Anti-CD19-Targeted Liposomal Doxorubicin Improves the Therapeutic Efficacy in Murine B-Cell Lymphoma and Ameliorates the Toxicity of Liposomes with Varying Drug Release Rates

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Abstract

Some formulations of liposomal doxorubicin with intermediate rates of drug release have shown increased levels of toxicity in mice. Because antibody-mediated targeting of liposomal drugs influences the pharmacokinetics, mechanism of uptake, and selectivity of the associated drugs, we hypothesized that anti-CD19-mediated targeting of liposomal doxorubicin might moderate the toxicity of the problem formulations. Pharmacokinetic and biodistribution studies and in vivo drug release rates were determined in mice using liposomes dual labeled with [3H]cholesteryl hexadecylether and [14C]doxorubicin. Therapeutic studies were done in xenograft models of human B lymphoma (Namalwa cells). The rate of clearance of the liposomal lipid was similar for all formulations (average t1/2, 18 hours), but the rate of clearance of doxorubicin was dependent on the release rate of the formulation (t1/2, 2-315 hours). Liposomes with the slowest drug release rates showed no toxicity and exhibited therapeutic activity that was superior to the other formulations when targeted with anti-CD19; liposomes with the most rapid drug release rates also showed no toxicity but showed little therapeutic effect even when targeted. Liposomes with intermediate drug release rates exhibited varying degrees of toxicity. The toxicities could be reduced and even overcome by targeting with anti-CD19 antibodies. For these formulations, therapeutic effects were intermediate between those found for liposomes with the fastest and slowest drug release rates.

Doxorubicin is an anthracycline antibiotic that is extensively used in anticancer therapy. An important dose-limiting toxicity for the drug is acute and chronic cardiomyopathy (1). Liposomal encapsulation of doxorubicin leads to decreased clearance, increased area under the time versus concentration curves (AUC), and other altered pharmacokinetic properties (2). A long circulating (Stealth), slow release formulation of doxorubicin (Doxil/Caelyx) has been approved for clinical use in the treatment of Kaposi’s sarcoma, ovarian cancer, and breast cancer (3–5). Liposomal formulations of doxorubicin that are in clinical use (i.e., Doxil/Caelyx and Myocet) result in reductions in cardiac toxicity because liposome encapsulation reduces doxorubicin levels in the heart (6, 7). Liposomal delivery of doxorubicin also leads to greater accumulation of the drug in solid tumors via the enhanced permeability and retention effect (8).

We have shown previously that nontargeted formulations of liposomal doxorubicin, having slow rates of drug release, showed superior therapeutic effects in the treatment of orthotopic models of murine breast cancer than liposomes with more rapid release rates (9). In these experiments, liposomes with intermediate drug release rates resulted in some unexpected deaths in mice, identified tentatively as due to gastrointestinal toxicities by pathologic examination. Variations in toxicity with different formulations of doxorubicin has also been observed by other researchers (10). We hypothesize that it may be possible to overcome toxicities associated with intermediate doxorubicin release rates by antibody-mediated targeting of the liposomes.

A suitable animal model for these studies is anti-CD19-targeted liposomal doxorubicin for the treatment of xenograft models of human B lymphoma (11, 12). B cells, including B lymphoma cells, such as the Namalwa cell line, express the CD19 antigen on their cell surface (13). Binding of anti-CD19-targeted liposomes to the target cells triggers the rapid receptor-mediated internalization of the liposome-drug package into the target cell interior; the binding and internalization of the liposomes to B cells increases their rate of clearance from...
the circulation (14). This mechanism works well for drugs like doxorubicin that escape degradation by lysosomal enzymes. Free doxorubicin has a large volume of distribution (15) and liposomal doxorubicin that is released more rapidly into the circulation may lead to redistribution of the released drug to sensitive tissues like the heart or gastrointestinal tract, resulting in toxicities (16). For targeted liposomes, rapid binding and internalization of the liposomal doxorubicin by malignant B cells in circulation may, we hypothesize, reduce the amount of free drug released into circulation, particularly for liposomes having more rapid release rates. This would in turn reduce the redistribution of doxorubicin to sensitive tissues, leading to reductions in drug toxicities for these formulations.

