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Review

Antivascular and Antitumor Activities of Liposome-associated Drugs

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Abstract. *Particulate drug carriers offer unique opportunities to improve tumor therapy through several different mechanisms. Liposomes may (1) assist in formulation of poorly-soluble therapeutic agents, (2) provide a slow-release vehicle to achieve pharmacokinetic profiles that maximize the therapeutic index, or (3) behave as long-circulating nano-particulates that can extravasate in the hyperpermeable regions of tumor vasculature. For paclitaxel, liposomes provide an aid to formulation. In the intracranial rat 9L brain tumor model, paclitaxel liposomes reduced dose-limiting toxicity and mediated a 40% increase in median survival. Free drug did not extend survival. Doxorubicin entrapped within sterically-stabilized liposomes (SSL-DXR) represents a long-circulating formulation that can extravasate within tumors and enhance drug deposition. Repetitive dosing with SSL-DXR mediated a 30% extension in median lifespan of animals bearing advanced 9L tumors. Fluorescence microscopic imaging revealed non-uniform, sporadic deposition of liposomes within the tumor. Magnetic resonance imaging showed that repetitive dosing with SSL-DXR, but not free drug, resulted in vascular collapse and microhemorrhage within tumors. Exploiting this antivascular effect may provide a new means to enhance tumor therapy, and suggests the utility of combination therapy with agents such as paclitaxel that have antiangiogenic effects on tumors.*

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The treatment of solid tumors frequently fails for both pathophysiological and pharmaceutical reasons. Poor perfusion, tortuous and impermeable vasculature and the cellular expression of drug efflux transporters are tumor properties that reduce drug penetration, deposition and retention. Pharmaceutical properties of the drug or formulation also can hinder effective therapy; these include poor drug permeability through cell membranes or tissue barriers, short circulating half-life, rapid metabolism, or instability in the biological milieu. The overall outcome is that repetitive exposure of tumor cells to sub-lethal concentrations of drug can promote the emergence of a drug-resistant phenotype, in which tumor cells are insensitive to broad classes of chemotherapeutic agents.

Nanoparticulate drug carriers such as liposomes (1) provide several avenues by which to attack the problem of tumor drug resistance (Figure 1). Incorporation of the drug in a carrier may result in a *generalized alteration of pharmacokinetics* (PK); modulation of the drug release rate can alter the drug exposure profile of tissues in such a way as to enhance lethality to tumor while sparing critical normal tissues. Drug carriers also can provide *specific alterations to the PK*; drug sequestration in a carrier that is restricted to the systemic circulation (or the compartment of administration) reduces the volume of distribution, thereby reducing drug exposure at sites of toxicity. In parallel, carrier deposition at the tumor site must be enhanced for the success of this strategy. Tumor deposition can be increased by passive exploitation of leaky tumor vasculature, or through active targeting strategies that employ tumor-selective ligands on the particle surface. Finally (Figure 1), carriers can function as a vehicle to enable the formulation of lipophilic drugs or lipid analogs that can modulate the function of drug transporters localized in cell membranes.

Here we discuss recent developments in liposome-based approaches to tumor therapy, and investigate the potential of this approach to target the tumor vasculature, a point of attack which has received increasing attention as its

Application of liposome drug carriers to drug resistance

1) **Generalized alteration of PK:** provide drug exposure profile that is more potent to tumor while sparing normal tissues
   - *Delayed/sustained release provides lower peak levels, longer exposure duration;*

2) **Specific alteration of PK:** enhance tumor deposition through:
   - *Limited volume of distribution, avoiding normal tissues;*
   - *Passive accumulation via regions of compromised tumor vasculature;*
   - *Active 'targeting' via ligands selective for tumor or tumor vasculature;*

3) **Formulation** and delivery
   - *specific drug transporter reversal agents*
   - *endogenous/semi-synthetic growth-modulating amphipaths (e.g.. specific lipids)*

