals for treatment of various maladies, namely, cancer and neurodegenerative disorders. In comparison with modern medicines, herbal analogues have better therapeutic value with fewer adverse effects. However, phytotherapeutics needs a scientific approach to deliver the components in a sustained manner to increase patient compliance and avoid repeated administration. This can be achieved by designing novel drug delivery systems for herbal constituents. Novel drug delivery systems not only reduce the repeated administration to overcome noncompliance but also help to increase the therapeutic value by reducing toxicity and increasing the bioavailability, and so on. This section summarises various topical drug delivery technologies for herbal actives that are gaining more attention due to better therapeutic response in treatment of cancer.

Curcumin, a major component from turmeric (Curcuma longa), possesses diverse anti-inflammatory, anti-tumour and antioxidant properties. The mechanisms implicated in the inhibition of tumourigenesis by curcumin are diverse and appear to involve a combination of anti-inflammatory, antioxidant, immunomodulatory, proapoptotic and antiangiogenic properties via pleiotropic effects on genes and cell-signalling pathways at multiple levels. The potentially adverse sequelae of curcumin’s effects on proapoptotic genes, particularly p53, represent a cause for current debate. When curcumin is combined with some cytotoxic drugs or certain other diet-derived polyphenols, synergistic effects have been demonstrated [57]. Patel and co-workers [58] have showed enhanced permeation of curcumin from curcumin-loaded transfersomes for transdermal delivery using phosphatidyl choline:Span 80 (85:15). Topical
and transdermal delivery of quercetin were evaluated in vitro using porcine ear skin mounted on a Franz diffusion cell and in vivo on hairless-skin mice. Skin irritation by topical application of the microemulsion containing quercetin as well as the protective effect of the formulation on UV-B-induced decrease of endogenous reduced GSH levels and increase of cutaneous proteinase secretion/activity were also investigated. The w/o microemulsion increased the penetration of quercetin into the SC and epidermis plus dermis at 3, 6, 9 and 12 hours post application in vitro and in vivo at 6 hours post application. No transdermal delivery of quercetin occurred. By evaluating established endpoints of skin irritation (erythema formation, epidermis thickening and infiltration of inflammatory cells), the study demonstrated that the daily application of the w/o microemulsion for up to 2 days did not cause skin irritation. The w/o microemulsion containing quercetin significantly prevented the UV-B irradiation-induced glutathione (GSH) depletion and secretion/activity of metalloproteinases [59].

Topical delivery of lycopene is a convenient way to supplement cutaneous levels of antioxidants. Microemulsions containing capric and caprylic acids based monoglycerides (MG) and triglycerides (TG) of the same fatty acids were isotropic, fluid and clear, with internal phase diameters of 27 and 52 nm, respectively. Both MG- or TG-containing microemulsions markedly increased lycopene penetration in the SC (6- and 3.6-fold, respectively) and in viable layers of porcine ear skin (from undetected to 172.6 ± 41.1 and 103.1 ± 7.2 ng/cm², respectively) compared to a control solution. To assure that lycopene delivered to the skin was active, the antioxidant activity of skin treated with MG-containing microemulsion was determined by CUPRAC assay and found to be 10-fold higher than in untreated skin. The cytotoxicity of MG-containing microemulsion in cultured fibroblasts was similar to that of propylene glycol (considered safe) and significantly less than that of sodium lauryl sulfate (a moderate-to-severe irritant) at 1–50 μg/ml. These results demonstrate that the MG-containing microemulsion is an efficient and safe system to increase lycopene delivery to the skin and the antioxidant activity in the tissue [60].

The effect of free and liposomal resveratrol on the viability of HEK 293 cells and their photoprotection after UV-B irradiation was assessed by the MTS method. Resveratrol decreased the cell viability at 100 μM concentration, while at 10 μM increased cell proliferation and also achieved the most effective photoprotection. Photomicrographs of the treated cells from inverted light and fluorescence microscopy demonstrated resveratrol effectiveness at 10 μM, as well as its toxicity at higher concentrations, based on changes in cell shape, detachment and apoptotic features. Interestingly, liposomes prevented the cytotoxicity of resveratrol at high concentrations, even at 100 μM, avoiding its immediate and massive intracellular distribution, and increased the ability of resveratrol to stimulate the proliferation of the cells and their ability to survive under stress conditions caused by UV-B light [61].
15.6 Lipid-based Nanocarriers for Gene Delivery for Cancer

In the past two decades, gene therapy is an emerging frontier of biotechnology attempting to treat diseases with DNA/RNA. To date, most gene delivery strategies have concentrated on the parenteral route of delivery, but recently the focus of gene delivery has been shifted to the topical route on account of the advantage of skin, namely, easy accessibility permitting gene delivery in a noninvasive and uncomplicated manner. The cutaneous delivery of genetic material is greatly hindered by the large molecular weight, low diffusion constant and highly hydrophilic nature of DNA. In addition to the limited penetration through the SC, gene expression is hindered by slow uptake of the plasmid DNA (pDNA) by the target cells, lack of release from endosomes, instability in cytoplasm and lack of nuclear uptake [62]. Also, naked therapeutic genes are rapidly degraded by nucleases and show poor cellular uptake; as a result, the development of safe and efficient gene carriers (vectors) is one of the prerequisites for the success of gene therapy [63].

The development of vectors for efficient cutaneous gene therapy has attracted a considerable attention in recent years. Numerous viral or nonviral vectors may be employed to deliver genes through the skin. Viral vectors are, however, difficult to produce and toxic (in particular immunogenic), besides having a limitation in terms of the size of the inserted genetic materials. In attempts to overcome these problems, nonviral vectors, such as cationic lipids and polymers, have been developed as gene carrier molecules. Nonviral vectors are advantageous due to the low immune response that enables repeated administration and the capability of large production with acceptable costs. They have the potential to be widely used in gene therapy clinics. Cationic liposome-mediated gene transfer or lipofection represents the most extensively investigated and commonly used nonviral gene delivery method. Currently, hundreds of lipids have been developed and tested for gene transfer. They share the common structure of positively charged hydrophilic head and hydrophobic tail that are connected via a linker structure. Transfection efficiency of cationic lipids varies dramatically depending on the structure of cationic lipids (the overall geometric shape, the number of charged groups per molecules, the nature of lipid anchor and linker bondages), the charge ratio used to form DNA-lipid complexes and the properties of the colipid. The most commonly used colipids are cholesterol and dioleoylphosphatidylethanolamine (DOPE). When mixed with the negatively charged DNA, the positively charged liposomes spontaneously form uniquely compacted structures called lipoplexes. In a lipoplex structure, DNA molecules are surrounded with positively charged lipids which grant them protection against extracellular or intracellular nucleases. Furthermore, lipoplexes, due to their positive charges, tend to electrostatically interact with the negatively charged molecules of the cell membrane (glycoproteins and proteoglycans) that may facilitate their cellular uptake [64].
Cationic gemini surfactants are a novel category of delivery agents with potential use in gene therapy. These surfactants are built from two ionic head groups that are attached to their hydrocarbon tails and also connected to each other with a spacer or linker. The gemini surfactants are capable of compacting DNA and have several advantages compared to classic monovalent counterparts: lower cellular toxicity, lower critical micelle concentration (generally, one or two orders of magnitude), higher efficiency in reducing surface tension, greater tendency to self-assemble and greater structural variety. DNA compaction is one of the most important requirements for efficient cellular delivery. In addition, for cutaneous gene therapy, the DNA has to partition into the viable layers of the skin, overcoming the barrier function of the SC. Researchers have evaluated the \textit{in vitro} transfection efficiency of a series of gemini surfactant-based delivery systems and demonstrated the feasibility of cutaneous gene delivery through intact skin in normal CD1 mice. Badea and co-workers [62] evaluated the potential of gemini nanoparticles as a topical formulation for the interferon gamma (IFN-\(\gamma\)) gene in an IFN-\(\gamma\)-deficient mouse model. Nanoparticles based on the gemini surfactant 16-3-16 (NP16-DNA) and another cationic lipid cholesteryl 3\(\beta\)-(-N-[dimethylaminoethyl] carbamate) [Dc-chol] (NPDC-DNA) were prepared and characterised. \textit{In vivo}, both topically applied nanoparticles induced higher gene expression compared to untreated control and naked DNA. However, NPDC nanoparticles caused skin irritation that compromised the integrity of the SC, which probably allowed pDNA to permeate through the damaged skin. Topical treatment with gemini nanoparticles thus has been found to be more effective (compared to naked DNA) and safer (compared to NPDC-DNA) and therefore, a feasible method for cutaneous noninvasive gene delivery in IFN-\(\gamma\)-deficient mice. Several studies have demonstrated the efficiency of lipid-mediated delivery of DNA \textit{in vitro}, including delivery into keratinocytes [65]. Ethanol-in-fluorocarbon microemulsion [66] and a biphasic formulation [67] were used for dermal delivery of genes \textit{in vivo} through intact skin.

Meykadeh and co-workers [68] studied the expression of pDNA \textit{in vivo} and \textit{in vitro} following topical application of pDNA in various liposomal spray formulations. Different concentrations of pDNA expressing enhanced green fluorescent protein (pEGFP-N1) were sprayed onto mouse or human skin once daily for three consecutive days and compared with direct injection. EGFP mRNA and protein were detectable by RT-PCR and Western blot, respectively, 24 hours after topical application. Foldvari and co-workers [69] evaluated biphasic lipid vesicle formulations, incorporating pDNA \textit{in vitro} using flow-through diffusion cells. It was found that biphasic vesicles delivered significant quantities of pDNA into the ‘viable’ layers of human skin \textit{in vitro} when determined by polymerase chain reaction. Thus, cutaneous gene therapy is an attractive approach, but it is still inadequate in its effectiveness. However, current results suggest that major breakthrough in this area is an imminent possibility.
15.7 Toxicity Considerations of Nanoparticles

Nanomaterial toxicology remains a challenge with regard to conducting a comprehensive safety evaluation of nanomaterials. As large-scale production of the nanomaterials increases, so does the threat of adverse health effects in humans and the potential for environmental damage [70]. An important route of exposure is by the skin, which could lead to skin cancer, skin sensitisation and skin irritation, or produce systemic effects after absorption. Skin is unique because it is a potential route of both occupational and/or environmental exposure to nanoparticles and also provides an environment within the vascular epidermis where particles could potentially lodge and not be susceptible to removal by phagocytosis. In the last few years, there has been an increasing awareness about the potential toxicity of nanosized materials. Due to their size, nanoparticles have completely different possibilities to interact with cells in the body; they can use ‘infiltration routes’ and utilise certain mechanisms, which are not accessible by micrometer material [71]. The outstanding advantage of lipid nanoparticles is their easy and complete biodegradation. Lipids are natural materials; glycosides are easily degraded by natural processes such as enzyme degradation. The time for degrading lipid nanoparticles depends on the nature of the lipid and the stabilisers used [72, 73]. To judge the safety or toxicity of carrier systems like lipid nanoparticles, it is important to perform studies comparing the toxicity of lipid nanoparticles with other nanoparticulate carrier systems and to compare lipid nanoparticles composed of different excipients with each other. Nanoparticles can cause cytotoxicity by adherence of the particle to the cell membrane, degradation and subsequent release of cytotoxic degradation products [74]. Another mechanism is the internalisation of nanoparticles by cells, intracellular degradation and subsequent toxic effects inside the cell. There are quite a number of different cell culture studies looking at the viability of cells evaluating either the damage of the cell membrane, for example, by neutral red uptake or LDH release or other factors like activity of the mitochondrial succinate dehydrogenases of living cells (MTT test).