Liposomal formulations were prepared with lipids having several different drug release profiles. The pharmacokinetics of the lipid and the drug components of the liposomal formulations were determined in vitro and in vivo, and the therapeutic activity of the nontargeted versus anti-CD19-targeted formulations was compared in severe combined immunodeficient (SCID) mice inoculated with human B-cell lymphoma (Namalwa) cells.

Materials and Methods

Materials. Dioleoyl phosphatidylcholine (DOPC), dipalmityl phosphatidylcholine (DPPC), palmitoyl-myristoyl phosphatidylcholine (PMPC), palmitoyl-oleoyl phosphatidylcholine (POPC), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Methoxy-polyethylene glycol (mPEG; M$_{r}$ 2,000) covalently linked via a carbamate bond to distearoyl phosphatidylethanolamine (DSPE), hydrogenated soy phosphatidylcholine (HSPC), and doxorubicin were generous gifts from ALZA Pharmaceuticals, Inc. (Mountain View, CA). Maleimide-derivatized PEG-DSPSE was custom synthesized by Shearwater Polymers, Inc. (Huntsville, AL). Nucleopore polycarbonate membranes (pore sizes, 0.2, 0.1, and 0.08 μm) were purchased from Northern Lipids (Vancouver, British Columbia, Canada). RPMI 1640 (without phenol red), penicillin-streptomycin, fetal bovine serum, and adult bovine serum (ABS) were purchased from Life Technologies, Inc. (Burlington, Ontario, Canada). 2-Iminothiolane (Traut’s reagent) was obtained from Sigma Chemical Co. (St. Louis, MO). Slide-a-Lyzer dialysis cassettes (M$_{r}$ cutoff of 10,000) and buoys were purchased from Pierce (Rockford, IL). The Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). 2-Iminothiolane (Traut’s reagent) was obtained from Sigma Chemical Co. (St. Louis, MO). Slide-a-Lyzer dialysis cassettes (M$_{r}$ cutoff of 10,000) and buoys were purchased from Pierce (Rockford, IL). The Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Sephadex G-50, Sepharose CL-4B, aqueous counting scintillant, and [14C]doxorubicin (1.85-2.29 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Baie d’Urfe, Quebec, Canada). Cholesteryl hexadecylether ([3H]CHE; 1.48-2.22 TBq/mmol), Solvable, and Ultima Gold were purchased from Perkin-Elmer Biosciences (Boston, MA). All other chemicals were of analytic grade purity or the highest available purity.

Animals, cell culture, and antibodies. Female 6- to 8-week-old CB.17 SCID mice were purchased from Taconic Farms (Germantown, NY) and housed in the virus antigen–free unit of the University Animal Services, University of Alberta (Alberta, Edmonton, Canada). The Health Sciences Animal Policy and Welfare Committee of the University of Alberta approved all experiments, which were in accordance with the Guidance to the Care and Use of Experimental Animals set forth by the Canadian Council on Animal Care.

The human Burkitt’s lymphoma cell line Namalwa (ATCC CRL 1432) was purchased from American Type Culture Collection (Manassas, VA). Suspension cultures of the cells were grown at 37°C in a humidified 5% CO$_2$ atmosphere in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, penicillin G (50 units/mL), streptomycin sulfate (50 μg/mL), and glucose. Only cells in the exponential phase of cell growth were used in experiments. Murine monoclonal antibody (mAb) anti-CD19 was produced from the FMC63 murine hybridoma cells (17) purified as described previously (18). [3H]-labeled antibody was used as a tracer to measure coupling efficiencies and to determine the amount of mAb attached to the liposomes as described previously (11).

