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EXPERT REVIEW

Targeting Anticancer Drugs to Tumor Vasculature Using Cationic Liposomes

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ABSTRACT Liposomal drug delivery systems improve the therapeutic index of chemotherapeutic agents, and the use of cationic liposomes to deliver anticancer drugs to solid tumors has recently been recognized as a promising therapeutic strategy to improve the effectiveness of conventional chemotherapeutics. This review summarizes the selective targeting of cationic liposomes to tumor vasculature, the merits of incorporating the polymer polyethylene-glycol (PEG), and the impact of the molar percent of the cationic lipid included in cationic liposomes on liposomal targeting efficacy. In addition, the discussion herein includes the therapeutic benefit of a dual targeting approach, using PEG-coated cationic liposomes in vascular targeting (of tumor endothelial cells), and tumor targeting (of tumor cells) of anticancer drugs. Cationic liposomes have shown considerable promise in preclinical xenograft models and are poised for clinical development.

KEY WORDS angiogenesis · anti-angiogenic therapy · anticancer drugs · dosing schedule · dual targeting · PEG-coated cationic liposome · vascular targeting

ABBREVIATIONS

5-FU	5-fluorouracil
A-Mel-3	Amelanotic melanoma
AUC	Area under the blood concentration *versus* time curve
bFGF	Basic fibroblast growth factor
CHOL	Cholesterol
DAS model	Dorsal air sac model
DC-6-14	O,O′-ditetradecanoyl-N-(alpha-trimethylammonioacetyl)diethanolamine chloride
DC-Chol	3β-(N-(N′,N′-dimethylaminoethane) carbamoyl) cholesterol
DOPE	Dioleoylphosphatidylethanolamine
DOTAP	Dioleoyl trimethylammonium propane
DXR	Doxorubicin
EPR	Enhanced permeability and retention
HSPC	Hydrogenated soy phosphatidylcholine
K14-HPV16	The oncogene from the human papilloma virus (HPV) is driven by a region of the keratin 14 (K14) promoter
LLC	Lewis lung carcinomas
l-OHP	Oxaliplatin
LS174T	Human epithelial colon cell line
MMPs	Matrix metalloproteinases
$mPEG_{2000}$-DSPE	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]
MPS	Mononuclear phagocyte system
NGR	Asn-Gly-Arg
PEG	Polyethylene glycol
RIP-Tag2	Expression of the SV-40 virus large T antigen (Tag)[1] oncogene is driven by the 5\ flanking region of the rat insulin gene including the promoter (RIP).
SCID	Severe combined immunodeficient
VEGF	Vascular endothelial growth factor

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INTRODUCTION

Tumor vasculature is the general route of entry by which chemotherapeutic agents gain access to tumor cells. The vasculature also represents the life support of these target cells. Therefore, interrupting the flow of oxygen and nutrients to tumor tissue may create an opportunity to effectively manage tumor growth and the progression of

disease (1–3). Targeting the tumor vasculature, instead of the tumor cells themselves, is assumed to have several advantages. Normal endothelial cells are quiescent, and, therefore, side effects in the non-target endothelium are expected to be minimal (4). Proliferating endothelial cells in solid tumors share similar phenotypes—even when different solid tumors are compared. This makes vascular targeting applicable to a wide variety of tumor types (5). Furthermore, endothelial cells are genetically stable (unlike tumor cells); thus, there is a reduced risk of developing drug resistance (6). Finally, endothelial cells in tumor vessels are more accessible to circulating chemotherapeutics than tumor cells are, because the vasculature of a tumor occupies a relatively small area in comparison with the tumor interstitium, and most anticancer agents are applied intravenously (7).

Nonetheless, despite the many advantages of the vascular targeting approach, one potential problem is the lack of specificity of free anticancer agents. It is, therefore, a formidable challenge to minimize the total amount of a drug being delivered to healthy tissues while improving selective delivery to tumor targets. The need for an effective way to overcome this problem is obvious. The loading of low-molecular-weight anticancer drugs onto nanomolecular drug delivery systems has been shown to promote selective delivery of anticancer drugs to solid tumors by altering the biodistribution of associated drugs.

Many approaches based on nanocarrier drug delivery systems have been applied to achieve the targeting of anticancer agents to tumor tissue, including vasculature (Fig. 1) (8–10). Cationic liposomes, the focus of this review, are a promising carrier system for the delivery of anticancer agents to tumor endothelial cells, which takes advantage of the natural affinity of cationic molecules at the surface of the carrier system for anionic molecules, such as glycoproteins, anionic phospholipids, and proteoglycans, in the tumor microvasculature.

TUMOR ANGIOGENESIS

Tumor angiogenesis, the formation of neovessels from pre-existing vessels in solid tumors, is critical for the support of tumor growth and progression, not only by providing nutrients, oxygen, growth factors and other substances to tumor cells, but also by allowing metastatic cells into circulation (11,12). Tumors can gain sufficient nutrients and oxygen by simple diffusion up to a size of 1–2 mm, at which point their further growth requires the elaboration of a vascular supply (13). The process of tumor angiogenesis involves recruitment of the neighboring host mature vasculature to begin sprouting new blood vessel capillaries, which grow toward, and subsequently infiltrate, the tumor mass (14). In addition, tumor angiogenesis might involve the recruitment of circulating endothelial precursor cells from the bone marrow to evoke neovascularization (15,16). Tumor angiogenesis is mainly triggered by growth factors in the microenvironment, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and the matrix metalloproteinases (MMPs). These activating factors are produced by the tumors themselves, by the surrounding host tissue, or by infiltrating macrophages and fibroblasts in the tumor (17,18). Most of these activating compounds exert their actions through endothelial cell surface receptors, for which they serve as ligands, leading to secretion of additional angiogenic factors (13). Suppression of the angiogenesis process leads to eradication of primary tumor cells and suppression of metastasis, which makes this a promising strategy for the treatment of solid tumors (anti-angiogenic therapy).