Figure 1. *Applications of liposomes to drug resistance. The effect of drug incorporation upon biodistribution and therapeutic index depends on the physicochemical properties of the drug and the characteristics of the liposomes.*

Distinct characteristics of liposome:drug formulations

1) ***Rapid-releasing* drug:liposome complexes:** relatively rapid release rates, but can alter PK:
   - *Lower peak free drug levels, sustained blood levels;*
   - *Key benefit may be pharmaceutical* (*e.g.* solubility);
   - ➢ ***Paclitaxel (Taxol) liposomes***

2) **High stability drug:liposome complexes**
   - *Slow release of drug from particle;*
   - *Drug deposition matches liposome deposition;*
   - *'Fortuitous' or active (ligand-directed) targeting possible;*
   - ➢ ***Remote-loaded sterically-stabilized doxorubicin liposomes (Doxil®/Caelix®)***

Figure 2. *Markedly differing formulation characteristics of promising liposome formulations. Liposomes containing paclitaxel, which is located in the membrane bilayer, release drug relatively rapidly after administration, but do alter pharmacology in a beneficial manner. Liposomes containing doxorubicin precipitated into the liposome core retain drug following administration, and drug biodistribution largely reflects that of the liposome carrier.*

potential importance has become better understood. Although the developments discussed below are applicable to solid tumors in general, the specific context of the studies is the development of improved therapies for brain tumors.

Brain tumors as a therapeutic problem. Although they represent only 1% of adult malignancies (2) brain tumors are highly fatal; of 17,000 cases of brain/CNS cancers diagnosed in the US annually, 13,000 are fatal (3). In childhood, brain tumors represent the leading cause of cancer-related morbidity and mortality (26% of cancer deaths), and their incidence is second only to leukemia (2-4). Gliomas account for approx. 45% of adult- and 70% of pediatric brain tumors. Advances in the detection and treatment of various cancers have increased remission- and survival time, but malignant primary and metastatic brain tumors were as lethal in the 1990's as in the 1970's (5). The median survival of malignant gliomas treated by surgery, surgery/radiation or surgery/chemotherapy was 14, 20 and 40-50 weeks, respectively (6). Thus, development of new therapies and improvement of existing treatments would have significant impact on those afflicted.

Barriers to tumor therapy. In order for systemic therapies to be effective, drugs must reach the tumor in therapeutically effective quantities, distribute uniformly to the minimum threshold concentration necessary to kill tumor cells and retain activity in the tumor microenvironment (7). Tumor blood flow and architecture, as well as the physical barrier properties of tumor microvasculature and interstitium, fluctuate spatially and temporally. Physiological barriers include elevated tumor interstitial pressure, which results in an outward convective flow that hinders drug extravasation and tumor penetration. In addition, the distance which drug must traverse from blood vessels to the tumor interior may be sufficiently large that diffusion cannot transport sufficient concentrations for tumor cell killing (7, 8). These physiological/anatomical variations hinder the delivery of chemotherapeutic agents. Heterogeneous intratumor deposition results in insufficient drug exposure and thus promotes the development of therapeutic resistance. A variety of approaches have been developed to improve the delivery of drugs using macromolecular carriers, but their ability to deliver adequate quantities of therapeutic agents uniformly throughout tumor is hampered by physiologic barriers.

Liposome-based formulations for brain tumor therapy. Liposomal formulations may possess markedly different properties depending on the physicochemical characteristics of the encapsulated drugs. A recent, comprehensive review describes the interplay of multiple factors in determining the performance of liposome formulations (1). Here we discuss two markedly different formulations (Figure 2); the first contains paclitaxel (taxol) and the second contains doxorubicin.

