Nanotechnologies in Cancer

Guest Editors: Giuseppe De Rosa, Michele Caraglia, Stefano Salmaso, and Tamer Elbayoumi
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Contents

**Nanotechnologies in Cancer**, Giuseppe De Rosa, Michele Caraglia, Stefano Salmasso, and Tamer Elbayoumi
Volume 2013, Article ID 604293, 3 pages

**Nanoparticle Albumin Bound Paclitaxel in the Treatment of Human Cancer: Nanodelivery Reaches Prime-Time?**, Iole Cucinotto, Lucia Fiorillo, Simona Gualtieri, Mariamena Arbitrio, Domenico Ciliberto, Nicoletta Staropoli, Anna Grimaldi, Amalia Luce, Pierfrancesco Tassone, Michele Caraglia, and Pierosandro Tagliaferri
Volume 2013, Article ID 905091, 10 pages

**Liposomal Doxorubicin in the Treatment of Breast Cancer Patients: A Review**, Juan Lao, Julia Madani, Teresa Puértolas, María Álvarez, Alba Hernández, Roberto Pazo-Cid, Ángel Artal, and Antonio Antón Torres
Volume 2013, Article ID 456409, 12 pages

**Gene Therapy for Advanced Melanoma: Selective Targeting and Therapeutic Nucleic Acids**, Joana R. Viola, Diana F. Rafael, Ernst Wagner, Robert Besch, and Manfred Ogris
Volume 2013, Article ID 897348, 15 pages

**Clinical Trials with Pegylated Liposomal Doxorubicin in the Treatment of Ovarian Cancer**, Carmela Pisano, Sabrina Chiara Cecere, Marilena Di Napoli, Carla Cavaliere, Rosa Tambaro, Gaetano Facchini, Cono Scaffa, Simona Losito, Antonio Pizzolorusso, and Sandro Pignata
Volume 2013, Article ID 898146, 12 pages

**Lipid-Based Nanovectors for Targeting of CD44-Overexpressing Tumor Cells**, Silvia Arpicco, Giuseppe De Rosa, and Elias Fattal
Volume 2013, Article ID 860780, 8 pages

**Recent Trends in Multifunctional Liposomal Nanocarriers for Enhanced Tumor Targeting**, Federico Perche and Vladimir P. Torchilin
Volume 2013, Article ID 705265, 32 pages

**Stealth Properties to Improve Therapeutic Efficacy of Drug Nanocarriers**, Stefano Salmasso and Paolo Caliceti
Volume 2013, Article ID 374252, 19 pages

**Bisphosphonates and Cancer: What Opportunities from Nanotechnology?**, Giuseppe De Rosa, Gabriella Misso, Giuseppina Salzano, and Michele Caraglia
Volume 2013, Article ID 637976, 17 pages

**Neoplastic Meningitis from Solid Tumors: A Prospective Clinical Study in Lombardia and a Literature Review on Therapeutic Approaches**, A. Silvani, M. Caroli, P. Gaviani, V. Feton, R. Merli, M. Riva, M. De Rossi, F. Imbesi, and A. Salmaggi
Volume 2013, Article ID 147325, 6 pages

**Nanomaterials Toxicity and Cell Death Modalities**, Daniela De Stefano, Rosa Carnuccio, and Maria Chiara Maiuri
Volume 2012, Article ID 167896, 14 pages

**Utilisation of Nanoparticle Technology in Cancer Chemoresistance**, Duncan Ayers and Alessandro Nasti
Volume 2012, Article ID 265691, 12 pages
Cancer is today the major cause of morbidity and mortality in western and industrialized countries. The use of drugs for the therapeutic treatment of cancer raises important issues about their toxicity on normal cells and, more in general, on their systemic side effects.

Issues about systemic toxicities have been faced with both first generation anticancer drugs and with more recent drugs that operate through specific targets, with the latter maintaining the homeostasis of several normal tissues. The emergence of the nanomedicine has opened a novel scenario in the use of all anticancer agents with the possibility to improve their efficacy and to reduce their side effects due to their distribution in normal tissues. Products based on nanotechnological carriers have entered the clinical practice, and a huge number of studies have been performed in order to optimize the application of nanomedicines in cancer treatment. Although these nanotechnology-based systems are still far to fully comply the idea of the "magic bullet," the advantages offered by this approach are clearly promising.

This special issue covers different aspects related to the exploitation of nanotechnology-based systems for cancer treatment, including the design and features of multifunctional nanocarriers, the drug targeting concept, the gene therapy, the toxicity of nanomaterials, and the more recent clinical studies that have determined a glimmer of hope for cancer patients.

Liposomes are among the first nanotechnological-based platforms ever developed for cancer therapy. One of the major limitations in the clinical use of liposomes and other nanoparticles is their short plasma half-life due to the rapid opsonization process that yields their removal from bloodstream and degradation by macrophages from reticular endothelial system. On the basis of these considerations, "stealth" nanocarriers have been promptly developed through conjugation of hydrophilic polymers, such as polyethylene glycol (PEG), on the particle surface. The review of S. Salmaso and P. Caliceti describes the basic concept underlining the "stealth" properties of drug nanocarriers, the parameters influencing the polymer coating performance in terms of opsonins/macrophages interaction with the colloid surface, the most commonly used materials for the coating process, and the outcomes of this peculiar procedure.

One of the first "stealth" nanocarriers loaded with anticancer drug that has achieved the clinical practice was the pegylated liposomal doxorubicin (PLD). The paper by C. Pisano et al. describes the role and clinical indications of PLD in ovarian cancer. PLD was firstly approved for platinum-refractory ovarian cancer and then received full approval for platinum-sensitive recurrent disease. Recently, it was demonstrated that the combination of PLD with platinum has similar activity but less toxicity than the combination containing free doxorubicin triggering new interest on PLD also in the first line of treatment of this tumour. Another clinical indication of PLD is the treatment of metastatic or locally advanced breast cancer when the maximal allowed cumulative doses of doxorubicin administered to patients is...
reached. The paper by J. Lao et al. summarizes the main results achieved with the use of PLD in this setting of patients underlining the loss of cardiotoxicity with the preservation of clinical activity if compared to free doxorubicin. Moreover, interesting results have been recorded by combining anti-HER-2 antibodies (trastuzumab) with PLD in the treatment of both locally advanced and metastatic breast cancer enlightening the potential advantages of the combination of these drugs (both cardiotoxic) in these two clinical settings.

An important concern that limits the therapeutic profile of doxorubicin and other anticancer agents is the development of innate or acquired tumour resistance that is mediated by several mechanisms. The paper by D. Ayers and A. Nasti describes the different mechanisms by which tumour cells generate the resistance to anticancer agents and the strategies to overcome the refractoriness of cancer cells. In details, the authors discuss the limits and advantages of different nanotechnological devices used to deliver cytotoxic drugs or nucleic acids (such as micro-RNAs or siRNAs) that target specific molecular resistance factors.

Another important limitation to the effective therapeutic activity of anticancer drugs is the inability of some molecules to overcome anatomic barriers, such as the blood-brain barrier, and to accumulate in the subarachnoidal or leptomeningeal spaces that can be sites of dissemination of brain or extra-brain tumours. The paper by A. Silvani et al. describes the role of liposomal arabinoside cytosine (AraC) in the treatment of neoplastic meningosis including an unpublished prospective trial performed in the Italian region Lombardia and a short review of the data reported by other already published clinical studies.

The paper from I. Cucinotto et al. reports and discusses the most recent findings on the clinical use of nanoparticle albumin-bound paclitaxel (nab-paclitaxel), also known with the commercial name of Abraxane. This drug is at the moment approved for the treatment of metastatic breast cancer and nonsmall cell lung cancer. However, this nanotechnology-based drug is very promising also for the treatment of other human neoplasms, such as pancreatic cancer or metastatic melanoma, which generally are considered refractory to treatment with conventional anticancer agents. In this view, the paper of J. R. Viola et al. provides a short introduction to the mechanisms of melanomagenesis, discussing the shortcomings of current therapeutic approaches ascribed to the existence of a wide range of mutations associated with this cancer. Authors highlight alternative approaches for treatment of melanomas based on the use of therapeutically active nucleic acids. The delivery of nucleic acid nanoparticles is brought into perspective as a novel highly selective antimelanoma therapeutic approach whilst avoiding unwanted and toxic side effects. The possibilities for melanoma selective targeting are discussed together with latest reports of advanced clinical applications.

Also target-based agents need to be specifically delivered to tumour tissues and in this regard G. De Rosa et al. provide a comprehensive article on the clinical applications of bisphosphonates (BPs) starting from their use as inhibitors of bone resorption up to their novel therapeutic indications as anticancer drugs. In detail, nitrogen-containing BPs (N-BPs) induce apoptosis in a variety of cancer cells in vitro and in preclinical settings and show a very intriguing antiangiogenic activity. Unfortunately, clinical anticancer activity of N-BPs is far to be demonstrated. In this light, the authors describe how nanotechnology can provide carriers to limit BP accumulation into the bone, thus increasing drug level in extra-skeletal sites of the body to directly kill cancer cells. On the other hand, BPs can also be used as targeting agents to specifically deliver nanocarriers loaded with anticancer drugs in the bone tissue for the treatment of bone tumours or metastases.

The active targeting of nanoparticles is an effective strategy to increase the uptake of anti-cancer drug-loaded vehicles by tumour cells. It is based on the decoration of nanoparticles with specific ligands such as peptides or antibodies raised against tumour-associated antigens (molecules with higher expression on tumour cells than in normal counterparts).

In this light, S. Arpicco et al. review the use of hyaluronic acid (HA) as a unique targeting agent for the recognition of cancer cells due to the high expression levels of its receptor (named CD44) on tumour cell surface. The CD44 receptor is found at low levels on the surface of epithelial, haematopoietic, and neuronal cells, but it is overexpressed in many cancer cells and on cancer stem cells. This review describes the approaches used for the preparation and investigation of lipid-based nanovectors decorated with HA for the active delivery of a variety of therapeutic molecules in the treatment of human cancer.

Other strategies in the development of nanotechnological devices include the multifunctional decoration with different moieties that allow both the detection and the treatment of cancer cells (theranostic devices). In this view, the paper by F. Perche and V. P. Torchilin describes multifunctional liposomal nanocarriers that combines long blood circulation and selective accumulation to the tumor lesions based upon remote-controlled or tumour stimuli-sensitive extravasation from blood to the tumour tissue and internalization motifs to move from tumour bounds and/or tumor intercellular space to the cytoplasm of cancer cells.

Finally, nanovectors are not completely inert materials and can be endowed with intrinsic cytotoxicity that causes, sometimes, potential deleterious effects in normal tissues. In this light, D. De Stefano et al. describe the main mechanisms by which nanosized materials can induce cell death, such as apoptosis, mitotic catastrophe, autophagy, necrosis, and pyroptosis. The understanding of these mechanisms is mandatory for a safe use of nanocarriers. The authors describe all the variables that can affect nanocarrier cytotoxicity underlining the need for generally accepted guidelines for the development and use of nanotechnological devices.

We believe that this special issue can be of great interest for the readers in depicting the most recent advances generated by basic, translational, and clinical research focused on the development and use of nanocarriers for the delivery of anticancer agents. The special issue thoroughly reports the outcomes derived from basic and preclinical studies and the main limitations emerged from both clinical trials and practice. The criticisms derived from the clinics need to be regarded as crucial starting points for the optimization
of the nanotechnological drug delivery systems. In other words, bidirectional flow of information from the bench to the bedside and back again to the bench is pivotal to offer improved nanomedicine-based strategies of treatment of cancer patients.

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Michele Caraglia
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Review Article

Nanoparticle Albumin Bound Paclitaxel in the Treatment of Human Cancer: Nanodelivery Reaches Prime-Time?

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Nanoparticle albumin bound paclitaxel (nab-paclitaxel) represents the first nanotechnology-based drug in cancer treatment. We discuss the development of this innovative compound and report the recent changing-practice results in breast and pancreatic cancer. A ground-breaking finding is the demonstration that nab-paclitaxel can not only enhance the activity and reduce the toxicity of chromophore-diluted compound, but also exert activity in diseases considered refractory to taxane-based treatment. This is the first clinical demonstration of major activity of nanotechnologically modified drugs in the treatment of human neoplasms.

1. Introduction

Current development of cancer treatment mainly relies on three avenues:

(a) the identification of molecular targets for selective blockade of driver pathways in cancer cells or in tumour microenvironment,

(b) immunemodulatory approaches which might enhance the antitumor specific immune response,

(c) new delivery approaches in order to achieve higher bioavailability of anticancer agents.

The topic of the current review is the nanoparticle albumin bound paclitaxel (nab-paclitaxel) development, which has opened a novel scenario in cancer treatment by the enhancement of paclitaxel delivery by the use of nanotechnology.

2. Taxane (First) Revolution of Cancer Therapy

Taxanes are an important class of antitumor agents using solvent-based delivery vehicles. Paclitaxel (Bristol-Myers Squibb (New York, NY)) was identified in 1966, as an extract from Taxus brevifolia, obtained in a pure form in 1969 but its structure was published in 1971. Investigators faced several problems due to low concentration and structure complexities for low water solubility [1, 2] (Figure 1).

In fact, only in 1979 Susan Horwitz discovered that paclitaxel has a unique mechanism of action and interest which was additionally stimulated when impressive activity was demonstrated in NCI tumor screening [3]. Paclitaxel is a diterpenoid pseudoalkaloid with formula C47H51NO14 (MW = 853 Da) whose activity was demonstrated in different preclinical models. For antitumor activity the presence of the entire taxane molecule is required (Figure 2) for the inactivity of the ester and the tetraol formed by a low temperature cleavage of paclitaxel [4].

Although the development of paclitaxel was hampered by limited availability of its primary source and the difficulties
of several important malignancies as lung cancer, breast and is presently of common use in the treatment of AIDS-related Kaposi’s sarcoma in preclinical models. Such studies led to the development of docetaxel (Taxotere), a semisynthetic taxane analogue extracted from the European yew. Docetaxel differs from paclitaxel in two positions in its chemical structure and this small alteration makes it more watersoluble. Taxanes disrupt microtubule dynamics by stabilizing the microtubule against depolymerization, enhancing their polymerization, promoting the nucleation and elongation phases of the polymerization reaction, and reducing the critical tubulin subunit concentration required for microtubule assembly. Moreover they alter the tubulin dissociation rate at both ends of the microtubule. This leads to reduced dynamic instability, whereas the association rate is not affected. After the treatment with taxanes, the microtubules are highly stable and resistant to depolymerization by cold, calcium ions, dilution, and other antimicrotubule agents. The final result is the impairment of dynamics of microtubule depolymerization, which is a critical event in the mitotic process [5].

Paclitaxel is active against primary epithelial ovarian carcinoma, breast cancer, colon, non-small-cell lung cancer, and AIDS-related Kaposi's sarcoma in preclinical models [3, 6, 7] and is presently of common use in the treatment of several important malignancies as lung cancer, breast cancer, Kaposi's sarcoma, squamous cell carcinoma of the head and neck, gastric cancer, esophageal cancer, bladder cancer, and other carcinomas. Despite being clinically very active, paclitaxel and docetaxel are associated with many serious side effects which often preclude the prolonged use in patients. A number of these side effects have been associated with the vehicles used for the formulation: the cremophor EL (CrEL-polyethoxylated castor oil) [8] for paclitaxel and polysorbate 80 (Tween 80) for docetaxel, respectively, that altered also their pharmacokinetic profiles; CrEL is considered to be responsible for the hypersensitivity reactions seen in patients during paclitaxel therapy. In vitro, CrEL caused axonal swelling, demyelination, and axonal degeneration, and, thus, it may also contribute to the development of neuropathy in patients receiving paclitaxel. The use of CrEL requires premedication with antihistamines and corticosteroids to prevent hypersensitivity reactions and, despite these premedications, approximately 40% of all patients will have minor reactions (e.g., flushing and rash) and 3% will have life threatening reactions. CrEL also causes leaching of the plasticizers from polyvinyl chloride (PVC) bags and infusions sets; thus paclitaxel must be infused via the use of special non-PVC infusion systems and in-line filtration. Another effect induced by CrEL is the alteration of lipoprotein pattern and the consequent hyperlipidemia. Moreover, CrEL and polysorbate 80 interfere with efficacy by limiting tumor penetration through the formation of large polar micelles, which for CrEL-paclitaxel can lead to nonlinear pharmacokinetics and decreased unbound drug fraction [9].

To overcome the ideal dosage form and bypass all the present limitations, novel “carrier delivery systems,” including liposomes, micelles, and particulate drug delivery systems, were formulated as common practice for novel drugs like microRNAs [10–15].

Some of them have already reached the clinical practice like liposomal doxorubicin or liposomal amphotericin B. Another example of nanotechnology applied to drug delivery is the preclinical development of stealth liposomes encapsulating zoledronic acid (LipoZOL) to reduce binding of ZOL to bone and increase its bioavailability in extraskeletal tumor sites [16]. Natural human protein based carrier can also be used to manufacture nanocarriers for drug delivery: this is the example of the paclitaxel albumin bound by which it is possible to selectively deliver larger amounts of drug to tumors, reducing the toxicities related to solvent-based formulations. Albumin is a natural carrier of hydrophobic endogenous molecules (such as vitamins, hormones, and other plasma constituents), in a noncovalent and reversible binding and allows for transport in the body and release at the cell surface [17].

Abraxane (nab-paclitaxel; ABI 007 or Abraxane; Celgene Inc, Odenton, MD, USA) was the first to receive FDA approval in 2005, for the treatment of breast cancer in patients who reported progressive disease after chemotherapy for metastatic cancer or relapse within 6 months of adjuvant chemotherapy.

Nab-paclitaxel is a colloidal suspension of 130 nanometer particles, solvent-free, homogenized with human serum albumin (3%-4%), by which it is possible to infuse higher

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**Figure 1:** Structure of paclitaxel (5β,20-epoxy-1,2α,4,7β,13α-hexahydroxytan-11-en-9-one-4,10-diacetate-13-ester with (2R,3S)-N-benzoyl-3-phenyllioserine).

**Figure 2:** Taxane nucleus.
doses of drug than the standard dose used in paclitaxel therapy, with fewer side effects, with less infusion time (30 minutes) and without premedication. The new formulation allows the delivery of paclitaxel to tumors with a 4.5-fold increase in its transport, coupled with albumin receptors, across endothelial cells [18] with an enhanced intracellular antitumor paclitaxel delivery and activity [19]. In the mechanism of drug delivery an albumin receptor (gp60) on endothelial cells seems to be involved which transports paclitaxel into the extravascular space with subsequent invagination of the cell membrane to form caveolae, transcytotic vesicles, and also tumor accumulation of nanoparticle bound to SPARC (secreted protein, acidic and rich in cysteine), which is overexpressed in many solid tumors, including bladder, prostate, and pancreas cancers [20]. Its intravenous infusion is more manageable and safe because it is performed by standard plastic intravenous infusion bags and can also be reconstituted in a much smaller volume of normal saline compared to paclitaxel. Preclinical studies have demonstrated that nab-paclitaxel achieved higher intratumor concentrations compared to CrEL-paclitaxel with a better bioavailability and showed an improved efficacy and therapeutic index in multiple animal models [21]. Other new technologies recently used to deliver paclitaxel have led to the development of innovative formulations such as Nanoxel and liposomal and polymeric paclitaxel.

Nanoxel-PM is efficacious and less toxic than free docetaxel formulation and was evaluated in comparison with Taxotere in preclinical studies. Nanoxel-PM can reduce side effects of hypersensitivity reactions and fluid retention while retaining antitumor efficacy in cancer patients [22].

Further studies led to the development of new formulations of liposomal paclitaxel. The special composition of the liposomal membrane which contains high doses of paclitaxel could reduce the aggregation giving the molecule higher stability and confers an increase of efficacy in animal models as in human tumors [23].

An hydrotropic polymer micelle system has also been developed for delivery of poorly water-soluble drugs as paclitaxel. This polymer showed not only higher loading capacity but also enhanced physical stability in aqueous media and provides an alternative approach for formulation of poorly soluble drugs [24,25].

### 3. Nab-Paclitaxel in Breast Cancer Treatment

Breast cancer (BC) is the most common cancer in female patients and follows lung cancer as the most common cause of female cancer death. While only 5–7% of BC patients present metastatic disease (mBC) at diagnosis and more than 30% presenting localized disease will eventually recur, 5 year survival of advanced disease is less than 20% [33]. Current treatment of advanced breast cancer is mainly aimed to ameliorate quality of life and prolong survival. Treatment choice is not an easy task in terms of drug selection and combination. Chemotherapy plays an essential role for the treatment of mBC. Among anticancer drugs, taxanes are considered the most effective, while their use involves long infusion time, neurotoxicity, and high risk of hypersensitivity reactions [8,34,35]. These latter effects are due to allergic reactions induced by the use of solubilizing agents (as chromophores) and today are less common due to the use in the clinical practice of corticosteroids and antihistamines [36]. In order to overcome these important limitations, a major interest is devoted to novel drugs as nab-paclitaxel, eribulin, ixabepilone, PARP inhibitors, and new HER 2 inhibitors as lapatinib, pertuzumab, TDM1, and neratinib [37–43].

Following phase I studies, by Ibrahim et al. in 2002 [19] and by Teng et al in 2004 [44], which led to MTD identification at 300 mg/m² in the three weekly schedule with neurotoxicity as dose limiting toxicity, Nyman et al. in 2005 [45] identify in the weekly schedule the MTD at 100 mg/sqm for highly pretreated patients and 150 mg/m² for nonhighly pretreated patients with grade 4 neutropenia and grade 3 neuropathy as DLT with earlier onset at higher dosages. The pivotal phase 3 study was published in 2005 where Gradishar et al. [30] compared nab-paclitaxel (260 mg/m²) at three week schedule with CrEL-paclitaxel 175 mg/m² also at three week schedule. The study clearly demonstrated a survival advantage for nab-paclitaxel with an improved toxicity profile.

In 2009 a phase II randomized study [26] compared three week docetaxel 100 mg/m² with three week nab-paclitaxel 300 mg/m², weekly nab-paclitaxel 100 mg/sqm and weekly nab-paclitaxel 150 mg/sqm. The 150 nab-paclitaxel weekly schedule provided the best PFS (>5 months) which resulted to be statistically significant. An update of this study published by Gradishar et al. in 2012 demonstrated a median overall survival (OS) of 33.8 months which statistically overcame the other treatment arms.

All together these data demonstrated that nab-paclitaxel is superior to CrEL-paclitaxel in the three week schedule and that nab-paclitaxel at weekly 150 schedule provides an impressive long term survival [27]. Recently, nab-paclitaxel was administered in combination with biological agents in the treatment of mBC. In detail, a safety analysis of the first ten enrolled patients treated for at least one cycle of the initial doses of nab-paclitaxel (125 mg/m² i.v. on days 1, 8, and 15 every 28 days) in combination with lapatinib (1,250 mg orally once daily on a continuous basis) in a 4-week cycle for a planned minimum of six cycles was performed. However, during the ongoing safety review of the first five patients, Grade 3 toxicities were observed in all five patients (four with neutropenia and one with neutropenic fever and diarrhea) and the decision was made to reduce the dose of both study drugs. All subsequent patients (n = 55) received nab-paclitaxel (100 mg/m² i.v. on days 1, 8, and 15 every 28 days) in combination with lapatinib (1,000 mg orally once daily on a continuous basis) in a 4-week cycle for a minimum of six cycles. RR was 53% with the majority of patient responses demonstrating a partial response (PR) (47%). Four (7%) patient responses demonstrated a complete response (CR), and ten (17%) demonstrated a stable disease. The progression-free survival (PFS) and time to progression (TTP) were 39.7 weeks (95% CI 34.1–63.9) and 41 weeks (95% CI 39.1–64.6), respectively. Lapatinib 1,000 mg with
Table 1: Randomized phase II and III trials with nab-paclitaxel in mBC.

(a) Phase II

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<th>Pts</th>
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<th>PFS (%)</th>
<th>PFS (%)</th>
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(b) Phase III

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<th>Arms</th>
<th>Pts</th>
<th>RR (%)</th>
<th>TTP weeks</th>
<th>Grade IV neutropenia</th>
<th>Grade III sensory neuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradishar et al., 2005 [30]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nab-paclitaxel</td>
<td>260 mg/sqm</td>
<td>229</td>
<td>33</td>
<td>23.0</td>
<td>9</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>175 mg/sqm</td>
<td>225</td>
<td>19</td>
<td>16.9</td>
<td>22</td>
</tr>
</tbody>
</table>

P: P value; nd: not done; AEs: adverse events; inv. rad.: investigator radiologist; ind. rad.: independent radiologist; ORR: overall response rate; RR: response rate; TTP: time to progression; PFS: progression-free survival; OS: overall survival; MDR: median duration of response.

4. Nab-Paclitaxel in Pancreatic Cancer Treatment

Pancreatic cancer (PC) is at present a big cancer killer, with an expected survival of 6 months in advanced stage PC (aPC). Till a recent report demonstrating good activity of oxaliplatin, irinotecan, and fluorofolate (FOLFIRINOX combination), gemcitabine is still the mainstay treatment. In a recent meta-analysis, Ciliberto et al. [51] described a statistically superiority in terms of survival.

nab-paclitaxel 100 mg/m² i.v. is feasible with manageable and predictable toxicity and an RR of 53% comparing favorably with other HER2-based combinations in this setting [50].

Two important points under investigation are the comparison of weekly nab-paclitaxel with CrEL-paclitaxel both at weekly schedules and the potential advantage of combination with bevacizumab. Finally nab-paclitaxel has shown some activity also in CrEL-paclitaxel heavily pretreated and resistant patients [28] (Table 1).
Table 2: Randomized phase I/II and III trials with nab-paclitaxel in aPC.

(a) Phase I/II

<table>
<thead>
<tr>
<th>Arms</th>
<th>Pts</th>
<th>MTD</th>
<th>RR (%)</th>
<th>Median OS (months)</th>
<th>1 year survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Hoff et al., 2011 [31]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gemcitabine 1000mg/sqm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nab-paclitaxel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mg/m² q3w</td>
<td>20</td>
<td>X</td>
<td>48</td>
<td>12.2</td>
<td>48</td>
</tr>
<tr>
<td>125 mg/m² q3w</td>
<td>44</td>
<td></td>
<td>48</td>
<td>11.8</td>
<td>48</td>
</tr>
<tr>
<td>150 mg/m² q3w</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) Phase III

<table>
<thead>
<tr>
<th>Arms</th>
<th>Pts</th>
<th>ORR (%)</th>
<th>Median TTP (MO)</th>
<th>PFS</th>
<th>OS</th>
<th>AEs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Von Hoff et al., 2011 [32]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nab-paclitaxel 125 mg/m² qw followed Gemcitabine 1000 mg/sqm qw</td>
<td>431</td>
<td>99</td>
<td>5.1</td>
<td>16</td>
<td>35</td>
<td>9</td>
</tr>
<tr>
<td>Gemcitabine 1000 mg/sqm qw</td>
<td>430</td>
<td>31</td>
<td>3.6</td>
<td>9</td>
<td>22</td>
<td>4</td>
</tr>
</tbody>
</table>

P: P value; nd: not done; AEs: adverse events; MTD: maximum tolerated dose; ORR: overall response rate; RR: response rate; TTP: time to progression; PFS: progression-free survival; OS: overall survival; MDR: median duration of response.

and response rate for gemcitabine-based combination compared to gemcitabine alone. Moreover, this advantage was marginal and at the cost of an increased toxicity. The authors concluded that in the era of targeted therapy new approaches were possible only in presence of solid preclinical findings.

A report by von Hoff et al. [31] demonstrated in a phase I/II study an interesting activity of gemcitabine/nab-paclitaxel combination at gemcitabine 1000 mg/m² and nab-paclitaxel at 125 mg/m² doses weekly for three doses in a 4 week schedule. A 48% response rate was achieved at MTD. The authors additionally demonstrated that SPARC-expressing tumors appeared more sensitive to the drug combination.

An interesting finding from a preclinical study reported that nab-paclitaxel demonstrated the capacity of increasing the gemcitabine bioavailability inside the tumors. These findings led to the design of a phase III study where gemcitabine/nab-paclitaxel was compared to gemcitabine alone showing an advantage in OS, PFS, and RR. This study, presented to ASCO GI 2013 (American Society of Clinical Oncology, Gastrointestinal Cancer Symposium) by von Hoff, is clearly a changing practice study and the gemcitabine/nab-paclitaxel, which led to an almost two month longer OS should be now compared to FOLFIRINOX combination (Table 2). The biological bases of the synergistic interaction between nab-paclitaxel and gemcitabine have recently been elucidated by an in vivo study in animal models. In detail, the combination treatment was administered to KPC mice that develop advanced and metastatic pancreas ductal adenocarcinoma. The authors have demonstrated an increase of intratumoral gemcitabine levels attributable to a marked decrease in the primary gemcitabine metabolizing enzyme, cytidine deaminase. Corresopondingly, paclitaxel reduced the levels of cytidine deaminase protein in cultured cells through reactive oxygen species-mediated degradation, resulting in the increased stabilization of gemcitabine. These findings support the concept that suboptimal intratumoral concentrations of gemcitabine represent a crucial mechanism of therapeutic resistance in PC [52]. This study provides mechanistic insight into the clinical cooperation observed between gemcitabine and nab-paclitaxel in the treatment of pancreatic cancer.

5. Other Areas of Nab-Paclitaxel Development

Melanoma represents 5% and 4% of all cancers in males in females, respectively. However, the rates of incidence of melanoma are steadily increasing in the USA as in most parts of Europe [53]. The survival rates of melanoma become worse
### Table 3: Randomized phase II and III trials with nab-paclitaxel in melanoma.

#### (a) Phase II

<table>
<thead>
<tr>
<th>Arms</th>
<th>Pts</th>
<th>RR (%)</th>
<th>PFS ( Median (MO) )</th>
<th>OS ( Median (MO) )</th>
<th>1 year (%)</th>
</tr>
</thead>
</table>
| Nab-paclitaxel  
150 mg/m² q3w | 37 | 21.6 | 4.5 | 34 | 9.6 | 41 |
| Nab-paclitaxel  
100 mg/m² q3w | 37 | 2.7 | 3.5 | 27 | 12.1 | 49 |

#### (b) Phase III

<table>
<thead>
<tr>
<th>Arms</th>
<th>Pts</th>
<th>ORR (%)</th>
<th>PFS ( Median (MO) )</th>
<th>OS ( Median (MO) )</th>
<th>AEs grade ≥ III (%)</th>
</tr>
</thead>
</table>
| Nab-paclitaxel  
150 mg/m² qw | 264 | 15 | 4.8 | 5.4 | 12.8 | 20 | 12 | 8 | 25 |
| Dacarbazine  
1000 mg/sqm q3w | 265 | 11 | 2.5 | 2.5 | 10.7 | 10 | 7 | 2 | 0 |

P: P value; nd: not done; AEs: adverse events; WT: wild type; V600Em: with mutation of V600E; Uk: unknown BRAF mutation; ORR: overall response rate; RR: response rate; PFS: progression-free survival; OS: overall survival.
Table 4: Randomized phase III trials with nab-paclitaxel in aNSCLC.

<table>
<thead>
<tr>
<th>Arms</th>
<th>Pts</th>
<th>ORR Median (%)</th>
<th>ORR SQ (%)</th>
<th>ORR NSQ (%)</th>
<th>Median PFS (MO)</th>
<th>Median OS (MO)</th>
<th>AEs grade III*-IV** (%) P = &lt;.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nab-paclitaxel 100 mg/m²</td>
<td>521</td>
<td>33</td>
<td>41</td>
<td>26</td>
<td>6.3</td>
<td>12.1</td>
<td>Neutropenia 13* Thrombocytopenia 14* Fatigue 32* Anemia 22*</td>
</tr>
<tr>
<td>Carboplatin AUC6 q3w (first line)</td>
<td></td>
<td>P = &lt;.005</td>
<td>P = &lt;.80</td>
<td>P = &lt;.214</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paclitaxel 200 mg/m²</td>
<td>531</td>
<td>25</td>
<td>24</td>
<td>25</td>
<td>5.8</td>
<td>11.1</td>
<td>Neutropenia 13* Thrombocytopenia 5* Fatigue 6* Anemia 5**</td>
</tr>
<tr>
<td>Carboplatin AUC6 q3w</td>
<td></td>
<td>P = &lt;.005</td>
<td>P = &lt;.80</td>
<td>P = &lt;.214</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P: P value; nd: not done; sq: squamous histology of NSCLC; nsq: non squamous histology of NSCLC; AEs: adverse events; ORR: overall response rate; RR: response rate; PFS: progression-free survival; OS: overall survival.
with advancing stage. Therefore, early diagnosis in addition
to surgical treatment before its spread is the most effective
treatment.

Melanomas are a heterogeneous group of tumors char-
acterized by specific genetic alterations, including mutations
in kinase, such as BRAF or c-kit. Dacarbazine is commonly
used as a treatment for metastatic melanoma and has been
for long time the standard of care for this disease. Recently,
new approaches have completely changed the diagnosis and
treatment of melanoma. New medications like vemurafenib
have been developed for the systemic therapy of advanced
melanomas in subpopulations identified by BRAF mutation
tests. Taxanes have been reported to have some limited
activity in malignant melanoma [54–58], due to the high
toxicity attributed to their waterinsolubility. In a phase II
clinical trial Hersh at al. in 2010 [46] demonstrated that
nab-paclitaxel has activity not only in chemotherapy-naïve
patients with metastatic melanoma administered at a dose of
150 mg/m² but also in previously treated patients adminis-
tered at a dose of 100 mg/m² for 3 of 4 weeks. In this study,
PFS and OS were longer than the previous results reported
with conventional standard of care. In previously treated and
chemotherapy-naïve patients, PFS was 4.5 months and 3.5
months, respectively, and similarly OS was 9.6 months and
12.1 months (in respect to 1.6 months of PFS reported in the
literature for treatment with dacarbazine and temozolomide).
In another phase II clinical trial, Kottschade et al. in 2011
[59] demonstrated that in patients with metastatic melanoma
the combination of nab-paclitaxel 100 mg/m² and carboplatin
AUC2 administered in days 1, 8, and 15 every 28 days is
moderately tolerated for the occurrence of adverse effects
that were fatigue, myelodepression, and gastrointestinal toxicity.
This study confirms that the efficacy and toxicity of nab-
paclitaxel are similar to those of paclitaxel when combined
with carboplatin for the treatment of patients with metastatic
melanoma. Even if such regimens have not been formally
compared in a randomized study, we can say that nab-
paclitaxel is a good alternative for patients who cannot
tolerate conventional therapy with paclitaxel. Last November
at the Society of Melanoma Research a preliminary analysis
of a Phase III study by Hersh was presented which shows
benefit in terms of PFS in favor of nab-paclitaxel compared
to dacarbazine (4.8 versus 2.5 months); the same trend
was observed in the interim analysis that shows a trend
for better OS (12.8 versus 10.7 months) (Table 3). Recently,
nab-paclitaxel was efficiently combined with temozolomide
and oblimersen in the treatment of melanoma patients.
In detail, in a phase I trial, chemotherapy-naïve patients with
metastatic melanoma and normal LDH levels were enrolled
in 3 cohorts. The treatment regimen consisted of 56-day
cycles of oblimersen (7 mg/kg/day continuous i.v. infusion
on days 1–7 and 22–28 in cohort 1 and 2; 900 mg fixed dose,
twice weekly in weeks 1-2, 4-5 for cohort 3), temozolomide
(75 mg/m², days 1–42), and nab-paclitaxel (175 mg/m² in
cohort 1 and 3, 260 mg/m² in cohort 2 on days 7 and 28).
The RR in the 32 treated patients was 40.6% (2 CR and 11
PR) and 11 patients had stable disease, for a disease control
rate of 75%. Haematological, renal, and neurologic toxicity
never exceeded grade 3 demonstrating a good tolerability of
the schedule [60].

Lung cancer (LC) is the first cause of cancer death all
over the world, with a 5 year survival of 5% for metastatic
disease. Treatment selection is based on different factors
like the performance status, comorbidities, histology, and,
in the last years, the molecular mutational profile, which is
now mandatory to assess before deciding treatment. The
most common chemotherapy approach is a platinum based
doublet which is commonly combined with gemcitabine,
vinorelbine, or pemetrexed [61] in Europe, while in the USA
the most common combination is carboplatin/paclitaxel dou-
blet (RR 15–32%); this combination is effective and relatively
well tolerated in the elderly [62–65]. Bevacizumab addition to
this combination led to improved survival [66]. Socinski et al.
reported in 2012 a phase III trial enrolling 1052 IIIb anSCLC
(advanced non-small-cell lung cancer) patients in the first
line of treatment which compared weekly nab-paclitaxel
100 mg/m² and carboplatin AUC6 every three weeks with car-
boptatin AUC6 and CrEL-paclitaxel 200 mg/m² every three
weeks [49]. The nab-paclitaxel/carboplatin combination was
more active in terms of RR with a trend in PFS and OS
improvement and was also better tolerated (Table 4).

6. Conclusions and Future Developments

Nab-paclitaxel has produced a paradigm change in the
treatment of tumors like breast cancer, pancreatic cancer,
and melanoma and a large use in these important diseases can
be predicted. Also in lung cancer, nab-paclitaxel has produced
a good safety profile and increase in RR.

We think that nab-paclitaxel has opened a new way to
human cancer treatment and indeed reached the prime-time.

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Review Article

Liposomal Doxorubicin in the Treatment of Breast Cancer Patients: A Review

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Drug delivery systems can provide enhanced efficacy and/or reduced toxicity for anticancer agents. Liposome drug delivery systems are able to modify the pharmacokinetics and biodistribution of cytostatic agents, increasing the concentration of the drug released to neoplastic tissue and reducing the exposure of normal tissue. Anthracyclines are a key drug in the treatment of both metastatic and early breast cancer, but one of their major limitations is cardiotoxicity. One of the strategies designed to minimize this side effect is liposome encapsulation. Liposomal anthracyclines have achieved highly efficient drug encapsulation and they have proven to be effective and with reduced cardiotoxicity, as a single agent or in combination with other drugs for the treatment of either anthracyclines-treated or naïve metastatic breast cancer patients. Of particular interest is the use of the combination of liposomal anthracyclines and trastuzumab in patients with HER2-overexpressing breast cancer. In this paper, we discuss the different studies on liposomal doxorubicin in metastatic and early breast cancer therapy.

1. Background

In the past years, we have seen significant advances in the understanding of neoplastic diseases and how they have been translated into improvements of therapy. An increasing number of more specific therapeutic options to manage different tumour types are now available, but classical chemotherapy (which is based on the administration of drugs that interfere with the cell’s cycle, prevent its division, and eventually destroy them) remains, in general, a backbone option for many tumours. Chemotherapy side effects must not, however, be underestimated because its mechanism of action affects both tumour and normal cells as well. That is the reason why efforts to improve chemotherapy treatments have focused on designing drugs that are more specific against cancer cells to minimize toxic side effects.

Liposomes were conceived as drug delivery systems to modify drug pharmacokinetics and distribution with the aim of reducing chemotherapy’s toxicity. These liposomes improve the pharmacological properties of some cytostatic agents, allowing an increased proportion of the drug that may be delivered within the tumour tissue whilst substantially reducing the exposure of normal tissues.

Liposomes as a vehicle for delivering cytostatic agents were first described in the 1960s. They were initially used as carriers for lipophilic cytostatic agents, but their suitability for both hydrophilic and hydrophobic drugs was soon assessed. Liposomes can be either a membrane-based closed structure able to incorporate lipophilic drugs or may be built from the direct encapsulation of hydrophilic compounds within the internal aqueous compartment of vesicles [1–3].

Phospholipids are the major component of liposomes, which make them to be less toxic, biodegradable, and biocompatible. The bilayer of phospholipids prevents also the active form of the drug from breaking down before it reaches the tumour tissue and in this way exposure of the normal tissue to the drug is minimized. The therapeutic index of the drug is then increased by two mechanisms: on one hand, a greater amount of the active drug reaches the tumour cells and an increased cytotoxic effect is obtained and, on the other
hand, side effects are also reduced as a consequence of the drug encapsulation. Liposomal formulations have an additional effect on drug metabolism by decreasing its enzymatic degradation [4].

Liposomes can be produced by different methods. Stability of both the bilayer and the incorporated drugs depends on lipid composition and cholesterol content. Their size ranges from 25 to 100 nM and is determined by the maximum quantity of drug stored within the membrane and its flexibility. The lower size limit avoiding liposomes may enter the normal capillary vessels whereas the upper limit is still within the tumour vasculature and enables the cytotoxic agent to reach the tumour bed; in order to produce its effect, the active drug needs to readily extravasate through the vascular defects present in the vessels surrounding cancer cells as a consequence of neoangiogenesis phenomena induced by neoplastic cells [5]. In this way, liposomes below this threshold have the potential to accumulate in the tumour bed after passive drug entry and boosted by impaired lymphatic drainage. This phenomenon has been described as “enhanced permeability plus retention effect” [6]. One more factor related to liposome’s size is that the bigger they are the greater the uptake by the reticuloendothelial system and, therefore, more rapid the drug is metabolized [7].

As the time liposomes are retained in the circulatory system is reduced, the drug they are carrying might not reach cytotoxic levels in the tumour tissue. The size of the nanotransporter could be reduced, but then less drug quantity should be transported. One method that has proven to be effective in overcoming this obstacle without compromising the quantity of the chemotherapeutic agent delivered to the tumour consists in coating these delivery systems with polymers, in particular, with polyethylene glycol (PEG) which allows liposomes to escape from the immune system and, therefore, increase “in vivo” circulating time [8]. Studies have shown that, when manufactured in this way, pegylated liposomes have a longer half-life than nonpegylated (ranging from a few hours to 45 hours) [9]. However, the presence of PEG may act as a barrier between the drug and the tumour cells hindering the delivery of the cytostatic. Therefore, future improvements should be directed to improve this aspect, particularly in the case of breast cancer.

In this cancer, new liposomal formulations have been developed to facilitate the supply of the confined cytostatic agent using thermosensitive molecules. These formulations have proven to be effective in this tumour and their design keep them stable at normal body temperature of 37∘C, but they become unstable at slightly higher temperatures as those existing inside the tumours. This system has also demonstrated a higher accumulation of the drug within the tumour and a facilitated release of the encapsulated drug [10].

An alternative strategy used to increase the therapeutic index of liposome-based drugs is based on improving the colocalization between the chemotherapeutic agent and the breast cancer cell. In some cases, this strategy can also include an improvement of the internalization of the drug into them as when cell surface receptors involved in endocytosis take part.

In general, these formulations involve modifications of the liposome surface to contain ligands that are specifically recognized by receptors overexpressed in the breast cancer cell surface. Several of these strategies have been recently published. For example, anti-HER2 immunoliposomes have proven much more effective against HER2-overexpressing breast cancer cells when compared with nontargeted liposomes. In one study, targeted liposomes were formulated with a Fab of recombinant humanized anti-HER2 monoclonal antibody [11].

Estrogen receptor is a particularly attractive target as it is overexpressed in a large amount of breast cancer cell lines [12]. Several studies incorporating either estradiol or estrone to liposomes to use them as a ligand against estrogen-expressing breast cancer have been reported. In one study, the accumulation of these estrogen-targeted liposomes was approximately six times higher than that observed with nontargeted liposomes [13].

2. Metastatic Breast Cancer Treatment and Liposomal Anthracyclines Pharmacology

Breast cancer is a heterogeneous disease that includes a variety of biological types with different treatment options and clinical outcomes. Metastatic breast cancer (MBC) is a chronic and incurable disease, with a median survival of approximately 2-3 years. Although advances have been made in the management of MBC, long-term survivors are rare, with 5-year survival rates varying from 5% to 10%.

At present, prognosis and treatment selection are based on tumor biology and molecular characterization. In particular, multigene array and expression analyses have provided a molecular classification for breast tumor. The most important subtypes are luminal A and B, Her2/neu, and basal like [14, 15].

Characterization of tumor biology (estrogen and progesterone receptors, Ki-67 and Her2) and clinical history (past treatment, patient symptoms, and functional status) is critical for selecting treatment in MBC. Quality of life is an important issue to consider when choosing a therapeutic option.

The targeted therapies, such as hormonal treatment of patients with hormone-sensitive tumors and trastuzumab in case of Her2 overexpression, represent a treatment of choice for a subset of selected patients. Nevertheless, cytotoxic chemotherapy remains the only therapeutic option in patients with triple negative condition or in those who progress after hormone therapy. Anthracyclines and taxanes are the most active drugs for the treatment of MBC. For many decades, conventional anthracyclines, doxorubicin, and epirubicin have been an important mainstay in the treatment of breast cancer. They have proven to be effective for both metastatic and early disease, but their use has been limited because of the intrinsic cardiotoxicity [16].

Many strategies have been designed to curtail this effect. Encapsulating anthracyclines into liposomes, which allowed patients to receive much higher doses of an anthracycline delivered mainly into the tumour tissue with fewer side effects, has been one of these. Several formulations of liposome-encapsulated doxorubicin are available for its use in
the clinical practice [17] which differ in pharmacological characteristics.

Pegylated liposomal doxorubicin (PLD) (Caelyx) is doxorubicin hydrochloride encapsulated in liposomes with surface-bound methoxypolyethylene glycol (MPEG). Doxorubicin hydrochloride is a cytotoxic anthracycline antibiotic derived from Streptomyces peucetius var. caesius. Pegylation avoiding liposomes may be detected by the monoclonal phagocyte system and thereby the blood circulating time is increased. Mean half-life of pegylated liposomes in humans is 55 hours. Its pharmacokinetic characteristics facilitate tissue accumulation and this has been demonstrated in tumour biopsies of Kaposi’s sarcoma (KS) and bone metastases from breast cancer [18, 19].

Plasmatic pharmacokinetics of PLD in humans significantly differ from the original doxorubicin. Caelyx has a linear pharmacokinetic profile at lower doses (10–20 mg/m²) while in the dose interval of 20–60 mg/m² PLD is nonlinear. Standard doxorubicin hydrochloride displays extensive tissue distribution (volume of distribution, 700–1,100 L/m²) and rapid clearance (24–73 L/h/m²). On the contrary, the distribution volume of PLD is limited mainly to the vascular fluid, and the elimination of doxorubicin from the blood depends on the liposomal carrier; doxorubicin becomes available for catabolism once the liposomes are extravasated and entered into the tissueal compartment.

At equivalent doses, plasma concentration and AUC values of PLD are significantly higher than those achieved with doxorubicin preparations. The pharmacokinetic profile of PLD determined in 18 patients with breast cancer (which was similar to a group of 120 patients with several tumour types) showed a mean half-life of 71.5 hours (range 45.2–98.5 hours).

As already has been mentioned, the pegylated liposomal doxorubicin hydrochloride formulation allows the liposomes to circulate in the blood for extended periods of time. These pegylated liposomes are small enough (mean diameter of approximately 100 nm) to pass intact through the defective blood vessels supplying tumours. The entry of pegylated liposomes from blood vessels and their accumulation in tumours have been tested in mice bearing C-26 colon carcinoma tumours and in transgenic mice with KS-like lesions. The pegylated liposomes also combine a low permeability lipid matrix with an internal aqueous buffer system that keeps doxorubicin hydrochloride encapsulated as long as liposomes remain in the blood stream.

Myocet (liposome-encapsulated doxorubicin citrate) is another form of encapsulated doxorubicin hydrochloride consisting of a drug delivery system with a highly rigid bilayer [20]. Myocet (LD) also provides a more prolonged circulating time than conventional doxorubicin and, in addition, liposome-encapsulation significantly modifies the biodistribution of doxorubicin, resulting in reduced toxicity. The clearance of LD was 5.1 ± 4.8 L/h and steady-state volume of distribution (Vss) was 56.6 ± 61.5 L whereas, after conventional doxorubicin elimination and (Vss) were 46.7 ± 9.6 L/h and 1.45 ± 258 L, respectively [21].

In animals (Table 1), liposome-encapsulated doxorubicin reduced the distribution to the heart and the gastrointestinal mucosa compared to conventional doxorubicin, while antitumor efficacy was maintained. However, when compared with conventional doxorubicin, LD did not prove to be more active in doxorubicin-resistant cell lines.

Doxorubicin plasma pharmacokinetics in patients receiving LD showed a high degree of interpatient variability. Nonetheless, as a rule, total doxorubicin plasma levels were significantly higher with LD than with conventional doxorubicin, while free doxorubicin peak plasma levels were lower. Similarly, the peak levels of the main circulating doxorubicin metabolite, doxorubicinol (synthesized via aldo-keto-reductase) appeared in plasma later with LD than with conventional doxorubicin. Available pharmacokinetic data preclude settling strong conclusions regarding the relationship between plasma levels of total/free doxorubicin and its influence on the efficacy/safety of LD.

3. Anthracycline Toxicity

Anthracyclines have a well-known toxicity profile. Their more frequent side effects include myelosuppression, mucositis, alopecia, and emesis. Other less frequent although highly relevant side effects are cardiotoxicity and the occurrence of secondary leukemias.

The emetogenic potential of anthracyclines is moderate even though it is potentiated by other agents when administered in combination. The lowest blood cell count (nadir) is reached between 10 and 14 days after administration. Doxorubicin is a potent vesicant agent and its extravasation may cause necrosis of the skin and soft tissue.

Anthracycline-induced cardiotoxicity was described for the first time in the 1970s [22]. Cardiac side effects can be divided into acute and late-onset events. Acute toxicity encompasses phenomena that are usually reversible and nonfatal, such as hypotension, tachycardia, and arrhythmias. The occurrence of symptoms of myocarditis (with or without accompanying pericarditis) in the immediate posttreatment days is less frequent but can lead to heart failure that is usually reversible.

However, late-onset cardiotoxicity is the most relevant problem. It results in dilated cardiomyopathy that causes lethal congestive heart failure (CHF) in 75% of cases in the following years and whose end-stage treatment may require a heart transplant [23]. This type of heart disease responds to a dosing and regimen-dependent pattern [22]. Toxicity is higher when anthracyclines are administered in bolus compared to regimens giving it as a continuous infusion and this seems to be related to the higher dose peak reached when administered in a short period of time.

A number of factors that predispose to this toxicity have been identified. Specifically, they are hypertension, age below 15 or over 70 years, a history of radiotherapy to the mediastinum, and the concomitant use with other drugs such as cyclophosphamide, paclitaxel, or trastuzumab. In particular, when given with paclitaxel the risk of cardiotoxicity is higher when doxorubicin is administered just after paclitaxel instead of the opposite sequence.
The earlier studies only recognized clinical-evident cardiac toxicity. 3–4% of patients treated with cumulative doses of 450 mg/m² and up to 18% of those who received 700 mg/m² presented with clinical heart failure [24]. The incidence of heart failure is lesser when epirubicin was used but occurred in a 0.7% of patients when cumulative doses of 660 mg/m² were reached [25].

Anthracyclines cause some pathological changes prior to the occurrence of clinical cardiomyopathy that can be detected by different techniques: myocardial biopsy (Billingham scale); isotope ventriculography (MUGA scan) and echocardiography. Billingham published in 1978 a histological classification based on the findings observed in myocardial biopsies. Biopsy findings correlated fairly well with the cumulative doses of anthracyclines and were able to detect early damage to the myocardial cells. Early histological changes secondary to anthracyclines include cytoplasmic vacuolization and loss of muscle fibres from myocytes due to dilated sarcoplasmic reticulum. In more advanced stages, changes occur in cellular remodelling leading to left ventricular failure [26]. Such an invasive method has had no widespread use in daily clinical practice.

Isotope ventriculography (MUGA scan) has proven to be an easily reproducible and accurate technique in detecting anthracycline-induced cardiotoxicity [27]. Echocardiography is another noninvasive test used in the study and followup of anthracyline-induced cardiotoxicity. It is less accurate than ventriculography in the early detection of systolic dysfunction but allows assessing diastolic function whose decline seems to be a good predictor of early cardiac toxicity [28]. Other techniques such as antimonyin antibody scintigraphy or biomarkers such as troponin have been unable to predict early cardiotoxicity.

The majority of recent studies accept as cardiotoxicity criteria a ≥20% reduction in the left ventricular ejection fraction (LVEF) as long as it remains above 50%, a >10% reduction if the resulting figure is below 50%, or when symptoms of CHF (congestive heart failure) occur [29]. Using these criteria, Swain calculated a 7.9% incidence of anthracycline-induced cardiotoxicity with a cumulative dose of 450 mg/m²; 15.7% with 500 mg/m²; 26% with 550 mg/m², and 48% with 700 mg/m² [30]. Shapiro et al. described cardiac toxicity incidence of 20% when the cumulative dose of doxorubicin in combination with cyclophosphamide reached 500 mg/m² [31]. Adjuvant chemotherapy studies in which cumulative doses of doxorubicin did not exceed 300 mg/m² showed an incidence of cardiomyopathy ranging from 0.2 to 0.9% [32]. Currently, cumulative doses that do not exceed 450–500 mg/m² of doxorubicin or 900–1000 mg/m² of epirubicin are accepted to be safe [25].

The simultaneous administration of other drugs potentiates anthracyline toxicity. The combined use of doxorubicin and paclitaxel was related to a rate of cardiotoxicity higher than predicted despite relatively low cumulative doses of doxorubicin [38]. This increased toxicity appeared to be caused by a pharmacokinetic interference between paclitaxel and doxorubicin resulting in higher doxorubicin and doxorubicin plasma concentrations [39].

The combination of anthracyclines and trastuzumab has also been correlated with a higher rate of cardiotoxicity. In the pivotal study that compared doxorubicin and cyclophosphamide with or without trastuzumab in patients with overexpression of HER-2, a 23% rate of cardiac toxicity was observed with the combination compared with 7% in the arm not receiving trastuzumab [40]. Another study of the combination of trastuzumab with epirubicin and cyclophosphamide found that the combination with epirubicin 90 mg/m² translated into 5% cardiac toxicity compared with only 1.7% when epirubicin was administered at 60 mg/m² [41].

### 4. Liposomal Anthracyclines and Metastatic Breast Cancer

In patients with MBC, liposomal anthracyclines have shown similar efficacy and less toxicity when compared with conventional anthracyclines. Currently, three formulations with liposomal anthracyclines are available:

(i) Myocet: formulated with conventional liposomes;
(ii) DaunoXome: liposomes with prolonged circulation half-lives;
(iii) Caelyx/Doxil: with pegylated liposomes.

According to their respective product labelling, liposomal doxorubicin (LD, Myocet) was approved for the treatment of metastatic breast cancer; pegylated liposomal doxorubicin

<table>
<thead>
<tr>
<th>Tissues</th>
<th>TLC D-99 AUC₀→𝑇_{last} (uM eq-h)</th>
<th>𝑇_{1/2} (h)</th>
<th>Doxorubicin AUC₀→𝑇_{last} (uM eq-h)</th>
<th>𝑇_{1/2} (h)</th>
<th>Ratio of AUC₀→𝑇_{last} (TLC D-99/Dox)</th>
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<tr>
<td>Myocardium (right ventricle)</td>
<td>189</td>
<td>62</td>
<td>282</td>
<td>54</td>
<td>0.67</td>
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* Due to short sampling intervals relating to apparent 𝑇_{1/2}, these values are estimated. TLC D-99: nonpegylated liposomal doxorubicin.
(PLD, Caelyx) for the treatment of advanced platinum-resistant ovarian cancer, advanced breast carcinoma, AIDS-related Kaposi’s sarcoma, and multiple myeloma.

In June 2000, Caelyx/Doxil received marketing authorisation in the US and subsequently in Europe, based on the results of a pivotal, randomised, controlled, and Phase III trial, which compared the efficacy of PLD with topotecan in the treatment of advanced ovarian cancer following failure of a platinum-containing regimen [42].

In MBC, both liposomal formulations have proven to be effective as single agent or in combination with other drugs for the treatment of either anthracycline-treated (progression-free interval of >6–12 months) or naïve patients [43–46].

Table 2 summarizes the trials that directly compared liposomal anthracyclines with conventional anthracyclines, either as monotherapy or combination. We shall review both, efficacy and toxicity, emphasizing data related to cardiac toxicity. Two Phase III studies have been published [33, 34] in which efficacy and toxicity of liposomal anthracyclines have been directly compared to conventional doxorubicin. There were no statistically significant differences between both treatments with respect to efficacy in terms of response rate, progression-free survival (PFS), or overall survival (OS).

O’Brien et al. [33] reported the results of a noninferiority Phase III study in which 509 patients (p) with metastatic breast cancer were randomized to receive PLD at a dose of 50 mg/m² every 4 weeks (254p) or conventional doxorubicin 60 mg/m² every 3 weeks (255p). The study met its objective of noninferiority with PFS being 6.9 versus 7.8 months, respectively (HR 1.00; 95% CI 0.82–1.22). OS was comparable: 21 and 22 months for PLD and doxorubicin, respectively (HR 0.94; 95% CI 0.74–1.19). The objective response rate was also similar for PLD (33%) and doxorubicin (38%). Remarkably, the risk of cardiotoxicity was significantly higher in the conventional doxorubicin group (HR 3.6; 95% CI 1.58–6.31): forty-eight patients (19.6%) treated with doxorubicin developed cardiac toxicity compared with only 10p among those receiving PLD (P < 0.001). There were no patients with clinical heart failure in the PLD arm, while 10 patients (4%) in the conventional doxorubicin arm developed clinical heart failure. The number of patients to treat with PLD to avoid a doxorubicin-related cardiac event was 7. Also significant is that 16% of patients in the PLD arm received treatment for more than 9 months compared with only 1% in the doxorubicin arm and this was not linked to an increase in cardiac toxicity with PLD. In contrast, hand-foot syndrome incidence was higher in the PLD group (48% versus 2%).

Harris et al. [34] compared the efficacy and safety of LD (75 mg/m² every 3 weeks) with conventional doxorubicin (75 mg/m² every 3 weeks) in 224 patients with metastatic breast cancer. Of them, 17% had received prior adjuvant or neoadjuvant treatment with anthracyclines. Response rate was 26% in both arms. PFS was 3.8 months in the LD arm compared to 4.3 in the conventional doxorubicin arm (P = 0.59). OS was 16 months in the LD arm versus 20 months in the conventional doxorubicin arm (P = 0.09). Myocardial biopsies were planned for patients with a LVEF reduction of >10% with absolute values above 50% or for those who had a LVEF reduction of >6% if the resulting LVEF was lower than 50%. In addition to the standard criteria for identifying cardiotoxicity, the presence of a grade of 2.5 or greater on the Billingham scale was included. The rate of cardiac events was favourable to the liposomal anthracycline arm (13 versus 29%, P = 0.0001) with a clinical heart failure rate of 5.9 versus 15%. When the heart biopsies performed were analyzed, the proportion of patients with a value of 2.5 on the Billingham scale was 26 versus 71% (P = 0.02) favouring the liposomal formulation. The mean cumulative dose until toxicity occurred was calculated at 570 mg/m² for doxorubicin and 785 mg/m² for liposomal doxorubicin.

Some other Phase III studies [35–37] compared efficacy and toxicity of liposomal anthracyclines in combination with other cytostatic agents (docetaxel or cyclophosphamide) with combinations with conventional anthracyclines or other drugs. Inclusion criteria for these studies were not identical, mainly regarding prior treatment allowed. Studies by Chan et al. and Batist et al. included patients not previously treated with anthracyclines; Sparano et al., however, randomized patients previously treated with anthracyclines during adjuvant or neoadjuvant therapy as long as progression-free interval was above 12 months. As Table 2 shows, we can see that overall efficacy of liposomal anthracyclines is similar to the efficacy of conventional formulations when combined with other cytostatic agents. Of note, in Chan’s study PFS was even higher in the group treated with Myocet plus Cyclophosphamide.

In Batist’s study [35], 30% of patients presented any cardiotoxicity risk factor and 10% had received prior anthracyclines (adjuvant) with a mean cumulative dose of 240 mg/m². Here, 21% of patients treated with conventional doxorubicin had some grade of cardiotoxicity compared to 6% in the group receiving liposomal doxorubicin (P = 0.0001). In the control arm, 3.2% of patients developed clinical heart failure compared with 0% in the liposomal doxorubicin arm. The analysis of patients with any cardiac risk factor showed an even greater difference between both drugs with a HR of 16.1. The mean cumulative dose calculated for 50% of patients presenting with cardiotoxicity was much higher in the group receiving liposomal doxorubicin (2.220 mg/m² versus 480 mg/m²).

Eventually, the same author published in 2006 [47] retrospective data from the analysis of 68 patients that had been included in the Phase III study and had been treated with adjuvant anthracyclines. Cardiac toxicity was lower in patients treated with liposomal doxorubicin (22 versus 39%, HR: 5.4, P = 0.001). Four patients developed congestive heart failure, 3 of them in the doxorubicin arm. The calculated mean cumulative dose until cardiotoxicity occurrence was 580 mg/m² for doxorubicin and 780 mg/m² for the liposomal formulation (HR: 4.8, P = 0.001).