In vitro data of ultradeformable liposomes containing bleomycin (Bleosome™) revealed that the LD50 of bleomycin encapsulated in Bleosome was approximately threefold higher than free bleomycin solution for SCC cells and nearly 30 times higher for NEB-1 cells. However, Bleosome containing 30 μg/ml of active bleomycin killed more than twice as many SCC cells than NEB-1 cells [75]. Liposomal formulation of copper palmitate was evaluated on porphyrin-photosensitised rats. Dorsal skin of rat was shaved and treated topically with a cream consisting of either empty or copper palmitate-encapsulated liposomal formulation. Histological studies revealed that no inflammatory cells were present at the skin sites treated with liposomal cream containing copper palmitate in the porphyrin-sensitized group while no reduction in the number of inflammatory cells was observed at the skin samples treated with the empty liposomes observed after being kept in a dimmed
light environment [76]. Vodovozova and co-workers have shown that liposomes bearing specific lipid-anchored glycoconjugates on a polymeric matrix bind *in vitro* to human malignant cells more effectively and, on being loaded with a lipophilic prodrug of merphalan, reveal higher cytotoxic activity compared with nonvectored liposomes [77].

It was shown by Muller and co-workers that the phagocytic uptake of SLN is low on applying them to human granulocytes. At 2.5% SLN concentration, no toxic effects were observed on these cells. This shows that SLN do not exhibit any extracellular toxic effect. Comparing the internalisation effect of SLN and poly(l-lactic acid)/γ-linoleic acid (PLA/GA) nanoparticles by human granulocytes, it was found that the effect is similar for both nanoparticles. Nevertheless, the toxicity of SLN was much lower. Comparing the toxicity of SLN composed of compritol and cetylpalmitate, the effect was similar for both systems indicating good tolerability [78]. Schölzer and co-workers found that the cytotoxicity of SLN assessed by MTT test on murine peritoneal macrophages is concentration dependent and influenced by the lipid matrix. SLN composed of stearic acid or dimethyl-dioctadecylammonium bromide showed toxic effects at concentrations of 0.01%, whereas SLN composed of triglycerides, cetylpalmitate and paraffin did not exhibit major cytotoxic effects at the same concentration [79]. Furthermore, it was found that the size of SLN did not affect the cytotoxicity [79, 80]. The nature of the lipid (Dynasan 114, Compritol ATO888) had no influence on the viability of HL60 cells. In a concentration range of 0.0150–1.5% SLN, no significant difference in viability was found compared to reference cells.

Weyenberg and co-workers [83] investigated the influence of SLN formulated using different lipids and different surfactants on cell viability of J774 macrophages, mouse 3T3 fibroblasts and HaCaT keratinocytes using the MTT test. The surfactant had a big impact on the toxicity of SLN. SLN formulated with lecithin, sodium taurocholate, phosphatidylserine and polysorbate 80 did not affect the viability of the three cell lines while the cell viability was significantly reduced by stearylamine. SLN formulated with stearic acid were toxic for all cell lines exhibiting the most...
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toxic effect on macrophages. Viability of >90% was observed when semisynthetic glycerides or hard fat was used to formulate SLN. Membrane damage and MTT reduction are relatively good parameters to assess cytotoxicity. However, it is more appropriate and more sensitive to look at the release of cytokines. Scholer and co-workers showed that the interaction of compritol and cetyl palmitate SLN with murine peritoneal macrophages does not cause stimulation of proinflammatory cytokines (IL-6, IL-12 and TNF-α) responses. At higher concentration levels of SLN, a significant decrease in IL-6 production caused by cytotoxic effects of SLN was observed [74]. This was confirmed in other studies, where it was also found that the size of SLN did not influence the cytokine production [79, 80]. Furthermore, it was found by Scholer and co-workers [84] that SLN preserved with thiomersal neither caused an increase in cytotoxic effects on the murine peritoneal macrophages nor did it lead to secretion of proinflammatory cytokines by these cells compared to unpreserved SLN. Comparing all these data, and considering the biodegradability without toxic degradation products, the lipid nanoparticles are really very well tolerated at the cellular level. In general, mucosal surfaces can be considered as being more sensitive towards toxic effects compared to the skin, being protected by the SC and having, in general, the function to protect against the environment. For topically applied nanoparticles, a frequently discussed question is if and to what extent the nanoparticles might penetrate deeply into the epidermis, maybe reaching the dermis and, finally, the systemic circulation. At present, there is an increased concern that very small nanoparticles might penetrate via the hydrophilic channels (about 50 nm sized) into the skin. Lipid nanoparticles typically are of the size range 150–200 nm and therefore well above the 50 nm. Even if smaller lipid nanoparticles should be present in the formulation, the material can be biodegraded. Assuming that some lipid nanoparticles might penetrate into the systemic blood circulation, they will be very well tolerated. Based on their composition, they are rather a kind of parenteral nutrition and their use in pharmacy is well documented [85, 86]. It is best advised to use the substances with accepted generally regarded as safe (GRAS) status. The choice of the lipid matrix and surfactant is essential in order to formulate an optimal safe and stable formulation [84]. Despite nanoparticles being biodegradable and leading to nontoxic degradation products, the particle itself might interact in an undesired manner with the body prior to its degradation. Lipid nanoparticles were investigated intensively regarding their effects on skin when the cosmetic products were introduced to the market. Animal tests like the Draize skin irritation test or Draize rabbit eye test are prohibited for cosmetic products in Europe [86]. To ensure the safety of cosmetic products, alternative tests like the Episkin test, the human patch test, the EPISKIN test, the HET-CAM test and cell culture tests [87–92] were reported. To evaluate the skin irritation potential and the eye irritation potential of SLN and dendritic core-multishell nanoparticles, the EPISKIN test and HET-CAM test were performed [93]. No irritation potential according to EU classification system R38
was found with the EPISKIN test. In the HETCAM test, no eye irritation potential was found for both SLN and dendritic core-multishell nanoparticles. The evaluation of cell viability by MTT test on human fibroblasts and keratinocytes after application of SLN and SLN containing prednicarbate showed a viability >90%. Cell viability evaluated by MTT test after application of prednicarbate containing SLN to reconstructed epidermis was 94.5% [93]. The excellent tolerability of the lipid nanoparticles is supported by many available cosmetic dermal products already being introduced to the market fulfilling the regulatory requirements towards tolerability and nanotoxicity.

15.8 Conclusions

Anti-cancer therapy is often plagued with potential adverse effects arising due to nonspecificity, poor bioavailability and extensive metabolism of the anti-cancer therapeutics. Circumventing these issues, formulation strategies have been revolutionised, albeit with minimal successful outcomes. With the inclusion of phytochemicals and gene therapy, the situation has been further complicated, owing to increase in the complexity of the delivery of these moieties. Though much focus has been laid on the formulation, the delivery route has been relatively less explored. Until now, oral and parenteral routes have been on the forefront of most of the delivery solutions for anti-cancer molecules. Notwithstanding, the benefits of these routes, the morbidity of the issue necessitates the probing of an alternative route of administration. In this context, the transdermal route is emerging as a very promising route for delivery of anti-cancer therapeutics. The advantage of this route is manifold; it enables localised delivery, avoids first-pass metabolism and permits systemic absorption. Moreover, as evident from numerous studies, this route facilitates delivery of lipid nanocarriers of anti-cancers with improved therapeutic activity. It has already been shown for various drugs that topical formulations containing lipid nanoparticles can enhance the penetration into the skin (thus increasing treatment efficiency), target the epidermis and reduce systemic absorption and side effects. Furthermore, an increased as well as prolonged activity was reported while the benefit/risk ratio was increased for many drugs. However, to fully leverage the benefits of this route, it is essential to combine the use of the colloidal drug delivery technology along with the invasive delivery tools available for this route. Nevertheless, with renewed interest in transdermal delivery, this approach could witness a surge in the marketed formulations in the near future.

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Lipid Nanocarriers for Topical Anti-cancer Therapy: An Update


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Lipid Nanocarriers for Topical Anti-cancer Therapy: An Update


16.1 Introduction

Skin cancer is commonly known to afflict Caucasians [1, 2]. Melanoma is a type of skin cancer that begins in melanocytes, and it accounts for 160,000 new cases each year worldwide. However, the situation in the US is worst with a high probability of death of 8700 people during 2010 (about 5670 men and 3030 women) due to melanoma [3]. Malignant melanoma is the leading cause of death from skin cancer, accounting for nearly 80% of deaths [3]. Historically, patients with metastatic melanoma live for less than 60 days without their disease worsening, and the median overall survival of these patients is less than 12 months [3]. The steadily increasing incidence of skin cancers has brought much attention to the process by which these tumours develop and how they can be prevented.

Skin is an organ that is constantly exposed to ultraviolet (UV) radiation [4] and to a series of environmental pollutants, such as cigarette smoke, which leads to the production of reactive oxygen species (ROS) [5]. The production of ROS is primarily responsible for photoageing (sun-induced cutaneous changes in the areas exposed to UV radiation). Moreover, ROS generation is the first step in tumour development as ROS are potent inducers of mutations in deoxyribonucleic acid (DNA) [6]. Thus, molecules that inhibit ROS formation or can enhance endogenous defence systems can prevent or reverse photodamage. Although clinical trials have provided contradictory findings concerning the efficacy of oral antioxidant pills, they have been proposed for the prevention of sunburn and for their supposed photoprotective properties [7].

16.2 Antioxidants in Skin Cancer

An antioxidant is a substance that when present in low concentrations relative to the oxidisable substrate significantly delays or reduces oxidation of the substrate [8, 9]. Antioxidants get their name because they combat oxidation. Some antioxidants
are produced endogenously, to protect the body from damaging oxidation reactions by reacting with free radicals and other ROS within the body, thereby hindering the process of oxidation. During this reaction, the antioxidant sacrifices itself by becoming oxidised. However, endogenous antioxidant supply is not unlimited as one antioxidant molecule can react with a single free radical. Therefore, there is a need to constantly replenish antioxidant resources, either endogenously or through supplementation [10].

The preventive role of dietary flavonoids/polyphenolic compounds at the initiation and the promotion stages of chemically induced carcinogenesis is well documented [11–13]. During cancer initiation stage in cell culture experiments, polyphenols have been shown to affect various carcinogen bioactivating steps, which are necessary for the covalent binding of the carcinogen to cellular DNA [14]. During the last few decades, studies have established the role of antioxidants and their mechanism of action in the prevention of skin tumours. A study on the skin cancer chemopreventive effects of silymarin proposes that its action is mediated via inhibition of epidermal growth factor receptor activation and its intrinsic kinase activity, which leads to impairment of downstream signalling pathway followed by perturbations in cell cycle progression and growth arrest [15].

There are many topical preparations that are now being used or are under investigation for amelioration of photodamaged skin. Dreher and Maibach [16] indicate that regular application of skincare products containing antioxidants may be of the utmost benefit in efficiently preparing skin against exogenous oxidative stressors to which it is exposed during daily life.

