0031-3998/10/6705-0514
PEDIATRIC RESEARCH

Vol. 67, No. 5, 2010
*Printed in U.S.A.*

# Targeting Liposomes Toward Novel Pediatric Anticancer Therapeutics

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**ABSTRACT:** Although modern multimodal treatment of pediatric cancer has resulted in long-term cure of many patients, clinical success has come with significant acute and chronic morbidity. Targeted therapy using anticancer agents encapsulated in nanoparticles holds considerable promise in further improving efficacy and reducing toxic side effects. This review highlights the current strategies toward developing such therapeutic tools with an emphasis on using liposomes as flexible delivery vehicles. Potential strengths and technical difficulties encountered in advancing this platform are summarized. Critical functional determinants of nanoparticle delivery systems and future strategies to improve efficacy and specificity are described. **(*Pediatr Res* 67: 514–519, 2010)**

## Pediatric Anticancer Therapies—Success at a Cost

Before 1950, the outcome for children and young adults diagnosed with cancer was dismal with cure rates as low as 5%. Today, the overall survival for children with malignancies approaches 80% and is truly one of the success stories in modern day medicine (1). The progress in the treatment of pediatric malignancy is partly because of the use of combination chemotherapy, multimodality treatments, a better understanding of the molecular pathogenesis of disease, improvements in supportive care, and interventions validated through large cooperative clinical trial groups, such as the Children's Oncology Group (COG). However, success has come with limitations. During the past few decades, the incremental improvement in overall survival has slowed. This is, in part, because of the poor prognosis of refractory, metastatic, and recurrent solid tumors where survival has not improved despite increasingly more aggressive therapies. For those patients who do survive into adulthood, treatment-related late onset toxicities are being recognized with greater frequency. These trends reinforce the need for development of therapeutic strategies that would be both more efficacious and more specific than current treatments.

Our current anticancer armamentarium does not lack for bombs, but the majority of our guidance systems are remarkably primitive. Systemic cytotoxic chemotherapy is administered with the implicit hope that enough of the active agent will percolate into tumor cells to kill them without wreaking irreversible havoc on the rest of the body. Strategies that improve the delivery of a particular anticancer agent to pediatric malignancies will hopefully not only increase the dose effectiveness of chemotherapy but also reduce the systemic toxicity to normal cells.

Nanotechnology provides a viable platform for the development of targeted therapeutic approaches to pediatric malignancies. Nanoparticles can be defined as synthetic structures, organic or inorganic, with loosely defined dimensions ranging from 1 to 1000 nm whose unique properties at least in part depend on size and component chemistry (2). There is now a wide variety of different nanoparticles that have been formulated as potential therapeutic delivery vehicles but none more intensively studied than liposomes. Liposomes are primarily organic nanoparticles composed of combinations of lipid molecules that self-assemble into hollow spherical structures into which a wide range of cargo molecules can be packaged. During the last several decades, the development of supporting chemistries and components has resulted in liposomes evolving into extremely flexible delivery vehicles. As a result, the liposome platform holds tremendous promise in the future of targeted cancer therapeutics to improve drug delivery while minimizing unwanted systemic bystander effects. This review will highlight our current knowledge, applications, challenges, and unanswered questions in the developing field of targeted anticancer liposomal nanoparticles.

## Optimizing Liposomal Formulations—To PEGylate or Not to PEGylate

Since the serendipitous discovery of highly purified phospholipid dispersions, now called liposomes, in 1965 by Alec Bangham (3), various liposomal formulations encapsulating cytotoxic chemotherapy drugs have been studied and are becoming widely available to adult cancer patients. Of these liposomal formulations, conventional liposomes ("naked" liposomes) and polyethylene glycol (PEG)ylated liposomes (coated with PEG) have been clinically used most extensively. More recently, there is a growing interest in immunolipo-

Received January 4, 2010; accepted January 5, 2010.

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Supported by the St. Baldrick's Foundation Career Development Award and the UCLA's Today's and Tomorrow's Childrens Fund [to N.F.].