Paclitaxel-containing liposomes. Paclitaxel is a complex diterpenoid natural product which has gained widespread use in the treatment of a variety of carcinomas, and has become a first line treatment for refractory ovarian, breast and non-small cell lung cancer (9-13). Because of the poor water

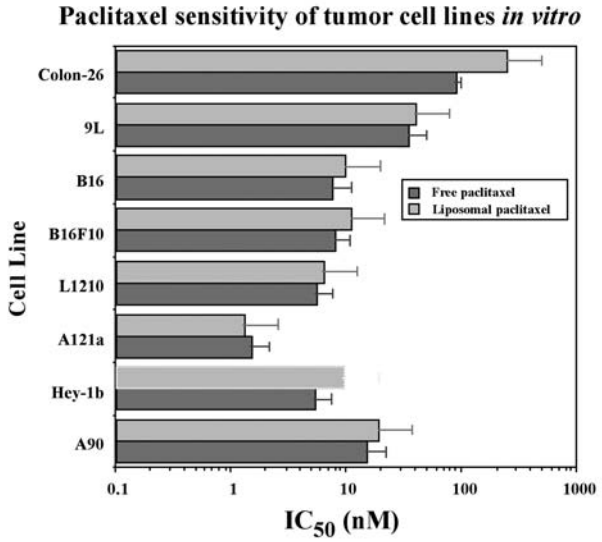

Figure 3. *Paclitaxel sensitivity of various cell lines. Cell lines in culture were treated with a range of paclitaxel concentrations for 72h, and the change in cell number was determined. The concentration inhibiting cell growth by 50% (IC_{50}) was calculated from the data. Colon 26: murine colon carcinoma; 9L: rat glioblastoma-like line; B16/B16F10: murine melanoma; L1210: murine leukemia; A121a, Hey-1b, A90: human ovarian carcinomas. Solid bars: cells were exposed to paclitaxel adsorbed to serum albumin; hatched bars: paclitaxel was incorporated at 3 mole% (relative to lipids) in liposomes of phosphatidylcholine:phosphaditylglycerol (9:1 mol:mol). Data plotted from results reported in (50).*

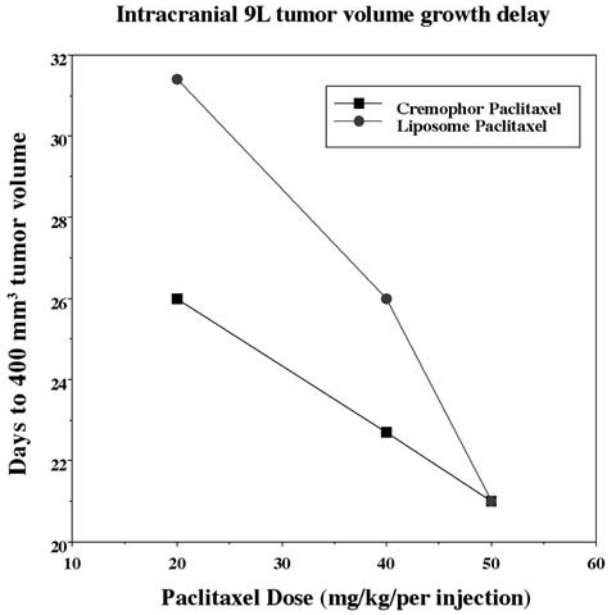

Figure 4. *Intracranial 9L tumor volume progression. Animals bearing intracranial 9L tumors were observed by Magnetic Resonance Imaging repetitively during treatment, and tumor volume was calculated at each time point from the 3D image set using Analyze (CN Software LTD, West Sussex, UK). Tumor progression was plotted as a function of time, and the data presented here show the extrapolation of the tumor growth rate to a volume of 400 mm^3, at which time animals appear symptomatic. Animals received paclitaxel in Cremophor EL or in liposomes composed of phosphatidylcholine:phosphatidylglycerol (9:1 mol:mol) according to these doses and schedules: a single 50 mg/kg bolus given at day 8 after tumor implantation, 40 mg/kg at day 8 and 15, and 20 mg/kg at day 8, 11 and 15. The effect of treatment on tumor volume was statistically significant for all treatments except the 50 mg/kg groups, for which tumor volume was no different than control. Data plotted from results reported in (47).*

