Pros and Cons of the Liposome Platform in Cancer Drug Targeting

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Coating of liposomes with polyethylene-glycol (PEG) by incorporation in the liposome bilayer of PEG-derivatized lipids results in inhibition of liposome uptake by the reticulo-endothelial system and significant prolongation of liposome residence time in the bloodstream. Parallel developments in drug loading technology have improved the efficiency and stability of drug entrapment in liposomes, particularly with regard to cationic amphiphiles such as anthracyclines. An example of this new generation of liposomes is a formulation of pegylated liposomal doxorubicin known as Doxil® or Caelyx®, whose clinical pharmacokinetic profile is characterized by slow plasma clearance and small volume of distribution. A hallmark of these long-circulating liposomal drug carriers is their enhanced accumulation in tumors. The mechanism underlying this passive targeting effect is the phenomenon known as enhanced permeability and retention (EPR) which has been described in a broad variety of experimental tumor types. Further to the passive targeting effect, the liposome drug delivery platform offers the possibility of grafting tumor-specific ligands on the liposome membrane for active targeting to tumor cells, and potentially intracellular drug delivery. The pros and cons of the liposome platform in cancer targeting are discussed vis-à-vis nontargeted drugs, using as an example a liposome drug delivery system targeted to the folate receptor.

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When considering the range of applications of drug delivery systems in cancer therapy, we can recognize three levels of increasing sophistication:

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• Level I consists of a system that provides a solubilization or dispersion vehicle for water-insoluble or lipophilic drugs. An example of this would be the tocol emulsions for delivery of paclitaxel (Constantinides et al., 2004).

• Level II refers to systems providing a slow and controlled release of the drug to improve its pharmacokinetic profile, and, thereby, its biologic activity, without a change in biodistribution. Implantable polymeric systems with controlled release of LH-RH partial agonists for the treatment of cancer of the prostate are one of the best examples of this approach.

• Level III implies a delivery system leading to changes in tissue drug distribution. These changes should result in a pharmacodynamic advantage by either sparing a tissue sensitive to the drug in question (site avoidance), or by enhancing drug deposition in the diseased tissue such as a tumor (tumor targeting).

Organ or tissue targeting, in turn, can occur at any of the following levels:

• Vascular targeting: The drug delivery vehicle concentrates in the endothelium of the blood vessels of the diseased tissue. In this case, the drug carrier has direct access to the target from the circulation.

• Passive tissue targeting: The drug carrier accumulates in the interstitial fluid of the diseased tissue by exploiting the enhanced permeability and retention (EPR) effect (Maeda H. 2001). Extravasation is critical in this setting. In addition, release of the drug from the carrier is required for bioavailability.

• Active cellular targeting: This is the holy grail of targeting. The drug carrier penetrates into the cellular compartment of the tumor. Here, the drug is released intracellularly as an intact carrier. Active targeting requires generally a ligand on the carrier directed against a receptor at the cell surface. The ligand-receptor interaction should result in internalization of the drug carrier and intracellular release of the drug.

In this review article, we will discuss passive and active targeting using as reference a liposomal formulation of doxorubicin known as Doxil® or Caelyx®, which is approved for clinical use in AIDS-related Kaposi’s sarcoma, recurrent breast cancer, and metastatic breast cancer (Gabizon, 2001). This is a well-known formulation of pegylated liposomes of an average diameter of 85–100 nm with doxorubicin encapsulated in the water phase of the liposome. Pegylation contributes to steric stabilization of the vesicles and provides important protection from opsonization resulting in delayed hepatic Kupffer cell clearance and greatly extended circulation time. Doxorubicin is encapsulated in the liposome water phase by remote loading mediated by an ammonium sulfate gradient. This method ensures a highly stable drug entrapment with negligible drug leakage in circulation, even after prolonged residence in the blood stream.