**Abbreviations: EPR,** enhanced permeability and retention; **BBB,** blood-brain barrier; **PEG,** polyethylene glycol; **RE,** reticuloendothelial system; **TF,** transferrin

somes and cationic liposomes as improvements on the conventional formulation to improve tumor targeting. Liposomal formulations of the anthracycline cytotoxic drugs, doxorubicin and daunorubicin, have been tested extensively in clinical trials and have shown both safety and efficacy, particularly in metastatic breast cancer (4). Both non-PEGylated and PEGylated versions of liposomal doxorubicin are in use although there are at least theoretically and, perhaps, clinically significant advantages to PEGylation.

The addition of PEG, a large synthetic hydrophilic polymer, to the liposomal surface reduces nonspecific interactions between liposomes and cells. PEGylation also decreases the binding of hydrophobic serum proteins that can act as opsonins, which can potentiate consumption of liposomes by phagocytes of the reticuloendothelial system (RE). In effect, PEGylation creates a "stealth" nanoparticle, which in comparison with its unPEGylated counterpart, has a prolonged circulation time, an increased bioavailability, and a greater potential for tumor targeting (5). For example, the PEGylated liposomal doxorubicin has a half life in children of 36 h (6). This is in contrast to the 2- to 3-h plasma half life of the non-PEGylated liposomal formulation (7). The optimal amount of surface PEG-lipid complex necessary for creating a stealth nanoparticle varies with different liposome formulations. In general, most PEGylated liposomes contain 5 to 10 mol% of PEG molecules with polymer lengths up to a molecular weight of 2000 D (8). Although PEG clearly extends the *in vivo* circulation time of liposomes, they are eventually removed and metabolized by the RE in the liver and spleen (9). Other synthetic polymers have been used in place of PEG to provide a liposomal cloak, such as poly(acryloyl) morpholine and poly(acrylamide) (10). However, there is such a bulk of safety information and clinical use of PEG in children and adults that this is the liposomal formulation with the greatest clinical applicability in the development of pediatric nanocancer therapeutics.

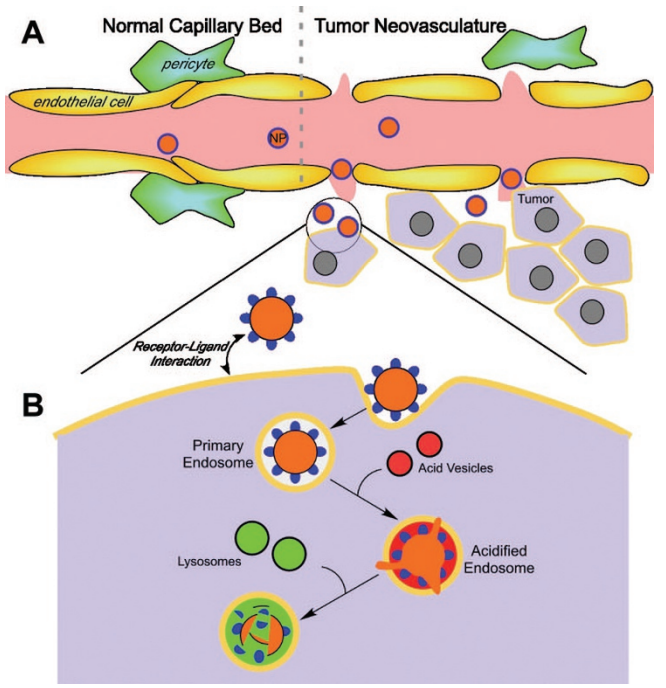


**Figure 1.** Schematic of targeted liposomal nanoparticle delivery to cancer cells. *A*, The nanoparticles circulate from the intact circulatory system into the disordered tumor vasculature. Nanoparticles are able to accumulate and extravasate through these large fenestrations, illustrating the EPR effect. *B*, Nanoparticles enter tumor cells predominantly through receptor-mediated endocytosis. Nanoparticles are internalized through receptor-ligand interactions in a primary endosome, then forming an acidified endosome initiating payload release. Fusion with lysosomes causes further enzymatic digestion of nanoparticles.