A further Phase III study [36] randomized 160 patients to receive cyclophosphamide 600 mg/m² plus either epirubicin 75 mg/m² or liposomal doxorubicin 75 mg/m². No significant differences were observed in the rate of asymptomatic reduction in LVEF (11 versus 10%). In this study, no patient developed clinical heart failure. It must be noted that epirubicin dosing was lower than the equipotent doxorubicin.
In 2010, the Cochrane Library reported a systematic review of the different anthracycline compounds and their cardiotoxicity [48]. Studies by Harris and Batist were analyzed together and authors concluded that nonpegylated liposomal anthracyclines reduced the overall risk of cardiotoxicity (RR = 0.38, P < 0.0001) and the risk of clinical heart failure (RR = 0.20, P = 0.02).

Efficacy and safety of pegylated liposomal doxorubicin (PLD) combined with other cytostatic agents were studied in two Phase III studies.

Sparano et al. [37] randomized 751 patients previously treated with anthracyclines (as adjuvant or neoadjuvant) with a PFI over 12 months to receive either docetaxel 75 mg/m² (373p) or the combination of PLD 30 mg/m² plus docetaxel 60 mg/m² every 21 days (378p) until disease progression or unacceptable toxicity occurred. Combined treatment improved PFS significantly from 7.0 to 9.8 months (HR 0.65; 95% CI, 0.55–0.77; P < 0.00001). OS was similar: 20.6 months in the docetaxel arm and 20.5 in the combined treatment arm (HR 1.02; 95% CI, 0.86–1.22). The incidence of hand-foot syndrome was higher in the combined treatment arm (24% versus 0%) and symptomatic cardiac toxicity was similar: 4% in the docetaxel group and 5% in the PLD-docetaxel group.

Patients with metastatic breast cancer progressing after taxanes and anthracyclines had fewer treatment options and often anthracyclines were not used again, due to the cumulative risk of cardiotoxicity. Based on the safety and efficacy data for PLD, a Phase III study was proposed [49] in which 301 patients with metastatic breast cancer progressing to taxanes (<6 months) were randomized to receive one of the following three alternatives: PLD 50 mg/m² every 4 weeks (150p); vinorelbine 30 mg/m² every week (129p); or mitomycin-C 10 mg/m², on days, on 1 and 28 plus vinblastine 5 mg/m² on days 1, 14, 28, and 42 every 6–8 weeks (22p). 83% of patients had received prior anthracyclines, in 10% of them cumulative doses above 450 mg/m² had been reached. No patient treated with PLD showed clinical symptoms of cardiotoxicity. PFS was similar (2.86 months in the PLD group versus 2.53 months in the other two control groups) (HR 1.26; 95% CI, 0.98–1.62). In the subgroup of patients not previously treated with anthracyclines (44p), PFS was higher in the PLD arm (5.8 months) compared with the control arms (2.1 months) (P = 0.01). OS was slightly higher with PLD (11 months) versus control arm (9 months), albeit not statistically significant (P = 0.93). The objective response rate was similar: 10% for PLD versus 12% for the control arm.

More recently an Austrian observational study was published [50] in which 129 patients with metastatic breast cancer treated with PLD were analyzed. 70% presented 2 or more cardiovascular risk factors. Despite this, only 4% of patients had some degree of cardiotoxicity and only 2 cases of clinical heart failure were reported.

Alba et al. [51], on behalf of GEICAM, published a Phase III study exploring the role of PLD as maintenance therapy. Eligible patients had previously received a sequential scheme based on 3 cycles of doxorubicin 75 mg/m² followed by 3 more cycles of docetaxel 100 mg/m². Patients, who had not progressed during this first part, were randomized to receive pegylated liposomal doxorubicin 40 mg/m² × 6 cycles or nothing. TTP from randomization of the 155 p was 8.4 versus 5.1 months favouring the maintenance treatment arm (P = 0.0002). No differences in OS were found. Six patients had reduced LVEF ≥ 10%, 5 of them in the arm of PLD. In 2 of the patients treated with PLD, a LVEF reduction below 50% during treatment was found, although both recovered within 6 months. There was no clinical cardiac toxicity.
5. Liposomal Anthracyclines and Trastuzumab

In HER2-positive breast cancer, the addition of trastuzumab to chemotherapy significantly increases response rate, time to progression, and overall survival compared with chemotherapy alone. However, when trastuzumab is combined with anthracyclines there is an increased risk of cardiac toxicity. Slamon et al. [40] randomized 469 patients with metastatic breast cancer and HER2 overexpression to receive standard treatment (anthracyclines/cyclophosphamide or paclitaxel) with or without trastuzumab. The addition of trastuzumab increased PFS (7.4 months versus 4.6 months, \( P < 0.001 \)) and OS (25.1 versus 20.3 months, \( P = 0.046 \)), but with an increased rate of cardiotoxicity in the group receiving the anthracycline and trastuzumab combination (27%). These results limited the use of anthracyclines in HER2-positive breast cancer, and in consequence non-anthracycline-based regimens such as TCH [52, 53] were designed. As anthracyclines showed a high level of activity in this subgroup of patients, other strategies were developed also to design regimens using less cardiotoxic anthracyclines such as epirubicin (a less cardiotoxic analog than doxorubicin) at limited doses or liposomal anthracyclines in combination with trastuzumab [54] which will be further analyzed.

Several studies with a small number of patients explored the viability of combination regimens with liposomal anthracyclines and trastuzumab in metastatic breast cancer. LD (Myocet) proved to be as effective as and less cardiotoxic than conventional anthracyclines when combined with trastuzumab in 4 Phase I/II studies.

The first was a Phase I/II study by Theodoulou et al. [55] that included 37 patients with HER2-positive metastatic breast cancer, 14 patients had been previously treated with adjuvant doxorubicin (<240 mg/m\(^2\)) and 17 patients with one or two lines of prior chemotherapy for advanced disease (II with trastuzumab). Myocet 60 mg/m\(^2\) was administered every 3 weeks plus trastuzumab 2 mg/Kg weekly. Response rate was 58% (95% CI 41–75%). A LVEF reduction of >10% was observed in 10 patients (25%). Five patients (12%) presented with a LVEF < 50%, 4 of them had been pretreated with anthracyclines; 2 patients (5%) withdrew from the trial due to cardiac toxicity.

Another Phase I/II trial [56] included 69 patients with locally advanced or metastatic disease who had received no prior treatment. The treatment regimen chosen for the Phase II was trastuzumab combined with liposomal doxorubicin 50 mg/m\(^2\) every 21 days and paclitaxel 80 mg/m\(^2\) weekly. Response rate was 98.1% (95% CI 90.1–99.9). Median time to progression was 22.1 months (95% CI 16.4–46.3) in metastatic patients and had not yet reached in locally advanced patients by the time of publication. No cases of treatment-related clinical heart failure were observed. Twelve patients presented with an asymptomatic reduced LVEF; 8 of them recovering up to values of 50% or greater within a mean of 9 weeks.

Venturini et al. [57] conducted a Phase II study in 31 patients with first-line metastatic disease to evaluate the safety and efficacy of combining trastuzumab, LD, and docetaxel. Eight cycles of chemotherapy were administered, followed by trastuzumab monotherapy to complete 52 weeks of treatment. The response rate was 65.5% with a TTP of 13 months. Five of the 31 patients experienced a ≥20% reduction from baseline or an absolute LVEF < 45%.

Another Phase I-II trial with LD in combination with trastuzumab and docetaxel was conducted by Amadori et al. [58]. Forty-five patients with metastatic breast cancer received weekly trastuzumab associated with LD 50 mg/m\(^2\) every 3 weeks and docetaxel 30 mg/m\(^2\) on days 2 and 9. The response rate was 55.6% with a TTP of 10.9 months. Only 2 patients had a decrease in LVEF below 50%.

Similarly, the use of PLD combined with trastuzumab may reduce the incidence of cardiotoxicity while maintaining a similar efficacy. We shall describe a series of small Phase II studies that investigated this alternative. Chia et al. [59] included 30 patients with HER2-positive metastatic breast cancer (MBC), 13 of them previously treated with adjuvant anthracyclines (<300 mg/m\(^2\)). PLD 50 mg/m\(^2\) was given every 4 weeks and trastuzumab 2 mg/Kg weekly for 6 cycles. Response rate was 52% and PFS 12 months. The most frequent toxicities were grade 3 hand-foot syndrome (30%) and grade 3/4 neutropenia (27%). Cardiac toxicity incidence was 10% and in no case was asymptomatic. Andreopoulou et al. [60] included 12 patients with MBC on first- and second-line therapy, 7 treated with adjuvant anthracyclines and 7 with prior trastuzumab for metastatic disease. They received treatment with PLD every three weeks and trastuzumab weekly achieving 66% disease stabilization. 25% presented with grade 2 cardiac toxicity. Stickeler et al. [61] enrolled 16 patients with HER2-positive metastatic breast cancer; 5 had received prior chemotherapy for advanced disease (2 of them received anthracyclines <400 mg/m\(^2\)). PLD 40 mg/m\(^2\) was administered every 4 weeks for 6–9 cycles plus trastuzumab weekly; response rate was 50%, PFS 9.67 months, and OS 16.23 months. Christodoulou et al. [62] studied trastuzumab combined with PLD administered at a dose of 30 mg/m\(^2\) every three weeks. All patients should have received first-line chemotherapy for advanced disease or have relapsed before the end of the year of taxane-based adjuvant treatment. The response rate was 22%, PFS 6.5 months, and OS 18.7 months. There were no episodes of LVEF reduction in any of the patients.

Wolff et al. [63] published a Phase II study (ECOG E3198) in which 84 patients with HER2-positive or negative MBC on first-line therapy were included and who had not been previously treated with anthracyclines. PLD was administered at a dose of 30 mg/m\(^2\) together with docetaxel 60 mg/m\(^2\) every three weeks (maximum of 8 cycles) plus trastuzumab (46p) or without it (38p) according to HER2 expression. Response rate was 47.4% in the arm without trastuzumab (95% CI 31.0–64.2%) and 45.7% in the arm with trastuzumab (95% CI 30.9–61%). PFS was 11 months (95% CI 8.6–12.8 months) and 10.6 months (95% CI 15.6–15.7), respectively. Median OS was 24.6 months (95% CI 14.7–37.3) and 31.8 months (95% CI: 23.7–44.9 months). There was only one case of heart failure who was a HER2-negative patient. The addition of trastuzumab in patients with HER2 overexpression was not associated with higher cardiac toxicity but was related to a higher incidence of hand-foot syndrome.
Recently, Martin et al. [64] published a Phase II study (GEICAM 2004/05) which included 48 patients in first-line metastatic disease. PLD was administered at doses of 50 mg/m<sup>2</sup> in combination with cyclophosphamide 600 mg/m<sup>2</sup> every 4 weeks along with weekly trastuzumab. The response rate was 68.8%, the TTP was 12 months and OS of 34.2 months. There were no symptomatic cardiac events. Eight patients (16.7%) had decreased LVEF grade 2; six of them had been previously treated with anthracyclines. Seven of the 8 patients recovered cardiac function.

6. Early Breast Cancer

A number of small studies of neoadjuvant treatment with liposomal anthracyclines for locally advanced breast cancer have been published. The Phase I study by Possinger et al. [65] included 20 patients receiving a combination of LD 60 mg/m<sup>2</sup> plus docetaxel 75 mg/m<sup>2</sup> on day 1 and gemcitabine 350 mg/m<sup>2</sup> on day 4, every 3 weeks. The use of colony-stimulating factors was mandatory. Response rate was 88%. No cardiotoxicity was observed, but there was significant haematological toxicity (29%) and stomatitis (28%). Another Phase II study published by Gogas et al. [66] included 35 patients receiving treatment with PLD 35 mg/m<sup>2</sup> in combination with paclitaxel 175 mg/m<sup>2</sup> every 3 weeks for 6 cycles. Response rate was 71%. Grade 3 toxicity was cutaneous (11%), hand-foot syndrome (9%), and leukopenia (11%). No cardiac toxicity was observed.

7. HER-2-Positive Early Breast Cancer

There has been a greater interest in the use of liposomal anthracyclines in early breast cancer overexpressing HER2 oncogene, as this subgroup of patients could obtain the greatest benefit from treatment with anthracyclines [67] and combining them with trastuzumab may be difficult due to the high cardiotoxicity that could be induced.

Our group designed a Phase I-II study (GEICAM 2003-03) in patients with early breast cancer to be given as neoadjuvant therapy to deal with the dose variability of LD (Myocet) in combination with other drugs and the lack of evidence for a maximum tolerated dose when combined with docetaxel and trastuzumab [68, 69]. The results for Phase I after the inclusion of 19 patients with stages II and IIIA HER2-positive breast cancer determined the recommended dose for Phase II to be LD 50 mg/m<sup>2</sup> plus docetaxel 60 mg/m<sup>2</sup> every three weeks with standard dose trastuzumab when prophylactic pegylated-filgrastim was administered. Only one of the 19 patients presented with cardiac toxicity and it was an asymptomatic grade 2 reduction in LVEF. Pathologic complete response rate in the primary tumour and axillary lymph nodes was 33%. With such stimulating data on activity and safety, Phase II of the study was completed. Fifty-nine patients with HER2-positive breast cancer were included: stages II, 40p and IIIA, 19p. The recommended dose from prior Phase I was administered every 21 days: liposomal doxorubicin 50 mg/m<sup>2</sup>, docetaxel 60 mg/m<sup>2</sup> and trastuzumab 2 mg/kg/weekly along with prophylactic pegylated-filgrastim. The clinical response rate was 86% and radiological response rate was 81%. No patient progressed during treatment. All patients underwent surgery which was conservative in 42 cases. Seventeen patients (29%, 95% CI 17.2–40.4) obtained a pathologic complete response in the breast tumour (G5 Miller and Payne) and 16 of them (27%, 95% CI 15.8–38.4) also obtained a pathologic complete response in the axillary lymph nodes. An additional 15% obtained a grade 4 Miller and Payne response in the primary tumour. Neutropenia was the most significant grade 3-4 haematological toxicity (17 patients, 29%), but only 3 developed neutropenic fever. Grade 3 nonhaematological toxicity was infrequent: asthenia in 5 patients, nausea in 3, diarrhea in 3, and stomatitis in one patient. Grade 2 (>20% reduction of the baseline value or reduction below the normal value of 50%) asymptomatic reduction of LEVF was observed in 5 patients (9%) and treatment was withheld in only one of them. By the end of treatment, 3 of the patients had recovered a LEVF greater than 50%. There were no episodes of clinical heart failure.

Finally, a Phase II randomized study published by Rayson et al. [70] provided us with information regarding cardiotoxicity of the combination of PLD plus trastuzumab used concomitantly in adjuvant therapy for intermediate-risk breast cancer with HER2 overexpression and either negative or positive lymph nodes. 181 patients with a baseline LVEF >55% were included. They were randomized (1:2) to arm A: doxorubicin 60 mg/m<sup>2</sup> plus cyclophosphamide 600 mg/m<sup>2</sup> every 21days, four cycles or arm B: PLD 35 mg/m<sup>2</sup> plus cyclophosphamide 600 mg/m<sup>2</sup> every 21 days, four cycles plus trastuzumab 2 mg/kg weekly for 12 weeks. Both groups subsequently received paclitaxel 80 mg/m<sup>2</sup> plus trastuzumab for 12 additional weeks, followed by trastuzumab in monotherapy to complete one-year therapy. The main objective of the study was cardiac toxicity: comparing the rate of cardiac events and/or the percentage of patients who were unable to complete one-year treatment with trastuzumab. The incidence of cardiac toxicity was 18.6% with doxorubicin (95% CI 9.7%–30.9%) versus 4.2% with PLD (95% CI 1.4%–9.5%) (P = 0.0036). Among the 16 patients who had a cardiac event (11 in the conventional doxorubicin arm and 5 in the PLD arm), 8 were over 55 years old. All the events occurred after the 4th course of therapy. One of the events was a myocardial infarction with subsequent clinical heart failure (this occurred in arm B). Of the remaining 15 cases, 7 were recorded as >10% reduction from baseline LVEF with absolut values of <50% (3 of them developing clinical symptoms were classified as NYHA class II heart failure). The other 8 cases were classified as asymptomatic (NYHA class I). There were no cardiotoxicity-related deaths. The LVEF mean value was similar in both groups (64.0%, PLD + C + H/T + H and 64.4%, A + C/T + H). Mean reduction of LVEF values after the 8th cycle (end of chemotherapy) was significantly higher in patients receiving conventional doxorubicin (5.6% versus 2.1%; P = 0.0014). Cardiac safety analysis for this study suggested that administering trastuzumab concomitantly with PLD in the tested regimen was feasible, caused less cardiotoxicity in the short term, and avoided the premature interruption of treatment.
with trastuzumab when compared with a standard regimen such as A + C/T + H. The authors concluded that this strategy of incorporating early and concomitantly a liposomal anthracycline plus trastuzumab was safe, but its possible clinical role should be properly investigated in a randomized Phase III trial versus a nonanthracycline regimen such as TCH.

8. Conclusions

Liposome-based drug delivery systems are able to modify the pharmacokinetics and pharmacodynamics of cytostatic agents, enabling us to increase the concentration of the drug released into the neoplastic tissue and, at the same time, reducing the exposure of normal tissue to the drug.

Anthracyclines are important agents in the treatment of both metastatic and early breast cancer, but cardiotoxicity remains one of the major limitations for their use. Liposome encapsulation is one of the strategies designed to minimize this side effect. There are several liposome-encapsulated doxorubicin formulations available which show different pharmacological characteristics. The most commonly used are liposomal doxorubicin (Myocet) and pegylated liposomal doxorubicin (Caelyx).

In patients with metastatic breast cancer, liposomal anthracyclines have proven to be as effective and less toxic when compared face to face with conventional anthracyclines, allowing a longer period of treatment and a higher cumulative dose of the anthracyclines. The combined analysis of available data indicates an overall reduction in risk for both cardiotoxicity (RR = 0.38, \( P < 0.0001 \)) and clinical heart failure (RR = 0.20, \( P = 0.02 \)). The safety of liposomal anthracyclines endorsed its use in patients with some cardiac risk factors.

In HER2-positive breast cancer, the addition of trastuzumab to chemotherapy significantly increased response rate, progression-free survival, and overall survival. Initial studies demonstrated synergy when trastuzumab was combined with anthracyclines, but their excessive cardiac toxicity limited their use and nonanthracycline therapeutic strategies were designed.

Liposomal anthracyclines have proven to be effective and safe when combined with trastuzumab both in advanced and early breast cancer. Of particular interest is the use of the combination of liposomal anthracyclines plus trastuzumab in patients with early and HER2-overexpressing breast cancer, as this is probably the subgroup that would benefit most from a treatment with anthracyclines. The potential clinical benefit of anthracyclines in this setting should be investigated in a clinical trial comparing a regimen with liposomal anthracyclines versus a nonanthracyclines combination.

Conflict of Interests

The authors declare no conflict of interests relating to the publication of this paper.

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Review Article
Gene Therapy for Advanced Melanoma: Selective Targeting and Therapeutic Nucleic Acids

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Despite recent advances, the treatment of malignant melanoma still results in the relapse of the disease, and second line treatment mostly fails due to the occurrence of resistance. A wide range of mutations are known to prevent effective treatment with chemotherapeutic drugs. Hence, approaches with biopharmaceuticals including proteins, like antibodies or cytokines, are applied. As an alternative, regimens with therapeutically active nucleic acids offer the possibility for highly selective cancer treatment whilst avoiding unwanted and toxic side effects. This paper gives a brief introduction into the mechanism of this devastating disease, discusses the shortcoming of current therapy approaches, and pinpoints anchor points which could be harnessed for therapeutic intervention with nucleic acids. We bring the delivery of nucleic acid nanopharmaceutics into perspective as a novel antimelanoma therapeutic approach and discuss the possibilities for melanoma specific targeting. The latest reports on preclinical and already clinical application of nucleic acids in melanoma are discussed.

1. Introduction

Melanoma derives from melanocytes—pigment cells of the skin. Melanoma most commonly arises from epidermal skin melanocytes (cutaneous melanoma), but primary tumors can also be found lining the choroidal layer of the eye (uveal melanoma) or the mucosal surfaces of the respiratory, genitourinary, and gastrointestinal surfaces. Similar to other tumors, the progression stage of melanoma is predictive for therapeutic success. Early stage melanomas (thin tumors) result in a 97% 5-year survival rate of the patients, after surgical removal [1]. Conversely, advanced melanoma patients, comprising metastasis in regional lymph nodes or other organs, face 5-year survival rates of less than 10% [1]. Due to the intrinsic tendency of melanoma to early metastasis, even small primary tumors have already led to metastasis and a substantial portion of diagnosed melanoma cases are of late progression stages. Treatment of advanced or metastatic melanoma has proven a challenge, as the conventional therapeutic approaches failed to translate into improved or significant survival rate in phase III clinical trials. Newer treatments were established in the last years that elicit unprecedented response rates in late stage melanoma, for example, up to 80% in the case of BRAF inhibitors. However, almost all tumors become resistant within months, and the treatment is available only for a subset of melanomas. Altogether, despite substantial improvements in therapeutic options during the last years, there is still an urgent need for alternative approaches.

Based on clinical and histopathological features melanoma cancer cells undergo four sequential phases before reaching metastasis [2]. These phases ensue from several genetic, epigenetic, and microenvironmental, modifications [3]. In the last decade, a number of reports have brought significant insight into melanoma genetics and molecular markers, which are essential for the development of therapies,
and in particular targeted regimens. This paper will focus on melanoma targeted gene delivery; we aim at providing a general view on melanoma-targeting ligands, and other forms of specifically driving gene expression, reported in the literature, as well as review the most recent and/or relevant nucleic acid therapeutics employed in this field. The current paper will not dwell upon melanoma mutations or relevant nucleic acid therapeuticsemployed in this field. The literature, as well as review the most recent and/or forms of specifically driving gene expression, reported in this field. The melanoma section serves rather as a comprehensive overview on the key players of the neoplasia, which is essential for the understanding of targeted therapies.

2. From Melanocytes to Metastatic Melanoma

2.1. Four Steps Separate Melanocytes from Metastatic Melanoma. Presently, it is generally believed that melanomagenesis instigates from alterations in multiple molecules or pathways rather than a single high-risk melanoma loci. Moreover, melanoma progression is a dynamic process involving several steps, each requiring the activation of different genes. First, normal melanocytes undergo genetic alterations that lead to their transformation into benign nevi. Benign nevi differ from normal melanocytes in that they have initially proliferated in the basal layer of the epidermis; however, they entered a long-term dormant status due to the lack of additional oncogenic alterations. For example, the most frequent activating mutation in the BRAF gene occurs in the same frequency in nevi, where it causes a dormant status called oncogene-induced senescence [6]. Additional alterations then allow bypassing senescence leading to continued tumor cell proliferation. This progression stage is characterized by noninvasive horizontal growth and spread through the epidermis and has been termed as radial growth phase (RGP). Further transformation is required for invasive tumor growth from the epidermis into the dermis. This phase has been termed as vertical growth phase (VGP). For invasion, alterations like loss of adhesive molecules together with an increase in extracellular matrix degrading enzymes are characteristic. For metastasis, cell populations have to migrate to distant locations. For this, cells have to acquire more alterations that enable the complex processes underlying metastasis. These processes involve tissue invasion, entering, and evasion of blood or lymphatic vessels to reach distant location but also survival and proliferation at distinct locations. Hence, melanocytic cells have to become largely independent from their normal microenvironment [7].

2.2. Melanoma Progression: Risk Factors and Biological Drivers. The most important risk factor for melanoma is UV irradiation upon sun exposure. Whole genome sequencing revealed that melanoma is the tumor type with the most DNA mutations—many being typical for UV-induced mutations [8]. Despite the plethora of DNA alterations, two gene mutations were found to be rather common in melanoma. A general overview on these mutations and their key players are schematically represented in Figure 1.

With respect to mutation frequency, the mitogen-activated protein kinase (MAPK) pathway plays a central role in melanoma. Activation of growth factor receptors leads to activation of RAS molecules which activate in a downstream phosphorylation cascade RAF, MEK, and ERK kinases. ERK kinase phosphorylates a panel of substrates leading to increased cell proliferation and survival. RAS molecules, comprising HRAS, KRAS, and NRAS, are small GTPases or G proteins, and activating mutations in NRAS are found in 10%–20% of melanomas. RAS molecules activate RAF family members consisting of ARAF, BRAF, and CRAF. A single nucleotide mutation in BRAF at amino acid 600—whereupon a valine (V) amino acid is replaced by glutamic acid (E)—represents the most common mutation in BRAF. This mutant V600EBRAF leads to an alternative protein structure and to a constitutive active protein. 50%–60% of melanomas contain an activating mutation in BRAF [9]. The outstanding importance of the RAS/RAF signaling pathway is documented by the observation that BRAF and NRAS mutations—exclusively NRAS or BRAF is mutated in a tumor—together are found in over 80% of melanomas and by inhibitors of mutated BRAF that are clearly effective in melanoma therapy.

Interestingly, V600EBRAF has also been reported in melanocytic nevi [10–12], which rarely develop into melanoma. Nevi are described to be senescent, and, similarly, expression of V600EBRAF in melanocytes induces oncogene-induced senescence [6]. These findings imply that BRAF mutations are involved in the first transition state of melanoma progression. Hence, this mutation per se is insufficient to drive tumorigenesis, rather additional alterations are required to avoid dormancy.

Several pathways have been shown to cooperate with RAS/RAF signaling and to reduce RAS/RAF-mediated senescence. DNA damage due to oncogene-induced DNA replication stress has been proposed as an important mechanism of senescence [13]. Accordingly, molecules involved in DNA damage signaling have been shown to promote oncogenesis together with BRAF; for example, the loss of p53 [14]. Most evidence for BRAF cooperation exists for phosphatase and tensin homolog (PTEN). PTEN is a tumor suppressor gene that negatively modulates signal transduction via phosphatidylinositol phosphatase (PIP3), a cytosolic second messenger. This gene encodes for a lipid protein phosphatase that regulates cell growth and survival. Allelic loss or altered expression of PTEN can be observed in tumors. In melanoma, this lost/modified expression is present in 20%/40% of melanoma tumors, respectively [15, 16]. In a mouse model, it was shown that expression of V600EBRAF in melanocytes leads to benign lesions that do not progress to melanoma. However, when PTEN was silenced, these mice developed metastatic tumors with high penetrance [17].

Regarding the family history of melanoma, a two-fold risk increase has been reported [18], and it was associated to the 9p12 chromosome [19]. In 1994, the cyclin-dependent kinase N2A (CDKN2A) gene was identified [20], and it is now hold as a high-risk melanoma locus. The CDKN2A gene encodes for two tumor suppressor proteins, p16INK4a and p14ARF,
involved in cell cycle and apoptosis, respectively. Explicitly, \(p^{14\text{ARF}}\) directly promotes the degradation of human double minute 2 (MDM2). MDM2 promotes ubiquitinylation and proteosomal degradation of p53. Accordingly, inactivation of \(p^{14\text{ARF}}\) leads to increased MDM2 levels leading to increased degradation of p53 [21]. The other product of the CDKN2A locus, \(p^{16\text{INK4a}}\), prevents cell cycle progression by binding to CDK4/6 and through a series of events prevents the release of E2F1 (a transcriptional inducer of S-phase genes) [22]. Mutations of \(p^{16\text{INK4a}}\), and similarly of \(CDK4\) gene [23, 24], can therefore lead to increased cell cycle progression. However, despite the contribution of CDKN2A mutations for oncogenesis, the absolute risk of melanoma in mutation carriers is still highly shaped by environmental and pedigree factors [25]. In close relation to pedigree structure is skin pigmentation; the positive connection between light skin color and melanoma risks is well known. Melanocortin-1 receptor (MC1-R) is responsible for the cutaneous pigmentation; and, interestingly, it has been reported as being overexpressed in both melanotic and amelanotic melanomas [26]. There are two forms of epidermal melanin: eumelanin (with a black-brown color) and pheomelanin (red-yellow color). The synthesis of eumelanin—in charge of UV attenuation—is stimulated by the activation of the MCI-R, through the binding of the tridecapeptide \(\alpha\)-MSH, or \(\alpha\)-melanocortin stimulating hormone [27–29]. The binding of \(\alpha\)-MSH results in an increment of cAMP, which in turn upregulates the microphthalmia-associated transcription factor (MITF) inducing the transcription of pigment synthetic genes and the production of eumelanin. In addition, some MC1-R variants have been associated to melanoma risk [30]. MITF, on the other hand, is also involved in the regulation of the cell cycle and proliferation, and few variants of the gene have been found in melanoma patients [31, 32]. In particular, MITF(E318K) was reported to represent a gain-of-function allele for the gene, supporting MITFs role as an oncogene. However, MITFs expression in melanoma metastasis is yet to be clarified, as there are also studies showing that
downregulation and ablation of this gene create a more invasive phenotype in vitro [33] and increase tumor growth in vivo [34], respectively.

The transcription factor activator protein-2α (AP2α) has been suggested as a major key player in the transition from RGP to VGP [4]. Similar to several other mediators, AP2α also modulates a variety of cellular processes, including cell growth and apoptosis. In tumors, AP2α acts as a tumor suppressor, and high cytoplasmic to nuclear expression ratio was shown to correlate with poor patients’ prognosis [35, 36]. In particular, the promoters for the adhesion molecule MCAM/MUC18 [37], which is overexpressed in tumors, and tyrosinase kinase receptor, c-KIT (silenced in 70% of metastatic tumors) [38], have AP2α binding sites. AP2α has been described to directly bind to MCAM/MUC18 promoter and to inhibit its transcription, whereas it promotes c-KIT expression. Therefore, the loss of this transcription factor during melanoma results in high MCAM/MUC18 levels and c-KIT downregulation. In addition, the loss of AP2α was also appointed as a probable cause for the upregulation of the G-protein-coupled receptor protease activated receptor-1, PAR-1 [10, 39]. In PAR-1 promoter region, there are two binding complexes for AP2α and SPI. In normal melanocytes, AP2α binds to PAR-1 inhibiting its transcription. However, upon melanoma progression, the levels of AP2α decrease, and SPI binds to the PAR-1 promoter instead, driving its expression. RAS, phosphoinositide-3 kinase (PI3 K), and MAPK pathways are all signaling events downstream PAR-1, and hence closely related to tumor progression [40].

During the metastatic process, following evasion into the blood circulation, tumor cells adhere to the endothelium at distant sites, and herein adhesion molecules are necessary. Together with selectins, integrins have been found to play crucial roles in these steps. Integrins are a family of transmembrane glycoproteins that mediate cell-cell and cell-matrix adhesion. It is therefore expected that their expression pattern changes during tumor growth, metastasis, and angiogenesis. In particular, αvβ3 and αvβ1 (very late activation antigen-4, VLA-4) have been reported as overexpressed in numerous cancer types [41, 42] and have served as therapeutic targets. VLA-4 has been shown to be used by malignant melanoma cells to adhere to the endothelium (binding to the ligand VCAM-1) [43, 44], and to promote transmigration [42, 45] and metastasis [46, 47].

3. Shortcomings of Current Melanoma Therapies

Overall, melanoma incidence has been increasing over the years, reaching an annually increase of 3.1% during the past two decades [48]. Early prognosis permits 90% survival rates by surgical removal. Yet, unresectable advanced melanoma is characterized by an aggressive behaviour, fast spread and metastasis, and a strong resistance to chemotherapy. Therefore, and in spite of the extensive research, the current prognosis for patients with advanced melanoma is limited. The earlier conventional chemotherapeutic treatment approved by US Food and Drug Administration (FDA), Dacarbazine, results in less than 10% response rate with median response durations of 4–8 months [49]. Alternative chemotherapeutic agents include Fotemustine, Temozolomide, Paclitaxel (often in combination with carboplatin), and Docetaxel [50]—all not yielding larger progression-free survival (PFS) or overall survival (OS) than Dacarbazine [50, 51]. Generally, chemotherapeutics suffer from a lack of targeting specificity; their low molecular mass results in easy and fast body secretion, and thus the need of increased doses, which leads to inevitable toxicity. Similarly, immunotherapy based on interleukine 2 (IL-2)—also FDA approved—has comparable response rates, and it is further restricted by the ensuing multiorgan toxicity, requiring management in specialized cancer centers. Although combined therapies resulted in higher response rates, they still failed to translate into improved survival, with no impact on PFS or OS compared to Dacarbazine alone [1, 52]. Another alternative is the combined treatment with the cytokine TNFα in combination with the alkylating drug melphalan. Although highly successful, this treatment is limited to local treatment of melanoma in-transit metastases in limbs by isolated limb perfusion due to live threatening systemic toxicity of therapeutically active TNFα doses [53].

In the last decade, much progress was achieved due to the discovery of mutations in the BRAF gene. This led to the development of therapies interfering with RAS/RAF signaling and to specific BRAF inhibitors. In August 2011, an alternative melanoma regimen, for patients positive for BRAF mutations, was brought into the market with the FDA approval of Vemurafenib (Zelboraf, Plexxikon/Roche). In Phase II and III studies, Vemurafenib showed a response rate up to 50%, yet the response duration varied between the phase studies [54–56]. In addition, Vemurafenib induces acanthopapillomas, keratoacanthomas, and cutaneous squamous cell carcinomas in the early treatment [57, 58]. Unfortunately, these unprecedented response rates are limited by the fact that almost all tumors become resistant to this therapy and the overall survival of patients was 6.7 months [59]. In addition, the treatment is only available for 50%–60% of patients with mutated tumors because it is not effective in tumors with wildtype BRAF. Nevertheless, this success has led to the development of other RAS/RAF pathway inhibitors, for example, for mutated BRAF or downstream kinases like MEK. Alternative activation of RAS/RAF pathway has been proposed as a resistance mechanism [60]. In line with this, the combination of BRAF inhibition with MEK inhibition led to an improved survival of 9.4 months [61].

Other new therapies that add to the therapeutic options for melanoma patients are immunotherapies. An anti-CTLA-4 antibody (ipilimumab) improved survival of stage II and IV melanoma patients (10.1 versus 6.4 months) [62]. Cytotoxic T-lymphocyte Antigen 4 (CTLA-4) inhibits T-cell responses and respectively, CTLA-4 blockade promotes immune responses and antitumor activity. In an early analysis of anti-PD-L1 antibody, a 20% response rate in melanoma was observed. Importantly, these responses lasted for more than 1 year [63]. Similar to CTLA4, PD-1 reduces immune activation, and its inhibition can lead to reactivation of immune responses.
Altogether, even with respect to the recent advances in melanoma therapy, the high resistance rates and the restriction to certain patient subgroups demonstrate that there is still an urgent need to develop alternative therapies.

4. Assets of Nucleic Acid Nanoparticles in Antitumoral Approaches

As also observed for other tumor entities, melanoma treatment with low molecular weight chemotherapeutic drugs often results in the rise of resistant cancers cells, especially in case of relapsed disease. A well-known mechanism of resistance is the elevated expression of multidrug transporter proteins, like p-glycoprotein, which actively pump chemotherapeutics out of the cell [64]. Here, macromolecular approaches can be a suitable approach to overcome such resistance. As an example, the attachment of chemotherapeutics to polymers via reversible covalent bonds helps to overcome this type of resistance (for a recent review see [65]). Also, biotherapeutics, such as antibodies, have been successfully applied in melanoma therapy (see above), but also here resistance can occur, for example, when blocking of one cellular pathway responsible for cancer cell proliferation can be replaced by another [66]. In this case, the application of therapeutically effective nucleic acids comes into play. Firstly, they exhibit a relatively high molecular weight, which prevents resistance mediated by p-glycoprotein upregulation. Secondly, nucleic acids can be designed to affect only malignant cells, for example, by using promoter elements being only activated in tumors, or as RNA oligonucleotides (like siRNA), which will enable the knockdown of a specific protein overexpressed in tumor tissue. Furthermore, the delivery of more than one siRNA targeting different pathways can prevent tumor resistance by blocking different resistance or escape strands. Last but not least, nucleic acid delivery permits systemic delivery of toxic agents, such as diphtheria toxin A [67] or tumor necrosis factor (TNF) [68], as they only become toxic after transcription in the target cell.

Solid tumors exceeding a certain size rely on a functional blood supply for access to nutrients and oxygen. In contrast to nonmalignant tissues, tumor vasculature often exhibits a leaky appearance, which in principle also allows nanosized particles to reach tumor cells [69]. Being packed into nanoparticles or polyplexes, nucleic acids can be protected from nucleases which are present in the bloodstream. Nevertheless, systemic delivery of nanopharmaceutics offers several pitfalls and obstacles, such as aggregation with blood cells, undesired adherence to the vessel wall, or opsonization with plasma proteins followed by clearance through tissue macrophages (a key component of the reticuloendothelial system). Blood proteins interact both with negatively and positively charged nanosystems, whereas a neutral surface charge enables, in principle, blood circulation, as it has been shown for small nanocrystals, so called quantum dots [70]. Alternatively, nanosystems can be decorated with hydrophilic polymers, which, owing to their excessive hydration, shield the particles’ surface charge, hereby preventing the aggregation with protein components. From the group of hydrophilic polymers, like N-(2-hydroxypropyl)methacrylamide (HPMA) [71], hydroxethyl starch (HES) [72], or polyethylene glycol (PEG) [73], PEG is the most commonly used one. In addition, targeting entities can be used to direct the nanocarrier to specific cells. Commonly, these are ligands that bind to receptors, or other cell surface molecules, that are overexpressed in tumor cells.

Macromolecular drugs, which exceed the renal excretion limit and are able to circulate in the blood stream, can benefit from the so-called enhanced permeability and retention (EPR) effect: nanopharmaceutics accumulate in tumor tissue as they can penetrate the leaky vasculature but are retained within the tumor tissue due to incomplete lymphatic drainage [98]. This tumor deposition is a prerequisite for all steps that follow: binding to and internalization of the particles into target cells. The latter can be promoted by the incorporation of the earlier mentioned cell-binding ligands into the carrier system. Figure 2 summarizes the limitations in nucleic acids delivery, the solutions for such limitations, and the therapeutic advantages of nucleic acid nanosystems.

5. On the Footsteps of Metastatic Melanoma: Cell Surface and Transcriptional Targeting

Directed approaches are of special interest as they have the potential to specifically distress malignant cells causing increased local concentrations of the active agent and avoiding undesired side effects. Tracking down melanoma-associated molecular targets involves identifying signaling pathways’ key players, earlier described, as much as cancer cell surface markers. In particular, for gene therapy, cell surface markers are important, and these abide with the conception of a treatment addressing multiple melanoma subgroups—as cells with different mutations can still exhibit common surface markers. Ergo, it is crucial to identify critical and idiosyncratic targets for these cells. Table 1 summarizes the most common melanoma-targeting tools herein described.

<table>
<thead>
<tr>
<th>Targeting tool</th>
<th>Target</th>
<th>Reference</th>
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<tbody>
<tr>
<td>[Nle⁴,dPhe⁷]-α-MSH</td>
<td>MCI-R</td>
<td>[74–85]</td>
</tr>
<tr>
<td>cRGD</td>
<td>αβ₁</td>
<td>[86–90]</td>
</tr>
<tr>
<td>LDV</td>
<td>αvβ₃</td>
<td>[91]</td>
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<tr>
<td>Transferrin</td>
<td>Transferrin receptor</td>
<td>[92]</td>
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<tr>
<td>Tyrosinase</td>
<td>—</td>
<td>[93–95]</td>
</tr>
<tr>
<td>MIA</td>
<td>—</td>
<td>[96, 97]</td>
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Table 1: Common melanoma-targeting tools: ligands for surface cellular targeting and promoters for tissue-specific transcription.
in position four by norleucine (Nle) and used MCR-1 ligand is the synthetic [Nle\textsuperscript{4}, D-Phe\textsuperscript{7}]-\textalpha-MSH or NDP-\textalpha-MSH [75]. The substitution of methionine in position four by norleucine (Nle\textsuperscript{4}) and of phenylalanine for its D-counterpart in position seven (D-Phe\textsuperscript{7}) renders this peptide with higher affinity and resistance to enzyme degradation than its native form. However, NDP-\textalpha-MSH was shown to have a strong nanomolar binding affinity towards MC3-R, MC4-R, and MC5-R [74], and, for gene delivery, it is crucial to decrease off-target effects. Aiming at the design of ligands suitable for micelle conjugation, and with an adequate selectivity to MCI-R, Barkey et al. have conducted a comparative study in which they screened several candidate ligands [74]. This paper allowed the following conclusions: (1) free rotation of carbons that compose the peptide’s binding motif seems to be required for MCI-R avidity; (2) alkyl modifications, for the attachment of triblock polymer micelle, at the N-terminal of the peptide, did not affect binding affinity in the short four amino acid peptide; (3) for peptides twice as long, C-terminal modifications for micelles’ attachment did not alter binding affinities. In addition, the authors have synthesized micelles conjugated to the short peptide version [4-phenylbutyryl-Hist-dPhe-Arg-Trp-Gly-Lys(hex-5ynoyl)-NH\textsubscript{2}], through a PEG linker. And importantly, in vitro cell-uptake studies showed the ability of conjugated micelles to selectively bind to MCI-R receptor, and, whether due to multivalent interactions or other factors, the micelles had higher avidity for the receptor than the ligand alone. Nevertheless, further studies (i.e., by flow cytometry or confocal laser microscopy) to quantify the uptake of these conjugated micelles are needed to better evaluate the delivery efficiency of this platform. More recently, \textalpha-MSH peptide has been conjugated to a nanoplateform based on the heavy chain of the human protein ferritin (HFt) [76]. Ferritin can be used to build a hollow nanocage that can transport materials such as Fe\textsubscript{3}O\textsubscript{4}, Co\textsubscript{3}O\textsubscript{4}, Mn\textsubscript{3}O\textsubscript{4}, Pt, and Au and hence be used for imaging and therapeutic purposes. The targeted ferritin nanocages have been evaluated in vitro and in vivo. Unfortunately, the authors have not analyzed the in vivo distribution of their nanoparticles, and the targeting efficiency was evaluated by immunohistochemistry in the tumor tissue in relation to normal skin. In a similar approach to that of HFt nanocages, Lu and collaborators have used hollow gold nanospheres, conjugated to NDP-\textalpha-MSH, aiming at cancer photothermal ablation [77]. In this study, nude mice were subcutaneously
inoculated with B16/F10 murine melanoma cells, and the nanoparticles were administered intravenously. The authors have collected different organs and were able to show the targeting effect by the NDP-α-MSH-gold nanospheres.

Interestingly, targeting of MC1-R by α-MSH peptide has been mostly used in radionuclide therapy studies and for diagnostic purposes. Currently, 2-[18F]fluoro-2-deoxy-d-glucose ([18F-FDG]) is the only radioactive probe used in the clinic to detect melanoma. Be that as it may, [18F-FDG] is an unspcific positron emission tomography (PET) imaging agent with poor sensitivity towards micrometastatic sites [78, 79], a fact that underlines the general insufficiency in melanoma targeting.

Regarding MC1-R targeting, Yubin Miao and Thomas P. Quinn's extensive work is of particular interest, reporting on two generations of an NDP-α-MSH-based peptide used for melanoma imaging by single-photon emission-computed tomography (PECT) and more recently by PET. What distinguishes the two α-MSH peptide generations is mostly the peptide's length, being twelve aminoacid-long in the first generation (CycMSH) [80–82] and six in the second (CycMSHhex) [83, 84]. In both generations, the peptide is cyclized (Cyc), and the MCI-R binding motif (His-pPhe-Arg-Trp) is conserved. The peptides have also undergone structural modifications concerning the aminoacid linkers, which are used to support the peptide cyclization and bridge the targeting ligand and the radiometal chelator. Interestingly, the authors have observed that the exchange of single aminoacids in these linkers [85], and the introduction of—GlyGly—linker between the chelator and the peptide [84] resulted in improved melanoma targeting, with decreased renal excretion and liver uptake of the radiolabelled peptide in B16/F1 melanoma-bearing C57 mice. These studies underscore the structural role of the targeting moiety but also of the integral component being delivered. In other words, the addition of a targeting entity to a carrier does not necessarily suffice for efficient deliver; the number of peptides conjugated to the delivery platform, the site of conjugation and the size and type of the linker play an important role.

Integrin targeting has also been extensively explored for cancer gene delivery in general. After the discovery of adhesion molecules as mediators of tumor metastasis, the identification of their binding motifs opened the possibilities for targeted therapies. Several peptide fragments have been employed to target these mediators, either as antagonists or as ligands for drug delivery purposes. One of the utmost targeted integrin is the αvβ3. αvβ3 plays a central role in angiogenesis—the formation of new vessels—and, by serving as receptor for extracellular matrix proteins, it mediates migration of endothelial cells into the basement membrane, and regulates their growth, survival, and differentiation. It is therefore no surprise that such integrin is found upregulated in different tumor cells, where it is involved in processes that govern metastasis. The integrin's binding peptide motif has been identified in 1990 [121]—Arginine-Glutamine-Aspartate or RGD—but studies that followed have shown that the cyclic version of RGD (cRGD) has higher binding affinities towards the integrin [86, 87]. Either alone or in combination with other ligands, cRGD has been conjugated to several nanocarriers for both diagnostic and therapeutic purposes [88–90].

Another integrin reported to have a dominant function in the metastatic spread is αvβ1, or VLA-4. Okumura, and more recently Schlesinger, have shown, in different settings, that inhibition of VLA-4 by natalizumab (an antibody against αv integrin) significantly decreased melanoma lung metastases in murine models [42, 44, 122]. In 1991, Makarem and Humphries have identified the Leucine-Aspartate-Valine (LDV) sequence as the integrin's motif [123], and a few years later, Vanderslice et al. have reported on a series of cyclized peptides based on LDV that were assayed for the inhibition of the integrin [124]. However, and despite the numerous reports relating this agent to tumor metastasis, and to melanoma in particular, most of the literature relies on the LDV sequence as an antagonist, rather than for deliver purposes, where, to our knowledge, there is only one paper reporting on in vitro studies [91]. Indeed, VLA-4 is found in multiple leukocyte populations; VLA-4 is a vital receptor of leukocytes, and it is involved in the immune response. Hence, a systemic application of VLA-4 inhibitors, or binding peptides, could induce undesired partially immunosuppressive effects. In this context, the application of transcriptional-targeting strategies could potentially prevent off-target effects and prove this ligand a promising tool. In fact, tissue-specific elements as components of the DNA vector can provide a tight control over gene expression and complement and strengthen targeted-delivery. Commonly, tumor cells' surface markers entail receptors that are also present in nontumor cells but are rather overexpressed in their malignant form. This is the case for both the integrins here described, but also the transferrin receptor [92]—all used as melanoma targets. Therefore, off-target effects can occur, and for gene delivery purposes, tissue-specific control elements are an elegant way to bypass undesired side effects. These control elements consist of nucleic acid sequences that are recognized by proteins or other nucleic acids, which hereby regulate gene expression. For the case of melanoma, tissue specific promoters have been described, and these include tyrosinase [93–95] and melanoma inhibitory activity (MIA) [96, 97]. Gene expression is hence to be accomplished in tissues where such promoters are activated.

MicroRNA (miR) binding sites can also serve as transcriptional control elements. MicroRNAs are a class of short (20–22 nucleotides long) regulatory RNAs, which are believed to regulate as many as 30% of all genes. Several microRNAs are tissue-specific and fine-tune genetic circuits, some of which are critical for normal development, cellular differentiation, and normal cellular homeostasis. If the target sequence and microRNA have perfect complementarity, the miRNA is eliminated by a RNA degradation pathway. In the context of transcriptional control, this means that a DNA vector that contains specific miR-binding sites is only translated in cells where the miR in question is absent [125, 126]. In tumor cells, several microRNAs are deregulated, while miRs enrolled in cell homeostasis are downregulated those involved in cell proliferation and differentiation are upregulated [127]. For the case of melanoma, miR let-7b,
miR-193b, miR-34a, miR-155, miR-205, miR148, miR-137, and miR-152 have been found downregulated (for a review on melanoma microRNAs, see [127]) and can therefore be suitable targets for transcriptional regulation when expressed in normal tissue.

6. Therapeutic Nucleic Acids in Melanoma

As opposed to conventional therapy, traditionally, that is, in the case of loss of function, gene therapy aims at permanent correction of a defective or missing gene by replacing with or providing, respectively, the corrected version—for example, by the introduction of plasmid DNA (pDNA). Ideally, this approach translates into a single treatment, or few initial treatments, rather than several (or life long) required to provide the patients with the functional form of the protein. However, this permanent correction treatment has proven very challenging.

In the last twenty years, new nucleic acids with attractive therapeutic properties were discovered, notably, siRNA and microRNAs. Small interference RNA (siRNA) has the ability to specifically silence protein expression—an asset particularly valuable for antiviral and cancer regimens. In general, also miRNA negatively regulates gene expression, although via two different mechanism depending on the degree of complementarity towards its mRNA target. Nucleic acid-based approaches offer several advantages when compared to treatment with small molecules or proteins. They can be seen as mostly inactive prodrugs, which are activated at the tumor site producing a therapeutically active protein or knocking down a specific target gene. Importantly, nucleic acid targeted delivery systems, preferably also relying in transfectional targeting, decreasing off-target effects and toxicity, and permitting a systemic administration otherwise not feasible with a therapeutic agent with toxic properties.

In parallel with new therapeutic nucleic acid tools, the last two decades brought insight into tumorigenesis in general and unveiled a plethora of therapeutic concepts against cancer (Figure 3). The following paragraphs will deal with different antimelanoma approaches based on nucleic acids.

Despite the apparent tumor tolerance, humoral and cellular immune responses are naturally generated against tumor antigens. Hence, whether the tumor grows as a result of stealth and nonrecognition or as the result of escape and immunological shaping [128], its recognition by the immune system can still be prompted. Indeed, at a later stage, during the progressive growth phase, tumors may become more immune-activating for various reasons: damage or disruption of surrounding tissue, generation of reactive oxygen species, upregulation of stress protective factors, or death by necrosis or apoptosis. However, at this stage, it is not known whether the tumor still needs to escape immune recognition, as it is unclear that these immune responses can cause tumor destruction [128]. Therefore, a number of studies have focused in eliciting earlier and suitable tumor recognition by the immune system. In a nucleic acid therapy context, this transliterates into genetic immunization or DNA vaccination: the delivery and transcription of a gene encoding antigens or immunestimulatory molecules that elicit an immune response. As an example, interleukine-12 (IL-12) has been used and studied in different animal models [104, 105]. IL-12 is originally produced by mononuclear phagocytes and dendritic cells and is responsible for activating NK and CD4+ T cells and inducing the production of high levels of interferon gamma (INF-γ). Interestingly, IL-12 has been described to increase antitumor immune responses [129, 130], and later studies investigated its suitability for a DNA vaccine approach against melanoma [106]. IL-12 effects appeared to be long lasting and efficient against tumor metastases, although not mainly mediated by INF-γ [106]. The murine studies also revealed moderate toxicity caused by IL-12, and while lower IL-12-encoding pDNA doses can be administered, ideally the gene expression should be controlled, regarding the tissue and the durability of the expression. Although DNA vaccination against a strong melanoma tumor antigen should be possible, the authors have not seen an effect on lung metastases when using melanoma-associated glycoprotein 100 (gp 100)/pmel17 pDNA alone. Adjuvants appear to be necessary for a successful DNA vaccination: the authors have seen an effect when the gp 100-pDNA was administered together with IL-12, similar to other murine study where granulocyte-macrophage colony-stimulating factor was used [107]. Alternatively, in a canine study, the developed vaccine was based on the human (rather than canine) gp 100 protein [108], where the human form of the antigen acted as adjuvant. Together with gp 100, and for the case of melanoma, two more tumor genes have been described for DNA vaccination: MART-1 and tyrosinase [108, 109].

Also, the expression of chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and interferon-inducible protein-10 (IP-10), can mediate an immune response. In particular, IP-10 as been described by Sgadari et al. as an antitumor agent and found to promote damage in established tumor vasculature as well as tissue necrosis in a murine model for the human Burkitt lymphomas [131]. Based on this, and after their studies with IL-12, Keyser and collaborators have investigated the efficiency of IP-10-encoding pDNA therapy in murine melanoma models [110]. The authors have used two murine tumor models, whereupon cells have been injected subcutaneously (originating a solid tumor) or intravenously, inducing lung metastases. When administered alone, and intramuscularly (resulting in systemic circulation), IP-10-encoding pDNA showed an antimitastatic effect, reducing the number of lung metastases as compared to the control-pDNA treated group. When administered with IL-12-encoding pDNA, IP-10 pDNA enhanced the IL-12 effect, and decreased its earlier observed toxicity. This anti-neoplastic effect of IP-10 has been attributed to the engagement of NK cells and the inhibition of angiogenesis and cell proliferation.

Alternative antitumor strategies aim at a direct destruction of cancer cells, through the delivery of pDNA encoding for a toxic protein—DNA-based strategies. This is referred to as a suicide gene therapy or gene-directed enzyme prodrug therapy (GDEPT), when the nucleic acid sequence encodes for an enzyme, which is not directly toxic but instead converts a nontoxic prodrug into a cytotoxic metabolite. The first proof of principle of GDEPT was presented in the mid-eighties and
Figure 3: Different strategies used in antitumor nucleic acid approaches. RNA-based strategies are commonly used to downregulate agents that are upregulated to favor cell proliferation or migration, such as Bcl-2. Alternatively, double stranded RNA (dsRNA) mimic polyinosinic-polycytidylic acid (pIC) can be used to engage the endosomal machinery, resulting in autophagy and apoptosis. Conversely, pDNA delivery aims at the expression of a protein that can (1) have toxic properties, directly causing tumor cell apoptosis (pDNA-based approaches); (2) be a chemokine, thus recruiting cell-mediated immunity; or (3) be a tumor antigen, recruiting humoral immunity (DNA vaccination-based strategies). Ultimately, all strategies aim at putting an end to tumor progression and eventually tumor cell destruction.

involved the herpes simplex thymidine kinase (HSV-tk) and the prodrug ganciclovir (GCV) [132]. Presently, HSV-tk as well as other approaches, such as Diptheria toxin A chain (DTA), have been employed in the clinics, the most successful cases being reported in ovarian and prostate cancers [67, 133]. As for melanoma treatments, HSV-tk has been the most commonly used [111–113], although there is no human clinical trial yet. Suicide gene therapy has also been proven effective when used in combined approaches, such as with cytokine-enhanced vaccine in a clinical trial involving canine melanoma patients [134]. Despite promising, this strategy is currently restrained by a poor delivery; most nanocarriers are not as target-specific and efficient as required, and the toxic gene does not reach the tumor cells in efficacious concentrations.

A number of studies have instead focused on mediators of cell proliferation and differentiation, which are upregulated during tumorigenesis, aiming at their downregulation by means of siRNA delivery [114, 135–137]—these are RNA-based approaches. As an example, based on the fact that in epithelial cells, N-cadherin induces changes in morphology of a fibroblastic phenotype (rendering the cells more motile and invasive), the laboratory of Laidler has investigated the outcome of N-cadherin silencing in human melanoma cell lines [114]. Although the results suggest that N-cadherin positively affects the regulation of the cell cycle and proliferation through activation of the AKT kinase pathway, further investigations are needed to describe the mechanism. Similarly, Villares et al., upon the observation that thrombin receptor (or protease-activated receptor-1, PAR-1) is overexpressed in highly metastatic melanoma cell lines, has evaluated the therapeutic potential of siRNA against PAR-1 [115]. The authors have observed a significant reduction of in vivo tumor growth as well as in the number of metastatic lung colonies. This report showed that downregulation of PAR-1 decreased the expression of matrix metallopeptidase-2 (MMP-2), interleukin 8 (IL-8), and vascular endothelial growth factor (VEGF), resulting in an overall decrease in angiogenesis and blood vessels. In 2010, Davis et al. reported on the first human clinical trial (including three melanoma patients) on siRNA therapy against melanoma [92]. The siRNA targeted the M2 subunit of ribonucleotide reductase (RRM2), and the protein knock down was confirmed at the mRNA level but not corroborated to the same extend by the protein analysis. Nevertheless, the fact that the authors used a delivery vector targeting the transferrin receptor without showing analysis of such receptor expression in melanoma cells was left to be explained [138].

Of special interests are combinatorial strategies involving siRNA delivery as these, similar to other combinatorial therapies, cause the most significant outcomes. Particularly, Poeck and coauthors have used a simple and elegant siRNA design [116]. The authors targeted Bcl2 (an apoptosis regulator protein), which was reported to play a central
role in the resistance of melanoma cells to chemotherapy [7, 116, 139, 140]. By adding 5'-triphosphate ends to their siRNA, the authors also activated innate immune cells, induced the expression of interferons, and caused specific cell tumor apoptosis. These actions are a consequence of the recognition of 5'-triphosphate ends by the cytosolic retinoic acid-induced protein-1 (Rig-1) and synergized with the silencing effects originated from siRNA resulting in massive tumor destruction in the murine lung metastases. Two years earlier, aiming at RNA-based vaccination, Tormo et al. first reported on a promising double stranded RNA (dsRNA) mimic polyynisine-polycytidylic acid (pIC) [117]. Importantly, the therapeutic effect of the dsRNA was significantly increased when delivered in the form of a complex, together with polyethyleneimine (PEI)-[pIC]PEI. Initially, the dsRNA mimic was thought to engage toll-like receptors (TLR), hereby mediating cellular tumor immunity [117]. In turn, further investigation studies showed that it mobilizes the endo/lysosomal machinery of melanoma cells, and through melanoma differentiation associated gene-5 (MDA-5) induces self-degradation by (macro) autophagy and apoptosis, following the MDA-5-mediated activation of proapoptotic factor NOXA [118]. Interestingly, at the exact same time, MDA-5 and NOXA were also reported to play a role in interferon-independent apoptosis in human melanoma cells by Besch and collaborators [141]. Not only were these findings meaningful, opening new windows for cancer therapy, but also, in particular in the Dam´ıa Tormo studies, was the murine model used very suited, whereupon mice overexpressing hepatocyte growth factor (HGF) and studies, was the murine model used very suited, whereupon mice overexpressing hepatocyte growth factor (HGF) and carrying an oncogenic mutation in the cyclin-dependent kinase-4 ([CDK4]R24C) developed invasive melanomas in the skin following neonatal exposure to carcinogens.

While a number of microRNA has been described to play relevant roles in melanoma progression [127], only few in vitro studies have reported on the miRNA potential for antimelanoma therapy [119, 120]. However, pertinent therapeutic approaches targeting miRNAs described for other tumor types [142, 143] foretell the potential and the therapeutic window opportunities entailing these nucleic acids in metastatic melanoma.

As an overview of this section, Table 2 presents the therapeutic nucleic acids herein described, and Figure 3 schematically summarizes the different strategies in nucleic acid therapies.

### 7. Conclusions and Future Perspectives

It is of general consensus that the last decade of cancer research significantly expanded our knowledge in tumor development and progression. Unfortunately—similar to the tumor escape shaped by the immune surveillance in an early growth phase—as new therapeutic strategies are applied, tumor cells undergo another round of selection, giving rise to therapy-resistant cells. It is therefore necessary to combine several approaches to attack different paths of tumor escape—a fact that is confirmed by the most significant results reported in studies where such strategies have been used.

<table>
<thead>
<tr>
<th>Therapeutic/silenced/ upregulated gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12</td>
<td>[104–106]</td>
</tr>
<tr>
<td>gp100</td>
<td>[107, 108]</td>
</tr>
</tbody>
</table>

**DNA-based approaches**

| MART-1                               | [108]     |
| Tyrrosinase                           | [109]     |
| IP-10                                | [110]     |
| HSV-tk                               | [111–113]|

**RNA-based approaches**

| RRM2                                 | [92]      |
| Bcl2                                 | [116]     |
| dsRNA pIC                            | [117, 118]|

**miR**

| Let-7b and miR 199a                  | [119, 120]|

On this note, nucleic acids deliveries are truly advantageous tools as they allow the systemic delivery of potentially toxic molecules that can be combined with chemotherapy aiming at terminating possible resistant-tumor cells. As an example, recently, Su and collaborators have reported on an antitumor strategy combining TNF-encoding pDNA and chemotherapy [68]. While systemically administered TNF is extremely toxic, in its genetic form, and when reaching specific target cells, TNF revealed to be a powerful antitumor agent. Specific and efficient are indeed key words in this type of targeted approaches, as in suicide gene delivery. It is thus of extreme importance to thoroughly evaluate the target options and to verify the levels of the target molecule in the cells of interest. The activation of possible target-receptors may be desired, such as in the case reported by Poeck et al. [116], but only when not hampering the therapeutic effect by activation of pathways that can lead to cell proliferation/differentiation, enhanced cell migration, or inhibition of apoptosis. As described by Schäfer et al., this can be the case when targeting the epidermal growth factor receptor (EGFR), and it is then desirable to design a ligand that targets the receptor circumventing its activation [144]. On the other hand, the relevance of analyzing the targeted receptor has been well exposed in the short letter of Perris in response to the work published by Davis et al. [138]. To avoid other pitfalls in nanovector development, also the in vivo distribution needs to be assessed, preferably by several approaches (e.g., bioluminescence imaging, positron emission tomography (PET), and magnetic resonance imaging (MRI)). To this end, immunohistochemistry studies may be suitable and very
convenient to corroborate and support data collected by different means, but also microscopy (mostly in vitro but also histochemistry analysis) has had its traps [145].