16.3 Need of Suitable Delivery Systems for Antioxidant Molecules

Low-molecular-weight antioxidants provide protection to skin against oxidative stress, and are depleted in the process; therefore, it is desirable to add these agents to the skin reservoir by applying them directly to skin. Although antioxidants can be supplied to skin through diet and oral supplementation, physiological processes related to absorption, solubility and transport limit the amount that can be delivered into skin. Direct application has the added advantage of targeting them to the area of skin needing protection. For topical application of antioxidants to be useful, however, several obstacles must be overcome.

Delivery systems help compounds to be delivered in an efficient manner without altering their chemical nature or biological activity. Suitable delivery systems represent one of the key enabling technologies for many skincare products. Although chemical modifications are recommended, they may not always help improve the efficiency of
the product; for example, shortening the lipophilic chain of coenzyme Q₁₀ resulted in compounds with good pharmacokinetic profiles, but raised certain doubts about the pro-oxidative properties of the derivatives [17]. Furthermore, the use of conventional delivery systems in several cases has shown little or no improvement in the drug’s properties/profile upon administration. Antioxidant molecules are inherently unstable in nature (e.g., presence of aqueous environment leads to oxidative degradation of ascorbic acid due to ionisation), which allows them to function in redox reactions. Also, many antioxidants are susceptible to photodegradation and presence of oxygen. Instability makes them difficult to formulate in an acceptable, stable composition for topical use. In addition, many antioxidants are deeply coloured, adding to the complexity of producing an aesthetically acceptable product. These observations facilitate the doorway of novel drug delivery systems (NDDS) in the development of antioxidants. NDDS are developed so as to work in all areas of the delivery and thus can be applied to improve solubility, permeability and stability of antioxidants. These delivery systems are further beneficial to the pharmaceutical industries as they serve as a strategic tool for expanding drug market and patent life. Initially developed to enhance stabilisation of the active ingredient, the key focus of development and application of delivery systems is the increased penetration of the active ingredient into the skin and retention therein [18].

Despite a large body of knowledge on cell culture systems, and animal models demonstrating substantial efficacy, previous studies of prevention strategies based on the administration of antioxidants to restore free radical homoeostasis have so far failed to provide unequivocal evidence that any antioxidant agent or strategy is effective in preventing oxidative injury-mediated carcinogenesis in human populations [19, 20]. In the light of these disappointing findings, it was subsequently shown in studies assessing the growth of various tumour cells in vitro that the efficacy of certain anti-tumoral drugs was actually diminished in the presence of antioxidants [21]. Since most antioxidants used for the treatment of cancers proceed through the production of ROS [22], it is possible that for cancers with a long preclinical development period, such as melanoma, antioxidants interfere with the scavenging of preclinical tumour cells by macrophages releasing ROS. However, ROS-containing pathways, genetic susceptibility to skin cancers and pharmacokinetics, skin bioavailability and concentration of the antioxidant are issues that still need to be addressed in order to elucidate the overall impact of ROS pathways on carcinogenesis [22]. It is thus proposed that a suitably delivered antioxidant molecule in a controlled amount and at a specific site wherein it will not hamper the functioning of the natural defence system (oxidative burst of macrophages to kill cancerous cells) needs to be evaluated. This will prevent any toxic (pro-oxidative) build-up of antioxidant(s) in the body, and at the same time provides an impetus to the normal cells to outgrow cancerous cells and prevent initiation of any new cancerous lesions due to induced oxidative stress. The usefulness of these natural molecules in anti-cancer clinical trials thus
needs to be re-evaluated from a new perspective proposed herein. Considering the volume of data claiming a protective role of these molecules, they deserve a fair trial and we suggest their evaluation only after their incorporation into a suitable tailor made system as explained above.

A very recent study of skin cancer risk reported an increased risk of skin cancers in women when antioxidant supplementation was stopped. The study did not reveal any delayed protective effects of antioxidant supplementation on skin cancer. The study also revealed a gender difference in oxidative stress related to melanoma as it is firmly established that males express lower amounts of antioxidant enzymes, resulting in a higher oxidative stress than females [23]. Other hypotheses put forward to explain this difference are that women may have more skin fat tissue, in which antioxidants and vitamins are stored [24], and that hormonal factors may also contribute to the lower susceptibility of women to skin cancers [25, 26].

16.4 ‘Open Sesame’

Sesame (*Sesamum indicum* Linn.) belongs to the family Pedaliaceae and is one of the oldest cultivated plants in the world. It was a highly prized oil crop of Babylon and Assyria at least 4000 years ago. Today, India and China are the world’s largest producers of sesame. Upon ripening, sesame capsules split, releasing the seed, hence the famous phrase, ‘open sesame’ ([Figure 16.1](#)). We however rephrase the sentence to open research on sesame in terms of developing and establishing an important antioxidant obtained from this plant for its therapeutic use especially in cancers.

Sesame seeds contain 50% oil and 25% proteins. Besides these, they also contain sesamolin, which is hydrolysed to give ‘Sesamol’. Sesamol is a natural antioxidant and is responsible for the long shelf life of sesame oil. In the field of cosmetics, sesame oil is used as a base in developing perfumes. In ancient folklore, sesame seed oil is

![Figure 16.1 The structure of sesamol](#)
said to remove wrinkles and prevent ageing, when applied to the skin through facial massage. This may be attributed to the presence of sesamol and vitamin E, both of which are known free radical scavengers. Sesame seeds are prone to rancidity but not the oil as it has the natural preservative sesamol. Sesame oil is used in cooking, soaps, pharmaceuticals, paints and so on. However, large doses of the oil can cause abortion and obesity. The composition of sesame seed indicates the presence of antioxidants (e.g., lignophenols and carboxyphenols) and bisepoxy lignans (e.g., sesamin and sesamolin).

The core of the multifaceted aspect of sesamum species is its antioxidant status. This is attributed mainly to the presence of a variety of polyphenolic substances including sesamol. In some ancient Chinese books, it is written that sesame increases energy levels and prevents ageing. Photoprotective effect of sesamol on UVB radiation-induced oxidative stress in human blood lymphocytes with increasing concentration of sesamol (1, 5 and 10 μg/ml) has been reported [27].

16.4.1 Pharmacology of Sesamol

Sesamol is a very effective inhibitor of lipid peroxidation of rat liver microsomes [28] and rat brain homogenates [29]. It has been shown to exhibit anti-mutagenic activity against oxygen species-mediated mutagenicity in Salmonella typhimurium TA100 and TA102 strains. This was attributed to its antioxidant activity [29, 30]. The antiageing property of sesamol was tested in our laboratory and encouraging results were obtained [31]. Sesamol has also been found to exert chemopreventive effect in the mouse skin two-stage carcinogenesis and the Epstein-Barr virus early antigen activation assay [32]. Sesamol has been reported to inhibit the excessive production of nitric oxide in lipopolysaccharide/γ-interferon-stimulated C6 astrocyte cells [33]. It also inhibited the formation of mutagenic/carcinogenic imidazoquinoline-type heterocyclic amines through the unstable free radical Maillard intermediates [34]. It inhibited the development of foci of preneoplastic hepatocytes in F344 rats [5]. Sesamol showed protective effects against carbon tetrachloride (CCL₄)-induced liver injury in rats [35].

16.4.2 Preliminary Physicochemical Studies on Sesamol

The physicochemical parameters of sesamol (solubility 38.8 ± 1.2 mg/ml; log P octanol/water 1.29 ± 0.01, molecular weight 138.34 g) indicate it to be an interesting and unique phenolic compound (due to its solubility in aqueous as well as oil phase) that has a potential for development as a therapeutic agent, especially considering its high superoxide and NO scavenging capacity [29], which was significantly better than that
of catechin and epicatechin (IC$_{50}$ for sesamol, catechin, epicatechin and ascorbic acid is 130.4, 188.3, 212.8 and 326.4 nmol, respectively). In view of the above-mentioned facts, the (super) sesamol molecule presented a very tempting avenue in the field of free radical scavenging research by us.

16.5 Solid Lipid Nanoparticles

Solid lipid nanoparticle (SLN) dispersions have been proposed as a new type of colloidal drug carrier system for intravenous administration [36], for cosmetic active ingredients [37] and also for an effective topical application of active substances with minimal systemic availability. SLN combine the advantages of polymeric nanoparticles, fat emulsions and liposomes but simultaneously avoid some of their disadvantages.

SLN system consists of spherical solid lipid particles in the nanometre range, dispersed in water or aqueous surfactant solution (Figure 16.2). Generally, they are made of a solid hydrophobic core having a monolayer of phospholipid coating. The solid core contains the drug dissolved or dispersed in the solid high-melting fat matrix. The hydrophobic chains of phospholipid are embedded in the fat matrix. They have the potential to carry hydrophilic or lipophilic drugs or diagnostics [38].

![Figure 16.2 Schematic illustration of the proposed structure of SLN](image)
16.5.1 Solid Lipid Nanoparticles in Topical Drug Delivery

SLN technology has the potential for being utilised for preparation of topical formulations for pharmaceutical as well as cosmetic purposes. Some of the salient features that make SLN promising carriers for pharmaceutically acceptable topical application are as follows:

1. Protection of labile compounds against chemical degradation has been reported, for example, retinol and tocopherol have been successfully incorporated into SLN [39, 40].

2. Depending on the type of SLN produced, controlled release of the active ingredients is possible. SLN with a drug-enriched shell show burst-release characteristics, whereas SLN with a drug-enriched core lead to sustained release [41]. For dermal application, both features are of interest. Burst release can be useful to improve the penetration of a drug. Sustained release is important with active ingredients that are irritating at high concentrations or to supply these ingredients into the skin over a prolonged period of time [42].

3. Due to the general adhesiveness of small particles, SLN applied to the skin form a film. This film of ultrafine particles has an occlusive effect, which promotes penetration of active ingredients into the upper part of the epidermis, mainly the stratum corneum, thus enhancing the pharmaceutical efficiency of incorporated ingredients. The occlusion of the skin increases skin hydration. This increased hydration improves the penetration of active ingredients [40].

4. Today, the public awareness regarding the harmful effects of UV radiation combined with the problem of ozone layer depletion is rising [43]. Particulate sunscreens like titanium dioxide have been reported to penetrate into the skin, which can lead to adverse effects like irritation. This can be avoided or minimised by entrapping molecular and particulate sunscreens in the SLN matrix. Surprisingly, it was found that the SLN themselves have a sun protective effect [44].

5. SLN have an advantage with regard to product registration for pharmaceuticals and also cosmetics. There is a need to prove these products qualitatively and quantitatively. Quantitative analysis of SLN in creams is very simple. Most of the cream bases do not exhibit a melting peak below 100 °C, which means that the content of SLN in a cream can be quantified by their melting peak determined by differential scanning calorimetry (DSC). The stability during storage can easily be monitored just by looking at the change in melting enthalpy. Analysis is even possible in cases where a cream contains a fraction that melts below
100 °C, provided the peaks are well separated. This special property of SLN opens new market for topical products containing colloidal carriers for active ingredients [45].

6. SLN dispersions are white in colour, for example, they are identical to aqueous suspensions of titanium dioxide pigments. The white colour of SLN conceals the colour of active ingredients or their coloured degradation products. Concealing of the colour helps achieve an aesthetic appearance and consequently a better acceptance by the customer.