VASCULAR STRUCTURE AND TARGETING

Vascular Structure of Solid Tumors

With the increasing promise of vascular targeting in solid tumors, a thorough understanding of the cellular structure and function of tumor vessels has become even more important. In comparison with blood vessels in normal tissues, tumor vessels are recognized as dynamic, both in terms of the formation of new vessels by angiogenesis and the remodeling of existing vessels (19,20). Tumor vessels are often dilated and irregular in distribution and shape and have abnormal pericytes and basement membrane coverage (21,22). As a result, vascular function is compromised. In addition, tumor blood vessels exhibit endothelial cell gaps, with an average size of ~100–600 nm (23). These pores are significantly larger than the gaps found in normal endothelium, which are typically <6 nm wide (24). This porous nature of tumor vasculature enables the preferential accumulation of macromolecules and polymeric drugs in tumor tissue, via a passive targeting phenomenon known as the enhanced permeability and retention (EPR) effect (25,26).

Tumor vessels are also characterized by the overexpression of specific surface receptors and antigens and by negatively charged macromolecules, such as glycoproteins, anionic phospholipids and proteoglycans (27–29). Such molecules can serve as exploitable selective targets to achieve active vascular targeting of chemotherapeutic agents by means of nanocarrier systems.

Vascular Targeting with Liposomes

As described above, tumor endothelial cells overexpress specific cell surface antigens, which are absent or barely

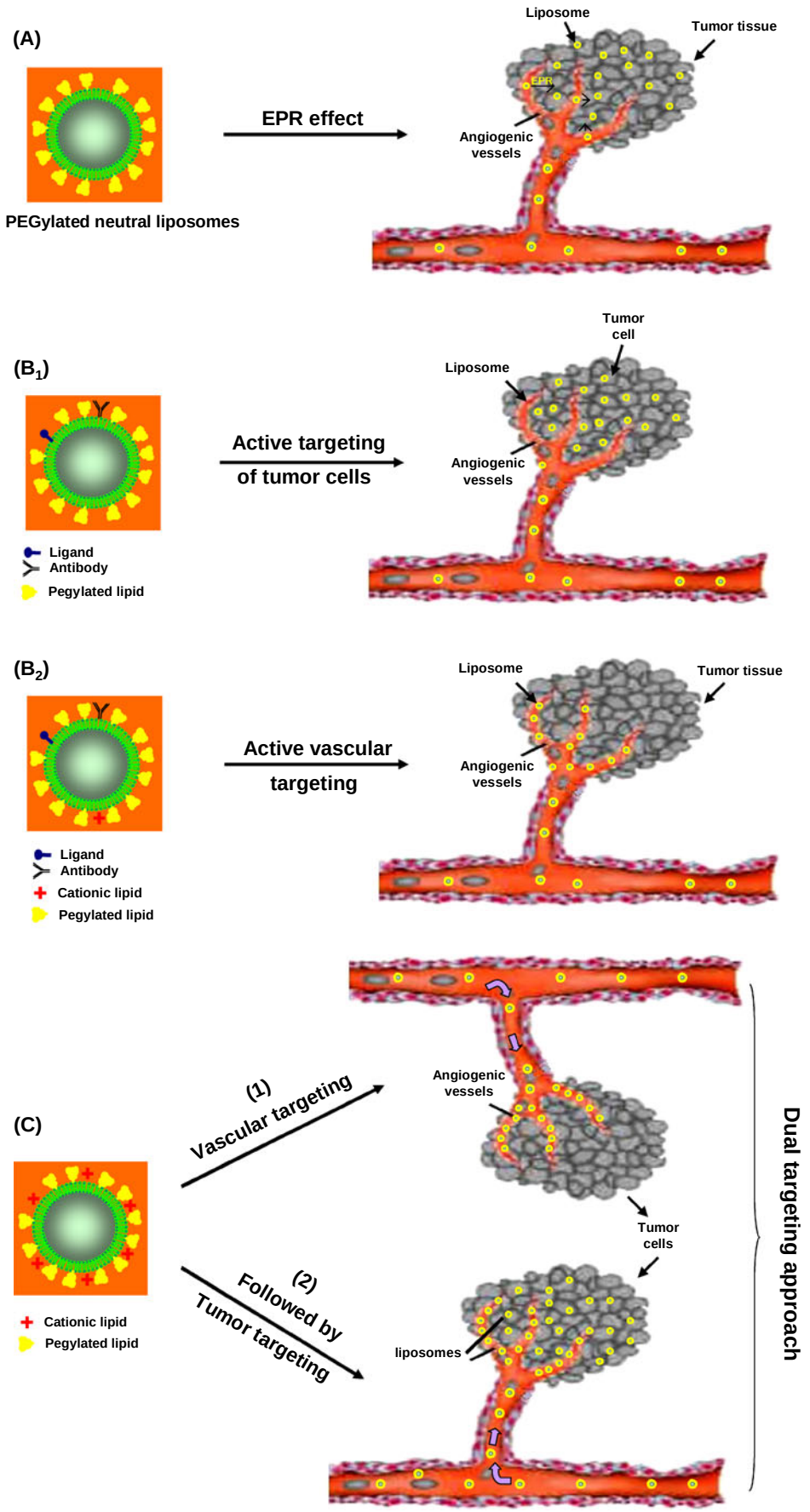

Fig. 1 Cartoon depicting different targeting approaches to tumor vasculature and/or tumor cells with liposomal drug delivery system. (A) Passive targeting of PEGylated liposomes via the leaky vasculature of solid tumor resulting in localization of the liposomes within the tumor interstitial space. (B_1) Active targeting of tumor cells with ligand (antibody or peptide)-targeted liposomes against cell surface antigens expressed selectively on tumor cells. (B_2) Active targeting of tumor vasculature with liposomes modified with ligand (antibodies, peptides or charge) against specific antigens overexpressed exclusively on the surface of tumor endothelial cells. (C) Dual targeting approach (*i.e.* targeting both tumor cells and tumor endothelial cells) with PEG-coated cationic liposomes. In dual targeting approach, PEG-coated cationic liposomes, by virtue of their surface positive charge, bind selectively to tumor endothelial cells via electrostatic interaction. Then, after the saturation of binding sites on the surface of tumor endothelial cells, the liposomes begin to extravasate through the leaky tumor vasculature into the tumor interstitial by EPR effect. Once being inside the tumor interstitium, PEG-coated cationic liposomes bind to tumor cells. Therefore, cytotoxic agents encapsulated in PEG-coated cationic liposomes will be allowed to exert their cytotoxic effect via a dual targeting approach as a result of affecting both tumor endothelial cells and tumor cells.