In summary, already a number of promising nucleic acid strategies exist, and these certainly present less hurdles for delivery than their protein counterpart, as they are smaller, less antigenic, and can bypass certain resistance mechanisms. Nevertheless, further improvements in nonviral targeted delivery appear required to increase the efficacy of such therapies. A small final note regarding the potential of miRNA approaches: microRNA therapies can aim at (1) miRNA upregulation, when the target nucleic acid is enrolled in cell homeostasis and is found silenced in tumor cells; (2) miRNA downregulation by anti-miRs, when it is upregulated in tumor cells due to its play in cell proliferation; (3) alternatively, miRNA can also have a role in cell-specific transcription in pDNA vectors containing miRNA binding-sites, allowing the expression of the gene of interest in cells, where the miRNA is silenced. All these assets make miRNA undoubtedly a very elegant and flexible tool.

Conflict of Interests

The authors state no conflict of interests.

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Review Article

Clinical Trials with Pegylated Liposomal Doxorubicin in the Treatment of Ovarian Cancer

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Among the pharmaceutical options available for treatment of ovarian cancer, increasing attention has been progressively focused on pegylated liposomal doxorubicin (PLD), whose unique formulation prolongs the persistence of the drug in the circulation and potentiates intratumor accumulation. Pegylated liposomal doxorubicin (PLD) has become a major component in the routine management of epithelial ovarian cancer. In 1999 it was first approved for platinum-refractory ovarian cancer and then received full approval for platinum-sensitive recurrent disease in 2005. PLD remains an important therapeutic tool in the management of recurrent ovarian cancer in 2012. Recent interest in PLD/carboplatin combination therapy has been the object of phase III trials in platinum-sensitive and chemonaive ovarian cancer patients reporting response rates, progressive-free survival, and overall survival similar to other platinum-based combinations, but with a more favorable toxicity profile and convenient dosing schedule. This paper summarizes data clarifying the role of pegylated liposomal doxorubicin (PLD) in ovarian cancer, as well as researches focusing on adding novel targeted drugs to this cytotoxic agent.

1. Introduction

Ovarian cancer (OvCa) is the leading cause of death from gynaecological malignancies with an estimated 65697 new cases and 41448 deaths every year in Europe [1]. Approximately 15% of women present with disease localized in the ovaries and in this group surgery allows a 5-year survival in more than 90% of the cases. However, the majority of women present at the diagnosis with advanced disease (International Federation of Gynaecological Oncology (FIGO) stage III-IV) and their survival at 5 years is poor, currently less than 30% [2].

The main reasons for the high mortality rate are the lack of symptoms accompanying this tumor, in addition to the lack of an effective screening strategy for the overall population, and, lastly, the limited results obtained with standard medical treatments.

The standard of care for the management of OvCa patients includes surgery for staging and optimal cytoreduction (no residual tumour) followed by a platinum/taxane chemotherapy combination [3, 4]. Recently bevacizumab has been approved in stage IIIB-IV cancer in combination and as a single-agent maintenance after carboplatin-paclitaxel [5, 6]. Although chemotherapy obtains high objective response rates even in patients with an advanced tumor stage, the vast majority of patients will experience tumor progression and require further therapy [7, 8].

Many strategies have been implemented in order to improve these unsatisfactory results and new drugs have been investigated.

In this context, among the pharmaceutical options currently available for medical treatment of ovarian cancer (OvCa), greater emphasis has been placed progressively on pegylated liposomal doxorubicin (PLD) (Doxil in the USA; Caelyx in Canada and Europe), which was approved in 1999 by the FDA and in 2000 by the European Medicines Evaluation Agency (EMA) as single agent for treatment of advanced OvCa patients failing first-line platinum-based treatment. Moreover, phase III trials have been already conducted and results suggest further role for PLD in salvage setting and in
front-line treatment in combination with other therapeutic drugs. The aim of this paper is to summarize data showing the role of pegylated liposomal doxorubicin (PLD) in the management of epithelial ovarian cancer.

2. Pegylated Liposomal Doxorubicin (PLD): Development, Structure and Pharmacokinetic Features

Anthracyclines have been for years among the drugs administered for the majority of gynecologic cancers. Before taxanes were introduced into first-line therapy of ovarian cancer, anthracyclines demonstrated a comparable efficacy, in monochemotherapy, with alkylating agents and superiority of the combination of both when compared to single-agent therapy. Furthermore, meta-analysis data suggest that the addition of anthracyclines to cisplatin might be advantageous compared to using cisplatin alone [9, 10].

Attempts have been made to introduce anthracyclines in combination with carboplatin-paclitaxel. In the randomized trial, conducted by the AGO group in collaboration with the French group GINECO, the addition of epirubicin (TEC arm) to the platinum/paclitaxel (TC arm) combination in first-line ovarian cancer treatment patients showed a not statistically significant advantage of about 5 months in median overall survival time (45.8 versus 41.0 months, HR 0.93) [11], with no progression-free survival benefit (18.4 versus 17.9 months, HR 0.95) at the price of a greater toxicity of TEC versus TC arm (grade 3/4 hematologic, nausea/emesis, mucositis, and infections). Despite the antitumor activity in ovarian cancer, the clinical use of conventional anthracyclines is limited by their associated side effects. The haematological toxicity and the cumulative and irreversible cardiac damage (congestive heart failure) are the more common side effects, dose limiting, of anthracyclines. As far as it is elucidated, cardiotoxic events take place by increasing oxidative stress, suppression of gene expression, and induction of apoptosis on cardiac tissue [12] with clinical manifestations reaching from acute cardiac heart failure to chronic cardiac insufficiency. Several treatment strategies, including the development of new formulations for delivering the cytotoxic agents (as liposomes encapsulation), have been proposed to improve the therapeutic index of anthracyclines [13]. The inclusion of anthracyclines in a liposomal structure has been proposed to reduce side effects and to enhance the antitumor activity. In this paper, we will focus on the pharmacologic properties of pegylated liposomal doxorubicin (PLD), a new available formulation of doxorubicin that is encapsulated in a pegylated liposome [14, 15]. The size of the liposomes, approximately 100 nm, prevents them from entering tissues with tight capillary junctions, such as the heart and gastrointestinal tract [16]. In contrast to other nanoparticles, the liposomal shell is surrounded by a polyethylene glycol (PEG) layer which represents a hydrophilic protective barrier between the liposome and the microenvironment, thus preventing the activation of the reticuloendothelial system, that leads to the destruction of the liposomal structure and release of the free drug. Liposomal drug delivery to cancer cells can occur in vivo by two different pathways: passive and active targeting. In contrast to normal vessels, the vessels of the tumor are tortuous, dilated, have morphologically abnormal endothelial cells, and are leaky due to large spaces between pericytes [17]. These physical characteristics allow more extravasation of the liposomes into the tumor, with higher cell concentration of the drug. The lack of functional lymphatic drainage in tumours prevents the outflow of extravasated liposomes, allowing doxorubicin accumulation in the tumour extracellular fluid. These liposomes will gradually release the entrapped drug in the vicinity of tumour cells, thus increasing the tumour-drug exposure [18]. This mechanism of passive targeting is known as “enhanced permeability and retention (EPR) effect” [19].

The efficacy and safety of PLD has been evaluated in a variety of different tumor models, including several human xenograft models supporting its introduction in cancer treatment [15]. In every model examined, PLD was more effective than the same dose of free doxorubicin in inhibiting or halting tumor growth, in preventing metastasis, and/or in prolonging survival of the tumor-bearing animals [20, 21]. The pharmacokinetic and tissue distribution studies in these models suggest that the greater persistence, particularly in tumor tissue, achieved with PLD compared with conventional doxorubicin offers a therapeutic advantage. PLD has well-known pharmacokinetic features, such as long circulation time, minimal (<5%) drug leakage from circulating liposomes, and half-lives of approximately 60–90 h for doses in the range of 35–70 mg/m² in patients with solid tumors [21]. This translates into a PLD AUC approximately 250–1000-fold higher than that of the free drug in humans [22]. PLD pharmacokinetics is best modeled as a one-compartment model displaying linear pharmacokinetics with C-max increasing proportionally with dose [23]. It has also been described as a two-compartment model with an initial half-life of several hours, followed by a more prolonged terminal decline with a half-life of 2-3 days, accounting for the majority of the AUC [22, 24]. After PLD administration, nearly 100% of the drug in the plasma is in the encapsulated form. Moreover, compared to free doxorubicin, PLD plasma clearance is dramatically slower, and its volume of distribution is very small and roughly equivalent to the intravascular volume [22, 24].

These properties, which represent the rational basis for the exploitation of nanoparticle technology, represent the major advantages of PLD compared to conventional doxorubicin in safety profile (lower cardiotoxicity and gastrointestinal toxicity compared to the free drug) [20–25].

Based on the previous evidences regarding the role of anthracyclines and the modified toxicity profile of PLD, this agent has been a rational choice for further evaluation as a single-agent and in combination with platinum agents in the treatment of ovarian cancer.

3. Pegylated Liposomal Doxorubicin: Activity in Ovarian Cancer

3.1. Phase II Studies with PLD as a Single-Agent or in Combination. The initial studies evaluating PLD have been conducted in recurrent ovarian cancer, as a single-agent monotherapy or
in combination with platinum (carboplatin) and later on with trabectedin or other new drugs.

A summary of phase II studies using PLD as a single agent or in combination regimens in ovarian cancer is presented in Table 1 [26–35].

Nonrandomized phase II trials of PLD in platinum-resistant ovarian cancer patients documented the biological activity of this agent in this clinical setting, with objective response rates of approximately 10–20% being reported in several trials [18, 25, 31]. Data indicated that palmar-plantar erythrodysesthesia (PPE; hand-foot syndrome, toxic acral erythema) and mucositis were the most common toxicities of PLD, reported in up to 50% of treated patients. PPE usually occurs after two or more courses of treatment and the risk of incidence increases with multiple repeated treatments. PPE is related to dose intensity and dose interval rather than to peak dose level. Although not life threatening, PPE can negatively impact the quality of life, and it is a major cause of both dose reduction and treatment discontinuation [61, 62]. As regards the cardiac toxicity, in several trials PLD formulation has been related to a better safety profile compared to conventional doxorubicin [63]. Compared to the 7.5% incidence of irreversible cardiotoxicity at cumulative doses of 400–550 mg/m² reported with doxorubicin [64], most of the studies of PLD showed a lower incidence of cardiac failure even at doses higher than 500 mg/m² [65, 66]. In a prospective trial performed on patients with advanced gynecological malignancies treated with PLD, the cardiac safety was further assessed at histology (endomyocardial biopsies), showing no myocardial damage in patients treated with PLD (median PLD dose of 708 mg/m²) [67]. Thus, the optimal cardiac safety profile of PLD may allow a prolonged treatment; encouraging results from a phase II trial in AIDS-related Kaposi’s sarcoma patients treated with PLD up to a 2360 mg/m² cumulative dose have been reported [68]. In metastatic breast cancer patients also doses greater than 450 mg/m² were not associated with a significant decrease in LVEF from baseline compared to conventional doxorubicin [69]. In relapsed ovarian cancer patient responding to second-line chemotherapy, a maintenance therapy with PLD for more than 1 year has been reported to be safe by Andreopoulou et al., with no cardiac event reported [70].

Different schedules and doses have been investigated in an effort to improve tolerability while maintaining antitumor efficacy [28, 35, 36, 71]. Several studies have shown that a more acceptable toxicity profile, in terms of decreased rates of hand-foot syndrome and stomatitis/mucositis, can be obtained with a PLD dose of 40 mg/m² every 28 days compared to the traditional dose of 50 mg/m², with comparable response rates and outcomes [26, 32, 33]. According to the studies published, the optimal dose intensity appears to range from 10 mg/m² to 12.5 mg/m² per week (given at doses of 40–50 mg/m² every 4 weeks) when used as a single-agent therapy.

The results obtained with a single-agent PLD in the subgroup of platinum-resistant patients were the basis for the development of PLD/platinum (cis-, carbo-, oxaliplatin) combinations.

The trials that evaluated the combination regimen of cisplatin or carboplatin with PLD showed an overall response rate ranging from 46 to 68% according to the platinum-free interval. In the Rapoport trial, the overall response rates were about 65% in a population including platinum-sensitive (81%) and partially sensitive patients (52.6%) [38].

Cisplatin combination regimen (PLD at 50 mg/m² dosage, plus cisplatin at 60 mg/m² q 1 q 28 days) was also developed showing a moderate tolerability profile (10% grade 2 neurotoxicity, 18% grade 3/4 anemia, 41% neutropenia, and 9% hand-foot syndrome) [34]. Due to these results, the PLD/carboplatin combination was considered more manageable due to the lower neurotoxicity [37–39, 72–74].

In two phase I-II trials PLD has been associated with carboplatin AUC 5–6 in sensitive or partially sensitive (>50%) ovarian or other gynecological cancer patients. In both studies, data of ORR (62 and 68%, resp.), PFS (9.2 and 11.6 months), and median overall survival (OS 23.4 and 32 months) substantially overlap [37, 39].

Based on toxicity results, the authors recommended a PLD dose of 40 mg/m² when given in combination with carboplatin AUC 5, both drugs administered on a 4-week schedule in epithelial ovarian or endometrial carcinoma.

Gemcitabine is another drug studied in combination with PLD. In several trials (PLD 30 mg/m²–gemcitabine 1000 mg/m² days 1–8 every 21 days) this combination has been associated with overall response rates of about 30–35% in the overall population (21–25% in platinum-resistant and 50–53% in platinum-sensitive diseases), with an acceptable toxicity profile. Myelosuppression was the most common toxicity and was found in 35% of patients [41, 42].

Combinations of PLD with oxaliplatin (OXA) have been also reported, with response rates that appear in the range of those reported with PLD/carboplatin. In these trials a very acceptable rate of stomatitis/mucositis and hand-foot syndrome has been shown, likely due to the use of the PLD at the dosage of 30 mg/m², every 21 or 28 days.

Nicoletto et al. [40] published a trial of pegylated liposomal doxorubicin, dosed between 30 and 35 mg/m² with oxaliplatin at 70 mg/m² every 28 days. The overall response rate was 54% with a median survival of 22.5 months. When evaluated according to platinum sensitivity, there was a response rate of 66.7% among the 29 platinum-sensitive patients and of 28.6% in the 14 platinum-resistant patients. There were 5 (12%) grade 3 or 4 toxicities and only 3 patients (7%) required dose reduction. Neutropenia was the treatment limiting toxicity.

Some phase II studies explored the efficacy of PLD associated with topotecan (TPT) [43], as well as paclitaxel (PTX) [44], vinorelbine (VNR) [45], and ifosfamide (IFO) [46]. Overall, response rates of about 28% to 37% with a median PFS of 5.5 to 7.5 months were found, figures which are quite comparable to those reported with other nonplatinum combinations. The association with weekly paclitaxel was well tolerated, as was the PLD/VNR combination [45]. In contrast, PLD/TPT, even if tested at different doses of the two drugs, was characterized by an unacceptable rate of severe anemia (48%), leukopenia (70%), and thrombocytopenia (44%) [43].
Table 1: Phase-II studies with pegylated liposomal doxorubicin (PLD) as a single agent or in combination regimens.

<table>
<thead>
<tr>
<th>Author</th>
<th>Dose/schedule</th>
<th>Clinical setting PFI (mts)</th>
<th>No. pts</th>
<th>RR (%)</th>
<th>PFS (median) (mts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muggia et al. [25]</td>
<td>50 mg/m², q21</td>
<td>≤6</td>
<td>35</td>
<td>25.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Gordon et al. [18]</td>
<td>50 mg/m², q21</td>
<td>ALL</td>
<td>89</td>
<td>16.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Rose et al. [26]</td>
<td>50 mg/m², q28</td>
<td>≤6</td>
<td>37</td>
<td>13.5</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>40 mg/m², q28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Katsumata et al. [28]</td>
<td>50 mg/m², q28</td>
<td>≤6</td>
<td>63</td>
<td>20.9</td>
<td>5.6</td>
</tr>
<tr>
<td>Markman et al. [31]</td>
<td>40 mg/m², q28</td>
<td>≤6</td>
<td>44</td>
<td>9.1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lorusso et al. [35]</td>
<td>35 mg/m², q21</td>
<td>≤6</td>
<td>17</td>
<td>18.9</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥6</td>
<td>20</td>
<td>10.0</td>
<td>—</td>
</tr>
<tr>
<td>Schoulli et al. [36]</td>
<td>20 mg/m², q15</td>
<td>ALL</td>
<td>64</td>
<td>10.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Du Bois et al. [37]</td>
<td>PLD (40 mg/m²) d1, CBDCA (AUC 6) d1, q28</td>
<td>≥6</td>
<td>67</td>
<td>68</td>
<td>11.6</td>
</tr>
<tr>
<td>Rapoport et al. [38]</td>
<td>PLD (50 mg/m²) d1, CBDCA (AUC 5) d1, q28</td>
<td>ALL</td>
<td>40</td>
<td>67.5</td>
<td>11.9</td>
</tr>
<tr>
<td>Ferrero et al. [39]</td>
<td>PLD (30 mg/m²) d1, CBDCA (AUC 5) d1, q28</td>
<td>7–12</td>
<td>19</td>
<td>52.6</td>
<td>9.7</td>
</tr>
<tr>
<td>Nicoletto et al. [40]</td>
<td>PLD (30 mg/m²) d1, OXA (70 mg/m²) d1, q28</td>
<td>≤6</td>
<td>14</td>
<td>28.6</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥6</td>
<td>29</td>
<td>66.7</td>
<td>9.9</td>
</tr>
<tr>
<td>D'Agostino et al. [41]</td>
<td>PLD (30 mg/m²), d1, GEM (1000 mg/m²), d1, 8 q21</td>
<td>≤12</td>
<td>36</td>
<td>25.0</td>
<td>—</td>
</tr>
<tr>
<td>Ferrandina et al. [42]</td>
<td>PLD (30 mg/m²), d1, GEM (1000 mg/m²), d1, 8 q21</td>
<td>≥12</td>
<td>31</td>
<td>45.2</td>
<td>—</td>
</tr>
<tr>
<td>Verhaar-Langereis et al. [43]</td>
<td>PLD (30 mg/m²) d1/TPT (1.0 mg/m²) d1–5 q21 and PLD (40 mg/m²), d1 TPT (0.75 mg/m²), d1–5 q21</td>
<td>≤12</td>
<td>27</td>
<td>28.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Campos et al. [44]</td>
<td>PLD (30 mg/m²), d1, q21 PTX (70 mg/m²), weekly</td>
<td>≤12</td>
<td>37</td>
<td>29.0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥12</td>
<td>24</td>
<td>17.0</td>
<td>—</td>
</tr>
<tr>
<td>Katsaros et al. [45]</td>
<td>PLD (30 mg/m²), d1, vinorelbine (30 mg/m²), d1, q21</td>
<td>ALL</td>
<td>30</td>
<td>37.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Joly et al. [46]</td>
<td>PLD (40 mg/m²), d1, ifosfamide (1700 mg/m²), d1–3 q28</td>
<td>RES</td>
<td>98</td>
<td>28.0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SEN</td>
<td>57</td>
<td>19.0</td>
<td>—</td>
</tr>
</tbody>
</table>

PFS: progression-free survival; RR: response rate; RES: platinum-resistant recurrent disease (platinum sensitivity according to the cutoff of 12-month platinum-free interval); SEN: platinum-sensitive recurrent disease; q: every; d: day; CDDP: cisplatin; CBDCA: carboplatin; PFI: platinum-free interval; GEM: gemcitabine; PTX: paclitaxel; TPT: topotecan; OS: overall survival.

3.2. PLD Single-Agent Phase III Randomized Trials. Table 2 summarizes the results from randomized trials using PLD alone or in combination in phase III studies [47–52].

In the first trial [48], Gordon randomized 474 ovarian cancer patients at first recurrence (stratified by PFI) to PLD (50 mg/m² every 4 weeks) or topotecan (1.5 mg/m²/day for 5 consecutive days every 3 weeks). In platinum-resistant disease (n = 255) no significant difference was seen in response rate, PFS, or OS between the two treatment arms, while in platinum-sensitive patients (n = 219), median PFS and OS were significantly prolonged in PLD-treated patients compared to TPT-treated patients (P value = 0.037 and P value = 0.008, resp.). More mature survival analysis confirmed the long-term advantage for platinum-sensitive patients receiving PLD versus TPT (median OS = 27 months versus 17.5 months, hazard ratio (HR) = 1.432, P value = 0.017) [49]. Moreover, for partially platinum-sensitive disease (n = 122), the HR favored PLD versus TPT (HR = 1.58, P value = 0.021). About the tolerability profile, grade 3/4 haematological toxicity occurred more frequently and more severely in TPT compared to PLD; in particular, severe neutropenia was documented in 77% of TPT-treated patients versus 12%
Table 2: Phase-III studies with pegylated liposomal doxorubicin (PLD) as a single agent or in combination regimens.

<table>
<thead>
<tr>
<th>Author</th>
<th>Dose/schedule</th>
<th>Clinical setting</th>
<th>No. pts</th>
<th>RR (%)</th>
<th>PFS (median) (mts)</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>O’Byrne et al. [47]</td>
<td>PLD (50 mg/m²) q28 versus PTX (175 mg/m²) q21</td>
<td>REC</td>
<td>214</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>107</td>
<td>17.8</td>
<td>5.4</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>107</td>
<td>22.4</td>
<td>6.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Gordon et al. [48, 49]</td>
<td>PLD (50 mg/m²) d1, q28 versus TPT (1.5 mg/m²) d1–5 q21</td>
<td>RES</td>
<td>255</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>130</td>
<td>12.3</td>
<td>2.3</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>125</td>
<td></td>
<td>3.4</td>
<td>10.3</td>
</tr>
<tr>
<td>Mutch et al. [50]</td>
<td>PLD (50 mg/m²) d1, q28 versus GEM (1,000 mg/m²) d1, 8, q21</td>
<td>RES</td>
<td>195</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96</td>
<td>8.3</td>
<td>3.6</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>99</td>
<td>6.1</td>
<td>3.1</td>
<td>13.5</td>
</tr>
<tr>
<td>Ferrandina et al. [51]</td>
<td>PLD (40 mg/m²) q28 versus GEM (1,000 mg/m²) d1, 8, 15 q28</td>
<td>RES</td>
<td>153</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>76</td>
<td>16</td>
<td>4.0</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>77</td>
<td></td>
<td>5.0</td>
<td>12.7*</td>
</tr>
<tr>
<td>Monk et al. [52]</td>
<td>TRAB (1.1 mg/m²) d1, q21 versus PLD (50 mg/m²) q28</td>
<td>ALL</td>
<td>672</td>
<td>28.0*</td>
<td>7.3*</td>
<td>20.5</td>
</tr>
<tr>
<td>OVA-301</td>
<td>PLD (50 mg/m²) d1</td>
<td>SEN</td>
<td>430</td>
<td>19.0</td>
<td>5.9</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>PLD (50 mg/m²) q28</td>
<td></td>
<td>335</td>
<td>35*</td>
<td>9.2*</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>TRAB (1.1 mg/m²) d1, q21 versus PLD (50 mg/m²) q28</td>
<td></td>
<td>337</td>
<td>23</td>
<td>7.5</td>
<td>—</td>
</tr>
<tr>
<td>Markman et al. [53]</td>
<td>PLD (50 mg/m²) d1/CBDCA (AUC 5) d1, q28 versus CBDCA (AUC 5) d1, q28</td>
<td>SEN</td>
<td>31</td>
<td>59*</td>
<td>12*</td>
<td>31</td>
</tr>
<tr>
<td>SWOG SO200</td>
<td></td>
<td>6–24 mts</td>
<td>30</td>
<td>28</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>Pujade-Lauraine et al.</td>
<td>PLD (50 mg/m²) d1</td>
<td>SEN</td>
<td>467</td>
<td></td>
<td>11.3*</td>
<td>—</td>
</tr>
<tr>
<td>[54]CALYPSO</td>
<td>JM8 (AUC 5) d1, q28</td>
<td></td>
<td>509</td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>PTX (175 mg/m²) d1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>—</td>
</tr>
</tbody>
</table>

GEM: gemcitabine; OS: overall survival; PFS: progression-free survival; PTX: paclitaxel; REC: not otherwise specified recurrent disease; RES: platinum-resistant recurrent disease; RR: response rate; SEN: platinum-sensitive recurrent disease; TRAB: trabectedin; q: every; d: day. *Statistically significant.

of PLD-treated patients ($P < 0.001$), and thrombocytopenia was found in 34% of TPT versus 1% of PLD cases ($P < 0.001$). No case of severe HFS was documented in the TPT arm while it was registered in 23% of PLD-treated patients ($P < 0.001$) with no difference in quality of life perceived by the patient.

In a second randomized trial conducted by O’Byrne et al. [47], 214 patients (not defined according to platinum sensitivity) were randomized to receive either PLD (50 mg/m² every 4 weeks) or paclitaxel (175 mg/m² every 3 weeks). A preliminary analysis of the data showed that there were no significant differences in response rates, PFS, OS, or rate of adverse events. The study was suspended due to poor accrual, as paclitaxel became incorporated into first-line therapy, so no definitive analysis was carried out.

Several additional phase III trials have been reported, which directly compared single-agent PLD to other single agents (paclitaxel, gemcitabine) in platinum-resistant and partially platinum-sensitive (platinum-free interval 6–12 months) ovarian cancer patients [47, 50, 51]. While side-effect profiles of the agents often differed substantially, these studies essentially revealed the therapeutic equivalence for these agents in this difficult clinical setting.

Two phase III trials compared PLD with gemcitabine in recurrent platinum-resistant or partially sensitive ovarian cancer patients [50, 51].

In both trials there was no difference in the response rates and median PFS between the two treatment arms. The median OS in the MITO3 trial was greater in the PLD arm (14 versus 12.7 months, respectively, $P$ value = 0.048). With the limits inherent in the small sample series, the survival advantage reported with PLD over GEM was maintained in the subgroup of partially sensitive patients ($P$ value = 0.016).

Based on these results PLD at 40 mg/m² seems to offer the most favourable toxicity profile, which is likely to sustain the achievement of better quality of life (QoL) scores (at least in comparison to GEM) and was adopted as a standard worldwide [50].

Other phase III trials have explored the combination of PLD with other nonplatinum agents. Among the most intriguing novel drugs, trabectedin (TRAB) (ET743; Yondelis) has become relevant for treatment of sarcomas and other solid tumors for its unique mechanism of action, in that, unlike most other agents, it binds to the minor groove of DNA thus affecting a variety of transcription factors, cell proliferation, and the nucleotide excision repair system and inhibits the MDR-1 gene coding for the protein responsible for chemoresistance [75–77].

Based on safety and efficacy results from phase-I/II studies, a phase-III trial (OVA-301, NCT00113607) has been performed to compare PLD (50 mg/m² every 28 days) with
the combination PLD (30 mg/m²) and TRAB (1.1 mg/m² every 21 days) in second-line relapsed ovarian cancer patients, unsuitable for platinum therapy, stratified according to the PFI (PFI < 6 months versus PFI > 6 months). After a median followup of 47.4 months, in the whole series, the response rate was significantly higher in the combination compared to the PLD arm, as was also median PFS (HR = 0.79, P value = 0.019) [52].

However, in platinum-resistant cases (n = 242) no statistically significant difference was observed with the doublet in terms of response rate (13.4% versus 12.2%, resp.) and PFS, while a clear advantage favouring the combination compared to single-agent PLD was evident in platinum-sensitive disease (RR 35.3% versus 22.6%, P = 0.0042; median PFS 9.2 months versus 7.5 months; HR = 0.73, P = 0.017) and partially sensitive disease with median PFS of 7.4 months versus 5.5 months in PLD/TRAB versus PLD arm (HR = 0.65, P = 0.0152).

An unplanned hypothesis-generating analysis adjusting for the PFI imbalance and other prognostic factors suggested an improvement in OS associated with the trabectedin/PLD arm (HR = 0.82; 95% CI: 0.69–0.98; P = 0.0285). In another unplanned exploratory analysis, the subset of patients with a PFI of 6–12 months had the largest difference in OS (HR = 0.64; 95% CI: 0.47–0.86; P = 0.0027). Data showed a longer time to the following platinum therapy, and this imbalance in platinum-free interval was suggested as a possible cause of the increased OS [78]. Thus, these data suggest that the treatment with an effective nonplatinum combination may artificially prolong the platinum-free interval giving more chance of activity to further platinum therapy. This hypothesis will be investigated in a phase III trial, called INNOVATYION.

As expected the combination regimen of TRAB/PLD has been associated to a greater haematological toxicity (grade 3/4 anaemia 14%, neutropenia and thrombocytopenia 63%). Among other toxicities, short-lived grade 3/4 hypertransam-minasemia (38%) and HFS were documented in 4% of the PLD/TRAB arm compared to 20% in the PLD alone arm [79]. In September 2009, based on these results, which support the PLD/TRAB combination as the most effective nonplatinum-based combination in platinum-sensitive disease, the PLD (30 mg/m²) and TRAB (1.1 mg/m²) association every 3 weeks has been approved by the EMA for treatment of patients with relapsed platinum-sensitive OvCa [80].

Based on the phase-II trials in platinum-sensitive OvCa the combination of PLD/carboplatin has been explored in phase-III trials [53]. Markman et al. compared single-agent carboplatin to its combination with PLD in recurrent ovarian cancer, showing a statistically significant improvement of PFS with carboplatin/PLD, without an overall survival benefit. Interestingly, for unknown reasons, the association drastically reduced the rate of hypersensitivity reactions compared to carboplatin alone (9% versus 0%, P = 0.0008) [53]. Later on the results of the CALYPSO trial have been reported [81, 82]. This international open-label phase-III trial compared carboplatin PLD (CD) with carboplatin-paclitaxel (CP) in patients with platinum-sensitive recurrent ovarian cancer (ROC). A total of 976 recurrent patients relapsing >6 months after first- or second-line therapy were randomized to receive CD or CP for six cycles.

Designed as a noninferiority trial, CALYPSO demonstrated that the combination of CD was not only noninferior to CP in terms of PFS, but indeed it was more effective (HR = 0.82, P = 0.005) in patients with platinum-sensitive recurrent ovarian cancer. Nevertheless, with a median followup of 49 months, no statistically significant difference in OS was observed (hazard ratio = 0.99 (95% confidence interval 0.85, 1.16) logrank P = 0.94), with median survival times of 30.7 (CD) and 33.0 months (CP). Treatment-related serious adverse events were more frequent in the CP arm (76 patients (30%) versus 44 patients (18%)), while the CD treatment was associated with more grade 3/4 thrombocytopenia and more grade ≥2 mucositis and PPE. Interestingly, even in this trial as in other phase-II studies there was a lower incidence of allergic reactions, alopecia, neuropathy, and arthralgia/myalgia. PLD/carboplatin represents a valid alternative to other platinum-based regimens in recurrent platinum-sensitive OvCa especially for patients whose QoL is recognized to be heavily compromised by alopecia or who had experienced or had not yet been rescued from taxane-induced neurotoxicity [81, 82].

Attempts to include PLD in a front-line treatment have also been made; in particular, with the aim of improving standard chemotherapy with carboplatin-paclitaxel, doublet or triplet combinations including PLD have been investigated based also on the very favourable and not overlapping toxicity profile. The potential efficacy of triplets and sequential doublets (with TPT, PLD, and gemcitabine) has been investigated in the GOG182/ICON5 trial that enrolled 4312 stage-III/IV patients who were randomized to 5-arm first-line chemotherapy regimens and sequences, with disappointing results. There was no PFS or OS advantage with sequential doublets or with triplets compared with the control arm. In this trial, PLD at a dosage of 30 mg/m² was added to carboplatin and paclitaxel at full dose every other cycle [83].

In the front-line setting, MITO-2 was the first trial investigating the PLD/carboplatin (30 mg/m², AUC = 5, every 21 days) combination compared to the standard treatment; this trial was designed to show a superiority for the carboplatin/PLD combination. Unfortunately, there were no statistically significant differences in either PFS or overall survival between the treatment arms with median PFS times of 19.0 months versus 16.8 months (HR, 0.95; 95% CI, 0.81 to 1.13; P = 0.58) and median overall survival times of about 61 and 53 months with carboplatin/PLD and carboplatin-paclitaxel, respectively, (HR, 0.89; 95% CI, 0.72 to 1.12; P = 0.32) [84]. Carboplatin/PLD also produced a similar response rate but different toxicities (less neurotoxicity and alopecia but more hematologic adverse effects).

Although the proposed combination has failed to undermine the primacy of the standard carboplatin-paclitaxel, given the observed confidence intervals and the different toxicity, carboplatin/PLD could be considered an alternative to standard first-line therapy, particularly in patients that cannot receive paclitaxel.
4. PLD in Epithelial Ovarian Cancer: Future Directions

Based on the excellent results obtained by the PLD alone or in combination with platinum as well as nonplatinum agents in almost all clinical settings of ovarian cancer, early phase trials have begun exploring the potential of adding PLD to a variety of alternative drugs, including bevacizumab (BEV) and other "targeted agents" in the management of epithelial ovarian cancer (Table 3).

Despite the encouraging results obtained in ovarian cancer, the combination of PLD with bevacizumab was introduced with caution because of the potential mechanism of interference. We know that the increased vascular permeability known as "EPR effect" greatly enhances liposome deposition in tumors enabling the increase of intratumoral delivering and concentration of PLD. Normalization of the vasculature induced by bevacizumab has been hypothesized to interfere with liposomal tumour entry, but a concomitant reduction in tumour interstitial pressure, on the other hand, could improve PLD delivery. In a trial conducted by Muggia et al. the pharmacokinetic of PLD alone or in combination with bevacizumab was investigated in order to evaluate the postulated interferences. Trial results show an increased PLD T3/4, C7d/Cmax, and PLD levels at day 21 after bevacizumab introduction, probably reflecting a greater delivery of PLD to tumors [55]. Preliminary results from a phase II study with the PLD/BEV combination in platinum-resistant patients have been presented by the same authors. The study was conducted on 48 patients. PLD (30mg/m² every 21 days) was administered alone at the first cycle, and then with BEV (15mg/kg every 21 days) for the following 6 cycles or until progression [85].

This proof-of-concept study was the first to report the efficacy and the tolerability of the combination of PLD and bevacizumab in the treatment of recurrent ovarian cancer. The ORR observed in this trial was 72.2% (95% CI: 58.4, 83.5). The safety profile was consistent with the known toxicities of these agents with no sign of overlapping toxicities nor any reports of cumulative-dose cardiotoxicity.

Following these data a large phase III randomized study (AURELIA) in platinum-resistant setting assessed the efficacy of bevacizumab (10mg/kg every 2 weeks or 15mg/kg every 3 weeks) combined to either dose-dense paclitaxel

<table>
<thead>
<tr>
<th>Author</th>
<th>Dose/schedule</th>
<th>Clinical setting</th>
<th>PFI (mts)</th>
<th>No. pts</th>
<th>RR (%)</th>
<th>PFS (median) (mts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muggia et al. [55]</td>
<td>PLD 30mg/m² and BEV 15mg/kg on cycles 2–7 (with option to continue)</td>
<td>≤6</td>
<td>48</td>
<td>Ongoing</td>
<td>Ongoing</td>
<td></td>
</tr>
<tr>
<td>Pujade-Lauraine et al. [56]</td>
<td>Arm1</td>
<td>PTX (80mg/m²) d1, 8, 15 and 22 q28 or TPT (4mg/m²) d1, 8, 15 q28 or PLD (40mg/m²) d1 q28</td>
<td>≤6</td>
<td>166</td>
<td>12.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Del Carmen et al. [57]</td>
<td>Arm2</td>
<td>BEV 10mg/kg d1 q15 or 15mg/kg d1 q21, PTX (80mg/m²) d1, 8, 15 and 22 q28 or TPT (4mg/m²) d1, 8, 15 q28 or PLD (40mg/m²) d1 q28</td>
<td>135</td>
<td>30.9</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>Steffensen et al. [58]</td>
<td>PLD (30mg/m²) d1 q28, CBDCA (AUC5) d1 q28, Beva 10mg/kg d1 q14</td>
<td>≥6</td>
<td>54</td>
<td>72.2</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>TRINOVA 2 [59]</td>
<td>Arm 1</td>
<td>PLD 50mg/m² d1 q28 and blinded AMG 386 15mg/kg qw</td>
<td>≤12</td>
<td>Ongoing</td>
<td>Ongoing</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Boers-Sonderen et al. [60]</td>
<td>T 15–20mg/m²/PLD 20–40mg/mq</td>
<td>ALL</td>
<td>20</td>
<td>3PR</td>
<td>9SD</td>
<td>4.9</td>
</tr>
</tbody>
</table>

PFS: progression-free survival; PTX: paclitaxel; TPT: topotecan; T: temsirolimus; PAN: panitumumab; BEV: bevacizumab; RR: response rate; SEN: platinum-sensitive recurrent disease; TRAB: trabectedin; q: every; d: day. *Statistically significant.
for platinum-sensitive ovarian cancer patients [57].

Chemotherapy alone compared to ORR 95% (CI: 58.4, 83.5), the median duration of response was 11.9 months, and median TTP was 13.9 months (95% CI: 11.4, 16.0). The safety profile was consistent with the known toxicities of these genetic drugs. A phase-III ongoing trial (TRINOVA2 study) compares PLD to PLD in association with AMG386, an angiopoietin inhibitor [59].

Panitumumab is a fully human monoclonal antibody specific to the epidermal growth factor receptor (EGFR). No previous studies have evaluated the effect of panitumumab in ovarian cancer (OC) based on KRAS mutation status. The main purpose of the PaLiDo study, a phase-II non-randomized multicenter trial presented at ASCO 2012 [58], was to investigate the response rate in platinum-resistant, KRAS wild-type OC patients treated with PLD and panitumumab. Patients with relapsed and pretreated (no more than two lines) ovarian cancer were treated with panitumumab (6 mg/kg days 1 and 15) and with PLD (40 mg/m² day 1) every 4 weeks. Progression-free and overall survival in the intention-to-treat population (N 543) was 2.7 months (2.5–3.2 months, 95% CI) and 8.1 months (5.6–11.7 months, 95% CI), respectively, with a considerable skin toxicity, grade 3 in about 40% of patients.

Other phase-I trials evaluated PLD in combination with the mTOR inhibitor temsirolimus [60] and with the folate receptor ligand farletuzumab [86] (humanized monoclonal antibody that binds to folate receptor-α, a target which is largely absent in normal epithelium and overexpressed in EOC) showing feasibility and activity.

Data regarding combinations are very preliminary, but, at least with antiangiogenic drugs, the combination seems tolerable and active.

Another field of development is that of the patients with BRCA mutation. BRCA1- or BRCA2-mutated ovarian cancer patients are defective of the mechanisms of DNA repairing. This determines an improved chemosensitivity to some DNA-damaging agents [87]. PLD that leads to DNA damage by inhibiting topoisomerase II may prove to be more effective in these patients [88]. In a recent study from Kaye et al. [89], the PARP inhibitor olaparib was compared with PLD in BRCA-mutated patients. The study showed significant single-agent olaparib activity while PFS was not significantly improved compared to PLD. Interestingly, this negative result was hypothesis generating based on the unexpected high PFS found in the control PLD arm. In fact, the 7.1-month PFS observed in this study with PLD was significantly higher than that expected for this drug in the general population. These results are in accordance with retrospective data published by Adams and colleagues on Gynecologic Oncology in 2011 confirming the higher activity of PLD in BRCA-mutated ovarian cancer patients. Although all these data are very preliminary, it seems that PLD may have a special role in patients with BRCA mutation or BRCAAness profile [90]. In the same direction are the results of a multicentre retrospective study in relapsed ovarian patients, BRCA mutation carriers, treated with PLD, where Safra et al. showed an improved outcome in terms of median time to treatment failure (15.8 months versus 8.1 months in nonhereditary OC) and overall survival (56.8 months versus 22.6 months) [91].

5. Conclusions

PLD plays an important role in the management of ovarian cancer. It represents the standard therapy in platinum-resistant recurrence and one of the standard options in platinum-sensitive patients. Between the combination regimes, due to the results of efficacy achieved in phase-II and -III trials and considering the favorable safety profile, carboplatin/PLD represents a valid alternative in both first-line (in patients that cannot receive paclitaxel) and recurrent ovarian cancer compared to actual standard options.

Combination with nonplatinum agents (trabectedin), and antiangiogenic drugs (bevacizumab) represents an alternative treatment option in the recurrent setting, associated in certain cases with remarkable toxicity. New target therapy is under evaluation in combination with PLD.

Acknowledgments

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References


Review Article

Lipid-Based Nanovectors for Targeting of CD44-Overexpressing Tumor Cells

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Hyaluronic acid (HA) is a naturally occurring glycosaminoglycan that exists in living systems, and it is a major component of the extracellular matrix. The hyaluronic acid receptor CD44 is found at low levels on the surface of epithelial, hematopoietic, and neuronal cells and is overexpressed in many cancer cells particularly in tumour initiating cells. HA has been therefore used as ligand attached to HA-lipid-based nanovectors for the active targeting of small or large active molecules for the treatment of cancer. This paper describes the different approaches employed for the preparation, characterization, and evaluation of these potent delivery systems.

1. CD44 Receptor

CD44 (cluster of differentiation 44) is a widely expressed cell surface hyaluronan receptor which consists in a single chain transmembrane glycoprotein with a size that varies between 80 and 200 kDa. It is moreover an acidic molecule with an isoelectric point between 4.2 and 5.8 [1]. CD44 receptor belongs to the family of cell adhesion molecules (CAMs) together with selectins, integrins, and cadherins. The CAMs control cell behavior by mediating contact between cells or between cells and the extracellular matrix and are essential for maintaining tissue integrity. Because of these important functions, they are also involved in pathological conditions including tumor progression and metastasis [2]. It is well known that various tumors, for example, epithelial, ovarian, colon, stomach, and acute leukemia, overexpress CD44 [3].

CD44 comprise a family of glycoproteins encoded by a single gene located on the short arm of chromosome 11 and composed of 20 exons [4]. Extensive alternative splicing generates multiple variant isoforms of CD44 receptor denoted as CD44v. The most abundant standard isoform of human CD44 protein is the smallest isoform that lacks any variant exons, designated CD44s, but some epithelial cells also express a larger isoform called CD44E [5]. The expression of CD44 isoforms containing combinations of the other variant exons is far more restricted in normal tissues. In particular, CD44s is abundantly expressed by both normal and cancer cells, whereas the variant CD44 isoforms (CD44v), that contain a variable number of exon insertions (v1–v10) at the proximal plasma membrane external region, are expressed mostly by cancer cells.

CD44 is endogenously expressed at low levels on various cell types of normal tissues [6, 7] but requires activation before binding to hyaluronic [8–11].

The CD44 structure of normal cells is distinct from that of cancer cells because pathological conditions promote alternate splicing and posttranslational modifications to produce diversified CD44 molecules with increased tumorigenicity [22, 23].

The effect of native hyaluronic as well as of the catabolic enzymes and the degradation products of this macromolecule on tumor progression is complex. Moreover, the amount of intratumoral hyaluronic also varies depending on the cell type and on the degree of tumor cell differentiation. There are some good reviews that describe the association of CD44 receptor with human cancer cells and underline the
receptor’s role in the progression of the disease [10, 24]; thus the overexpression of CD44 could be a good tool in drug delivery approaches using the receptor as an anchor to attach, through a ligand, prodrugs or nanomedicine-based delivery systems to increase the efficiency of anticancer drugs [25].

2. Hyaluronic Acid

Hyaluronic acid (hyaluronan, HA) is a nonsulfated glycosaminoglycan polymer. It is ubiquitous, being the main component of extracellular matrix [26]. HA is composed of disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine linked together through alternating $\beta_{1,3}$ and $\beta_{1,4}$ glycosidic bonds (Figure 1). HA is a biodegradable polymer with a molecular weight of $10^6$–$10^7$ Da that is biocompatible, nontoxic, hydrophilic, and nonimmunogenic [27]. Moreover, HA molecules have a number of sites suitable for chemical modification such as hydroxyl, carboxyl, and $N$-acetyl groups.

In adult tissues such as the vitreous, synovial fluid and dermis, hyaluronan plays an extracellular, structural role that depends on its hydrodynamic properties as well as on its interactions with other extracellular matrix components. However, it is also concentrated in regions of high cell division and invasion (during embryonic morphogenesis, inflammation, wound repair, and cancer). Hyaluronic acid is thus also involved in tumorigenesis, and its role is complex and depends on various factors such as, for example, its molecular weight. In fact lower molecular weight HA (10–100 kDa) stimulates angiogenesis but high molecular weight hyaluronan (>1,000 kDa) is inhibitory [28–30]. High amount of HA production usually promotes tumor progression, but it was observed that extremely high levels of hyaluronan production can be inhibitory [31]. As also reported, tumor progression is often correlated with both hyaluronan and hyaluronidase levels in human cancers [32]. These considerations led to the hypothesis that the turnover of HA is strictly involved in the promotion of tumor progression by HA [33–35].

In addition to its principal and previously described receptor, CD44, HA also interacts with other cell surface receptors such as RHAMM (receptor for hyaluronan-mediated motility, CD168), ICAM-1 (intracellular adhesion molecule-1), TLR-4 (toll-like receptor-4), HARE (HA receptor for endocytosis), and LYVE-1 (lymphatic vessel endocytic receptor).

The mechanism of HA-CD44 binding is still not fully understood, but it has been reported that the CD44 receptor contains the specific binding domain for HA, which consists of 160 amino acid residues. The binding affinity of CD44 to HA was found to be dependent on the size of HA oligomers. In fact, hexamer and decamer are considered to be the minimum size able to bind to CD44 while larger oligomers (20) have higher binding affinity because of their multiple interactions with more than one CD44 receptor simultaneously [3, 8, 36, 37]. It has also been reported that all the CD44 isoforms have uniform affinity for HA [38]; therefore HA can be used as vector for the active targeting of anticancer drugs. Different strategies have been exploited with interesting results, for example, in the preparation of bioconjugates obtained by covalently linking HA to a cytotoxic drug such as, for example paclitaxel [39, 40] or doxorubicin [41, 42]. These topics are out of the scope of this paper where only strategies consisting in the design of HA decorated nanosystems will be discussed in depth.

3. Chemical Conjugation of HA to Lipid-Based Nanocarriers

Different approaches can be used to bind HA to the lipid-based nanocarriers, depending on the molecular weight of the HA as well as on the need to start from preformed nanocarriers or from pure lipids that will be then used to prepare particles.

HA binding to preformed nanocarriers was the firstly used method [43] and offers the advantage to conjugate the HA only on the external surface of the particle. Of course, this approach makes difficult the control of the density of attachment of HA on the carrier surface. Moreover, the lower specificity of the linkage, due to the possibility to bind different amino groups, results in a consequent multipoint attachment of the polymer on the nanocarrier that is then difficult to characterize.
Alternatively, HA can be previously conjugated to a pure lipid and then added in the lipid mixture during the preparation of the nanoparticles. This procedure permits the introduction of a controlled amount of HA on nanocarriers, but could require a more elaborated synthetic method.

3.1. HA Binding to Preformed Nanocarrier. High molecular weight (HMW) HA was attached to the surface of preformed liposomes through amidation reaction between the aminoreactive group of a lipid on the liposome surface, generally a phosphatidylethanolamine (PE), and HA glucuronic carboxylate (Figure 2) [13, 14, 43]. The amidation reaction was performed preactivating HA by incubation with the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) condensing agent in acidic medium and then adding the activated HA to the nanocarrier suspension in a basic medium. Elimination of the excess of reagent and reaction byproducts was obtained by centrifugation and repeated washing.

3.2. Preparation of HA-PE Preformed Conjugates. HA conjugation to the lipid before nanocarrier preparation was carried out with both high and low molecular weight (LMW) polymers [12, 19]. In all cases, HA reacted with an aminoreactive group present on the lipid that was PE, also in this case (Figure 2). Two different conjugation methods have been proposed depending on the HA molecular weight. Eliaz and Szoka attached a mixture of oligosaccharide HA to PE by reductive amination using sodium cyanoborohydride as reducing agent [12]. Reductive amination is a chemical reaction widely used in polysaccharide conjugation and consists in two steps. In the first step, the aldehydic group of the terminal residue of HA, generated by opening the sugar ring, reacts, in acidic medium, with the amino group of PE forming the unstable imine. Then, the imine is reduced in the presence of a reducing agent to a secondary amine leading to the formation of the conjugate. An improvement of this reaction was proposed by the same group in 2006 [44]. The authors developed a methodology for the preparation of aldehyde functionalized HA and reported that the reductive amidination with this derivative is more efficient than that performed using the classical approach consisting in the reaction at the sugar reducing end.

In these reactions involving LMW-HA, only one PE molecule was linked to the polymer. Both kinds of conjugates were purified by silica column chromatography, and the latter was characterized by MALDI and $^1$HNMR.

HMW-HA-dioleoylphosphatidylethanolamine (DOPE) conjugate was prepared by EDC-mediated amidation reaction [19]. In this conjugate the DOPE amino group is randomly linked to the carboxylic residues of HA. The conjugate was purified by ultrafiltration and dialysis and its purity was assessed by capillary electrophoresis [20]. This conjugate was introduced into cationic lipids during liposome formation [19–21].

A similar synthetic approach was used by Toriyabe et al. [45] for the preparation of a conjugate between HA and stearylamine (HA-SA conjugate). SA was linked via an amide linkage using EDC and NHS as coupling agents; then the solution of conjugate was added and incubated to the liposome suspension.

Recently Cho et al. described the preparation of an amphiphilic polymer obtained conjugating HA oligomers to a cellular component, ceramide (CE). To obtain HA-CE conjugate, HA was first activated by reaction with tetra-n-butylammoniumhydroxide (HA-TBA), and CE was previously modified by esterification reaction with chloromethylbenzoyl chloride, used as linker. Then linker CE was conjugated to HA-TBA by ether bond formation [17].

4. Lipid-Based Nanocarriers for Targeting of CD44-Rich Cells

First evidence of powerful delivery of chemotherapeutics to cancer cells by HA-modified liposomes was provided by the group of Eliaz and Szoka [12] (Table 1). In this study, a low LMW-HA was bound onto the liposome surface. The authors demonstrated B16F10 cells expressing high levels of CD44, an avid cell-liposome binding followed by internalization in a temperature-dependent manner. Lower uptake was found in cells expressing low levels of CD44 (CV-1). B16F10 cell association of the unilamellar vesicles was found to depend critically on the density of HA on liposome surface. These findings were observed after exposing cells to HA-modified liposomes in both transient (3 h and replacement with fresh cell medium) and continuous conditions for periods going up to 24 h [12]. Moreover, for given amounts of intracellular-delivered chemotherapeutic agent, namely, doxorubicin (DOX), the encapsulated form was more efficient in killing B16F10 cells than the free form [12]. Due to the enhanced potency of DOX encapsulated into HA-modified liposomes, it was hypothesized that the
drug reaches a critical compartment more efficiently, when compared with the free form. In particular, the authors hypothesized that an uptake of the delivery system via a non-clathrin-coated endosome, as already reported in the case of hyaluronan catabolism, could occur [46]. This hypothesis was recently confirmed by our group after incubating HA-modified cationic liposomes with CD44-expressing A549 cells with different endocytosis inhibitors [20]. It was found that the transfection efficiency of HA-modified cationic liposomes was not affected by a clathrin-mediated endocytosis inhibitor, while it was significantly decreased by inhibitors of caveolae-mediated endocytosis, demonstrating that the latter is the main endocytosis pathway of HA-bearing lipoplexes. It is worthy of note that in the studies of Eliaz et al. [47] and Dufaży Wojcicki et al. [20] an LMW and an HMW-HA were used, respectively, although a similar endocytotic pathway can be reasonably hypothesized.

The targeting of cancer cells using HMW-HA bound to liposomes was firstly demonstrated by Peer and Margalit [13, 14]. HMW-HA should offer advantages such as to bind the CD44 receptors with a higher affinity than hyaluronan fragments, to provide long-term circulation through its many

### Table 1: Examples of HA-decorated lipid-based nanocarriers for targeting of CD44.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Drug</th>
<th>HA</th>
<th>Main findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes</td>
<td>DOX</td>
<td>LMW-HA</td>
<td>Avid cell-liposome binding followed by internalization in cells overexpressing CD44.</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Higher cytotoxicity compared with free drug on CD44-overexpressing cells.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Higher affinity of HMW-HA to bind the CD44 receptors, compared to hyaluronan fragments.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Long-term circulation of HMW-HA liposomes. HMW-HA can act as cryoprotectant, thus allowing liposome lyophilization.</td>
<td></td>
</tr>
<tr>
<td>Liposomes</td>
<td>MMC DOX</td>
<td>HMW-HA</td>
<td>Loading into the HA-modified liposomes generates a 100-fold increase in drug potency in tumor cells overexpressing CD44 receptors.</td>
<td>[13, 14]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Higher drug accumulation in tumor, compared to free drug or drug in unmodified liposomes.</td>
<td></td>
</tr>
<tr>
<td>Self-assembled lipid nanoparticles</td>
<td>PTX</td>
<td>HMW-HA</td>
<td>Reduced PTX accumulation in liver and spleen and increased drug accumulation in the tumor, compared to Taxol. Prolonged PTX half-life. Reduced PTX toxicity.</td>
<td>[15]</td>
</tr>
<tr>
<td>HA-coated nanostructured lipid carriers</td>
<td>PTX</td>
<td>HMW-HA</td>
<td>More effective than Taxol with fewer side effects. Prolonged PTX half-life. Increased PTX accumulation in tumors.</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enhanced intracellular DCT uptake in the CD44-overexpressing cell lines. MDR-overcoming effects.</td>
<td></td>
</tr>
<tr>
<td>Self-assembled nanoparticles</td>
<td>DCT</td>
<td>LMW-HA</td>
<td>In vivo specific CD44-mediated tumor targeting.</td>
<td>[17]</td>
</tr>
<tr>
<td>PEGylated self-assembled nanoparticles</td>
<td>DOX</td>
<td></td>
<td>Improved retention time in the bloodstream and nanoparticle accumulation at the tumor site. PEGylation resulted in prolonged nanoparticle circulation and reduced DOX clearance rate. Higher in vivo antitumor efficacy in the tumor xenograft mouse model in comparison to non-PEGylated nanoparticles and DOX alone.</td>
<td>[18]</td>
</tr>
<tr>
<td>Cationic liposomes</td>
<td>DNA and siRNA</td>
<td>HMW-HA</td>
<td>The presence of HA-DOPE lipid conjugate in the liposome composition did not affect the lipoplex formation. Increased nucleic acid protection against enzymatic degradation. Increased the level of transfection on CD44-highly expressing cells.</td>
<td>[19–21]</td>
</tr>
<tr>
<td>Nanoparticles</td>
<td>Different molecular weights</td>
<td></td>
<td>No induction of complement activation.</td>
<td>[18]</td>
</tr>
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</table>
hydroxyl residues, and to allow liposome lyophilization, due to the properties of HA to act as a cryoprotectant [48]. In particular, in an in vivo study, HA-modified liposomes resulted in long-circulating species, over a time frame at least equal to those reported for PEG-coated liposomes [13]. Mitomycin C (MMC), a chemotherapeutic agent used in different form of tumors but also characterized by severe side effects, was encapsulated into HA-modified liposomes and tested in vitro and in two experimental models of lung metastases. The in vitro studies showed that loading into the HA-modified liposomes generates a 100-fold increase in MMC potency in tumor cells that overexpress hyaluronan receptors, but not in cells with poor expression of these receptors. Moreover, when using HA-modified liposomes, MMC accumulated in the tumor 30-fold higher than when the drug was administered in free form and 4-fold higher than when delivered via unmodified liposomes. Interestingly, liver uptake was significantly reduced when the drug was delivered via the HA-modified liposomes that should contribute to reducing the subacute toxicity associated with MMC administered as free drug [13]. It is worthy of note that, in the case of MMC free or encapsulated in unmodified liposomes, tumor size, metastatic burden, and survival time were not much different than those observed in untreated mice. High positive responses were only reported in the case of mice treated with MMC HA-modified liposomes. Similar results were obtained from different experimental model of tumors with HA-modified liposomes, but replacing the MMC with DOX, thus demonstrating that the targeting is carrier-specific, rather than drug-specific [14]. In this study, the HA-modified formulation was compared to free DOX, DOX encapsulated in unmodified liposomes, and pegylated liposomes (Doxil). Drug accumulation in tumor-bearing lungs, as well as key indicators of therapeutic responses such as tumor progression, metastatic burden, and survival, was superior in animals receiving DOX-loaded HA-modified liposomes, compared to the controls.

HA-modified lipid-based nanoparticles encapsulating paclitaxel (PXT) were also proposed. PXT is a chemotherapeutic agent largely used in the treatment of solid tumors. However, its poor water solubility as well as the lack of selective delivery approach represents important clinical limitations. In vivo evidence of CD44 targeting by HA-modified lipid-based nanoparticles was also obtained by encapsulating paclitaxel (PXT) into self-assembled lipid nanoparticle-like “clusters” [15]. Thus, HA-coated PXT-encapsulating clusters were administered in an experimental mice model of colon adenocarcinoma, and their antitumor effect, as well as the toxicity, was compared with that of FDA approved PXT formulations, namely, Taxol (PTX solubilized in the detergent Cremophor EL and in ethanol) and Abraxane (PXT encapsulated into albumin nanoparticles). Safety of the new HA-targeted formulation was demonstrated by any change in blood levels of enzymes released from the liver, namely, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively, regarded as reliable indicators of liver tissue damage and, more generally, systemic tissue damage. This effect was not associated with any change in body weight. On the contrary, multiple i.v. administrations of Taxol resulted in changes of body weight and release of high amounts of liver enzymes [15]. Moreover, when using Taxol, PTX was eliminated from the circulation within less than 1 h after i.v. injection, while PTX, administered within HA-modified lipid clusters, was still circulating even 24 h after i.v. injection. These findings still support the hypothesis that HMW-HA, when used as targeting moieties, also confers stealth properties on the nanoparticles. Interestingly, the HA-modified nanoparticles reduced PTX liver and spleen accumulation by almost 2-fold and increased PTX accumulation in the tumor by 10-fold compared to Taxol. Finally, tumor progression was exponential in the case of 5 mg/Kg body Taxol or Abraxane, while it was arrested at the same dose of PXT administered in HA-modified lipid clusters. This effect was also obtained with 20 mg/Kg body of Taxol, although it was associated with a significant loss of body weight indicating global toxicity [15]. Recently, Yang et al. proposed the preparation of HA-coated nanostructured lipid carriers (HA-NLCs) for tumor targeting via electrostatic attraction [16]. In this approach, cationic NLCs loaded with PTX were prepared by melt emulsion technology, followed by coating with HA (300 kDa); the process of electrostatic attraction was simple and controllable, and no chemical reagents were needed. The in vitro cytotoxicity and in vivo antitumoral activity studies showed that HA-PTX-NLCs were more effective than Taxol with fewer side effects. HA-NCL also prolonged the blood circulation time of PTX and increased its accumulation in tumors.

HA-modified nanoparticles have been proposed to overcome clinical limits of chemotherapeutics, such as Doxorubicin (DCT). DTC is a semisynthetic taxane derivative very effective against different tumors, but its clinical use causes several side effects and other limitations regarding the appearance of multidrug resistance (MDR) and its insolubility. Recently Cho et al. described the preparation of HA-ceramide (CE) self-assembled nanoparticles for DCT and DOX active targeting [17, 49]. The in vitro cellular uptake studies showed that nanoparticles enhanced intracellular DCT uptake in the CD44-overexpressing cell lines MCF-7. MDR-overcoming effects of DCT nanoparticles were observed in cytotoxicity test in CD44-positive MCF-7 breast cancer cells resistant to doxorubicin. The in vivo tumor targetability was evaluated using a noninvasive fluorescence imaging system in the same cells xenografted in a mouse model. To assess the uptake mechanism by a competitive inhibition assay, CD44 receptors were blocked with preinjection of high doses of HA. The fluorescence signal in the HA preinjected animal group was lower than that in no preinjection group for 24 h, indicating a probable reduction in nanoparticle uptake due to the blocking of CD44. The real-time imaging data showed that the fluorescent signal increased for the first 6 h and was maintained for 1 day. Then the tumors were dissected 24 h following injection, and the observed fluorescence intensity of HA pre-injection group was only 43.9% of the no preinjection group.

The same research team described the preparation of DOX-loaded, self-assembled, HA-CE-PEG-based nanoparticles [18]. In vitro tests were performed on two different cell lines with different CD44 expression: NIH3T3 (mouse...
embryonic fibroblast cells, CD44-negative) and SCC7 (mouse squamous cell carcinoma cells, CD44-positive). The cytotoxicity studies showed that HA-CE-based nanoparticles can be used as vehicle without important toxicity. The cellular uptake efficacy of DOX from nanoparticles via HA and CD44 interaction was demonstrated by confocal microscopy analysis. In vivo studies on SCC7 tumor xenograft mouse model showed improved retention time in the bloodstream and nanoparticle accumulation at the tumor site. The pharmacokinetics evaluation confirmed that PEGylation resulted in prolonged nanoparticle circulation and reduced DOX clearance rate. Improved half-life of DOX, when formulated as HA-CE-PEG nanoparticles, led to higher in vivo antitumor efficacy in the tumor xenograft mouse model in comparison to non-PEGylated nanoparticles and DOX alone.

