Kupffer Cells Do Not Play a Role in Governing the Efficacy of Liposomal Mitoxantrone Used to Treat a Tumor Model Designed to Assess Drug Delivery to Liver

Howard J. Lim, Michael J. Parr, Dana Masin, Natasha L. McIntosh, Thomas D. Madden, Guoyang Zhang, Sharon Johnstone, and Marcel B. Bally

ABSTRACT

A tumor model designed to assess liposome-mediated drug delivery to liver has been used in an attempt to better understand the mechanism of activity of liposomal mitoxantrone, a liposomal anticancer drug formulation that appears to be uniquely effective in treating this tumor model. Reductions in liposomal mitoxantrone accumulation in the liver were achieved either by use of poly(ethylene)glycol (PEG)-modified lipids or by methods designed to deplete liver phagocytes, a method referred to as hepatic mononuclear phagocytic system (MPS) blockade. A 2-fold reduction in mitoxantrone delivery to the liver was obtained using a mitoxantrone formulation with PEG-modified lipids, and a 3-fold reduction was obtained when liposomal mitoxantrone was given to animals pretreated to induce hepatic MPS blockade. Results demonstrate that the liposomal mitoxantrone formulation prepared with PEG-modified lipids was significantly less active than the formulations that did not contain PEG lipids, with median survival times of 17 days and 100% 60-day survival, respectively. In contrast, hepatic MPS blockade had no effect on the therapeutic activity of 1,2-dimyristoyl phosphatidylcholine/cholesterol (DMPC/Chol) mitoxantrone (100% 60-day survival). These data suggest that the hepatic MPS does not play a role in mediating the therapeutic activity of DMPC/Chol mitoxantrone in the treatment of liver localized disease. Results with formulations prepared with a PEG-stabilized surface, however, suggest that nonspecific methods to decrease liposome cell interactions inhibit the therapeutic activity of DMPC/Chol mitoxantrone.

INTRODUCTION

Liposomes can increase the therapeutic efficacy of anticancer drugs, notably the anthracycline doxorubicin (1, 2) and the Vinca alkaloid, vincristine (3, 4). Allegedly, this improved therapy is achieved by increasing drug exposure at the site of the tumor. Evidence to support this has come from studies documenting that the maximum drug concentration achieved in a region of tumor growth is increased when an anticancer drug is administered inside an appropriately designed liposomal carrier (5–8). In addition, these high drug concentrations in regions of tumor growth are maintained over an extended length of time. This improvement in tumor delivery with liposome-encapsulated drugs has been attributed to extended circulation longevity (5) and the presence of blood vessels in the vicinity of tumors that are hyperpermeable to circulating macromolecules (9–11). Tumor drug levels are, however, low in comparison with those that can be obtained in the liver after parenteral administration of a liposomal anticancer drug.

Liposomes have a tendency to localize in sites that contain fenestrated blood vessels and high levels of tissue-associated macrophages, such as the liver (12–14). Investigators have shown that liver drug exposure can be at least 5-fold greater with liposomal drug compared with free drug (15). Higher drug levels and increased exposure of the liver would imply that liposomal anticancer drugs should be well suited for treatment of liver cancer. This has, however, not been demonstrated.

There are many possible explanations for why liposomal anticancer drugs have not been more successful in treating liver cancer. These include: (a) an inherent insensitivity or resistance to cytotoxic drugs in tumor cells that arise in or metastasize to the liver (16); (b) liver phagocytic cell uptake of the liposomal drug and subsequent inactivation of the agent; and (c) vascular density in liver-localized disease may be lower than in other extrahepatic sites (17) and may exhibit an altered vascular permeability to circulating macromolecules that is dependent on the tumor microenvironment (18). The latter two points emphasize that the regional and cellular distribution of the drug may be critical for therapeutic activity against liver neoplasms.
In this study, we evaluated the influence of hepatic MPS avoidance and blockade strategies on the activity of a DMPC/Chol mitoxantrone formulation. The basis for this study rests on three observations: (a) we demonstrate that the efficacy of DMPC/Chol mitoxantrone is exceptional in treatment of the liver-localized L1210 tumor, even when compared with liposomal doxorubicin or liposomal vincristine; (b) previous studies have shown that liposomal vincristine and liposomal doxorubicin are very effective when given i.v. to treat animals with i.p. L1210 tumors (2, 4) but ineffective when used to treat animals with tumors after i.v. administration of L1210 cells; (c) the most significant difference between liposomal formulations of vincristine, doxorubicin, and mitoxantrone is that the vincristine and doxorubicin formulations induce hepatic MPS blockade (3, 19, 20). It is important to note that we are not attempting to suggest that the i.v. L1210 tumor model is a relevant model of liver cancer; rather, we use this model as a tool to gain a better understanding of the role of drug delivery to the liver in controlling the therapeutic activity of liposomal mitoxantrone. Two strategies designed to decrease liposomal delivery to the liver were used. The first included PEG-modified lipids in the liposomes to prevent recognition and uptake by the mononuclear cell phagocytic system (21). The second method used drug-loaded (nontherapeutic) liposomes to eliminate or impair Kupffer cells of the liver (19, 20, 22). The results suggest that the therapeutic activity of liposomal mitoxantrone used to treat liver-localized cancer is not dependent on the presence of Kupffer cells. However, strategies that nonspecifically inhibit liposome-cell interactions (e.g., use of liposomes with PEG-modified lipids) significantly inhibit the therapeutic activity of DMPC/Chol liposomal mitoxantrone.