Preparation of liposomes. Nontargeted liposomes were prepared with various phosphatidylcholines, cholesterol, and mPEG-DSPSE at a 2:1:0.1 molar ratio to phospholipid. The phosphatidylcholines were DOPC, POPC, DPPC, PMPC, and HSPC. All liposomes were prepared by hydration of thin lipid films as described previously and extruded to mean diameters in the range of 100 ± 10 nm as determined by laser light scattering (11). Doxorubicin was loaded into liposomes using the ammonium sulfate loading method (19). Targeted liposomes were composed of HSPC/cholesterol/mPEG-DSPSE/maleimide-derivatized PEG-DSPSE at a 2:1:0:0.08:0.02 molar ratio were prepared by a direct coupling technique. Anti-CD19 mAb was coupled to the terminus of the maleimide-derivatized PEG-DSPSE at 2.000:1 (lipid/protein) molar ratio using the coupling procedure described previously (14). Briefly, mAb (10 mg/mL) was incubated with 2-iminothiolane in O$_2$-free HEPES-buffered saline (pH 8.0) at a ratio of 20:1 mol/mol for 1 hour at room temperature to thiolate the amino groups. At the end of the incubation, the sample was chromatographed on a Sephadex G-50 column, equilibrated with O$_2$-free HEPES-buffered saline (pH 7.4), and immediately incubated with liposomes in an O$_2$-free environment overnight with continuous stirring. To reduce loss of contents during coupling, targeted formulations of liposomes containing DOPC, POPC, DPPC, or PMPC (i.e., those with higher doxorubicin release rates) were prepared using the post-insertion method (20). All liposomes were radiolabeled with [14C]CHE as a lipid tracer. To assess coupling efficiency of the antibodies, a trace amount of [3H]-labeled anti-CD19 was added to the unlabeled antibody before thiolation (11). For animal experiments, liposomes were exchanged into pyrogen-free HEPES-buffered saline by chromatography down Sephadex CL-4B columns and were sterile filtered before injection. Sephadex CL-4B column chromatography also served to remove any uncoupled mAb from the liposomes.

Antibody-coupled micelles for the postinsertion method were prepared from maleimide-derivatized PEG-DSPSE and mPEG-DSPSE at a 4:1 molar ratio. The dried lipid films were hydrated immediately before coupling to a concentration of 10 mmol/L in deoxygenated 25 mmol/L HEPES (pH 7.4) by heating in a 65°C water bath. Antibodies were coupled to the PEG termini of micelles as described previously (18). After overnight coupling, the micelles were incubated with preformed liposomes at a molar ratio of 0.05:1 (micelle lipid/liposomal lipid) for 1 hour at 60°C. Following transfer, the lipid/micelle mixture was cooled and chromatographed over a Sepharose CL-4B column and equilibrated with pyrogen-free HEPES-buffered saline (pH 7.4) to separate the immunoliposomes from PEG micelles and free antibody.

Leakage assays, pharmacokinetics, and biodistribution. The release rate of doxorubicin from the various liposomal formulations was determined in vitro in 50% ABS. Liposomes (0.5 mmol/L) containing trace amounts of [14C]doxorubicin were diluted in ABS (1:1 by volume), loaded in a dialysis cassette (M$_{r}$ cutoff of 10,000), and dialyzed against 50% ABS in HEPES-buffered saline at 37°C. At different time points, aliquots were counted for [14C]doxorubicin. Results are expressed as t$_{1/2}$ (time in which 50% of drug leak out from liposomes).

For determining in vivo leakage rates and for pharmacokinetic and biodistribution experiments, liposomes were prepared using [3H]CHE as a lipid label and [14C]doxorubicin as a drug label. Naive SCID mice (three mice per time point) were injected with liposomal formulations of doxorubicin at 3 mg/kg doxorubicin, the same drug dose used in the therapeutic studies. At selected time points, mice were euthanized by cervical dislocation. Whole blood (100 μL) was collected via cardiac puncture with a heparinized syringe. The liver, kidney, spleen, and heart were dissected out, weighed, and then dissolved in Solvable (1.0 mL). For blood, an equal volume of Solvable (100 μL) was added to the whole blood. The tissues were then digested for 3 hours at 50°C, with the exception of blood, which was digested for 1 hour. After the vials had cooled to room temperature, 50 μL of 200 mmol/L EDTA were added and the samples were bleached with 1,200 μL of 30% (v/v)
H₂O₂. Ultima Gold (5 mL) was added to the samples and they were left overnight in the dark at room temperature. The samples were counted in a Beckman LS 6500 liquid scintillation counter for ³H and ¹⁴C counts. Results are expressed as percentage of injected doxorubicin or lipid concentration present in blood or organs at each time point. To provide an estimate of drug leakage in vivo, the ratio of [¹⁴C]doxorubicin drug counts to [²H]CHE lipid counts was normalized to 1 at time 0. The rate of decrease of the drug/lipid ratio at subsequent time points indicates the rate of release of the drug from the liposomes. Pharmacokinetic variables for blood were calculated in WinNonLin version 4.1 (Pharsight, Cary, NC) using model 201 (i.v. bolus input) for blood and model 200 (extravascular input) for the heart.