The EPR effect, initially described by Maeda et al. to account for increased deposition of macromolecular drug carriers in tumors (2001), also applies to liposomes and other nanoparticles. The mechanism underlying the EPR effect is related to the increased vascular permeability of tumor vessels characteristic of the tumor neoangiogenic process. Tumor microvessels have, among other abnormalities, large fenestrations that enable extravasation of macromolecules and liposomes. In addition, the lack of functional lymphatic drainage prevents the outflow of the extravasated liposomes. This creates a dead-end street situation whereby liposomes accumulate in the tumor extracellular fluid. These liposomes will gradually release the entrapped drug in the vicinity of tumor cells. Data obtained with lipid vesicles of different mean size in an experimental tumor model suggest that the threshold vesicle size above which major hindrance to extravasation occurs is
around 400 nm (Yuan et al., 1995). The occurrence of EPR in tumors coupled with the low permeability of most normal tissues to nanoparticles results in a high tumor:normal tissue ratio for liposomal drug concentration. This represents passive targeting that can be exploited to achieve an enhanced therapeutic effect of liposomal drugs. It should be emphasized that in this scenario, the drug enters the tumor cells as free drug. The rate-limiting step of drug bioavailability is the release from liposomes. Although the factors controlling the drug release rate from liposomes in the interstitial fluid are not well known, drug measurement studies in tumor tissue show peak concentrations between 48 to 72h, followed by a gradual drop in the ensuing days (Gabizon et al., 1997) indicating that the drug is becoming bioavailable and thereafter cleared from the tumor tissue. According to experiments using fluorescently labeled liposomes in the mouse skin-fold tumor chamber model, most liposomes appear to accumulate in the immediate perivascular area with little or no penetration into the tumor cell layers (Yuan et al., 1994).

One direct result of the enhanced deposition of liposomal drug in tumors is the observation of a greater anti-tumor effect of Doxil®, referred heretofore as pegylated liposomal doxorubicin (PLD), when given at lower dose than free doxorubicin (Fig. 1). Thus, PLD is at least four times more effective than free drug on a mg/dose basis (Gabizon et al., 2002), an observation also made in other tumor models (Colbern et al., 1999).

Therapeutic studies have also shown that PLD is also active against tumors completely refractory to doxorubicin as in the case of C26 carcinoma (Gabizon A. et al., 2002) (Fig. 2) indicating that the biokinetic changes of the drug when given in this liposome formulation are such that a certain level of drug resistance can be overcome.

The delivery of liposomal drugs to tumors is affected by a number of factors related to the liposome formulation and to a number of tumor characteristics. The following liposome factors have been identified to play a role: circulation time (the longer, the better); vesicle size (the smaller, the better); drug leakage from circulating liposomes (the slower, the better); RES (Kupffer cell) saturation (reaching saturation diminishes competition of liver vs. tumor for liposome uptake).

**Figure 1.** Growth inhibitory effect of PLD is >4-fold greater than that of Doxorubicin (DXR). BALB/c mice inoculated with 10⁶ M109 carcinoma cells s.c.. Treatment given I.V. on day 15 at the indicated doses (mg/kg). Three weeks later, on day 36, mice were sacrificed, and tumors dissected and weighed.
The following tumor factors are of relevance: blood flow—tumor areas poorly perfused will be less exposed to liposomes; vascular permeability—fenestrations in the endothelium are critical to enable liposomes to extravasate; interstitial pressure—a high pressure will reduce the hydrostatic pressure gradient that contributes to convective flow and liposome extravasation; phagocytic activity—liposome trapping by tumor or host macrophages in tumor tissue will increase the retention of liposomal material reaching the tumor.

Following the development of PLD, attempts have been made to extend the pegylated liposome platform to the delivery of other cytotoxic drugs. Recently, we have described a liposomal prodrug approach to deliver Mitomycin C (MMC). MMC, a highly potent cytotoxic compound, is unsuitable for passive entrapment in liposomes because of its facile transbilayer migration, leading to rapid leakage. Attachment of drugs to bilayer-compatible lipids forming lipophilic prodrugs is an established method of ensuring prolonged association with a liposome. The prodrug approach should retain the drug payload in vivo, and take full advantage of the pharmacokinetic benefits of a long-circulating liposome. MMC is a good candidate for the prodrug approach. We have synthesized an MMC prodrug interlinked via a dithiobenzyl group with di-stearic acid lipid anchor as promoiety. This liposomal prodrug is activated by reductive/thiolytic cleavage (Fig. 3), which is thought to take place preferentially in tumors due to overexpression of various reductive/thiolytic factors. We have recently reported on the pharmaceutical and pharmacologic features of this prodrug formulation (Gabizon et al., 2006). The main features are:

- Ease of formulation in pegylated (Stealth®) liposomes
- Stability and lack of toxicity in absence of reducing agents
- Thiolytic cleavage, by endogenous biologic factors or by exogenous pharmacologic agents, generates active MMC
- High plasma levels and prolonged circulation
- Reduced toxicity in comparison to free MMC
- Effective therapy for multidrug resistant and other tumors

Figure 2. Doxil® is more effective in the C26 tumor model where free doxorubicin is totally ineffective. BALB/c mice inoculated with 10^6 C26 carcinoma cells i.p. Treatment given i.v. on day 5. Mice followed up for survival.
This formulation of MMC prodrug in pegylated liposomes deserves further testing and may be an attractive formulation for clinical development covering a spectrum of tumors hitherto difficult to treat with chemotherapy.

**Ligand-directed Targeting of Liposomes to Tumors – Folate Targeting as an Example**

A number of targeting ligands have potential for selective delivery of liposomes to tumor cells. These include antibodies to cell surface determinants and growth factor receptors such as anti-CD20 and anti-Her2, folic acid, transferrin, and others (Sapra et al., 2004).

The vitamin folic acid has attracted our attention as a targeting device since the folate receptor, a 38kDa glycosyl-phosphatidylinositol (GPI)-anchored glycoprotein, is highly over-expressed in a number of human tumors including ovarian (Parker et al., 2005), lung, brain, head and neck, renal cell, and breast (Elnakat, Ratnam, 2004), whereas in normal tissue its expression is significantly lower and limited mainly to kidney tubuli, lung epithelium in the apical (luminal) cell pole, choroid plexus, and placenta for folate transport to CNS and to fetus (Antony, 2004).

We and others have demonstrated in vitro uptake of folate-targeted liposomes by tumor cells—reviewed in (Gabizon et al., 2004). In our studies using rhodamine-PE labeled liposomes (Goren et al., 2000), or dextran-FITC encapsulated in liposomes (Shmeeda et al., 2006), we demonstrated rapid and specific uptake leading to internalization of the vesicles and release of the markers in the cell cytoplasm. In the case of doxorubicin-containing liposomes, release of the drug and rapid movement of the drug to the nucleus was also shown. The binding and uptake of folate-targeted liposomes were specifically inhibited by an excess of free folic acid. It is worth noting that a large concentration

![Figure 3. Thiolytic cleavage of MMC prodrug by cysteine.](image-url)
of free folate (500–1,000-fold over liposomal folate) was required for inhibition of lipo-
some binding, an observation that is explained by the higher affinity of multivalent lipo-
some binding to cell surface receptors.

Intra-cavitary administration of targeted liposomes is a simple in vivo model to test
the putative advantage of a regional targeting approach. In an ascitic mouse tumor model,
we examined the distribution of folate-targeted liposomal doxorubicin vs. that of non-tar-
geted liposomal doxorubicin (PLD) given by intra-peritoneal injection (Shmeeda et al.,
2006). In mice injected with folate-targeted liposomes, we found that the drug levels were
17-fold greater in ascitic J6456 folate receptor-expressing tumor cells, and 14-fold lower
in plasma as compared to mice injected with nontargeted PLD. Thus there is a clear phar-
macologic advantage (increased drug in target, with decreased systemic drug exposure)
for targeted liposomes in this model of intra-cavity therapy.