16.6 In Vitro Evaluation of Sesamol

Sesamol has been shown earlier to exhibit anti-mutagenic (reactive oxygen mediated) and anti-ageing activity [30, 31] in our laboratory and it has also been reported to exert chemopreventive effect. We have also reported the in vitro antioxidant activity of sesamol. As most of the antioxidants act due to their property to auto-oxidise and the pro- or antioxidant activity would depend on the concentration of the agent used and the free radical source, at least six dilutions in the concentration range of 5–1000 nmol of sesamol were selected for each test system. Furthermore, the antioxidant activity was compared with a water-soluble antioxidant (ascorbic acid). Sesamol was found to be an efficient scavenger of the entire range of ROS against which its activity was evaluated [29].

Furthermore, in most of the test systems, it was found to be more active than ascorbic acid. The total antioxidant capacity of sesamol, determined on the basis of the phosphomolybdenum complex formed, was significantly higher (1.8 times) than that of ascorbic acid.

Similarly, sesamol showed a significantly better scavenging of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical (1.5 times), superoxide anion (6 times) and nitric oxide (2.5 times), whereas its hydroxyl radical and hydrogen peroxide scavenging capacity was equivalent to that of ascorbic acid.

16.6.1 Anti-cancer Studies in Molt-4 and HL-60 Cancer Cell Lines

Apoptosis (‘normal’ or ‘programmed’ cell death) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Induction of apoptosis of cancer cells is a useful method or a valuable tool for cancer treatment [46]. A number of methods have now been developed to study apoptosis in cell populations. Apoptosis and cell-mediated cytotoxicity are characterised by cleavage of the genomic DNA into discrete fragments prior to membrane disintegration (Figure 16.3). Because DNA cleavage is a hallmark of
apoptosis, assays that measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death.

In many systems, this DNA fragmentation has been shown to result from the activation of an endogenous Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA), generating mono- and oligonucleosomal DNA fragments. These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit. Radioactive as well as nonradioactive methods to detect and quantify DNA fragmentation in cell populations have been developed. In general, these methods are based on the detection and/or quantification of either low-molecular-weight DNA (LMW DNA), which is increased in apoptotic cells, or high-molecular-weight DNA (HMW DNA), which is reduced in apoptotic cells. The underlying principle of these methods is that DNA that has undergone extensive double-stranded fragmentation (LMW DNA) may easily be separated from very large, chromosomal length DNA (HMW DNA), for example, by centrifugation and filtration. To detect fragmented DNA in cells that do not replicate in vitro, the DNA has to be isolated and analysed by agarose gel electrophoresis.

### 16.6.2 Deoxyribonucleic Acid Fragmentation Assay

Molt-4 and HL-60 cell lines were used for the DNA fragmentation assay. Etoposide, a standard anti-cancer agent, was used as the positive control. The cells were collected by centrifugation at 5000 rpm for 5 minutes. The pellet was washed with cold phosphate-buffered saline (PBS) and the cells were resuspended in the lysis buffer (sodium dodecyl sulfate, 0.5%; Tris-HCl, 50 mM; ethylenediaminetetraacetic acid (EDTA), 10 mM). Proteinase K at a concentration of 50 μg/ml was added to the suspension and the mixture was incubated at 50 °C for 1–3 hours, till the pellet dissolved completely. All the proteins were then precipitated out by adding an equal volume of a mixture of phenol:chloroform:isoamyl alcohol in the ratio of 25:24:1. The contents of the microcentrifuge tube were mixed gently and then centrifuged at a speed of 10,000 rpm for
10 minutes. The supernatant was separated and the pellet was suspended in 100 μl of TE (Tris-EDTA; 1×). The suspension was centrifuged again at the same speed, and the supernatant obtained was combined with the previous one. This step is included to obtain a maximum recovery of DNA. The combined supernatants were mixed with an equal volume of chloroform, vortexed gently till a suspension was formed and centrifuged at 5000 rpm for 5 minutes. The supernatant was recovered and the DNA was precipitated by adding two volumes of absolute ethanol and sodium acetate at a concentration of 0.3 M. Then the tubes were placed at -70 °C for 1–2 hours. Again, the tubes were centrifuged at the speed of 15,000 rpm for 15 minutes. The supernatant was aspirated and the precipitates were washed with 70% ethanol and air dried for 15 minutes. Then they were dissolved in TE (1×) using 40 μl of TE per two million cells. The samples mixed with loading buffer (5×, 250 mg of bromophenol blue in 33 ml of 150 mM tris + 60 ml of glycerol + 7 ml of water) were run on 1.8% agarose gel (voltage according to the length of the gel). Ethidium bromide at the concentration of 0.5 μg/ml of agarose gel was added during the preparation of the gel. The gel was visualised under UV [47]. During the last step of centrifugation, around 40–50% of the DNA sometimes remains sticking to the walls of the microcentrifuge tube after precipitation. So the walls of the tube were rinsed gently with TE several times (8–10 times) to recover maximum DNA such that there is sufficient DNA available in the gel to be observed in the form of bands. All the procedures were carried out in ice.

Sesamol showed DNA fragmentation in only HL-60 cell lines (Figure 16.4). Two forms of DNA cleavage have been observed during apoptosis [48]. One is the generation of 300 or 50 kbp DNA fragments detected by field gel electrophoresis [49]. The other is single-strand breaks [50]. Generation of single-strand DNA breaks is presumed to be one of the major causes of cell death by apoptosis in leukaemia cell lines.

16.6.3 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium Bromide Assay

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is another colorimetric assay used to determine cell viability after exposure of cancer cells to anti-cancer agents. Varying concentrations of sesamol were added to a 96-well plate, containing around 10,000 cells/100 μl/well, and the contents were incubated for 24 hours. Then 20 μl of MTT solution (5 mg/ml in PBS) was added into each well and incubated for 2–3 hours at 37 °C. After centrifugation at 2500 rpm, the supernatant was removed and 100 μl of dimethylsulfoxide was added into each well. After incubation for 15 minutes, the absorbance was read at 540 nm [51].

The above procedure is a highly automated, and a rapid test system to determine cell survival. The tetrazolium salt, MTT, is taken up into cells and reduced by a mitochondrial dehydrogenase enzyme to yield a purple formazan product, which
is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells results in the liberation of the product, which can readily be detected using a simple colorimetric assay. The ability of cells to reduce MTT serves as an indication of mitochondrial integrity and activity, which, in turn, may be interpreted as a measure of viability and/or cell number [52].

MTT assay clearly indicated sesamol to show a dose-dependent activity in both the cell lines, such that as the dose was increased from 10 to 100 μg, there was an increase in apoptotic activity by almost 60% (Figure 16.5).

16.6.3.1 Skin Retention Studies – Rationale for Development of Solid Lipid Nanocarriers

A known amount of sesamol solution (5 mg per application) or formulation (0.5 g; equivalent to 5 mg sesamol) was applied on the dorsal shaved skin surface of mice as a single topical application. The formulations were rubbed thoroughly on a marked
area of skin until no residue remained. Due caution was exercised while applying the formulation on the marked area.

The mice were sacrificed at different time intervals (1, 2, 4, 6, 10, 16 and 24 hours) after application of a suitable amount of sesamol solution (control) or sesamol in ointment (formulation B) and sesamol-loaded SLN (S-SLN) incorporated into a cream base (formulation I). For each sample, corresponding blanks where only the vehicle or the base was applied for suitable time periods were also run. The amount remaining in the skin was determined by homogenising the skin samples on which the drug solution or its formulations were applied. The homogenised samples were centrifuged and filtered, and then the drug content was determined spectrophotometrically.

On evaluating the data obtained for different time periods, in the case of aqueous sesamol solution (Table 16.1), the amount of drug present in the skin at 2 hours was

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>Amount of drug in μg (n = 3) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1877.00 ± 378.04</td>
</tr>
<tr>
<td>1</td>
<td>2293.00 ± 421.56</td>
</tr>
<tr>
<td>2</td>
<td>2751.66 ± 460.02</td>
</tr>
<tr>
<td>4</td>
<td>348.33 ± 60.50</td>
</tr>
</tbody>
</table>

Figure 16.5 MTT assay results of sesamol in Molt-4 (■) and HL-60 (□) cell lines. All values are significantly different from the other values (p < 0.05)
found to be significantly higher ($p < 0.05$) than that at 0.5, 1 and 4 hours. It was concluded that it takes almost 2 hours for the drug solution to attain a maximum concentration (>50% of that applied) within the skin, which shall depend upon its rate of penetration. The rate of penetration in turn depends on various physicochemical properties of the drug, including its molecular weight, structure, log $P$, hydrophobicity and aqueous solubility [53]. The significant reduction in concentration at 4 hours can be attributed to the loss of sesamol into the systemic circulation, which was attributed to its favourable log $P$ (1.29) and solubility (38.8 mg/ml).

In the case of sesamol ointment (formulation B), the drug concentration in the skin increases up to 6 hours, after which it declines (Table 16.2). The peak concentration achieved is almost 25% of that applied and out of that approximately 95% of the drug is lost into the systemic circulation at 10 hours. The reason could be a preferential partitioning of the drug into the ointment base because of which the drug release from the ointment is slow and less (only 25%). It seems that after 6 hours, whatever drug enters the skin is drained into the systemic circulation, again accounted for by a sufficient aqueous solubility and log $P$ of 1.29 of sesamol. Application of S-SLN incorporated into a cream base confirmed the usefulness of the developed SLN system. Upon application of the SLN cream (formulation I; Table 16.2), peak concentrations at 6 hours were much higher (1.6 times that achieved with formulation B) and this concentration was maintained up to 10 hours, indicating a sustained effect. Furthermore, it may be noted that approximately 40% of the peak concentration is still retained in the skin at 16 hours and the amount penetrating into the skin at 2 and 4 hours is significantly higher than that from the ointment base. On comparing

| Table 16.2 The amount of drug (μg) in the skin homogenates of animals sacrificed at various time intervals, after application of sesamol ointment (B) and SLN cream (I) |
|----------------------------------|-----------------|-----------------|
| Time in hours | Amount of drug in μg (n = 3) ± SD | Formulation B | Formulation I |
| 2 | 99.320 ± 8.465 | 566.167 ± 88.385 |
| 4 | 623.233 ± 25.350 | 813.7 ± 65.277 |
| 6 | 1190.033 ± 149.722 | 1875.333 ± 13.429 |
| 10 | 46.587 ± 10.191 | 1859.333 ± 25.541 |
| 16 | 14.710 ± 5.069 | 750.317 ± 55.090 |
| 24 | 7.723 ± 2.090 | 36.673 ± 9.511 |

$^a$ 0.5 g of formulation was applied, It contained 4790 ± 190 μg of drug

$^b$ 0.5 g of cream was equivalent to 4480 ± 170 μg of drug
the data of sesamol solution, ointment and SLN cream, it can be concluded that the drug is retained for a longer time and at a sufficiently higher concentration in skin in case of SLN cream.

The results indicate that sesamol is a highly permeable molecule (the solution applied probably enters systemic circulation with no lag time) as shown by high flux values in the *in vitro* studies; however, high permeability promotes high flux and subsequent loss into systemic circulation. This betokens incorporation of sesamol into a suitable system that will retain sesamol in the skin without compromising its permeability. For this purpose, SLN were developed.