MATERIALS AND METHODS

Materials. Novantrone (mitoxantrone hydrochloride) was obtained from the British Columbia Cancer Agency and is a product of Wyeth Ayerst Canada (Montreal, Quebec, Canada). 1,2-Clodronate (dichloromethylene-bisphosphonate) was generously donated by Boehringer Mannheim. DSPC was purchased from Northern Lipids (Vancouver, British Columbia, Canada). DMPC and 1,2-distearoyl phosphatidylethanolamine-PED 2000 were purchased from Avanti Polar Lipids (Alabaster, AL). DiI was purchased from Molecular Probes (Eugene, OR). MTT, HEPES, collagenase, citric acid, and CHOL were purchased from Sigma Chemical Co. (St. Louis, MO). Dibasic sodium phosphate was obtained from Fisher Scientific (Fair Lawn, NJ). [3H]CDE, a lipid marker that is not exchanged or metabolized in vivo (23), was purchased from Amersham (Oakville, Ontario, Canada). [3H]Mitoxantrone used as tracer was generously provided by the American Cyanamid Company (Montreal, Quebec, Canada). Pico-Fluor 40 scintillation fluid was purchased from Canberra-Packard (Meriden, CT). Solvable was obtained from NEN Research Products (DuPont Canada, Mississauga, Ontario, Canada). OCT was purchased from Sakura Finetek (Torrance, CA). F4/80 antibody and FITC-conjugated goat-antirat antibodies were purchased from Serotec (Cedarlane, Mississauga, Ontario, Canada). The L1210 tumor cell line was originally purchased from the National Cancer Institute tumor repository (Bethesda, MD), and cells were obtained from ascites fluid generated weekly by passage in BDF1 mice. Cells were used for experiments between the third and twentieth passage. Female CD1, DBA2, and BDF1 mice (8–10 weeks of age) were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). RPMI 1640 was purchased from Stem Cell Technologies (Vancouver, British Columbia, Canada). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT).

Preparation of Liposomes. DSPC/Chol (55:45; mol/mol), DMPC/Chol (55:45; mol/mol), DMPC/Chol/DSPC-PED 2000 (50:45:5; mol/mol/mol), and DMPC/Chol/DiI (DiI was added at a ratio of 0.4 mg to 100 mg of DMPC/Chol 55:45) liposomes were prepared with a Lipex Extruder (Lipex Biomembranes, Inc., Vancouver, British Columbia) using established extrusion technology (24). Briefly, phospholipid and cholesterol at the indicated mole ratios were dissolved in chloroform with [3H]CDE added as lipid tracer (23). Lipids were dried under nitrogen and then under vacuum. The resultant lipid film was hydrated to a concentration of 100 mg of lipid/ml in 300 mM citric acid buffer (pH 4.0). The multimamellar vesicle mixture was frozen and thawed five times (25) and then extruded through three stacked 100-nm polycarbonate filters (Nuclepore, Pleasanton, CA). Large unilamellar vesicles generated had a mean diameter of 100–120 nm as determined by quasielastic light scattering using a Nicomp 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA) operating at 632.8 nm.

Transmembrane pH Gradient Loading. Vincristine, mitoxantrone, and doxorubicin were encapsulated using transmembrane pH gradient-driven loading procedures. Vincristine and mitoxantrone were added, at a final drug:lipid weight ratio of 0.1, to liposomes that had been preincubated at 65°C for 10 min (26–28). The pH gradient was generated by raising the external pH to 7.2 by the addition of 350 μl of 0.5 M NaHPO4 for each 1.0 ml of drug/liposome mixture. Encapsulation efficiency after a 15-min incubation at 65°C was ~95% for both vincristine and mitoxantrone. Liposomes for plasma elimination and liver accumulation studies were generated with [3H]Mitoxantrone added as a marker.

To encapsulate doxorubicin, the pH gradient was generated by addition of 0.5 M sodium carbonate (to a final external pH of 7.8–8.0) to liposomes with an interior pH of 4.0 (300 mM citrate buffer; Ref. 29). Doxorubicin, solubilized in HBS, and liposomes were preheated at 65°C for 2 min prior to being combined at a doxorubicin:lipid weight ratio of 0.2:1. The mixture was vortexed for 2–3 min at 65°C and then maintained at this temperature for an additional 10 min to complete the drug loading. Liposomal doxorubicin preparations were diluted with saline prior to in vivo administration.