## Passive Tumor Targeting and the EPR Effect—Close Enough for Government Work?

In 1991, Maeda et al. (11) discovered that in animal model systems, high molecular weight drug conjugates preferentially accumulated in tumors to greater levels than free drug did. To describe this effect, these investigators coined the term the "enhanced permeability and retention (EPR)" effect. Although primarily studied in animal model systems, there is preliminary evidence that the EPR might also exist in human solid tumors (12). Although precise mechanisms responsible for the EPR effects are still be elucidated, it is currently thought that inherent differences in tumor vascular organization from normal tissues play a dominant role.

Similar to the tumors that they feed, tumor vasculature is frequently immature, disorganized, and chaotic (Fig. 1). In many solid tumors, the capillary bed is haphazardly constructed with an assortment of malformed branching structures juxtaposing vessels of random caliber and dimension. The vessels themselves are immature, consisting of loosely fitted endothelial cells lacking pericyte support. Tumor vessels have large endothelial fenestrations ranging in size from 100 to 600 nm (13). As a result, there is an increased capillary permeability leading to extravasation of plasma proteins and a proportionate increase in extravascular pressure within many solid tumors. It is still unclear that which of these features is important to establish an EPR effect.

Nanoparticles, like other high–molecular-weight bioconjugates, can be designed to exploit the EPR effect and preferentially enter the tumors (14). At least two critical parameters need to be optimized for this to happen: (i) nanoparticles must be able to exist in the circulation for a sufficient period of time; (ii) nanoparticles need to be able to cross the vascular endothelial barrier into the tumor interstitium. From this perspective, nanoparticle size matters. Liposomes that are too large (>250 nm) will not be able to pass through the fenestrations between the tumor endothelial cells. Liposomes that are too small (10 nm) are rapidly filtered out of the circulation by the kidney (15).

Charge is also important in the design of these tumor-targeting liposomes because it can effect the ultimate circulation life of the nanoparticles and the potential for EPR. Although anionic liposomes have the benefit of decreased self-aggregation in suspension, they seem to have increased nonspecific cellular uptake. Cationic liposomes, on the other hand, can be cleared by the kidney having the ability to filter positively charged particles. Furthermore, large amounts of cationic liposomes may cause a tissue inflammatory response

(16). Highly charged liposomes, whether positively or negatively charged, can trigger opsonization by fixing complement proteins and, hence, increasing RE clearance (17). Neutrally charged liposomes have the longest circulation times and least amount of RE uptake but greatest aggregation, which may limit tumor penetration. Thus, it seems that the optimal configuration of liposomal nanoparticles is maintaining a size somewhere between 50 and 100 nm with either a neutral or slightly anionic charge (18).

## Active Tumor Targeting—Pursuing Anticancer's Holy Grail

When it comes to targeting tumors with therapeutic nanoparticles, the EPR effect is probably necessary but may not be sufficient to accomplish the task. First, there is variability to the EPR. It is anticipated that across a wide range of solid malignancies there will be a differential susceptibility to the EPR effect, and even the permeability of the vasculature may be heterogeneous within the tumor itself (19,20). Certainly, the mere feat of achieving passive targeting of a liposome to a tumor does not necessarily mean that the particle will enter the cancer cell, nor that it will deliver its cytotoxic payload. Simply put, the EPR may get a particular nanoparticle into tumor tissue but not necessarily into tumor cells. To achieve the latter, a strategy of active targeting needs to be used.

The most widely studied strategy of active liposomal targeting has been attaching ligands or specific binding molecules, such as antibodies, to the surface lipids of the nanoparticle. These liposomal-ligand complexes are then internalized within the tumor cell by receptor-mediated endocytosis. Whether these "immunoliposomes" improve tumor localization *in vivo* is debated, it seems that, at least, the receptor-ligand interactions facilitates internalization and payload delivery into tumor cells (21). A summary of some of the targeting ligands that have been used in liposomal nanoparticles to achieve active targeting in malignancies is listed in Table 1.