In vivo survival experiments. Nalmlaw cells (5 × 10⁶) in 0.2 mL PBS were injected i.v. in the tail vein of SCID mice (8-18 mice per group). Treatments were given at 24 hours after tumor inoculation as a single bolus i.v. dose of 3 mg/kg doxorubicin (20 µmol/kg phospholipid; 40-60 µg CD19/µmol phospholipid) as free doxorubicin, untargeted doxorubicin liposomes, or anti-CD19 doxorubicin liposomes. Mice were monitored daily and euthanized when they lost weight and looked scrawny or when they developed hind leg paralysis.

Results

In vitro and in vivo doxorubicin release characteristics, pharmacokinetics, and biodistribution. Studies evaluating in vitro drug release, by dialysis at 37°C against 50% ABS, from the various formulations of liposomal doxorubicin are shown in Table 1. Liposomes prepared from phosphatidylcholines with unsaturated or shorter fatty acyl chains exhibited increased drug leakage rates (decreased t½) compared with liposomes with longer chain, saturated fatty acyl chains. t½ in vitro ranged from 11.2 to 85.5 hours with the rank order HSPC > DPPC > PMPC > DOPC > POPC.

In vivo pharmacokinetics and biodistribution for the various liposome formulations were determined in naïve SCID mice as a function of time after injection of 3 mg doxorubicin/kg mouse weight in the form of liposomal doxorubicin (10.4-13.6 mg/kg phospholipid). Radiolabeling of both the drug ([¹⁴C]doxorubicin) and the liposomal lipid ([³H]CHE) also allowed in vivo doxorubicin release rates to be determined from the ratios of drug/lipid at each time point, normalized to 1.0 at time 0 (Fig. 1). Clearance of lipid from blood was similar for all formulations, whereas clearance of doxorubicin from blood increased with increased rates of doxorubicin release, with doxorubicin in DOPC liposomes having the most rapid clearance (Fig. 2). The t½ for doxorubicin release in vivo, calculated from the normalized lipid/drug ratios, ranged from 1.9 to 315 hours (Table 1) and, with the exception of DOPC-containing liposomes, were slower than the in vitro release rates. The rank order of release was also similar to the in vitro results, again with the exception of DOPC-containing liposomes. The t½ for the clearance of liposomal lipid from blood were similar for all of the formulations, ranging from 15.7 to 20.6 hours, with a tendency for slightly faster clearance rates for liposomes that were composed of unsaturated phospholipids (e.g., DOPC).

![Graph showing drug/lipid ratios](image)

**Table 1.** Pharmacokinetic variables of doxorubicin liposomes

<table>
<thead>
<tr>
<th>Phospholipid component in doxorubicin-loaded liposomes</th>
<th>HSPC</th>
<th>DPPC</th>
<th>PMPC</th>
<th>POPC</th>
<th>DOPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin release in vitro (t½, h)</td>
<td>85.5</td>
<td>43.6</td>
<td>22.1</td>
<td>11.2</td>
<td>13.6</td>
</tr>
<tr>
<td>Doxorubicin release in vivo (t½, h)</td>
<td>315.0</td>
<td>125.0</td>
<td>65.0</td>
<td>40.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Phospholipid clearance (t½, h)</td>
<td>20.6</td>
<td>19.1</td>
<td>17.6</td>
<td>17.7</td>
<td>15.7</td>
</tr>
<tr>
<td>Doxorubicin clearance (t½, h)</td>
<td>19.2</td>
<td>16.9</td>
<td>14.4</td>
<td>13.1</td>
<td>7.6</td>
</tr>
<tr>
<td>Phospholipid AUC (blood, mg h/kg)</td>
<td>318.4</td>
<td>229.0</td>
<td>239.6</td>
<td>233.0</td>
<td>182.0</td>
</tr>
<tr>
<td>Doxorubicin AUC (blood, mg h/kg)</td>
<td>60.0</td>
<td>56.5</td>
<td>40.4</td>
<td>48.8</td>
<td>19.5</td>
</tr>
<tr>
<td>Phospholipid AUC (heart, mg h/kg)</td>
<td>9.07</td>
<td>5.58</td>
<td>6.41</td>
<td>6.17</td>
<td>5.50</td>
</tr>
</tbody>
</table>