detectable in normal blood vessels. This unique characteristic of tumor endothelial cells can be exploited to achieve active vascular targeting by means of nanocarrier drug delivery systems, such as liposomes. Many approaches have been applied to enhance the targeting efficiency of liposomes to tumor endothelial cells (8,9,30). They include the coupling of specific molecules, such as antibodies, specific peptides, or growth factors, or incorporating cationic charges on the surface of liposomes. This review, however, is focused on cationic liposomes as one of the most promising carriers for targeting tumor vasculature.

CATIONIC LIPOSOMES

Nearly two decades have passed since the introduction of cationic liposomes in gene therapy (31). Cationic liposomes have proved to be an effective tool for gene delivery, both *in vitro* and *in vivo* (32–34). They have several advantages over viruses as gene transfer vectors. Unlike viral vectors, they can be used to transfer DNA of essentially unlimited size. In addition, they are technically simple and quick to formulate, have low immunogenicity, and are readily available commercially (35). There are, however, some drawbacks with lipid vectors, including lower efficiency than viral vectors in gene transfer, and transient gene expression (36–38). Such drawbacks prompted a re-evaluation of their use as gene vectors.

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

In Vivo Toxicity of Cationic Liposomes

Cationic liposomes possess many physical characteristics that make them attractive as candidates for gene/anticancer drug delivery to solid tumors. However, a major limitation is their toxicity. It is assumed that their toxicity depends mainly on the type of cationic lipid incorporated in their composition. For example, biodegradable ester lipids are less toxic in cell culture than ether lipids, which are more resistant to enzymatic hydrolysis (39,40). Lipids that do not form micelles, such as double-chain lipids, are less toxic in cell culture than single-chain cationic surfactants, which do adopt a micellar structure (41). When administered to animals, cationic liposomes tend to aggregate with serum proteins or blood cells (42) and adhere electrostatically to vascular endothelium cells after intravenous injection, resulting in their rapid clearance from the bloodstream or their localization in the lungs with the risk of causing lung embolisms (43).

In addition, inflammatory reactions have been observed upon *in vivo* use of cationic liposomes. However, the mechanism by which cationic liposomes elicit such inflammation is still not completely understood. Freimark *et al.* (44) reported that intratracheal instillation of cationic liposomes induced cytokine production and cellular influx in the lung airways, leading to severe lung inflammation. Dokka *et al.* (45) demonstrated that instillation of cationic liposomes elicited dose-dependent toxicity and pulmonary inflammation. They found that the inflammatory reaction was correlated with an oxidative burst that resulted from a dose-dependent increase in the generation of reactive oxygen intermediates induced by cationic liposome instillation.

Immune responses to cationic lipids that are frequently used to formulate cationic liposomes are much more enigmatic. However, Zelphati *et al.* (46) have shown that the serum complement system can be activated by cationic lipids incorporated in the liposomal membrane. Complement activation could result in complement components binding to the liposomes, thereby leading the liposomes to be recognized by the receptors for complement components found in lungs or liver (47). This could also explain the rapid clearance of cationic liposomes from the blood and enhanced uptake by lungs or liver. These studies made the assumption that PEGylation is necessary for *in vivo* use to protect cationic liposomes against immune reactions, aggregation with blood components, and enhanced uptake by the lungs and/or a mononuclear phagocyte system (MPS).

Inclusion of PEG in Cationic Liposomes

As described above, cationic liposomes have attracted attention as potential carriers to deliver therapeutic genes (48–50) and cytotoxic drugs specifically to the tumor vasculature (neovascular therapy) (51–53). A potential limitation, however, is the propensity of cationic liposomes to be rapidly eliminated from circulation by the "first-pass" organs, such as the lungs, the liver and the spleen (54). The inclusion of high-molecular-weight polymers, such as polyethylene glycol (PEG), in the liposome surface is considered an efficient approach to limit the interaction of conventional liposomes with circulating blood proteins, blood cells or cells of MPS, and thus to prolong their blood circulation time (55,56). The mechanism for the protective effect imparted by the inclusion of PEG at the surface of the liposome is believed to be the formation of a physical barrier, a hydration zone, around the liposomes, related to the hydrophilic nature of the grafted PEG at the

surface of the liposomes. This zone of exclusion diminishes liposome-protein interaction as a result of long PEG polymer chain constrictions (57,58).

Many studies have focused on elucidating the optimal concentration of PEG that should be incorporated in the liposomal membrane to provide liposomes with long-circulating characteristics. Levchenko *et al.* (59) demonstrated that PEG concentrations of ≥6 mol% shield the electric surface potential of cationic liposomes, while higher concentrations (≥15 mol%) were found to cause unfavorable structural changes in the liposomal bilayer, and thus enhance the rapid clearance of liposomes from circulation. For this reason, approximately 5–10 mol% of PEG-lipid derivative is included in the preparation of PEG-coated cationic liposomes. It should be noted that the partial coating of cationic liposomes with PEG does not alter their affinity to bind to tumor vascular surfaces (60). Campbell *et al.* (61) reported that although the zeta potential of cationic liposomes coated with 5 mol% of PEG was lower than that of their uncoated counterparts, PEG-coated cationic liposomes still retained the ability to associate with tumor endothelial cells. Furthermore, the inclusion of PEG-lipid derivatives on the liposome surface imparts long-circulating characteristics and, thus, enhances the therapeutic efficiency of the encapsulated drugs.