solubility of paclitaxel, the clinical formulation Taxol® contains the organic cosolvents ethanol and polyethoxylated castor oil (Cremophor EL) in a 1:1 volume ratio. Cremophor EL has been shown to cause toxic effects such as life-threatening anaphylaxis (14-16). High doses of antihistamines and glucocorticoids are administered to manage these adverse effects (17, 18), but these co-administered drugs have raised the possibility of additional pharmacokinetic and pharmacodynamic interactions with paclitaxel. The Cremophor EL vehicle also exerts a range of effects on the biodistribution of the drug (19-22), modulating multidrug resistance through the P-glycoprotein efflux system and contributing to the nonlinear pharmacokinetics of paclitaxel.

Paclitaxel shows activity on a wide range of human and animal brain tumor lines (23, 24), including *in vivo* activity on human gliomas xenografted in nude mice (25, 26). However, it shows little clinical activity in malignant glioma (27-30). Such a finding may be rationalized by limited CNS penetration (31, 32) and frequent resistance of brain tumors to paclitaxel (33).

Paclitaxel interferes with cell cycle progression by interfering with microtubule dynamics; a series of events ensue that result in cell death (34, 35). Cells blocked at G2/M by paclitaxel are most sensitive to radiation (36), suggesting utility as a radiation sensitizer (37, 38). Paclitaxel also possesses strong antiangiogenic activity at low concentration and may synergize with antiangiogenic agents such as TNP-470 (39, 40).

Incorporation of paclitaxel in liposomes has been achieved, and this not only eliminates the hypersensitivity reactions associated with the Cremophor EL vehicle, but also reduces drug toxicity to critical normal tissues (12, 41-44). The antitumor potency in a variety of model systems equals or slightly exceeds that of the clinical Taxol® formulation (12, 25, 26, 41-43, 45). Drug is released from the liposomal particle comparatively rapidly, based on pharmacokinetics in blood that are similar to the Cremophor EL-based formulation (43, 46). However, drug release is not instantaneous, based on the liposome-mediated alterations in therapeutic index and the drug biodistribution.

In a number of model systems, a substantial elevation of the maximum tolerated dose (MTD) has been observed as a result of liposome-mediated reduction of the dose-limiting toxicity of paclitaxel. The impact of these changes on the therapeutic index are striking; in a paclitaxel-resistant colon tumor model (12), no dose of paclitaxel administered in the Cremophor EL-based formulation had an effect on tumor growth, up to and including high doses that caused delayed (*i.e.*, non-vehicle-related) lethality in 100% of the animals. In contrast, paclitaxel in liposomes arrested tumor growth and did so at doses that would be lethal to 100% of animals if administered in Cremophor EL (12).

Preservation of drug antitumor activity in the face of reduced toxicity to non-target tissues is a hallmark of delayed-release formulations, and we undertook to investigate the potential of paclitaxel-containing liposomes in a rat model for drug-resistant intracranial brain tumors (47). We chose as a tumor target the intracranial 9L tumor cell line, which is moderately drug-resistant (48, 49) and displays several characteristics of authentic human brain tumors. Figure 3 compares the paclitaxel sensitivity of 9L brain tumor cells *in vitro* to other tumor cell lines in our laboratory. The IC_{50} (concentration inhibiting cell growth by 50%) for 9L is approx. 40 nM, considerably higher than for the most sensitive tumor lines we have tested (2 nM for the A121a human ovarian carcinoma), but lower than the most paclitaxel-resistant line we have used (100 nM for the murine Colon-26 carcinoma) (50).