16.7 Development and Characterisation of Sesamol-loaded Solid Lipid Nanoparticles

16.7.1 Preparation of Solid Lipid Nanoparticles by Microemulsification-solidification Method

SLN (Figure 16.6) were prepared from oil-in-water microemulsion prepared at 65–70 °C. The microemulsion was prepared using two different lipids, that is, glyceryl monostearate (GMS) or cetyl alcohol (2 g) as the internal phase, α-phosphatidylcholine (egg lecithin; 100 mg) as the surfactant, sodium deoxycholate or

![Figure 16.6](image)

*Figure 16.6* Transmission electron microscopic picture illustrating the morphology of S-SLN prepared with GMS
Tween-80 (100 mg) as the cosurfactant and distilled water (5 ml) as the continuous phase. Drug-loaded SLN were prepared by adding the drug (sesamol; 100 mg) to melted lipid at about 65–70 °C. Surfactant and cosurfactant dissolved in warm water were added successively to the melted mixture and a transparent microemulsion was obtained by stirring at about 65–70 °C. SLN were then obtained by dispersing the warm microemulsion (5 ml) in cold distilled water (100 ml; 2–3 °C) under mechanical stirring; the resulting dispersion was washed with distilled water, by dialysis (2 hours), to remove any free lipids and the unentrapped drug.

### 16.7.2 Particle Size Analysis

The average diameter of the formed SLN was determined by photon correlation spectroscopy (PCS) using Malvern Zetasizer. SLN were prepared using two different lipids, that is, GMS or cetyl alcohol, and two different cosurfactants, that is, sodium deoxycholate or Tween-80, along with egg lecithin as surfactant. GMS is a partial glyceride and cetyl alcohol is a wax, and both are considered lipids [54, 55]. The average particle sizes of the SLN dispersions measured by PCS are given in Table 16.3. It has been reported that the presence of ionic surfactants (sodium deoxycholate) decreases the particle size of SLN [55]. Similar observations can be made from Table 16.4. In all the cases, egg lecithin was used as the surfactant.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Co-surfactant</th>
<th>Size (nm)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMS</td>
<td>Sodium deoxycholate</td>
<td>127.9</td>
<td>0.256</td>
</tr>
<tr>
<td>GMS</td>
<td>Tween 80</td>
<td>174.9</td>
<td>0.299</td>
</tr>
<tr>
<td>Cetyl alcohol</td>
<td>Sodium deoxycholate</td>
<td>229.4</td>
<td>1.000</td>
</tr>
<tr>
<td>Cetyl alcohol</td>
<td>Tween 80</td>
<td>484.5</td>
<td>1.000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMS</td>
<td>88.21 ± 0.096</td>
</tr>
<tr>
<td>Cetyl alcohol</td>
<td>58.43 ± 0.126</td>
</tr>
</tbody>
</table>
The polydispersity index value of less than 1.00 indicates a narrow particle size distribution. SLN prepared with cetyl alcohol had greater particle size distribution (229.4 and 484.5) than those prepared with GMS (127.9 and 174.9), and the SLN dispersions so formed were not very stable in terms of dispersibility. Also the polydispersibility index is high for SLN prepared with cetyl alcohol, indicating a wide range of particle size. The critical parameters for nanoparticle formation are different for different lipids. The important parameters are the velocity of lipid crystallisation, the lipid hydrophilicity [56] and the shape of the lipid crystals. Several reports in literature indicate an influence of lipid composition on the particle size of SLN [57]. For example, the particle size of SLN prepared with Witepsol was 117 ± 1.8 nm and that with Dynasan was 175.1 ± 3.5 nm, which is due to the presence of shorter fatty acid chains and the surface-active nature of Witepsol. GMS is also reported to possess a surface-active property that facilitates emulsification and probably helps it to form more rigid surfactant films and therefore improve the long-term stability of SLN prepared with it [58].

The choice of the emulsifiers and their concentration has a great impact on the quality of the SLN dispersion [41]. It has been reported that SLN stabilised with surfactant mixtures (Lipoid S 75/polyoxamer 188 or tyloxapol/lecithin) [59] have lower particle sizes and higher storage stability. Addition of sodium glycocholate to the aqueous phase as a coemulsifying agent has been reported to decrease the particle size of SLN [59]. The particle size of SLN produced with ionic surfactants is also reported to be smaller than that of nonionic formulation [60]. A combination of the above-mentioned factors could be responsible for the smaller size (127.9 nm) of the SLN prepared with GMS, egg lecithin and sodium deoxycholate, and hence were selected for subsequent studies.

16.7.3 Encapsulation Efficiency

SLN dispersions (4 ml) were ultracentrifuged at 60,000 rpm for 1 hour at 4 °C. The amount of drug in the pellet gave a direct measure of the extent of encapsulated drug. Considering that in general the maximum drug-loading capacity for SLN prepared by microemulsification-solidification method is 20% [61], we used 100 mg of drug/2 g of lipid. The encapsulation efficiency of S-SLN as obtained by experimental studies is presented in Table 16.4.

\[
\text{encapsulation efficiency} = \left( \frac{\text{amount of drug/ml of SLN dispersion} \times \text{total volume of dispersion}}{\text{total drug incorporated}} \right) \times 100
\]

(16.1)

An encapsulation efficiency of almost 90% was achieved with SLN prepared from GMS, which was almost 1.5 times more than that achieved with cetyl alcohol.
SLN. The encapsulation efficiency of the drug in SLN mainly depends on the solubility of the drug in the lipid melt. For this reason and for reasons discussed earlier, GMS SLN containing sodium deoxycholate were used for the subsequent in vivo studies.

16.7.4 Differential Scanning Calorimetry

For thermal analysis, a sample of the sesamol, all the excipients and S-SLN were scanned using DSC and the thermograms so generated were observed for any significant shift or disappearance/appearance of new peaks. DSC is a tool for investigating the melting and recrystallisation behaviour of crystalline materials like SLN. Figure 16.7 depicts DSC thermograms of pure sesamol, GMS, cetyl alcohol and the SLN dispersion of sesamol. The DSC curve for pure sesamol shows a single fusion endotherm, representing the melting point at 65.3 °C. Pure GMS exhibits the endothermic peak at 58.7 °C and pure cetyl alcohol at 53.7 °C while no peak was observed at 65.3, 58.7 or 53.7 °C in the case of SLN dispersion.

Figure 16.7 DSC thermographs of (a) sesamol; (b) cetyl alcohol; (c) GMS; and (d) S-SLN
If the bulk material is turned into SLN, the melting point is depressed irrespective of the presence of drug. The decrease of the onset and maximum temperature observed for S-SLN could be due to the effect of small size [59]. Organic materials usually show a melting range, and an increase in the melting range is correlated with the presence of impurities or less-ordered crystals. Thus melting enthalpy is another tool to characterise the crystal order. If the melting enthalpy is higher, there is higher ordered lattice arrangement and vice versa. Therefore, the lipids within nanoparticles should be in a less-ordered arrangement compared to the bulk materials so that the melting peak is depressed. It may thus be inferred that sesamol is entrapped within the GMS matrix indicating the successful formulation of SLN.

16.8 Skin Permeation Studies

Jacketed Franz glass diffusion cells were used for the determination of permeability of sesamol solution and S-SLN through mice skin. The cells consisted of donor and receptor chambers between which mice skin was positioned. The area for diffusion varied between 0.785 ± 0.15 cm² and the receptor chamber volume varied from 6.8 to 7.0 ml. These values were predetermined for each diffusion cell and the respective values were subsequently used for calculations. LACA mice, 6–8 weeks old, were sacrificed by cervical dislocation. After depilation, a section of the dorsal skin surface was excised from the animals with surgical scissors. The skin was lifted and the adhering fat and other visceral debris were removed carefully from the surface underneath by wiping with isopropanol.

The receptor medium used consisted of phosphate buffer, pH 7.4. Circular skin pieces equal to the size of external circumference of donor compartment were cut and mounted onto the diffusion cell assembly by keeping the stratum corneum side towards the donor compartment, and the dermal side of the skin was kept in contact with the receptor fluid. The whole system was water jacketed and thermostatically controlled by an external circulating water bath at 37.0 ± 1.0 °C. The receptor medium was stirred, throughout the experiment, using a magnetic stirrer. The skin was mounted and was allowed to equilibrate for 48 hours. The receptor phase was changed 3 times during this period, so as to remove any water-soluble UV-absorbing materials present in or released from the skin, which could interfere with the UV spectrophotometric readings of the drug. Before applying the formulation, the receptor medium was replaced with fresh medium. Then pure sesamol solution (2 mg/ml sesamol) and its SLN formulations; 100 mg of cream with the incorporated 1 ml of SLN (containing 2 mg of sesamol); as well as blank SLN were applied evenly onto the donor side of the skin surface and the donor cell was covered with parafilm to prevent evaporation. Aliquots (0.5 ml) of the samples were withdrawn from the receptor compartment at different time intervals starting at 15 and 30 minutes, 1, 2,
4, 6, 8, 12, 24, 36 to 48 hours and replaced by an equal volume of fresh phosphate buffer. The experiments were carried out for 48 hours. The samples were suitably diluted and analysed spectrophotometrically at the $\lambda_{\text{max}}$ of 290 nm.

After 48 hours, the skin was wiped with a moist cotton swab several times (4–6 times) to remove any residual formulation sticking to the skin. The skin samples were minced thoroughly and homogenised in 5 ml of phosphate buffer (ensuring the absence of solid particles) to a viscous turbid mixture. The homogenised samples were centrifuged (4000 rpm for 10 minutes) to get almost clear supernatants. The supernatants were further clarified by passing through membrane filters. Drug content in the supernatants was determined spectrophotometrically after appropriate dilutions. The raw data obtained from permeation studies were analysed by applying a correction factor for volume and drug losses during sampling using the ZOREL software [62]. The flux was obtained by plotting the cumulative amount of drug(s) in the receptor phase per square centimetre of the skin against time. Steady-state slope obtained by linear regression of cumulative amount per square centimetre versus time plot gives the flux value.

The flux values obtained with sesamol when applied as such are significantly higher. This is attributed to the small size of the molecule and its optimum lipophilic and hydrophilic nature. Incorporation of sesamol into SLN resulted in controlled release and an increased retention in skin as shown by the lag time of 1.5 hours and a 3 times higher skin retention (Figure 16.8).

**Figure 16.8** Cumulative amount of sesamol permeated (µg) per square centimetre versus time for SLN and SLN incorporated into the cream base
Furthermore, the SLN were incorporated into a cream base due to the following reasons: it has been reported in the literature that the stability of SLN increases if incorporated into a cream base, because the mixing of aqueous SLN dispersion with cream reduces the probability of particle collision and subsequent aggregation [40]. Furthermore, the surfactants present in creams are expected to protect and stabilise the lipids [39, 63]. Also, incorporation of SLN into a cream would help to retard drug release such that the duration of action increases [42] as confirmed in our studies also (Table 16.5). Hence, the SLN dispersions were incorporated into a cream base to reach an optimum concentration and to achieve better stability, desirable spreadability and a prolonged duration of action. Flux values, lag time and the amount retained in skin after 48 hours of application of sesamol solutions, S-SLN dispersions and S-SLN in cream base are given in Table 16.5.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Flux (μg/sq.cm/h)</th>
<th>Lag time (hours)</th>
<th>Skin retention (μg) (after 48 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sesamol</td>
<td>38.92 ± 0.62</td>
<td>0</td>
<td>200 ± 0.16</td>
</tr>
<tr>
<td>S-SLN</td>
<td>23.06 ± 0.17</td>
<td>1.5 ± 0.01</td>
<td>600 ± 0.34</td>
</tr>
<tr>
<td>S-SLN cream</td>
<td>15.76 ± 0.63</td>
<td>2 ± 0.02</td>
<td>750 ± 0.52</td>
</tr>
</tbody>
</table>

### 16.9 Pharmacokinetic Studies

Healthy male Wistar rats (200–250 g, 3 months old) were used for the pharmacokinetic studies. After 1 week of quarantine, they were housed three per cage in a room with normal light and dark cycle. They were given a commercial rat chow and water *ad libitum*. The experiments started after acclimatisation for at least 1 week. Each group consisted of six animals. Sesamol was administered orally as a solution, and its SLN were applied topically. The dose of sesamol used was 4 mg/kg. Blood samples (500 μl) were collected from the cannulated femoral artery into heparinised tubes at 5, 15, 30 and 45 minutes, 1, 2, 3, 4, 6, 8, 12, 24 and 48 hours. After sample collection, blood was immediately transferred to the tubes containing 50 μl of heparin to avoid coagulation. The plasma was obtained after centrifugation for 10 minutes at 5000 rpm. The plasma samples were stored at −80 °C until used.