Once affixed to the tumor cell surface through multivalent ligand-receptor interactions, liposomes can enter the cell through a variety of internalization pathways, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and other clathrin- and caveolae-independent pathways. Receptor-dependent, clathrin-mediated endocytosis is a major mechanism for internalization of ligand-receptor complexes (32). Ligand-conjugated liposomes bind to their cognate receptors and endocytosis takes place in clathrin-rich areas of the cell's cytoplasmic membrane forming an endocytic vesicle. Sequential fusion with cytoplasmic vesicles produces a harsher acidic environment that initiates liposomal degradation and payload release. Acidified endosomes can go on to fuse with lysosomes resulting in enzymatic dissolution of the liposomes and potentially destruction of payload molecules as well (33). For this reason, strategies that facilitate payload release before lysosomal attack have been investigated for particular nanoparticles. Clathrin-mediated endocytosis of liposomal nanoparticles can also occur in a receptor-independent manner although in a nonspecific fashion with a much slower internalization rate (33,34).

Although endocytosis through clathrin-coated pits seems to be the most common mechanism of nanoparticle entry into cells, other mechanisms exist (Fig. 1). Caveolae-mediated endocytosis is an alternative pathway that uses regions of the cytoplasmic membrane rich in the calveolin-1 protein. Finally, there are less well-characterized endocytic mechanisms that are independent of either clathrin or caveolin. The potential benefit of nonclathrin endocytic pathways is that, under certain circumstances, they can bypass harsh lysosomal degradation. This may be a preferable route for liposomal delivery of pH sensitive cytotoxic agents, nucleic acids, and peptides.

Although basic pathways by which nanoparticles bind and enter cells are well described, the critical determinants dictating the effectiveness of a particular liposomal formulation are less well understood. For example, the characteristics of specific cell-surface targets may play a role in how efficiently a liposome enters a cell. Doxorubicin-loaded anti-CD19 immunoliposomes proved more effective in antagonizing the growth of a mouse lymphoma model than similarly loaded immunoliposomes directed against the noninternalizing CD20 epitope (35). On this basis, actively targeting liposomes using ligands or receptors that get internalized as part of normal physiologic processes [*e.g.* folate, transferrin (TF), or receptor tyrosine kinases] have been favored by many. Finally, devising hard and fast rules that dictate how a nanoparticle is processed once it enters a cell have proved elusive. It seems that particles with a size of $<$200 nm are endocytosed primarily through clathrin-mediated processes, whereas larger particles are increasingly endocytosed through caveolae vesicles (36). However, size is likely to be only one of many factors influencing liposomal fate in cells.

## Penetrating the Blood-Brain Barrier—Breaking on Through to the Other Side

As a group, few tumors conjure more frustration, even despair, among pediatric oncologists than brain tumors. The

**Table 1.** *Ligands conjugated to liposomal nanoparticles to achieve active targeting*

| Targeting ligand | Ligand class | Liposomal vehicle | Therapeutic cargo |
|---|---|---|---|
| Folic acid | Organic compound | PEG liposomes | Doxorubicin (22), arsenic trioxide (23) |
| Anti-Her2 (ErbB2) mAb, and ScFv fragment | Antibody | PEG liposomes | Doxorubicin (24,25) |
| Tryptophan, threonine, and tyrosine (WTY) | Peptide | PEG liposomes | Doxorubicin and vinorelbine (26) |
| Transferrin | Glycoprotein | PEG liposomes | Doxorubicin (27) |
| Anti-CEA mAb and Fab fragment | Antibody | PEG liposomes | Doxorubicin (28) |
| Anti-EGFR mAb ScFv, and Fab | Antibody | PEG liposomes | Doxorubicin, vinorelbine, and methotrexate (29) |
| RGD | Peptide | PEG liposomes | Doxorubicin (30) |
| PR$\beta$ | Peptide | PEG liposomes | 5-fluorouracil (31) |