Effect of Surface Charge of Cationic Liposomes on Tumor Vascular Targeting

The targeting efficiency of cationic liposomes to tumor endothelial cells is strongly governed by the degree of surface cationic charge. Krasnici *et al.* (62) demonstrated that intravenously applied cationic liposomes, but not anionic or neutral liposomes, preferentially accumulate in the amelanotic (A-Mel-3) melanoma of the hamster *in vivo*, in comparison with normal surrounding host tissue. This preferential accumulation of cationic liposomes in the solid tumor was caused mainly by binding of the liposomes to angiogenic tumor microvessels, whereas neutral and anionic liposomes extravasated passively into the parenchyma. Campbell *et al.* (61) showed that an increase in cationic lipid from 10 to 50 mol% in PEG-coated cationic liposomes led to a 2-fold increase in liposomal accumulation in tumor vessels.

Besides binding to angiogenic endothelial cells, intratumoral clearance of liposomes is also dependent on the liposomal surface charge. Karsnici *et al.* (62) demonstrated that, in a tumor-bearing mouse model, anionic liposomes were almost cleared from the tumor, as well as the tumor tissue, at 3 h after intravenous injection, while cationic liposomes were retained within the tumor vasculature for up to 6 h after injection. Nomura *et al.* (63) reported that, in tissue-isolated tumor perfusion systems, clearance of positively charged liposomes was greatly retarded in comparison to neutral liposomes, which immediately appeared in the venous outflow perfusate following intra-tumoral injection. Recently, using a tumor-bearing mouse model, Abu Lila *et al.* (64) emphasized that PEG-coated cationic liposomes showed 2–3-fold higher accumulation in tumor tissue than PEG-coated neutral liposomes. It is interesting that tumor accumulation of PEG-coated cationic liposomes tended to increase up to 24 h after injection and remained at this level at 48 h after injection. Tumor accumulation of PEG-coated neutral liposomes, on the other hand, was substantially lower, and reached a maximum at 24 h post-injection followed by a gradual decline during the next 24 h. They ascribed this enhanced intra-tumor accumulation of PEG-coated cationic liposomes to the selective binding of PEG-coated cationic liposomes—not only to tumor angiogenic vessels, but to tumor cells as well. The preferential and prolonged accumulation of cationic liposomes in angiogenic tumor vessels, therefore, seems to be a general feature of cationic liposomes, and is independent of tumor type.

Pharmacokinetics of Cationic Liposomes

Although many publications discuss the *in vivo* fate of neutral and negatively charged liposomes, surprisingly little is known about the *in vivo* fate of cationic liposomes. Only a few reports about the pharmacokinetics of cationic liposomes exist, and their results are ambiguous or conflicting (65). Generally, cationic liposomes have been viewed as incompatible *in vivo*, giving accelerated clearance from blood circulation (66,67). Litzinger *et al.* (68) studied the biodistribution pattern of cationic liposomes consisting of 3β-(N-(N′,N′-dimethylaminoethane) carbamoyl) cholesterol (DC-Chol)/dioleoylphosphatidyl ethanolamine (DOPE) (1:4, molar ratio). They reported that the injected liposomes accumulated primarily in the liver. More than 60% of the injected dose had accumulated in the liver at 5 min post-injection, while only ~18% of the injected dose remained in blood circulation. Comparatively little accumulation occurred in the lungs (less than 10% of the injected dose). They postulated that the interaction of cationic liposomes with plasma components, including opsonin(s), immediately following injection, may have hindered non-specific binding with cell membranes and accumulation within tissues. This would explain the relatively low accumulation of cationic liposomes in the lungs, the first capillary bed encountered following tail vein injection, and enhanced accumulation in the liver, presumably due to enhanced uptake by Kupffer cells in the liver. In contrast, Ishiwata *et al.* (69) studied the *in vivo* fate of cationic liposomes composed of O,O′-ditetradecanoyl-N-(alpha-trimethylammonioacetyl) diethanol amine chloride (DC-6-14)/DOPE/cholesterol (CHOL)

(4:3:3, molar ratio). A dramatic and transient accumulation of liposomes in the lungs was observed. At 3 min post-injection, ~60% of the injected dose was present in the lungs. The accumulated liposomes were then slowly released from the lungs by the flow of blood and were immediately removed from circulation and accumulated in the liver. This may account for the inverse pharmacokinetic relationship between accumulation in the lungs and the liver.

Some studies have focused on the effect of the molar percent of cationic lipid incorporated into liposomes and the presence of PEG on the *in vivo* fate of cationic liposomes (69,70). Generally, PEG-coated cationic liposomes showed enhanced pharmacokinetic profiles, with longer circulation half-lives than cationic liposomes without PEGylation, as observed in PEG-coated neutral liposomes. In addition, incorporating a high mol% of cationic lipid in the liposomal composition triggers rapid clearance of the liposomes from blood circulation, even though they were coated with PEG. Stuart *et al.* (70) demonstrated that in the absence of PEGylation [1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-n-[methoxy (polyethylene glycol)-2000 [mPEG$_{2000}$-DSPE]]], cationic liposomes consisting of 5–50 mol% cationic lipid, dioleoyl trimethylammonium propane (DOTAP), were rapidly cleared from circulation, resulting in <5% of liposomes present in blood at 24 h post-injection. In addition, PEGylation did not increase the blood levels of liposomes containing 50 mol% cationic lipid (DOTAP/hydrogenated soy phosphatidylcholine (HSPC) in a molar ratio of 1:1). However, when the cationic lipid content was reduced to 20 mol%, inclusion of 5 mol% mPEG$_{2000}$-DSPE significantly increased blood levels of the liposomes. At ≤10 mol% cationic lipid, the inclusion of 5 mol% of mPEG$_{2000}$-DSPE was found to exert a maximal protective