NOTE: Doxorubicin liposomes composed of phosphatidylcholine/cholesterol/mPEG-DSPE (2:1:0.1), where phosphatidylcholine was HSPC, DOPC, POPC, PMPC, or DPPC, were radiolabeled with [²H]CHE and [¹⁴C]doxorubicin. In vitro doxorubicin release rates were determined in ABS. In vivo release rates were determined from the lipid/drug ratios after injecting naïve SCID mice (three mice per time point) with 3 mg/kg liposomal doxorubicin. At selected time points, mice were euthanized, whole blood or tissues were analyzed for radioactivity, and in vivo leakage and pharmacokinetic variables were calculated.
when the liposomes have higher release rates drug-depleted liposomes are in the circulation.

The AUC\(_{0-48}\) varied very little for the lipid component of the various formulations (182-318 mg \(h/\)kg), with the lowest AUC\(_{0-48}\) found in liposomes composed of DOPC. The plasma AUC for the drug component decreased as the release rate of doxorubicin from the formulations increased (Table 1). HSPC liposomes had the highest doxorubicin AUC (60 mg \(h/\)kg), whereas DOPC liposomes had the lowest AUC (19.5 mg \(h/\)kg).

The biodistribution of liposomes in naïve SCID mice showed that the concentration of lipid in the liver was very similar for all formulations and reached maximal levels at 24 hours for all formulations, except DOPC, which was still increasing at 48 hours (Fig. 3). The highest liver accumulation of liposomal doxorubicin at 0.5 hour postinjection was found for liposomes containing HSPC liposomes, whereas DOPC liposomes had the lowest AUC (19.5 mg \(h/\)kg).

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Because the heart is a site of both acute and cumulative dose-limiting doxorubicin toxicity, liposomal lipid and doxorubicin levels in the heart were measured. Table 1 shows that the AUC for doxorubicin in the heart varied relatively little, with DOPC liposomes being the lowest (1.5 mg \(h/\)kg) and HSPC liposomes the highest (2.1 mg \(h/\)kg). The AUC for lipid showed a similar trend. The levels of uptake of lipid and drug into the heart as a function of time is shown in Fig. 4. Although the highest heart levels of both phospholipid and drug were found for HSPC liposomes likely due to drug that was still associated with liposomes, there were not large differences between any of the formulations.

### In vivo survival studies in xenograft model of human B-cell lymphoma

The results of pooled \textit{in vivo} survival experiments in a xenograft model of human B-cell lymphoma in SCID mice were used to examine differences in the therapeutic effects of the liposomal formulations with respect to toxicity, drug release rates, and targeting (Table 2). Liposomes with the fastest (DOPC) and slowest (HSPC) release rates lacked toxicity. However, formulations with intermediate drug release rates showed varying degrees of toxicity with PMPC > DPPC > POPC. Premature deaths were observed in animals treated with nontargeted PMPC and DPPC liposomes (Table 2) and necropsy showed congestion of organs with blood. Animals treated with nontargeted POPC liposomes showed less toxicity, losing up to 20% of their weight between days 7 and 13, but no premature deaths were observed. Fatal toxicity had been observed in our previous studies with nontargeted POPC...
liposomes, so in this study mice receiving this treatment were carefully observed (9). The premature deaths in the PMPC and DPPC groups were not related to lipid toxicity because drug-free formulations of PMPC and DPPC liposomes showed no toxicity, nor did empty liposomes given together with free doxorubicin cause toxicity in the mice (Table 2).