Intracranial 9L model tumors were initiated by stereotaxic injection of $4x10^4$ cells (in approx. 4 μL) into a specific location in the caudate putamen region of Fisher 344 rats. Treatment was initiated at day 8 after tumor implantation, at which time the tumor is well established and vascularized, based on histological analysis. The dosing regimens included a single 50 mg/kg bolus given at day 8 after tumor implantation, 40 mg/kg at days 8 and 15, and 20 mg/kg at days 8, 11 and 15. Each treatment was given by tail vein injection. Neutrophil counts and body weight were measured to evaluate treatment toxicity. The therapeutic effect of the drug was determined by survival time of tumor-bearing animals and tumor volumes were determined noninvasively by magnetic resonance (MR) imaging.

Liposomal paclitaxel at a dose of 20 mg/kg x3 doses conferred the greatest increase in median lifespan (approx 40% greater than for saline-treated animals), and the equivalent dose and schedule of paclitaxel in Cremophor EL resulted in nearly a 10% reduction in median lifespan (data not shown; (47)). This is the greatest extension of lifespan we have observed with this advanced tumor model (49).

Tumor progression was observed noninvasively by repetitively acquiring T2-weighted proton spin echo images (TR/TE=2000/120 ms) for individual rats during treatment.

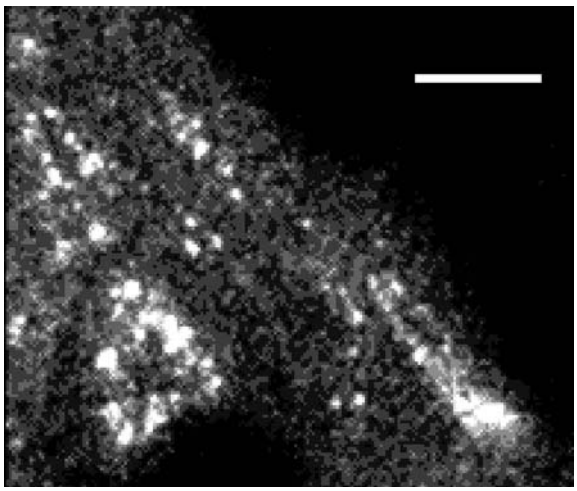

Figure 5. *Deposition of liposomes in 9L brain tumors. 9L tumor cells were implanted stereotaxically in rat brains. Liposomes of distearoylphosphatidylcholine:cholesterol:polyethyleneglycol-modified distearoylphosphatidylethanolamine (DSPC:Chol:PEG-DSPE; 9:5:1 mole ratio) were labeled with 1 mole% of the fluorescent phospholipid analog Rhodamine-DPPE (dipalmitoylphosphatidylethanolamine), and injected intravenously. Rats were sacrificed 24 hours later and frozen sections (<10 μm thick) were imaged using a laser scanning confocal microscope. Data was processed using NIH_Image (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Panel shows projection of fluorescence in 13 sequential optical sections into a single plane. Dark regions correspond to areas of normal brain tissue. Accretions of punctate fluorescence surround blood vessels. Bar: 20 μm. Reprinted with permission from (49).*

Tumor volumes were computed using an image processing tool (Analyze, CN Software LTD, West Sussex, UK), and plotted as a function of time (47); from these plots, a rate of tumor progression was calculated by regression analysis. The growth rate was extrapolated to a volume of 400 mm^3, the volume that appeared to correlate with the onset of symptoms and impending death (Figure 4). Significant tumor growth delay (compared to saline controls) was observed for all treatment groups except those treated with 50 mg/kg (paclitaxel liposomes or free drug). At each of the other dose levels, the greatest effect was observed for groups treated with liposomes (Figure 4).

The tumor progression results are consistent with the greater extension of lifespan mediated by paclitaxel liposomes. However, the observation of significant tumor growth delay mediated by the Cremophor EL-based formulation, in the absence of an extension in survival, suggests that the doses necessary to achieve any retardation of 9L tumor growth are toxic to the animal. In contrast, liposome-based formulations mediated both a retardation in tumor growth and an increase in lifespan.