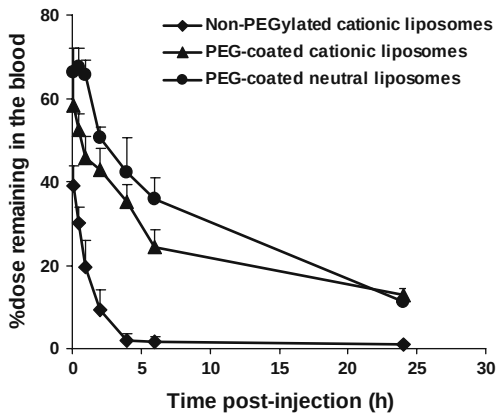

Fig. 2 Blood clearance of various liposomal formulations. On day 12 after tumor inoculation, LLCC tumor-bearing mice ($n=4$) were injected intravenously with either radio-labeled non-PEGylated cationic liposomes (*filled diamond*), PEG-coated cationic (*filled triangle*) or PEG-coated neutral liposomes (*filled circle*). At selected time points (0.083, 0.5, 1, 2, 4, 6, and 24 h), blood was collected and analyzed for radioactivity.

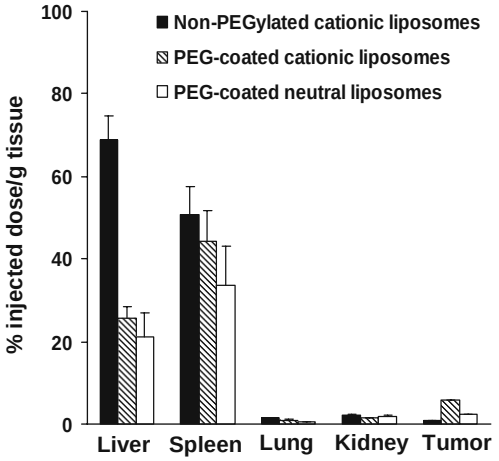

Fig. 3 Organ distribution of different liposomal formulations. On day 12 after tumor inoculation, LLCC tumor-bearing mice ($n=4$) were injected intravenously with either radio-labeled non-PEGylated cationic liposomes (*closed columns*), PEG-coated cationic (*hatched columns*) or PEG-coated neutral liposomes (*open columns*). At 24 h post-injection, the animals were euthanized and the radioactivity in each organ was determined. Data are presented as mean±SD. (In the case of the spleen, the value was per 500 mg instead of per gram).

effect against the rapid clearance of cationic liposomes from blood circulation. Abu Lila *et al.* (71) also recently confirmed that, in tumor-bearing mice, PEG-coated cationic liposomes consisting of HSPC/CHOL/DC-6-14/mPEG$_{2000}$-DSPE (2:1:0.2:0.2 molar ratio) showed more enhanced pharmacokinetic profiles than non-PEGylated cationic liposomes (Figs. 2 and 3). In addition, PEGylation significantly delayed the rapid clearance of cationic liposomes from blood circulation to an extent similar to that of PEG-coated neutral liposomes consisting of HSPC/CHOL/mPEG$_{2000}$-DSPE (2:1:0.2 molar ratio). However, PEG-coated cationic liposomes showed a lower area under the blood concentration *versus* time curve (AUC) compared to PEG-coated neutral liposomes. It is likely that the lower AUC was a result of the relatively enhanced blood clearance and tissue distribution of PEG-coated cationic liposomes (Table I), due to the presence of a positive charge on the liposomal surface. Interestingly, PEG-coated cationic liposomes and PEG-coated neutral liposomes accumulated in liver and spleen to similar extents (Fig. 3). This demonstrates that PEGylation of the cationic liposomes effectively prevents the rapid clearance of cationic liposomes from the blood circulation as is observed for non-PEGylated cationic liposomes (Fig. 2).

Selective Delivery of Cationic Liposomes to Tumor Vasculature

It is well known that cationic liposomes quite selectively target the vasculature of tumors, a phenomenon not noted with anionic (negatively charged) or electroneutral (zero

Table I Pharmacokinetic Parameters of PEG-Coated Liposomes in Tumor-Bearing Mice

Liposome	Half-life (h)	Clearance (ml/h)	AUC $_{t=0\rightarrow\alpha}$ (%dose·h/ml)	V_d (ml)
PEG-coated cationic liposomes	10.4 ± 0.7^{ns}	0.133 ± 0.007*	750.6 ± 35.2*	1.99 ± 0.10**
PEG-coated neutral liposomes	9.4 ± 0.5	0.113 ± 0.005	884.9 ± 41.7	1.53 ± 0.07

LLCC tumor-bearing mice received a single intravenous injection of either radio-labeled PEG-coated cationic liposomes or radio-labeled PEG-coated neutral liposomes. Pharmacokinetic variables were determined using PK Analyst software. Data represent the mean±SD ($n = 4$ mice per time point). ns > 0.05,*$p < 0.05$, **$p < 0.01$ against PEG-coated neutral liposomes.

charge potential) liposomes. Campbell *et al.* (61) demonstrated that after the intravenous application of PEG-coated anionic and neutral liposomes in LS174T-bearing mice, there was no selective accumulation in the tumor vasculature. By contrast, PEG-coated cationic liposomes accumulated extensively in tumor vessels. They assumed that the electrostatic interaction between the positively charged surface of cationic liposomes and the negatively charged glycoprotein layer of the tumor endothelium was partly responsible for this preferential accumulation in tumor vasculature. In addition, they proposed that the sluggish and stunted blood flow in tumor vessels, in contrast to the normal continuous flow in most healthy tissues, enhances the interaction between the anionic sites on the dynamic vasculature of the tumor and the cationic liposomes. Chang *et al.* (72) demonstrated that in the case of mosaic tumor vessels, vessels comprised of both vascular endothelial cells and tumor cells themselves, the tumor cells may come in direct contact with cationic liposomes, and uptake may occur in both vascular endothelial cells and tumor cells.