HA was also used to increase transfection efficiency of cationic liposomes. Plasmid DNA and siRNA were successfully delivered to CD44-expressing cancer cells with this approach [19, 21]. The use of a lipid conjugate HA-DPPE into the liposome composition did not affect the lipoplex formation upon liposome mixing with DNA [19] or siRNA [21]. On the contrary, the lipoplex zeta potential was strongly affected shifting from a positive to a negative value. This was consistent with the presence of HA at lipoplex surface. Moreover, the presence of HA in the liposome formulation led to increased nucleic acid protection from degradation against DNase I or RNAse V1, probably because the HMW-HA and cationic lipids prevent access of these enzymes to the whole colloidal system [19, 21]. The presence of HA-DPPE did not modify the in vitro cytotoxicity, on the MDA-MB-231 and MCF-7 breast cancer cell lines, characterized by high and low expressions of CD44, respectively. On the contrary, the use of HA strongly reduced the cytotoxic profile of DOTAP/DPPE liposomes in combination with siRNA on A549 CD44-expressing cells [21]. This effect was attributed to the endogenous nature of HA that should be biocompatible and, when located on the lipoplex surface, might avoid the direct contact of the cationic liposome with the negatively charged cell surface and hence reduce its cytotoxic potential. Finally, HA-DPPE increased the level of transfection on CD44-highly expressing cells (MDA-MB-231 or A549) compared to the cells expressing low levels of CD44 (MCF-7 or Calu-3). The involvement of the CD44 receptors was confirmed by using anti-CD44 Hermes-1 antibody that highly inhibited transfection efficiency; this effect was not observed by nonspecific anti-ErbB2 antibody [19, 20].

HA-coated cationic liposomes were also prepared using an HA-stearylamine (SA) conjugate, and their ability to reach liver endothelial cells was evaluated [45]. The pharmacokinetics and biodistribution studies on HA-SA modified liposomes showed that liver accumulation was higher than the corresponding value for nonmodified liposomes at every time point and increased depending on the extent of modification of HA-SA. On the contrary, if free HA was introduced on liposomes surface, via electrostatic interactions, liver accumulation decreased indicating that HA alone did not fully function as targeting ligand. From confocal microscopy analysis, HA-SA modified liposomes accumulated along the blood vessels to a greater extent than nonmodified liposomes, suggesting that the HA-coated liposomes are distributed within endothelial cells in the liver.

Recently, the complement activation capacity of HA nanoparticles has been investigated [20, 50]. Complement activation is an important aspect to consider since it may initiate adverse reactions among sensitive individuals and could represent an obstacle for the clinical application of HA-decorated nanovectors. Mizrahi et al. evaluated the level of the terminal complement pathway activation markers C5a and SC5b-9 by ELISA on a panel of nanoparticles decorated with HA of different molecular weights (ranging from 6.4 kDa to 1500 kDa). In these experiments, no induction of complement activation was observed, and the marker levels remained comparable with the baseline value [50]. Dufaÿ Wojcicki et al. [20] evaluated the behavior of HA lipoplexes made with increasing lipids:DNA ratio (2, 4, and 6) and the activation of a protein of complement cascade, the protein C3, were determined by 2D immunoelectrophoresis. Low activation of complement was observed in all the formulations although lipoplexes containing HA with, DNA ratios of 4 and 6, give higher values than the respective nonhyaluronate samples [20]. These data suggest that HA-coated nanosystems could be an interesting alternative to PEG grafted particles since the latter were shown to activate complement after intravenous administration [51].

The impact of HA size and density of HA-grafted nanoparticles on affinity toward CD44 was evaluated in a systematic manner [50, 52]. Qhattal and Liu prepared liposomes decorated with HA of both low and high molecular weights (5–8, 10–12, 175–350, and 1600 kDa) and with different degree of grafting density. They performed in vitro studies (fluorescence microscopy analysis, flow cytometric analysis, and competitive binding experiments) and stated that cellular targeting efficiency of HA liposomes depends on HA molecular weight, grafting density, and cell surface CD44 receptor density. In particular, the HA liposomes binding and internalization increased with increasing polymer molecular weight and/or the grafting density [52]. A small library of HA-coated nanoparticles distinguished by the size of their surface HA was also described [50]. The authors used HA of 5 different molecular weights (6.4 kDa, 31 kDa, 132 kDa, 700 kDa, and 1500 kDa) and evaluated the nanoparticles interaction with CD44 receptor through surface plasmon resonance analysis. Also in this case, the affinity towards CD44 was low for LMW-HA and increased with the polymer molecular weight [50].

## 5. Conclusions

HA represents a promising opportunity to develop new cancer therapies. A growing number of scientific works explored the possibility to target cancer cells overexpressing CD44 receptor by using HA-modified vectors. HA is biocompatible, biodegradable, nontoxic and noninflammatory. Moreover, it can easily undergo chemical modifications and conjugates with drugs or other ligands. HA targeting of cancer cells overexpressing CD44 receptor has been largely demonstrated. In
addition, HA coating has been recently proposed as a safer alternative to PEG grafting in order to increase the particles’ half-life. The success of this strategy is demonstrated by an HA conjugate at the moment in clinical trials. A phase III clinical trial based on a hyaluronic acid–Irinotecan conjugate is in the recruitment state, and the final data collection is scheduled for January 2014. The possibility to conjugate HA to lipid-based nanocarriers, such liposomes that are on long time in the clinical practice, should open new opportunities to target cancer cells also with drug that cannot be easily conjugated to HA. Further studies are certainly needed to understand the relations between the molecular weight and “biological” properties of HA, especially in the interaction of HA-modified nanoparticles with the target.

Moreover, further information on the in vivo distribution of HA conjugated nanocarries as well as their tumor localization should be useful to design new anticancer therapies based on CD44 targeting.

References


Review Article

Recent Trends in Multifunctional Liposomal Nanocarriers for Enhanced Tumor Targeting

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Liposomes are delivery systems that have been used to formulate a vast variety of therapeutic and imaging agents for the past several decades. They have significant advantages over their free forms in terms of pharmacokinetics, sensitivity for cancer diagnosis and therapeutic efficacy. The multifactorial nature of cancer and the complex physiology of the tumor microenvironment require the development of multifunctional nanocarriers. Multifunctional liposomal nanocarriers should combine long blood circulation to improve pharmacokinetics of the loaded agent and selective distribution to the tumor lesion relative to healthy tissues, remote-controlled or tumor stimuli-sensitive extravasation from blood at the tumor's vicinity, internalization motifs to move from tumor bounds and/or tumor intercellular space to the cytoplasm of cancer cells for effective tumor cell killing. This review will focus on current strategies used for cancer detection and therapy using liposomes with special attention to combination therapies.

1. Introduction

Liposomes, first described in 1965 [1, 2], are established drug and gene delivery carriers with clinical evidence of efficacy [3–5] and several commercially available approved clinical formulations [6]. Liposomes are lipid vesicles either unilamellar or multilamellar with an aqueous compartment. The structure of liposomes allows for delivery of a cargo loaded in the aqueous compartment or embedded in the lipid bilayer for cancer therapy, noninvasive cancer imaging, or therapy [7, 8]. As recently reviewed [9], the most important property of liposomal nanocarriers is protection from the degradation and optimization of the pharmacokinetics of the encapsulated drug to improve tumor accumulation and therapeutic efficacy while reducing the adverse effects associated with bolus administration [7, 10, 11]. This paper will focus on the use of liposomal nanocarriers in cancer therapy and diagnosis. Cancer therapy targets the hallmark traits of cancer: deregulated cell growth, evasion from apoptosis, sustained angiogenesis, tissue, invasion and metastasis [12]. Liposomes remain one of the first drug delivery carrier tested for improvement of pharmacokinetics of new anticancer drugs with more than 2000 papers and 200 reviews published in 2011 and many liposomal drugs approved for cancer therapy notably Doxil for doxorubicin (Johnson & Johnson, New Brunswick, USA), Lipusu for paclitaxel (Luye Pharma Group, Yantai, China), and Marqibo for vincristine (Talon Therapeutics, South San Francisco, USA) [7, 13–15]. The liposomal platform has undergone continuous optimization for improved stability in vivo, high drug and/or imaging agent loading, stimuli-targeted delivery of the cargo at the tumor site for efficient uptake by cancer cells, and intracellular payload release to engineer multifunctional liposomal nanocarriers (Table 1, Figures 1–3) [16]. We will describe the main axes of design of multifunctional liposomal nanocarriers.

2. Stealth Targeted Liposomes

2.1. Stealth Liposomes. Effective cancer treatment generally implies drug delivery to cancer cells after systemic administration by taking advantage of the leaky tumor vasculature
to deposit at the tumor site [17]. Indeed, liposome uptake by tumors relies primarily on the enhanced permeability and retention (EPR) effect [13, 17–19]. EPR is dependent on large endothelial fenestrations in the tumor endothelial vasculature coupled with the incomplete pericyte coverage that permits extravasation of large molecules and liposomes of size below 200 nm into tumors with an impaired lymphatic drainage that is responsible for their retention [17, 18, 20]. However, after parenteral administration, most liposomes are captured by the mononuclear phagocyte system (MPS) in the liver and spleen [21]. This elimination is due to the recognition by serum proteins (opsonins) and complement components which prime liposomes for macrophage removal from the circulation [21, 22]. The step, required to increase the probability of extravasation at the tumor site, involves extended stabilization, decreased blood clearance, and capture by the MPS to favor their accumulation in tumors (Figure 2) [7, 8, 23].

To achieve this, two approaches are currently used in preclinical and clinical liposomal drug carriers [44]. Decrease of membrane fluidity through incorporation of cholesterol to impede lipid extraction by high density lipoproteins in the blood associated with liposome breakdown (approved formulations DaunoXome, Myocet, Depocyt, Mariqibo, Doxil) [44, 45]. The second approach is the incorporation of flexible hydrophilic moieties, mainly polyethylene glycol (PEG), since this component is approved for use by the United States Food and Drug Administration and is currently used in several approved formulations (Doxil, SPI-077, S-CDK602) [7, 10, 44, 46], but also polyvinyl pyrrolidones [8] or Poly[N-(2-hydroxypropyl)methacrylamide] [47]. The inclusion of flexible hydrophobic inert and biocompatible polyethylene glycol, (PEG) with a lipid anchor in liposome allows the formation of an hydrated steric barrier decreasing liposome interaction with blood-borne component, increasing their blood circulation time, decreasing their spleen and liver capture [48, 49], and their resistance to serum degradation [50]. This lack of recognition by the MPS and decreased elimination of PEGylated liposomes led to the term "stealth" liposomes to qualify them [44].

Protection by PEG was shown to be dependent on both the PEG molecular weight and density on the liposome surface with ~5% by weight, allowing the maximal decrease in protein adsorption and enhanced blood circulation time [51]. Longer blood circulation time, decreased spleen and liver capture, and increased tumor accumulation after intravenous injection have been reported for In-labeled liposomes containing 6% PEG compared to 0.9% PEG [52]. Lee et al. compared the liver and spleen accumulation of Tc-labeled liposomes containing 0, 5, 9.6, or 13.7% PEG (molar ratio) [53]. While 5 or 9.6% PEG decreased spleen and liver accumulation compared to unPEGylated liposomes, spleen accumulation increased again with 13.7% PEG, indicating an upper limit to the effect of PEGylation. When PEG chains of different lengths were appended to the surface of immunoliposomes, as short (750 Da), intermediate (2000 Da), or long PEG (5000 Da), DSPE-PEG2000 was the best compromise for extended blood circulation and target binding in vivo. PEG750 did not improve blood circulation and PEG5000 decreased ligand binding [54].

Similarly, superior interaction of cell penetrating peptide-modified PEGylated liposomes with cells was evidenced in vitro after coupling of the peptide to PEG1000 over PEG750 or PEG3400 and was correlated with the architecture of ligand presentation [55]. The longer blood residency of PEGylated liposomes associated with their lower elimination by the MPS has been correlated with increased tumor accumulation and efficacy [19, 21, 23, 56]. However, liver, spleen, and bone marrow remain the final destinations of empty or drug-loaded PEGylated liposomes [23, 56]. Improvement of drug pharmacokinetics and therapeutic efficacy after encapsulation in PEGylated liposomes was well illustrated by Yang et al. [57]. Indeed, PEGylation of paclitaxel-loaded liposomes led to increased plasma and tumor levels of paclitaxel, in parallel decreased liver and spleen paclitaxel levels over Taxol or conventional paclitaxel liposomes and resulted in the best tumor growth inhibition [57].

Interestingly, albumin conjugation to drug-loaded PEGylated liposomes further enhanced their circulation time and resulting therapeutic activity [58, 59]. Indeed the blood clearance of doxorubicin after intravenous administration in rats decreased from 131 mL/h for free doxorubicin to 17.9 mL/h for PEGylated liposomal doxorubicin and decreased further to 7 mL/h for PEGylated and albumin-conjugated doxorubicin-loaded liposomes. Albumin also decreased opsonin binding to PEGylated liposomes and improved the therapeutic activity of doxorubicin-loaded liposomes against sarcoma.

Inclusion of PEG in the liposome is achieved either by mixing a lipid-anchored PEG with the liposome forming lipids prior to liposome formation (preinsertion) or by insertion of PEG-lipid in already formed liposomes (postinsertion). These two approaches are currently used in clinically approved formulations [44]. Postinsertion of DSPE-PEG2000 compared to its preinsertion in irinotecan-loaded liposomes revealed higher plasma concentration and slower drug release in rats [60]. Of note, this longer blood circulation time was correlated with better therapeutic efficacy of postinserted DSPE-PEG2000 drug-loaded liposomes. Although the lipid-PEG conjugates can be incorporated in liposomes before their formation (preinsertion) or inserted into preformed liposomes, the former strategy induces presentation of the PEG groups both at the liposomal surface and in reverse orientation at the inner side of the lipid bilayer. This results in decreased drug loading and stealth properties of the liposomes. Indeed, when both strategies of PEGylation were compared, higher blood circulation and higher therapeutic efficacy in vivo of postinsertion over preinsertion modification were demonstrated [60, 61].

A new alternative to increase the circulation time of drug-loaded liposomes is the use of superhydrophilic zwitterionic polymers to create a hydrated shell around the liposome [62]. Cao et al. compared the therapeutic activity of two doxorubicin formulations, Doxil where DSPE-PEG2000 imparts blood stability and doxorubicin-loaded liposomes containing the zwitterionic lipid DSPE-poly(carboxybetaine) for the same function. Similar doxorubicin accumulation in tumors after intravenous administration was detected.
Table 1: Examples of multifunctional liposomal nanocarriers.

<table>
<thead>
<tr>
<th>Encapsulated agent</th>
<th>Targeting ligand</th>
<th>Development stage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>None</td>
<td>Approved (Doxil/Caelyx)</td>
<td>[13]</td>
</tr>
<tr>
<td>Vincristine</td>
<td>None</td>
<td>Approved (Marqibo)</td>
<td>[14]</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>None</td>
<td>Approved (Lipusu)</td>
<td>[15]</td>
</tr>
<tr>
<td>Cytarabine and daunorubicin</td>
<td>None</td>
<td>Phase I (CPX-351)</td>
<td>[24]</td>
</tr>
<tr>
<td>Irinotecan and floxuridine</td>
<td>None</td>
<td>Phase I (CPX-1)</td>
<td>[25]</td>
</tr>
<tr>
<td>PKN3 siRNA</td>
<td>None</td>
<td>Phase I (Atu-027)</td>
<td>[26]</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>None</td>
<td>Phase I (NL CPT-11)</td>
<td>[27]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Stomach cancer-specific anti-GAH mAb</td>
<td>Phase I (MCC-465)</td>
<td>[28]</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>Transferrin</td>
<td>Phase II (MBP-426)</td>
<td>[29]</td>
</tr>
<tr>
<td>Liposomal p53 DNA and docetaxel</td>
<td>Anti-Transferrin receptor scFv</td>
<td>Phase I (SGT53-01)</td>
<td>[30]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Thermoresponsive liposomes</td>
<td>Phase III (ThermoDox)</td>
<td>[31]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Cancer-specific 2C5 mAb</td>
<td>preclinical</td>
<td>[32]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Anti-CD22 mAb</td>
<td>preclinical</td>
<td>[33]</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Anti-HER2 mAb</td>
<td>preclinical</td>
<td>[34]</td>
</tr>
<tr>
<td>Vincristine</td>
<td>mBAFF</td>
<td>preclinical</td>
<td>[35]</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>Transferrin</td>
<td>preclinical</td>
<td>[36]</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Transferrin and mannose</td>
<td>preclinical</td>
<td>[37]</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>NSCLC-specific peptide</td>
<td>preclinical</td>
<td>[38]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Metastasis-specific peptide</td>
<td>preclinical</td>
<td>[39]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>MMP-2/9 detachable PEG</td>
<td>preclinical</td>
<td>[40]</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>Folic acid</td>
<td>preclinical</td>
<td>[41]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Estrone</td>
<td>preclinical</td>
<td>[42]</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Chondroitin sulfate</td>
<td>preclinical</td>
<td>[43]</td>
</tr>
</tbody>
</table>

for both formulations, but poly(carboxybetaine) containing liposomes led to an earlier cure of tumor-bearing mice validating this chemistry.

2.1.1. Importance of Charge Neutralization for Passive Targeting. Although neutral non-PEGylated radiolabeled liposomes were shown to accumulate in human tumors [63], PEGylation is required for effective tumor localization. PEGylation protected against aggregation of assemblies made with cationic lipids, enhanced their tumor uptake, and decreased their accumulation in the liver [64]. Campbell et al. compared the biodistribution of negatively charged liposomes (−20 mV) and positively charged liposomes (+31 mV) after intravenous injection to tumor-bearing mice [65]. While liver was the major destination for both formulations with more than 50% of the injected dose, positively charged liposomes showed lower spleen accumulation and higher lung accumulation. Interestingly, in tumors, positively charged liposomes showed higher association with tumor blood vessels than negatively charged ones. Levchenko et al. proposed the modulation of positively and negatively charged liposomes biodistribution by different opsonins [66]. Moreover, neutral PEGylated liposomes encapsulating doxorubicin showed superior therapeutic activity compared to cationic ones the decreased antitumor efficacy was correlated with reduced blood circulation and tumor accumulation of cationic liposomes [67]. A critical correlation between negative liposome charge and uptake by liver and spleen has been reported [66]; charge shielding by PEG decreased liver uptake and prolonged blood circulation. Finally, Huang and coworkers reported abolishment of liver uptake of cationic liposomes after their neutralization by postinsertion of DSPE-PEG leading to an increased tumor accumulation [68].

2.1.2. Importance of Prior Administration/Accelerated Blood Clearance (ABC). Cancer treatments usually imply repeated administration of the same therapeutic agent to previously treated (predosed) patients. Administration of radiolabeled PEGylated liposomes to animals pretreated with a first dose of PEGylated liposomes revealed a drastic decrease of their blood concentration 4 h after injection from 50% of the injected dose for naive animals to 0.6% of the injected dose for predosed animals [69]. Noteworthy, after the second administration, PEGylated liposomes were cleared from the circulation very rapidly (decrease in half-life from 2.4 h to 0.1 h) and this decreased blood residency was mirrored by increased accumulation in liver and spleen, supporting the accelerated blood clearance of liposomes after their second administration. This phenomenon is termed accelerated blood clearance (ABC). ABC is dependent on the time after initial injection: no ABC was reported for PEGylated liposomes injected daily or with injection intervals less than 5 days in rats whereas a one week interval induced accelerated blood clearance in the same study [69]. This delay reflects the two phases of ABC [70, 71]. First, anti-PEG IgM is secreted in the spleen during the effectuation phase [72, 73], an organ where both drug-loaded PEGylated and non-PEGylated liposomes accumulate
Second, during the effectuation phase, opsonisation of PEGylated liposomes by anti-PEG IgM primes them for elimination by liver macrophages [75]. Tagami et al. recently demonstrated that production of anti-PEG2000-DSP IgM in mice after administration of PEGylated lipoplexes was higher with PEGylated liposomes harboring siRNA on their surface over PEGylated liposome-wrapped siRNA lipoplexes [76]. Moreover, the same group reported higher anti-PEG IgM production after parenteral injection of PEGylated DNA lipoplexes prepared with adjuvant CpG motifs-containing pDNA over PEGylated lipoplexes prepared with pDNA devoid of CpG motifs [77]. This lower anti-PEG IgM production from CpG-free lipoplexes was correlated with lower accelerated blood clearance. Both of these studies suggest an important effect of the liposome cargo in anti-PEG IgM production and the ABC phenomenon.

Anti-PEG IgM production is not limited to PEGylated liposomes; anti-PEG IgM was also detected in rats injected with PEGylated adenovirus, bovine serum albumin, or ovalbumin [78]. Interestingly, Laverman et al. reported no ABC induction of Doxil when rats were preinjected with Doxil one week before administration, whereas preinjection with empty PEGylated liposomes induced ABC of Doxil [70]. These data suggest prevention of ABC by doxorubicin entrapment in liposomes. This has been attributed to a decreased clearance capacity of Doxil-injected rats due to toxicity of doxorubicin for liver macrophages [79]. By contrast, Van Etten et al. reported no decrease in bacterial clearance after Doxil injection [80] suggesting a macrophage-independent mechanism. Kiwada and coworkers reported the induction of anti-PEG IgM production in the spleen after administration of PEGylated liposomes priming them for elimination by liver macrophages and also demonstrated decreased ABC in splenectomized rats which was correlated with lower anti-PEG IgM titers [72].

Longer blood circulation of doxorubicin-loaded PEGylated liposomes after a second administration has been observed in mice, dogs, rats, and patients [70, 81–83] and was proposed to be due to toxicity towards splenic B cells [70]. The importance of toxicity in resistance to ABC by Doxil liposomes is supported by the suppression of IgM production after a second administration of oxalipatin-loaded PEGylated liposomes compared to empty PEGylated liposomes [84] and by the evidence of ABC induction with PEGylated topotecan-loaded liposomes that have a fast drug release rate [85]. Additionally, blood clearance of radiolabeled liposomes was inhibited by a preadministration of Doxil whereas preinjection of free doxorubicin or empty liposomes did not inhibit blood clearance [82] further supporting inhibition of the MPS as the mechanism of decreased blood clearance of drug-loaded liposomes.

However, as pointed out recently by Suzuki et al., there is no report yet of ABC in patients [86] although PEGylated liposomes such as Doxil have been in clinical use for more than 20 years suggesting caution in interpretation of the preclinical model data [86]. Indeed, Gabizon et al. recently reported decreased blood clearance of Doxil after repeated administration in cancer patients [81]. The high variability of pharmacokinetics of drug-loaded PEGylated liposomes in cancer patients [87] should also be considered as it may render an ABC phenomenon difficult to detect without a very large cohort. Although complement activation by PEGylated drug-loaded liposomes has been reported both in animal models and in patients (reviewed in [88]), its correlation with accelerated blood clearance is still controversial [89]. Finally, ABC could be decreased after methylation of the anionic charge on the phosphate group of PEG [90] further improving pharmacokinetics of PEGylated liposomes.

2.2. Targeted Stealth Liposomes. As recently reviewed, PEGylation fails to lead to more than 5% of the administered formulation accumulation in the tumor [23, 91]. Furthermore, although radiolabeled liposomes were shown to accumulate in solid tumors in patients, they also distributed to normal organs, revealing the need for tumor targeting [63]. Moreover, most macromolecules, free drugs, and liposomes without an internalization moiety have an accumulation limited to the periphery of a tumor due to the poor vascular density in tumors and the high tumor interstitial fluid pressure impeding transport of macromolecules [92–94]. In a direct comparison of doxorubicin-loaded PEGylated and non-PEGylated liposomes, PEGylation did not improve doxorubicin accumulation in tumors, with comparable therapeutic efficacy of PEGylated and non-PEGylated doxorubicin-loaded liposomes [95]. On the contrary, conjugation of internalizing antibodies with the surface of doxorubicin-loaded PEGylated liposomes dramatically improved their therapeutic efficacy [96, 97] demonstrating the need for improved internalization of antineoplastic agents for effective therapy [98]. Similarly, while Bartlett et al. reported identical tumor distribution of untargeted and transferrin-targeted siRNA nanoparticles, the latter achieved superior in vivo silencing [99].

To increase liposomal drug accumulation in the cancer cells, liposomes must combine small size and long circulation to reach the tumor (tumor site targeting), a targeting ligand to discriminate between cancer cells and supportive cells (cancer cell targeting), and an internalizing moiety for intracellular delivery (Figure 3, Table 2). For a combination of long blood circulation and targeting, the ligand must be accessible to the target for recognition while the liposomal surface should be coated with PEG for long blood circulation [117] (Figure 1). Thus, in addition to protection from steric hindrance of the liposome surface by the PEG chains, presentation of the ligand at the distal end of PEG allows better ligand recognition [117, 118] and multivalent binding thanks to the flexibility of PEG [119]. Such a combination allowed ultimately superior therapeutic activity compared to PEGylated drug-loaded liposomes without ligand [32–34, 118, 120, 121]. The rationale of targeting plus PEGylation for antitumor efficacy has been well demonstrated by Yamada et al. using folate-linked PEGylated liposomal doxorubicin [122]. They compared the in vitro cytotoxicity and in vivo anti-tumor efficacy of untargeted PEGylated doxorubicin-loaded liposomes, non-PEGylated liposomes harboring folate, and PEGylated liposomes with folate exposure at the liposomal surface. While the non-PEGylated folate-modified liposomes
showed the highest toxicity in vitro, the highest antitumor efficacy was reported with PE-Gylated, folate-modified doxorubicin-loaded liposomes. The need for targeted drug delivery for the best antitumor efficacy is not limited to liposomes. Indeed, when Saad et al. compared the therapeutic efficacy of targeted or untargeted paclitaxel delivery using a linear polymer, dendrimer or PE-Gylated liposomes, the best tumor accumulation and tumor suppression were obtained with targeted delivery systems over untargeted ones and free paclitaxel for the three types of carriers [107]. In agreement with targeted delivery systems over untargeted ones and free paclitaxel for the three types of carriers [107]. In agreement with this study, addition of a targeting moiety to PE-Gylated liposomes containing the near infrared probe NIR-797 or [111] in improved tumor accumulation of the imaging agent, suggesting the benefit of targeting stealth liposomes for cancer therapy and monitoring [123]. Several ligands, including antibodies and peptides directed against molecular markers of tumor cells or their supportive endothelial cells present in the tumor microenvironment, have been employed for targeted drug delivery [124] (Table 2).

2.2.1. Antibody-Targeted PE-Gylated Liposomes. Targeted liposomes are obtained either by incorporation of ligand-lipid conjugates during liposome preparation, incorporation of lipids with reactive groups during liposome preparation and subsequent ligand coupling, and finally by insertion of ligand-lipid conjugates into preformed liposomes (postinsertion) [125, 126]. For a comparison of techniques available for antibody conjugation to liposomes we refer the reader to recent reviews [97, 127].

Coupling of the humanized anti-CD22 antibody targeting the lymphocyte marker CD22 to PE-Gyalted doxorubicin-loaded liposomes increased doxorubicin accumulation in Non-Hodgkin’s Lymphoma xenografts and increased survival over untargeted doxorubicin-loaded liposomes [33]. The p185HER2 (human epidermal growth factor receptor 2) receptor is upregulated in human cancers of several histology (breast, ovarian, and prostate) with a low basal expression in normal tissues allows cancer-specific delivery with HER2 monoclonal antibody conjugation [128, 129]. Conjugation of a single-chain fragment antibody against HER2 to doxorubicin-loaded liposomes led to higher doxorubicin accumulation in breast cancer xenografts and better tumor control than untargeted PE-Glylated doxorubicin-loaded liposomes [100]. Conjugation of the recombinant humanized anti-HER2 antibody Herceptin (Genentech, San Francisco, CA, USA) to paclitaxel-loaded PE-Gylated liposomes also increased drug accumulation in tumors and therapeutic efficacy over untargeted paclitaxel-loaded liposomes [34]. The potentiation of paclitaxel-loaded liposomes by HER2 antibody was due to enhanced drug uptake by receptor-mediated endocytosis since a similar tissue distribution and antitumor activity were reported against breast xenografts expressing low levels of HER2. Indeed, in a seminal study, Kirpotin et al. demonstrated that although HER2 antibody-targeted liposomes and untargeted liposomes had similar accumulation profiles in tumors after intravenous injection, they showed, by flow cytometry and histological analysis of disaggregated tumors, a 5.9-fold higher cancer cell accumulation of immunoliposomes versus untargeted liposomes [98]. Antinuclear autoantibodies are present in both healthy elderly individuals and cancer patients [32]. One of these antibodies, 2C5 monoclonal antibody recognizing cell surface-bound nucleosomes specifically recognizes multiple tumor cell lines [32]. Liposomes conjugated with 2C5 antibody at the distal end of PEG3400-DSPE were preferentially accumulated in tumors [32, 130] and increased the therapeutic activity of doxorubicin-loaded (Doxil) liposomes [102].

<table>
<thead>
<tr>
<th>Type of ligand</th>
<th>Ligand</th>
<th>Target</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Anti-HER2</td>
<td>HER2 receptor overexpressed by cancer cells</td>
<td>[34, 98, 100]</td>
</tr>
<tr>
<td></td>
<td>Anti-CD19</td>
<td>CD19 overexpressed in B cell Lymphoma</td>
<td>[101]</td>
</tr>
<tr>
<td></td>
<td>Nucleosome-specific 2C5 mAb</td>
<td>Cancer cells surface-bound nucleosomes</td>
<td>[32, 102]</td>
</tr>
<tr>
<td>Protein</td>
<td>Transferrin</td>
<td>Transferrin receptor overexpressed by cancer cells</td>
<td>[36, 103]</td>
</tr>
<tr>
<td></td>
<td>Interleukin 13 (IL-13)</td>
<td>IL-13 receptor overexpressed in human gliomas</td>
<td>[104]</td>
</tr>
<tr>
<td>Peptide</td>
<td>Octreotide</td>
<td>Somatostatin receptor type 2 overexpressed by cancer cells</td>
<td>[105, 106]</td>
</tr>
<tr>
<td></td>
<td>LHRH-derived peptide</td>
<td>LHRH receptors overabundant on cancer cells</td>
<td>[107]</td>
</tr>
<tr>
<td></td>
<td>Arg-Gly-Asp (RGD)</td>
<td>αVβ3 overexpressed by endothelial tumor cells</td>
<td>[108–110]</td>
</tr>
<tr>
<td>Small molecule</td>
<td>Folate</td>
<td>Folate receptor on cancer cells</td>
<td>[41, 111]</td>
</tr>
<tr>
<td></td>
<td>Estrone</td>
<td>Estrogen receptors overexpressed in ovarian and breast cancers</td>
<td>[42, 112]</td>
</tr>
<tr>
<td></td>
<td>Anisamide</td>
<td>Sigma receptors overexpressed by cancer cells</td>
<td>[113]</td>
</tr>
<tr>
<td>Sugar</td>
<td>Mannose</td>
<td>Dendritic cells and macrophages to induce an immune response</td>
<td>[114, 115]</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td>Asialoglycoprotein receptors overexpressed by hepatocellular carcinomas</td>
<td>[116]</td>
</tr>
</tbody>
</table>

HER2: human epidermal growth factor receptor 2, mAb: monoclonal antibody, LHRH: luteinizing hormone releasing hormone.
lipoosomes was similar, the MT1-MMP-targeted doxorubicin-loaded lipoosomes showed superior tumor protection thanks to enhanced uptake of the drug by tumor cells, in agreement with the results of Kirpotin et al. with anti-HER2 targeted lipoosomes [98].

The conjugation of whole antibodies to the liposome surface can induce complement activation and decrease their blood circulation since the Fc fraction of immunoglobulins is recognized by macrophages [45, 131]. Thus conjugation of Fab' fragments instead of the whole antibody was proposed. While doxorubicin-loaded PEGylated immunoliposomes harboring Fab' fragments of an anti-CD19 antibody had similar blood circulation and MPS accumulation than untargeted lipoosomes, immunoliposomes harboring the anti-CD19 IgG showed faster blood clearance and a threefold accumulation in liver and spleen over untargeted or Fab' lipoosomes [101]. Fab' immunoliposomes also resulted in superior therapeutic efficacy over untargeted or anti-CD19 antibody-decorated immunoliposomes [101]. Analogous with their results, the blood circulation of pH-sensitive 1-D-arabinofuranosylcytosine-loaded lipo-somes harboring Fab' fragments against CD33 was superior to those decorated with the whole monoclonal antibody [121].

2.2.2. Protein-Targeted Liposomes. Qi et al. described a novel antineoplastic liposomal agent, liposomal saposin C [132]. Development of this agent is based on the observation that patients suffering from lysosomal storage diseases frequently have saposin C deficiencies leading to accumulation of toxic glycosylceramide sphingolipids [133] and that saposin C inserts into negatively charged membranes at acidic pH [134]. They prepared a saposin C-DOPS conjugate which assembled as 190 nm lipo-somes under sonication at acidic pH. Tumor targeting is based on activation of membrane fusion domains of saposin C at the acidic pH in tumors leading to its internalization and glycosylceramide-induced apoptosis. Intravenous injection into neuroblasto-toma xenograft-bearing mice led to apoptosis induction in tumors and tumor growth inhibition without systemic toxicity. BAFF (B cell activating factor) is a cytokine whose receptor is overexpressed in B-cell lymphomas, conjugation of a BAFF mutant to vincristine-loaded PEGylated liposomes increased the survival of lymphoma-bearing mice over untargeted vincristine-loaded lipo-somes or free drug [35]. Cancer cells overexpress transferrin receptors [135] making the glycprotein, transferrin or antibodies to transferrin receptor, suitable ligands for tumor targeting [136]. Addition of transferrin to the surface of PEGylated oxaliplatin-loaded lipo-somes increased tumor accumulation over free oxaliplatin or untargeted lipo-somes leading to the highest tumor growth inhibition against C26 colon carcinoma-bearing mice [36]. In parallel to these studies, conjugation of transferrin to doxorubicin-loaded lipo-somes resulted in higher doxorubicin delivery to tumors and tumor growth inhibition over untargeted doxorubicin-loaded lipo-somes [103].

2.2.3. Peptide-Targeted Liposomes. More and more tumor-specific ligands are being identified by combinatorial screening of bacteriophage-borne peptide libraries, phage display biopanning. This is a strategy whereby the recombinant virions able to bind cancer cells in vitro or tumors in vivo are purified before identification of the peptide and its use for targeted drug delivery, allowing identification of peptides specific for cancer cells, tumor vasculature or both (reviewed in [137]).

We previously described the selective exposure of nucleohistones by cancer cells effective cancer therapy of antinuclear-targeted doxorubicin-loaded liposomes [32]. In good agreement with these studies, Wang et al. reported tumor targeting of doxorubicin-loaded liposomes harboring the histone H1-specific peptide ApoPep-1 [138]. This peptide is selectively presented at the surface of tumor cells due to spontaneous apoptosis in avascular tumors. ApoPep-1 conjugation to doxorubicin-loaded lipo-somes led to superior doxorubicin distribution in lung xenografts and better tumor growth inhibition over untargeted liposomes. Somatostatin receptors, particularly somatostatin receptor type 2, are over-expressed by cancer cells and endothelial cells of the tumor vasculature [139]. Coupling of the somatostatin receptor type 2 agonist to irinotecan-loaded liposomes improved their anti-tumor activity in a medullary thyroid carcinoma model [105]. Its coupling to PEGylated doxorubicin-loaded liposomes led to superior doxorubicin accumulation in tumors and enhanced anticancer efficacy against small cell lung cancer tumors compared to untargeted liposomes [106].

Han and coworkers selected a peptide (HVGGSSV) by phage display which selectively bound to the tumor vasculature of tumors that were regressing after radiotherapy, while no binding was detected before irradiation or in areas of tumor necrosis factor alpha-induced inflammation in mice [140]. They proposed the peptide that recognized a protein displayed only on tumor endothelial cells that were responding to therapy. Interestingly, they conjugated this peptide to the surface of doxorubicin-loaded lipo-somes for "radiation-guided tumor-targeted drug delivery" [141]. Higher tumor accumulation of doxorubicin was achieved with targeted liposomes after irradiation over untargeted doxorubicin-loaded liposomes with or without irradiation and resulted in higher therapeutic efficacy in both Lewis lung carcinoma and non-small cell lung carcinoma (HL460) tumors. Identification of a non-small cell lung cancer-specific peptide also identified by phage display to doxorubicin or vinorelbine-loaded PEGylated liposomes enhanced drug distribution to tumors and resulted in increased therapeutic efficacy over untargeted drug-loaded liposomes [38]. Another group reported higher therapeutic efficacy against lung cancer xenografts of PEGylated doxorubicin-loaded liposomes conjugated with a large-cell cancer-specific peptide over untargeted doxorubicin-loaded liposomes [142].

Breast cancer-specific peptide/phage fusion coat protein pVIII chimeras have been used for tumor-targeted drug delivery [143, 144]. Membranophic major phage coat protein pVIII fused with a targeting peptide identified by phage display spontaneously inserts into liposomes. The insertion of a
of doxorubicin-loaded liposomes increased doxorubicin accumulation in ovarian xenografts by 2.86-fold over untargeted doxorubicin-loaded liposomes with decreased liver and spleen capture though binding to the LHRH receptors highly overexpressed in ovarian cancer [152]. The basic fibroblast growth factor (bFGF) receptor is also overexpressed in several cancers [153]. Electrostatic coating of cationic liposomes encapsulating doxorubicin or paclitaxel with a negatively charged bFGF-derived peptide resulted in increased survival of melanoma or prostate tumor-bearing mice over untargeted liposomal formulations, respectively [154]. The use of chondroitin sulfate which binds CD44 overexpressed by tumor cells has recently been introduced [43]. Coupling of chondroitin sulfate to the surface of etoposide-loaded liposomes increased etoposide accumulation in breast cancer xenografts after intravenous injection 40-fold compared to free drug and by 8-fold compared to untargeted liposomes. Presentation of lactose at the surface of doxorubicin-loaded PEGylated liposomes using a lactose-DOPC conjugate to target the asialoglycoprotein receptors overexpressed in hepatocellular carcinomas increased doxorubicin accumulation in tumors and resulted in tumor growth inhibition over untargeted doxorubicin-loaded liposomes [116].

Tan and coworkers introduced ternary nucleic acid complexes, Liposome Polycation DNA (LPD) where nucleic acids are complexed by protamine before interaction with cationic liposomes to form a core nucleic acid complex surrounded by two lipid bilayers [155]. Sigma receptors are ion channel regulators overexpressed in several cancer types [156]. Conjugation of the small molecular weight sigma receptor ligand anisamide, [157] to the distal end of PEG2000-DSPe led to higher 70–80% luciferase silencing in an experimental lung metastasis model [113]. Moreover, parenteral injection of anisamide-armed LPD prepared with a combination of siRNA against the inhibitor of p53, MDM2 (Murine Double Minute 2), against the Cmyc oncogene and the other against the angiogenesis regulator, VEGF (Vascular Endothelial Growth Factor) were localized in tumors and allowed a 70–80% decrease in tumor load [68]. However, while the common sigma receptor agonist haloperidol and anisamide recognize sigma receptor type 1 and 2, only sigma receptor type 2 overexpression has been reported to be a prognostic indicator [158]. The latter has low expression in healthy tissues, suggesting a higher therapeutic index of sigma receptor 2 targeted therapies [158]. Indeed, binding of the sigma 2 receptor agonist SV119 to its receptor induced cell death in vivo in a pancreatic cancer model, and conjugation of SV119 to the surface of liposomes increased their uptake in vitro in cell lines including lung, breast, and prostate cancer carcinoma whereas no increased uptake in normal cells was reported [158].

3. Biological Targets

3.1. Brain Tumor Targeting. Brain tumors are a major concern for both primary brain and brain metastases from primary lung, melanoma, breast, and kidney cancers [159]. Therapy against brain cancers is challenging since the brain is largely accessible. Therefore, searching for effective therapeutic approaches is essential. Several strategies have been explored to target the brain, including the use of tumor-specific markers and the development of deliverable systems. In this regard, ligand-targeted drug delivery systems, such as PEGylated liposomes, have shown promise. The use of folate-targeted liposomes for the treatment of brain tumors has been extensively studied, as folate receptors are overexpressed in many primary brain tumors. Folate-targeted liposomes have been shown to exhibit increased binding and accumulation in tumor tissues compared to free drug. A similar approach has been taken with the use of ligands targeting other markers overexpressed in brain tumors, such as 

\[\text{LHRH} \rightarrow \text{LHRH receptor} \rightarrow \text{tumor cells} \]

This interaction allows for the selective delivery of the encapsulated drug to the tumor site, resulting in improved therapeutic efficacy. However, the development of ligands with high specificity and affinity towards brain tumor targets remains a challenge. The use of combinatorial approaches, such as the use of multiple ligands, has shown promise in increasing the efficiency of targeted drug delivery. The combination of different ligands targeting different brain tumor markers can further increase the selectivity and efficacy of targeted drug delivery systems. Other strategies for brain tumor targeting include the use of the EGF (Epidermal Growth Factor) receptor, which is overexpressed in many brain tumors, and the use of small molecule ligands such as anisamide, which targets the sigma receptor. These ligands can be coupled to liposomes or nanoparticles to enhance their targeting ability and improve therapeutic efficacy.

Another approach involves the use of charged bFGF-derived peptides to target the surface of liposomes. These peptides can increase doxorubicin accumulation in breast cancer xenografts after intravenous injection, resulting in increased survival of mice over untreated liposomes. The use of chondroitin sulfate, a ligand that binds CD44, has also been shown to increase the accumulation of liposomal drugs in tumor tissues. These ligands can be coupled to the liposomes to increase their specificity and efficacy.

In summary, the development of targeted drug delivery systems for the treatment of brain tumors is an active area of research. The use of ligands targeting specific brain tumor markers, such as folate, EGF, and sigma receptors, has shown promise in increasing the selectivity and efficacy of targeted drug delivery. However, further development is needed to improve the specificity and affinity of these ligands, as well as to develop new ligands with high specificity towards brain tumor targets. The combination of different ligands and the use of charged bFGF-derived peptides are promising approaches that could improve the targeted delivery of drugs to brain tumors.

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2.2.4. Small Molecule-Mediated Tumor Targeting. Aberrant tumor growth is correlated with a greater demand for nutrients relative to healthy organs and has been exploited for tumor targeting. To sustain their rapid growth, tumor cells overexpress folate receptor to capture the folate required for DNA synthesis [149]. The overexpression of folate receptor in cancers of several histology relative to normal tissues, the low cost of folic acid (FA), and the vast library of conjugation reactions available make it one of the most used ligands for tumor-targeted drug delivery and tumor imaging (reviewed in [150]). Inclusion of a FA-PEG-DSPe conjugate into irinotecan-loaded liposomes enhanced drug concentration in tumors after intravenous injection over untargeted liposomes or free irinotecan resulting in the highest anticancer activity without detected side toxicity [41]. Similarly, folate-targeting of doxorubicin-loaded liposomes increased the survival of tumor-bearing mice by 50% over untargeted liposomes [111]. Lee et al. used tetraiodothyrocyclic acid, a competitive inhibitor of thyroid hormone binding to the endothelial cell integrin αvβ3, as a new ligand for tumor-targeted drug delivery. This ligand increased liposomal accumulation in tumors after intravenous injection and enhanced anticancer activity of the encapsulated anticancer drug edelfosine [151].

Estrogen receptors are often overexpressed in breast and ovarian cancers and conjugation of the ovarian estrogenic hormone estrone to doxorubicin-loaded liposomes resulted in a dramatic increase in doxorubicin accumulation in breast tumors after intravenous injection over free drug or untargeted PEGylated doxorubicin-loaded liposomes (24.3 and 6.0-fold, resp.) resulting in the highest therapeutic activity [42, 112]. Similarly, conjugation of a luteinizing hormone-releasing hormone (LHRH) analog to the surface of docetaxel-loaded liposomes increased docetaxel accumulation in ovarian xenografts by 2.86-fold over untargeted docetaxel-loaded liposomes with decreased liver and spleen capture though binding to the LHRH receptors highly overexpressed in ovarian cancer [152]. The basic fibroblast growth factor (bFGF) receptor is also overexpressed in several cancers [153]. Electrostatic coating of cationic liposomes encapsulating doxorubicin or paclitaxel with a negatively charged bFGF-derived peptide resulted in increased survival of melanoma or prostate tumor-bearing mice over untargeted liposomal formulations, respectively [154]. The use of chondroitin sulfate which binds CD44 overexpressed by tumor cells has recently been introduced [43]. Coupling of chondroitin sulfate to the surface of etoposide-loaded liposomes increased etoposide accumulation in breast cancer xenografts after intravenous injection 40-fold compared to free drug and by 8-fold compared to untargeted liposomes. Presentation of lactose at the surface of doxorubicin-loaded PEGylated liposomes using a lactose-DOPC conjugate to target the asialoglycoprotein receptors overexpressed in hepatocellular carcinomas increased doxorubicin accumulation in tumors and resulted in tumor growth inhibition over untargeted doxorubicin-loaded liposomes [116].

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isolated from the rest of the body by the blood brain barrier (BBB), a dense barrier of endothelial cells, pericytes, astrocytes, and extracellular matrix which limits molecular transport into the brain [160]. Several strategies to overcome this barrier have been proposed for the treatment of brain tumors, either by targeted delivery of drug-loaded liposomes to the brain or by remote-controlled drug release within the brain.

Overexpression of IL-13 receptors has been reported in human gliomas [161], and conjugation of IL-13 to doxorubicin-loaded liposomes allowed a 5-fold reduction in tumor volume and extended survival of intracranial glioma tumor-bearing mice over untargeted doxorubicin-loaded liposomes [104]. In the same vein, the conjugation of IL-13 to PEGylated doxorubicin-loaded liposomes for astrocytoma targeting dramatically improved brain delivery of doxorubicin compared to untargeted liposomes and resulted in increased survival of intracranial U87 glioma-bearing mice after intraperitoneal administration [104]. To reinforce brain drug delivery, Du et al. armed PEGylated topotecan-loaded liposomes with both wheat germ agglutinin for brain capillary targeting and tamoxifen to decrease drug efflux [162]. These dual-targeted liposomes crossed a model BBB in vitro and increased the survival of brain tumor-bearing rats over free topotecan or untargeted topotecan-loaded liposomes [162]. The need for dual-targeting for effective BBB crossing in vivo is also exemplified in a study by Ying et al. [163]. They took advantage of the expression of glucose transporter 1 and transferrin receptor by endothelial cells of the BBB for intracranial glioma therapy using mannose and transferrin dual-targeted daunorubicin-loaded liposomes. Dual-targeting led to superior tumor growth inhibition and increased life span over untargeted or single-targeted daunorubicin-loaded liposomes.

Gong et al. used thermosensitive doxorubicin-loaded PEGylated liposomes capable of releasing 90% of drug after 30 min at 42°C compared to less than 3% for unsensitive liposomes [164]. They reported improved doxorubicin delivery to the brain after intravenous injection (3.4-fold over nonsensitive liposomes) and increased survival of C6 glioma-bearing mice when heads of mice were heated in a water bath to 42°C after injection [164]. Another physically controlled content release strategy has been described by the group of Yang using focused ultrasounds for reversible disruption of the BBB as evidenced by higher brain accumulation of Evan's blue or gadolinium in ultrasound-treated animals over untreated ones [165]. Administration of brain tumor-targeted doxorubicin-loaded liposomes followed by ultrasound-mediated BBB disruption allowed higher levels of intracranial liposomes and doxorubicin accumulation over untargeted liposomes in an intracranial glioblastoma model [166].

3.2. Vasculature Targeting. The “angiogenic switch,” when tumors establish their own blood supply by extensive neoangiogenesis, is critical for the progression of tumors from a dormant avascular nodule to an invasive carcinoma [167, 168]. This dependence on blood supply for tumor growth and the correlation between vascular permeability and accumulation of liposomal drug and therapeutic efficacy [169–171] supports research on liposomal tumor vasculature-targeting for cancer therapy (reviewed in [172]). After intravenous injection in mice, PEGylated liposomes were shown to accumulate in the perivascular space with limited tumor penetration [94, 173, 174]. Moreover, when the tumor accumulation and therapeutic efficacy of PEGylated liposomal oxaliplatin were compared in animals bearing C26 colon carcinoma, Lewis lung carcinoma and B16BL6 melanoma, a correlation among tumor blood vessel permeability, tumor drug accumulation and the resulting therapeutic efficacy have been reported [171]. In vitro results were not predictive of in vivo activity: the least tumor accumulation and tumor growth were detected in B16BL6 tumors, whereas this cell line was the most sensitive to liposomal oxaliplatin in vitro, [171]. Of note, the lower tumor vessel permeability of melanoma xenografts compared to colon or lung carcinoma is clinically relevant. When the microvessel density of biopsies from cancer patients was determined, melanoma was also the least vascularized (~35 vessels/field) compared to colon (~70) or lung tumors (~127), stressing the point that extravasation of agents from the tumor vasculature is a major barrier for liposomal drug delivery [175].

Targeting of selectin on endothelial cells with P-selectin glycoprotein ligand 1 allowed a 3-fold higher luciferin delivery to B16F10 tumors after intravenous injection over untargeted liposomes [176]. The α5β3 integrin is overexpressed by endothelial cells in the tumor vasculature [177]. The tripeptide Arg-Gly-Asp (RGD) and the cyclic RGD (Arg-Gly-Asp-D-Phe-Lys) are α5β3 ligands used for tumor-targeted drug delivery [108]. RGD-targeted paclitaxel or doxorubicin-loaded PEGylated liposomes showed superior therapeutic activity over free drug or untargeted liposomes [109, 110]. Antitumor activity of RGD-targeted liposomes is consistent with tumor microvessel destruction after injection of RGD-targeted paclitaxel-loaded liposomes reported by another group [178]. Functionalization of doxorubicin-loaded liposomes with a peptide targeted to bombesin receptors overexpressed in cancers improved therapeutic efficacy over untargeted liposomes [179]. α5β1 integrin is another integrin overexpressed in cancer in which the fibronectin-derived peptide antagonist ATN-161 showed antineoplastic and antimetastatic properties [180]. Coupling of ATN-161 to doxorubicin-loaded PEGylated liposomes increased their therapeutic activity in a melanoma model [181]. Doxorubicin-loaded PEGylated liposomes were functionalized with a NGR peptide at the distal end of PEG to target a CD13 isofrom overexpressed in the tumor neovasculature [182–184]. In the study by Pastorino et al., vasculature-targeted Caelyx showed superior apoptosis induction in tumor xenografts and decreased blood vessel density leading to increased survival of mice bearing lung, ovarian, or neuroblastoma xenografts compared to untargeted Caelyx [182].

To further improve the destruction of blood vessel support of tumors, Takara and coworkers recently developed a dual-ligand approach for antiangiogenic therapy using
lipoosomes targeted to CD13 (NGR-PEG2000-DSP) functionalized with the stearylated cell penetrating peptide tetra-arginine at the liposome surface [183]. They first compared endothelial cell association in vivo in tumor-bearing mice after intravenous injection of PEGylated doxorubicin-loaded liposomes measuring either 100 nm (small liposomes) or 300 nm (large liposomes). Since a superior association with tumor blood vessels and lower extravasation was observed with large liposomes over small ones, they used the former for ligand conjugation. Dual-ligand labeled liposomes accumulated ~3-fold more in tumors than unmodified or single ligand-modified liposomes, revealing synergy of the two ligands. Consistent with the tumor accumulation and blood vessel association results, only the dual-ligand doxorubicin-loaded liposomes allowed protection against tumor growth and induced tumor blood vessel destruction that revealed a synergy of endothelial cell targeting and enhanced uptake for antiangiogenic therapy.

Cationic liposomes selectively bind to endothelial cells in vivo with superior internalization over anionic or neutral liposomes due to the enrichment of tumor endothelial cell membranes with negatively charged lipids and heparan sulfate proteoglycan [172, 185, 186]. Superior accumulation of oxaliplatin in lung tumors was obtained after intravenous injection of PEG-coated cationic drug-loaded liposomes over neutral liposomes [187]. The same group used cationic liposomes for delivery of siRNA against the neoangiogenesis regulator, Argonaute 2 (Ago2) which resulted in Ago silencing in tumors together with apoptosis of tumor blood vessels and decreased tumor growth while no therapeutic effect was observed with cationic lipoplexes prepared with an irrelevant siRNA [188, 189]. In support of the effect of the negative charge of angiogenic vessels, paclitaxel-loaded cationic liposomes (EndoTAG-1) induced endothelial cell apoptosis in vivo, retarded melanoma and pancreatic carcinoma tumor growth, and decreased the number of melanoma lung metastases in vivo [190–192]. Recently, targeting of tumor vasculature by an aptamer directed against the tumor vasculature marker E-selectin has been reported [193]. E-selectin aptamer conjugated liposomes accumulated in the tumor vasculature of breast cancer xenografts after intravenous injection, whereas untargeted liposomes were detected in tumors, supporting use of this selective approach for vasculature-targeted drug delivery. The vasculature-targeting group used may be relevant only to a particular histology. Indeed, while the p15-RGD peptide which recognizes platelet-derived growth factor receptor β expressed by pericytes of the tumor vasculature identified by phage display against pancreatic cancer increased delivery of liposomes to pancreatic tumors in vivo, it did not direct liposomes to tumors in a melanoma model [194, 195]. In the same study, liposomes harboring p46-RGD α5-integrin binding peptide targeting tumor endothelial cells allowed a significant tumor accumulation over controls with higher therapeutic efficacy [195]. Chang et al. also used phage display to identify neovasculature peptides which when conjugated to doxorubicin-loaded liposomes increased doxorubicin delivery to tumors and therapeutic efficacy over untargeted PEGylated doxorubicin-loaded liposomes [196].

Pericytes are a critical conjunctive component of vasculature; aminopeptidase A (APA) has been identified as a marker of pericytes from orthotopic primary and metastatic (ovary) neuroblastoma in mice [197]. Coupling of a peptide ligand of APA to doxorubicin-loaded liposomes increased doxorubicin accumulation in neuroblastoma tumors over untargeted doxorubicin with better therapeutic activity demonstrating that pericytes are another critical target within the vasculature [198]. Moreover, coadministration of APA-targeted doxorubicin-loaded liposomes and aminopeptidase N (APN, a marker of tumor endothelial cells) targeted doxorubicin-loaded liposomes led to superior doxorubicin accumulation in tumors over either targeted formulation alone [198]. The destruction of perivascular and endothelial cells in tumors resulted in a significant increase in survival of neuroblastoma-bearing mice over either endothelial cell-targeted or pericycle-targeted liposomes alone [198].

Tumor lymphatics are also a therapeutic target since they support lymph node metastasis [199]. Indeed, lymph node invasion is frequent in melanoma and is an indicator of poor prognosis [200]. Laakkonen and coworkers identified a tumor lymphatics-binding peptide (LyP-1) which, after intravenous injection in breast carcinoma-bearing mice, was shown to accumulate in hypoxic areas of primary tumors, colocalize with lymphatic markers in primary tumors and lymph node metastases leading to tumor growth reduction and a decreased number of lymphatic vessels [201, 202]. Interestingly, presentation of this peptide on doxorubicin-loaded liposomes increased tumor accumulation and therapeutic efficacy over untargeted liposomes and decreased lymph node metastasis rate and growth [201, 203–205].

A combination of targeting ligands may be needed for effective antiangiogenic therapy. Murase et al. demonstrated synergy in association with endothelial cells in vitro by liposomes modified with two angiogenic vessel-targeted peptides (APRPG and GNGRG) identified by phage display and revealed the more intense association with tumor blood vessels in vivo of dual-targeted liposomes over single-modified liposomes [206]. Similarly, Meng et al. demonstrated synergy in tumor growth inhibition of non-small cell lung cancer of PEGylated paclitaxel-loaded liposomes targeted to tumor vasculature by both RGD and a neuropilin 1-specific peptide over untargeted or single-targeted liposomes [207]. These results are in accordance with the increased detection of
3.3. Targeting and Inhibition of Metastasis. Metastasis is the ultimate stage of clinical cancer and is the stage with the least survival. Treatment of metastasis is challenging because micrometastatic foci are hard to detect and more aggressive than the primary tumors [208]. Elimination of metastases is thus of utmost importance to prevent cancer recurrence after chemotherapy or surgical removal of the primary tumor. Platelets have been proposed as shuttles for tumor cell metastasis by formation of platelets-tumor cell aggregates [209, 210]. This is consistent with the elevated platelet counts in patients with advanced cancer [210]. Therefore, Wenzel et al. used PEGylated liposomes to code deliver the haemostatic inhibitor dipyridamole (DIP) and the cytotoxic drug perifosine (OPP) to inhibit platelet-tumor cell aggregate formation and kill tumor cells, respectively [211]. OPP/DIP coloaded liposomes inhibited aggregation of platelets, decreased formation of platelet-tumor cell aggregates in vitro and decreased the number of experimental lung metastases when intravenously injected 6 h before parenteral injection of tumor cells. The metastasis-specific peptide TMPT1 [212] recognizes highly metastatic primary tumors and metastases of prostate, breast, and lung cancers relative to their nonmetastatic counterparts. Conjugation of this peptide to doxorubicin-loaded liposomes led to deeper tumor penetration and greater induction of apoptosis with superior tumor growth inhibition against highly metastatic breast cancer xenografts [39]. PAR-1 (Protease Activated Receptor 1), a thrombin receptor, is a major regulator of metastasis in melanoma through its roles in matrix degradation and angiogenesis [213]. Villares et al. reported for the first time a dramatic antimelanoma therapeutic activity after systemic delivery of PAR-1 siRNA-loaded neutral DOPC liposomes with tumor weight reduction and a decrease in experimental lung metastatic colonies [214]. This was achieved via down-regulation of promoters of angiogenesis (VEGF and IL-8) and invasion (MMP-2) together with decreased tumor blood vessel density (decreased CD31 staining).

3.4. Immune Cell Targeting. For therapeutic vaccination against cancer, patient’s immune cells are stimulated by tumor cell antigens. Since the development of effective adaptive immune responses by CD4+ T cells or CD8+ T cells with cytotoxic activity (Cytotoxic T Lymphocytes, CTL) requires their activation by dendritic cells (DCs) that present tumor antigen peptides [215], their targeting is of therapeutic relevance [215–217]. Altin’s group used a chelator lipid [Nickel/3(nitrilotriacetic acid)-ditetradecylamine], (Ni-NTA3-DTDA) for functionalization of liposomes with histidine-tagged peptides though polyhistidine binding to nitrilotriacetic acid in the presence of nickel [218, 219]. For antigen delivery, Ni-NTA3-DTDA functionalized liposomes were prepared by preinsertion before conjugation with histidine-tagged peptides derived from ICAM4 (Intercellular Cell Adhesion Molecule 4), a ligand of the murine dendritic cell (DC) integrin CD11c/CD18 [220]. Ovalbumin-loaded PEGylated liposomes decorated with DC-targeting peptides distributed to splenic DC in vivo, induced an adaptive immune response against, ovalbumin and exhibited dramatic therapeutic activity against established B16-OVA melanoma...
tumors with complete tumor regression in 80% of treated mice [218].

In other studies Altin’s group reported on DC-targeted gene delivery in vivo and potent antitumor effects in the B16-OVA melanoma model after liposome functionalization with histidylated flagellin, the major constituent of the bacterial flagella, recognized by the Toll Like Receptor 5 that leads to their activation [221, 222]. LPR (Lipid-Polymer-RNA) mannosylated and histidylated lipopolyplexes loaded with MART1 (Melanoma Antigen Recognized by T cells 1) mRNA delayed the progression of B16F10 melanoma more effectively than untargeted LPR [223]. This study also illustrated the importance of cytosolic delivery of nucleic acids for in vivo transfection of DC. The authors used a ternary formulation of mRNA or pDNA coding for the reporter gene EGFP (Enhanced Green Fluorescent Protein) complexed with PEGylated histidylated poly-L-Lysine and imidazole-rich liposomes, both of which promote endosomal escape [224, 225]. While no in vivo transfection of splenic DC was observed with pDNA, 12% were transfected with mRNA mannosylated LPR and 3% with untargeted LPR demonstrating that nuclear delivery is a limiting step for DC transfection. Liposomes targeted to dendritic cells by mannosylated ligands have recently been used as a platform for effective cancer immunotherapy [114]. The liposomes used harbored mannosylated ligands at their surface for targeting of antigen presenting cells with a cytotoxic T lymphocyte peptide of the renal carcinoma antigen ErbB2 for induction of an adaptive immune response, Toll Like Receptors (TLRs) agonists as adjuvants and a T helper lymphocyte epitope peptide for improved immune activation. Of note, the authors developed new functionalized lipid anchors devoid of adjuvant activity for their study: dipalmitoylglyceryl maleimide and dipalmitoylglycerol bromoacetate. These liposomes induced an adaptive immune response against the ErbB2 antigen with high therapeutic activity. Targeting of intraperitoneal macrophages by ovalbumin-loaded liposomes armed with dipalmitoylphosphatidylethanolamine conjugated mannose increased antigen-specific cell lysis induction by splenocytes over untargeted liposomes resulting in therapeutic efficacy both as a preventive and therapeutic cancer vaccine [115]. In addition to carrying tumor antigens, liposomal vaccines are armed with immunostimulatory lipids, usually derived from microorganisms, recognized by pathogen recognition receptors leading to immunostimulation (reviewed in [226]). Zhong et al. compared the antitumor efficacy of a basic Fibroblast Growth Factor (bFGF) vaccine in a mouse melanoma model when administered as a Freund's adjuvant mixture, in cationic liposomes, or cationic liposomes containing 0.25% of monophosphoryl lipid A as adjuvant [227]. They reported higher anti-bFGF IgG titers and higher pulmonary metastasis inhibition in mice treated with monophosphoryl lipid A bFGF-loaded liposomes over cationic liposomes or a bFGF/Freund's adjuvant mixture without the toxicity associated with administration of free adjuvants.