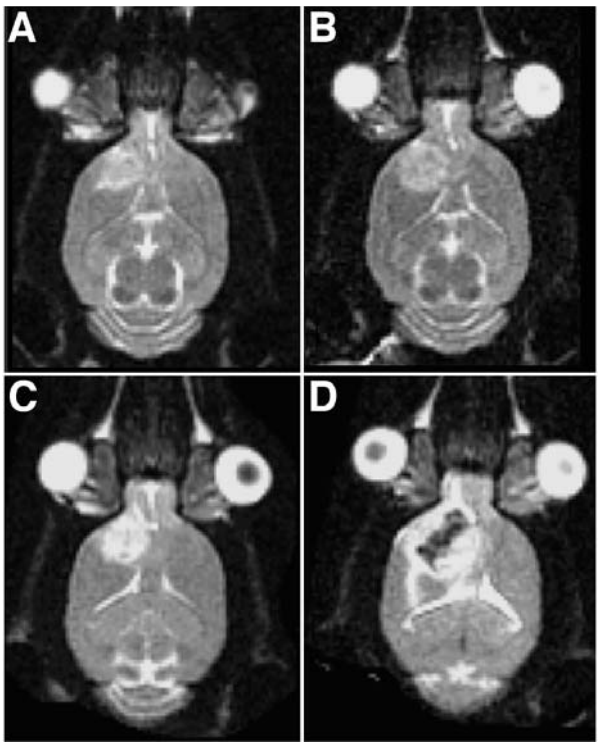

Figure 6. *MR images of advanced 9L brain tumors before and after treatment with DXR. T2-weighted spin echo MR images of rat brain were acquired 24 h before (A,C) and 48 h after (B,D) the second of two weekly injections of saline, DXR, or DXR encapsulated in sterically stabilized liposomes (SSL-DXR). (A) Control rat 24 h before and (B) 48 h after saline injection (day 16 after tumor implantation). (C) Representative image of a rat treated 6 days prior with 1 injection of 5.67 mg/kg L-DXR; image was acquired on day 13 after tumor implantation, 24 h before treatment with second injection of L-DXR; (D) image acquired from same animal as in (C) except 48 h after treatment with 5.67 mg/kg L-DXR (day 16 after tumor implantation). The parameters for the T2-weighted spin echo images are as follows: (TR/TE= 2000/120 ms, slice thickness = 1 mm). Reprinted with permission from (67).*

Recent work by others indicates that repetitive dosing with taxanes can increase drug penetration into tumors by reducing cell density (51, 52) and microvascular/interstitial fluid pressures, resulting in the dilation and re-opening of collapsed vessels (53). These effects, coupled with observations of antiangiogenic activity of the taxanes (39, 40, 54), suggests the potential for liposomal forms of this drug in combination with antiangiogenic therapies (54).

Doxorubicin-containing liposomes. Most liposome compositions are cleared relatively rapidly by the liver and spleen (tissues of the reticuloendothelial system), limiting their utility as drug carriers. However, the development of sterically stabilized "Stealth" liposomes (SSL), which bear hydrated polymers such as PEG (polyethylene glycol) on their external surface (55, 56), represents a major advancement in the field. SSL show markedly altered pharmacokinetics (*i.e.*, increased circulation time and decreased release rate) and improved therapeutic efficacy. An SSL formulation containing doxorubicin (DXR) in a semisolid state within the liposome core (57-60) is now a clinically-approved product under the names Doxil® or Caelix®. This drug:liposome formulation is highly stable (Figure 2), and drug deposition reflects liposome deposition, providing an opportunity to target drug to specific sites such as tumors. Highly stable liposome-encapsulated anthracyclines thus represent a new class of therapeutic entity, with pharmacology altered significantly from that of the parent drug (61, 62).

We have examined the antitumor efficacy of doxorubicin in sterically stabilized liposomes (SSL-DXR) in the 9L advanced tumor model (49). Treatment was administered starting 7 days after tumor implantation, as described above for paclitaxel liposomes. SSL-DXR mediated a substantial (30%) increase in median lifespan, whereas free DXR was ineffective in prolonging lifespan, and appeared to accelerate death due to toxicity.