Thurston *et al.* (73) previously demonstrated that, in the RIP-Tag2 and K14-HPV16 tumor models, the amount of cationic liposomes accumulated in tumor vasculature was up to 33-fold higher than that accumulated in vessels in normal tissue of non-tumor-bearing (normal) mice. Recently, Abu Lila *et al.* (51) developed a PEG-coated cationic liposome composed of HSPC/CHOL/DC-6-14/$mPEG_{2000}$-DSPE (2:1:0.2:0.2 molar ratio) and confirmed that, in the dorsal air sac (DAS) model, the PEG-coated cationic liposomes accumulated preferentially and selectively in tumor angiogenic vessels induced in mouse skin. In addition, no selective accumulation/binding to pre-existing blood vessels in the skin was observed. Collectively, these studies provide many lines of evidence that cationic liposomes have the inherent potential to bind selectively to tumor vascular endothelial cells, which may be exploited to achieve successful anti-angiogenic therapy.

Selective Delivery of Anticancer Drugs to Tumors and Their Vasculature

The idea of exploiting accessible anionic sites, along with tumor vessels, by means of PEG-coated cationic liposomes containing anticancer drugs is promising for cancer therapy. Many preclinical studies (see Table II) have addressed the utilization of cationic liposomes to selectively deliver anticancer drugs to tumor vasculature. Some of the studies that illustrate the vascular targeting of anticancer drugs using a cationic liposome-based drug delivery system are described below.

Sengupta *et al.* (74) studied the therapeutic efficacy of etoposide encapsulated in cationic liposomes using a murine fibrosarcoma model. Liposomal etoposide significantly delayed tumor growth when compared with non-liposomal etoposide. In addition, *in vivo* survival studies demonstrated a significant increase in the lifespan of mice treated with etoposide-containing cationic liposomes, compared to mice treated with free (non-liposomal) etoposide. However, the exact mechanism of this enhanced antitumor activity was not completely elucidated in this study. Kunstfeld and co-workers (75) demonstrated that paclitaxel encapsulated in cationic liposomes potentially suppresses tumor angiogenesis and inhibits orthotopic melanoma growth in SCID mice. By contrast, free paclitaxel, while showing an inhibitory effect in *in vitro* cell culture, was unable to significantly suppress angiogenesis and tumor growth *in vivo*. Strieth *et al.* (76) evaluated the therapeutic efficacy of cationic liposomes containing paclitaxel (EndoTAG™-1) in Meth A sarcoma-bearing mice. Drug-containing EndoTAG™-1 resulted in a significant suppression of tumor growth, compared to free paclitaxel. The authors attributed the higher therapeutic efficacy of paclitaxel-containing EndoTAG™-1 to the delivery of more drug to angiogenic blood vessels. This might result in a decrease in tumor vessel density and a reduction in tumor microcirculatory perfusion index in these animals. Schmitt-Sody and colleagues (77) also emphasized that, in a dorsal skinfold model, vascular targeting of paclitaxel was achieved by encapsulating the drug in cationic liposomes. They also showed that paclitaxel-containing cationic liposomes were able to reduce *in vivo* growth and metastasis of A-Mel-3 mouse melanoma to a significantly greater extent than free paclitaxel. Eichhorn *et al.* (78) investigated the therapeutic efficiency of camptothecin encapsulated in cationic liposomes (EndoTAG™-2) and demonstrated that EndoTAG™-2 showed remarkable antitumor efficiency in

Table II Selected List of Cationic Liposomes that Have Received Evaluation *In Vivo* for Delivery of Anticancer Drugs

Encapsulated anticancer drug	Liposomal composition (molar ratio)	Tumor	Therapeutic effect
Paclitaxel	DOTAP/DOPC (25/23.5)	Humanized A-375 melanoma	Suppression of tumor angiogenesis Inhibition of tumor growth Increased survival time (75)
Paclitaxel	DOTAP/DOPC (25/23.5)	Amelanotic melanoma (A-Mel-3)	Enhanced tumor accumulation Tumor growth suppression Inhibition of local lymph node metastasis (77)
Paclitaxel	DOTAP/DOPC (100/94)	A-Mel-3	Retardation of tumor growth Decreased tumor vessel density Reduction in the microcirculatory perfusion index (76)
Paclitaxel	DOTAP/DOPC (100/94)	A-Mel-3	Increase of platelet adherence in tumor microvessels Acute impairment of the microcirculation Induction of microthromboses within the tumor microcirculation Decreased microcirculatory perfusion index (89)
Etoposide	Lecithin/CHOL/Stearylamine/ α-tocopherol (7/2/2/1)	Solid fibrosarcoma	Decreased tumor growth Prolongation of survival time Decreased cytotoxicity (74)
Cisplatin	HSPC/CHOL/TRX-20 (50/42/8)	Osteocarcinoma (Chondroitin Sulfate-expressing tumors)	Suppression of tumor growth Enhanced intra-tumoral accumulation Reduced systemic toxicity Prolongation of the survival time (88)
Doxorubicin	EPC/CHOL/DDAB (40/40/20)	Human oral carcinoma	Increased intra-tumoral accumulation Increased lifespan (79)
Doxorubicin	DOPC/DOTAB/CHOL/DOPE-PEG (50/35/10/5)	Human pancreatic cancer	Improved uptake of cationic liposomes by tumor endothelium Enhanced growth inhibitory properties (1)
Camptothecin	DOTAP	Lewis lung carcinoma (LLC)	Tumor growth suppression Decreased metastasis Reduction of tumor microvessel density Impairment of tumor microcirculation function (78)
Oxaliplatin	HSPC/CHOL/DC-6-14/mPEG$_{2000}$-DSPE (2/1/0.2/0.2)	Mouse melanoma B16Bl6	Selective binding to tumor angiogenic vessels Inhibition of tumor angiogenesis Decreased binding to erythrocytes (51)
Oxaliplatin	HSPC/CHOL/DC-6-14/mPEG$_{2000}$-DSPE (2/1/0.2/0.2)	LLC	Enhanced uptake by both tumor endothelial cells and tumor cells Tumor growth suppression Prolongation of the survival time (64)

Lewis lung carcinomas (LLC) implanted in mice. In addition, they correlated such efficient antitumor activity with a significant reduction of microvessel density. Wu *et al.* (79) demonstrated the therapeutic efficacy of doxorubicin (DXR)-containing cationic liposomes in a murine leukemia model. DXR-containing cationic liposomes showed antitumor activity superior to either DXR-containing neutral liposomes or free DXR. The authors attributed this superior antitumor activity to the higher accumulation of cationic liposomal DXR in tumors compared with that of free DXR and DXR-containing neutral liposomes.