Plasma samples were analysed for sesamol using a validated high-performance liquid chromatography (HPLC) method developed by us. For the analysis of sesamol in plasma, 100 μl of plasma sample was mixed with 100 μl of methanol to precipitate...
Figure 16.9 Plasma concentration-time profiles of sesamol when administered by oral route (a) and as SLN topically (b)

The proteins. Then the tubes were centrifuged at 5000 rpm for 15 minutes and the clear supernatant obtained was injected into the HPLC.

The plasma-concentration time curves for orally administered sesamol and topically applied S-SLN are shown in Figure 16.9. When the data were analysed, the best fit was achieved with a two-compartment model, which is described by the following mathematical equation:

\[
C(t) = B e^{-\beta t} + A e^{-\alpha t} - C_0 e^{-K_a t}
\]  

(16.2)

where \(C(t)\) is the concentration at time \(t\), \(C_0\) the concentration at time 0, \(\beta\) the elimination rate constant, \(\alpha\) the distribution rate constant, \(B\) the intercept of back-extrapolated monoexponential elimination slope \(\beta\) with the ordinate, \(A\) the intercept of distribution slope \(\alpha\) with the ordinate and \(K_a\) the absorption rate constant.

The pharmacokinetic parameters of sesamol and its SLN are listed in Table 16.6. The elimination half-life for free sesamol was 10.9 ± 0.06 hours while it was 17.03 ± 0.15 hours after SLN formulation was applied topically. The \(C_{\text{max}}\) value was maximum (0.44 µg/ml) when sesamol was administered orally, while the \(t_{\text{max}}\) was 2 hours. However, in case of SLN, wherein the amount of drug appearing in blood is almost half that of sesamol and it reaches almost 2 hours later than sesamol (\(C_{\text{max}}\; 0.22 ± 0.001\; \mu\text{g/ml at 4 hours in case of topically applied SLN); it may be said that a large amount of drug was retained in the skin and for a longer period of time, thus improving its therapeutic action as was aimed at when developing the formulation and establishing potential of S-SLN as a topical formulation for skin cancers.

Elimination half life \(t_{\frac{1}{2}}\); \(C_{\text{max}}\) – Maximum concentration; \(t_{\text{max}}\) – Time to reach maximum concentration; F – Absolute bioavailability; \(V_{\text{dss}}\) – Volume of distribution at steady state; AUC – Area under the curve; \(CL_{\text{tot}}\) – Total clearance.
In Vivo Skin Anti-cancer Studies

Carcinogenesis is a multistep process involving the sequential accumulation of mutations that allow cells to gain a selective growth advantage, have the phenotypic attributes of local invasiveness and acquire the ability to form distant metastasis. At the molecular level, progression results from accumulation of genetic lesions. Chemoprevention is the use of one or several agents to prevent the occurrence of cancer [64, 65]. The mouse skin carcinogenesis model is very useful for studying the genetic and biological changes involved in tumour promotion [66, 67]. Some of the genetic changes associated with the chemical initiation of benign papillomas and the transition to squamous cell carcinoma have been characterised in a mouse skin carcinogenesis model [68].

Many tumour promoters have been shown to exert their action by production of ROS, and many compounds that possess antioxidant activity have been reported to inhibit tumour promotion [69, 70]. Several compounds possessing antioxidant or anti-inflammatory

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Oral solution</th>
<th>S-SLN applied topically</th>
</tr>
</thead>
<tbody>
<tr>
<td>t½ (h)</td>
<td>10.9 ± 0.06</td>
<td>17.03 ± 0.15</td>
</tr>
<tr>
<td>Cmax (μg/ml)</td>
<td>0.44 ± 0.001</td>
<td>0.22 ± 0.001</td>
</tr>
<tr>
<td>tmax (h)</td>
<td>2.00 ± 0.04</td>
<td>4.00 ± 0.08</td>
</tr>
<tr>
<td>F (%) up to 24 h</td>
<td>95.61 ± 1.65</td>
<td>75.19 ± 1.9</td>
</tr>
<tr>
<td>F (%) up to 48 h</td>
<td>–</td>
<td>104.29 ± 2.5</td>
</tr>
<tr>
<td>Kd (h⁻¹)</td>
<td>5.15 ± 0.016</td>
<td>1.39 ± 0.016</td>
</tr>
<tr>
<td>Vds (L)</td>
<td>2.02 ± 0.002</td>
<td>6.59 ± 0.002</td>
</tr>
<tr>
<td>AUC (μg/ml).h-24 h</td>
<td>6.01 ± 0.004</td>
<td>4.73 ± 0.005</td>
</tr>
<tr>
<td>AUC (μg/ml).h-4 8 h</td>
<td>–</td>
<td>6.57 ± 0.05</td>
</tr>
<tr>
<td>Cltot (L/h)</td>
<td>0.127 ± 0.002</td>
<td>0.237 ± 0.001</td>
</tr>
</tbody>
</table>

AUC: Area under the curve
CLtot: Total clearance
Cmax: Maximum concentration
F: Absolute bioavailability
t½: Elimination half life
tmax: Time to reach maximum concentration
Vds: Volume of distribution at steady state
activity are able to inhibit 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced tumour promotion in mouse skin [71–74], and it is expected that natural phenolic antioxidants may have a modifying or inhibitory effect on tumour promotion /carcinogenesis.

*In vitro* studies in our laboratory have indicated sesamol to possess good antioxidant activity (1.48 ascorbic acid equivalent and 1.14 vitamin E equivalent). The two-stage mouse skin carcinogenesis model was used to test the efficacy of sesamol and S-SLN to prevent, alter or reverse the promotional changes that occur during the process of neoplastic development, under *in vivo* conditions.

**16.10.1 Animal Model and Treatment Groups**

Male Laca mice, 4–6 weeks old, were used in the study. The animals were kept in well-ventilated polypropylene cages. The mice were fed standard laboratory diet and water *ad libitum*. The backs of the animals were shaved 2 days prior to the start of the experiment and an area of 4 cm² was marked for application of various treatments (carcinogen and test or control drug and S-SLN). The animals were divided into seven groups (*n* = 10 for each group). Group I animals received three topical applications of 150 μg benzo(α)pyrene (B(α)P) in acetone (50 μl per mouse) over a period of 2 weeks (once a week starting from 0 day), followed by 5 nmol TPA in acetone (50 μl per mouse) twice weekly for 12 weeks (*Figure 16.10*).

![Figure 16.10](image)

**Figure 16.10** Experimental protocol for the present short-term skin cancer studies in mice. B(α)P (∆), 150 μg/50 μl acetone/mouse topically once a week for 3 weeks, and TPA (•), 5 nmol/50 μl acetone/mouse topically twice a week up to 12 weeks. The control group received 50 μl acetone/mouse (o) at all the corresponding times.
The mice of groups II–IV were treated as group I mice, except that they were treated with sesamol (10 mg/kg), S-SLN and tretinoin cream (15 nmol/30 mg cream), respectively, 30 minutes after the application of the above dose of TPA twice weekly for 12 weeks. Group V animals (per se group) were treated topically with the drug alone (sesamol 10 mg/kg). Group VI animals served as naive controls. These animals received acetone (50 μl per mouse) application only. At the end of 12 weeks, the animals were killed for histopathological and biochemical studies. The marked area on the dorsal skin was quickly excised and washed thoroughly with chilled PBS (pH 7.4). A 10% tissue homogenate (w/v) was prepared from part of the samples in 0.15 M Tris-HCl (pH 7.4) and the homogenates were then centrifuged at 12,000 g for 15 minutes. The supernatant thus obtained was taken for estimation of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA) and protein. The remaining part of the skin samples were used for histopathological evaluations.

16.10.2 Macroscopic Evaluation of Cancer

The number of papillomas that developed on the dorsal skin surface was enumerated before the histological examination (Figure 16.11). The percentage of skin tumours in mice treated with B(a)P/TPA was 33.33% (group I). The onset of tumours (>2 mm) commenced at 9 weeks in B(a)P/TPA-treated mice, whereas in drug/formulation/B(a)P/TPA-treated mice, the onset of tumours (i.e., >2 mm) was delayed until 12 weeks, that is, there was 0% incidence of skin tumours during the tenure of present study (groups II–IV).

16.10.3 Biochemical Analysis of Skin Homogenates

The skin of individual mice was homogenised in cold 1.15% potassium chloride (KCl) and the various biochemical estimations were performed (n = 4). The significance of the difference in the values of GSH, MDA, CAT and SOD with respect to the control and the test groups (Table 16.7) was determined using Tukey’s test. p-Values <0.05 were considered significant.

Lipid peroxidation was found to be significantly higher in the carcinogen-treated (positive control) group (p < 0.05) as compared to the sesamol- or S-SLN-treated mice skin homogenates (Figure 16.12). The above may be attributed to the lipid peroxidation induced by either free radicals participating in B(a)P epoxidation, or the B(a)P epoxides or the tumour necrosis factor produced by the malignant cells [75]. Similarly, significant differences (reduction in levels) were also observed in GSH, CAT and SOD levels (p < 0.05) (Figure 16.10). A statistically significant difference (p < 0.05) was observed in CAT activity when the treatment groups were compared with the positive control animals (Figure 16.12). GSH activity of carcinogen-treated mice was significantly lower than that of the naive control, and the sesamol- and S-SLN-treated
Figure 16.11 Photographs of normal skin and skin given different treatments

Table 16.7 Effect of sesamol and its SLN on LPO (MDA, nmoles/mg of protein), SOD (units/mg of protein), CAT (units/mg protein) and GSH levels (nmoles/mg protein) in cancer induced mice skin homogenates ($n = 4$)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LPO</th>
<th>CAT</th>
<th>GSH</th>
<th>SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>2.09 ± 0.09*</td>
<td>6.85 ± 0.6</td>
<td>31.95 ± 0.14‡</td>
<td>20.24 ± 1.2</td>
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<tr>
<td>Carcinogen</td>
<td>15.31 ± 0.07</td>
<td>2.01 ± 0.2</td>
<td>7.27 ± 0.02</td>
<td>8.71 ± 0.45</td>
</tr>
<tr>
<td>Sesamol</td>
<td>4.40 ± 0.13</td>
<td>3.69 ± 0.3</td>
<td>11.97 ± 0.19*</td>
<td>14.68 ± 1.50</td>
</tr>
<tr>
<td>Sesamol-SLN</td>
<td>3.84 ± 0.04</td>
<td>5.51 ± 0.5</td>
<td>13.74 ± 0.08†</td>
<td>18.48 ± 1.12*</td>
</tr>
<tr>
<td>Tretinoin</td>
<td>4.83 ± 0.40</td>
<td>4.63 ± 0.2*</td>
<td>12.21 ± 0.02*</td>
<td>13.18 ± 1.01</td>
</tr>
<tr>
<td>Sesamol-per se</td>
<td>2.05 ± 0.09*</td>
<td>5.92 ± 0.4</td>
<td>31.68 ± 0.04‡</td>
<td>20.47 ± 1.17</td>
</tr>
</tbody>
</table>

All the groups are significantly different from other groups except those marked with *, ‡ and †.
mice skin homogenates (Figure 16.12) reverted the levels towards normal ($p < 0.05$). S-SLN showed a better anti-cancer activity than tretinoin ($p < 0.05$).