Preparation of EPC/Chol Clodronate Liposomes. Clodronate liposomes were prepared as outlined by van Rooijen et

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3 The abbreviations used are: MPS, mononuclear phagocytic system; DSPC, 1,2-distearoyl phosphatidylcholine; DMPC, 1,2-dimyristoyl phosphatidylcholine; EPC, egg phosphatidylcholine; DSPE-PED 2000, distearoylphosphatidylethanolamine-poly(ethylene)glycol 2000; Chol, cholesterol; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocya- nine perchlorate; CDE, cholesteryl hexadecyl ether; ILS, increased life span; IC50, 5% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
al. (30) with minor modifications. The EPC/Chol (11:2 mol/mol) mixture was prepared in chloroform and dried down first under nitrogen and then under vacuum for 3 h. The EPC/Chol film was hydrated in 5 ml of clodronate (2 mg/ml) and then subjected to five freeze-thaw cycles to increase encapsulation efficiency (25). The resulting solution was centrifuged at 30,000 × g for 20 min. The liposomes were recovered in the pellet and then resuspended in PBS and centrifuged at 20,000 × g for 30 min four times to remove any unencapsulated clodronate. The clodronate multilamellar vesicles were resuspended in 4 ml of PBS.

MTT Assay. A modified MTT cytotoxicity assay (31) was used to measure the IC50 of mitoxantrone, doxorubicin, and vincristine on L1210 cells. Briefly, L1210 cells were obtained through in vivo cultivation in the mouse peritoneum. Cells were collected from the ascitic fluid into EDTA-containing tube cells and then separated from lymphocytes and RBCs by Ficoll-Hypaque density gradient centrifugation. L1012 cells were collected and washed in RPMI 1640 containing 10% fetal bovine serum three times and then transferred to a T75 culture flask. Cells were incubated for 4 h at 37°C in a humidified incubator with 5% CO2, at which time nonadherent cells were collected. Cells were maintained in culture for 24 h prior to use in cytotoxicity studies. Cells were seeded at 104 cells/well in 96-well, flat-bottomed Costar culture plates (Cambridge, MA) in a volume of 100 μl. Drug was then added to a final volume of 200 μl/well. Cells were incubated for 24 h prior to addition of 50 μl of 1 mg/ml MTT to each well. After 4 h, plates were centrifuged at 1800 rpm for 15 min, the medium was removed, and the assay was developed by addition of 150 μl of DMSO. The absorbance at 570 nm, measured with a Titertek Multiskan plate reader (Flow Laboratories, Mississauga, Ontario, Canada), was used to compare relative viability of treated cells to untreated cells. Each assay was performed in triplicate and replicated at least three times. The IC50, the concentration of drug giving 50% of the viability of untreated cells, was determined for mitoxantrone, doxorubicin, and vincristine.

Tumor Model. In our previous studies, therapeutically active liposomal formulations of mitoxantrone for the treatment of liver-localized disease were described (32, 33). The tumor model used in this study was generated by i.v. administration of L1210 cells into immune-competent BDF1 mice (F1 DBA2/C57-BL6 crosses) or DBA2 mice. Two mouse strains were used in these studies because they were conducted by two groups of investigators, one that worked with DBA mice and the other with the BDF1 crosses. The control data obtained using the two strains had no therapeutic activity (ILS) at the doses administered.

Efficacy of Liposomal Mitoxantrone in the i.v. L1210 Tumor Model. Twenty-four h after tumor cell inoculation of female BDF1, or DBA2 mice, animals were given the specified drug dose in a volume of 200 μl. To assess the impact of hepatic MPS blockade on the therapeutic activity of DMPC/Chol mitoxantrone, mice were injected i.v. with either DSPC/Chol doxorubicin (2 mg/kg drug), DSPC/Chol vincristine (1 mg/kg drug), or EPC/Chol clodronate 2 h after tumor cell inoculation. Agents used to blockade the hepatic MPS had no therapeutic activity (ILS) at the doses administered.