Abu Lila *et al.* (51) recently addressed the utilization of PEG-coated cationic liposomes to selectively deliver an encapsulated anticancer drug to tumor angiogenic vessels,

and showed that PEG-coated cationic liposomes loaded with oxaliplatin (l-OHP) strongly suppressed tumor angiogenesis in a murine dorsal air sac model. Neither free l-OHP nor l-OHP encapsulated in PEG-coated neutral liposomes showed such a strong suppressive effect. In another study (64), they investigated the therapeutic efficacy of l-OHP-containing PEG-coated cationic liposomes in an LLC-bearing mice model. Treatment of the mice with l-OHP-containing PEG-coated cationic liposomes resulted in a significant suppression of tumor growth and a significant increase in survival times, relative to either free l-OHP- or l-OHP-containing neutral liposomes. Such enhanced antitumor activity was attributed to the preferential accumulation of l-OHP-containing cationic liposomes in both tumor endothelial cells and tumor cells, compared to either free l-OHP- or l-OHP-containing neutral liposomes.

Effect of Dosing Schedule on the Antitumor Efficacy of Cationic Liposomal Formulations

Despite the fact that the antitumor efficacy of anticancer drugs encapsulated in neutral or anionic liposomes has been confirmed as dependent on the dosing schedule, little is known about the impact of the dosing schedule on the anti-angiogenic efficacy of cationic liposomal formulations. Eichhorn *et al.* (80) were the first to investigate the impact of the dosing schedule on the anti-angiogenic activity of EndoTAG™-1 (paclitaxel-containing cationic liposomes). They showed that a single weekly dose was less efficient, compared to a metronomic dosing, with three to five intravenous applications per week at a lower dose. This strongly relates to the endothelial cell turnover time in solid tumors. The minimal doubling time of the tumor endothelium is approximately 2.5 days in solid mouse tumors (81). Such rapid endothelial cell turnover was assumed to compensate for the anti-vascular effect of EndoTAG™-1, administered only once a week. In fact, on this dosing schedule, tumor microvessel density was unchanged. An improved therapeutic effect with EndoTAG™-1 was achieved by drug application every 2–4 days, which is in accordance with the endothelial turnover time (~2.5 days).

Recently, Abu Lila *et al.* (71) addressed the impact of the dosing schedule on the antitumor activity of l-OHP-containing PEG-coated cationic liposomes. They emphasized that the intra-tumoral accumulation of l-OHP-containing PEG-coated cationic liposomes is dependent on the dosing schedule. Administering liposomal l-OHP every 4 days significantly enhanced the intra-tumoral accumulation of subsequently injected PEG-coated cationic liposomes, and thereby increased the therapeutic efficacy. In contrast, administration of liposomal l-OHP once a week resulted in lower antitumor activity, compared to a 4-day administration schedule. They assumed that this difference in the therapeutic efficacy between the dosing regimens (*i.e.*, 4-day *vs.* 1-week dosing schedules) may be correlated with the degree of tumor angiogenic vessel maturation. As shown earlier, cationic liposomes could selectively bind to the newly formed (immature) tumor angiogenic vessels, but not to the pre-existing mature blood vessels (1,51). One week might be enough for the maturation of tumor angiogenic vessels. Cationic liposomes might, therefore, lose their binding sites in the solid tumor. Consequently, the therapeutic efficacy of l-OHP-containing PEG-coated cationic liposomes administered once a week was lower than that administered every 4 days.

Dual Targeting of Both Tumor Endothelial Cells and Tumor Cells

Many studies have focused on the application of various combination treatment regimens that include cytotoxic and anti-angiogenic agents to improve the overall antitumor response in preclinical models (82–85). However, preclinical and clinical studies with such combinations have indicated that their toxicity profile differs from that of conventional single chemotherapy, thus ruling out additive toxicity as a major limitation of combination chemotherapy (86,87). One successful approach to improve the therapeutic outcome of either cytotoxic or anti-angiogenic agents, while minimizing the associated side effects, was to encapsulate either agent into a liposomal drug delivery system. Targeting of an anticancer agent to tumor vasculature by means of a liposomal drug delivery system has been proven to increase the therapeutic index of the agent without increasing the side effects (75,76,88,89). Moreover, the targeting of liposomal anticancer drugs directly to tumor cells by targeted liposomal delivery has also been confirmed to enhance the therapeutic efficacy and reduce side effects (90–93). Accordingly, it is easy to imagine that a strategy that targets both the tumor vasculature and the tumor cells using targeted liposomes would be more effective than a strategy that targets either tumor vasculature or tumor cells alone, which can leave a cuff of unaffected tumor cells.