Selective depletion of tumor supporting cells represents another approach to cell-specific cancer therapy [228]. The tumor environment is enriched in tumor supporting cells among the tumor-associated macrophages that constitute a predominant inflammatory population involved both in resistance to therapy and metastasis [228]. Dichloromethylenediphosphonate (DMDP) liposomes induced macrophage depletion after intravenous injection in mice [229]. Intradermal injection of DMDP liposomes into the tissues surrounding melanoma or squamous cell carcinoma tumors led to a decrease in tumor-associated macrophages content and tumor rejection [230].

Ligand density was shown to influence both drug retention and target recognition. Zhang et al. demonstrated increase in liposome uptake in vitro as the ligand density was increased from 0% to 1, 3, and 5% demonstrating enhanced ligand recognition [231]. However, increase of in vitro drug release as a function of DSPE-PEG-RGD ligand moiety has been reported by others [232]. Moreover, Saul et al. evidenced increase of nonspecific uptake in vitro with ligand density [233]. Consistent with their results, lower tumor accumulation of NGR (Asparagine-Glycine-Arginine) vasculature targeted liposomes has been evidenced in vivo with liposomes harboring 2.56% mole NGR-PEG-DSPe than 0.64% mole NGR-PEG-DSPe [234]. Altogether, these data suggest the use of the lowest targeting ligand density allowing target binding for effective anticancer therapy.

4. Liposomes for Combination Therapy

The prevalence of drug resistance in cancer patients, both prior to treatment and de novo [235, 236], fueled the application of drug combinations to treat cancer as an alternative to increased doses of chemotherapeutics associated with life threatening sideeffects [237–239].

Codelivery was well illustrated in a study by Chen et al. [240]. Using LPH-NP (liposome-polyconjugated-hyaluronic acid) nanoparticles targeted by postinsertion of DSPE-PEG-GC4 (scFv selected by phage display against ovarian tumors [241]), they codelivered 3 different siRNA and one miRNA and obtained a 80% decrease in tumor load after treatment. They simultaneously targeted proliferation pathways with Cmuc siRNA and miR34a miRNA [242, 243], apoptosis with MDM2 siRNA [244], and angiogenesis using VEGF siRNA [245]. Liposomal codelivery of siRNA against the apoptosis regulator Mcl-1 (Myeloid cell leukemia sequence 1) and of the MEK (Mitogen-activated Extracellular Kinase) and apoptosis resistance inhibitor PD0325901 enhanced tumor growth inhibition compared to each treatment alone [246]. The same group also developed trilysinoyl oleyamide (trily-sine peptide linked to oleyamine by a peptide bond) based PEGylated liposomes for codelivery of Mcl-1 siRNA and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) [247]. Intravenous administration increased the tumor growth delay compared to liposomes with SAHA and an irrelevant siRNA. Likewise, Xiao and coworkers used targeted liposomes to codeliver doxorubicin and DNA encoding a dominant mutant of survivin [248]. Liposomes were targeted by a truncated basic fibroblast growth factor (tbFGF) peptide recognizing the bFGF receptor upregulated
in lung cancers and contained doxorubicin and pDNA encoding for a dominant negative mutant of survivin to counter survivin-mediated apoptosis resistance [249]. Their codelivery produced a higher therapeutic efficacy against Lewis lung carcinoma tumors than liposomes with either agent alone.

A further step in combination of an antineoplastic agent with modulation of drug resistance was achieved recently by Minko and coworkers [250] by formulation of peptide-targeted liposomes containing doxorubicin or cisplatin together with oligonucleotides against the two main drug resistance mechanisms Bcl-2 and MDRI. The efficacy of this “combined targeted chemo and gene therapy” system was evaluated in xenografts established from human ovarian malignant ascites. While inclusion of either Bcl-2 or MDRI antisense oligonucleotides in cisplatin or doxorubicin-loaded targeted liposomes decreased primary tumor volume and intraperitoneal metastases load, further inhibition of tumor growth inhibition was obtained with targeted liposomes containing doxorubicin or cisplatin, Bcl-2 and MDRI antisense oligonucleotides together with complete prevention of the development of detectable intraperitoneal metastases or ascites. Interestingly, Minko et al. proposed this system as a platform for personalized cancer therapy with liposomal formulations containing antisense oligonucleotides targeting individually relevant resistance mechanism. Savant et al. combined PEGylated liposomes with a palmitoyl-ascorbate conjugate and paclitaxel [251]. The therapeutic benefit of the coloading against 4T1 mammary carcinoma was evident at 10 mg/kg compared to palmitoyl-ascorbate or paclitaxel-loaded liposomes. Atu027 (Silence Therapeutics, London, UK) is a liposomal formulation of siRNA against protein kinase N3, a downstream effector of the mitogenic PI3K/PTEN pathway involved in prostate cancer metastasis [252, 253]. This formulation was composed of 2'-O-methyl-stabilized siRNA encapsulated in cationic liposomes (50 mol% cationic lipid –L-argininyl-2,3-L-diaminopropionic acid-N-palmitoyl-N-oleylamido trihydrochloride (AtuFECT01), 49 mol% co-lipid 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (DPhyPE), and 1 mol% DSPE-PEG2000) [253]. This formulation showed very promising results in phase I clinical trial with tumor regressions in neuroendocrine and breast cancer patients [254].

Dai et al. combined targeted delivery with antineoplastic and antiangiogenic agent delivery in PEGylated liposomes [255]. Coloading of the antangiogenic agent combrestatin A-4 in the lipid biler and the anticancer drug doxorubicin in the aqueous core of PEGylated liposomes resulted in increased therapeutic activity. Hu et al. also combined liposomal delivery of the antineoplastic and antiangiogenic agent, honokiol with irradiation for maximal therapeutic efficacy [256]. They hypothesized that this protocol would combine the destruction of tumor cells by irradiation with inhibition of irradiation-induced neoangiogenesis by honokiol [257]. The combination of PEGylated honokiol-loaded and radiotherapy showed increased survival of Lewis lung carcinoma-bearing mice compared to radiotherapy or honokiol liposomes alone, resulting in decreased angiogenesis in vivo. Maitani et al. also combined an antineoplastic drug (irinotecan) and an antiangiogenic agent (sunitinib) [258]. The drug combination had more therapeutic efficacy against pheochromocytoma neuroendocrine tumors in vivo when they were administered as sunitinib liposomes plus irinotecan liposomes or as coloaded liposomes than the combination of the free drugs, with higher drug accumulation as liposomes than as free drug. In a similar fashion, folate-targeted doxorubicin-loaded liposomes coloaded with a bifunctional peptide capable of vascular disruption and antitumor activity were more effective against KB human nasopharyngeal carcinoma in vivo than untargeted coloaded liposomes with either monotherapy [259]. RGD-targeted liposomes coloaded with doxorubicin and the vascular disrupting drug combrestatin A-4 increased tumor regression of BT6F10 melanoma compared to untargeted coloaded liposomes or targeted liposomes with either drug [260].

Zucker and coworkers have optimized the simultaneous loading of vincristine and topotecan into PEGylated liposomes (LipoViTo liposomes) and provided the reader with the methods needed to characterize a liposomal drug combination [261]. Use of LipoViTo increased 100-fold the drug distribution to tumors compared to free drug and led to superior therapeutic efficacy over a free drug combination or liposomes with a single drug. PEGylated liposomes containing both vincristine and quercetin allowed reduced blood clearance of both drugs in mice, increased the therapeutic activity over a combination of free drugs and decreased side-toxicity [262].

Celator Pharmaceuticals Inc. (Princeton, NJ) developed a liposomal formulation of cytarabine: daunorubicin (CPX-351, 5:1 molar ratio) [24, 263, 264]. These PEGylated liposomes coloaded with the weak acid drug, 5-fluoroorotic acid (FOA) and the amphiphatic drug, irinotecan (CPT-II) at a 5:1 ratio revealed a synergy between the two drugs with higher therapeutic efficacy than the free drug cocktails in animal models [264, 265]. To encapsulate both drugs, they first prepared liposomes before active loading of CPT-II by a pH gradient method, with the protonated CPT-II retained in liposomes after complex formation with FOA. Mice treated with coloaded liposomes had increased survival compared to the combination with separate liposomes. However, the therapeutic efficacy was lower than with liposomes loaded with FOA only, probably because the FOA content had to be lowered for CPT-II coloading, further demonstrating the difficulty of reproducing a synergy with liposomes relative to free drugs. When tested in phase I trial with acute leukemia patients, the 5:1 ratio was maintained in plasma for 24 h, and CPX-351 induced complete responses in 9 out of 43 patients [24]. The same group developed irinotecan: floxuridine liposomes (CPX-1, 1:1 molar ratio). In phase I clinical trial they demonstrated that the drug ratio was maintained in plasma up to 12 h after infusion and showed positive clinical responses in patients with colorectal cancer [25]. It is noteworthy that the high therapeutic efficacy of liposomes encapsulating two anticancer drugs was always correlated with the maintenance of their synergistic molar ratio in plasma, in animal models [266] as well as in cancer patients [24, 25, 264] indicating optimization of drug loading and
liposomal stability as primary concerns for effective combination therapy. Ko et al. codelivered the proapoptotic peptide D-(KLAKKLAK)2 and the Bcl-2 antisense oligodeoxynucleotide G3139 [267]. The authors took the advantage of the electrostatic properties of these therapeutic molecules to codeliver them by formation of a negatively charged complex between the peptide and G3139 before mixing with positively charged liposomes. Intratumoral injection of coloaded liposomes led to an enhanced tumor growth suppression.

Finally, the combined liposomal delivery of magnetic fluid hyperthermia and photodynamic therapy using magnetic fluid and zinc phthalocyanine as the photosensitizer demonstrated superior toxicity in vitro of combined light and magnetic stimuli over their separate applications suggesting a new treatment modality for enhanced tumor therapy [268].

5. Tumor Stimuli-Triggered PEG Release

The addition of PEG to the liposome surface was reported to decrease the interaction of the ligand-targeted liposomes with their ligand, either when small molecules were conjugated to the liposome surface [269] or with antibody-targeted liposomes [48, 118] by steric hindrance of the surface ligand. Moreover, PEGylation decreases targeted liposomal accumulation and drug release [270]. Finally, for gene delivery, PEGylation has been shown to decrease intracellular trafficking of DNA [271]. These drawbacks and the extensive research in PEGylation chemistry (recently reviewed in [272, 273]) have led to the preparation of new multifunctional carriers where PEG release is promoted at the tumor’s vicinity after a stimulus either by physiological stimuli (pH, altered redox potential, sensitivity to an enzyme overabundant in the tumor microenvironment) or by physical external stimuli (light, heat, and ultrasound) [8, 274] (Figure 2).

5.1. pH-Sensitive PEG Release. While normal tissues and blood have a physiological pH near 7.4, human tumors have lower pH values (~6.0/6.5) because of an elevated rate of glycolysis [275, 276]. pH-sensitive bonds have been developed for the coupling of PEG to liposomes [277] (Figure 1). pH-sensitive liposomes achieved a higher concentration of cargo in the cytoplasm and nucleus than non-pH-sensitive PEGylated liposomes in vitro and allowed faster intratumoral content release in vivo [278, 279]. In addition to tumor sensitivity, pH-sensitive groups can potentiate the efficacy of targeted drug-loaded liposomes.

Folate-targeting of daunorubicin-loaded liposomes by incorporation of a pH-sensitive folate-PEG-cholesterol hemisuccinate (CHEMS) conjugate combined tumor targeting and increased drug release at the tumor site with improved chemotherapeutic activity over untargeted liposomes [280]. Similarly, untargeted cisplatin-loaded liposomes or EGFR-targeted gemicitabine-loaded liposomes incorporating CHEMS had superior antitumor activity over untargeted drug-loaded liposomes or free drugs [281, 282]. Obata et al. used a glutamic acid-based zwitterionic lipid (1,5-dihexadecyl N,N-diglutamyl-lysyl-L-glutamate) as titratable lipid for doxorubicin delivery [283]. These liposomes showed a charge inversion from negative to positive at acidic pH with endosomal escape leading to higher doxorubicin delivery in the cytoplasm and higher toxicity in vitro over conventional liposomes. This resulted in superior antitumor activity in vivo. Biswas et al. developed a new pH-sensitive DSPE-PEG-hydrazone-PEG2000 conjugate for attachment of ligands to the liposome surface [284]. In their work, the cell penetrating peptide (TATp) was unmasked after PEG release at acidic pH allowing efficient cellular uptake.

Recently, three new approaches for generation of pH sensitivity have been reported. First, by electrostatic adsorption of negatively charged carboxyl-modified gold nanoparticles to the surface of cationic liposomes (egg dipalmitoylphosphatidylcholine/DOTAP 9:1 weight ratio) at pH 7 (pKa of 5 for the carboxylic group) [285]. Authors reported detachment of gold nanoparticles at acidic pH due to protonation of the carboxyl groups and speculated that a similar strategy could be applied with negative charged liposomes and amine-modified gold nanoparticles. Second, a platform for finely tuned pH-induced PEG release was introduced using phenyl-substituted-vinyl-ether-(PIVE)-PEG lipid conjugates [286]. Liposomes containing PIVE showed pH-induced PEGylation and content release at acidic pH whereas they were stable at physiological pH. Third, ligand unmasking by acidic pH-induced membrane reorganization has been introduced as a reversible ligand-masking strategy. Sofou and coworkers developed a new platform for pH-triggered liposomal drug delivery [287, 288]. The rationale for their design involves the increased permeability at the boundaries between lipid domains [289]. Using lipid pairs of phosphatidic acid as a titrable headgroup and phosphatidylcholine as the colipid headgroup with mismatched hydrophobic chain lengths (dipalmitoyl and distearoyl) they demonstrated that formation of heterogeneous domains in PEGylated liposomes containing 5% of cholesterol allowed faster pH-dependent content release than liposomes with matched chains [288]. They showed a pH-dependent membrane transition due to the protonation of phosphatidylserine at lower pH in cholesterol-rich membranes, with protonation favoring their homologous interaction, leading to the formation of DOPS (1,2-distearyl-sn-glycero-3-phosphor-L-serine) lipid domains. PEG-lipid conjugates of matching hydrophobic anchor (DSPE-PEG) also segregated to these domains at acidic pH, whereas no redistribution of unmatched chain DPPE-PEG was in evidence [290]. The liposomes contained a ligand (biotin or an anti-HER2 peptide) harbored by an unmatched lipid (DPPE) which was masked by PEG at physiological pH but freed from PEG shielding at acidic pH after formation of the lipid heterogeneities. Application of this strategy to doxorubicin-loaded PEGylated (DSPE-PEG2000) liposomes harboring an HER2-specific peptide led to pH-dependent doxorubicin release in vitro and superior tumor growth inhibition than did untargeted vesicles or targeted vesicles devoid of pH-responsiveness [291].

5.2. MMP-Sensitive PEG Release. Hatakeyama and coworkers introduced coupling of PEG to DOPE by an MMP-cleavable linker, since MMPs are overexpressed in the tumor
environment [292, 293]. Transfection efficiency in vitro was correlated with MMP levels and lipoplexes prepared with a MMP-responsive PEG-lipid conjugate showed tumorm-specific transgene expression when compared to PEGylated lipoplexes with higher transgene expression for the same quantity of delivered lipoplexes. To enhance tumor targeting, Zhu et al. combined an MMP-sensitive PEG-lipid conjugate with antibody targeting and an intracellular penetrating moiety (TaT peptide) [294] combining long circulation by PEGylation, tumor targeting via antibody antibody 2C5, and selective internalization by tumor cells through MMP-2 triggered exposure of TaT peptide.

5.3. Redox-Sensitive PEG Release. Tumor cells have a higher concentration of reductases than the extracellular environment or normal cells and this feature has promoted the use of disulfide linkers both for the design of reduction-sensitive PEG-lipid conjugates and crosslinked nanoparticles, since the linker is stable in the circulation and normal tissues but reduced in the tumor cells [295, 296]. Goldenbogen et al. developed a versatile reduction-sensitive conjugate for targeted delivery [297]. Biotin was conjugated to a lipid anchor via a disulfide linker to prepare biotin-decorated liposomes; conjugation of streptavidin-HER2 monoclonal antibody allowed superior cellular uptake of doxorubicin in vitro over untargeted liposomes. Interestingly, less intracellular doxorubicin was detected after incubation with unsensitive HER2 targeted doxorubicin-loaded liposomes than reduction-sensitive targeted liposomes, further demonstrating the need for multifunctional liposomes. A combination of enhanced uptake and reduction-sensitivity was also done using reduction-detachable PEG and TAT [298]. Cleavage of DOPE-S-S-PEG5000 allowed unmasking of DOPE-PEG600-TAT and superior uptake of calcein in vitro over uncleavable TAT-modified liposomes together with stability in the presence of serum. Reduction-sensitive liposomes have also been used for gene delivery and a linear correlation between intracellular glutathione content and transfection efficiency has been recently demonstrated [299].

6. Intracellular Delivery

Internalization of anticancer drugs by cancer cells in tumors was shown to be a barrier to be overcome for cancer therapy [98, 101]. The use of internalization modifications at the liposomal surface or exposed after release of a PEG corona in the tumor-environment for active transport into cells and even subcellular delivery increased therapeutic activity [7, 17, 96, 300]. The influence of lipid composition on drug release and internalization, endosomal escape strategies, and mitochondria targeting is discussed below (Figure 4).

6.1. Importance of Lipid Composition. The presence of cholesterol or rigid saturated lipids (DSPC, HSPC) stabilizes the liposomal membrane against liposomal dissociation by plasma proteins and limits drug leakage, and thus most drug-loaded liposomes include cholesterol in the lipid bilayer [45, 288, 301]. These lipids have high gel-to-liquid crystalline phase transition temperatures (55–58°C) compared to physiological temperature (37°C) which prevents coexistence of the two phases and contributes to improved drug pharmacokinetics [13, 45, 302]. In some studies, the couple sphingomyelin/cholesterol is used to further rigidify the membrane through hydrogen bonding [303]. However, cholesterol inclusion can decrease drug loading. Indeed, paclitaxel loading decreased form 99.3% at a 5% molar content of cholesterol to 66.5% at 17% cholesterol content and 6.2% at a 37% molar content as a result of the hindered drug penetration in the increasingly rigid lipid bilayer [304]. The lipid composition is also important for the choice of the PEG-lipid conjugate used for PEGylation. Indeed, Kusumoto et al. reported a 10-fold higher transfection using liposomes armed with an endosomal-escape peptide (IFN7) harboring cholesterol-PEG2000 over DSPE-PEG2000 [305]. The superior endosomal escape of liposomes prepared with the former was attributed to the higher fluidity of cholesterol over DSPE, a superior fluidity favoring interaction with endosomal membranes and the resulting endosomal escape and transfection efficiency. Hydrophobicity was also shown to be a determinant for the design of smart multifunctional nanocarriers. Hansen et al. compared UV-triggered TaT peptide-mediated liposome internalization with a 16 or 12 carbons lipid anchor [306]. In addition to better internalization, liposomes with a C16 anchor were less prone to aggregation than those with a C12 anchor. The authors suggested the more hydrophobic alkyl chain favored liposomal insertion and the burial of the TaT peptide in a PEG-loop for the best UV-responsiveness and stability in cell culture media with bovine serum albumin.

Insertion of negatively charged lipids such as cardiolipin has been used to increase the retention of positively charged drugs in liposomes [45]. This was recently well illustrated for the preparation of mitoxantrone liposomes (mitoxantrone-complexed liposomes) by electrostatic complexation between anionic cardiolipin-based liposomes and cationic mitoxantrone [307]. While loading efficiencies of 95% were obtained with anionic liposomes using cardiolipin (CA), cholesteryl hemisuccinate (CHEMS), egg L-α-phosphatidylglycerol (PG), or L-α-phosphatidylserine (PS), only 3.8% loading was achieved with neutral liposomes. The therapeutic activity of the different anionic liposomal mitoxantrone preparations was in good agreement with release of mitoxantrone, that is, with the mitoxantrone release in vitro after heparin treatment. CHEMS liposomes had the lowest retention capacity and had virtually no impact on the survival of peritoneal carcinoma-bearing mice, and both PS and PG liposomes had intermediate mitoxantrone retention and exhibited higher therapeutic activity than free drug, albeit still inferior to that of CA liposomes capable of the highest mitoxantrone retention in vitro. Inclusion of anionic lipids should be coupled with PEGylation, since a negative charge directs liposomes to liver and spleen [308].

Lipid composition is also determinant for stimuliresponsive drug release. Goldenbogen et al. reported no calcine release from disulfide conjugated dipalmitoylphosphatidylcholine liposomes after treatment with a reducing agent, whereas reduction-induced release was observed
from liposomes including 55% of unsaturated dioleylphosphatidylethanolamine [297]. Note that Candiani et al. also incorporated DOPE in the lipid composition for bioreducible gene delivery, stressing the importance of DOPE as a helper lipid for membrane destabilization [299]. Increased permeability for thermosensitive drug release has been addressed by inclusion of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (P-lyso-PC) due to its tendency to form micelles and allow therapeutic efficacy in vitro of doxorubicin-loaded thermosensitive liposomes [309]. Nevertheless, the pharmacokinetics after administration in dogs was more similar to free doxorubicin than Doxil, which demonstrates the need to further optimize the lipid composition. Although liposomal cisplatin with 80% hydrogenated soy phosphatidylcholine (HSPC) showed increased cisplatin accumulation in preclinical tumors over free drug [21], this did not translate into therapeutic activity in patients [310, 311]. Absence of clinical activity was correlated with a lack of detectable released drug in the serum of treated patients, revealing the need for a balance between modifying the free drug pharmacokinetics for improved biodistribution to the diseased site and bioavailability [96]. PEGylation is required for enhanced blood residency and therapeutic efficacy, but postinsertion of DSPE-PEG6000 into preformulated siRNA lipoplexes was reported to induce siRNA release in vitro [312] and was nicely overcome by the use of cholesterol grafted siRNA for increased retention in liposomes. The combination of cellular uptake and targeting using a cholesterol-siRNA conjugate and cyclic RGD peptide allowed luciferase silencing in a B16F10-luc 2 experimental lung metastasis model, validating this new system [313].

6.2. Cell Penetrating Peptides. Cell penetrating peptides (CPPs) are amphiphatic peptides, usually cationic, either derived from viruses or synthetic that are able to improve the cellular internalization of the attached cargo [314] (Figure 4). The most frequently used CPPs are the Tat peptide derived from the transcription-activating protein of human immunodeficiency virus type 1 and synthetic polyarginine [315, 316]. Tat peptide is a powerful internalization moiety. However its endocytosis lacks cell-specificity and Tat peptide exposure at the liposome surface can lead to MPS elimination after opsonin binding as well [317]. For Tat-mediated internalization only in the tumor environment, masking strategies have been proposed. This concept was proved by Kale and Torchilin using masked Tat peptide surface-functionalized lipoplexes prepared with a plasmid coding for GFP (DSPE-PEG1000-TAT) by a pH-sensitive PEG corona (DSPE-hydrazone-PEG2000), leading to higher transgene expression in tumor tissue after intratumoral injection of pH-sensitive formulations [318]. Kuai et al. masked Tat peptide at the liposome surface (TAT-PEG2000-DSPE) by a reduction-sensitive PEG corona (PEG5000-S-S-DSPE) to take advantage of the higher concentration of reductive enzymes in tumors [319]. This allowed higher tumor accumulation and less liver uptake than unmasked Tat peptide-modified liposomes after intravenous administration.

More recently, UV-triggered CPPs have been proposed [306]. They added a CPP through incorporation of a Tat peptide-lipid-conjugate with two lipid anchors, a Tat peptide-PEG2000-DSPE conjugate linked to a less stable single chain hydrophobic group of 12 or 16 carbons via a UV-cleavable linker. They demonstrated a UV-dependent internalization of liposomes (a 15-fold increase in cellular adhesion and internalization only after irradiation), not observed with an uncleavable linker, that reached levels comparable to DSPE-PEG2000-Tat peptide liposomes. For the same purpose of cell-type selective CPP-mediated uptake, Kibria et al. functionalized liposomes with either RGD peptide or the tumor endothelial cell-specific peptide KYND and the octaarginine CPP and showed synergy of the combination of targeting peptide and cell penetrating peptide for liposome uptake in vitro with higher cell selectivity [320]. The same group later demonstrated superior antitumor activity of doxorubicin-loaded liposomes harboring both the tumor endothelial cell-specific peptide NGR and the cell penetrating peptide tetraarginine over untargeted liposomes or single-modified doxorubicin-loaded liposomes [183]. Presentation of octaarginine at the surface of bleomycin-loaded liposomes increased apoptosis induction in tumors and tumor growth inhibition over bleomycin-loaded liposomes devoid of the CPP [321]. Superior tumor growth inhibition was evidenced over untargeted RTN (receptor-targeted nanocomplexes, RTN) using lipopolypelexes decorated with an integrin-targeting peptide for delivery of pDNA encoding IL-2 and IL-12 to promote antitumor immunity [322, 323]. In their study, the complexes were optimized for disassembly in the target cell [323, 324]. The PEG-lipid conjugates used had an esterase-cleavable bond for endosomal escape and the integrin-targeting peptide was coupled to the polycation used for pDNA condensation by a linker cleavable by both cathepsin B and along with furin for intracellular release of the nucleic acid and high transfection efficiency.

In addition to enhancing cellular uptake, Tat peptide conjugation allowed crossing of the blood brain barrier in in vitro models and increased drug delivery of doxorubicin-loaded liposomes, resulting in prolonged survival of orthotopic glioma-bearing animals after intravenous administration [325].

6.3. Endosomal Escape. After the endocytosis, the cargo is transferred from endosomes (pH 6.5–6) to lysosomes (pH < 5) [326] in which enzymatic degradation occurs. Although PEGylation is required for extended blood circulation and tumor accumulation [7], this modification decreases cellular uptake and further increases endosomal degradation of the cargo, thereby reducing its activity [327, 328]. These conflicting properties of PEG have been referred to as the “PEG dilemma” [292]. The decreased endosomal pH has been exploited as a means to escape degradation using either fusogenic lipids or peptides which destabilize membranes after conformational activation at low pH, amines protonable at acidic pH for endosome swelling and rupture by a buffer effect [329–338] (Figure 4). The peptides used are either
derived from viruses such as TATp from Human Immunodeficiency Virus [339], IFN7 from the haemagglutinin of influenza virus [340], or artificial peptides like GALA [341]. Inclusion of these peptides leads to superior intracellular drug accumulation and resulting in higher cytotoxicity than liposomes devoid of endosomolysis properties. As a new approach, Kullberg et al. attached the pore-forming protein listeriolysin O to HER2-targeted bleomycin-loaded liposomes, resulting in a higher toxicity in vitro over targeted bleomycin-loaded liposomes without listeriolysin O [342].

6.4. Mitochondrial Targeting. Effective treatment of cancer faces problems due to limited drug penetration and drug resistance [343–345]. Since resistance to antineoplastic agents induced cell death is frequently associated with altered mitochondrial function and/or altered expression of mitochondrial regulators of apoptosis [300, 343], subcellular accumulation of anticancer drugs in mitochondria can give a therapeutic advantage and has been exploited [300, 346] (Figure 4).

Mitochondria targeting of epirubicin-loaded liposomes by inclusion of the positively charged electrolyte dequalinium increased their cytotoxicity in vitro and antitumor activity in vivo over untargeted liposomes [347]. Hatakeyama and coworkers developed a Mito-Porter multifunctional envelope-type nanodevice (MEND) nanocarrier with sequential activation of essential functions necessary for mitochondria delivery [292, 346, 348]. These formulations have a “programmed packaging”; their surface is functionalized with a targeting moiety (transferrin or antibody), a PEG-lipid conjugate for long blood circulation; and a PEG-lipid bond that is cleaved in the tumor environment by matrix metalloproteinases leading to exposure of a CPP (octaarginine, tetraarginine) for tumor-selective endocytosis. Once inside the cell, a fusogenic peptide (KALA or GALA) allows endosomal escape of positively charged liposomes by membrane fusion, the positive charge favoring their interaction with the largely negative outer mitochondrial membrane, and finally the fusogenic lipid DOPE allows internalization of the cargo by the mitochondria [346]. Although complex, such nanocarriers are produced in GMP conditions warranting their clinical evaluation [348].

Instead of using one moiety for each step of intracellular targeting, Zhang and coworkers designed a smart, pH-responsive lipid (1,5-dioctadecyl-L-glutamyl-2-histidyl-hexahydroxybenzoic acid, HHG2C18) [349]. The liposomes generated are negatively charged at physiological pH and have a sharp charge inversion at acidic pH (from −22.9 mV at pH 7.4 to +6.3 mV at pH 6.5) for tumor-selective uptake. After uptake, hexahydroxybenzoic acid is released by cleavage of the β-carboxylic acid linker in the endosomes leading to exposure of histidine and the endosomal escape of positively charged liposomes electrostatically targeted to the outer mitochondrial membrane. Liposomes containing the HHG2C18 lipid and encapsulating the anticancer drug Temsirolimus showed higher renal cancer tumor growth inhibition than free drug or nonresponsive liposomes. Targeting of topotecan-loaded PEGylated liposomes to mitochondria by inclusion of dequalinium, a lipophilic cation with a delocalized charge center that is attracted by the mitochondrial transmembrane potential [350], showed higher therapeutic efficacy than untargeted drug-loaded liposomes or free drug in two animal tumor models.

In another study [351], postinsertion of the mitochondriotropic dye Rh123-PEG2000-DSPE conjugate into PEGylated liposomes permitted their mitochondrial accumulation and increased the toxicity of paclitaxel-loaded liposomes over untargeted liposomes or free drug. This result is in line with the activation of the intrinsic apoptosis pathway by paclitaxel [352]. Although these modifications lead to superior cytotoxicity, the lack of cancer cell specificity can decrease their therapeutic index. To address this challenge, the same authors modified paclitaxel-loaded liposomes with a mitochondriotropic lipid (triphenylphosphonium, TPP) TPP-PEG-PE conjugate [353]. While the PEGylation of liposomes leads to their extravasation into the tumor by the EPR effect, TPP modification allowed superior therapeutic activity of mitochondria-targeted liposomes since more drug was intracellularly available. Malhi et al. developed “mitocancerotropic” doxorubicin-loaded liposomes combining tumor targeting by folic acid and mitochondriotropism by TPP [354]. Dual-targeted liposomes led to higher doxorubicin accumulation in mitochondria and superior toxicity than single-targeted doxorubicin-loaded liposomes, thus warranting further evaluation of this strategy.

7. Remote-Controlled Payload Release

To achieve release of the therapeutic agent at the tumor site, several strategies have been explored including ultrasound-triggered, photo-triggered, thermotriggered content release after controlled destabilization of the lipid bilayer (Figure 2).

7.1. Ultrasonication. Ultrasound-induced membrane permeabilization has been used for external stimuli-triggered drug release form liposomes by thermal or nonthermal effects (reviewed in [355]). Using PEGylated cisplatin-loaded liposomes, a 70% drug release after external ultrasound heating and a 2.7-fold increase in drug content occurred in vivo whereas only 3% cisplatin was released without ultrasound exposure, leading to the superior therapeutic activity of the formulation in ultrasound-treated mice [356]. A correlation between DSPE content in liposome membranes and sonosensitivity has also been reported [357].

7.2. Photo-Sensitive Release and Photodynamic Therapy. Photo-sensitive liposomal drug delivery relies on photodestabilization of the liposomal bilayer to release the encapsulated drug [358]. The liposomes used should be able to route the drug to the tumor and protect it from photodynamic damage [359]. Photodynamic therapy (PDT) consists of the destruction of tumors by light-activation of a photosensitizer, resulting in liberation of singlet oxygen that destroys the tumor by apoptosis, necrosis, or autophagy-induced cell death mechanisms [360]. Although the limited light diffusion
of this approach has been challenged by coupling of a light source to diffusing tips to treat deeper tumors [361], the area of cell death induction is still restrained due to the short lifetime of singlet oxygen (nanoseconds) [360]. Moreover, as these agents are mainly hydrophobic, their administration is limited by their aggregation, and the technique is limited to detectable tumors due to the nonspecific photosensitization [360, 362, 363]. Liposomal delivery of photosensitizers would allow treatment of both primary tumors and metastases by enhanced uptake of the photosensitizer by tumor cells. Yavlovich et al. reported for the first time light-triggered release of doxorubicin from PEGylated liposomes after laser irradiation including 10% of the photopolymerizable diacetylene phospholipid (1,2bis-(tricosa-10, 12-diynoyl)-sn-glycero-3-phosphocholine, DCa3 PC) resulting in photo-triggered cell killing in vitro [359]. The encapsulation of zinc tetraphenylporphyrin into PEGylated, folate-targeted liposomes improved its uptake and cytotoxicity after irradiation compared to untargeted liposomes in vitro [364]. Bovis et al. compared the pharmacokinetics of m-THPC [5,10,15,20-tetra-(m-hydroxyphenyl)chlorin] administered either in its clinically approved ethanol/propylene glycol formulation (Foscan) or in PEGylated liposomes [363]. Formulation of m-THPC in liposomes decreased its blood clearance and decreased skin photosensitivity compared to Foscan. Furthermore, m-THPC showed superior tumor accumulation and higher tumor necrosis than Foscan supporting its preclinical evaluation. Using another m-THPC un-PEGylated liposomal formulation (dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol, 9:1 molar ratio) Lasalle et al. stressed the importance of optimization of the delay between photosensitizer administration and irradiation [365]. Indeed, while no increase in survival of mammary carcinoma-bearing mice was observed compared to control for 1 h and 3 h drug-light intervals, 6 h and 15 h intervals cured 79% and 63% of mice, respectively.

7.3. Thermoresponsive Preparations. While lipids with high transition temperatures (above 55 °C) are required for blood stability and to decrease blood leakage, inclusion of lipids with transition temperatures closer to physiological body temperature (40–45 °C) allows induction of drug release after external localized heating [45]. Inclusion of low transition temperature lipids is a strategy used in tumor therapy for more than 30 years since the pioneering study of Weinstein et al. who used dipalmitoylphosphatidylcholine [366]. Doxorubicin-loaded liposomes containing 2% of poly [2-(2-ethoxy)ethoxethyl vinyl ether (EOEOVE)], (transition temperature 40 °C) exhibited a rapid doxorubicin release after heating to 45 °C with limited release at 37 °C, and allowed tumor growth suppression only after heating [367]. Interestingly, in their study thermoresponsiveness of poly (EOEOVE) liposomes was improved by inclusion of DSPE-PEG5000 in the liposome formulation and revealed an advantage of multifunctional lipidic PEGylation. Encapsulation of the doxorubicin analog, epirubicin into PEGylated thermoresponsive liposomes increased blood residency and tumor accumulation over unresponsive liposomes or free drug, resulting in a 20% higher tumor growth inhibition in animals treated with thermoresponsive liposomes over unresponsive epirubicin-loaded liposomes [368].

Paasonen et al. used gold-nanoparticles as “energy collectors” to lower the threshold energy required to induce photo-sensitive drug release [369]. After heat transfer from gold nanoparticles to lipids promoting liquid crystal-to-gel phase transition, a UV-induced liberation of the model compound calcine was evidenced with virtually no release without irradiation. Magnetic fluid hyperthermia involves heat transfer from magnetic particles after exposure to a magnetic field that results in localized elevation of temperature and induction of cell death [370]. To improve the selectivity, doxorubicin thermo-responsive liposomes coloaded with doxorubicin and magnetic nanoparticles were armed with folic acid and resulted in improved cytotoxicity in vitro over nonresponsive liposomes or untargeted thermo-responsive doxorubicin-loaded liposomes [371]. Intra-tumoral injection of anti-HER2 immunoliposomes containing magnetite followed by alternate magnetic field heating promoted iron retention in tumors in a HER2-specific manner 48 h after injection [372]. A 3-fold higher iron content was detected in HER2-overexpressing BT474 breast cancer xenografts over low HER2-expressing SKOV3 ovarian cancer xenografts, and magnetite retention in BT474 xenografts correlated with stable tumor regression [372]. In line with these studies, conjugation of HER2 antibody to thermo-sensitive doxorubicin-loaded liposomes improved the doxorubicin-mediated toxicity over controls [373].

Boron capture neutron therapy relies on delivery of 10B boron followed by γ-irradiation and capture of neutrons by 10B, leading to the production of toxic α-particles, 4H and 7Li for cell death induction [374]. Maruyama encapsulated 10B into PEGylated transferrin-armed liposomes for targeted delivery to colon carcinoma xenografts, this led to higher 10B tumor accumulation compared to the free isotope or untargeted liposomes and resulted in superior therapeutic efficacy after irradiation over free isotope or untreated 10B liposomes [36]. Lastly, the group led by Miyata reported a 3.6-fold higher 10B tumor concentration in orthotopic gliomas after intratumoral convection-enhanced delivery using PEGylated transferrin armed liposomes over untargeted liposomes with a lower retention in normal brains [375]. Superior therapeutic activity was observed against intracranial gliomas after intravenous injection of transferrin-targeted liposomes encapsulating sodium borocaptate over untargeted ones after neutron irradiation [376].

8. Theranostic Liposomes

Simultaneous therapy and diagnosis following codeelivery of therapeutic and imaging agents, theranostic, are determinant for the development of personalized medicine since it would allow clinicians to detect and characterize lesions and rapidly evaluate tumor response and modify treatment accordingly (increase dose, stop treatment, or use an alternate drug) [377–379]. Indeed, liposomes are currently widely used for diagnosis (see recent reviews) [380–382].
Kenny et al. designed PEGylated liposome-entrapped siRNA nanoparticles (LESiRNA) loaded with gadolinium (III) for magnetic resonance imaging, siRNA against the apoptosis inhibitor survivin for tumor therapy, and labeled with DOPE-rhodamine for fluorescence detection [383]. Accumulation of LESiRNA in ovarian cancer xenografts after intravenous injection was demonstrated by MRI and confirmed post mortem in tumor biopsies by fluorescence with in vivo survivin silencing and tumor weight reduction. Gd-labeled, doxorubicin-loaded thermo-responsive liposomes allowed detection of both tumor imaging by MRI and tumor regression after localized heating [384]. Note that to retain thermoresponsiveness after Gd-labeling a new Gd-chelate-dendron-based lipid was included in the lipid bilayer instead of a standard Gd-lipid conjugate to decrease Gd-lipid content to enhance thermosensitivity.

The use of magnetic resonance imaging (MRI) to allow both tumor visualization and temperature feedback for imaging-guided thermo-responsive drug delivery showed improved therapy of the image-guided, thermallyinduced drug release [385, 386]. Labeling of prednisolone-labeled liposomes did not decrease its therapeutic activity, allowed evaluation of in vivo drug biodistribution and response monitoring simultaneously, with MRI signal detection 1 week after injection [387]. To combine the advantages of three imaging modalities (optical imaging, CT imaging, and MRI), Li et al. and Mitchell et al. developed liposomes labeled with a fluorophore tracer, with $^{99m}$Tc, $^{111}$In or $^{64}$Cu, and Gd [388, 389]. Since most facilities do not possess all the imaging equipment, this system would allow a more flexible followup of therapeutic activity by optical imaging, while in depth studies would use CT or MRI without the need of administration of another imaging agent. Spatially controlled thermallyinduced drug release was achieved with MRI-guided high intensity focused ultrasound heating of the targeted tumor region resulting in deep tumor penetration of doxorubicin-loaded thermo-sensitive liposomes, coloring of liposomes with doxorubicin and gadolinium allowing tumor visualization and therapy [385, 386, 390].

The contrast agent used for the preparation of theranostic siRNA liposomes must be chosen with care. Mikhailova et al. reported nonspecific protein downregulation in vitro after incorporation of gadolinium of Magnevist into COX-2 (cyclooxygenase 2) siRNA-loaded liposomes, while COX-2 silencing without nonspecific downregulation was detected with liposomes coloaded with COX-2 siRNA and Feridex [391]. Targeting drug-loaded liposomes, in addition to enhancing their therapeutic activity, enhances tumor detection and response monitoring when they are coloaded with an imaging agent. Addition of transferrin to $^{10}$B plus iodine contrast agent coloaded liposomes allowed a 3.6-fold higher $^{10}$B concentration in tumor tissues over untargeted coloaded liposomes [375]. The selective retention of transferrin-targeted formulations led to better tumor detection 72 h after administration of liposomes, a period during which the signal from untargeted liposomes had washed out, thus combining monitoring of drug delivery and tumor response with boron neutron capture therapy [375]. Combined delivery of Gd and doxorubicin in liposomes targeted with a neural cell adhesion molecule-specific peptide allowed higher concentration of doxorubicin in tumor tissues correlated with increased tumor growth inhibition over untargeted coloaded liposomes together with better visualization of tumors by MRI [392]. Targeting of iron oxide and doxorubicin coloaded liposomes to pancreatic tumors by conjugation of an antimesothelin antibody improved the antitumor activity and tumor signal enhancement over untargeted liposomes [393]. Folate targeting of doxorubicin-loaded liposomes encapsulating iron oxide resulted in superior tumor growth inhibition of liver cancer tumors than the standard formulation Doxil and simultaneously allowed tumor imaging by MRI with higher sensitivity than the commercial contrast agent, Resovist [394].

9. Conclusions
In addition to the need for extended blood circulation and stimuli-controlled extravasation to the tumor’s niche, multifunctional liposomal nanocarriers must target at least one hallmark of cancer (aberrant cell growth, drug resistance, sustained angiogenesis, and tissue invasion) for enhancement of tumor therapy and/or diagnosis. As described throughout the paper, this requires coordinated action of stealth, targeting, and internalizing moieties to achieve intracellular delivery to cancer cells in tumors. Moreover, combined targeting of tumor cells and related neangiogenesis is becoming a focus of research that allows destruction of both primary and distant tumor nodules. However, targeted therapies rely on ligands presented by a few types of tumors and must face up to the fact of the heterogeneity of tumor cells and their surface markers [175, 395, 396]. A possible direction may be the coupling of ligands of different natures (antibody, protein, peptides and chimikine, hormone analogs) to target at least two tumor cell populations for relapse-free cancer therapy and more sensitive malignant lesion detection.

Conflict of Interests
The authors declare that they have no conflict of interests.

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Review Article

Stealth Properties to Improve Therapeutic Efficacy of Drug Nanocarriers

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Over the last few decades, nanocarriers for drug delivery have emerged as powerful tools with unquestionable potential to improve the therapeutic efficacy of anticancer drugs. Many colloidal drug delivery systems are underdevelopment to ameliorate the site specificity of drug action and reduce the systemic side effects. By virtue of their small size they can be injected intravenously and disposed into the target tissues where they release the drug. Nanocarriers interact massively with the surrounding environment, namely, endothelium vessels as well as cells and blood proteins. Consequently, they are rapidly removed from the circulation mostly by the mononuclear phagocyte system. In order to endow nanosystems with long circulation properties, new technologies aimed at the surface modification of their physicochemical features have been developed. In particular, stealth nanocarriers can be obtained by polymeric coating. In this paper, the basic concept underlining the “stealth” properties of drug nanocarriers, the parameters influencing the polymer coating performance in terms of opsonins/macrophages interaction with the colloid surface, the most commonly used materials for the coating process and the outcomes of this peculiar procedure are thoroughly discussed.

1. Introduction

Cancer is a leading cause of death worldwide as accounted for 7.6 million deaths (around 13% of all deaths) in 2008 (source: WHO Fact sheet N°297 February 2012). About 70% of all cancer deaths occurred in low- and middle-income countries. Deaths caused by cancer are forecasted to rise to over 13.1 millions in 2030 (Globocan, 2008, IARC, 2010).

Nevertheless, over the past few decades, significant advances have been made in fundamental cancer biology, allowing for remarkable improvements in diagnosis and therapy for cancer. Beside the development of new drugs with potent and selective activities, nanotechnology offers novel opportunities to cancer fighting by providing adequate tools for early detection and personalized treatments.

Over the last decades, a number of different long circulating vehicles have been developed for theranostic purposes. These carriers are in the nanometer range size and most of them have been intended for the delivery of anticancer drugs to tissues affected by this pathology.

The aim of this paper is to examine the features of “stealth” long circulating nanocarriers and the pharmacokinetic outcomes of stealthiness, and it will showcase the most investigated approaches yielding prolonged circulation of surface-engineered nanocarriers.

2. The Opsonisation Process

The selective and controlled delivery of anticancer drugs to disease tissues is a requisite to prevent systemic toxicity, enhance the pharmacological profiles, and improve the patient compliance, which in turn provide for amelioration of antitumour therapy.

Due to the leaky vasculature and low lymph drainage, solid tumours present erratic fluid and molecular transport dynamics. These features can yield specific accumulation of colloidal anticancer drug delivery systems into the tumour tissue by enhanced permeation and retention (EPR) effect [1]. However, in order to exploit the physiopathological and anatomical peculiarities of the tumour tissues, the nanovehicles need prolonged circulation in the bloodstream, ideally over 6 hours [2].
The permanence in the bloodstream of nanovehicles is strongly affected by physical interactions with specific blood circulating components, opsonins. These components prevalently include complement proteins such as C3, C4, and C5, laminin, fibronectin, C-reactive protein, type I collagen, and immunoglobulins [3].

Surface opsonisation promotes the removal of particles from the circulation within seconds to minutes through the mononuclear phagocytic system (MPS), also known as reticuloendothelial system (RES), and by Kupffer cells, phagocytic macrophages permanently located in the liver [4]. The natural role of opsonins is to promote the bacteria and viruses approach by the phagocytic cells, both systems having the same negative charge that inhibits the interaction between bacteria/viruses and the phagocytes due to charge repulsion [5]. After bacteria and virus coating, opsonins undergo conformational rearrangements that induce the biorecognition by phagocytes through specific membrane receptors. The xenoparticle opsonisation by complement proteins, over 30 soluble and membrane-bound proteins, induces the complement activation through a cascade of physiological events. The opsonisation finally promotes the removal process by phagocytes [4].

The complement is a key component of innate immunity that naturally monitors host invaders through three distinct activation pathways described in Figure 1 [6].

The classical pathway is activated after the fixation of C1q proteins to antibodies or to C1q receptors on the cell surface. The alternative pathway is spontaneously activated by the binding of C3 fragments to the surface of the pathogen. The lectin pathway is activated by the binding of mannose-binding lectin on mannose contained on the surface corona of bacteria and viruses. Although a few hypotheses have been proposed to explain the existence of supplementary activation pathways, they have not been fully elucidated.

 Regardless of the activation pathway, the enzymatic cascade of the complement activation leads to the formation of a common enzyme, C3 convertase, which cleaves the central protein of the complement system, the third component C3 [7]. The fragment C3b of C3 is the crucial active component that triggers the cleavage of a variety of complement proteins (C5–C9). The assembly of these proteins contributes to the formation of the membrane attack complex (MAC) that is able to destabilize bacteria, viruses, and nanocarriers for drug delivery. C3b and its inactive fragment iC3b can be recognised by specific receptors on phagocytic cells leading to the engulfing of opsonised particles and their removal from the bloodstream.

Additionally, the complement activation triggers a cascade of inflammatory and adverse complex reactions, named complement activation-related pseudoallergy (CARPA), that...
reflect in symptoms of transient cardiopulmonary distress. These effects have been detailed by the literature [8–11].

The complement system is also finely regulated by the presence of inhibitor proteins such as CI INH, Factor I and H [12].

Even though the natural role of opsonisation is directed to the body protection from xenogeneic nanosystems, this process promotes the removal of circulating drug nanocarriers. This represents a major obstacle to achieve adequate systemic and local therapeutic drug concentrations.

2.1. Steric Shielding and Stealth Properties of Nanocarriers. In the bloodstream, opsonins interact with nanoparticles by van der Waals, electrostatic, ionic, and hydrophobic/hydrophilic forces. Therefore, the surface features of the nanocarriers have a key role in the opsonisation process. Hydrophobic and charged particles undergo higher opsonisation as compared to hydrophilic and neutrally charged particles [13–16].

In the last decades, different theories have been attempted to describe the pharmacokinetic profiles of nanosized drug delivery systems, namely, liposomes and polymeric nanoparticles. It is now recognised that long circulating nanocarriers, “stealth” systems, can be obtained by surface coating with hydrophilic polymers that prevent the opsonisation process [17–19]. The consequence of avoiding opsonisation is the prolongation of the liposome and particle permanence in the bloodstream from few seconds to several hours [17, 20, 21].

Peppas described the effect of the hydrophilic polymer shell on nanoparticle surface in terms of elastic forces. He focused the attention on PEG that is the most representative of the materials used to produce stealth nanocarriers. According to their hydrophilic and flexible nature, the PEG chains can acquire an extended conformation on particle surface. Opsonins attracted to the particle surface compress the extended PEG chains that shift to a more condensed and higher energy conformation. As a consequence, the repulsive forces counterbalance the attractive forces between opsonins and the particle surface [22].

At low polymer density on the particle surface, when the polymer chains cannot interact with the surrounding chains and may freely collapse on the surface, the polymer chains provide for steric repulsion at a distance \( h \) according to the equation

\[
F_{st}^m = \frac{(kT)}{(D^2h_c)(h_c/h)^{8/3}}.
\]  

(1)

In the equation \( F_{st}^m \) is the sterically repulsive force referred to the “mushroom” model \( m \), \( h_c \) is the extension of a polymer above the surface \( = Na(a/D)^{2/3} \), \( D \) is the average distance between adjacent grafting points, \( a \) is the size of the segment, and \( N \) is the degree of polymerization.

At high polymer densities, the polymer chains extend and interact with each other exerting the sterically repulsive force \( F_{st}^{br} \) referred to the “brush” model \( br \):

\[
F_{st}^{br} = \frac{(kT)}{D^3}\left[ (h_c/h)^{9/4} - (h/h_c)^{3/4} \right].
\]  

(2)

These equations describe repulsive phenomena occurring on flat surfaces. However, they can be properly elaborated to gain information about repulsive steric barriers endowed by adsorbed polymers on curved surfaces of stealth nanoparticles [23].

2.2. Polymers Used to Coat Nanocarriers. Long circulating nanocarriers are usually obtained by polymer surface coating that endows systems with stealth properties [24]. In drug delivery, the term “stealth,” translated from the “low observable technology” applied to military tactics, refers to nanovehicles that are invisible to the biological system involved in clearance of particle from the bloodstream, namely, RES and Kupffer cells.

So far, many efforts have been done to yield stealth products by modification of the surface properties of nanocarriers with polymers that prevent opsonin interactions [25] and subsequent phagocyte clearance [26–28].

The polymers used to confer stealth properties to nanoparticles and nanovesicles have few basic common features: high flexibility and high hydrophilicity. Either natural and semisynthetic polysaccharides or synthetic polymers have been used for these purposes. Dextran (Dex), polysialic acid (PSA), hyaluronic acid (HA), chitosan (CH), and heparin are the most used natural polysaccharides. Synthetic polymers include polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), polyacrylamide (Pam), poly(ethylene glycol) (PEG), and PEG-based copolymers such as poloxamers, poloxamines, and polysorbates.

2.2.1. PEG. Poly(ethylene glycol) (PEG) is the polymer of choice to produce stealth nanocarriers. This neutral, flexible, and hydrophilic material can in fact properly produce surface barrier layers that reduce the adhesion of opsonins present in the blood serum on the nanoparticles making them “invisible” to phagocytic cells. The protein repulsion operated by PEG was also visualized by freeze-fracture transmission electron microscopy (TEM) [29].

A few physical protocols have been adopted to coat nanoparticle with PEG [22], even though these procedures entail the risk of polymer desorption in the blood with consequent loss of the beneficial contribution of the polymer [30]. In order to overcome this problem, covalent PEG conjugation protocols have been developed [31, 32]. Biodegradable nanoparticles with PEG covalently bound to the surface have been produced using PEG derivatives of poly(lactic acid), poly(lactic acid-co-glycolic acid) [33], or poly(alkylcyanoacrylates) [34]. The nanoparticles are prepared by emulsion, precipitation, or dispersion protocols in aqueous media. These procedures allow for the PEG orientation toward the water phase, while the biodegradable hydrophobic polymer fraction is physically entangled in the inner nanoparticle matrix [22]. Alternatively, PEG chains may be covalently conjugated to preformed nanoparticles through surface functional groups [35, 36].

2.2.2. Poloxamine and Poloxamer. Poloxamines (Tetronics) and poloxamers (Pluronics) are amphiphilic block copolymers consisting of hydrophilic blocks of ethylene oxide (EO)
and hydrophobic blocks of propylene oxide (PO) monomer units. Poloxamers are a-b-a type triblock copolymers (PEO-PPO-PEO) while poloxamines are tetrablock copolymers of PEO-PPO connected through ethylenediamine bridges [(PEO-PPO)\(_2\)–N–CH\(_2\)–CH\(_2\)–N–(PPO-PEO)\(_2\)] [37–39].

These polymers can be physically adsorbed on the nanocarrier surface through the hydrophobic PPO fraction [22].

Following intravenous injection to mice and rats, poloxamer- or poloxamine-coated sub-200 nm poly(ethylene oxide) [40], PLGA nanoparticles [41], and liposomes [42, 43] did not show prolonged circulation time as compared to the uncoated counterparts. This unexpected behaviour was ascribed to the desorption of the polymers from the nanocarrier surface [30] as well as to the polymer capacity to adsorb opsonins [44]. Indeed, the polymer composition has been found to affect the particle opsonisation as opsonins can associate with the hydrophobic polymer fraction that may be partially exposed on the particle surface [45, 46]. This possible effect can further contribute to the clearance of the polymer-coated nanocarriers.

For a given triblock polymer, it was found that both surface polymer density and coating layer thickness are affected by the particle size: smaller particles (below 100 nm) adsorb fewer polymer molecules per unit area than larger particles. Therefore, the polymer surface density decreases as the particle size decreases. Additionally, Pluronic adsorption on larger particles is relatively weaker than on smaller particles, which can affect the rate and extent of displacement of adsorbed polymers by blood components [47].

The surface adsorption efficiency and the stability of the polymer coating are strictly related to the polymer composition, namely, PEO/PO molar ratio and PPO and PEO chain length [44].

Pluronic F-108 NF (poloxamer 338) has a bulkier central hydrophobic block and longer side hydrophilic arms (122 monomers of PEO; 56 monomers of PPO) as compared to Pluronic F-68 NF (76 monomers of PEO; 30 monomers of PPO). Accordingly, Pluronic F-108 NF forms more stable coating layers than Pluronic F-68 NF. In vivo, Pluronic F-68 NF-modified nanoparticles accumulate at 74% of the dose in the liver in 1 h, while the liver accumulation of Pluronic F-108 NF-modified nanoparticles was 67% [48].

2.2.3. Dextran. Dextran is a polysaccharide largely used for biomedical applications including for the decoration of nanoparticle drug delivery systems [49].

Dextran coating was found to bestow long circulating properties on liposomes [50]. Similarly to PEG, the steric brush of the dextran on the vesicle surface reduces the protein adsorption. This effect results in enhanced liposome stability in the blood [50], which depends on the density of dextran molecules.

Interestingly, 70 kDa dextran coating was also found to reduce the burst of drug release from liposomes [50].

Dextran was used to coat superparamagnetic iron oxide nanoparticles for magnetic resonance imaging [51, 52]. Particles of 4 to 5 nm were coated with 20 to 30 dextran chains organized in “brush-like” structures, which reduced the removal from the bloodstream by Kupffer cells and splenic macrophages. The circulation half-life was prolonged to 3-4 hours [52]. The slight macrophage recognition of the dextran-coated superparamagnetic iron oxide nanoparticles was attributed to antidextran antibody opsonisation.

2.2.4. Sialic Acid Derivatives to Mimic the Nature. Sialic acid derivatives received considerable interest as potential materials to confer stealth properties to nanoparticles for drug delivery applications. Sialic acid is a component of eukaryotic cell surface and plays an important role in preventing the removal of self-tissue by low level of complement activation through the alternative pathway. Desialylation of erythrocyte membranes results in reduction of factor H binding on their membrane that switches them from nonactivators to activators of the alternative complement pathway [53, 54]. Plasmatic circulating factor H adsorbed on bacteria or the surface of colloidal systems physiologically inhibits their complement-mediated destruction. This result is ascribable to factor H action as cofactor for the inactivation of the complement C3b factor and the alternative pathway convertase [55]. Therefore, factor H behaves as a dysopsonin.

Surolia and Bachhawat demonstrated that liposomes coated with sialic acid derivatives are poorly recognised by the macrophages as they mimic the mammalian cell surface [56].

Stealth nanocarriers have been obtained using a variety of polysialic acid derivatives, including gangliosides [57–61], ganglioside derivatives, and glycoporphin [62–64]. On the contrary, the coating with orosomucoid protein, a sialic acid rich protein, did not yield stealth poly(isobutylcyanoacrylate) nanoparticles. This effect was ascribed to the poor density of the sialic acid on the particle surface that does not allow for proper coating or to the inefficient conformation of the clustered glycans [65].

The liposome coating with the monosialolganglioside GM1 (Figure 2), a brain-tissue-derived monosialoganglioside, was found to inhibit the alternative complement pathway by promoting the association of factor H to C3b factor on the vesicle surface [66]. In mice, the liposome decoration with 5–7 mol% of GM1 was found to increase the vesicle stability and inhibit the complement activation cascade, which resulted in prolonged permanence in the circulation [67]. As the molar ratio of GM1 in liposomes increases, the macrophage uptake inhibition increases up to 90% with 10 mol% GM1 [64].

Few studies postulated that the shielding of the negative charges of GM1 by the bulky, neutral hydrophilic sugar moieties is paramount to its stealth activity [58]. Nevertheless, other investigations showed that macromolecules bearing unshielded negative charges, namely, the ganglioside GM3, a sialic acid synthetic derivative, and a GM1 semisynthetic compound, increase the blood circulation time of sub-200 nm liposomes in mice [63]. Therefore, it can be concluded that the sterical organization of the ganglioside residues is primarily responsible for preventing the opsonisation of liposome containing glycolipids.

Interestingly, studies performed with mice and rats showed that the gangliosides have a specie-specific activity.
Indeed, the GM1 decoration was effective in mice while it did not have any beneficial effect on the circulation time of liposomes in rats [63].

2.2.5. Zwitterionic Polymers. Zwitterionic phospholipid derivatives have been demonstrated to reduce the complement activation induced by liposomes [68].

Based on this evidence, synthetic zwitterionic polymers have been used to produce stealth drug delivery systems. These materials bind water molecules more strongly than polymers forming hydrogen bridges such as PEG. Furthermore, they provide electrostatically induced hydration [69] that decreases the rate of adsorption of proteins, cells, and bacteria on surfaces [70, 71]. Conversely than amphiphilic polymers, namely, PEG, that can partially insert itself in the lipid bilayer of liposomes [72, 73], zwitterionic polymers enhance the hydration of lipid polar group regions on the surface of liposomes and do not perturb the lipidic bilayer stability [74].

Liposomes coated with poly(zwitterionic) 2 and 5 kDa poly(carboxybetaine)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (poly(carboxybetaine)-DSPE) (Figure 3) possess similar stability of PEGylated liposomes. After 4 days of incubation at 37°C, no aggregation was observed. The enhanced hydration and fluidity of the liposome membrane provided by the poly(zwitterionic) component reduced its permeability and accounted for prolonged drug release as compared to the PEGylated counterparts. In vivo, poly (zwitterionic) polymer and PEG-coated liposomes showed similar pharmacokinetic profiles suggesting that the former may be used as an alternative to PEG [75].

Poly(carboxybetaine) is more chemically stable than PEG and has lower interactions with proteins over short and long time [76]. This material has been used to coat a variety of nanoparticles including silica [77], gold [78], iron oxide [79], PLGA [80], and hydrogel nanoparticles [81, 82]. In serum, the coated nanoparticles showed excellent stability to aggregation indicating that negligible opsonisation occurred as compared to other stealth particles [83]. This behaviour translates in exceptionally low unspecific cellular uptake. As an example, internalization of cross-linked poly(carboxybetaine)/iron oxide nanogels by HUVEC cells and macrophages was barely detectable [79].

2.2.6. Polyglycerols. Polyglycerols (PGs) are biocompatible and flexible hydrophilic aliphatic polyether polyols, with an antifouling effect comparable to PEG [84]. By virtue of their multivalency that allows for the conjugation of targeting agents, drugs, labels, and physical modifiers [85], these polymers have been extensively studied as drug carriers.

Liposomes decorated with PGs exhibit extended blood circulation time and decreased uptake by liver and spleen [86].