Plasma Elimination and Biodistribution Studies. Female CD1 mice (20–25 g, four mice/group) received injections via the lateral tail vein with a single dose of 10 mg/kg DMPC/Chol mitoxantrone or DMPC/Chol/DSPC-PEG mitoxantrone. When hepatic MPS blockade was used to alter the plasma elimination and biodistribution of DMPC/Chol liposomal mitoxantrone, animals were injected i.v. with a 2-mg/kg drug dose of DSPC/Chol doxorubicin 24 h prior to injection of the DMPC/Chol mitoxantrone (10 mg/kg lipid dose). At 1 and 4 h, 25 μl of blood were collected from the tail vein into EDTA-coated microcapillary tubes. Blood was mixed with 250 μl of 5% EDTA and centrifuged for 15 min at 500 × g. The supernatant was reserved, and the pellet was washed once by resuspending in HBSS (250 μl) and then centrifuging at 500 × g. The two supernatants were pooled, and the radioactivity in the sample ([3H]CDE and [14C]mitoxantrone) was determined using a Packard 1900 liquid scintillation counter. Mice were terminated by CO2 asphyxiation 24 h after injection of liposomal mitoxantrone, and whole blood was collected via cardiac puncture into EDTA-coated Microtainer tubes. The blood was centrifuged at 500 × g for 10 min, and plasma radioactivity was assessed by scintillation counting.

Isolated, saline washed livers were weighed and then frozen at −70°C. To measure liver drug levels, distilled water was added to concentration of 10% (w/v), and tissue was minced with a Polytron tissue homogenizer (Kinematica, Lucerne, Switzerland). A 200-μl aliquot of the homogenate was mixed with 500 μl of Solvable and incubated at 50°C for 3 h. This was then cooled to room temperature, and 50 μl of 200 μl EDTA, 200 μl of 30% H2O2, and 25 μl of 10 N HCl were added. Five ml of scintillation fluid were added to the samples, and radioactivity ([3H]CDE and [14C]mitoxantrone tracer) was determined by liquid scintillation counting.

Hepatocyte Isolation. Hepatocytes were extracted from female CD1 mice as described by Klaunig et al. (37), with slight modification. Mice were terminated via CO2 asphyxiation, and livers were harvested and kept in ice-cold HBSS. The livers
were finely minced using two scalpel blades and then transferred to a 15-ml culture tube. HBSS was added to final volume of 5 ml. Three hundred μl of collagenase (4 mg/ml) were then added to the solution and incubated on a rotating tube rack at 37°C for 30 min. The cell suspension was then strained through a 40-μm nylon filter, and 40 ml of HBSS were added. This was spun for 1 min at 500	. The cell suspension was then translated through a 40-μm nylon filter, and 40 ml of HBSS were added. This was spun for 1 min at 500	. The cell suspension was then translated through a 40-μm nylon filter, and 40 ml of HBSS were added. This was spun for 1 min at 500	. The cell suspension was then translated through a 40-μm nylon filter, and 40 ml of HBSS were added. This was spun for 1 min at 500	. The cell suspension was then translated through a 40-μm nylon filter, and 40 ml of HBSS were added. This was spun for 1 min at 500	. 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The image was captured to yield xyz dimensions 0.4 μm cubed (×20) and 0.2-μm pixel (×60). Image analysis was performed with NIH Image version 1.61, and all images were based on maximum intensity projection. Projections were saved in TIFF format and then imported and merged in Adobe Photoshop version 4.0 to generate the final image. Identical settings on the confocal microscope and identical processing times were used to facilitate comparison of DI fluorescent intensity.

### Statistical Analysis

ANOVA was performed on the results obtained after administration of the two liposomal formulations and free mitoxantrone. Common time points were compared using the Post Hoc Comparison of Means, Scheffé test. Differences were considered significant at $P < 0.05$. Therapeutic effect was considered to be significant when the ILS was >25%.

### RESULTS

**Therapeutic Activity of Free and Liposomal Anticancer Drugs Given i.v. to Mice Bearing the L1210 i.v. Tumor Model.**

The L1210 i.v. tumor model was used to evaluate the efficacy of mitoxantrone, vincristine, and doxorubicin administered i.v. in free form or encapsulated in liposomes. The results in Table 1 were obtained after a single injection at a drug dose that was either the maximum tolerated dose (free and DSPC/Chol vincristine; free and DSPC/Chol mitoxantrone, EPC/Chol doxorubicin) or at the lowest drug dose required to give maximum therapeutic effect (free and DSPC/Chol doxorubicin and DMPC/Chol mitoxantrone). Untreated animals (both BDF1 mice and DBA mice) and animals treated with empty liposomes (EPC/Chol or DSPC/Chol liposomes with encapsulated citrate buffer and pH 7.5 HBS outside and administered at a lipid dose of 150 mg/kg total lipid) were terminated as a result of significant tumor related disease within 10 days. The most significant point made from the data in Table 1 is that the therapeutic influences of liposomal mitoxantrone and drug delivery to liver.
activity of DMPC/Chol liposomal mitoxantrone (~80% of the 32 animals treated at 10 mg/kg drug survived beyond day 60) is unequalled by the other drugs.