Given the previous observations that SSL-DXR mediate large increases in tumor deposition of drug (63), presumably through flaws in the tumor vasculature (64, 65), one interpretation is that liposomes may extend survival by providing a localized, intratumor sustained-release depot. We examined intratumor deposition of SSL in rats bearing advanced intracranial 9L brain tumors (49), using liposomes that were labeled with a fluorescent phospholipid analog. Fluorescence was distributed non-uniformly and sporadically within the tumor 24 h after injection (Figure 5). Regions of normal brain in close proximity to the tumor were devoid of fluorescence. Confocal imaging allowed optical sectioning of tissue to a depth of approx. 20 μm in these experiments. Stereo projections of the optical slices enabled the visualization of intense fluorescence accretions lining tumor capillaries or blood vessels (Figure 4), but little spread of liposomes within tumor. Such non-uniform deposition of liposomes raises the possibility that some regions of the tumor may be under-dosed.

Our most recent work suggests an alternative to the drug-depot hypothesis for explaining enhanced antitumor efficacy of SSL-DXR. Tumor growth and the effects of therapy with free DXR or SSL-DXR were observed noninvasively in rats bearing advanced intracranial 9L tumors by using repetitive MR imaging (66, 67). We observed that the repetitive dosing scheme which mediated the maximal extension of median lifespan (49) also mediated drastic changes in the tumor, as observed by MR imaging. Two days after a second weekly dose of SSL-DXR, a large, hypointense region was observed in the tumors of animals treated with SSL-DXR (Figure 6D). No such changes were observed in animals treated with the

equivalent regimen of free DXR or saline (Figure 6B), or in animals treated with only one dose (Figure 6C). Histological examination of the brain tumors (67) revealed extensive regions of microhemorrhage, and confirmed that the hypointense regions appearing in MR images resulted from the extravasation and breakdown of erythrocytes within the tumor (not shown). Thus we hypothesize that extravasation of drug-loaded particulate carriers such as liposomes can deposit large doses of drug in the few hyperpermeable vascular regions of naïve tumor. There, the drug may exert localized cytotoxic effects on either the vascular endothelium or nearby tumor cells. As a result, the endothelium may be denuded, or the underlying tumor cells killed. Either of these effects may open the tumor stroma by reduction of the cell density (68, 69) and could result in localized collapse of the tumor vasculature. This sporadic, localized damage could open larger areas of the vasculature to subsequent doses of liposomes, with each cycle of treatment expanding the hyperpermeable areas, thereby increasing the penetration of the next dose.

Conclusion

Particulate carriers such as liposomes provide unique opportunities to improve tumor therapy, either as formulation aids for poorly-soluble compounds, as delayed-release vehicles for modulating pharmacokinetics, or as stable drug/carrier complexes that may be targeted to tumors through regions of hyperpermeable vasculature. Clinically-approved formulations such as sterically-stabilized doxorubicin-containing liposomes represent the first in a new class of therapeutic agent that may enable the selective targeting of tumors. The antivascular effects discussed here suggest a novel mechanism of action which may be exploited to enhance the penetration and deposition of subsequent doses or of other therapeutic agents. The repair processes resulting from such antivascular effects may involve angiogenic activities, and therefore combination therapy with antiangiogenic agents may further enhance therapeutic effect.

Other formulations that are under development, such as those containing paclitaxel, also may find clinical application owing to the observed reduction in toxicity to critical normal tissues. The newly-recognized antiangiogenic action of the taxanes, which may be enhanced by sustained delivery of low drug concentrations, suggests an additional mechanism by which liposomes may enhance therapy. Overall, the observations discussed here suggest that combination of multiple carrier-based therapies, involving an initial permeability-enhancing sequence of treatments and followed by treatment with antiangiogenic or cytotoxic agents, may be a means to enhance the efficacy of treatment for difficult tumor targets.

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