Obviously, the most important challenge for targeted therapy is systemic treatment
for disseminated tumors. Experiments examining the fate of folate-targeted liposomes
after i.v. injection point to faster plasma clearance, higher liver uptake, and similar tumor
uptake when compared to nontargeted liposomes (Fig. 4) (Gabizon et al., 2003). To exam-
ine the effect of targeting on liposome localization in tumor cells as opposed to extracellu-
lar tumor fluid, radiolabeled liposomes were injected i.v. into mice bearing ascitic tumors.
Malignant ascites was generated by prior i.p. injection of J6456-FR lymphoma cells.
Ascites was drawn from the peritoneal cavity 72h following liposome injection and ascitic
cells were separated from the ascitic fluid by centrifugation. Folate targeted liposomes
were found to associate with tumor cells in significantly larger amounts than nontargeted
liposomes, while the latter were found to be present in greater concentrations in the ascitic
fluid (Fig. 5). Overall liposome concentration in ascites was roughly similar for both tar-
geted and nontargeted liposomes. Thus, targeting has a clear effect on the intra-tumoral
distribution of liposomes in ascitic models (Gabizon et al., 2003). However, caution
should be exerted before generalizing this observation to solid tumors, especially if the
interstitial fluid pressure is high and liposome movement is limited.

From this and other observations, we can conclude that the rate-limiting step of lipo-
some localization in tumors is extravasation, whether liposomes are nontargeted or
actively targeted, as in the case of folate. However, once liposome extravasation takes
place, targeting may play a role in the ultimate fate of the liposome: extracellular or

Figure 4. Tissue distribution of nontargeted and folate-targeted radiolabeled liposomes 48h after
i.v. injection. Folate targeting leads to higher liver uptake, faster plasma clearance, and similar
tumor uptake (M109).
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Intracellular compartment. The therapeutic value of actively targeted liposomes carrying anticancer drugs remains difficult to predict and may vary depending on the tumor model used and on the ligand-receptor interaction. Encouraging results have been published targeting of PLD with an anti-Her2 single chain Fv in Her2-expressing breast cancer models (Park et al., 2002). However, in many cases, ligand-bearing liposomes failed to produce the expected efficacy benefits compared to the nontargeted PEGylated liposomes (Goren et al., 1996). There are many variables that can influence this outcome including ligand size, charge, and density in the bilayer, pharmacokinetic changes, and potential immunogenicity (Harding et al., 1997; Zalipsky et al., 1997).

Finally, ligand-targeted liposomal preparations also face significant pharmaceutical challenges:

- Insertion of ligands conjugated to a PEG-lipid anchor into preformed liposomes (Zalipsky et al., 1997; Allen et al., 2002): This is the approach of choice for formulation of targeted liposomes. Provided the ligand-PEG-lipid is pure, this approach does not add any extraneous residues to the external surface of the liposome. The production line of the liposomal drug formulation should remain unaltered. The ligand should be added as a final step without disrupting the stability of the formulation. In addition to offering the most economical utilization of often precious ligand-PEG-lipid conjugates, this also provides flexibility as to the choice of liposomal drug and ligand (Zalipsky et al., 2004).

- Maintaining prolonged circulation time: Ligand attachment may affect the circulation time of the liposomal formulation. Since long circulation time is critical for liposome tumor accumulation, this is a critical property of pegylated (Stealth®) liposomes that should be maintained as much as possible. In some cases,
the ligand-liposome ratio will have to be fine-tuned to preserve a good affinity of
the particle to the receptor on the one hand, and to minimize any change in clearance rate on the other hand.

- Ligand retention during circulation: If the ligand is lost in vivo during circulation, then the particles reaching the tumor will have lost the ability to bind to the tumor cell receptors. It is important to test the robustness of ligand-liposome association in animal models. We have tested the ligand-liposome association of folate-targeted liposomes using an ascitic tumor similar to the above mentioned model. However, this time, the tumor did not express folate receptors. When the liposomes are collected from the ascitic fluid 48 h after i.v. injection, they are tested ex vivo for binding to FR-expressing cells grown in vitro. These experiments have clearly indicated that folate-targeted liposomes retain the ability to bind to FR-expressing cells after in vivo passage and extravasation to ascitic fluid, to an extent similar to that of the same formulation taken from the shelf (Gabizon et al., 2003).

Conclusion
Long-circulating, pegylated liposomes provide an attractive platform to improve the therapeutic index of a variety of anticancer drugs. The conceptual approach, the challenges, and limitations of active targeting of liposomes to tumor cells have been clearly defined in recent years, from both the formulation and pharmacology viewpoints. It remains to be seen whether ligand-mediated targeting can provide an added value in clinical applications.

References