Several parameters in Table 1 should be considered when reviewing these data. Two mouse strains were used, as a consequence of studies being completed at two different experimental locations. For this reason, no attempt has been made to statistically compare data obtained in one mouse strain to data obtained in the other strain. The control (saline-treated) data are summarized individually for each mouse strain, and the %ILS was determined in comparison with the appropriate control group. It is also important to note that the studies summarized in Table 1 included liposomes selected for comparable drug release rates and/or optimal therapeutic activity. DMPC/Chol mitoxantrone has a higher drug release rate than DSPC/Chol mitoxantrone (33). The EPC/Chol doxorubicin formulation (6, 38) and the DSPC/Chol liposomal vincristine (4) have also been characterized as formulations that support release of entrapped contents after i.v. administration. When doxorubicin was encapsulated in DMPC/Chol liposomes, the drug was four to five times more toxic than free drug and less active (29); therefore, a comparison of DMPC/Chol mitoxantrone to doxorubicin encapsulated in DMPC/Chol liposomes was not completed. Vincristine encapsulated in liposomes that rapidly release drug, such as EPC/Chol or DSPC/Chol, was no more active than free vincristine (4); therefore, this formulation was not included in this study. Furthermore, as indicated in Table 2, we believe that the in vivo studies have not been biased by use of drugs that exhibit broad differences in cytotoxic/cytostatic activity. These results suggest that isolated L1210 cells were most sensitive to free mitoxantrone relative to doxorubicin and vincristine; however, the IC\textsubscript{50}s of mitoxantrone and vincristine were comparable.

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC\textsubscript{50} (nm)\textsuperscript{a}</th>
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<tr>
<td>Doxorubicin</td>
<td>820</td>
</tr>
<tr>
<td>Vincristine</td>
<td>70</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>55</td>
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\textsuperscript{a}IC\textsubscript{50} is defined as the concentration of drug where cell growth and/or viability is 50% of that observed in control (drug) free cultures in the MTT assay.

Reducing DMPC/Chol Liposomal Mitoxantrone Delivery to the Liver. To investigate the role of mitoxantrone accumulation in the liver, two methods (PEG-modified lipid incorporation and hepatic MPS blockade) were used to effect reductions in the liposomal mitoxantrone delivery to the liver. Both strategies caused a decrease in the rate of liposomal lipid (Fig. 1A) and mitoxantrone (Fig. 1B) elimination from the plasma compartment, and this correlated with decreased drug accumulation in the liver (Fig. 2A). For example, 24 h after i.v. administration of DMPC/Chol mitoxantrone, the level of mitoxantrone measured (determined with [\textsuperscript{14}C]mitoxantrone as a marker) in the liver was 27 µg/g of liver; however, administered in DMPC/Chol liposomes with 5 mol% PEG 2000-modified lipids, the drug level in the liver at 24 h was reduced to 12.2 µg/g of liver (P < 0.01). After hepatic MPS blockade, the level of mitoxantrone 24 h after administration of DMPC/Chol mitoxantrone was <8 µg/g of liver (P < 0.01). The >2-fold reduction in liver mitoxantrone levels measured at 24 h was associated with an approximately 3- and 5-fold increase in plasma concentrations of drug and liposomal lipid, respectively. The plasma elimination rates over the first 24 h after administration were comparable for the PEG-containing liposomes and the DMPC/Chol mitoxantrone formulations given to mice after hepatic MPS blockade. Results from these plasma elimination studies were comparable when using mice that received i.v. injections previously of L1210 cells (results not shown). This was not unexpected, considering it has been established in previous studies (33) that there are no significant histological changes in the liver and spleen of mice given i.v. tumors until 4 days after cell administration. We have not determined whether the presence of established tumor (day 4 or later after cell injection) effects the plasma elimination or tissue distribution of the liposomal drugs.

Influence of Reducing Liver Mitoxantrone Levels on the Therapeutic Activity of DMPC/Chol Mitoxantrone. Fig. 2B illustrates how the two strategies for reducing DMPC/Chol mitoxantrone delivery to the liver affected its therapeutic activity against the L1210 i.v. tumor model. The results obtained...
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were surprising. Incorporation of PEG 2000-modified lipids into the DMPC/Chol mitoxantrone resulted in a significant reduction in the therapeutic activity. In dramatic contrast, the reduction in liver accumulation resulting from pretreatment with DSPC/Chol doxorubicin had no impact on the therapeutic activity of the DMPC/Chol mitoxantrone formulation.

In an effort to explain this phenomenon, additional MPS blockade methods were used to effect decreases in liposomal mitoxantrone delivery to the liver. Specifically, mice were given liposomal doxorubicin at a dose of 2 mg/kg to deplete liver macrophage cells. Although this formulation had minimal activity when used to treat the L1210 i.v. tumor model at doses of 30 mg/kg (Table 1) and the activity was not detectable at doses of <20 mg/kg, the activity of this formulation could be increased if it worked synergistically with mitoxantrone. For this reason, hepatic MPS blockade was also induced with two other agents, liposomal vincristine and liposomal clodronate. Although vincristine is also an anticancer agent, its mechanism of activity is distinct from doxorubicin, and liposomal vincristine is also not active against the L1210 i.v. tumor model (see Table 1). Clodronate is a bisphosphonate used for treatment of osteoporosis (39, 40) and is known to eliminate macrophages when given in liposomal form (22, 41).