Self-assembled monolayers (SAMs) of dendritic PGs were deposited on gold surface through a disulfide linker group (thiolic acid). Surface Plasmon resonance (SPR) measurements showed that PGs monolayers efficiently prevent the adsorption of proteins. It was concluded that dendritic PGs...
behave as antiopsonic materials because they combine the characteristic structural features of several protein-resistant materials: flexible aliphatic polyether structure, hydrophilic surface groups, and a highly branched architecture [84]. The inhibition of protein adsorption of hyperbranched polyglycerol was more efficient than linear PEG of similar molecular weight [87] and dextran. Furthermore, PGs have enhanced resistance to heat and oxidative stress as compared to PEG, which makes them potential candidates for biomedical applications [84].

2.2.7. Polyacrylic and Polyvinyl Polymers. Synthetic polyacrylic and polyvinyl polymers bearing hydrophobic moieties have been prepared to coat liposomes. The hydrophobic function allows for the polymer anchoring on the particle surface.

Palmitoyl- or phosphatidylethanolamine- (PE-) terminated derivatives of poly(acryl amide) (PAA), poly(vinyl pyrrolidone) (PVP), and poly(acryloyl morpholine) (PAcM) have been found to exert comparable stealth effects on liposomes in vivo. This behaviour depends on the length of the hydrophobic alkyl function, the polymer molecular weight, and its surface density [88, 89]. Comparative studies performed with palmitoyl-or PE-functionalized 6–8 kDa PAA, PVP, and PEG showed that the PEG derivative has slightly better performance as compared to the other polymers. Macromolecules containing shorter hydrophobic moieties than palmitoyl- or phosphatidylethanolamine-, namely, dodecyl alkyl chains, or higher polymer molecular weight (12–15 kDa) showed a lower effect on circulation time of liposomes. Short hydrophobic moieties cannot efficiently anchor the polymer on the liposome surface as the energy of the polymeric chain motion is higher than the energy of the anchoring alkyl chain interaction with the liposomal phospholipid bilayer [88, 90]. The higher the polymer molecular weight, the higher the free energy of the exposed polymer chains. Therefore, the polymer can detach in vivo inducing liposome opsonisation and removal by the RES [91].

The layer thickness of poly(vinyl alcohol)s (6, 9, and 20 kDa PVA) derivatized with C_{16}H_{33}S– as hydrophobic anchor (PVA-R) on the liposome surface was directly proportional to the polymer molecular weight and to the concentration of the polymer solution used for the coating process. Furthermore, it was found that the PVA-R density on the liposome surface increased as the molecular weight of the polymer decreased. The PVA-R on liposomes was not detached by dilution or in presence of serum while preventing the adsorption of plasma proteins. In vivo the PVA-R-coated liposomes showed prolonged permanence in the circulation, which increased as the PVA molecular weight increased. The circulation time of liposomes coated with 1.3% mol of 20 kDa PVA-R was comparable to that of liposomes coated with 8% mol of 2 kDa PEG-1,2-diesteroyl-sn-glycero-3-phosphoethanolamine (PEG-DSPE). Detailed investigations showed that the increased permanence in the bloodstream was strictly related to the PVA-R stability on the liposome surface that was higher compared to PEG-DSPE [92].

2.3. Surface Requirements to Set Up Long Circulating Nanocarriers. The capacity of hydrophilic polymers to repel proteins is strictly related to the polymer composition, polymer molecular weight, density on the carrier surface, thickness of the coating, conformation, flexibility, and architecture of the chains. Furthermore, this capacity depends also on the physicochemical properties of the anchoring moieties that allow for the attachment of the polymer on the particle surface.

2.3.1. Architecture and Molecular Weight of PEG Derivatives. The length of the polymer chains on stealth particle surface must exceed the range of the van der Waals attraction forces with soluble proteins in the bulk and phagocytic cells [93]. In the case of PEG, 2 kDa molecular weight is considered the lower threshold to guarantee macrophage avoidance. As the polymer molecular weight increases, the blood circulation half-life of the PEGylated particles increases [34, 94]. A study
carried out with nanoparticles assembled using PEG-PLA block copolymer demonstrated that the 5 kDa PEG has the maximal capacity to reduce protein adsorption that yields to the uptake by phagocytic cells [33, 95]. High sensitivity differential scanning calorimetry was used to evaluate the effect of PEG size and acyl chain length of the PEG-phospholipid conjugate on the physical stability of liposomes [96]. The study was carried out with liposomes obtained using PEG-dipalmitoyl phosphatidylethanolamine (PEG-DPPE) and dipalmitoyl phosphatidylcholine (DPPC). A mixed lamellar/micellar phase was obtained with compositions containing more than 7% mol of 1–3 kDa PEG-DPPE while the complete conversion to micelles was achieved above 17% mol of PEG-DPPE. High molecular weight PEG-DPPE derivatives (12 kDa PEG-DPPE) could not be incorporated in the DPPC bilayer at all concentrations. The 5 kDa PEG-DPPE, which has an intermediate molecular weight, was partially miscible with DPPC at concentrations below 7% mol. Phase separation occurred above 7% mol 5 kDa PEG-DPPE while above 11% transition to micellar state was observed together with phase separation. In conclusion, stable stealth liposomes can be obtained with low ratio of 3–5 kDa PEG-DPPE.

Concerning the hydrophobic anchoring moiety, longer alkyl chains than DPPE yielded unstable liposomes. PEG-DPPE embedded in a liposome distearoyl phosphatidylcholine (DSPC) bilayer promoted the phase separation even at low PEG-DPPE molar ratio (5%). This is ascribable to the steric restriction of the DSPC moiety within the bilayer due to high van der Waals cohesive forces that limit its mobility. This enhances dramatically the PEG chain/chain interactions that result in high mixing energy and favour demixing of the PEG-DPPE accompanied by structural rearrangements of the bilayer. Lipid phase separation generates domains on the liposome surface with low PEG-DPPE density that yields inhomogeneous PEG coating and poor sterical stability with rapid opsonin-mediated clearance. The phase separation would also lead to the leakage of encapsulated drug. On the other hand, short phospholipid alkyl chains, namely, PEG-dimyristoyl phosphatidylethanolamine (PEG-DMPE), embedded in liposome dimyristoyl phosphatidylcholine (DMPC) bilayer slightly delayed the formation of mixed lamellae/micelles at higher PEG-DMPE molar ratio (above 10%) than PEG-DPPE. The extent of demixing of PEG-phospholipid from bilayers decreases as the phospholipid alkyl chain decreases in the order of C18:0 > C16:0 > C14:0.

2.3.2. PEG Density. The polymer density on the nanocarrier surface is as much relevant as polymer molecular weight. Few authors showed that the high polymer surface density can compensate the low polymer molecular weight in obtaining stealth particles [25, 95, 97]. Vittaz et al. investigated complement consumption of PEGylated PLA nanoparticles. The authors concluded that a distance between two chains of 2 kDa PEG of 2.2 nm corresponding to 0.2 PEG molecules/nm² could achieve efficient 100 nm particle coating with minimum complement consumption [98]. Studies carried out using human phagocytes demonstrated that a distance of 1.4 nm between 5 kDa-PEG chains optimally yielded stealth 190–270 nm PEG-PLA nanoparticles [33]. However, it is worth to note that the polymer density threshold depends on a number of parameters, including particle size and surface curvature.

Investigations carried out by decorating gold-coated silica particles with 750 and 2000 Da methoxy-PEG suggested that a polymer density of 0.5 chain/nm² is a critical threshold to prevent the adsorption of plasma proteins [99].

Low complement consumption was observed in the case of 1.5 kDa PEG-stearate-coated 26 nm nanocapsules. The protein repulsion was found to depend on the polymer density rather than the polymer chain length [25, 100]. The nanocapsule surface covered by one PEG 1.5 kDa-stearate molecule was estimated to be about 2.8 nm², corresponding to about 1.7 nm distance between two PEG chains, which is in fair agreement with the results described above. As a result of the low opsonisation and complement consumption, these nanoparticles displayed prolonged residence time in the blood with 20% of the dose still present in the blood 24 h after injection [101].

The homogeneous surface polymer coating is, together with the polymer density, a key parameter to obtain stealth particles. A study showed that 30% of PEGylated polystyrene nanoparticles underwent phagocytosis as a consequence of the inhomogeneous physical adsorption of the polymer on the particle surface [102].

2.3.3. Liposome Rigidity and Cholesterol Effect. Phospholipid membrane rigidity is paramount to produce liposomes with stealth properties as well as to prevent rapid drug release. Decreased rigidity due to the use of phospholipids with low melting temperature (Tm) for the preparation of liposomal formulation can lead to drug leakage and opsonin adsorption.

The liposome membrane rigidity, homogeneity, and stability can be optimised by selecting phospholipids with proper Tm and by introducing cholesterol in the phospholipid bilayer. A minimum content of 30% mol cholesterol ratio is required to prevent the formation of phase separated lamellas and mixed micelles. It also reduces the leakage of encapsulated drug from liposomes [42, 103] and decreases the interaction of liposome surface with plasma components [96, 104].

2.3.4. Surface Polymer Conformation. The polymer chain conformation on the particle surface plays a critical role in conferring improved stealth properties to nanocarriers.

It was found that the optimal surface coverage to confer adequate stealth properties is the one that allows for a polymer chain conformation in between the “mushroom” and “brush” configurations. In this specific condition most of the chains are in a slightly constricted configuration, at a density to ensure no uncoated gaps on the particle surface. It is conceivable that predominant brush-like PEG configurations would sterically suppress the deposition of large proteins such as C3 convertase [25]. However, even when PEG is in the brush-like conformation on the surface of
nanoparticles, its capacity to prohibit the protein adsorption on the surface is again affected by the obstruction capacity of the protecting layer. Small molecules can, in fact, slide in between the polymeric chains. For such a reason, Papisov et al. [105] highlighted the influence of (i) brush density, (ii) brush rigidity, (iii) brush molecular length, (iv) substrate size, and (v) cooperative character of interaction on steric repulsion and obstruction.

The polymer chains conformation is dictated by the distance of the anchorage site of two polymer chains ($D$) and by the gyration radius of the polymer known as Flory radius ($R_g = an^{3/5}$, where $n$ is the number of monomers per polymer chain and $a$ is the length of one monomer in angstroms which corresponds to 3.5 Å for PEG) [106]. The $R_g$ of 2 kDa PEG is approximately 5.6 nm, which can be compressed depending on the surface grafting density. At low surface density, the PEG chains have higher mobility. In the case of $R_g < D < 2R_g$, the polymer chain conformation corresponds to an intermingled “mushroom” configuration. This conformation allows the polymer chain for closer interactions to the surface of the particle and formation of gaps in the PEG protective layer that yields nanoparticle opsonisation [107]. High PEG density results in $D \sim R_g$ and limited polymer chain motion that yields the transition from mushroom-like to mushroom/brush conformation. When $D \ll R_g$, the polymer chains convert to a brush-like conformation. The resulting low PEG chain mobility and flexibility reduces the ability of the polymer to repulse opsonins [23]. The polymer chain movement, due to its high flexibility and mobility, reduces both of the accessible surface of the nanoparticles and the interaction of the polymer with the cryptic pockets of the opsonins [108].

Studies performed with 100 nm liposomes coated with 2 kDa PEG-DSPE showed that below 4% PEG-DSPE molar ratio, the PEG chains were arranged in a mushroom conformation while a brush conformation was obtained above 8% PEG-DSPE molar ratio [109].

2.3.5. Polymeric Corona Thickness. PEG layer thickness is paramount to obtain stealth nanoparticles. The minimum coating layer thickness required to guarantee efficient particle coating depends on a number of parameters including the potential absorbable proteins and the nanocarrier size [110].

Studies have shown that a minimum effective hydrodynamic layer thickness is about 5% of the particle diameter [111]. Moghimi et al. demonstrated that efficient protection of 60–200 nm polystyrene particles from complement activation and protein adsorption can be obtained with 4 kDa PEG that provides for a coating thickness of 5 nm [17].

The thickness of the polymer coating depends on the polymer chemical composition. In aqueous medium, PEG can provide for a maximum thickness corresponding to its full chain length. For copolymer such as poloxamers and poloxamines instead the thickness is linearly related to the number of EO monomers since only this function of the polymer can extend outward from the nanocarrier surface [93].

A hydrophilic polymer can provide for a surface coating thickness of $h_c = aN(a/D)^{1/5}$, where $N$ is the degree of polymerization, $a$ is the size of the monomer, and $D$ is the mean distance between grafting points [112]. For a good solvent the exponent is 3/5.

In general, proper particle stabilization is achieved when $A(b/h_c) < T$ where $T = \text{temperature}$, $A = \text{Hamaker constant}$, and $b = \text{particle radius}$. As $A/T$ is typically in the order of 1/10, a coating with a thickness corresponding to 10% of the particle diameter is conventionally considered adequate to provide for efficient steric stability [23].

2.3.6. Polymer Flexibility. Studies have demonstrated that polymer chain mobility is required for repelling proteins from polymer chains on particle surface yielding stealth nanocarrier [113]. Accordingly, the lower complement activation of PEG as compared to dextran can be explained on the basis of polymer chain flexibility. In a CH50 assay, an in vitro haemolytic complement consumption assay, 10% complement activation was obtained with 20 cm$^2$ of 5 kDa dextran coated and 120 cm$^2$ 5 kDa PEG-coated polycaprolactone nanoparticles [114]. The results normalized by the particle surface area show that the PEG coated particle surface induces a lower complement activation as compared to the dextran-coated surface. This is due to continuous change of the well-hydrated PEG chain conformation that reduces the exposure of fixation sites for complement proteins. The rapid movement of the flexible chains allows for the polymer to occupy a high number of possible conformations and leads to a temporary squeezing out of water molecules, making the surface impermeable for other solutes such as plasma proteins [108]. Therefore, the water cloud surrounding the PEG chains confers an interfacial free energy on the particle surface that protects the nanocarriers from opsonisation and recognition by macrophages.

2.3.7. Amphiphilic Polymer Architecture. The coating polymer conformation on the nanocarrier surface is strongly affected by the polymer architecture which influences the plasma protein adsorption and interactions with cells.

Nanoparticles obtained with multiblock (PLA-PEG-PLA)$_n$ copolymers were found to adsorb higher amounts of proteins compared to nanoparticles obtained with polyethylene-glycol-grafted poly-$(D,L)$ lactide (PEG-g-PLA) [115]. The low protein adsorption on PEG-g-PLA nanoparticles was ascribed to a higher surface PEG density. Similarly, nanoparticles obtained with copolymers with a PCL backbone and PEO grafts (PCL-g-PEO) were more effective in preventing protein adsorption as compared to PEO-b-PCL diblock copolymer nanoparticles [116].

The PEG attached through both terminal groups to the nanoparticle surface formed a single-turned-coil arrangement, which was found to provide compact conformational structures that endowed particles with high resistance against blood protein adsorption [117].

The effect of linear and branched PEGs on stealth properties of nanocarriers was also investigated by using liposomes decorated with PEG-PE and PEG$_2$-PE. PEG$_2$-PE was more
efficient in improving the blood circulation time than PEG-PE at a low content (3% mol), whereas at high molar ratio (7% mol) their effect on liposome blood clearance is almost identical. At higher ratio of protecting polymer (7% mol), even PEG-PE can provide complete coating of the liposome surface that does not take place at low molar PEG-PE ratio [108].

2.4. Controversial Effect of Polymer Coating. Many studies have demonstrated that the particle opsonisation can be reduced by surface coating with hydrophilic flexible polymers and mathematical elaborations have been developed to describe this effect. However, it should be noted that several controversial results have been reported in the literature.

In vitro studies showed that stealth vesicles obtained by PEG coating can associate with a pool of opsonic proteins of serum and plasma such as components of the complement system and immunoglobulins. Nevertheless, it was not clear if the protein interaction occurred with the exposed or internal part of the coating polymer [14, 29, 33, 60, 118–124]. In vivo, 2.5–10% of the dose of PEG-coated vesicles and nanoparticles has been found to dispose in the liver and spleen in the first hour after intravenous administration [125–130]. The limited removal of stealth particles from the bloodstream seems to indicate that a small amount of specific opsonic proteins can target PEG-coated nanocarriers [124]. This hypothesis is supported by the evidence that low doses (20 nmol/kg body weight) of PEGylated liposomes are rapidly cleared by macrophages, while the cleared dose fraction decreases as the amount of the injected PEG-coated liposomes increased [125–127].

Stealth nanocarriers were found to display long circulation profiles even after extensive opsonisation. A typical example is Doxil, the PEGylated doxorubicin loaded liposome formulation, which is efficiently opsonised by the C3b factor and activates the complement. Nonetheless, Doxil presents a biphasic circulation half-life with prolonged permanence in the circulation [21].

Overall these data show that the stealth behaviour of long circulating nanocarriers is a very complex mechanism and it cannot be reduced to the simple opsonin repulsion underlining some additional and relevant effects operated by the steric coating on the nanocarrier surface.

2.4.1. PEG Induced Complement Activation. PEG coating on one side reduces the opsonisation process, while on the other can induce the complement activation that is involved in the nanoparticle removal. Liposomes are a typical example of the double effect of particle PEGylation.

Liposomes with low surface charge obtained with saturated phospholipids and high cholesterol content, which endows rigid and uniform bilayer without surface defects, are poorly prone to opsonisation and structural destabilisation by C3 adsorption [121, 128, 131, 132]. On the contrary, negatively charged and flexible liposomes undergo rapid opsonisation and phagocytosis. The incorporation of 5–7.5 mol% of PEG 2 kDa-DSPE into the bilayer of anionic liposomes formed by egg phosphatidyl-choline, cholesterol, and cardiolipin (35:45:20 mole ratio) was found to dramatically reduce the complement activation of these vesicles. However, the degree of complement activation also depended on the liposomes concentration. Indeed, in vitro studies showed that 15 mM PEGylated liposomes concentration induced 40% complement consumption [133].

Studies carried out with Doxil showed that 0.4 mg/mL of PEGylated liposomes elicited the rapid complement activation and generate the soluble terminal complement complex (SC5b-9) in 7 out of 10 human sera [134]. These results underline the individual effect of PEGylated liposomes on the complement activation.

The complement activation by PEGylated liposomes was found to be responsible for several side effects. In pigs Doxil was demonstrated to activate the complement through both the C1q-dependent classical and the alternative complement activation pathways [135], which was responsible for the cardiopulmonary distress [136].

In few cases, a transient in vivo response was observed in rabbits as a drop in the systemic arterial pressure at 10 min after liposome injection which is typical of the complement activation [137]. On the contrary, no complement activation after PEGylated liposome administration was evidenced by the in vitro assay. These evidences highlight that in vitro complement activation tests should be carefully evaluated for what concerns their sensitivity and response threshold in order to obtain results that can be correlated with the in vivo data.

Studies performed with PEGylated polymeric nanoparticles confirmed that PEG-coated systems can induce the complement activation regardless of the PEG chain length and surface density. The complement activation was inversely correlated with the PEG molecular weight suggesting that steric hindrance on the particle surface due to the polymer coating reduces the approach and association of large proteins such as the C3 convertase [97, 138].

Studies carried out using PEGylated erythrocytes showed that the complement activation may be mediated by anti-PEG IgG and IgM [139].

Anti-PEG IgM elicited by a first administration of PEGylated liposome forms immunocomplexes with the second dose of liposomes [140]. These complexes activate the complement and convert the C3 component into C3b. The complex formed by C3b with other complement components is involved in the antibody-mediated complement activation pathway [134, 141] that yields C3b fragmentation to iC3b operated by factors H and I. iC3b is a proteolytically inactive product of the complement fragment C3b that can still opsonise. However, it cannot participate in the complement cascade since it does not associate with factor B, a component of the alternative activation pathway in the early stage of the activation. The generation of iC3b prevents the amplification of the complement cascade. Overall the PEG molecules on the liposome surface do not interfere with production of opsonic components from the C3 component.

Complement activation has been suggested to account for the clearance of PEGylated liposomes by the macrophage uptake of the RES [142].
Furthermore, the extent of the accelerated blood clearance (ABC) of PEGylated liposomes is inversely proportional to the dose probably because of the saturation of the mononuclear phagocytic system [143].

2.4.2. Poloxamine Induced Complement Activation. Similarly to PEG, Poloxamines and Poloxamers have been extensively used to endow nanocarriers with stealth properties. Nonetheless, even these materials have been found to activate the complement to some extent thus reducing the beneficial effect on particle opsonisation.

Poloxamine-908-coated polystyrene nanoparticles were found to activate the complement through a complicated pathway. The adsorbed poloxamine-908 on the polystyrene nanoparticles rearranges from flat mushroom-like to brush-like conformation as the density of the polymer on the particle surface increases. As the polymer packs on particle surface, the surface area occupied by poloxamine decreases from 45 to 15 nm²/poloxamine chain. The intermediate mushroom-brush poloxamine conformation induced remarkable complement activation that decreased when the polymer rearranged to a brush-like structure. Uncoated nanoparticles and particles coated with poloxamine in the mushroom-like conformation promote surface association of the Clq fragment of the complement protein C1 and activate the complement through the classical pathway. Naked and poloxamine-coated nanoparticles in the mushroom and mushroom-brush conformation also activate the complement through the alternative pathway by covalent conjugation of properdin to poloxamine and the C3 component adsorption. Conversely, particles coated with poloxamine in the mushroom-brush and fully brush conformation activate the complement via the lectin pathway, which involves the opsonisation of mannose-binding lectin protein (MBL) and/or ficolins. This complement activation pathway was attributed to the structural similarities between the EO monomers of poloxamine and a region of D-mannose [144]. The brush-like conformation minimizes the MBL and ficolin binding to PEG backbone and consequently reduces the complement activation via the lectin pathway [145].

Thus, the conformation and the mobility of surface projected PEO chains of poloxamine on nanoparticles are paramount to modulate the complement activation pathway [146].

2.5. “Long Circulation” Revealed. PEG-and poloxamine-coated nanocarriers have been demonstrated to undergo immunoglobulin, fibronectin, and apolipoprotein association [14, 29, 33, 118, 122–124, 147] as well as C3 opsonisation that mediates the biorecognition by macrophages through specific complement receptors (CRI and CR3, CD11b/CD18) [18]. However, these systems possess long-lasting profiles in blood [148]. The prolonged circulation in the bloodstream is due to the steric hindrance of the surface polymers [134] that prevents the macrophage approach [124]. Furthermore, the C3b adsorbed on the polymer corona of the particle surface can be proteolytically degraded to fragments that by assembling with other cofactors inhibit the recognition by the macrophage receptors [149]. The factor C3bn of the complement adsorbed on PEG-coated liposomes may also bind CRI receptor associated with the erythrocytes membrane, which can also explain the prolonged circulation time of PEGylated liposomes [150].

The steric shielding effect conveyed by polymer coating on long circulation properties of stealth nanocarriers was demonstrated by Moghimi using poloxamine-908-coated particles. These particles, incubated with serum obtained from a poloxamine-908 preinjected animal, showed a higher protein adsorption as compared to particles incubated with serum obtained from animals that were not preexposed to poloxamine. The protein-coated nanoparticles showed similar pharmacokinetic profiles when administered to animals never exposed to poloxamine. This evidence reinforces the explanation that the improved circulation time of stealth nanoparticles is not solely ascribable to reduced protein adsorption on particle surface [151] which surely takes place for sterically stabilized nanocarriers. Improved circulation time can be mainly attributable to the prohibited biorecognition of the adsorbed opsonic proteins by the macrophages.

2.6. Nanocarrier Coating with Hydrophilic Polymers: Physical and Chemical Strategies. Sterically protective polymer can be physically or chemically conjugated to the nanocarrier surface. Physically conjugation involves the hydrophobic adsorption of polymer fragments on the particle surface while the chemical conjugation is obtained by chemical reaction of polymers with surface functions to yield covalent bonds.

So far a variety of protocols have been set up to conjugate PEG to small molecules and biologically active proteins. These methods have been translated to obtain stealth nanoparticles with other materials [152, 153].

2.6.1. Physical Coating of Polymeric Nanoparticles and Liposomes. Surface PEG coating of PLGA nanoparticles was carried out using 2kDa PEG-DSPE as emulsifier during oil-in-water microemulsion nanoparticle preparation. The process allows for the embedding of the PEG-DSPE phospholipid fraction in the PLGA matrix by hydrophobic interactions, whereas the hydrophilic PEG chain extends outward the nanoparticle surface, forming a polymeric brush that stabilizes the system. Drug loaded 120 nm PEGylated PLGA nanoparticles were successfully used for the treatment of a cystic fibrosis murine model by intranasal administration [154].

An original multistep technique for physical PEGylation of doxorubicin loaded PLGA nanoparticles involves the surface adsorption of palmitate-avidin on the particles through the avidin alkyl chain anchor during the particle preparation by emulsion. The avidinated particles are subsequently PEGylated by exposure to PEG-biotin. The particle coating with 5 and 10kDa PEG reduced protein adsorption by 50, and 75%, respectively, compared to the non-PEGylated PLGA nanoparticles. Approximately 3% of the initial dose of the doxorubicin loaded nanoparticles intravenously administered was detected in the serum after 48 hours from administration. This corresponds to a twofold
residual doxorubicin plasma concentration as compared to that obtained with non-PEGylated particles [155].

Protective PEG layer on liposomes can be achieved through two very conventional strategies. In the first approach PEG is conjugated with a hydrophobic moiety (usually the residue of PE or a long chain fatty acid is reacted with methoxy-PEG-hydroxy succinimide ester) [156, 157] (Figure 4). Subsequently a dry mixture film of phospholipids and the mPEG-PE is rehydrated to yield liposomes that spontaneously expose the PEG chains on their surface [158].

A second approach to coat liposomes with PEG is called the “postinsertion method” and consists in the conjugation of activated PEG to preformed liposomes.

2.6.2. Polymer Coating of Magnetic Iron Oxide Nanoparticles. Specific coating protocols have been set up to produce stealth inorganic nanoparticles.

The incorporation of a polymer coating on the nanoparticle surface can be achieved either via “one-pot” methods, where the nanoparticles are coated by a polymer dissolved in the particle production mixture, or by “two-step” or “postproduction” method, where nanoparticles are first generated and then coated with a polymer. Magnetic nanoparticles coated with PEG-based copolymers have been prepared in one pot by Fe$_3$O$_4$ nucleation and growth. Poly(ethylene glycol) monomethyl ether-b-poly(glycerol monoacrylate) (PEG-b-PGA) was added to Fe$^{2+}$/Fe$^{3+}$ solutions and the coprecipitation of the iron ions was induced. The iron atoms on the nanoparticle surface were coordinated via the 1,2-diols of the PGA block, which resulted in particle stabilization [159].

Iron oxide nanoparticles stabilized by carboxyl coordination of the surface oxide molecules were prepared by high-temperature decomposition of tris(acetylacetonate) iron(III) [Fe(acac)$_3$]$_3$ in the presence of monocarboxyl-terminated PEG [160].

Postproduction iron oxide nanoparticle decoration was performed using silane-terminating PEG. The silane group strongly interact with the oxide on the nanoparticle surface [161]. PEGs derivatised with amino propyl trimethoxy silane (APTMS) or amino propyl triethoxy silane (APTES) were used.

Phosphonic acid-terminated poly(oligoethylene glycol acrylate) [poly(OEGA)] was grafted to iron oxide nanoparticles through the phosphonic acid end group that provide strong interaction with iron oxide nanoparticles. The poly(OEGA-) stabilized iron oxide nanoparticles showed significant stealth properties and exhibited low BSA adsorption (<30 mg g$^{-1}$ nanoparticles) over a wide range of protein concentration (0.05 to 10 g L$^{-1}$) [162].

Iron oxide nanoparticles synthesized by Fe(acac)$_3$ decomposition in high-boiling organic solvents were postproduction PEGylated by the ligand exchange method. The nanoparticles produced with oleic acid, hexane, or triol phosphine oxide (TOPO) coating were combined with PEG-silanes, PEG-PEI, PEG-PAMAM, PEG-fatty acid to allow for the coating exchange in aqueous medium [163–168].

Dopamine has been proposed as an alternative anchoring group to silane to coat magnetic nanoparticles. Dopamine has high affinity for the iron oxide and can be conjugated to PEG through the amino group. PEG-dopamine was used to displace the oleate/oleylamine coating on the particles produced by high-temperature decomposition of Fe(acac)$_3$ thereby converting the particle surface from hydrophobic to hydrophilic according to a postproduction protocol [169].

“Growing from” approaches based on living radical polymerization techniques such as Atom-Transfer Radical-Polymerization (ATRP) and Reversible Addition-Fragmentation chain-Transfer (RAFT) polymerization have been largely investigated to coat preformed iron oxide nanoparticles with PEG copolymers. ATRP polymerization of PEG-methacrylate (PEG-MA) was performed in aqueous solvent after a silane initiator (4-(chloromethyl) phenyl trichlorosilane) immobilization on iron oxide nanoparticle surface. After poly(PEG-MA) grafting, the uptake of the nanoparticles by macrophages was reduced from 158 to less than 2 pg per cell confirming the excellent shielding capacity of this novel material [170].

Alternatively, the ATRP polymerization of the PEGMA was performed according to a solvent-free protocol. The macroinitiator on the surface of the magnetic iron oxide nanoparticles was introduced by exchanging the surfactant (oleic acid) on the nanoparticle surface with 3-chloropropionic acid. The exchange made the nanoparticles soluble in PEG-MA that was then polymerized by ATRP. No difference in terms of capacity to evade macrophage uptake was detected when poly(PEG-MA-) coated iron oxide nanoparticles were prepared in water or by the solvent-free method [171].

Hyperbranched polyglycerol (HPG) has recently emerged as a biocompatible and resistant material to protein adsorption, which was ascribed to its hyperbranched nature [84]. HPG-grafted magnetic iron oxide nanoparticles have been
prepared by surface-initiated anionic polymerization of glycidol. Iron oxide nanoparticles were first functionalized with 3-mercaptopropyltrimethoxysilane that, in the anionic form, promotes the ring opening polymerization of glycidol in toluene. A 13 wt% HPG coating was obtained by this procedure. The protein adsorption was very low and comparable to that of nanoparticles prepared with silanated methoxy-PEG (MW = 750 Da) at a similar grafting density [172]. Glycidol polymerization can be also initiated by aluminum isopropoxide grafted to 6-hydroxycaproic acid coated iron oxide nanoparticles. The resulting 24 nm HPG-grafted nanoparticles are very stable in PBS and culture media and their uptake by macrophages was very low (<3 pg Fe/cell), over a 3-day contact time [173].

2.6.3. Polymer Coating of Gold Nanoparticles. Gold nanoparticles have been PEGylated according to “one-pot” methods. AuCl₄⁻ in solution can in fact be reduced by the amino groups of the PEI block of poly(ethylenimine)-poly(ethylene glycol) block copolymer (PEI-b-PEG) [174].

Postproduction PEGylation strategies have relied mostly on the use of thiol (-SH) terminated PEGs because of the very high specific binding affinity of thiol groups to metal gold (S-Au bond energy = 47 kcal mol⁻¹). Thiol-PEG can react in solution with gold nanoparticles providing covalently stable and biocompatible gold nanoparticles [175].

Bidentate PEGs (PEG-thiolic acid and PEG-dihydroxylic acid) conjugated on gold nanoparticle surface substantially improved the stability in biological media [176]. Gold nanoparticles PEGylated with thiolic-modified 5 kDa PEG were shown to perform better in vivo than gold nanoparticles coated with thiol-PEG since the latter can release the PEG by exchange with thiolated compounds in the body [177].

The in vivo performance of gold nanorods stabilized with thiol-PEG depends on the polymer molecular weight. Accordingly, stable nanorods for blood circulation were obtained with 5 and 10 kDa PEGs while smaller or larger PEGs were poorly flexible or bend into a mushroom-like configuration, respectively [34, 178].

The maximum achievable density of PEG chains on gold nanoparticles was 2.2 nm² per chain, which is comparable to the hydrodynamic size of the mPEG-thiol molecule [179]. At saturation, the PEG molecules are so tightly packed that opsonins will be prevented from adsorbing on the coating layer thus prohibiting the binding to macrophage receptors. Layer-by-layer (LBL) coating approaches relying on electrostatic interactions between polymer chains and gold nanoparticle surface have been investigated to build up a hydrophilic polymer corona on gold nanoparticles. The colloidal core of gold nanoparticles was coated with layers of poly(allylamine) (PAH) and poly-(styrenesulfonate) (PSS). F-HPMA, a hydrophilic terpolymer composed by 90% mol of N-(2-hydroxypropyl) methacrylamide, was then conjugated to the amino groups of PAH to yield core/shell multifunctional nanoparticles. The terpolymer provides a highly water-solvated corona layer that minimizes the opsonisation process and bestows remarkable stealth properties on nanoparticles. The multifunctional nanoparticles did not show a significant degree of adsorption on the macrophage membrane or internalization by the cells [180].

PEG was grafted on gold nanoparticle surface according to a process named physisorption. PEG-NH₂ and 1,2-distearyl-sn-glycero-3-phosphoethanolamine (DSPE) were conjugated to the backbone of polyglutamic acid (PGA) at 60% and 10% mol ratio with respect to the PGA monomers, respectively. Gold nanoparticle coating was achieved by exchanging the citrate adsorbed on gold particles, obtained by tetrachloroauric acid reduction, with the multifunctional polymer PGA-DSPE-mPEG. These functionalized colloidal systems showed high stability to aggregation over 48 hours of incubation in 50% fetal calf serum [181].

Polyethylene glycol-block-poly(2,N,N-dimethylamino) ethyl methacrylate (PEG-b-PAMA) was shown to improve the long-term stability of gold nanoparticles. The tertiary amino group of PAMA can strongly adsorb to the surface of gold nanoparticles even though the mechanism of immobilization is not clear yet. The alkylation of pendant amino groups along the polymer backbone seems to favour the interaction of the nitrogen atom with gold. The colloidal system was physically stable over 4 days of storage in 95% human serum [182].

Gold nanoshell can also be coated with a variety of polymers according to the same postproduction strategies reported for gold nanoparticles and nanorods.

2.6.4. Polymer Coating of Silica Nanoparticles. Silica nanoparticles possessing an organosilica core and a PEG shell were prepared according to a one-pot procedure. The process includes the co-hydrolysis and copolycondensation reactions of ω-methoxy-(polyethyleneoxy)propyltrimethoxysilane and hydroxymethylriethoxysiloxane mixtures in the presence of sodium hydroxide and a surfactant [183].

Alternatively, silica nanoparticles were also PEGylated by a postproduction procedure by mesoporous silica nanoparticle reaction with PEG-silanes. It was reported that the PEG coating inhibits the nonspecific binding of human serum proteins to PEGylated silica nanoparticles. This is a guarantee if the molecular weight of the polymer is higher than 10 kDa and the polymer density (defined as wt% of the coating on the mesoporous silica nanoparticles) is 0.75 wt% and 0.075 wt% for PEG 10 kDa and PEG 20 kDa, respectively. The human serum albumin adsorption was only 2.5 wt% when PEGylated silica nanoparticles were tested compared to 18.7% for non-PEGylated nanoparticles [184].

PEG coating on silica nanoparticles can also be achieved via electrostatic adsorption of polyethyleneimine-polyethylene glycol (PEI-PEG) copolymer. The polymeric coating was stable and tightly associated with the particle surface by virtue of the strong electrostatic interactions between the polyamino backbone of the copolymer and the negatively charged silica surface. The PEI-PEG copolymer investigated had 34 PEG chains (5 kDa) per PEI chain. The efficiency of the PEG coating in preventing the adsorption of serum proteins on the nanoparticle surface was remarkably high. Protein adsorption was at the limit of sensitivity for X-ray photoelectron spectroscopy (XPS) detection and no aggregation was observed for the coated nanoparticles [185].
The synthesis of PEO on silica nanoparticles has also been performed resulting in a 40 wt% of grafted PEO. The method has been carried out first by a two-step conjugation process of prehydrolyzed 3-glycidoxypropyl trimethoxysilane and aluminium isopropoxide to the particle surface. The subsequent polymerization of ethylene oxide was carried out at 55°C. The density of the polymer chains was found to be strictly dependent on the conjugation efficiency of the metal alkoxide on the particle surface [186, 187].

3. Conclusions

The therapeutic advantages of nanotechnology-based drug delivery systems include improved drug bioavailability, extended duration of action, reduced frequency of administration, and lower systemic toxicity with beneficial effects on the patient acceptance. The medical management of malignancies has already benefited from the outcomes of few nanotechnology-based delivery systems. However, following intravenous administration, drug-loaded nanocarriers are rapidly opsonised by a variety of proteins, most of them belonging to the complement system, and undergo very rapid clearance via the MPS cells.

In this paper, the main aspects of polymer coating technology applied to colloidal drug delivery systems have been reviewed. A number of studies and examples reported in the literature showing that stealthiness can be conferred to nanocarriers by a proper formulation design and predicated by precise physicochemical determinants have been detailed and critically discussed.

The evidence reported in the literature shows that the residence time in the blood of nanocarriers can be prolonged by surface coating with neutral or zwitterionic polymers characterized by high hydrophilicity and high flexibility. Furthermore, the stealth character of the nanocarriers depends on the polymer organization on the particle surface, namely, density, thickness, and association stability. The beneficial effect of nanocarrier polymer coating in promoting stealth properties generates predominantly from the polymer ability to confer a physical barrier to the biorecognition of adsorbed opsonins by macrophages. On the other hand, the paper underlines that the components of the hydrated polymeric corona are not completely inert to the biological environment and these materials do not totally prohibit the protein opsonisation [124].

In conclusion, while many discoveries in the field of nanotechnology have allowed to clearly improve the performances of stealth nanocarriers, a significant amount of work needs to be done before these systems achieve the required level of safety for use in humans. Studies are required to fully profile at the molecular level the interactions of the nanocarriers with the biological environment and the MPS cell response that is triggered upon contact with a specific nanocarrier.

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Review Article

Bisphosphonates and Cancer: What Opportunities from Nanotechnology?

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Bisphosphonates (BPs) are synthetic analogues of naturally occurring pyrophosphate compounds. They are used in clinical practice to inhibit bone resorption in bone metastases, osteoporosis, and Paget’s disease. BPs induce apoptosis because they can be metabolically incorporated into nonhydrolyzable analogues of adenosine triphosphate. In addition, the nitrogen-containing BPs (N-BPs), second-generation BPs, act by inhibiting farnesyl diphosphate (FPP) synthase, a key enzyme of the mevalonate pathway. These molecules are able to induce apoptosis of a number of cancer cells in vitro. Moreover, antiangiogenic effect of BPs has also been reported. However, despite these promising properties, BPs rapidly accumulate into the bone, thus hampering their use to treat extraskeletal tumors. Nanotechnologies can represent an opportunity to limit BP accumulation into the bone, thus increasing drug level in extraskeletal sites of the body. Thus, nanocarriers encapsulating BPs can be used to target macrophages, to reduce angiogenesis, and to directly kill cancer cell. Moreover, nanocarriers can be conjugated with BPs to specifically deliver anticancer agent to bone tumors. This paper describes, in the first part, the state-of-art on the BPs, and, in the following part, the main studies in which nanotechnologies have been proposed to investigate new indications for BPs in cancer therapy.

1. The Bisphosphonates

Bisphosphonates (BPs), synthetic analogues of naturally occurring pyrophosphate compounds, represent the treatment of choice for different diseases, such as metabolic bone disease, osteoporosis, Paget’s disease, and bone metastases [1]. In the 1960s Fleisch et al. proposed that inorganic pyrophosphate, a naturally occurring polyphosphate and a known product of many biosynthetic reactions in the body, might be the body’s own natural “water softener” that normally prevents calcification of soft tissues and regulates bone mineralization by binding to newly forming crystals of hydroxyapatite [2, 3]. It subsequently became clear that calcification disorders might be linked to disturbances in inorganic pyrophosphate (PPI) metabolism [2, 3]. Alkaline phosphatase present in bone destroys pyrophosphate locally, thereby allowing amorphous phase calcium phosphate to crystallize and inducing mineralization of bone [2]. The major limitation of pyrophosphate is that, when orally administered, it is inactive because of its hydrolysis in the gastrointestinal tract. During the search for more stable analogues of pyrophosphate that might also have the antimineralization properties of pyrophosphate but would be resistant to hydrolysis, several different chemical classes were studied. The bisphosphonates (at that time called diphosphonates), characterized by P–C–P motifs, were among these classes [1–4]. The fundamental property of BPs, which has been exploited by industry and medicine, is their ability to form bonds with crystal surfaces and to form complexes with cations in solution or at a solid-liquid interface. Since BPs are synthetic analogues of pyrophosphates, they have the same chemical activity, but greater stability [1–4]. Like pyrophosphates, BPs had high affinity for bone mineral and they were found to prevent calcification both in vitro and in vivo but, unlike pyrophosphate, they were also able to prevent experimentally induced pathologic calcification when given orally to rats in vivo. This
property of being active orally was key to their subsequent use in humans [4]. Perhaps the most important step toward the successful use of BPs occurred when their ability to inhibit hydroxyapatite crystals dissolution was demonstrated. This finding led to following studies designed to determine if they might also inhibit bone resorption [5]. The clarification of this property made BPs the most widely used and effective antiresorptive agents for the treatment of diseases in which there was an increase in the number or activity of osteoclasts, including tumor-associated osteolysis and hypercalcemia [6]. After more than three decades of research, first-, second-, and third-generation bisphosphonates have been developed. Changes in chemical structure have resulted in increased potency, without demineralization of bone [1]. There is now a growing body of evidence regarding the efficacy of these drugs in clinical settings. All BPs that act significantly on the skeleton are characterized, as stated above, by P–C–P bond (Figure 1(a)), in contrast to pyrophosphate, which has a P–O–P bond (Figure 1(b)).

This peculiarity confers stability both to heat and to most chemical reagents and is one of the most important properties of these compounds [4]. Extensive chemical research programs have produced a wide range of molecules with various substituents attached to the carbon atom. Variations in potency and in the ability of the compounds to bind to crystals in bone one determined by the chemical and three-dimensional structure of the two side chains, R₁ and R₂, attached to the central, geminal carbon atom [1–4]. The bioactive moiety comprising the R₁ chain of the molecule is considered primarily responsible for BPs’ effect on resorption, and small changes in this part of the structure can result in large differences in their antiresorptive potencies [4]. The uptake and binding to bone mineral is determined by the bi- or tridentate ligand (hydroxybisphosphonate) of the molecule, which is also thought to be responsible for the physicochemical effects, the most important being the inhibition of growth of calcium crystals. The most effective structures for binding to bone mineral consist of the two phosphonate groups attached to the central carbon and the substitution at R₁ with a hydroxyl or amino group that provides tridentate binding [4]. In fact, the addition of a hydroxyl (OH) or primary amino (NH₂) group increases the affinity for calcium ions, resulting in preferential localization of these drugs to sites of bone remodelling. Increasing the number of carbon atoms in the side chain initially increases and then decreases the magnitude of the effect on bone resorption [1–4]. The early compounds, clodronate (CLO) and etidronate (ETI), contained simple substituents (H, OH, Cl, CH₃) and lacked a nitrogen atom (Figure 2).

Subsequently, more complex and potent compounds were produced by the insertion of a primary, secondary, or tertiary nitrogen function in the R₂ side chain, for example, pamidronate (PAM), alendronate (ALN), ibandronate (IBA), and incadronate (INC), which have an alkyl R₂ side chain, or risedronate (RIS), zoledronate (ZOL), and minodronate (MIN), which have heterocyclic rings in the R₂ side chain (Figure 2). Variation of the substituents modulates the pharmacologic properties and gives each molecule its unique profile [7].

2. Intracellular Effect and Pharmacodynamics of Bisphosphonates

Extensive structure/activity studies have resulted in several very useful drugs that combine potent inhibition of osteoclastic bone resorption with good clinical tolerability [5–8]. The pronounced selectivity of BPs for bone rather than other tissues is the basis for their value in clinical practice. The antiresorptive effect cannot be accounted simply by adsorption of BPs to bone mineral and prevention of hydroxyapatite dissolution. It became clear that BPs must inhibit bone resorption by cellular effects on osteoclasts rather than simply by physicochemical mechanisms [5]. Bisphosphonate moiety and R₁ group are both essential for hydroxyapatite affinity [8]. The BPs bind to hydroxyapatite crystals in the area of osteoclast-mediated bone erosion; during resorption, the dissolution of hydroxyapatite crystals by osteoclast determines the consequent release of the bisphosphonate that may indeed come into contact with osteoclasts and inhibit their absorption capacity [8]. Incorporation of an aminoalkyl side chain at R₁ increases antiresorptive potency by 10-fold; also, the length of carbon chain is important (alendronate is about 1000-fold more potent than etidronate while pamidronate is only 100-fold more active than etidronate) [4, 8]. In addition, incorporation of a nitrogen heterocycle (third-generation agents) further enhances antiresorptive potency: the most active compound in this class is ZOL, a BP containing an imidazole ring, which is up to 10000-fold more potent than both CLO and ETI in some experimental systems. During bone resorption, BPs are probably internalized by endocytosis along with other products of resorption [4, 8]. Many studies have shown that BPs can affect osteoclast-mediated bone resorption in a variety of ways, including effects on osteoclast recruitment, differentiation, and resorptive activity, and may induce apoptosis [7]. Because mature, multinucleated osteoclasts are formed by the fusion of mononuclear precursors of hematopoietic origin, BPs could also inhibit bone resorption by preventing osteoclast formation, in addition to affecting mature osteoclasts. In vitro, BPs can inhibit dose-dependently the formation of osteoclast-like cells in long-term cultures of
human bone marrow [7]. In organ culture, also, some BPs can inhibit the generation of mature osteoclasts, possibly by preventing the fusion of osteoclast precursors [5]. In contrast to their ability to induce apoptosis in osteoclasts, which contributes to the inhibition of resorptive activity, some experimental studies suggest that BPs may protect osteocytes and osteoblasts from apoptosis induced by glucocorticoids [9].

Since the early 1990s there has been a systematic effort to elucidate the molecular mechanisms of action of BPs, and, not surprisingly, it has been found that they could be divided into 2 structural subgroups [10, 11]. The first group comprises the nonnitrogen-containing bisphosphonates, such as CLO and ETI, that perhaps most closely resemble pyrophosphate. These can be metabolically incorporated into nonhydrolyzable analogues of adenosine triphosphate (ATP) methylene-containing (AppCp) nucleotides, by reversing the reactions of aminoacyl-transfer RNA synthetases [12]. The resulting metabolites contain the P–C–P moiety in place of the β,γ-phosphate groups of ATP [13]. Intracellular accumulation of these metabolites within osteoclasts inhibits their function and may cause osteoclast cell death, most likely by inhibiting ATP-dependent enzymes, such as the adenine nucleotide translocase, a component of the mitochondrial permeability transition pore [14]. Induction of osteoclast apoptosis seems to be the primary mechanism by which the simple BPs inhibit bone resorption, since the ability of CLO and ETI to inhibit resorption in vitro can be overcome when osteoclast apoptosis is prevented using a caspase inhibitor [15].

In contrast, the second group, comprising the nitrogen-containing bisphosphonates (N-BPs), which are several orders of magnitude more potent at inhibiting bone resorption in vivo than the simple bisphosphonates, is not
Figure 3: Isoprenoids are synthesized from the mevalonate pathway that starts from reaction catalyzed by the 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (the rate-limiting reaction in cholesterol biosynthesis) which catalyzes the conversion of HMG-CoA to mevalonic acid. The pathway triggered by this reaction can lead to the synthesis of a key isoprenoid molecule, the farnesyl-pyrophosphate (Farnesyl-PP), whose formation is catalyzed by the farnesylpyrophosphate synthase (FPPS). Farnesyl-PP can be either converted by a series of reactions in cholesterol or can be transferred on target cellular proteins as Farnesyl-PP itself (reaction catalyzed by farnesyltransferase, FTase) or firstly converted in geranyl-geranyl-pyrophosphate (Geranyl-Geranyl-PP) and then transferred on cellular proteins by type I or type II geranylgeranyl-transferase (GGTase). FTase and GGTase-I catalyze the prenylation of substrates with a carboxy-terminal tetrapeptide sequence called a CAAX box, where C refers to cysteine, A refers to an aliphatic residue, and X typically refers to methionine, serine, alanine, or glutamine for FTase or to leucine for GGTase-I. Following prenylation of physiological substrates, the terminal three residues (AAX) are subsequently removed by a CAAX endoprotease, and the carboxyl group of the terminal cysteine is methyl esterified by a methyltransferase. At this moment prenyl substrates, such as Ras, are ready to be located on the inner side of the biological membranes to receive signals mediated by external factors. ZOL specifically inhibits the FPPS activity required for the synthesis of farnesyl and geranylgeranyl lipidic residues blocking prenylation of Ras that regulates the proliferation, invasive properties, and prosangiogenic activity of human tumour cells.

metabolized to toxic analogues of ATP [16]. N-BPs act by inhibiting farnesyl diphosphate (FPP) synthase, a key enzyme of the mevalonate pathway (Figure 3). This enzyme is inhibited by nanomolar concentrations of N-BPs. ZOL and the structurally similar MIN are extremely potent inhibitors of FPP synthase [6] and inhibit the enzyme even at picomolar concentrations. Importantly, studies with recombinant human FPP synthase revealed that minor modifications to the structure and conformation of the R2 side chain that are known to affect antiresorptive potency also affect the ability to inhibit FPP synthase. These studies strongly suggest that FPP synthase is the major pharmacologic target of N-BPs in osteoclasts in vivo and help to explain the relationship between bisphosphonate structure and antiresorptive potency [6]. Clinical and experimental evidence indicates that N-BPs suppress the progression of bone metastases, and recent observations suggest that this effect may be independent of the inhibition of bone resorption [17]. Tumour progression and metastasis formation are critically dependent on tumour angiogenesis [18]. Antiangiogenic treatments suppress tumour progression in animal models, and many antiangiogenic substances are currently being tested in clinical trials for their therapeutic efficacy against human cancer [19]. Recent research indicates that ZOL possesses antiangiogenic activities [20].

The exact mechanism by which N-BPs inhibit FPP synthase is only just becoming clear. The recent generation of X-ray crystal structures of the human FPP synthase enzyme, co-crystallized with RIS or ZOL [51], revealed that N-BPs bind the geranyl diphosphate (GPP) binding site of the enzyme, with stabilizing interactions occurring between the nitrogen moiety of the N-BP and a conserved threonine and lysine residue in the enzyme. Enzyme kinetic analysis with human FPP synthase indicates that the interaction with N-BPs is highly complex and characteristic of "slow tight binding" inhibition [51]. By inhibiting FPP synthase, N-BPs
prevent the synthesis of FPP and its downstream metabolite geranylgeranyl diphosphate [11]. These isoprenoid lipids are the building blocks for the production of a variety of metabolites, such as dolichol and ubiquinone, but are also required for posttranslational modification (prenylation) of proteins, including small GTPases [11]. The loss of synthesis of FPP and geranylgeranyl diphosphate therefore prevents the prenylation at a cysteine residue in characteristic C-terminal motifs of small GTPases, such as Ras, Rab, Rhos, and Rac (Figure 3). Small GTPases are important signaling proteins that regulate a variety of cell processes important for osteoclast function, including cell morphology, cytoskeletal arrangement, membrane ruffling, trafficking of vesicles, and apoptosis. Prenylation is required for the correct function of these proteins because the lipid prenyl group serves to anchor the proteins in cell membranes and may also participate in protein-protein interactions [3, 20].

3. Pharmacokinetics of Bisphosphonates

Recent studies with a fluorescently labelled bisphosphonate have shown that macrophages and osteoclasts internalize bisphosphonates into membrane-bound vesicles by fluid-phase endocytosis; endosomal acidification then seems to be absolutely required for exit of bisphosphonate from vesicles and entry into the cytosol [52]. This mechanism of uptake suggests that large amounts of N-BPs are poorly absorbed in the intestine due to their negative charge hindering their transport across the lipophilic cell membrane; they are therefore given mainly intravenously. A pharmacokinetic evaluation of ZOL for treatment of multiple myeloma and bone metastases, carried out by Ibrahim et al., exhibited a three-compartment model [53]. The distribution half-life (\( t_{1/2} \)) was 14 min, followed by a \( \beta \)-phase of 1.9 h. A prolonged terminal phase, with a half-life of at least 146 h, might indicate a slow release of ZOL from the bone back into the plasma. ZOL pharmacokinetics were dose proportional from 2 to 16 mg based on peak plasma concentration (\( C_{\text{max}} \)) and area under the curve (\( \text{AUC}_{0-\infty} \)). ZOL dosed every 21 days did not demonstrate significant plasma accumulation. In vitro studies indicated that 22% of ZOL is protein bound. The excretion of ZOL was primarily renal. Approximately 40% of the radiolabeled ZOL dose was recovered in urine within 24 h. Only traces of ZOL were observed in the urine after two days, suggesting a prolonged period of ZOL binding to bone. Population modeling described the ZOL clearance as a function of creatinine clearance. On the basis of a comparison of \( \text{AUC}_{0-\infty} \), patients with mild or moderate renal impairment had 15 and 43% higher exposure, respectively, than patients with normal renal function. However, no significant relationship between ZOL exposure (AUC) and adverse events might be established. The use of ZOL in patients with severe renal failure was not recommended. In vitro studies showed no inhibition of or metabolism by cytochrome P-450 enzymes [53].

One of the most important limits of N-BPs, which makes the direct anticaner activity difficult to demonstrate in vivo, is just their pharmacokinetic profile. This issue is demonstrated by also other pharmacological studies performed on different N-BPs. In fact, after intravenous administration (4 mg over 15 min) of ZOL, an immediate increase of its concentration in peripheral blood was recorded, as shown by estimations of the early distribution and elimination of the drug, which resulted in plasma half-lives of the drug of about 15 min (\( t_{1/2a} \)) and of 105 min (\( t_{1/2b} \)), respectively. The maximum plasma concentration (\( C_{\text{max}} \)) of ZOL was about 1 \( \mu \)M, that was from 10- to 100-fold less than that required in in vitro studies to induce apoptosis and growth inhibition in tumour cell lines, while the concentrations required for anti-invasive effects were in the range of those achieved after in vivo administration. Moreover, approximately 55% of the initially administered dose of the drug was retained in the skeleton and was slowly released back into circulation, resulting in a terminal elimination half-life (\( t_{1/2b} \)) of about 7 days [54, 55]. Other studies performed on ALN demonstrate that N-BP concentration in noncalcified tissues declined rapidly at 1 h (5% of the initial concentration). On the other hand, its concentration in the bone continuously increased, reaching its peak at 1 h, demonstrating that a significant redistribution of the drug from noncalcified tissues to bone occurred. The drug was retained in bone tissue for a long time and was slowly released into plasma, with a terminal half-life of about 200 days [56]. Similar data were obtained with IBA and ZOL [54–57] demonstrating that long-lasting accumulation in bone is a common feature of N-BPs. The rapid redistribution of N-BPs results not only in a short exposure of noncalcified tissues to the drug, but also in a prolonged accumulation in bone where N-BPs can also reach higher and tumoricidal concentrations. These considerations explain the relative efficacy of N-BPs on tumours placed in bone tissues [20]. In biodistribution studies by Weiss et al. performed in rats and dogs administered with single or multiple intravenous doses of \(^{14}\text{C}\)-labeled ZOL, its levels rapidly decreased in plasma and noncalcified tissue, but higher levels persisted in bone and slowly diminished with a half-life of approximately 240 days. In contrast, the terminal half-lives (50 to 200 days) were similar in bone and noncalcified tissues, consistent with ZOL rapidly but reversibly binding to bone, being rapidly cleared from the plasma, and then slowly released from bone surfaces back into circulation over a longer time. The results suggested that a fraction of ZOL is reversibly taken up by the skeleton, the elimination of drug is mainly by renal excretion, and the disposition in blood and noncalcified tissue is governed by extensive uptake into and slow release from bone [58]. It is important to consider that ZOL is not taken up by tumor cells but prevalently by cells with increased endocytosis processes such as osteoclasts and macrophages. However, owing to the intrinsic pharmacokinetics limitations of ZOL, more efforts were required to increase the anticancer activity of both this drug and the other members of N-BPs family.
4. Bisphosphonate and Cancer:  
**In Vitro Studies**

FPP synthase is a highly conserved, ubiquitous enzyme; therefore, N-BPs have the potential to affect any cell type in vitro. Among BPs recent advances suggest that ZOL, beyond the strongest activity of antboneresorption, has direct anticancer effects. In fact, extensive in vitro preclinical studies support that ZOL can inhibit tumor cell adhesion to extra-cellular matrix proteins, thereby impairing the process of tumour-cell invasion and metastasis [59]; moreover, it was demonstrated that ZOL has a direct effect on angiogenesis in vitro [60, 61] and an in vitro stimulation of γ/δ T lymphocytes, which play important roles in innate immunity against cancer [62]. One of the crucial mechanisms responsible for the antitumor activity of ZOL is the induction of tumor cell apoptosis [63].

Inhibition of protein prenylation by N-BPs can be shown by measuring the incorporation of 14C mevalonate into farnesylated and geranylgeranylated proteins [64]. The most potent FPP synthase inhibitor, ZOL, almost completely inhibits protein prenylation in J774 cells at a concentration of 10 μmol/L, which is similar to the concentration that affects osteoclast viability in vitro [65]. Alternatively, the inhibitory effect of N-BPs on the mevalonate pathway can be shown by detecting accumulation of the unprenylated form of the small GTPase Rap1A, which acts as a surrogate marker for inhibition of FPP synthase and which accumulates in cells exposed to N-BPs. Roeofs et al. have shown the ability of N-BPs to inhibit the prenylation of Rap1A in a wide range of cultures of different types of primary cells and cell lines such as osteoclasts, osteoblasts, macrophages, epithelial, and endothelial cells, and breast, myeloma, and prostate tumor cells [16]. Macrophages and osteoclasts were the most sensitive to low concentrations of N-BPs (1–10 μM) in vitro. Moreover, treatment with 100 μM N-BP caused a detectable accumulation of unprenylated Rap1A already after few hours. Concerning myeloma cells, in order to detect the unprenylated form of Rap1A, longer times of in vitro treatments and higher concentrations were required [16].

BPs have also been shown to inhibit adhesion of tumor cells to extracellular matrix (ECM) proteins and to promote invasion and metastasis. Inhibition of the mevalonate pathway and induction of caspase activity are important mechanisms in explaining the inhibitory effects of N-BPs on tumor cells adhesion to the ECM and on invasiveness [66]. In vitro findings have demonstrated that N-BPs, particularly ZOL, can affect endothelial cells exerting a suppressive effect on angiogenesis [67, 68]. In fact, N-BPs inhibit the expression of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) that induce the proliferation of endothelial cells and enhance the formation of capillary-like tubes.

Recent evidence suggests that ZOL is a potent inducer of apoptosis in several cancer cell types [69]. It has recently been demonstrated in vitro that N-BPs, PAM and ZOL, induce apoptosis and growth inhibition in human epithemoid cancer cells, together with depression of Ras-dependent Erk and Akt survival pathways. These effects occurred together with poly(ADP-ribose) polymerase (PARP) fragmentation and the activation of caspase 3 [70]. Moreover, the latter seems to be essential for apoptosis induced by N-BPs in this experimental model. Furthermore, it was reported that ZOL induced growth inhibition on both androgen-dependent LnCaP and androgen-independent PC3 prostate cancer cell lines with GI accumulation. Recent studies showed that the effects of ZOL were caspase dependent. In human breast cancer cell lines, ZOL induced a modulating expression of Bcl-2 and subsequent caspase 3 activation. These events might be precipitated by inhibition of Ras activation, which requires protein farnesylation [71].

In human colon carcinoma HCT-116 cells, ZOL strongly inhibited the proliferation paralleled by a GI cell cycle accumulation and induction of apoptosis via a caspase-dependent mechanism [72]. Recent studies by Fujita et al. demonstrated the involvement of the mevalonate pathway in the antiproliferative and proapoptotic effects of ZOL on ACHN renal cell carcinoma cells [73].

The sensitivity of different cell types to N-BPs most likely depends largely on their ability to internalize sufficient amounts of N-BPs to inhibit FPP synthase. In view of the pharmacokinetic concerns that limit the anticancer activity of ZOL, in the last decade the scientists have defined a series of pharmacological and molecular strategies. Some approach was represented by the design of rationale-based drug combinations and the improvement of the pharmacokinetic profile. Evidence from both in vitro and in vivo models indicated a synergistic antitumor activity of N-BPs when used in combination with either cytotoxic drugs or targeted molecular therapies [69]. Based on the relevance of the farnesylation inhibitory effects on antitumour activity of N-BPs, the farnesyl transferase inhibitor (FTI) R115777 was used together with PAM or ZOL, and the effects of the combination treatment on growth inhibition and apoptosis were evaluated. N-BPs and FTI given in combination were strongly synergistic [70]. Notably, low concentrations of FTI induced a strong increase of Ras expression with only a moderate reduction of Ras activity that was, on the other hand, significantly reduced by the combined treatment [70]. These data suggested that escape mechanisms for the inhibition of isoprenylation of Ras might be based on the geranylgeranylation or other prenylation processes [74]. The addition of farnesol to cells treated with the combination abolished the effects of the N-BPs/FTI combination on apoptosis and on the activity of the signaling molecules, suggesting that the synergistic growth-inhibitory and proapoptotic effects produced by the N-BPs/FTI combination involved the inhibition of both Erk and Akt survival pathways acting in these cells in a Ras-dependent fashion [70].