dramatic responses to chemotherapy, which can be seen with many pediatric cancers, rarely occur with the majority of primary CNS tumors. In part, this may be due to the relative resistance of brain tumors to current cytotoxic agents. However, a major problem is surmounting the blood-brain barrier (BBB), a physiologic system that normally protects the brain from potentially noxious exposures. The inherent problem of transducing cytotoxic chemotherapeutic agents across the BBB and maintenance of therapeutic drug concentrations in the CNS has been a major concern in pediatric primary CNS malignancies. This has required the use of direct intrathecal administration of chemotherapy in some cases and suboptimal CNS concentrations of chemotherapeutic agents in others. A non-PEGylated liposomal formulation of the chemotherapeutic agent, cytarabine, has been available and tested in children and adults. This agent does not effectively cross the BBB but, when given intrathecally, results in sustained drug levels in the cerebrospinal fluid for ~8 d (37,38). However, traversing the BBB is another issue.

There are several ways through which molecules can cross the BBB: carrier-mediated transport, active efflux transport (from brain to blood), and receptor-mediated transport (39). Liposomes, whether PEGylated or not, generally have a limited ability to cross the BBB in most situations. However, by targeting the large number of TF receptors on the endothelial capillary surfaces of the BBB with a number of TF-receptor ligands has resulted in efficient transport of liposomes across this barrier into the CNS (40,41). Other strategies using immunoliposomes to cross the BBB have used coating the liposome surface with ligand to the insulin receptor and E-selectin also highly expressed on the endothelial surface participating in receptor-mediated transport (42,43). Targeted immunoliposomes with TF ligands or other ligands to BBB endothelial receptors is a developing field and promising strategy for delivering traditionally impenetrable molecules, such as nucleic acids, peptides, proteins, and small and large molecule inhibitors, into the pediatric CNS.

## Liposomal Therapeutic Payloads—Getting the Most Bang for the Buck

Liposomes have a vast potential for delivering therapeutic payloads to malignancies. Much of the early development of liposomal formulations to cancers have centered on the delivery of well-known and widely applicable cytotoxic agents, such as doxorubicin, daunorubicin, vinorelbine, paclitaxel, cytarabine, and vincristine. Some of the available liposomal chemotherapeutic agents approved by the Food and Drug Administration in adult cancers, currently in pediatric clinical trials, and others that have received orphan drug designation are listed in Table 2.

Liposomes can be loaded with drug either passively or actively. In passive loading or entrapment, the liposomal formulation is prepared with the chemotherapeutic agent of choice in the incubation mixture. Drug is then encapsulated into the liposome as the nanoparticle is formed. The free, nonentrapped drug is then washed away by gel-filtration or other dialysis method. Lipophilic and ampiphilic cytotoxic drugs, such as paclitaxel (highly lipophilic), and doxorubicin (ampiphilic), are loaded somewhat more efficiently because they partition stably in both the lipid membrane and internal compartment of the liposomes. However, drug loading through passive entrapment is less efficient for water soluble drugs, such as methotrexate and cytarabine, in which the concentration in the liposome is directly proportional to the external concentration of the solution (44,45). As expected, chemotherapies with limited biphasic solubility (*e.g.* 6-Mercaptopurine) are poorly incorporated into the liposome aqueous and lipid compartments (45).

Active entrapment, also termed "remote loading," is a more efficient and preferable strategy for liposomal drug loading. In this method, a pH or ion gradient is created, which efficiently drives a molecule of choice across the lipid membrane leading to up to 100% loading efficiency of chemotherapeutic agents, such as doxorubicin and vincristine, with stable retention (46,47). In the end, efficiency and ability to actively load a liposomal nanoparticle depends on the individual chemotherapeutic drug characteristics and reaction conditions.