The results presented in Table 3 are unambiguous: (a) hepatic MPS blockade achieved by pretreating animals with liposomal doxorubicin, vincristine, or clodronate had no impact on the median survival time of mice bearing the i.v. L1210 tumors; (b) regardless of what agent was used to achieve hepatic MPS blockade, mice treated with DMPC/Chol mitoxantrone exhibited 100% long-term (>60 day) survival; and (c) hepatic MPS blockade, by any of the pretreatment strategies, did not affect the therapeutic activity of the DMPC/Chol/PEG mitoxantrone formulation.

**Influence of Hepatic MPS Avoidance and Blockade Strategies on Mitoxantrone Release.** The therapeutic activity of liposomal mitoxantrone markedly correlated with the rate of release of mitoxantrone from liposomes after administration (33); therefore, it was important to determine whether the hepatic MPS avoidance and blockade strategies affected drug release rates. As shown in Fig. 3, there was a significantly higher drug:lipid ratio observed 24 h after injection of DMPC/Chol/PEG mitoxantrone in comparison to DMPC/Chol mitoxantrone, suggesting that drug release was inhibited in liposomes with the PEG-modified lipid. This was surprising considering results with other drugs, such as vincristine, suggest drug release rates increases when the liposomes used contain PEG-modified lipids (3). As expected, hepatic MPS blockade had no effect on drug release from the DMPC/Chol liposomes (Fig. 3).

**Influence of Hepatic MPS Avoidance and Blockade Strategies on Liposome Distribution in the Liver and on Kupffer Cell Depletion.** Confirmation that the hepatic MPS blockade strategies did deplete Kupffer cells is provided in the micrographs shown in Fig. 4. These micrographs were obtained by staining liver cryosections with an antibody (F4/80) toward mature macrophages (42–44). Sections from livers of untreated mice (Fig. 4A) are strongly F4/80 positive, denoting the presence of cells presumed to be liver Kupffer cells. The population of labeled cells was significantly reduced in liver sections obtained from mice after MPS blockade (Fig. 4, B and C). The reduction in F4/80-positive cells was most significant when hepatic blockade was achieved with liposomal clodronate. The observed macrophage depletion presented in Fig. 4 is consistent with other reports (45).

To examine how macrophage blockade and macrophage avoidance (PEG-liposomes) impact the distribution of liposomal mitoxantrone in the liver, two approaches were taken; fluorescently labeled lipid was used to determine the distribution pattern of liposomes in the liver, and radioactively labeled mitoxantrone was used to quantify drug levels in hepatocytes isolated from treated animals. DMPC/Chol or DMPC/Chol/PEG mitoxantrone formulations were prepared with the fluorescent lipid DiI. This fluorescent lipid does not exchange with neighboring membranes (46, 47) and thus is considered as a useful marker for liposomes in vivo. Twenty-four h after i.v. administr-
Table 3 Influence of PEG-lipid incorporation and hepatic MPS blockade on the L1210 antitumor activity of DMPC/Chol mitoxantrone

<table>
<thead>
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<th>Pretreatment</th>
<th>Treatment</th>
<th>Median survival time</th>
<th>%ILS</th>
<th>% survival</th>
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<tr>
<td>None</td>
<td>Untreated</td>
<td>9.5</td>
<td>0</td>
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<td>Doxorubicin blockade</td>
<td>DMPC/Chol mitoxantrone</td>
<td>&gt;60 days</td>
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<td>DMPC/Chol/PEG mitoxantrone</td>
<td>17</td>
<td>79</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>DMPC/Chol mitoxantrone</td>
<td>20</td>
<td>111</td>
<td>0</td>
</tr>
<tr>
<td>Doxorubicin blockade</td>
<td>DMPC/Chol/PEG mitoxantrone</td>
<td>15</td>
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<td>0</td>
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<tr>
<td>Vincristine blockade</td>
<td>DMPC/Chol/PEG mitoxantrone</td>
<td>18.5</td>
<td>94</td>
<td>0</td>
</tr>
</tbody>
</table>

* Pretreatment was administered two h after tumor cell inoculation.
* Treatment dose was at drug dose of 10 mg/kg at a drug:lipid ratio of 0.1 (wt:wt).
* Percentage increase in life span (ILS) values were determined from median survival times of treated and untreated control groups.
* Cannot be determined because more than half the animals survived past 60 days.