A synergistic interaction between R115777 and ZOL was also found on both androgen-independent PC3 and androgen-dependent LnCaP prostate cancer cell lines [70], and the effects were attributed to enhanced apoptosis and inactivation of Erk and Akt. Several papers reported the significant cytostatic and cytotoxic effects of docetaxel (DTX) and ZOL on the hormone-sensitive prostate cancer cell line, LnCaP [17, 75, 76]. In details, the highest inhibition of cell proliferation was observed after DTX exposure and was already evident...
at concentrations 200-fold lower than the plasma peak level. Fabbri et al. hypothesized the use of low DTX doses in concomitance with and followed by a prolonged ZOL exposure to reduce the prostatic tumour cell population and to rapidly induce eradication of hormone-resistant cells present in hormone-responsive tumours, without compromising the use of conventional-dose DTX for the first-line treatment for hormone-sensitive prostate cancer. The principal molecular mechanisms involved were found to be apoptosis and decreased pMEK and Mcl-1 expression [77]. Furthermore, Karabulut et al. found that the combination treatment of DTX and ZOL in hormone and drug refractory, PC-3 and DU-145 prostate cancer cells, synergistically inhibited cell growth by inducing the apoptotic pathways through the downregulation of the antiapoptotic protein Bcl-2 [78].

A further strategy for the implementation of ZOL activity is the interference of its molecular targets. The recent analysis—performed by CDNA microarray platform—of gene modulation induced by ZOL in androgen-resistant prostate PC3 cell line showed a significant dose- and time-dependent reduction of transcriptional activity of CYR61 after exposure to ZOL, as demonstrated by the reduction of the transcriptional activity of Cyr61 promoter [79]. This result is considered of interest in designing new therapeutic approaches in androgen-independent prostate cancer.

5. Bisphosphonate and Cancer: In Vivo Studies

In addition to the established in vitro induction of tumor cell apoptosis, also emerging in vivo evidence supports N-BP anticancer activity. Preclinical studies support that ZOL displays an antitumor activity, including direct antitumor in vivo effects such as inhibition of tumor cell adhesion to mineralized bone, invasion and effects on angiogenesis (animal models) probably due to the modification of various angiogenic properties of endothelial cells [59–61]; effects on the metastatic process (animal models) [60]; stimulation of γ/δ T lymphocytes in humans [62]. N-BPs may target several steps involved in the metastatic process, extracellular matrix, extravasation into distant tissues, angiogenesis, and avoidance of immune surveillance [80].

Roelofs et al. detected the unprenylated form of Rap1A in osteoclasts purified from ALN-treated rabbits using immunomagnetic beads, thereby showing that N-BPs inhibit protein prenylation in vivo [16].

Many animal studies have focused on models of multiple myeloma, breast cancer, and prostate cancer showing that the newer N-BPs can significantly reduce the number and size of osteolytic lesions in tumor-bearing mice, reduce skeletal tumor burden, induce tumor cell apoptosis in bone lesions, reduce serum levels of tumor markers, and prevent formation of bone metastases [81–83].

A recent study, utilizing a plasmacytoma xenograft model without complicating skeletal lesions, demonstrated that treatment with ZOL led to significant prolongation of survival in severe combined immunodeficiency mice inoculated with human INA-6 plasma cells. Following treatment with ZOL, histological analysis of tumors revealed extensive areas of apoptosis associated with poly(ADP-ribose) polymerase cleavage. Furthermore, western blot analysis of tumor homogenates demonstrated the accumulation of unprenylated Rap1A, indicative of the uptake of ZOL by nonskeletal tumors and inhibition of farnesyl pyrophosphate synthase [84]. This is one of the few evidence of direct antitumor effects of N-BPs in plasma cell tumors in vivo. In fact, it is generally believed that the reduction in tumor burden observed in some animal models may be due to inhibition of osteoclast activity [85]. For example, bisphosphonates, including IBA and ZOL acid, were shown to inhibit the development of osteolytic bone lesions in the 5T2MM model and alternative models of myeloma bone disease [86]. Moreover, the effect of bisphosphonates on the osteoclast stimulatory activity (OSA) was evaluated in the marrow of patients with multiple myeloma. For this purpose, the effects of IBA treatment prior to the development of bone disease were examined in a murine model of human myeloma. Sublethally irradiated severe combined immunodeficient (SCID) mice were transplanted with ARH-77 cells on day 0. These ARH-77 mice were treated daily with subcutaneous injections of N-BP started before or at different times after tumor injection. ARH-77 mice were sacrificed after they developed paraplegia, and the data demonstrated that early treatment of ARH-77 mice with IBA prior to development of myeloma bone disease decreases OSA and possibly retards the development of lytic lesions but not eventual tumor burden [87]. Numerous studies in breast cancer models have also been reported. A study using MDA-MB-231 human breast tumour cells injected directly into the femoral artery of male athymic rats also showed that IBA (10 μg/kg/day, days 18 to 30) reduced the extent of the osteolytic lesions [88]. This study also provided evidence that once tumours have reached a certain size (>6 mm in this model) they become less dependent on the bone microenvironment for their further expansion, and hence less sensitive to BP therapy. A study by van der Pluijm and colleagues showed that BPs modify tumour growth primarily through effects on bone, rather than through targeting tumour cells directly [89]. MDA-231-B/luc+ breast cancer cells were implanted by intracardiac injection, and olpadronate given as a preventive (subcutaneous 1.6 μmol/kg/day from 2 days before implantation) or a treatment (days 3 to 43) schedule. Effects on the formation of new bone metastases and osteolysis were assessed, as well as tumour burden, both inside and outside the bone marrow cavity. However, the reduction in tumour growth was only transient and did not affect progression of established tumours. Studies in a prostate cancer model have also recently been reported. In those studies PC-3 and LuCaP cells were injected directly into the tibia of mice [81], PC-3 cells form osteolytic lesions, and LuCaP cells form osteoblastic lesions. The treatment group receiving ZOL (5 μg s.c. twice weekly) either at the time of tumor cell injection or after tibial tumors was established (7 days for PC-3 tumors and 33 days for LuCaP tumors). Treatment with ZOL significantly inhibited growth of both osteolytic and osteoblastic metastases by radiographic analysis and also reduced skeletal tumor burden, as evidenced by a significant decrease in serum levels of
prostate-specific antigen in animals bearing LuCaP tumors. The observed reduction in serum prostate-specific antigen levels provides compelling direct evidence of the antitumor activity of ZOL in this animal model. The potential of ZOL to prevent bone metastasis was also demonstrated in an animal model of prostate cancer [90].

In order to separate the direct antitumour effects of BPs from those mediated via bone, the sequential or combined treatment with other antitumor agents were investigated.

The synergistic interaction between R115777 and ZOL on both androgen-independent PC3 and androgen-dependent LNCaP prostate cancer cell lines was also found to induce cooperative effects in vivo on tumour growth inhibition of prostate cancer xenografts in nude mice with a significant survival increase [70]. These in vivo and in vitro effects were in both cases attributed to enhanced apoptosis and inactivation of Erk and Akt.

On the basis of preliminary results about sequence-dependent synergistic effects of ZOL and DTX combination on growth inhibition and apoptosis of human prostate cancer cells, the closely related taxane, paclitaxel (PTX), has shown synergistic inhibitory activity with ZOL in animal models for lung cancer. Compared with vehicle and ZOL alone, cancerous cells in the bone of mice with PTX + ZOL expressed higher levels of Bax and lower levels of Bcl-2 and Bcl-xL. Moreover, this drug combination produced a significant reduction in serum n-telopeptide of type I collagen which levels correlate with the rate of bone resorption. The results of this study indicated that ZOL enhanced the efficacy of PTX synergistically, by reducing the incidence of bone metastasis from lung cancer and prolonging survival [91]. These in vivo and in vitro effects were in both cases attributed to enhanced apoptosis and inactivation of Erk and Akt.

Ottewell et al. also showed that the treatment with ZOL after exposure to doxorubicin (DOX) elicited substantial antitumor effects in a mouse model of breast cancer. Interestingly, the treatment induced an increase in the number of caspase-3-positive cells paralleled by a decrease in the number of tumour cells positive for the proliferation marker Ki-67. Moreover, the sequential treatment with clinically relevant doses of DOX, followed by ZOL, reduced intraosseous but not extraosseous growth of breast tumours in mice injected with a clone of MDA-MB-231 [92].

The findings of synergy of interaction between ZOL and other agents could reduce the ZOL concentrations required for antitumour activity and then could allow the achievement of its effective in vivo levels, overcoming the limits associated with the pharmacokinetics of ZOL.

Another strategy to potentiate the antitumor effects of chemotherapeutic agents and ZOL could be also the administration of the drugs at repeated low doses (“metronomic” way). Santini et al. recently demonstrated that weekly administration of ZOL has higher antitumor effects as compared with conventional 3 weekly administration in nude mice xenografted with breast cancer cells, even if the total administered dose is the same [93]. Moreover, a single dose of 1 mg ZOL is able to induce a significant reduction of circulating VEGF in patients with bone metastases suggesting an in vivo biological activity of low ZOL concentrations in humans [93].

6. Nanotechnology and BPs: Macrophage Targeting

Macrophages are the major differentiating cell of the mononuclear phagocyte system (MPS). They derive from monocytes that migrate from the peripheral blood to extravascular tissue where they differentiate into macrophages [94]. Macrophages play a critical role in host defense because they migrated to an infected focus following attraction by a variety of substances, such as components from bacteria, complement components, immune complexes, and collagen fragments. Once at the infected focus, macrophages may phagocytose and kill infectious agents by a variety of mechanisms [95]. Moreover, following uptake of protein antigens, macrophages generated immunogenic fragments activating and regulating the immune response [96]. Finally, macrophages infiltrate tumors, where they represent an important mechanism of host defense against tumor cells, either inhibiting tumor cell division or killing the cells following secretion of soluble mediators or by other means [97, 98]. However, most tumors can be infiltrated by a different macrophage phenotype, which provides an immunosuppressive microenvironment for tumor growth. Furthermore, these tumor-associated macrophages (TAM) secrete many cytokines, chemokines, and proteases, which promote tumor angiogenesis, growth, metastasis, and immunosuppression [99].

Thus, due to their pivotal role in a number of physiological and pathological processes including tumors, macrophages represent an attractive target for therapy. While in the case of small soluble drug, only a small fraction can reach the macrophages, these latter can be the preferential accumulation site for intravenously injected colloidal carriers. Indeed, once into the bloodstream plasma proteins adsorb on particle surface and this process, also named opsonization, facilitates particle recognition and clearance from the blood by circulating phagocytes as well as tissue macrophages that are in direct contact with the blood [100]. Thus, the localization of intravenously injected nanocarriers in cells of the mononuclear phagocyte system (MPS) offers a potential and powerful method to target therapeutic agents to these cells. Nowadays, various lipid and polymeric carriers such as liposomes and nanoparticle are under investigation to deliver drugs to macrophages. However, nanocarrier characteristics, in terms of size, shape, and particle surface, affect the pharmacokinetics of the nanocarrier and need to be carefully evaluated when designing nanocarriers for macrophage targeting. For more details, the readers are directed to more specific reviews on this theme, for example, an excellent review by Moghimi [100].

The powerful effect of BPs against osteoclasts suggests a possible activity on cells with a common lineage, such as the macrophages. However, pharmacokinetics of BPs require delivery method to escape bone and to target macrophages. Liposomes encapsulating CLO were successfully used to achieve temporary macrophage depletion in the spleen [21]. The authors demonstrated that once phagocytosed, the liposomal membranes were disrupted by the phospholipases of the lysosomes, and the drug is released into the cell. Other
and free ZOL also led to reduced tumor angiogenesis, with the highest effects found with ZOL. This effect could be surprising when considering that zoledronic acid was used as free; however, the strong activity of ZOL at very low concentrations, compared with CLO, could explain the highest effect found in this study. In the same study, the authors found toxic effects in animals treated with liposomes encapsulating CLO, while ZOL appeared as more promising, especially because already in the clinical practice. Macrophage depletion by using BP-containing liposomes has also been proposed as adjuvant agent in the cancer radiotherapy. Indeed, radiotherapy, although directly inducing tumor cell death, may upregulate proangiogenic and prosurvival factors within the tumor microenvironment. In particular, upon radiation, upregulation of tumor cells and cells of the myeloid lineage can occur, with consequent TNFα production [35] followed by the induction of macrophage-secreted vascular endothelial growth factor (VEGF) with consequent radioprotective effect. Radiotherapy association with the treatment with CLO-containing liposomes resulted in the improvements in the therapeutic index, as determined by a delay of tumor regrowth [36]. The use of CLO-containing liposomes was also useful to reduce metastasis of human prostate cancer in bone, thus confirming the role of TAM in regulation of tumor tissue homeostasis [37]. The effect was potentiated when mice were inoculated with cancer cells, previously knocked down of IL-6, thus confirming the role of IL-6 as a strong chemotactic factor that recruits TAM to the tumor lesion.

7. Nanotechnology and BPs: Targeting of Cancer Cells

Although many research papers are focused on the use of nanocarriers targeting macrophages, the delivery of bisphosphonates directly to cancer cells has been recently investigated.

Tumors characterized by cells derived from myeloid lineage cells can be targeted with BP. This has been recently demonstrated in a model of malignant histiocytosis [38]. CLO-containing liposomes were firstly assayed in vitro on canine malignant histiocytosis cells, demonstrating a significant inhibition of cell growth. This effect was also found even in nonphagocytic cells, although, for these cells, free CLO was more efficient. In vivo, dogs with spontaneous malignant histiocytosis and treated with CLO-containing liposomes elicited significant tumor regression in two of five treated animals. The authors also reported an antitumor activity following i.v. administration of CLO-containing liposomes in several different nonhistiocytic mouse tumor models, thus suggesting the antitumor activity may have been mediated by a combination of both direct and indirect tumor effects [38].

Liposomes have been used to deliver BPs directly to cancer cells (Table I). Neridronate (NER) encapsulated into liposomes increased the inhibition activity on cell growth on human breast cancer cells (MDA-MB-231) by 50 times, compared to the free drug [39].

Liposome encapsulating BP was also confirmed with other BPs, namely, PAM and ETI, on RAW 264 and CV1 cells [24]. In this study, free BPs were found to be even 1000 times less active, compared with the corresponding liposome-based formulations. Interestingly, the use of high calcium extracellular concentration resulted in a stronger macrophage depletion, suggesting the role of calcium to mediate BP cell uptake [24, 27]. The liposome type affected macrophage depletion, which was higher when using negatively charged unilamellar liposomes [27]; however, this effect was found only in the case of CLO and ETI but not in the case of PAM. Finally, the use of calcium/bisphosphonate complex was found to lead to an inhibitory effect on the cytokine production by macrophages [27]. BP-encapsulating liposomes, when intravenously administered, led to elimination of macrophages from spleen and liver [25] but not those in other organs [23], reflecting the pharmacokinetics of the carrier. Accordingly, subcutaneous footpad administration of the BP-encapsulating liposomes resulted in macrophage elimination in draining lymph nodes [28] while intratracheal administration exclusively eliminates macrophages from lung tissues [29].

Liposome encapsulating BPs were used to enhance tumor growth in an experimental model of liver metastasis [30]. Rat inoculation with colon carcinoma cells resulted in a strong enhanced tumor growth in the liver only when the animals were pretreated with an i.v. injection of CLO-encapsulating liposomes. This effect was attributed to the effective elimination of all Kupffer cells that are preferential accumulation site for colloidal carriers. Accordingly, in the same experiment, nonphagocytic cells into the liver were not affected [30]. In contrast, liposome encapsulating CLO have been successfully used to inhibit the tumor growth. In different experimental animal models of cancer, this effect was accompanied by drastic reduction of the blood vessel density in the tumor tissue [31–33, 101]. CLO-encapsulating liposomes were also used in combination therapy with VEGF-neutralizing antibody. The treatment led to significant reduction of angiogenesis, as demonstrated by blood vessel staining and vessel quantification, that was associated to a significant reduction of the TAM and tumor-associated dendritic cells [31]. Liposomes encapsulating CLO were also investigated in combination with sorafenib in two human hepatocellular carcinoma xenograft nude mouse models [34]. Mice treated with sorafenib showed a significant inhibition of tumor growth and lung metastasis but associated to significant increase of macrophage recruitment in peripheral blood as well as increased intratumoral infiltration. A combination therapy with sorafenib and liposome containing CLO or sorafenib and free ZOL also led to reduced tumor angiogenesis, with the highest effects found with ZOL. This effect could be surprising when considering that zoledronic acid was used as free; however, the strong activity of ZOL at very low concentrations, compared with CLO, could explain the highest effect found in this study. In the same study, the authors found toxic effects in animals treated with liposomes encapsulating CLO, while ZOL appeared as more promising, especially because already in the clinical practice. Macrophage depletion by using BP-containing liposomes has also been proposed as adjuvant agent in the cancer radiotherapy. Indeed, radiotherapy, although directly inducing tumor cell death, may upregulate proangiogenic and prosurvival factors within the tumor microenvironment. In particular, upon radiation, upregulation of tumor cells and cells of the myeloid lineage can occur, with consequent TNFα production [35] followed by the induction of macrophage-secreted vascular endothelial growth factor (VEGF) with consequent radioprotective effect. Radiotherapy association with the treatment with CLO-containing liposomes resulted in the improvements in the therapeutic index, as determined by a delay of tumor regrowth [36]. The use of CLO-containing liposomes was also useful to reduce metastasis of human prostate cancer in bone, thus confirming the role of TAM in regulation of tumor tissue homeostasis [37]. The effect was potentiased when mice were inoculated with cancer cells, previously knocked down of IL-6, thus confirming the role of IL-6 as a strong chemotactic factor that recruits TAM to the tumor lesion.

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<thead>
<tr>
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<th>Strategy</th>
<th>Bisphosphonate</th>
<th>Main findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes</td>
<td>Macrophage depletion</td>
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<td>Macrophage elimination in the spleen and liver following i.v. administration.</td>
<td>[21–25]</td>
</tr>
<tr>
<td>Liposomes</td>
<td>Macrophage depletion</td>
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</tr>
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<td>Clodronate</td>
<td>Enhanced tumor growth in an experimental model of liver metastasis.</td>
<td>[28]</td>
</tr>
<tr>
<td>Liposomes</td>
<td>Macrophage depletion</td>
<td>Clodronate</td>
<td>Inhibition of the tumor growth in different experimental animal models of cancer; reduction of the blood vessel density in the tumor tissue; reduction of the tumor-associated macrophages and tumor-associated dendritic cells.</td>
<td>[30–33]</td>
</tr>
<tr>
<td>Liposomes</td>
<td>Macrophage depletion</td>
<td>Clodronate in combination with sorafenib</td>
<td>Significant inhibition of tumor growth and lung metastasis; reduced tumor angiogenesis.</td>
<td>[34]</td>
</tr>
<tr>
<td>Liposomes</td>
<td>Macrophage depletion</td>
<td>Clodronate as adjuvant agent in radiotherapy</td>
<td>Adjuvant agent in the cancer radiotherapy with delayed tumor regrowth.</td>
<td>[35, 36]</td>
</tr>
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<td>Reduced metastasis of human prostate cancer in bone.</td>
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</tr>
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<td>Inhibition of cell growth.</td>
<td>[39]</td>
</tr>
<tr>
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<td>Targeting of extraskeletal tumors</td>
<td>Zoledronate</td>
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</tr>
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<td>Targeting of extraskeletal tumors</td>
<td>Zoledronate</td>
<td>Enhanced cytotoxic effect in vitro.</td>
<td>[42]</td>
</tr>
<tr>
<td>Self-assembling NPs</td>
<td>Targeting of extraskeletal tumors</td>
<td>Zoledronate</td>
<td>Enhanced cytotoxic effect in vitro; enhanced inhibition of tumor growth (prostate cancer).</td>
<td>[41, 43]</td>
</tr>
<tr>
<td>Superparamagnetic iron oxide nanocrystals</td>
<td>Theranostic purposes</td>
<td>Alendronate, zoledronate</td>
<td>Decrease cell proliferation in vivo and inhibition of tumour growth in vivo, only in combination with a magnetic field.</td>
<td>[44–46]</td>
</tr>
<tr>
<td>Liposomes</td>
<td>Targeting of doxorubicin to bone tumors</td>
<td>Bisphosphonate head group in a novel amphiphatic molecule</td>
<td>Increased cytotoxicity in vitro on human osteosarcoma cell line associated to hydroxyapatite.</td>
<td>[47]</td>
</tr>
<tr>
<td>Poly(lactide-co-glycolide) NPs</td>
<td>Targeting of doxorubicin to bone tumors</td>
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<td>[48]</td>
</tr>
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Moreover, even at a lower concentration, liposomal NER showed a suppressive effect on tumor cell mobility in vitro, whereas free NER showed almost no effect. Reasonably, liposomes should mediate the enhanced bisphosphonate uptake into the cells, although this hypothesis was demonstrated only by indirect evidence by co-encapsulation of fluorescent dye together with the drug.

In order to directly deliver BP in tumor cells, accumulation in MPS should be avoided. Thus, nanocarriers with stealth properties able to avoid opsonization should be preferred. In the light of this consideration, stealth liposomes encapsulating ZOL (lipoZOL) designed for tumor targeting were developed [40, 42]. ZOL was encapsulated into liposomes by different strategies, and the reverse-phase evaporation technique was selected to achieve the highest encapsulation efficiency (unpublished data). With this technique, the use of an alkaline buffer improved the ZOL solubility into the aqueous phase of liposomes, thus increased the drug encapsulation efficiency up to about 5% [40]. Liposomes were able to significantly prolong ZOL circulation time. Free ZOL was quickly cleared from blood, with 0.1-0.2% of the injected dose still present 1h after injection. ZOL encapsulation into liposomes, especially PEGylated liposomes, significantly increased ZOL circulation time, with more than 10% of the injected dose still present into the blood 24h following the injection [42]. Concerning the in vitro activity of lipoZOL, contrasting results have been found. In particular, our group demonstrated that the use of lipoZOL, compared with free ZOL, increased the cytotoxic effect until a potentiation factor of about 20 [40]. The effect was confirmed in cell lines of different cancer, namely, prostate, breast, head/neck, lung and pancreas, and multiple myeloma, with an IC50 ranging from 4 to about 200 μM. These data are in contrast with those reported by other authors who found that stealth liposomes containing ZOL did not elicit any significant inhibitory effect on cell from 0.01 to 200 μM [42]. Significant cytotoxicity was found only by using folate-conjugated lipoZOL, especially in cell overexpressing the folate receptor. The discrepancy among the two studies could be ascribed to the different formulations used as well as to the different cell lines.

The in vivo antitumor activity of lipoZOL was demonstrated in two different model of tumors, namely, prostate cancer and multiple myeloma [40, 41]. In these experiments, mice treated with lipoZOL, compared to animal with free ZOL, showed a higher tumor weight inhibition and tumor growth delay, together with increased mice survival. As in the case of non-stealth nanocarriers, also stealth liposomes allowed to obtain reduced number of TAM as well as inhibition of the neoangiogenesis [40, 41]. Moreover, no significant changes were found in serum creatinine, urea, and calcium in animals treated with lipoZOL, suggesting the absence of potential adverse effects [40]. In order to overcome technological limits of the lipoZOL, such as low encapsulation efficiency and stability issue of the liposomal formulation, our group recently developed a new nanovector to deliver ZOL in extraskeletal tumor. The new system consists of self-assembling NPs encapsulating ZOL and designed to be prepared before use, thus avoiding storage issues [43, 102]. In particular, the formulation can be prepared by mixing two components, namely, an aqueous solution of ZOL, Ca2+/PO43− NPs, and cationic PEGylated liposomes. Ca2+/PO43− have already been used to deliver other negatively charged molecules, such as nucleic acids [103]. In the case of BPs, an encapsulation process driven by ionic interactions allowed to overcome the loading issues observed with liposomes. Indeed, in the case of self-assembling NPs, a ZOL encapsulation efficiency 12-fold greater, compared with that obtained with ZOL-containing liposomes, was achieved. The self-assembling NPs increased the growth inhibition of ZOL on different cancer cell lines, compared to free ZOL. The highest cell growth inhibition was observed on breast cancer cells. The anticancer activity of this formulation was also demonstrated in vivo in an animal model of prostate cancer. ZOL encapsulated into self-assembling NPs elicited a marked antitumor activity, while free ZOL did not show a significant reduction of tumor growth [43]. The in vivo anticancer activities of two different ZOL-containing nanocarriers, namely, lipoZOL and self-assembling NPs, were compared [41]. In this study, self-assembling NPs encapsulating ZOL induced the complete remission of tumour xenografts and an increase of survival time higher than that observed with lipoZOL. This effect was paralleled by a significant increase of both necrotic and apoptotic indexes. NPs, more than lipoZOL, also caused a statistically significant reduction of TAM and displayed a higher neoangiogenesis inhibition. With both nanovectors, toxic effects affecting the mice weight or inducing deaths were not found. Finally, the histological examination of some vital organs such as liver, kidney, and spleen did not find any changes in terms of necrotic effects or modifications in the inflammatory infiltrate [41].

The ability of BPs to bind metal ions was used to prepare BP-complexing superparamagnetic iron oxide nanocrystals with theranostic purposes [44–46]. In a first study, a 5-hydroxy-5,5-bis(phosphono) pentanoic acid was used, while in the following works more powerful BPs, such as ALE and ZOL, were used. Amino fluorescein or rhodamine were covalently coupled with the nanocrystal, thus allowing to visualize an efficient uptake of the nanovector into two different cell lines [44, 104]. However, cell viability assays demonstrated that ZOL alone had an IC50 at 48 h that was 1 order of

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**Table 1: Continued.**

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<th>References</th>
</tr>
</thead>
<tbody>
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<td>Targeting of docetaxel to bone tumors</td>
<td>Zoledronate conjugated on the nanocarrier surface</td>
<td>Enhanced cytotoxic effect in vitro.</td>
<td>[49]</td>
</tr>
<tr>
<td>Poly(ethylene glycol)-dendrimer</td>
<td>Targeting of paclitaxel to bone tumors</td>
<td>Alendronate conjugated to the nanocarrier</td>
<td>Significant improvement of paclitaxel in vivo half-life.</td>
<td>[50]</td>
</tr>
</tbody>
</table>
magnitude lower than with $\gamma\text{Fe}_2\text{O}_3$-ZOL nanocrystals. According to the authors, cell proliferation decreases to 75% under an applied magnetic field, compared to 40% without magnetic field [45]. $\gamma\text{Fe}_2\text{O}_3$-ALE NPs were investigated on different cell lines; however, a clear advantage of the NPs was found only on breast cancer cell [104]. These NPs were also investigated in vivo in an experimental model of breast cancer [104]. In this study, tumour growth in animals treated with free ALE and $\gamma\text{Fe}_2\text{O}_3$-ALE NPs was not significantly different than in control group. NPs used in combination with a magnetic field significantly inhibited tumour growth by about 60% after 5 weeks, with all mice treated that were alive 5 weeks after treatment and did not present significant loss of body weight. However, the lack of control experiments with $\gamma\text{Fe}_2\text{O}_3$ NPs (NPs without ALE) hampers to affirm that ALE could be responsible for the antitumor affect, while the physical effect of NPs under the magnetic field could be the main responsible of anticancer effect described by the authors.

8. Nanotechnology and BPs: Targeting of Bone Tumors

Bone metastasis, especially originating by breast and prostate cancer, are the most frequent form of skeletal neoplasia. In the majority of patients, treatments of bone metastasis are palliative, being aimed to relieve pain, improve function, and prevent complications such as spinal cord compression and pathological fracture. The development of anticancer therapies with high affinity for bone and reduced distribution to other sites is certainly attractive. To this aim, nanovectors targeting hydroxyapatite have been proposed. Hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$) is the major inorganic mineral phase present in bone and teeth and not found in other tissues under normal circumstances. Thus, the use of nanocarriers conjugated to BPs that are characterized by high affinity for hydroxyapatite have been proposed.

A novel amphipathic molecule bearing a bisphosphonate head group, 4-N-(3,5-ditetradecyloxybenzoyl)-aminobutane-1-hydroxy-1,1-bisphosphonic acid disodium salt (BPA), was synthesized and used at different concentrations to prepare liposomes [47]. The presence of the bisphosphonates on the liposome surface was suggested by a zeta potential that was as negative as high the amount of the BPA used in the preparation. BPA-containing liposomes bound hydroxyapatite in vitro, depending on the BPA concentration into the carrier, while no binding was found in the case of liposomes prepared without BPA. In vitro studies on human osteosarcoma cell line associated to hydroxyapatite demonstrated an increased cytotoxicity of BPA-containing liposomes encapsulating doxorubicin, compared to liposome not containing BPA, this effect being dependant on the amount of BPA used in the preparation [47]. Liposomes containing doxorubicin (DOX) were also conjugated to CLO to target osteosarcoma [105], DOX-encapsulating BP-conjugated liposomes showed similar antitumor effect on two different osteosarcoma cell lines, compared to DOX in free form or encapsulated into PEGylated liposomes. Moreover, in an experimental model of osteosarcoma, a higher inhibition rate of tumor growth, together with a prolonged survival, was observed when comparing mice treated with DOX-encapsulating BP-conjugated liposomes with the other groups.

ALE has also been coupled to poly(lactide-co-glycolide) (PLGA) NPs encapsulating doxorubicin [48]. These NPs were investigated in a panel of human cell lines, representative of primary and metastatic bone tumors on which doxorubicin, as free or encapsulated in ALE-conjugated NPs, induced a concentration-dependent inhibition of cell proliferation. In vivo studies on an orthotopic mouse model of breast cancer bone metastases demonstrated a reduced incidence of metastases in the case of mice treated with doxorubicin, as free or encapsulated in ALE-conjugated NPs. However, in the case of ALE-conjugated NPs, independently on the presence of doxorubicin, a significant reduction of the osteoclast number was found at the tumor site, reasonably attributed to the ALE activity [48]. PLGA NPs conjugated with ZOL have been recently developed to deliver docetaxel (DCX) to bone [49]. ZOL was conjugated to PLGA-PEG-NH2 and the resulting PLGA-PEG-ZOL was used to prepare the NPs. In vitro bone binding affinity showed that PLGA-PEG-ZOL NPs have affinity with human bone powder comparable to that observed for ZOL in solution. On two different breast cancer cell lines, PLGA-PEG-ZOL NPs exhibited significantly higher cytotoxicity compared to DCX, DCX associated to ZOL, and unconjugated NPs at all drug concentrations and different time points. Interestingly, the authors demonstrated that the presence of ZOL on the NP surface affected the pathway for the intracellular uptake. In particular, PEGylated PLGA NPs predominantly followed lysosome through early endosomes which displayed significant colocalization of NPs and lysosomes. On the other hand, ZOL-modified NPs were endocytosed by both clathrin-mediated and caveolae-mediated endocytosis mechanism, where caveolae pathway followed a non-lysosomal route. The different intracellular trafficking of ZOL-coupled and ZOL-free NPs was also confirmed by the prolonged time needed for the exocytosis [49]. Finally, ZOL-coupled NPs showed an enhanced cytotoxic effect that has been attributed to the higher uptake via ZOL-mediated endocytosis. Finally, ALE was also conjugated to a poly(ethylene glycol) (PEG) dendrimer, in combination with paclitaxel to target bone tumors [50]. The pharmacological activity of paclitaxel, in terms of inhibition of cell growth and cell migration, was not altered by conjugation with PEG dendrimer. Moreover, in vivo half-life of paclitaxel was significantly improved when administering the conjugate ALE-dendrimer-paclitaxel, compared with free paclitaxel.

9. Concluding Remarks

In vitro results have clearly demonstrated that BPs, in addition to inhibiting osteoclast-mediated bone resorption, can exert marked proapoptotic and antiproliferative effects on tumor cells, especially when combined with other standard antineoplastic therapy. In vivo, this antitumor effect appears to be better experienced in tumor cells of bone metastases, at least
in the majority of experiments performed to date. This may be explained by the high local concentration of BPs in bone relative to the much lower one in other organs and plasma; this feature makes bisphosphonates the drugs of choice in the treatment of bone problems associated with malignancy. However, large-scale clinical trials have investigated the benefit of bisphosphonate therapy in reducing the incidence of SRE in myeloma, in breast cancer metastases, in metastatic prostate cancer, in lung cancer, in renal cell carcinoma, and in other solid tumors. Many in vivo tumor models have demonstrated ZOL, Pam, CLO, and IBA antitumor efficacy compared with control.

The use of nanotechnology can open new therapeutic scenario for BPs. Nanocarriers such as conventional liposomes allow to use the BP as potent agent for macrophage depletion. Preferential accumulation of BP in extraskeletal tissue can be achieved by using long circulating nanocarriers, such as lipoliposome, PLGA NPs or PEG dendrimer conjugated with the anticancer agent, to be used as targeting moieties, for the treatment of bone cancers.

Taking together all the scientific papers cited in this paper, the role of BPs in therapy appears underestimated. This class of molecules, especially the third-generation N-BPs as ZOL, can certainly represent a new weapon against cancer, although today they are approved only as antiresorption agent. Of course, new therapeutic indications cannot leave aside the design of a specific delivery system able to change biopharmaceutical characteristics of BPs. In line with this, nanotechnology can certainly represent an attractive opportunity.

10. Future Perspectives

Several strategies could be developed in the next future: the rational use of N-BPs in combination with other target-based agents to overcome escape mechanism occurring in cancer cells; the sequential combination of N-BPs with conventional cytotoxic agents to strengthen their apoptotic and antiangiogenic potential; the administration of N-BPs in metronomic-like modality (low doses for protracted time); the discovery and the targeting of new intracellular molecules found through the use of new advanced molecular technologies, such as DNA microarray. In all these possible perspectives nanotechnology will represent a valid support, also contributing to make these molecules more specific, thus reducing contraindications, for example, osteonecrosis of the jaw, due to the excessive N-BP accumulation in sites where their action is not required. Studies in progress in our labs suggest future applications of BPs also in form of cancer hard to kill, like glioma, and for other applications in the central nervous system, like the treatment of neuropathic pain (data submitted for publication).

Authors’ Contribution

G. D. Rosa and G. Misso equally contributed to the paper.

References


Review Article

Neoplastic Meningitis from Solid Tumors: A Prospective Clinical Study in Lombardia and a Literature Review on Therapeutic Approaches

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Neoplastic dissemination to the leptomeninges is an increasingly common occurrence in patients with both haematological and solid tumors arising outside the central nervous system. Both refinement of diagnostic techniques (Magnetic resonance imaging) and increased survival in patients treated with targeted therapies for systemic tumors account for this increased frequency. Cerebrospinal fluid cytological analysis and MRI confirm clinical diagnosis based on multifocal central nervous system signs/symptoms in a patient with known malignancy. Overall survival in patients with leptomeningeal neoplastic dissemination from solid tumors is short, rarely exceeding 3–4 months. However, selected patients may benefit from aggressive therapies. Apart from symptomatic treatment, intrathecal chemotherapy is used, with both free (methotrexate, thiotepa, AraC) and liposomal antitumor agents (liposomal AraC). Palliative radiotherapy is indicated only in cases of symptomatic bulky disease, surgery is limited to positioning of Ommaya recervoirs or CSF shunting. We report clinical data on a cohort of 26 prospectively followed patients with neoplastic leptomeningitis followed in Lombardia, Italy, in 2011. Prognostic factors and pattern of care are reported.

1. Introduction

Neoplastic meningitis is due to dissemination of malignant cells to the leptomeninges and the subarachnoid space. It occurs in 10–15% of haemolymphoproliferative malignancies and in 5–10% of solid cancers [1].

It more frequently represents late complication of long-standing neoplastic disease, but in 10–15% of patients may be the first-ever manifestation of otherwise occult cancer [1]. The pathways for tumor dissemination to the leptomeninges and subarachnoid space include haematogenous route, perineural blood/lymphatic vessels, and direct infiltration from contiguous sites (for instance, dural and/or bone metastases close to the brain and spinal cord/root surface).

Not only extra-CNS tumors, but also tumors arising within the CNS (among which gliomas, ependymomas, medulloblastomas, and germinomas) display relapses and/or multifocal presentations with distant foci and a supposedly intra-CSF pathway of dissemination of neoplastic cells.

Guidelines for effective treatment of neoplastic meningitis are lacking, due to the low levels of evidence, which is mostly present for haemolymphoproliferative disease.
In meningeal dissemination from solid extra-CNS tumors, and more so in distant spread of primitive CNS tumors, there is a lack of uniform approach due to a number of factors: among these, the belief of oncologists that neoplastic meningitis invariably implies a dismal prognosis in the short-term has limited patient recruitment in clinical trials.

Although this assumption holds true in a high number of cases, it does not apply to the totality of patients, however. This consideration, together with the more widespread availability of MRI facilities in neurooncological diagnosis and with the progress in survival in extra-CNS cancers achieved by chemotherapy and molecularly targeted therapies [2], increases the need for accurate diagnosis of neoplastic meningitis, as a prerequisite for accurate validation of prognostic factors and for enrollment of patients in clinical trials.

2. Diagnosis of Neoplastic Meningitis

The clinical signs and symptoms of neoplastic meningitis are classically subdivided in those pointing to cerebral, cranial nerve, or spinal cord/roots involvement. Typically, in a high proportion of patients symptoms are present suggesting simultaneous involvement of both cerebral and spinal levels, but some patients present with isolated deficits (for instance, an isolated cranial nerve defect).

Cerebral signs and symptoms may either be localized (as in the case of focal seizures) or suggestive of a widespread brain dysfunction (for instance, drowsiness in hydrocephalus or encephalopathic features in diffuse sulcal enhancement), or be even more unspecific, such as headache.

The literature reports that the presence of signs at the neurological examination is more frequent as compared to the reporting of symptoms by the patients during history collection.

Neoplastic meningitis not infrequently coexists with intraparenchymal or dural metastases, especially in the case of breast cancer and leukemia/lymphoma.

The diagnosis of neoplastic meningitis is straightforward in the majority of cases, but a number of cases may pose diagnostic challenges.

This happens more frequently when the gold standard for diagnosis (i.e., CSF cytology) does not yield unequivocally positive results. This may be the case—according to the literature—in a proportion of patients ranging from 20 to 50–60%; reasons for this include too little volume of CSF analyzed, distance of the CSF sampling site from the bulk of leptomeningeal disease, and delay in CSF processing and analysis [3, 4]. The diagnostic yield of CSF cytology increase significantly from the first to the second lumbar puncture, to rise only negligibly thereafter [5].

In such cases, CSF analysis may yield negative results for malignant cells, yet display other abnormal features (however, less specific), such as increase in total proteins and reduced glucose levels, as well as moderate reactive pleocytosis.

Such CSF pattern may pose serious difficulties in differential diagnosis with CNS infections, which may mimic the neuroradiological picture of NM and are not unexpected in heavily treated cancer patients (for instance, chronic fungal and/or mycobacterial meningitis).

Some reports have stressed that the closer the CSF sampling to the site of disease, the higher the percentage of positivity for CSF malignant cells; ventricular CSF or lumbar CSF may thus provide different information as far as cytology is concerned.

In exceptional cases, leptomeningeal biopsy is deemed necessary.

In neoplastic meningitis from hematological malignancies, CSF cytofluorimeter analysis has been reported to be more often diagnostic as compared to standard cytomorphological analysis [6, 7].

As far as the role of MRI is concerned, the features of leptomeningeal dissemination include both indirect and direct evidence of neoplastic cell CSF seeding. Among the former, hydrocephalus is not rare, due mostly to alterations in the CSF flow and particularly in CSF reabsorption at the skull vault. Direct evidence of neoplastic dissemination includes linear or nodular enhancement at leptomeningeal/ependymal level.

More subtle signs of alterations in the CSF dynamics include exclusion of part of cerebral sulci, with limited volumes with increased protein content.

3. Management of Neoplastic Meningitis

The role of surgery is limited to resection of symptomatic, bulky disease, and/or biopsy in order to achieve diagnosis in selected cases; in some patients, positioning of an Ommaya reservoir may allow intraventricular chemotherapy without the need for repeated lumbar punctures, but the dynamics of CSF flow need to be carefully assessed in order to possibly achieve tumoricidal drug concentrations in the sites of disease. Ventriculoperitoneal shunting procedures to relieve symptomatic hydrocephalus carry a risk for the development of neoplastic dissemination to the peritoneum and are often complicated by shunt dysfunction/occlusion.

Intrathecal chemotherapy should preferably be delivered in patients with good PS (see below), with limited extra-CNS disease and with linear contrast enhancement at MRI (the penetration of drugs within bulky disease areas is limited to 2-3 mm).

The NCCN 2012 Guidelines for diagnosis and management of CNS tumors include brain and spine MRI as well as CSF examination in the workup of patients with suspected leptomeningeal tumor dissemination. According to these guidelines, either positivity of CSF cytology alone or positive radiologic findings with supportive clinical findings or else signs and symptoms with suggestive CSF in a patient known to have a malignancy may be sufficient for diagnosis.

After diagnosis, patients are stratified in either poor risk (low KPS, multiple, serious, major neurologic deficits, extensive systemic disease with few treatment options, bulky CNS disease, and encephalopathy), or else good risk (high KPS, no major neurologic deficits, minimal systemic disease, and reasonable systemic treatment options).
In the former group, only fractionated external beam RT is considered to symptomatic sites, and palliative care is the standard. An exception is possible in patients with highly chemosensitive tumors such as lymphoma and SCLC.

On the other hand, in good risk patients both radiotherapy to bulky disease or symptomatic sites may be delivered and intrathecal chemotherapy is a worthwhile option.

Of note, assessment of CSF flow is strongly recommended before initiating intrathecal chemotherapy. This assessment is more frequently performed in northern America, while it is less a frequent practice in Europe.

With normal CSF flow, either craniospinal irradiation—in the case of breast cancer or lymphoma—or high dose methotrexate i.v in the case of breast cancer or lymphoma or intrathecal chemotherapy with methotrexate or AraC or liposomal AraC are the treatment of choice.

Unless an Ommaya reservoir is positioned by the neurosurgeon, repeated intrathecal administration of antineoplastic drugs is usually performed via lumbar punctures. With methotrexate, twice weekly administrations are performed during the induction phase, due to the short half life of the drug in the CSF.

Analogous schedules are needed with nonliposomal cytarabine, whereas a pegylated formulation of cytarabine allows sustained tumoridical concentrations in the CSF which make once every 2 weeks treatment possible. The development of cytarabine encapsulated in multivesicular liposomes has led to detection of CSF concentrations of more than 0.1 μG/mL persisting at 14 days.

In this technology, microscopic particles made of aqueous chambers separated from each other by bilayer lipid membranes (with synthetic analogs of natural lipids), deliver gradually the incorporated drug, with subsequent metabolism of the membrane remnants via normal pathways. Cytarabine, a highly hydrophilic compound, is an ideal molecule for this approach [8].

The achievement of tumoridical concentrations of cytarabine in the CSF is of crucial importance since cytarabine is a phase-specific drug affecting only cells in the S phase. In the CSF, very little activity of the inactivating enzyme cytidine deaminase enables cytarabine to persist in its biologically active form for longer time as compared to systemic delivery [9].

Only few randomized trials have been conducted on the effectiveness and toxicity of intrathecal chemotherapy in neoplastic meningitis (reviewed in [10]).

In the 1999 published trial by Glantz et al. on neoplastic meningitis from solid tumors [11], intrathecal methotrexate was compared to liposomal cytarabine in 61 patients. After the induction phase, a slight increase in the frequency of patients attaining a response in the liposomal AraC group (26% versus 20%) was seen. Overall, median survival reached 73 days in the latter group and 105 in the former, with a nonsignificant advantage. The only parameter displaying a definite benefit in the liposomal AraC group was the time to neurological progression, which was of 58 versus 30 days with a statistically significant difference. It remains to be seen whether this statistically significant improvement translates into a clinically meaningful effect, but in this respect the studies conducted so far lack detailed quality of life data and this makes conclusions difficult.

Also the 2006 trial by Shapiro and colleagues provides data pointing to a nonsignificantly different effect of liposomal AraC versus methotrexate in 103 patients with neoplastic meningitis from solid tumors [12].

In the other 1999 paper by Glantz et al. [13], liposomal AraC was compared to AraC in the treatment of neoplastic meningitis in a low number (28) of patients with lymphomatosus meningitis. This trial showed an increase in time to tumor progression, in survival time and in response rate in the liposomal AraC treated subgroup.

Other nonrandomized studies have been performed [14, 15] investigating the effectiveness and side effects of liposomal cytarabine in neoplastic meningitis. Overall, a fair tolerability profile has emerged. The frequent occurrence of chemical meningitis may be prevented by concomitant steroid treatment.

The main reason for continuing use of liposomal AraC in these patients—apart from the lack of a consolidated and effective standard of care—is the need for less frequent lumbar punctures in often severely ill patients. However, the levels of evidence in favour of this approach are weak. A recent determination of EMA has temporarily suggested to consider alternative therapies to liposomal AraC after an inspection to the production site of the drug in California; treating physicians are waiting for a solution of this possibly temporary problem.

Other widely adopted intrathecal treatments apart from liposomal AraC include methotrexate and thiopeta.

Preliminary experiences show the feasibility of associating rituximab with liposomal cytarabine in patients with recurrent neoplastic meningitis [16]. Also systemic bevacizumab may be effective in some cases on neoplastic meningitis [17], in combination with other systemic chemotherapy agents.

Some effect has been reported for systemic treatment with systemic gefitinib or erlotinib in NSCLC with neoplastic meningitis. The effectiveness and toxicity of intrathecal chemotherapy in solid tumors is reviewed in [10], with normal CSF flow, either craniospinal irradiation in the case of breast cancer or lymphoma—or high dose methotrexate i.v in the case of breast cancer or lymphoma or intrathecal chemotherapy with methotrexate or AraC or liposomal AraC are the treatment of choice.

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meningitis, and with sorafenib in renal cancer, whereas the role of trastuzumab in breast cancer with neoplastic meningitis is still debatable (reviewed in [18]).

4. Prospective Collection of Newly Diagnosed Neoplastic Meningitis Cases from Solid Tumors in Lombardia

In 2011 a prospective collection of patients diagnosed with neoplastic meningitis from solid tumors was started in a number of Centers in Lombardia. The aim of this study is to assess the pattern of care in this often underdiagnosed and undertreated condition. Previous work from an analogous initiative in Piedmont [19] supports the concept that a higher index of suspect for diagnosis may lead to earlier diagnosis of this condition. Increase in frequency of neoplastic meningitis may indeed be a consequence of survival increase in a number of systemic malignancies thanks to advances in targeted therapies, as well as of more widespread use of MRI in the followup of these patients.

In 12 months, 26 patients with neoplastic meningitis from solid extra-CNS tumors have been diagnosed. Their clinical features are reported in Tables 1 and 2.

Cerebrospinal fluid analysis was performed in 22 out of 26 patients, yielding the following results: in 18/22 patients, CSF analysis revealed malignant cells. Mean values of CSF total protein were 152 mg\% (normal values 10–45 mg\%), whereas mean CSF glucose was 51.5 mg/dL (normal values 40–80 mg/dL for normal glycemic levels). Lower than normal glucose levels were only seen in 3 patients out of 22.

As reported in Table 3, 11 out of the 26 patients were treated by intrathecal liposomal AraC and 2 by systemic chemotherapy.

In this cohort, no patient was treated by radiotherapy after diagnosis of neoplastic meningitis.

Figure 1 reports overall survival in the entire cohort. This attained a median value of 22 weeks, in line with data from the literature.

Assessment of possible prognostic factors showed that at univariate analysis, higher performance status, primary histology (breast versus others), less elevated CSF protein, and linear contrast enhancement at MRI versus nodular disease, as well as intrathecal chemotherapy versus no intrathecal chemotherapy were associated with more prolonged survival.

However, probably due to the low number of patients, no statistically significant differences were detected in subgroups at multivariate analysis.

In Figure 2 the MRI images of a young female affected by neoplastic meningitis from breast cancer are reported; this 28-yr-old woman had a 2-year history of ductal carcinoma Her2-, hormone receptor-negative with positive lymph nodes at diagnosis. She had been treated with systemic chemotherapy, surgery, second-line chemotherapy associated with antiangiogenic therapy for relapse, and with RT
Table 1: Demographic features, site of primary tumor and PS.

<table>
<thead>
<tr>
<th>Extra CNS tumor</th>
<th>26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>13</td>
</tr>
<tr>
<td>Lung</td>
<td>7* (* 1 pt lung and colon tumor)</td>
</tr>
<tr>
<td>Digestive system</td>
<td>3*</td>
</tr>
<tr>
<td>Melanoma</td>
<td>2</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>53 yrs (30–82)</td>
</tr>
<tr>
<td>Median KPS (range)</td>
<td>60 (20–100)</td>
</tr>
</tbody>
</table>

Table 2: Clinical signs and symptoms at onset of neoplastic meningitis.

<table>
<thead>
<tr>
<th>Signs and symptoms and PS in extra CNS tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinal cord and root symptoms and signs</td>
</tr>
<tr>
<td>Headache, Mental status change</td>
</tr>
<tr>
<td>Meningeal signs and headache</td>
</tr>
<tr>
<td>Cranial nerve symptoms and signs</td>
</tr>
<tr>
<td>Seizures</td>
</tr>
</tbody>
</table>

Table 3: Therapeutic management in the 26 patients of the cohort.

<table>
<thead>
<tr>
<th>Control at primary site of disease</th>
<th>16 yes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 no</td>
</tr>
<tr>
<td>Steroids</td>
<td>22/26</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>0/26</td>
</tr>
<tr>
<td>Systemic Chemotherapy</td>
<td>2/26</td>
</tr>
<tr>
<td>Intrathecal Depocyte</td>
<td>11/26</td>
</tr>
</tbody>
</table>

( median 3 injections)

When considering the toxicity profile, only one grade 4 toxicity occurred. In a melanoma patient, an inflammatory encephalopathy picture with seizures, stupor, signs of meningeal irritation, nausea, moderate increase in temperature took place starting 24 hours after intraventricular administration of 50 mg of liposomal AraC; concomitantly, a slight intraventricular CSF lymphocytosis was detected. The encephalopathy improved progressively leading to recovery of the premorbid status within 72 hours. CSF culture was negative for infectious complications.

4 more patients displayed moderate postinjection headache and slight fever, usually starting within 24 hours from intrathecal delivery of liposomal AraC and receding in 1 to 2 days.

2 patients—both affected by metastatic breast cancer—are alive at a followup ranging from 11 to 23 months.

5. Future Developments

Intrathecal chemotherapy for neoplastic meningitis may be a worthwhile option for a number of patients with this very serious disease. Technological developments allowing slow-release delivery of potentially active drugs may in the future be combined with targeted treatments (monoclonal antibodies, small molecule inhibitors) focused on multistep inhibition of neoplastic cell survival, growth, and spreading within the neuraxis.

However, a better basic knowledge of the biological mechanisms underlying selective homing of neoplastic cells to the leptomeninges, together with strict monitoring of the risk/benefit ratio [20, 21], will be needed before routine adoption of these approaches becomes a standard of care.

This is very important, since increased survival times are (also) the consequence of more aggressive systemic treatments, which may significantly enhance the neurotoxicity of intrathecal therapies [22–24].

References


Review Article

Nanomaterials Toxicity and Cell Death Modalities

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In the last decade, the nanotechnology advancement has developed a plethora of novel and intriguing nanomaterial application in many sectors, including research and medicine. However, many risks have been highlighted in their use, particularly related to their unexpected toxicity in vitro and in vivo experimental models. This paper proposes an overview concerning the cell death modalities induced by the major nanomaterials.

1. Introduction

Nanotechnologies are emerging for important new applications of nanomaterials in various fields. Nanomaterials are defined as substances which have one or more external dimension in the nanoscale (1–100 nm). Nanomaterials, especially nanoparticles and nanofibres, show higher physical and chemical activities per unit weight. These properties explain their large application not only in industry but also in the scientific and medical researches. In fact, in these areas, the use of many kinds of manufactured nanoparticles products is in development, such as metal oxide nanoparticles (cerium dioxide, cupric oxide, titanium dioxide, zinc oxide, etc.), metal nanoparticles (gold, silver, platinum, palladium, etc.), C60 fullerenes nanocrystals, carbon nanotubes (CNTs), and quantum dots. Initially, the nanomaterials were believed to be biologically inert, but a growing literature has highlighted the toxicity and potential risks of their use. Extrapolations from the field of toxicology of particulate matter (less than 10 nm) confirm that nanoparticles present a range of harmful effects [1, 2]. In most cases, enhanced generation of reactive oxygen species (ROS), leading to oxidative stress which in turn may trigger proinflammatory responses, is assumed to be responsible for nanomaterials toxicity, although nonoxidative stress-related mechanisms have also been recently reported (see the extensive and interesting reviews [3–10]). However, despite intensive investigations, the understanding of nanomaterials-induced cellular damage remains to be clarified. The literature in the field suggests correlations between different physicochemical properties and the biological and toxicological effects of cells and tissues exposure to nanomaterials. First of all, nanomaterials are characterized by high specific surface area that correlates with high interfacial chemical and physical reactivity that, in turn, translates to biological reactivity [11]. The addition of different types of nanoparticles to various primary cell cultures or transformed cell lines may result in cell death or other toxicological outcomes, depending on the size of the nanomaterial. Quantum dots were reported to localize to different cellular compartment in a size-dependent manner [12]. Silica nanoparticles (40–80 nm) can enter into the nucleus and localize to distinct subnuclear domains in the nucleoplasm, whereas thin and coarse ones located exclusively in the cytoplasm [13]. Gold nanocluster (1.4 nm) intercalates within the major groove of DNA and is a potent inducer of cell death in human cancer cells [14]. Growing evidence suggests that the state of nanoparticles aggregation cannot be ignored; in fact, the toxicity may depend on the size of the agglomerate and not on the original nanoparticle size itself [15, 16]. For example, in rats exposed by inhalation to 20 nm or 250 nm titanium dioxide (TiO2) particles, the half-times for alveolar clearance of polystyrene test particles...
were proportional to the TiO$_2$ particle surface area per million of macrophages [17, 18]. Clearly, a surface impurity, resulting from air or water contaminants such as bacterial endotoxin, could contribute to the cellular responses induced by nanomaterials, in particular immunological responses [16]. The same consideration is true for residual materials (surfactants or transition metals) arising from the synthetic process [6, 19, 20]. Nevertheless, the adsorption ability and surface activity are also involved in cellular influences of nanomaterials. When dispersed in culture medium, some metal oxide nanoparticles and CNTs could adsorb proteins, often called “protein corona” such as serum albumin, or calcium, which could change the biological activity of nanomaterials. This adsorption could be particle size and time dependent. In these conditions, many nanoparticles form secondary particles, which are a complex of nanoparticles dependent. In these conditions, many nanoparticles form secondary particles, which are a complex of nanoparticles and medium components [21–26]. For example, adsorbed albumin on the CNT was involved in phagocytosis of the macrophage via scavenger receptor [27]. A surface-engineered functionalization also may be linked with the biological nanomaterials activity, although in this item that is a wanted effect. Moreover, examples of dose-dependent toxicity also are evaluated [6, 28, 29]. As pointed out in a recent review [6], the degree of recognition and internalization of nanomaterials likely influences their distribution and may determine also their toxic potential. It has been reported that the number of internalized quantum dots (the intracellular dose) correlates with the toxicity in human breast cancer cell line [30]. Furthermore, the toxicity and cell death fate appear to correlate with the type of crystal structures [16, 31]. Finally, the nanomaterials degradability should also be taken into account (Figure 1). Nondegradable nanomaterials can accumulate into the cells and/or organs and exert damage effect as well as their degradation products [32–34]. However, it is not yet clear which of these parameters mainly influences the nanomaterials toxicity or if all of these features act together [35]. It is important to note that in the literature conflicting results are present. These are likely caused by variations in type, composition, size, shape, surface charge, and modifications of nanoparticles employed; use of various in vivo and in vitro models (the cell death mode may be also cell type dependent); experimental procedures (different methods to evaluate cell death; nanomaterials dose, concentrations and efficiency of cellular uptake, and time of exposure). This paper aims to give a critical overview concerning the different cell death modalities induced by nanomaterials.

Deregulated cell death is a common element of several human diseases, including cancer, stroke, and neurodegeneration, and the modulation of this cellular response can be an optimal target for an effective therapeutic strategy. Many cytotoxic agents are potent anticancer therapeutics, whereas cytoprotective compounds may be used to elude unwanted cell death in the context of stroke, myocardial infarction or neurodegenerative disorders [36, 37]. The complex molecular mechanisms and signalling pathways that control cell death are increasingly becoming understood, and it is now clear that different cell death subroutines play a critical role in multiple diseases. In many instances, the modality by which cells die is crucial to the cell death achievement at the organism level. The Nomenclature Committee on Cell Death (NCCD) has recently formulated a novel systematic classification of cell death based on morphological characteristics, measurable biochemical features and functional considerations [38]. We will consider these definitions of cell death in order to summarize and organize the molecular mechanisms underlying the nanomaterials toxicity. We could not report all the studies, and we apologize for this; we will describe the most recently, accurate, and representative ones in term of the described molecular mechanisms.

2. Nanomaterials and Apoptosis

Apoptosis is a form of cellular suicide that can be classified into extrinsic and intrinsic apoptosis. Extrinsic apoptosis indicates the cell death, caspase dependent, stimulated by extracellular stress signals that are sensed and propagated by specific transmembrane receptors. Three major lethal signalling cascades have been reported: (i) death receptor signalling and activation of the caspase-8 (or -10) and then caspase-3 cascade; (ii) death receptor signalling and activation of the caspase-8 then BH3-interacting domain

**Figure 1**

| Cell-site specific accumulation (nucleus/cytoplasm) | Biodegradability |
| Aggregation | Chemical nature |
| Nanomaterials features leading to toxicity | Surface changes |

- **Chemical nature**
  - Aggregation
  - Adsorption of proteins, ions, etc.
- **Biodegradability**
- **Size/shape**
- **Surface changes**

- **Figure 1**

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<thead>
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<th>Aggregation</th>
<th>Adsorption of proteins, ions, etc.</th>
<th>Biodegradability</th>
<th>Size/shape</th>
<th>Nanomaterials features leading to toxicity</th>
<th>Surface changes</th>
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death agonist (BID), mitochondrial outer membrane permeabilization (MOMP), caspase-9 and caspase-3 pathways; and (iii) ligand deprivation-induced dependence receptor signalling followed by (direct or MOMP-dependent) activation of the caspase-9 and after caspase-3 cascade [38]. Intrinsic apoptosis can be triggered by a plethora of intracellular stress conditions, such as DNA damage, oxidative stress, and many others. It results from a bioenergetic and metabolic catastrophe coupled to multiple active executioner mechanisms. This process could be caspase-dependent or independent and is mediated by MOMP associated with the generalized and irreversible dissipation of the mitochondrial transmembrane potential, release of mitochondrial intermembrane space proteins into the cytosol (and their possible relocation to other subcellular compartments), and the respiratory chain inhibition [38]. Apoptosis plays a fundamental role in development and for maintenance of tissue homeostasis in the adult organism. In addition, impairment of apoptosis may contribute to tumour progression.

Nanomaterials are described as triggers of extrinsic and intrinsic apoptotic pathways; however, the oxidative stress paradigm of nanomaterials-induced cell death linked to intrinsic apoptotic network is by far the most accepted, in fact many in vitro studies have identified increased ROS generation as an initiating factor of toxicity in nanomaterials exposed cells [3, 6, 7, 10, 39]. Although it is well established that the mode of cell death depends on the severity of the cellular insult (which may, in turn, be linked to mitochondrial function and intracellular energy), it has been difficult to set up a comprehensive mechanism of nanomaterials cell death based on conflicting observations present in the literature. Furthermore, in most of the studies, the molecular mechanisms underlying cell death are not investigated. Finally, another problem is the nonhomogeneity of the studies, in terms of materials and experimental methods used, which makes it difficult to compare.

Sarkar and colleagues showed that the nano-copper induces intrinsic apoptotic cell death in mice kidney tissue (via the increase of ROS and reactive nitrogen species production, regulation of Bcl-2 family protein expression, release of cytochrome c from mitochondria to cytosol, and activation of caspase-3), but, in addition, they observed the activation of FAS, caspase-8, and tBID, suggesting also the involvement of extrinsic pathways [40]. The exposure to nano-copper dose-dependently caused oxidative stress and led to hepatic dysfunction in vivo. Nano-copper caused the reciprocal regulation of Bcl-2 family proteins, disruption of mitochondrial membrane potential, release of cytochrome c, formation of apoptosome, and activation of caspase-3. These results indicate that nano-copper induces hepatic dysfunction and cell death via the oxidative stress-dependent signalling cascades and mitochondrial event [41].

Metallic nickel nanoparticles induced apoptotic cell death through an FAS/caspase-8/BID mediated, cytochrome c-independent pathway in mouse epidermal cells [42]. Nickel oxide nanoparticles excited in dose-dependent manner the increase of ROS production, lipid peroxidation, and caspase-3 activation in human airway epithelial and breast cancer cells [43]. Moreover, nickel ferrite nanoparticles provoked apoptosis in human lung epithelial cells through ROS generation via upregulation of p53 and Bax as well as the activation of caspases cascade [44].