The discovery of RNAi a decade ago by Fire *et al.* (48) opened up an exciting field of cancer therapeutics with vast clinical application theoretically being able to silence any cancer-related gene pathway. In pediatric bone and soft tissue sarcomas associated with distinct fusion genes, such as Ewing sarcoma, rhabdomyosarcoma, and synovial sarcoma, the potential therapeutic use of siRNA holds promise for tumor-

**Table 2.** *Representative examples of liposomal formulations that are available and currently in pediatric clinical trials*

| FDA-approved liposomal formulation | Cytotoxic agent | Indication/pediatric phase trial |
|---|---|---|
| Doxil | Doxorubicin | Recurrent/refractory ovarian cancer, multiple myeloma in combination with bortezomib, AIDS-related Kaposi sarcoma, and pediatric phase I/II in combination with temsirolimus in recurrent sarcoma |
| Depocyte | Cytarabine | Lymphomatous meningitis and pediatric phase III of depocyte *vs* intrathecal triple therapy in ALL |
| DaunoXome | Daunorubicin citrate | AIDS-related Kaposi's sarcoma and pediatric phase III in refractory/relapsed |
| FDA orphan drug designation | | |
| l-Annamycin | Annamycin | Recurrent/refractory adult ALL and pediatric phase I in refractory/relapsed ALL or AML |
| Marqibo | Vincristine sulfate | Recurrent/refractory adult lymphocytic leukemia |
| ThermoDox | Doxorubicin | Unresectable hepatocellular carcinoma |
| CPX-351 | Daunorubicin:cytarabine | Advanced adult lymphocytic and myeloid leukemias |

ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia.

specific therapy. However, the safe and effective delivery of siRNA to malignancies has been the major hurdle in the translation of this technology to clinical application. "Naked" siRNAs are subjected to immune system activation and recognition, endogenous enzymatic degradation, and generally are too negatively charged to cross the cell membrane (49).

Liposomes are attractive delivery vehicles for siRNA in their inherent ability to protect the nucleic acid payload, camouflage the siRNA from the RE system, and inhibit potentially harmful nonspecific delivery to normal tissues. There is substantial literature and experience during the past several decades using liposomes as nanocarriers for nucleic acids both *in vitro* and *in vivo* (50). In models of adult-type malignancies, melanoma, lung cancer, breast cancer, and ovarian cancer, targeted liposomes have been regularly used to deliver siRNA constructs (51–53). Currently, exciting applications to pediatric cancer therapeutics using liposomes as nanocarriers for siRNA involve neuroblastoma, chronic myeloid leukemia, and hepatoblastoma. In neuroblastoma, an aggressive small round blue cell cancer of childhood, antisense oligonucleotides to c-myb have been encapsulated in anti-GD2 coated liposomes. These liposomes target neuroblastoma cells expressing the disialoganglioside GD2 and result in inhibition of growth and increased apoptosis (54). With *in vitro* chronic myeloid leukemia cell lines, TF receptor-targeted liposomes containing anti-BCR-ABL siRNA have been used to efficiently knockdown BCR-ABL mRNA resulting in increased cytotoxicity. However, there were reported off target effects related to nonspecific gene silencing (55). In an *in vitro* model of hepatoblastoma, a liver cancer diagnosed almost exclusively in young children and liposomal transfer of anti-BCL2 siRNA resulted in improved sensitivity of hepatoblastoma to cisplatin (56).

## Concluding Remarks—"Candygram for Mongo . . . "

In Mel Brooks' ground breaking film "Blazing Saddles," the indestructible villain is brought to heel not by brute force but by using an irresistible package to penetrate his defenses. Targeted anticancer therapy is aiming for the same outcome. In constructing targeted nanoparticles, it is hoped that anticancer agents can be delivered directly to tumor cells with greater efficiency and precision. To achieve this goal, successful nanoparticle-based therapies will rely on optimizing two effects: (i) passive accumulation of nanoparticles in tumor tissues by the EPR effect and (ii) active targeting of nanoparticles to tumor surface markers to promote intracellular delivery of therapeutics. It seems doubtful that a single nanoparticle preparation will be optimal for all tumors. However, with greater understanding, the rules for successfully tuning specific formulations to particular cancers will be forthcoming.

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