dil-labeled DMPC/Chol mitoxantrone and DMPC/Chol/PEG mitoxantrone (10 mg/kg drug dose), livers were removed, and cryosections were prepared for viewing using confocal microscopy. As seen in Fig. 5, incorporation of PEG-modified lipids caused a reduction in Dil accumulation in the liver (Fig. 5, compare A with B). Changes in the distribution of Dil-labeled DMPC/Chol mitoxantrone in the liver were more dramatic in livers isolated from mice after hepatic MPS blockade (Fig. 6). Hepatic MPS blockade significantly reduced the amount of fluorescently labeled DMPC/Chol mitoxantrone delivered to the liver (Fig. 6, compare A with B–D). Note that in addition to the decreased liposome accumulation, the liposome distribution pattern was changed when comparing the effect of hepatic MPS blockade induced by liposomal doxorubicin (Fig. 6B) and vincristine (Fig. 6C) to that induced by liposomal clodronate (Fig. 6D). After hepatic MPS blockade with liposomal doxorubicin and vincristine, Dil-labeled DMPC/Chol mitoxantrone was distributed in discrete patches. Numerous vacuoles were seen in the micrographs of livers from liposomal vincristine pretreated mice, possibly a consequence of autophagocytic vacuoles induced by vincristine in hepatocytes (48). The distribution pattern observed in animals pretreated with liposomal clodronate (Fig. 6D) was comparable with that of animals that did not undergo pretreatment, except that in the former there was less Dil label present.

Mitoxantrone delivery to liver hepatocytes was measured in an effort to resolve differences between the DMPC/Chol mitoxantrone efficacy in the presence of hepatic MPS blockade with formulations prepared with PEG-modified lipids. The level of mitoxantrone (14C-labeled) was measured in isolated hepatocytes, and drug levels were standardized to cell number. It should be noted that hepatocyte drug levels may be attributable, in part, to drug that was taken up during the hepatocyte isolation procedure. It was anticipated on the basis of the data presented in Fig. 4 that the 90% reduction in Kupffer cells would significantly increase hepatocyte drug levels. As shown in Fig. 7, this was not the case. Hepatic MPS blockade effected a 2-fold reduction in hepatocyte delivery, a reduction that was comparable with that observed in the whole liver (Fig. 2A). When hepatocyte mitoxantrone levels were determined in animals given i.v. DMPC/Chol/PEG mitoxantrone, the values also decreased by a factor of 2. The differences in the antitumor activity of DMPC/Chol liposomal mitoxantrone attributable to PEG-lipid incorporation or hepatic MPS blockade cannot be attributed to altered drug delivery to hepatocytes. We did not undertake studies to determine drug delivery to cells in the livers of tumor-bearing animals for two reasons. At the time point used (24 h after drug injection and 48 h after L1210 cell injection), the presence of L1210 cells in the liver could not be determined histologically. Although attempts to identify L1210 cells in cell suspensions generated from tumor-bearing animals by flow cytometry were initiated, a procedure that could selectively label the L1210 cells could not be defined. Tumor cell delivery is an important aspect of studies such as these; however, drug delivery to the tumor cells within the liver needs to be completed using a model that is different from that described here.
DISCUSSION

The observation that the i.v. L1210 tumor model was exquisitely sensitive to DMPC/Chol mitoxantrone provided an opportunity to investigate the role of drug accumulation in liver in governing therapeutic activity. There are two very simple conclusions that can be made on the basis of the data presented in this study: (a) Kupffer cells do not play a role in governing the therapeutic activity of DMPC/Chol liposomal mitoxantrone; and (b) incorporation of PEG-modified lipids significantly inhibits the therapeutic activity of DMPC/Chol liposomal mitoxantrone. The question that needs to be addressed on the basis of these conclusions is equally simple: why should one strategy designed to reduce drug accumulation by liposomes be correlated with increased therapeutic activity has been demonstrated by previous investigators (5). However, liposomal anticancer drugs have not been as effective in the treatment of liver cancer models, suggesting something unique about the liver as a target. This has been attributed to liver drug metabolism and detoxification of drugs (48–50) and to inherent drug resistance of colon cancer and hepatocellular carcinomas (51). The later is perhaps not an issue in this murine liver tumor model, because L1210 cells are quite sensitive to the drugs selected (see Table 2). However, drug metabolism may be a critical factor. For instance, although in vitro cytotoxicity assay results suggest that L1210 cells are ~10-fold less sensitive to doxorubicin than to mitoxantrone, free doxorubicin and liposomal doxorubicin were quite effective in treating animals bearing i.v. L1210 tumors in the peritoneal cavity (2). In contrast, the L1210 cells localized in liver were less responsive to these drugs. Differences in drug metabolism, in the liver and elsewhere, may account for the different therapeutic sensitivities of results, whether a result of PEG-lipids or hepatic MPS blockade. The three assumptions, in retrospect, seem quite naïve.