In vitro, silicon dioxide (SiO2) nanoparticles increased ROS and RNS (reactive nitrogen species) production that, in turn, can induce the intrinsic apoptotic machinery [45]. Furthermore, Wang and collaborators showed that p53 plays a key role in silica-induced apoptosis in vitro (mouse preneoplastic epidermal cells and fibroblasts) and in vivo (p53 wild-type and deficient mice) [46].

TiO2 nanoparticles, sized less than 100 nm, triggered apoptotic cell death through ROS-dependent upregulation of FAS and activation of Bax in normal human lung fibroblast and breast epithelial cell lines [47]. Moreover, it was also demonstrated that TiO2 nanoparticles induced apoptosis through the caspase-8/BID pathway in human bronchial epithelial cells and lymphocytes as well as in mouse preneoplastic epidermal cells [48, 49]. Some reports indicated that TiO2 induced also lipid peroxidation, p53-mediated damage response, and caspase activation [50, 51]. In contrast, there are also reports demonstrating that TiO2 nanoparticles did not induce oxidative stress on mouse macrophages [52] as well as did not shown cytotoxicity in human dermal fibroblasts and lung epithelial cells [31].

A number of studies have been published concerning the effects of CNTs on apoptosis. Multiwall carbon nanotubes (MWCNTs) induced an increase of ROS, cell cycle arrest, decrease in mitochondrial membrane potential, determining apoptosis in different in vitro models [53–56]. In contrast, another study reported that these nanotubes were nontoxic [57]. Accordingly, it has been observed that MWCNTs did not stimulate cell death in vitro after acute exposure and neither after the continuous presence of their low amounts for 6 months [58]. Instead, apoptotic macrophages have been observed in the airways of mice after inhalation of SWCNTs (single-walled carbon nanotubes) [6]. Accordingly, several studies in vivo suggest that the exposure to SWCNTs leads to the activation of specific apoptosis signalling pathways [59, 60]. For more details, recent interesting reviews focus on the nanomaterials toxicity in vivo studies [6, 34].

Nanoparticles are frequently detected in lysosomes upon internalization, and a variety of nanomaterials have been associated with lysosomal dysfunction [61]. It has been established that lysosomal destabilization triggers the mitochondrial pathway of apoptosis [62, 63]. Carbon nanotubes were shown to induce lysosomal membrane permeabilization and apoptotic cell death in murine macrophages and human fibroblasts [64, 65]. Carbon black nanoparticles elicited intrinsic apoptosis in human bronchial epithelial cells with activation of Bax and release of cytochrome c from mitochondria, whereas TiO2 nanoparticles induced apoptosis through lysosomal membrane destabilization and cathepsin B release, suggesting that the pathway of apoptosis differs depending on the nanomaterials chemical nature [66]. The lysosomal destabilization induced by TiO2 is also confirmed in mouse fibroblasts [67]. SiO2 and several cationic nanoparticles, such as cationic polystyrene nanospheres and cationic polyamidoamine (PAMAM) dendrimers, have also shown the same mode of action [68–70]. However,
also the micromaterials are able to destabilize lysosomes, in fact silica microparticles have been demonstrated to induce apoptosis in mouse alveolar macrophages by this molecular mechanism [70]. A comparative study of nano- versus microscale gold particles demonstrated that nanoparticles present a higher potency in the induction of lysosomal membrane destabilization [71].

Chronic or unresolved endoplasmic reticulum (ER) stress can also cause apoptosis [72, 73]. Zhang and colleagues reported that the ER stress signalling is involved in silver nanoparticles-induced apoptosis in human Chang liver cells and Chinese hamster lung fibroblasts [74]. Using omic techniques and systems biology analysis, Tsai and collaborators demonstrated that upon ER stress, cellular responses, including ROS increase, mitochondrial cytochrome c release, and mitochondria damage, chronologically occurred in the gold nanoparticles-treated human leukemia cells. This treatment did not induce apoptosis in the normal human peripheral blood mononuclear cells [75]. It has been shown that poly(ethylene glycol)-phosphoethanolamine (PEG-PE), an FDA-approved nonionic diblock copolymer widely used in drug delivery systems, accumulated in the ER and induced ER stress and apoptosis only in cancer cells (human adenocarcinoma alveolar basal epithelial), whereas it did not have effect in normal cells (secondary human lung fibroblasts and embryonic kidney cells) [76].

The predisposition of some nanoparticles to target mitochondria, ER, or lysosomes and initiate cell death could be used as a new cancer chemotherapy principle. Interestingly, nanoparticles (polystyrene nanoparticles of 20–40 nm with two different surface chemistries, carboxylic acid, and amines) may also induce apoptosis in individual cells (differentiated human colorectal adenocarcinoma) that then propagates to other neighbouring cells through a “bystander killing effect.” The authors of this study suggest that ingested nanoparticles represent a potential health risk due to their detrimental impact on the intestinal membrane by destroying their barrier protection capability over time [77].

Surely in this context, a common incentive to synchronize the studies and research efforts is needed. The understand why cancer cells and distinctive normal cells have different cell fates as a result of nanomaterials exposure, focusing on the underlying mechanisms, will allow a better prediction of the consequences of exposure to nanomaterials and a safer assessment of the risks (Figure 2).

### 3. Nanomaterials and Mitotic Catastrophe

Recently, Vitale and colleagues suggested a novel definition of mitotic catastrophe based on functional consideration [78]. They proposed to consider mitotic catastrophe not a “pure” cell death executioner pathway but as an oncosuppressive mechanism that is triggered by perturbations of the mitotic apparatus, is initiated during the M phase of the cell cycle, is paralleled by some degree of mitotic arrest, and induces cell death (apoptosis or necrosis) and senescence [78].

It has been reported that several nanomaterials, such as SiO$_2$, TiO$_2$, cobalt-chrome (CoCr) metal particles, and carbon nanotubes, interact with structural elements of the cell, with an apparent binding to the cytoskeleton and in particular the tubulins [79, 80]. In this setting, some evidence in vitro demonstrated that carbon nanotubes mimic or interfere with the cellular microtubule system, thereby disrupting the mitotic spindle apparatus and leading to aberrant cell division [81–83]. In particular, the perturbation of centrosomes and mitotic spindles dynamics caused by these nanoparticles results in monopolar, tripolar, and quadrupolar divisions, that, in turn, could determinate aneuploidy [78], an event closely linked to the carcinogenesis. Tsao and collaborators found that alumina ceramic particles increase significantly in micronucleated binucleate cells [84], which is considered a morphological marker of mitotic catastrophe [78]. Interestingly, this increase was much greater after exposure of primary human fibroblasts to CoCr metal particles, suggesting that these nanoparticles are particularly efficient in affecting the mitotic machinery [84]. Apparently, the genotoxic effect of CoCr nanoparticles is size dependent. Indeed, CoCr nanoparticles induced more DNA damage than microsized ones in human fibroblasts (Figure 3). In fact, the mechanism of cell damage appears to be different after nano- or microparticles exposure. The enhanced oxidative DNA damage by the microparticles may result from a stronger ability of large particles to activate endogenous pathways of reactive oxygen species formation, for example, involving NADPH oxidases or mitochondrial activation. It also suggests that the observed genotoxic effect of the nanoparticles in the comet assay and the micronucleus assay (i.e., stronger aneugenic effect) is due to mechanisms other than oxidative DNA attack. A different mechanism of DNA damage by nanoparticles and microparticles is further suggested by measures of DNA damage from the comet and micronucleus assays. The comet assay revealed more damage in nanoparticle-exposed than in microparticle cells. In contrast, the micronucleus assay revealed slightly less centromere-negative micronuclei in nanoparticle exposed than in microparticle-exposed cells. This assay measures clastogenic, that is, double strand breakage events. Although some micronuclei in nanoparticle-exposed cells might not have been seen as a result of inhibition of cell division from greater cytotoxicity, these results point to a greater complexity of DNA damage caused by exposure to nanoparticles compared to microparticles [85]. A genotoxic effect has also been described for silver nanoparticles that induced chromosomal aberrations, damage of metaphases, and aneuploidy in medaka (Oryzias latipes) cell line [86].

Further studies are needed to validate this dangerous potential effect of the nanomaterials. Obviously, close attention to safety issues will be required, also in the light of the potential interference between engineered nanomaterials and the environment.

### 4. Nanomaterials and Autophagy or “Autophagic Cell Death”

Autophagy is a highly conserved homeostatic process, involved in the recognition and turnover of damaged/aged
proteins and organelles. During autophagy, parts of the cytoplasm are sequestered within characteristic double- or multi-membraned autophagic vacuoles (named autophagosomes) and are finally delivered to lysosomes for bulk degradation. This process is dynamically regulated by ATG (Autophagy-related gene) gene family and is finely controlled by several signalling pathways [87]. Autophagy constitutes a cytoprotective response activated by cells in the challenge to cope with stress. In this setting, pharmacological or genetic inhibition of autophagy accelerates cell death. On the basis of morphological features, the term “autophagic cell death” has widely been used to indicate instances of cell death that are accompanied by a massive cytoplasmic vacuolization [38]. The expression “autophagic cell death” is highly prone to misinterpretation and hence must be used with caution, but, discussion this problem is beyond the scope of this paper, and an excellent paper concerning this subject has been published [88]. In any case, “autophagic cell death” is used to imply that autophagy would execute the cell demise. In the literature, it has been reported that several classes of nanomaterials induce elevated levels of autophagic vacuoles in different animals and human cell culture as well as in vivo models (masterfully summarized in two recent reviews [10, 61]). Such nanomaterials include alumina, europium oxide, gadolinium oxide, gold, iron oxide, manganese, neodymium oxide, palladium, samarium oxide, silica, terbium oxide, titanium dioxide, ytterbium oxide, and yttrium oxide nanoparticles; nanoscale carbon black; fullerene and fullerene derivate; and protein-coated quantum dots. The induction of autophagy was evaluated using panoply of established methods, including the electron microscopy detection of autophagic vacuoles, the immunoblot detection of autophagic vacuoles, the immunoblot detection of ATG expression level and/or LC3-I to LC3-II conversion (an established marker of autophagy activity) and/or cellular immunolabeling of punctate LC3-I in cytoplasmic vacuoles. These studies were performed in vivo but mainly in primary cells and/or cell lines from rat (alveolar macrophages, kidney, dopaminergic neuron, and glioma), mouse (macrophages and neuroblasts), porcine (kidney), and human (lung, oral, colon, breast, cervical and epithelial cancer cells as well as fibroblasts, peripheral blood mononuclear, and endothelial and mesenchymal stem cells). Nanomaterials may induce
autophagy via an oxidative stress mechanism, such as accumulation of damaged proteins and subsequent endoplasmic reticulum or mitochondrial stress [39, 89–92] and altering gene/protein expression and/or regulation, and interfering with the kinase-mediated regulatory cascades [93–103]. The increase in autophagic vacuoles in response to nanomaterials may be an adaptive cellular response. There is evidence that autophagy can selectively compartmentalize nanomaterials. In fact, nanoparticles are commonly observed within the autophagosome compartment, suggesting that activation of autophagy is a targeted exertion to sequester and degrade these materials following entrance into the cytoplasm [104]. It is possible that the cells might perceive nanomaterials as an endosomal pathogen or an aggregation-prone protein (both commonly degraded by the autophagy machinery). Recent evidence supports ubiquitination of nanomaterials directly or indirectly via colocalization with ubiquitinated protein aggregates, suggesting that cells may indeed select nanomaterials for autophagy through a pathway similar to invading pathogens [13, 98, 105]. Additionally, ubiquitinated proteins accumulate concomitantly with nanomaterial-induced autophagic vacuoles [106].

It is important to underline that nanoscale was a significant factor in eliciting the autophagic response. Autophagy was not induced by quantum dots that had a tendency to aggregate to microscale particles into the cells [107]. Nanoscale size dependence was also reported for neodymium oxide nanoparticle, with larger particles inducing less autophagy [108]. Apparently, modifications of the surface properties might be able to alter the autophagy-inducing activity of the nanomaterials. Cationic PAMAM dendrimers elicited autophagy more than anionic ones in vitro [94]. Carbon nanotubes with carboxylic acid group could induce autophagy, while those functionalized with poly-aminobenzene sulfonic acid and polyethylene glycol groups were not [100]. Recently, it has been published that a short synthetic peptide, RE1, binds to lanthanide-based nanocrystals, forms a stable coating layer on the nanoparticles surface, and significantly abolishes their autophagy-inducing activity. Furthermore, the addition of an arginine-glycine-aspartic acid motif to RE1 enhances autophagy induced by lanthanide-based nanocrystals [109].

It is also possible that nanomaterials cause a state of autophagic dysfunction, correlated with a blockade of autophagy flux, and this may be involved in their mechanism of toxicity [110, 111]. Nanoparticles could give rise to autophagy dysfunction by overloading or directly inhibiting lysosomal enzymes or disrupting cytoskeleton-mediated vesicle trafficking, resulting in diminished autophagosome-lysosome fusion [112]. Nanoparticles could also directly affect lysosomal stability by inducing lysosomal oxidative stress, alkalization, osmotic swelling, or causing detergent-like disruption of the lysosomal membrane (see the complete review of Stern and colleagues [61] about this subject). Disruption in autophagosome trafficking to the lysosome has been implicated in several human pathologies, including cancer development and progression as well as neurodegenerative diseases. As exposure to airborne pollution has been associated with Alzheimer and Parkinson-like pathologies, and nanoparticles are the primary particle number and surface area component of pollution-derived particulates, Stern and Johnson have recently postulated a relationship between nanoparticle-induced autophagy dysfunction and pollution-associated neurodegeneration [113].

Several studies have been suggested also that the nanomaterial-induced autophagy dysfunction is correlated with mitochondrial damage [102, 114–118]. In the majority of the studies, autophagosome accumulation induced by nanomaterials treatment was associated with cell death, unfortunately the possibility of autophagy inhibition was not often investigated (the block of autophagy flux and autophagy induction both can determine autophagosome accumulation) [119], and the mechanism of nanomaterial-induced autophagy accumulation in many cases is unclear.

Interestingly, nanomaterials have been proposed also as tools to monitor autophagy [120, 121]. In conclusion, a growing body of the literature indicates that nanomaterials impact the autophagy pathways, then the possible autophagic response should be always taken into consideration in the development of novel nanomaterials systems (Figure 4). Moreover, further studies should be performed to clarify the molecular mechanisms underlying the interaction between nanomaterials and the autophagy machinery as well as to expand the knowledge of the implications and biological significance of this modulation.

5. Nanomaterials and Necrosis

Necrosis was, for a long time, considered as an accidental form of cell death, but in recent years several studies clarified that this process is regulated and may play a role in multiple physiological and pathological settings [122]. Several triggers can induce regulated necrosis, including alkylating DNA damage, excitotoxins, and the ligation of death receptors [38, 122]. Indeed, when caspases are genetically or pharmacologically inhibited, RIP1 (receptor-interacting protein kinase 1) and its homolog RIP3 are not degraded and engage in physical and functional interactions that ultimately activate the execution of necrotic cell death [38, 122]. It should be noted that RIP3-dependent and RIP1-independent cases of necrosis have been described, suggesting that there are several subprograms of regulated necrosis [38, 122–124]. In a genome-wide siRNA screen, Hitomi and colleagues elucidated the relationship between apoptosis and necrosis pointing out that some components of the apoptotic pathway (e.g., the BH3-only protein Bmf) are also crucial in the necrotic machinery [125]. Moreover, recent studies provide evidence that apoptosis and necrosis are closely linked [126–128]. The term “necroptosis” has been used as a synonym of regulated necrosis, but it was originally introduced to indicate a specific case of necrosis, which is induced by death receptor ligation and can be inhibited by the RIP-1 targeting chemical necrostatin-1 [38, 122, 129].

In the literature, there are confused and inconsistent examples of necrosis induced by nanomaterials, because on one hand only the loss of cell viability is often evaluated without focalising into the cell death modalities and on
the other hand, there are no single discriminative biochemical markers available yet. Moreover, it should not be underestimated that the induction of apoptosis in cell culture is inevitably followed by secondary necrosis, and this could lead to a misinterpretation of results. However, a recent study demonstrated that water-soluble germanium nanoparticles with allylamine-conjugated surfaces (4 nm) induce necrotic cell death that is not inhibited by necrostatin-1 in Chinese hamster ovary cells [130]. Although the mechanisms of ligand and surface chemistry, surface charge, and crystallinity-based toxicity are complex, studies are beginning to elucidate certain surface functional groups and properties that can effectively alter biological responses. In fact, the crystal structure, with the different forms, of nanomaterials can dictate its cytotoxic potential. Braydich-Stolle and coworkers identify that both size and crystal structure (rutile, anatase, and amorphous) of TiO2 nanoparticles affect the mechanism of cell death in mouse keratinocyte cell line [131]. They found that 100% anatase TiO2 nanoparticles induced necrosis in size-independent manner, whereas the rutile TiO2 nanoparticles elicited apoptosis. Pan and collaborators investigated the size-dependent cytotoxicity exhibited by gold nanoparticles (stabilized with triphenylphosphine derivatives) in several human cell lines. All cell types internalised gold nanoparticles (<1.4 nm) were more toxic than their larger equivalents. However, 1.4 nm nanoparticles cause predominantly rapid cell death by necrosis, while closely related particles 1.2 nm in diameter affect predominantly apoptosis [132, 133]. Besides, it has been reported that small (10 nm) silver nanoparticles had a greater ability to induce apoptosis than other-sized ones (50 and 100 nm) in mouse osteoblastic cell line and induce necrosis in rat phaeochromocytoma cells [134]. The shape-dependent toxicity of polyaniline (PANI) nanomaterials with four different aspect ratios on human lung fibroblast cells was evaluated. The toxicity increased with decreasing aspect ratio of PANI nanomaterials; low aspect ratio PANI nanomaterials induced more necrosis than others [135]. Furthermore, the surface charge seems to be a major factor of how nanoparticles impact cellular processes. It has been demonstrated that charged gold nanoparticles induced cell death via apoptosis, whereas neutral nanoparticles caused necrosis [136]. Clearly, other parameters may influence the cell death modalities induced by nanomaterials, such as the dose or the time of exposure. Depending on the concentration, nano-C60 fullerene caused ROS-mediated necrosis (high dose), or ROS-independent autophagy (low dose) in rat and human glioma cell cultures [137]. The type of cell death induced by silver ions (Ag⁺) and silver nanoparticle coated with polyvinylpyrrolidone were also dependent on the dose and the exposure time, with Ag⁺ being the most toxic in a human monocytic cell line [138]. The silver nanoparticles concentrations required to elicit apoptosis were found to be much lower than the concentrations required for necrosis in human fibrosarcoma, skin, and testicular embryonal carcinoma cells [139, 140]. In conclusion, although the reports are often contradictory, the cell death appears roughly cell type, material composition, and concentration dependent. For instance, it has been reported that TiO2 (5–10 nm), SiO2 (30 nm), and MWCNTs (with different size: <8 nm, 20–30 nm, and >50 nm, but same length 0.5–2 μm) induce cell-specific responses resulting in variable toxicity and subsequent cell fate in mouse fibroblasts and macrophages as well as telomerase-immortalized human bronchiolar epithelial cells. Precisely, the macrophages were very susceptible to nanomaterial toxicity, while fibroblasts are more resistant at all the treatments, whereas only the exposure of SiO2 and MWCNT (<8 nm) induce apoptosis in human bronchiolar epithelial cells. In the experimental conditions of this study, the investigated nanomaterials did not trigger necrosis [65]. In the same mouse macrophage cell line, it has been demonstrated that MWCNT (10–25 nm) and SWCNTs (1.2–1.5 nm) induced necrosis in a concentration-dependent manner [141]. CNTs have been demonstrated to induce

![Figure 4](image-url)
both necrosis and apoptosis in human fibroblasts [142]. In contrast, Cui and co-workers found that SWNTs upregulate apoptosis-associated genes in human embryo kidney cells [143], and Zhu and colleagues showed that MWCNTs induce apoptosis in mouse embryonic stem cells [144], while Pulskamp and collaborators assert that commercial CNTs do not induce necrosis or apoptosis in rat macrophages [145]. Recently, a multilevel approach, including different toxicity tests and gene-expression determinations, was used to evaluate the toxicity of two lanthanide-based luminescent nanoparticles, complexes with the chelating agent EDTA. The study revealed that these nanomaterials induced necrosis in human lymphoblasts and erythromyeloblastoid leukemia cell lines, while no toxicity was observed in human breast cancer cell line. Moreover, no in vivo effects have been observed. The comparative analysis of the nanomaterials and their separated components showed that the toxicity was mainly due to the presence of EDTA [146].

The knowledge advances concerning the molecular characterization of necrosis will make necessary more precise and accurate studies to confirm the ways in which nanomaterials might cause necrotic death.

6. Nanomaterials and Pyroptosis

Pyroptosis described the peculiar death of macrophages infected by Salmonella typhimurium [147]. Several other bacteria triggering this atypical cell death modality have been identified. Pyroptosis neither constitutes a macrophage-specific process nor a cell death subroutine that only results from bacterial infection. Pyroptotic cells can exhibit apoptotic and/or necrotic morphological features. The most distinctive biochemical feature of pyroptosis is the early caspase-1 activation associated with the generation of pyrogenic mediators, such as Interleukin-1β (IL-1β) [38].

Recently, it has been shown that the exposure of macrophages (both a mouse macrophage cell line and primary human alveolar macrophages) to carbon black nanoparticles resulted in inflammasome activation as defined by cleavage of caspase-1 to its active form and downstream IL-1β release. The carbon black nanoparticles-induced cell death was identified as pyroptosis through the inhibition of caspase-1 and pyroptosis by specific pharmacological inhibitors. The authors showed that, in this setting, TiO₂ particles did not induce pyroptosis or significantly activate the inflammasome [148]. In contrast, it has been shown that nano-TiO₂ and nano-SiO₂, but not nano-ZnO (zinc oxide) and carbon nanotubes, induced inflammasome activation but not cell death in murine bone marrow-derived macrophages and human macrophages cell line. Although the caspase-1 cleavage and IL-1β release was induced, the inflammation caused by nanoparticles was largely caused by the biological effect of IL-1α [149]. This apparent discrepancy could be explained considering the different concentration and kind of nanomaterials used in these studies; moreover, it is possible that different macrophages perform differently in response to nanomaterials. Future studies should address this issue. However, the identification of pyroptosis as a cellular response to carbon nanoparticles exposure is novel and relates to health impacts of carbon-based particulates.

7. Conclusions and Perspectives

The continued expansion of the nanotechnology field requires a thorough understanding of the potential mechanisms of nanomaterial toxicity for proper safety assessment and identification of exposure biomarkers. With increasing research into nanomaterial safety, details on the biological effects of nanomaterials have begun to emerge. The nanomaterials intrinsic toxicity has been attributed to their physicochemical characteristics, that is, their smallness and the remarkably large surface area per unit mass and high surface reactivity. In fact, their type, composition and modifications, size, shape, and surface charge should be considered. However, the complex death paradigms may also be explained by activation of different death pathways in a context-dependent manner. In vitro experiments could be influenced by a cell type-specific response, and ones in vivo could be affected by the animal species and the model used or by pharmacokinetic parameters (administration, distribution, metabolism, etc.). Moreover, the dose, concentrations, and the time of exposure of a nanomaterial employed are essential. In effect, the efficiency of cellular uptake of nanomaterials and the resultant intracellular concentration may determine the cytotoxic potential. Elucidating the molecular mechanisms by which nanosized particles induce activation of cell death signalling pathways will be critical for the development of prevention strategies to minimize the cytotoxicity of nanomaterials. Unfortunately, in the literature, there are many conflicting data; the most plausible reason is certainly the discrepancy of nanomaterials and experimental models engaged. Although some authors have recently alerted colleagues on these issues [3, 5, 8, 9, 150–152], it has not yet been put in place a guideline, generally accepted by the scientific community in the field, to address these matters. In fact, harmonization of protocols for material characterization and for cytotoxicity testing of nanomaterials is needed. In addition, parallel profiling of several classes of nanomaterials, combined with detailed characterization of their physicochemical properties, could provide a model for safety assessment of novel nanomaterials [153]. During the past decade, owing to major technological advances in the field of combinatorial chemistry in addition to the sequencing of an ever increasing number of genomes, high-content chemical and genetic libraries have become available, raising the need for high-throughput screening (HTS) and high-content screening (HCS) approaches. In response to this demand, multiple conventional cell death detection methods have been adapted to HTS/HCS, and many novel HTS/HCS- amenable techniques have been developed [37, 154]. In the last years, several authors started to study the nanotoxicity with this tools and highlighted the potential of these approaches [9, 60, 75, 155–161]. An overall aim should identify HTS/HCS assays that can be used routinely to screen nanomaterials for interaction with the cell death modalities system. HTS/HCS may accelerated the analysis on a scale that commensurates with the rate of expansion.
of new nanomaterials but in any case is a first validation step, then it remains to confirm whether the same identified mechanisms in vitro are responsible for their in vivo toxicity. In conclusion, a multilevel-integrated uniform and consistent approach should contemplate for nanomaterial toxicity characterization.

In spite of the recent advances in our understanding of cell death mechanisms and associated signalling networks, much work remains to be done before we can fully elucidate the toxicological behaviour of the nanomaterials as well as understand their participation in the determination of cell fate. More and accurate results are needed for apoptosis, autophagy, and necrosis induction by nanomaterials; further studies are necessary to test if the novel strategic targets identified could be affected either directly or indirectly by nanomaterials. Moreover, no data are present in the literature concerning the nanomaterials exposure and other forms of cell death including anoikis, entosis, parthanatos, netosis, and cornification. For example, although numerous studies have been performed on keratinocytes, none of these has rated cornification, a cell death subroutine restricted to keratinocytes and functionally linked to the generation of the stratum corneum of the epidermis [38]. It will be of considerable interest to establish whether these various cell death modalities are associated with the intent of identifying a structure-activity relationship and delineating the mechanisms by which these interactions occur. In addition to the established paradigms of nanomaterials toxicity, the study of their interactions with the death signalling pathways could potentially have many important human pathological outcomes, including cancer, metabolic disorders, and neurodegenerative disorders.

**Abbreviations**

Ag⁺: Silver ions  
ATG: Autophagy-related gene  
Bcl-2: B-cell lymphoma 2  
BH3: Bcl-2 homology domain 3  
BID: BH3-interacting domain death agonist  
Bmf: Bcl-2-modifying factor  
CNTs: Carbon nanotubes  
CoCr: Cobalt-chrome  
DNA: Deoxyribonucleic acid  
EDTA: Ethylenediaminetetraacetic acid  
ER: Endoplasmic reticulum  
FDA: Food and Drug Administration  
HCS: High-content screening  
HTS: High-throughput screening  
IL: Interleukin  
MOMP: Mitochondrial outer membrane permeabilization  
MWNTs: Multiwall carbon nanotubes  
NADPH: Nicotinamide adenine dinucleotide phosphate  
NCCD: Nomenclature Committee on Cell Death  
PAMAM: Cationic polyamidoamine  
PANI: Polyaniline  
PEG-PE: Poly(ethylene glycol)-phosphoethanolamine  
RIP: Receptor-interacting protein kinase  
RNA: Ribonucleic acid  
RNS: Reactive nitrogen species  
ROS: Reactive oxygen species  
SiO₂: Silicon dioxide  
siRNA: Small interfering RNA  
SWCNTs: Single-walled carbon nanotubes  
tBID: Truncated BID  
TiO₂: Titanium dioxide  
ZnO: Zinc oxide.

**Conflict of Interests**

The authors declare that they have no conflict of interests.

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Review Article

Utilisation of Nanoparticle Technology in Cancer Chemoresistance

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The implementation of cytotoxic chemotherapeutic drugs in the fight against cancer has played an invariably essential role for minimizing the extent of tumour progression and/or metastases in the patient and thus allowing for longer event free survival periods following chemotherapy. However, such therapeutics are nonspecific and bring with them dose-dependent cumulative adverse effects which can severely exacerbate patient suffering. In addition, the emergence of innate and/or acquired chemoresistance to the exposed cytotoxic agents undoubtedly serves to thwart effective clinical efficacy of chemotherapy in the cancer patient. The advent of nanotechnology has led to the development of a myriad of nanoparticle-based strategies with the specific goal to overcome such therapeutic hurdles in multiple cancer conditions. This paper aims to provide a brief overview and recollection of all the latest advances in the last few years concerning the application of nanoparticle technology to enhance the safe and effective delivery of chemotherapeutic agents to the tumour site, together with providing possible solutions to circumvent cancer chemoresistance in the clinical setting.

1. Introduction

It is definitely not a matter of dispute that chemotherapy and its constituent cytotoxic agents play a vital role in the clinical management of the vast majority of cancer conditions. Chemotherapy measures focus on eradication of tumour presence or (at least) control the degree of tumour progression and metastasis. However, this therapy has its own critical flaws due to two major issues, namely, dose-dependent adverse conditions and the emergence of chemoresistance properties within the tumour.

2. Dose-Dependent Cumulative Adverse Effects

The issue of dose-dependent cumulative adverse effects derives from the pharmacological properties of cytotoxic chemotherapeutic agents, which are not tissue-specific and thus affect all tissues in a widespread manner. In addition, tissues having increased turnover rates, such as the gastro-intestinal system and skin, are more vulnerable to cytotoxic drug activity and are the most prevalent dose-limiting cumulative adverse effects in patients undergoing chemotherapy. Table 1 describes in brief the pharmacology and adverse effects of a few of the most commonly prescribed chemotherapeutic agents that are implemented in many cancer chemotherapy strategies.

3. Tumour Chemoresistance Properties

The emergence of chemoresistance within tumour cells of solid tissues is sadly one of the main reasons for treatment failure and relapse in patients suffering from metastatic cancer conditions [1]. Resistance of the tumour cell to chemotherapeutic agent exposure may be innate, whereby the genetic characteristics of the tumour cells are naturally resistant to chemotherapeutic drug exposure [2]. Alternatively, chemoresistance can be acquired through development of a drug resistant phenotype over a defined time period of exposure of the tumour cell to individual/multiple chemotherapy combinations [1, 2] (see Figure 1).
Table 1: Overview of a selection of cytotoxic drugs commonly used in chemotherapy.

<table>
<thead>
<tr>
<th>Cytotoxic drug</th>
<th>Mechanism of action</th>
<th>Major adverse effects</th>
<th>References</th>
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<tbody>
<tr>
<td>Cisplatin</td>
<td>Inter/intrastrand cross-link formation on nucleophilic N7 sites of adjacent adenine and guanine bases, leading to apoptosis.</td>
<td>Dose-dependent ototoxicity, nephrotoxicity, neurotoxicity, and myelosuppression.</td>
<td>[3–9]</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>Inter/intrastrand cross-link formation on nucleophilic N7 sites of adjacent adenine and guanine bases, leading to apoptosis.</td>
<td>Dose-dependent myelosuppression.</td>
<td>[3, 4]</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Oxazaphosphorine DNA-alkylating pro-drug activated by liver P450 cytochrome-induced 4-hydroxylation., thus forming DNA cross-linking phosphoramide mustard.</td>
<td>Neurotoxicity and nephrotoxicity due to chloroacetaldehyde formation by P450 cytochrome-induced oxidation.</td>
<td>[10]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Anthracycline-glucuronide conjugate prodrug activated by tumour β-glucuronidase, whereby the drug/DNA adduct possibly induces apoptosis by topoisomerase 2 inhibition or by a caspase cascade.</td>
<td>Dose-dependent cardiotoxicity, hepatotoxicity, and myelosuppression.</td>
<td>[11–15]</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topoisomerase II inhibitor, by raising the stability of the enzyme/DNA cleavage complex, ultimately leading to DNA strand breaks and apoptosis.</td>
<td>Possible secondary leukaemia due to chromosomal translocations induced by etoposide strand break activity, myelosuppression.</td>
<td>[16–22]</td>
</tr>
<tr>
<td>Ifosfamide (in severe NB cases)</td>
<td>Oxazaphosphorine DNA-alkylating prodrug, activated by liver P450 cytochrome-induced 4-hydroxylation, thus forming DNA cross-linking phosphoramide mustard.</td>
<td>Marked neurotoxicity and nephrotoxicity due to increased chloroacetaldehyde formation by P450 cytochrome-induced oxidation.</td>
<td>[10]</td>
</tr>
</tbody>
</table>

Figure 1: Overview of chemoresistance emergence, using cisplatin as an example for a conventional chemotherapeutic drug. Intrinsic chemoresistance (a) demonstrates the presence of tumour cell colonies that possess the optimal genetic and phenotypic characteristics to withstand exposure to cytotoxic agent activity. These characteristics were present in such cells prior to initial chemotherapy exposure and hence the term intrinsic chemoresistance. In acquired chemoresistance (b), the tumour cell line develops chemoresistance due to mutational driving forces following prolonged exposure to chemotherapeutic agents.

The biological routes by which the tumour cell is able to escape death by chemotherapy are numerous and complex. However, the major pathways enabling chemoresistance in cancer have been studied in detail and are summarised in Table 2.

4. Nanoparticle Technology

The introduction of nanotechnology in the last few decades has led to an undisputed boom in the conception and development of innovative methods for effective and safe delivery of small-molecule drugs and gene-based therapies to their intended target tissues. The advantages of exploiting nanoparticle delivery systems are many, such as the possibility to protect nucleosidic drug therapies, such as short interfering RNAs (siRNAs) and microRNAs (miRNAs) during transit within the bloodstream [87, 88]. In addition, implementation of nanoparticle-based delivery systems has led to improved pharmacokinetic profiles for the specific drug being carried within such a system, together with enhanced targeting of the site of action of the drug [89–91]. The excellent review by Hu and Zhang [92] highlighted that nanoparticles also have the capacity to carry combination therapies of two drugs/small molecules and have demonstrated to be particularly effective in circumventing multidrug resistance (MDR) issues in multiple cancer models.
Table 2: Overview of methods adopted by tumour cells for acquiring chemoresistance properties.

<table>
<thead>
<tr>
<th>Chemoresistance method</th>
<th>Description</th>
<th>Key player genes, proteins and/or signalling pathways</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug efflux mechanisms</td>
<td>Utilisation of drug efflux active pump proteins for expulsion of multiple cytotoxics from tumour cell cytoplasm, thus inducing multidrug resistance (MDR).</td>
<td>ATP-dependent binding cassette (ABC) transporter proteins, multidrug resistance 1 (MDR1) gene, P-glycoprotein (P-gp), multidrug resistance 1 protein (MRP1), ABCG2.</td>
<td>[23–26]</td>
</tr>
<tr>
<td>Drug modulation</td>
<td>Tumour cell ability to inactivate, or at least attenuate, drug activation through the modulation of expression of key enzyme/s involved in the target cytotoxic drug’s pharmacological and pharmacokinetic pathways.</td>
<td>Decreased expression or impairment of polyglycopoly-gamma glutamate-synthetase activity, resulting in antifolate drug resistance. Effect of glutathione on cisplatin inactivation-mediated chemoresistance.</td>
<td>[27–29]</td>
</tr>
<tr>
<td>Modification of drug targets</td>
<td>Upregulated expression or amplification of a target protein/enzyme, which may prove crucial for drug potency and effectiveness.</td>
<td>β-catenin, thymidylate synthase.</td>
<td>[30, 31]</td>
</tr>
<tr>
<td>Repair mechanisms following DNA damage</td>
<td>Exacerbated activity of components of the nucleotide excision repair pathway following tumour cell DNA damage.</td>
<td>Excision repair cross complementing 1 protein, microsatellite instability phenotype due to mutations in DNA mismatch repair genes.</td>
<td>[32–37]</td>
</tr>
<tr>
<td>DNA methylation mechanisms</td>
<td>Inhibition of key tumour suppressor genes leading to DNA methylations.</td>
<td>Caspase-8 promoter hypermethylation in neuroblastoma.</td>
<td>[38, 39]</td>
</tr>
<tr>
<td>Apoptotic pathway defects</td>
<td>Dysfunction or inactivation of the cytotoxic drug targeted intrinsic/extrinsic prosapoptotic pathways in tumour cells.</td>
<td>Bcl-2 protein family, cellular FADD-like interleukin 1 beta converting enzyme-inhibitory protein (c-FLIP), cellular inhibitors of apoptosis proteins (cIAPs).</td>
<td>[47–59]</td>
</tr>
<tr>
<td>Proliferative pathway activation</td>
<td>Stimulation of cell proliferation through modulation of the PI3K and extracellular signal-regulated kinase (ERK) survival signalling pathways</td>
<td>Protein tyrosine kinases (PTKs) families, epidermal growth factor receptor (EGFR) family, transcription factor kappa B (NFκB), Sirtuins (SIRTs).</td>
<td>[60–68]</td>
</tr>
</tbody>
</table>

Figure 2: Representative example of a chitosan-based nanoparticle designed for the loading of individual siRNAs within the electrostatic network created by the nanoparticle internal infrastructure.

The chemical composition of nanoparticles, both from natural occurring compounds (see Figure 2) and synthetic ones (see Table 3), is varied and the selection of which nanoparticle to utilize for any individual drug delivery system is very much dependent on a multitude of factors such as the chemical nature of the drug to be transported, the loading capacity of the nanoparticle, and resultant pharmacokinetic and pharmacodynamics properties of the nanoparticle following drug loading [93].

It is beyond the scope of this review to delve into the specific technical details regarding each individual type of nanoparticle utilized at present, as this has been already discussed extensively in other technical reviews and research articles within the literature [83, 84, 94, 95]. However, a brief summary encompassing the spectrum of varying nanoparticle compositions, key advantages together with toxicity profiles can be viewed in Table 3 and Figure 3.
Table 3: Overview of the major classes of nanoparticles utilised for chemotherapeutic drug delivery.

<table>
<thead>
<tr>
<th>Nanoparticle (NP) composition</th>
<th>Unique characteristics and advantages</th>
<th>Adverse effects/toxicity of nanoparticle components</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid lipid</td>
<td>Acidic pH of MDR tumour cells favours drug release from NP.</td>
<td>No haemolytic activity in human erythrocytes.</td>
<td>[69]</td>
</tr>
<tr>
<td>Polymer-based</td>
<td>Versatile acid-responsive drug release kinetics.</td>
<td>Minimal cytotoxicity observed on ovarian cancer cell lines.</td>
<td>[70]</td>
</tr>
<tr>
<td>Hydrogels</td>
<td>Easy synthesis, peptide-attachment facility for targeted delivery.</td>
<td>Nontoxic.</td>
<td>[71]</td>
</tr>
<tr>
<td>Magnetic (iron oxide)</td>
<td>Allows for physical (magnetic) enhancement of the passive mechanisms implemented for the extravastation and accumulation within the tumour microenvironment.</td>
<td>L-glutamic acid coated iron oxide nanoparticles demonstrated in vitro biocompatibility.</td>
<td>[72–74]</td>
</tr>
<tr>
<td>Micelle-based</td>
<td>Capable of solubilizing a wide range of water-insoluble drugs.</td>
<td>Relatively safe, though elevated doses can induce dose-dependent adverse effects such as hyperlipidaemia, hepatosplenomegaly, and gastrointestinal disorders.</td>
<td>[75–77]</td>
</tr>
<tr>
<td>Gold</td>
<td>Lack of complexity in their synthesis, characterization, and surface functionality. Gold nanoparticles also have shape/size-dependent optoelectronic characteristics.</td>
<td>Can induce cellular DNA damage.</td>
<td>[78–80]</td>
</tr>
<tr>
<td>Quantum dots</td>
<td>Capacity to be tracked in real time within specific areas of the target cells, due to their intrinsic fluorescence properties.</td>
<td>Potential long-term toxicity due to release of toxic components (e.g., Cadmium) and generation of reactive oxygen species.</td>
<td>[81, 82]</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Naturally occurring compound, derived from crustacean shells.</td>
<td>High biocompatibility properties.</td>
<td>[83, 84]</td>
</tr>
<tr>
<td>Mesoporous silica</td>
<td>Physical characteristics (e.g., size, shape) can be easily modified to induce bespoke pharmacokinetic/pharmacodynamics profiles.</td>
<td>Possible membrane peroxidation, glutathione depletion, mitochondrial dysfunction, and/or DNA damage.</td>
<td>[85, 86]</td>
</tr>
</tbody>
</table>

5. Recent Advances in Nanoparticle-Based Cancer Chemoresistance Circumvention Methodologies

The study carried out by Kang et al. [69] demonstrated that administration of solid lipid nanoparticles containing doxorubicin (SLN-Dox) to the adriamycin-resistant breast cancer cell line MCF-7/ADR, which also overexpressed P-glycoprotein (P-gp), allowed for chemosensitisation of the cell line. This was induced due to enhanced accumulation of doxorubicin within the cell line, contributed by the nanoparticle-based delivery method, and thus the degree of apoptosis was enhanced [69].

The same principle of exploiting nanoparticle delivery to substantiate chemotherapeutic drug accumulation within the target cancer cell, with the ultimate goal of enhancing tumour chemosensitivity, was adopted in the study by Aryal et al. [70]. Polymer-cisplatin conjugate nanoparticles were developed and consequently delivered to A2780 human ovarian carcinoma cell line [70]. The added potential of this delivery system relied on the cisplatin analogue prodrug covalently linked to a poly(ethylene glycol)-based polymer, which only released its therapeutic payload in a low pH environment [70]. Consequently, clinical administration of such a delivery system would ensure that the drug will remain complexed whilst in transit within the bloodstream due to its neutral pH environment [70].

Additionally, RNAi therapeutics have come to rely much further on the utilization of nanoparticle delivery systems to exert their biological effects. The study by Dickerson et al. [71] elucidated the efficiency to knock-down genes such as epidermal growth factor receptor (EGFR) by the delivery of EGFR-specific siRNAs contained within core/shell hydrogel nanoparticles (nanogels). The nanogels were also coated with peptides targeting the EphA2 receptor to enhance delivery of anti-EGFR siRNAs within the targeted Hey tumour cells [71]. Consequently, the knock-down effect on EGFR led to enhanced chemosensitivity of cancer cells to taxane chemotherapy [71].

The implementation of nanoparticle technology has also demonstrated to aid the clinical effect of other therapies that were previously unsuccessful due to poor drug delivery issues. Jin et al. [98] developed transferrin conjugated pH-sensitive lipopolyplex nanoparticles with the capacity to bind specific oligodeoxynucleotides (GTI-2040 in this case). This delivery system allowed GTI-2040 to exert its effect on the R2 subunit of the chemoresistance factor ribonucleotide reductase in acute myeloid leukaemia cell line models [98]. The influence of utilising such a delivery system was evident in that the 50% inhibitory concentration (IC(50)) for 1 μM GTI-2040 decreased from 47.69 nM to 9.05 nM [98].
Figure 3: Visual representation of a selection of varying nanoparticle-based drug (Rx) delivery systems adopted for averting cancer chemoresistance properties. Polymer-based [70] and solid lipid nanoparticle-based [69] delivery systems (blue) allow for bypass of the drug efflux pump, acquired chemoresistance pathways and allow for enhanced drug accumulation within the target cell cytoplasm, together with P-gp downregulation [96]. RNA interference methods utilising short interfering RNAs (purple) have been incorporated in hydrogel nanoparticles for targeting of epidermal growth factor receptor, a key player in mediating cell adhesion methods of chemoresistance [71]. Another major MDR gene targeted by short interfering RNAs includes P-gp [97]. Lipopolyplex nanoparticles were successful in enhancing the pharmacodynamic properties of the GTI-2040 oligonucleotide, targeting ribonucleotide reductase [98]. Ferromagnetic nanoparticles (black) have also been deployed for downregulation of the major chemoresistance gene MDR1 [72]. Micelle-based nanoparticles (orange) were found to be effective in delivering doxorubicin and VLA-4-specific peptides in multiple myeloma cells [76]. Quantum dots (green) containing siRNAs were also successfully deployed for downregulating MDR1 and P-gp expression in HeLa cell lines [81]. Chitosan nanoparticles (grey) incorporating Jagged1 siRNAs were also highly effective in circumventing MDR properties in taxane-resistant ovarian cell lines [99].

An additional nanoparticle delivery system, adopted against MDR in leukaemic conditions, was investigated by Cheng et al. [72]. This system combined magnetic iron oxide nanoparticles together with daunorubicin and 5-bromotetrandrin, which proved to possess a sustained release pharmacokinetic drug profile when administered to K562/A02 multidrug resistant leukaemic cell lines [72]. The principle behind the utilization of magnetic nanoparticles is due to the effects of magnetic field gradients positioned in a non-parallel manner with respect to flow direction within the tumour vasculature [73]. This allows for physical (magnetic) enhancement of the passive mechanisms implemented for the extravastation and accumulation of such magnetically responsive nanoparticles within the tumour microenvironment, followed by cellular uptake of the nanoparticles within the target tumour cell cytoplasm [73]. The magnetically responsive nanoparticle itself is composed of one or a combination of the three ferromagnetically active elements at physiological temperature, namely, iron, nickel, and cobalt [73]. The delivery system described by Cheng et al. [72] also aided in providing a dose-dependent antiproliferative effect on such cell lines, together with enhanced intracellular accumulation of daunorubicin and downregulated transcript expression of MDR1 gene, the main factor for induction of MDR in most cancer models [72]. These factors all contributed to a reduction in MDR and were directed by the level of endosomal-mediated cellular uptake properties of such nanoparticles [100].

In chronic myelogenous leukaemia (CML), a Bcr-Abl positive status induces MDR properties through multiple pathways, including resistance to p53 and Fas ligand-induced apoptotic pathways [101]. The delivery system devised by Singh et al. [101] consisted of magnetic nanoparticles combined with paclitaxel and was consequently administered...
to Bcr-Abl positive K562 leukemic cell lines [101]. The addition of lectin functional groups to the nanoparticle complex served to aid cellular uptake by the target K562 cell line and also demonstrated a reduction in the IC(50) for paclitaxel within this cell line model [101].

Multiple myeloma is an additional tumour model that has seen benefit from the exploitation of nanoparticle technology in its therapeutic avenues [76]. The study by Kiziltepe et al. [76] succeeded in developing a micelle-based nanoparticle delivery system containing doxorubicin and very late antigen-4 (VLA-4) antagonist peptides [76]. This delivery method not only accomplished enhanced cytotoxic activity when compared to doxorubicin alone, but also the addition of VLA-4 antagonist peptides served well in circumventing the phenomenon of cell-adhesion-mediated drug resistance due to the resultant impaired VLA-4 mediated adhesion of multiple myeloma cells to the stroma of bone marrow within CB.17 SCID murine multiple myeloma xenograft models [76]. Additionally, drug accumulation within the stroma of the multiple myeloma murine xenograft models was also tenfold higher than the control murine model [76].

Yet another tumour model that has been investigated for the application of nanoparticle-based chemotherapy, for the purpose of avoidance of chemoresistance, is prostate cancer [102]. Gold nanoparticles are an attractive avenue for drug delivery researchers primarily due to their lack of complexity in their synthesis, characterization, and surface functionality [78]. Gold nanoparticles also have shape/size-dependent optoelectronic characteristics [78]. The endosomal-based route for gold nanoparticle cellular uptake can be viewed as the primary advantage for circumventing MDR within the tumour cell, since the drug efflux pump is bypassed and the nanoparticle-held chemotherapeutic agent is released within the acidic environment of the endosome and allowed to penetrate the tumour cell cytoplasm [79]. Consequently, tumour progression phenotypes such as cell proliferation and level of apoptosis are affected to direct an amelioration of patient prognosis.

Gold nanoparticle/antiandrogen conjugates were developed by Dreaden et al. [102], with the capacity to selectively bind to two surface receptors which are upregulated in prostate tumour cell surface. Thus allowing accumulation of the nanoparticle conjugate specifically within treatment-resistant prostate tumour cells [102]. Gold nanoparticles were also exploited in the study conducted by Tomuleasa et al. [103] for the purpose of reducing MDR hepatocellular carcinoma-derived cancer cells. The gold nanoparticles were loaded with doxorubicin, capcetabine, and cisplatin, followed by nanoparticle stabilization by L-aspartate [103]. The resultant cellular proliferation rates of the hepatocellular carcinoma cells treated with this nanoparticle-based therapy were found to be lowered drastically [103].

In the study carried out by Punfa et al. [104], the cytotoxic properties of curcumin on multidrug resistant cervical tumours were maximized through the development of a nanoparticle-curcumin drug delivery system. Curcumin was successfully entrapped within poly (DL-lactide-co-glycolide) (PLGA) nanoparticles, followed by the incorporation of the amino-terminal of anti-P-gp [104]. Consequently, the curcumin-nanoparticle conjugates were deployed onto the KB-V1 cervical cancer cell line, having upregulated P-gp expression, together with the KB-3-1 cell line that has a reduced P-gp expression level [104]. The results of this study demonstrated that nanoparticle conjugates bearing anti-P-gp surface markers were highly efficient in binding to the MDR-inducing surface protein, allowing enhanced cellular uptake and ultimately aid in the cytotoxic efficacy of curcumin due to increased accumulation of the drug, particularly within the KB-V1 cell line due to its exacerbated P-gp expression status [104].

Curcumin/doxorubicin-laden composite polymer nanoparticles were also developed in other studies [105] as a means of enhancing the pharmacokinetic and pharmacodynamics properties of curcumin, thus enhancing its MDR-modulating effect in the target tumour cells. The resultant nanoparticle complex was deployed onto several MDR tumour models such as acute leukaemia, multiple myeloma, and ovarian cancers, both in vitro and in vivo [105]. The results of this study highlighted the possibility of administration of lower doses of doxorubicin due to the circumvention of tumour MDR by efficient curcumin activity, thus enhancing the toxicity profile for doxorubicin in clinical use stemming from the reduction in cardiotoxicity and haematological toxicity dose-dependent adverse effects [105].

Retinoblastoma therapeutic avenues have also been increased due to the introduction of nanoparticle drug delivery technology. The study by Das and Sahoo demonstrated the effectiveness of utilising a nanoparticle delivery system which was dual loaded with curcumin together with nutlin-3a (which has been proven to stimulate the activity of the tumour suppressor protein p53) [106]. The results of this particular investigation highlighted an enhanced level of therapeutic efficacy on utilizing the nanoparticle-curcumin-nutlin-3a conjugates on the target retinoblastoma Y79 cell lines [106]. In addition, a downregulation of bcl2 and NFκB was also observed following cell line exposure to the nanoparticle conjugates [106].

The nanoparticle-based drug delivery system designed by Saxena and Hussain [96] for its application against multidrug resistant breast tumours was novel in that the actual components of the nanoparticle biomaterials, namely, poloxamer 407 and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), are both known to exert pharmacological activity against P-gp [96]. The drug utilized for nanoparticle loading in this case was gambogic acid, a naturally occurring cytotoxic agent though laden with issues of poor bioavailability and severe dose-limiting adverse effects [96]. Similarly to other studies mentioned above, the incorporation of a nanoparticle-based drug delivery system allowed for enhanced cellular uptake by the target breast cancer cell line MCF-7, thus leading to elevated drug accumulation on the intracellular level and ultimately inducing enhanced cytotoxic effects in the target breast cancer cell line [96].

A separate nanoparticle-based drug delivery system for use in circumventing MDR effects in breast cancer is the one developed by Li et al. [107]. In this study, the nanoparticle drug delivery system consisted of a dimethylidodecylammonium bromide (DMAB)-modified poly(lactic-co-glycolic
acid) (PLGA) nanoparticle core that was conjugated to doxorubicin, then consequently coated with a 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) shell [107]. This system has been described to be specifically effective against MCF-7 breast cancer cell lines overexpressing P-gp [107]. The results obtained from this particular study indicated an elevated accumulation of doxorubicin released from the nanoparticle complex, within the nuclei of the drug resistant MCF-7 cell line [107]. In comparison, the level of accumulation of freely administered (i.e., not utilising a nanoparticle-based drug delivery system) doxorubicin attained lower drug concentration levels within the same cell line [107]. Finally, the IC(50) levels for doxorubicin on Adriamycin-resistant MCF-7 have been observed to be lowered by 30-fold following the incorporation of this nanoparticle delivery system [107].

Apart from delivery of conventional chemotherapy drugs in drug resistant breast cancer cell line models, researchers also delved into the possibility of adopting siRNA therapeutic approaches, using the aid of nanoparticle drug delivery systems [97]. The study conducted by Navarro et al. [97] developed a nanoparticle-based delivery system for siRNAs targeting P-gp expression, with the nanoparticle constituent biomaterials being dioleoylphosphatidylethanolamine and polyethyleneimine (PEI) [97]. Again, the reduction in P-gp expression led the path to enhanced cytotoxic effects brought about by the exposure of the MCF-7 cell line to doxorubicin, thus this nanoparticle-siRNA therapy was successful in drastically reducing MDR in this cancer model [97].

Quantum dots have also been implemented as novel and effective drug delivery systems for circumventing multidrug resistance in cancer chemotherapy [81]. Researchers in this study developed a quantum dot-based drug delivery system that allowed anti-MDR1 siRNA and doxorubicin incorporation to two cadmium-selenium/zinc-selenium quantum dots that were eventually functionalized by β-cyclodextrin coupling to L-arginine or L-histamine [81]. Following deployment of these dual loaded quantum dots in the HeLa cervical cancer cell line model, elevated accumulation of doxorubicin within the tumour cells was denoted, together with a marked reduction in MDR1 and P-gp expression on analysis by reverse transcription real time quantitative polymerase chain reaction and western blotting [81]. In line with magnetic and gold nanoparticle platforms, quantum dots rely mainly on the endosomal method of tumour cellular uptake and therefore the drug efflux pump system is bypassed, with consequent reduction in MDR properties by the tumour cells [82]. Finally, the additional benefit of utilizing quantum dots as a drug delivery system is their capacity to be tracked in real time within specific areas of the target cells, due to their intrinsic fluorescence properties [81].

Apart from cell line studies, researchers have also looked into the feasibility of implementing nanoparticle-based drug delivery systems within in vivo models [108]. The study by Milane et al. [108] investigated the efficacy of utilising a EGFR-targeting polymer blend nanoparticles, loaded with paclitaxel and the mitochondrial hexokinase 2 inhibitor lonidamine. The nanoparticle polymer blend consisted of 70% polycaprolactone (PCL) incorporating a PLGA-polylethylene glycol-EGFR specific peptide that helped enable nanoparticle active targeting efficiency [108].

Following nanoparticle development, four groups of orthotopic MDR breast cancer murine models (MDA-MB-231 in nude mice) were treated with free paclitaxel, free lonidamine, free paclitaxel/lonidamine combination, or nanoparticle complexes containing paclitaxel/lonidamine combination [108]. The degree of toxicity of such treatments was also monitored through body weight change measurements, liver enzyme plasma levels, and white blood cell/platelet counts, together with H & E staining of tumour sections was carried out [108].

Tumour weight and other clinical parameters such as MDR protein marker (P-gp, Hypoxia Inducible factor α, Hexokinase 2, EGFR, Stem Cell factor) were observed over the course of 28 days after-treatment [108]. Following this 28-day period, the results demonstrated that only the murine model sample group exposed to the nanoparticle-based paclitaxel/lonidamine combination treatment was the only group to experience statistically significant tumour volume and density reduction, together with overall alteration of the MDR phenotype [108]. Toxicity effects due to paclitaxel and lonidamine were also drastically reduced when administered within the nanoparticle-based delivery system, which can ultimately provide enhanced tolerance by the cancer patient [108].

Other in vivo studies in this field include the investigations carried out by Shen et al. [109], which focused on the codelivery of paclitaxel and survivin short hairpin RNA (shRNA) for circumventing chemoresistance in lung cancer. The investigators utilized the pluronic block co-polymer P85 combined with D-α-Tocopheryl polyethylene glycol 1000 succinate (P85-PEI/TPGS) for developing the nanoparticles to be implemented in this study [109]. These nanoparticles were based upon triblock structural formation of hydrophilic poly(ethylene oxide) (PEO) blocks and hydrophobic poly(propylene oxide) (PPO) blocks, which also gives enhanced capacity to revert chemoresistance due to drug efflux pump inhibition properties, downregulation of ATPase activity and P85-induced inhibition of the glutathione S-transferase compound detoxification enzyme at the subcellular level [109]. Paclitaxel and surviving shRNA were selected as the ideal drugs for nanoparticle delivery due to the former having poor efficacy due to chemoresistance within the tumour, and survivin was identified as highly expressed within chemoresistant tumours [109]. The in vivo activity of such nanoparticle systems (with/without paclitaxel and survivin shRNA) was evaluated on BALB/c nude mice injected with viable, paclitaxel-resistant, A549/T lung adenocarcinoma epithelial cells [109]. The results of this study demonstrated that deployment of the nanoparticle-based chemotherapeutic drug proved to have distinct enhancement of antitumour efficacy, when compared to deployment of the drug/s alone [109].

Chemoresistance to the aromatase inhibitor letrozole in postmenopausal breast cancer is another major therapeutic hurdle which was investigated in vivo [110]. Biodegradable PLGA-polylethylene glycol copolymer nanoparticles were
developed by nanoprecipitation and designed to incorporate hyaluronic acid-bound letrozole (HA-Letr-NPs) [110]. The addition of hyaluronic acid served to enhance letrozole binding specificity to CD44 on the target tumour cell surface, with the expected consequences of enhanced drug accumulation within the target tumour cell cytoplasm and resultant re-sensitization of the target tumour cells to letrozole activity [110]. Such HA-Letr-NPs, once produced at a size of less than 100 nm diameter, were deployed within a letrozole-resistant murine xenograft tumour model [110]. The results of this study demonstrated a highly efficient nanoparticle-based drug delivery system, with the IC(50) for HA-Letr-NPs within the murine xenograft model being only 5 μM when compared to the control groups, thus enhancing the in vivo aromatase enzyme activity within the xenograft and ultimately inducing a prolonged resensitising of the breast cancer tumour to letrozole activity [110].

The naturally occurring compound chitosan was also utilized for the development of in vivo nanoparticle-based therapies to circumvent ovarian cancer chemoresistance properties induced by overexpression of the Jagged1 notch ligand [99]. Murine orthotopic models, utilising female athymic nude mice, were injected with SKOV3Trip2 taxane-resistant ovarian cancer cell line and consequently, following one week, subjected to anti-Jagged1 siRNA/chitosan nanoparticle complexes (5 μg dose of siRNA) with/without taxane, applied via intraperitoneal route twice weekly for a total period of five weeks [99]. The results of this study indicated that such nanoparticle-based complexes had the capacity to reduce tumour weight by over 70% within such murine models and also induced taxane sensitization within the tumour [99].

In a similar study, cationic liposome-polycation-DNA (LPD) and anionic liposome-polycation-DNA (LPD II) nanoparticle systems were developed to incorporate doxorubicin and VEGF siRNA within a murine ovarian cancer animal model [111]. Female, athymic nude mice were treated with 5 × 10⁶ cells of the MDR ovarian cancer cell line NCI/ADR-RES [111]. Once the murine tumours reached a size of approximately 16–25 mm², the mice were consequently injected with individual nanoparticle complexes bearing either siRNA or doxorubicin at a dose of 1.2 mg/Kg in both cases, once daily for three consecutive days [111]. The results of this study demonstrated the effectiveness of such nanoparticle complexes for inhibiting tumour progression within the treated murine model groups, mainly due to impaired VEGF expression-related MDR [111].

Other human cancer conditions which were investigated for circumvention of tumour MDR properties through nanoparticle delivery include uterine sarcomas [112]. In the study carried out by Huang et al. [112], pH-sensitive mesoporous silica nanoparticles incorporating hydrazine and doxorubicin were developed for in vivo testing on murine models of doxorubicin-resistant uterine sarcoma. Since the composition of such nanoparticles specifically allow for cellular uptake through endocytosis, bypassing of the P-gp efflux pump induced a marked reduction in P-gp dependent MDR properties [112]. Consequently, the murine MDR tumour model treated with such nanoparticles demonstrated enhanced tumour apoptotic effects which were clearly confirmed by active caspase-3 immunohistochemical validation analysis [112].

6. Conclusion

The latest studies described above undoubtedly serve as a testament to the immense clinical value represented by nanoparticle technology. The ability of such nanoparticles, irrelevant of biomaterial composition to efficiently load individual or combinations of chemotherapeutic drugs and/or chemosensitising agents (such as curcumin) and novel RNA interference-based therapies has been clearly demonstrated above. This property provides an excellent escape mechanism for circumventing target tumour cell multidrug resistance properties based on drug efflux pump activity on the tumour cell surface, such as that exerted by P-gp. The overall advantage of deploying nanoparticles includes the drastic reduction in the IC(50) parameter for most of the carried chemotherapy agents, due to marked intracellular accumulation pharmacodynamics. This in turn would lead to a reduction in the clinical doses of the conventional cytotoxic agents required for chemotherapy, ultimately demonstrating a striking reduction in dose-dependent adverse effects in the oncology patient.

Presently, this does not mean that nanotechnology-based translational therapies are not fraught with challenges, such as biocompatibility issues of the nanoparticle components and the level of complexity required for cost-effectively translating these novel therapies to the patient bedside. However, it is the firm belief of the authors that through constant accumulation of marginal gains in knowledge, derived from persistent and motivated researchers on a global scale, will ultimately overcome such scientific hurdles, thus nanoparticle-based drug delivery aided therapies will eventually become commonplace in the oncology clinic in the near future.

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References