In multistep carcinogenesis, ROS have been shown to play a role mostly in the promotion phase. Antioxidants are reported to act as protective agents against cancer [73, 76, 77]. The same is evident with sesamol treatment. Sesamol treatment seems to reinstate the losses in GSH levels provoked by $B(a)P/TPA$ treatment. The low levels of antioxidant enzymes SOD and CAT in $B(a)P/TPA$-treated mice show a poor endogenous antioxidant status. The increase in antioxidant enzymes by treatment with sesamol or its SLN reflects that they inhibit the process of oxidative stress-induced carcinogenesis. Several reports suggest that GSH is a more efficient antioxidant agent than SOD or CAT [78]. GSH alters the profile of lipoxygenase and cyclooxygenase [79, 80], which are involved in tumourigenesis. Oberley and Oberley [81] reported decreased SOD and CAT activity in papillomas and squamous cell carcinoma leading to a pro-oxidant state of cells, facilitating tumourigenesis. But GSH levels have been found to be highly variable and contradictory, depending on the cell type and nature of the carcinogen and its modulatory pathways [82, 83]. An increase in the level of GSH by the chemopreventive action of flavonoids in mouse skin has been reported [84].

During oxidative stress, MDA and/or other aldehydes are formed in biological systems. These can react with amino acids and DNA and introduce crosslinks between proteins and nucleic acids, resulting in alterations in replication and transcription [85] leading to tumour formation. Elevated levels of MDA were observed in skin tumours of animals
treated with B(a)P/TPA, suggesting oxidative stress in B(a)P/TPA-induced mouse skin carcinogenesis. Reduction in the level of lipid peroxides in the mouse skin model in response to certain flavonoids has also been reported earlier [84]. Thus, a significant decrease in MDA levels induced by sesamol treatment indicates its ability to reduce endogenous oxidative stress, thus indicating its protective potential against skin carcinogenesis.

### 16.10.4 Histopathological Analysis

Mice skin was fixed in 10% formalin and embedded in paraffin. Sections 2–3 mm in thickness were cut on a rotary microtome and stained with haematoxylin and eosin. Histopathology of group I at 12 weeks showed necrotic, thick keratinised squamous pearls, suggesting squamous cell carcinoma (Figure 16.13). Epidermal layer had

![Histopathology images](image-url)

**Figure 16.13** Histopathology of carcinogen-treated and drug-treated mice skin
proliferated and was up to 12 cells thick (2–4 layers of cells in normal mice skin). The basal cells were malignant showing active mitotic figures. The deeper subcutaneous tissue showed reactive inflammation (Figure 16.13). The group treated with sesamol solution [86] showed occasional foci of squamous cell proliferation with nuclear atypia. The epidermal layer had again proliferated but was only 4–6 cells thick, with mild fibrosis in the superficial dermis (Figure 16.13). But in the S-SLN-treated mice (group III), the skin was more towards normal, with tiny squamous pearls. The tretinoin-treated mice skin (group IV) had a slight epidermal hyperplasia with nuclear atypia (Figure 16.13). In free sesamol-treated group (group V), some focal epidermal hyperplasia with nuclear atypia could be observed. This may be due to some local irritation caused by sesamol solution, probably due to its high and immediate permeability with no lag.

16.11 Conclusions

Proposing a scientific rationale for the purported anti-cancer effects of sesamol (as seen in \textit{in vitro} anti-cancer studies) followed by its pharmaceutical development forms the genesis of this study. Physicochemical characterisation of sesamol (small molecule with sufficient aqueous solubility and log $P$ of 1.29) coupled with the \textit{ex vivo} skin permeation and \textit{in vivo} skin retention studies indicates the need to design a controlled-release system for sesamol, so as to maximise its retention in the skin for prolonged periods to elicit a physiological effect. Thus, its incorporation into SLN not only enhanced its bioavailability in the skin but also helped achieve the desired pharmacodynamic effect. In addition, the study established the usefulness of S-SLN as a therapeutic for the treatment of cancers after induction. Most of the phytochemical/polyphenolic antioxidants show a preventive effect. As they may interfere with the macrophage-induced oxidative burst for kill of cancer cells, their use during initiation is not recommended. This may be responsible for the failure of clinical trials using chronic antioxidant supplementation. The use of SLN helps target sesamol to skin layers for longer periods and the slow release ensures a therapeutic effect.

References


Indu Pal Kaur, Thanga M. Geetha and Vandita Kakkar


Treatment of Skin Cancer


82. L.W. Oberley and T.D. Oberley, Molecular and Cellular Biochemistry, 1988, 84, 147.


Abbreviations

Chapter 1
ICH International Conference on Harmonisation
ROS reactive oxygen species

Chapter 2
5-FU 5-fluorouracil
ATRA all-trans retinoic acid
AUC area under the curve
BBB blood-brain barrier
CDDS controlled drug delivery system
CPC cetylpyridinium chloride
CTAB cetyltrimethylammonium bromide
EPR enhanced permeability and retention
FMLP f-Met-Leu-Phe
HLB hydrophilic-lipophilic balance
HUVEC human umbilical vein endothelial cell
IV intravenous
LNP lipid nanoparticle
MDR multidrug resistance
PEG polyethylene glycol
P-gp P-glycoprotein
PMN polymorphonuclear
RES reticuloendothelial system
SC subcutaneous
### Abbreviations

#### Chapter 3

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>adenosine triphosphate-binding cassette</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMEA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EPR</td>
<td>enhanced permeability and retention</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>$^{111}$In-2C5-LCL</td>
<td>$^{111}$In-radiolabelled long-circulating tumour-targeted liposome</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HPV</td>
<td>human papillomavirus</td>
</tr>
<tr>
<td>ISCOM</td>
<td>immune stimulating complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>QD-IL</td>
<td>quantum dot-conjugated immunoliposomes</td>
</tr>
<tr>
<td>scFv</td>
<td>single-chain variable fragment</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SLN</td>
<td>solid lipid nanoparticles</td>
</tr>
<tr>
<td>SSL</td>
<td>sterically stabilised liposomes</td>
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</tbody>
</table>

#### Chapter 4

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>ATRA</td>
<td>all-trans retinoic acid</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>CA</td>
<td>camptothecin</td>
</tr>
<tr>
<td>CA-SLN</td>
<td>camptothecin SLN</td>
</tr>
<tr>
<td>CLB</td>
<td>chlorambucil</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DDAB</td>
<td>dimethyl dioctadecyl ammonium bromide</td>
</tr>
</tbody>
</table>
Abbreviations

DO-FUdR 3′,5′-dioctanoyl-5-fluoro-2′-deoxyuridine
DOX  doxorubicin
DXM  dexamethasone
EPR  enhanced permeability and retention
FUdR  5-fluoro-2′-deoxyuridine
IC_{50}  half-maximal inhibitory concentration
IP  intraperitoneal
IV  intravenous
MDR  multidrug resistance
MMC  mitomycin C
MPS  mononuclear phagocytic system
MRI  magnetic resonance imaging
MRT  mean residence time
MTX-INJ  MTX injection
MTX-SLN  methotrexate SLN
NLC  nanostructured lipid carrier
o/w  oil-in-water
PACA  polyalkylcyanoacrylate
PEG  polyethylene glycol
PEI  polyethylenimine
P-gp  P-glycoprotein
PLGA  polylactic/glycolic acid
PLN  polymer-lipid hybrid nanoparticle
PTX  paclitaxel
PTX-INJ  PTX injection
PTX-SLN  PTX-loaded tristearin SLN
RES  reticuloendothelial system
SC  subcutaneous
SLN  solid lipid nanoparticle
StA  stearic acid
STAT3  signal transducer and activator of transcription 3
Tc-99m  technetium-99m
TMZ  temozolomide
Abbreviations

Chapter 5

AIDS	 acquired immune deficiency syndrome
ALL	 acute lymphoblastic leukaemia
AML	 acute myeloid leukaemia
APRPG	 Ala-Pro-Arg-Pro-Gly
AUC	 area under the drug concentration–time curve
CML	 chronic myeloblastic leukaemias
EGFR	 epidermal growth factor receptor
EPR	 enhanced permeability and retention
FDA	 US Food and Drug Administration
FR	 folate receptors
HCC	 hepatocellular carcinoma
IFP	 interstitial fluid pressure
IT	 intrathecal
IV	 intravenous
LEP-ETU	 Liposome-entrapped paclitaxel easy-to-use
MDR	 Multidrug resistance
MMP	 matrix metalloproteinase
MPS	 mononuclear phagocyte system
MTD	 maximum tolerated dose
NB	 neuroblastoma
NDA	 New Drug Application
NGR	 asparagine-glycine-arginine
NSCLC	 non-small cell lung cancer
PEG	 polyethylene glycol
PLD	 PEGylated liposomal doxorubicin
RES	 reticulo-endothelial system
RGD	 arginine-glycine-aspartic acid
RNAi	 RNA interference
SCID	 severe combined immunodeficiency
siRNA	 small interfering RNA
Tf	 Transferrin
TfR	 Transferrin receptors
VIP	 vasoactive intestinal peptide
VSLI	 vincristine sulfate liposome infusion
Abbreviations

Chapter 6

CHOL  cholesterol
DAS  dorsal air sac
DC-6-14  O,O'-ditetradecanoyl-N-(α-trimethylammonioacetyl) diethanolamine chloride
DOPE  1,2-dioleoyl-sn-glycero-3-phosphoethanol-amine
DOTAP  1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl]-3-trimethylammonium propane
EPR  enhanced permeability and retention
FDA  U.S. Food and Drug Administration
HSPC  hydrogenated soy phosphatidylcholine
l-OHP  oxaliplatin
mPEG$_{2000}$-DSPE  1,2-distearoyl-sn-glycero-3-phosphoethanolamine-$n$-[methoxy (polyethylene glycol)-2000]
MPS  mononuclear phagocyte system
PEG  polyethylene glycol
PHSCN  N-acetyl-proline-histidine-serine-cysteine-asparagine-amide
VEGF  vascular endothelial growth factor
VEGF-A  vascular endothelial growth factor-A