The first assumption that improved drug delivery by liposomes is correlated with increased therapeutic activity has been demonstrated by previous investigators (5). However, liposomal anticancer drugs have not been as effective in the treatment of liver cancer models, suggesting something unique about the liver as a target. This has been attributed to liver drug metabolism and detoxification of drugs (48–50) and to inherent drug resistance of colon cancer and hepatocellular carcinomas (51).
L1210 cells to doxorubicin in vitro and in various in vivo disease models.

A previous study suggested that Kupffer cells play a role in processing liposomal anticancer drugs (52), releasing drugs back into the systemic circulation and/or locally in the liver. Although this hypothesis was developed using liposomal doxorubicin, it was not known at that time that this drug caused elimination of Kupffer cells. This led us to speculate that the absence of Kupffer cells and lack of processing by these cells was the reason why liposomal formulations of doxorubicin and vincristine were not active in the treatment of liver localized disease. Conversely, we anticipated that liposomal mitoxantrone activity is attributable, in part, to Kupffer cell processing. The data presented in Fig. 2B and Table 3 demonstrate clearly that the therapeutic activity of liposomal mitoxantrone was not affected under conditions where Kupffer cells were eliminated. A compensatory increase in hepatocyte drug accumulation was also not observed (Fig. 6), and therefore the preservation of DMPC/Chol mitoxantrone anticancer activity is not attributable to a redistribution of drug in the liver.

There are evidently attributes of mitoxantrone that may make it better suited for treatment of the liver-localized L1210 cells than, for instance, doxorubicin. Although we have shown that Kupffer cells are not essential to the cytotoxic activity of mitoxantrone, a functional cytochrome P-450-dependent mixed function oxidase is necessary (53) to generate a mitoxantrone metabolite that is the effector of cytotoxicity (54).

The importance of mitoxantrone metabolism could also be used to explain differences between the DMPC/Chol and the DMPC/Chol/PEG formulations, in the presence and absence of hepatic MPS blockade. Although liver accumulation of mitoxantrone was reduced by hepatic MPS blockade, cell internalization and processing by different liver cell populations likely contributes to the activity of DMPC/Chol mitoxantrone. Several types of liver cells, for example, may be responsible for removal of particles from the blood compartment in the absence of Kupffer cells, including sinusoidal endothelial monocytes or monocyte-derived macrophage precursors in liver (56–61). If these cells are important in terms of regulating the therapeutic activity of the PEG-free systems, then reduced therapeutic activity of DMPC/Chol/PEG mitoxantrone may be attributable to inhibition of liposome-cell interactions by the surface-grafted PEG (21). Inhibition of cell binding by PEG was observed, even when targeting ligands were attached to the liposomes (62), and if cell binding is obtained, the presence of PEG-modified lipids may prevent endocytosis (63).

We also cannot entirely eliminate the possibility that the reduced activity of DMPC/Chol/PEG mitoxantrone was attrib-

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Fig. 6 Confocal imaging of biodistribution of DiI-labeled DMPC/Chol mitoxantrone liposomes with and without MPS blockade. MPS blockaded mice were pretreated with a 2 mg/kg drug dose of DSPC/Chol doxorubicin or 1 mg/kg DSPC/Chol vincristine or with EPC/Clodronate. Control mice were untreated. Twenty-four h later, mice received injections of a 10 mg/kg drug dose of DiI-labeled DMPC/Chol mitoxantrone. Twenty-four h after this injection, mice were terminated via CO2 asphyxiation, and livers were collected and processed as outlined in “Materials and Methods.” Images were obtained using a Bio-Rad 6000Z Confocal Imaging System at ×20. A, representative image from a control mouse. B, representative image from a mouse with hepatic MPS blockade generated using DSPC/Chol doxorubicin. C, representative image from a mouse with MPS blockade achieved using DSPC/Chol vincristine. D, representative image from a mouse with MPS blockade generated using EPC/Chol clodronate. ×20.
Drug delivery to hepatocytes. Female CD1 mice received injections of a 10-mg/kg drug dose of DMPC/Chol mitoxantrone (A) or DMPC/Chol/PEG mitoxantrone (D). Hepatic MPS blockaded mice were pretreated with either DSPC/Chol doxorubicin (B) or EPC/Chol clorozonate (C). Twenty-four h later, mice were treated with a 10-mg/kg drug dose of DMPC/Chol mitoxantrone. Livers were extracted, and hepatocytes were isolated as described in “Materials and Methods.” Lipid and drug concentrations were assessed via scintillation counting for $^3$H and $^{14}$C. Columns, averages; bars, SE.

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Kupffer Cells Do Not Play a Role in Governing the Efficacy of Liposomal Mitoxantrone Used to Treat a Tumor Model Designed to Assess Drug Delivery to Liver

Howard J. Lim, Michael J. Parr, Dana Masin, et al.


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