Pastorino *et al.* (94) provided the proof-of-principle study for the hypothesis that the combined administration of liposomal anticancer drugs, which target tumor cells and tumor vasculature, improves therapeutic efficacy relative to each therapy used individually. To target tumor vasculature, DXR-loaded liposomes were modified with NGR peptides that target the angiogenic endothelial cell marker aminopeptidase N (2). To target tumor cells, they used anti-GD2 monoclonal antibody against the disialoganglioside receptor GD2, which is widely expressed on cancer cells of neuronal origin (95). In an orthotopic neuroblastoma xenograft model, the combined formulations showed

superior antitumor efficiency over both liposomal formulations when administered separately. They attributed such enhanced antitumor activity to the complementary modes of action of the two therapeutic approaches: DXR-loaded liposomes modified with NGR peptides acting primarily on the tumor vasculature, and DXR-loaded liposomes modified with anti-GD2 monoclonal antibody mainly affecting tumor cells. In this way, an effective 'two-compartment' tumor therapy was realized, which affected both the tumor cell and the vascular compartment within the tumor.

As described earlier, the authors recently developed a PEG-coated cationic liposome and confirmed that it is a promising carrier for the delivery of an encapsulated chemotherapeutic agent to tumor endothelium (51). Later, in another study (64), they demonstrated that in a murine solid tumor model, l-OHP-containing PEG-coated cationic liposomes showed antitumor activity superior to either free l-OHP- or l-OHP-containing PEG-coated neutral liposomes. This superior antitumor activity was confirmed to be due to the delivery of l-OHP to dual targets, tumor endothelium and tumor cells, by means of PEG-coated cationic liposomes (Fig. 1C). Such a dual targeting approach, with a single liposomal anticancer drug formulation, has the potential to overcome some of the major shortcomings of conventional strategies.

Clinical Applications of Cationic Liposomes in Cancer Therapy

The success achieved in the preclinical models provides a strong rationale for the use of cationic liposomal cytotoxic therapeutic agents for the treatment of human cancer. To date, cationic liposomal paclitaxel has been the most extensively evaluated in the clinical setting (96). The first clinical trial (97) was performed to evaluate the safety of EndoTAG™-1 in patients suffering from advanced metastatic colorectal cancer. Approximately 13% of the patients under study showed stable disease, and the treatment was well tolerated. A phase 1b clinical trial (98) was conducted to evaluate the safety and antitumor efficacy of EndoTAG™-1 in patients with metastatic breast cancer and tumor progression after anthracycline-based chemotherapy. The overall assessment of tumor response showed 6% partial response and 28% stable disease in patients receiving a dose of 0.55 mg/kg on days 1, 3, and 5 of a 3-week cycle. Nausea and vomiting were the major side effects associated with the treatment. A clinical phase II study was conducted to investigate the safety and efficacy of EndoTAG™-1 in combination with standard gemcitabine treatment in patients with locally advanced and/or metastatic pancreatic cancer (99). Two-hundred patients have been enrolled in this phase II study, and preliminary data confirm a favorable safety profile for EndoTAG™-1 in combination with gemcitabine treatment. Moreover, the study has shown promising preliminary therapeutic results, as the median overall survival was increased by EndoTAG™-1 combination therapy compared to gemcitabine standard monotherapy (100). Such data extracted from the preclinical and clinical studies could potentially serve as a basis for the future development of cationic liposomal drug delivery systems for cancer treatment. The achievements, and any limitations, of these clinical trials should encourage researchers to invest their efforts in the development of cationic liposomal formulations amenable to clinical applications.

SUMMARY AND FUTURE PERSPECTIVES

Targeting of tumor vasculature is considered a rational alternative to interstitial tumor targeting, because several factors favor this approach. First, tumor vasculature is more accessible to circulating therapeutics than tumor cells (53). Second, many cancer cells depend upon a few endothelial cells for their growth and survival, and, therefore, the death of a single endothelial cell may result in the death of more than 100 tumor cells (101,102). Third, since vascular-targeted agents need not penetrate deeply within the tumor interstitium to exert their therapeutic effect, physiological barriers to tumor targeting, such as high interstitial fluid pressure and tumor hypoxia, pose little threat to vascular targeting strategies (60,103). Fourth, endothelial cells are genetically stable; hence, multidrug resistance is not a competing factor (14). Finally, the therapeutic target, tumor vasculature, is independent of the type of solid tumor, so the killing of proliferating endothelial cells in the tumor microenvironment can be effective against a variety of malignancies. Nonetheless, despite the many advantages of vascular targeting, a strategy that targets both the tumor vasculature and the tumor cells themselves must be more effective than strategies that target only the tumor vasculature, because this strategy can leave a cuff of unaffected tumor cells at the tumor periphery that can subsequently re-grow and kill the animal (104).

Cationic liposomes have been shown to preferentially target the tumor angiogenic microvessels of solid tumors (61–63). Therefore, cationic liposomes appear to be one of the most promising drug carriers to direct chemotherapeutic agents to the tumor endothelium to realize the vascular targeting therapy concept (75–78). This novel therapeutic strategy was first realized by the synthesis of EndoTAG™-1 (formerly known as Lipopac/MBT-0206), comprised of paclitaxel encapsulated in cationic liposomes. EndoTAG™-1 has been shown to induce endothelial cell apoptosis and severe impairment of functional tumor microvasculature (105), by triggering intravascular thrombosis within treated

tumors (89). Moreover, treatment with EndoTAG™-1 significantly retarded tumor growth and delayed the incidence of metastatic disease in subcutaneously growing experimental tumors (80). Because of these promising results, EndoTAG™-1 has entered clinical phase II for the treatment of different tumor entities. In addition to paclitaxel, to realize vascular targeting therapy in preclinical animal models, DXR, 5-FU, camptothecin, and l-OHP also have been successfully encapsulated in cationic liposomes (51,76,78,79). To date, anti-vascular tumor therapy, as monotherapy, has failed to provide convincing results in clinical trials. Anti-angiogenic drugs and vascular targeting agents cannot completely eradicate tumors, and remarkable antitumoral effects can be achieved, in the clinical situation, only by combining anti-vascular tumor therapy with conventional cytotoxic radiotherapy or chemotherapy directly targeting the tumor cell compartment. Accordingly, the recently proposed dual targeting approach—vascular targeting and tumor targeting with a single liposomal anticancer drug formulation—may have the potential to overcome some of the major limitations of conventional strategies.

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