Chapter 7

β-gal  β-galactosidase
Ad  adenovirus
Bik  Bcl2-interacting killer
CAT  chloramphenicol acetyltransferase
CMV  cytomegalovirus
DC-Chol  3β[N-(N′N′-dimethylaminoethane)-carbamoyl]cholesterol
DOPE  dioleoylphosphatidylethanolamine
DOTAP:Chol  DOTAP:cholesterol
FDA  Food and Drug Administration
IL  interleukin
IP  intraperitoneal
IT  intratumoral
IV  intravenous
Luc  luciferase
Abbreviations

mda-7  melanoma differentiation-associated gene-7  
MTD  maximum tolerated dose  
NSCLC  non-small cell lung cancer  
NTB  non-tumour-bearing  
PEG  polyethylene glycol  
RES  reticuloendothelial system  
SC  subcutaneous  
TB  tumour-bearing  
TSG  tumour suppressor gene  
VISA  VP16-GAL4-WPRE integrated systemic amplifier  

Chapter 8

AFM  atomic force microscopy  
BBB  blood-brain barrier  
CTX  chlorotoxin  
DHP  sodium dihexadecylphosphate  
DODAB  dioctadecyldimethylammonium bromide  
DODAC  dioctadecyldimethylammonium chloride  
DOX  doxorubicin  
DPPC  1, 2-dipalmitoyl-sn-glycero-3-phosphocholine  
DPPC  Dipalmitoylphosphatidylcholine  
DSPE  diesteroylphosphatidylamine  
EPL  egg phospholipid  
FR  folate receptor  
HA  haemagglutinin  
HAV  hepatitis A virus  
HDL  high-density lipoproteins  
HEPES  [N-(2-hydroxyethyl) piperazine-N9-(2-ethanesulfonic acid)]  
LbL  layer-by-layer  
MDR  Multidrug resistance  
PC  phosphatidylcholine  
PDDA  poly(diallyldimethylammonium chloride)  
PE  polyelectrolyte  

532
Abbreviations

PEG Poly(ethylene glycol)
PEI P-glycoproteins
PI phosphatidylinositol
PNP polymeric nanoparticles
POPC 1-palmitoyl-2-oleyl-sn-glycero-3-phosphatidylcholine
Ps phosphatidylserine
PS polystyrene sulfate
PSA polystyrene amidine
PSS poly(sodium 4-styrenesulfonate)
RES reticuloendothelial system
RNAi RNA interference
QCM-D quartz crystal microbalance with dissipation monitoring
siRNA small-interfering RNA
SLB supported lipid bilayers
SLN solid lipid nanoparticles
TH transhydrogenase
Tris Tris-hydroxymethyl aminomethane

Chapter 9

DPPG dipalmitoyl phosphatidyl glycerol
EGFR Epidermal growth factor receptor
EPR enhanced permeability and retention
LTT Ligand-targeted therapy
NSCLC non-small cell lung carcinoma
PEG Polyethylene glycol
RES reticuloendothelial system
SCLC small cell lung carcinoma
VEGF vascular endothelial growth factor

Chapter 10

AE antioestrogens
AI aromatase inhibitors
Abbreviations

BC breast cancers
CBP (CREB-binding protein)
CDK cyclin-dependent kinase
COX-2 cyclooxygenase-2
EGFR Epidermal growth factor receptor
EPR enhanced permeability and retention
ER oestradiol receptors
FA folic acid
FDA Food and Drug Administration
FR folate receptor
FTI Farnesyl transferase inhibitors
HAT histone acetyltransferase
HDAC histone deacetylase
HDACi histone deacetylase inhibitors
IV intravenous
LBD ligand-binding domains
LHRH luteinising hormone-releasing hormone
MFL Magnetic-fluid-loaded liposomes
MPS mononuclear phagocyte system
NAD+ nicotinamide
NS nanospheres
NTS neurotensin
PEG polyethylene glycol
PLA poly(D,L-lactic acid)
PLGA poly (D,L-lactic acid/co-glycolic acid)
SAHA suberoyl anilide hydroxamic acid
SERD selective E₂ receptor down regulator
SERM selective oestrogen receptor modulator
shRNA short hairpin RNA
siRNA small interfering RNA
SRC steroid hormone receptor coactivator
VEGF vascular endothelial growth factor
TF transcription factors
TSA trichostatin A
Chapter 11

Abbreviations

ACC adrenocortical carcinoma
ACTH adrenocorticotropic hormone
AIMAH ACTH-independent macronodular adrenal hyperplasia
BWS Beckwith-Wiedemann syndrome
Chol cholesterol
CNC Carney’s complex
DHSM dihydrosphingomyelin
ESM egg sphingomyelin
IGF1R IGF1 receptor
LFS Li-Fraumeni syndrome
MEN1 multiple endocrine neoplasia type 1
o,p’-DDA o,p’-dichlorodiphenyl acetic acid
PEG polyethylene glycol
PPNAD primary pigmented nodular adrenocortical disease
SLN solid lipid nanoparticle
TEM tumour endothelial marker
VEGF vascular endothelial growth factor

Chapter 12

ABC ATP-binding cassette
ALA α-linolenic acid
ARA arachidonic acid
ATP adenosine triphosphate
BBB blood-brain barrier
BCRP breast cancer resistance protein
BNC bionanocapsule
CED convection-enhanced diffusion
CNS central nervous system
CSF cerebrospinal fluid
DHA docosahexaenoic acid
DOX doxorubicin
EGFR epidermal growth factor receptor
EPA eicosapentaenoic acid
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GBM</td>
<td>glioblastoma multiforme</td>
</tr>
<tr>
<td>GLUT</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>HLB</td>
<td>hydrophilic-lipophilic balance</td>
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<td>IAC</td>
<td>infiltrative astrocytoma</td>
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<td>intracerebroventricular</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>LA</td>
<td>linoleic acid</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>ME</td>
<td>microemulsion</td>
</tr>
<tr>
<td>MPS</td>
<td>mononuclear phagocyte system</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance-associated protein</td>
</tr>
<tr>
<td>MTX</td>
<td>methotrexate</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NE</td>
<td>nanoemulsion</td>
</tr>
<tr>
<td>NP</td>
<td>nanoparticle</td>
</tr>
<tr>
<td>o/w</td>
<td>oil-in-water</td>
</tr>
<tr>
<td>OAT</td>
<td>organic anion transporter</td>
</tr>
<tr>
<td>OATP</td>
<td>organic anion-transporting polypeptide</td>
</tr>
<tr>
<td>OCT</td>
<td>organic cation transporter</td>
</tr>
<tr>
<td>PBCA</td>
<td>poly(butylcyanoacrylate)</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEG-DSPE</td>
<td>PEG-modified distearoylphosphatidyl ethanolamine</td>
</tr>
<tr>
<td>PEG-PE</td>
<td>polyethylene glycol-phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEO</td>
<td>polyethylene oxide</td>
</tr>
<tr>
<td>PEPE</td>
<td>polyether-co-polyester</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PIT</td>
<td>phase inversion temperature</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PPG</td>
<td>poly(propylene glycol)</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>RME</td>
<td>receptor-mediated endocytosis</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SLC</td>
<td>solute carrier</td>
</tr>
<tr>
<td>SLN</td>
<td>solid lipid nanoparticle</td>
</tr>
<tr>
<td>SMEDDDS</td>
<td>self-microemulsifying drug delivery system</td>
</tr>
<tr>
<td>vitamin E TPGS</td>
<td>d-α-tocopheryl polyethylene glycol 1000 succinate</td>
</tr>
<tr>
<td>w/o</td>
<td>water-in-oil</td>
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<tbody>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>CIN</td>
<td>cervical intraepithelial neoplasm</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>C-SLN</td>
<td>curcumin-loaded solid lipid nanoparticle</td>
</tr>
<tr>
<td>curcumin-EV</td>
<td>curcumin entrapped in elastic vesicles</td>
</tr>
<tr>
<td>DDS</td>
<td>drug delivery system</td>
</tr>
<tr>
<td>DENA</td>
<td>N-nitrosodiethylamine</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DO-FUdR</td>
<td>3’,5’-dioctanoyl-5-fluoro-2’-deoxyuridine</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FUdR</td>
<td>5’-fluoro-2’-deoxyuridine</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>LC/MS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>MDS</td>
<td>myelodysplastic syndrome</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-l-cysteine</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>PB</td>
<td>phenobarbital</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<th>Definition</th>
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<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td>SLCP</td>
<td>solid lipid curcumin particle</td>
</tr>
<tr>
<td>SLN</td>
<td>solid lipid nanoparticle</td>
</tr>
<tr>
<td>SMEDDS</td>
<td>self-microemulsifying drug delivery system</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>THC</td>
<td>tetrahydrocurcumin</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>NLC</td>
<td>nanostructured lipid carriers</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigmented epithelium</td>
</tr>
<tr>
<td>SLN</td>
<td>solid lipid nanoparticles</td>
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**Chapter 15**

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<tr>
<td>ALA</td>
<td>aminolevulinic acid</td>
</tr>
<tr>
<td>DAC</td>
<td>dacarbazine</td>
</tr>
<tr>
<td>DQA</td>
<td>dequalinium</td>
</tr>
<tr>
<td>DTX</td>
<td>docetaxel</td>
</tr>
<tr>
<td>EGCG</td>
<td>epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>FNS</td>
<td>finasteride</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>PCL</td>
<td>paclitaxel</td>
</tr>
<tr>
<td>PpIX</td>
<td>photosensitiser protoporphyrin IX</td>
</tr>
<tr>
<td>SLN</td>
<td>Solid lipid nanoparticles</td>
</tr>
<tr>
<td>TAM</td>
<td>tamoxifen</td>
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**Chapter 16**

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<tr>
<td>B(a)P</td>
<td>benzo(a)pyrene</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
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<table>
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<th>Full Form</th>
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<tr>
<td>GMS</td>
<td>glyceryl monostearate</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HMW DNA</td>
<td>high-molecular-weight DNA</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>LMW DNA</td>
<td>low-molecular-weight DNA</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NDDS</td>
<td>novel drug delivery system</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCS</td>
<td>photon correlation spectroscopy</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SLN</td>
<td>solid lipid nanoparticle</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>S-SLN</td>
<td>sesamol-loaded SLN</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl-phorbol-13-acetate</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
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</table>
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This book introduces the fundamental principles of lipid nanocarriers science relevant to cancer therapy. The first in this field, it fills a need for an accurate, coherent and authoritative introduction to lipid nanocarriers focusing on cancer therapy, both because of the growing popularity of these modern drug delivery systems and also because of the emergent need for dealing with cancer treatment.

Active drug delivery and targeting using nanobiotechnologies has become one of the most successful strategies to approach chemotherapy. This book deals with lipid nanocarriers for targeted delivery to tumours of various organs and combination of these with other methods of treatment of cancer such as radiotherapy, and diagnostic imaging analysis. Lipid nanocarriers are also used for gene therapy for cancer.

Also addressed is how diagnostic strategies with lipid nanocarriers can be combined with therapeutics, which will be important for the personalised management of cancer.

Cancer chemotherapeutic drugs are also being used as immunosuppressive agents in the chronic treatment of non-neoplastic diseases. The scientific research in this field has been focused on the development of strategies to target and deliver several cancer chemotherapeutic drugs to decrease cytotoxicity. These include both carrier- and receptor-mediated nanocarriers. Being biodegradable and biocompatible systems, lipid nanocarriers have been extensively explored to increase the therapeutic index of drugs, reducing adverse side effects to ultimately extend the lifespan of patients.

Furthermore, a familiarity with lipid materials science will enable scientists to conduct rational performance studies, and to understand and optimize formulations, drug delivery systems and their manufacturing processes as well as associated process analytical technologies and quality by design.