Nontargeted formulations containing HSPC, DOPC, and POPC resulted in increased life spans that were not different than free doxorubicin. When formulations containing these phosphatidylcholines were targeted with anti-CD19 mAbs, they all resulted in a significant increase in life span (P < 0.01 for DOPC and P < 0.0001 for POPC and HSPC). No signs of toxicity were observed in mice receiving targeted POPC-containing formulations. For the nontargeted DPPC formulation, which caused premature deaths due to toxicity, targeting with anti-CD19 increased the survival of mice to levels that were significantly higher than free doxorubicin (P < 0.0001) and no weight loss was observed. The life span increased by 3 days in mice receiving targeted versus nontargeted PMPC-containing liposomes (P < 0.0001). Therefore, anti-CD19 targeting decreased the toxicity of the formulations in the order POPC ≥ DPPC > PMPC. Anti-CD19-targeted HSPC liposomes with the slowest doxorubicin release rates resulted in the greatest increase life span (ILS), whereas anti-CD19-targeted DOPC liposomes with the fastest release rates resulted in the smallest ILS. The ILS for targeted formulations of POPC and DPPC was similar and intermediate between the ILS for HSPC and DOPC.

### Table 2. Survival of SCID mice bearing B-cell lymphoma after treatment with various formulations of targeted versus nontargeted liposomal doxorubicin

<table>
<thead>
<tr>
<th>Formulation</th>
<th>No. animals</th>
<th>Mean survival time (d)</th>
<th>ILS (%)</th>
<th>Long-term survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>18</td>
<td>26 ± 1</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Free doxorubicin</td>
<td>18</td>
<td>29 ± 2</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Anti-CD19-PMPC[doxorubicin]</td>
<td>13</td>
<td>10 ± 1</td>
<td>—62</td>
<td></td>
</tr>
<tr>
<td>PMPC[doxorubicin]</td>
<td>13</td>
<td>7 ± 0</td>
<td>—73</td>
<td></td>
</tr>
<tr>
<td>Anti-CD19-DPPC[doxorubicin]</td>
<td>10</td>
<td>38 ± 6</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>DPPC[doxorubicin]</td>
<td>13</td>
<td>9 ± 1</td>
<td>—65</td>
<td></td>
</tr>
<tr>
<td>Anti-CD19-POPC[doxorubicin]</td>
<td>10</td>
<td>29 ± 3</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Anti-CD19-DOPC[doxorubicin]</td>
<td>10</td>
<td>31 ± 3</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>DOPC[doxorubicin]</td>
<td>18</td>
<td>28 ± 2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Anti-CD19-HSPC[doxorubicin]</td>
<td>18</td>
<td>47 ± 15</td>
<td>88</td>
<td>1</td>
</tr>
<tr>
<td>HSPC[doxorubicin]</td>
<td>18</td>
<td>29 ± 3</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>PMPC (empty)</td>
<td>8</td>
<td>26 ± 0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DPPC (empty)</td>
<td>8</td>
<td>27 ± 2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>PMPC (empty) + free doxorubicin</td>
<td>8</td>
<td>27 ± 2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>DPPC (empty) + free doxorubicin</td>
<td>8</td>
<td>25 ± 1</td>
<td>—4</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** SCID mice (two to three replicate groups of six to eight mice per group) were injected i.v. with 5 × 10⁶ Namalwa cells in 0.2 mL PBS, and 24 hours post-inoculation, the animals were injected with the following treatments: saline (control); free doxorubicin; nontargeted liposomal doxorubicin composed of phosphatidylcholine/cholesterol/mPEG-DSPE (2:1:0.1), where the phosphatidylcholine component was HSPC, DPPC, PMPC, POPC, or DOPC; or anti-CD19-targeted liposomal doxorubicin composed of phosphatidylcholine/cholesterol/mPEG-DSPE/maleimide-derivatized PEG-DSPE (2:1:0.08:0.02), where the phosphatidylcholine component was the same as above. The concentration of anti-CD19 on the liposomes was between 54 and 78 A²g mAb/A mol phospholipid. All doxorubicin formulations were given at a doxorubicin dose of 3 mg/kg. The mean survival time, ILS, and long-term survivors (90 days) were calculated.

### Discussion

Toxic side effects of doxorubicin include acute and chronic cardiotoxicity, myelosuppression, and gastrointestinal toxicities (21, 22). Commercial liposomal drug delivery systems, such as Doxil/Caelyx and Myocet, have improved the side effect profile of doxorubicin, particularly by reducing its acute and chronic cardiotoxicity (7, 23). Interestingly, although the doxorubicin release profile of Doxil/Caelyx and Myocet are very different, with Doxil/Caelyx having very slow release rates in vivo and Myocet fast drug release rates (24), both formulations moderate the cardiotoxicity of doxorubicin compared with conventional doxorubicin, with Doxil/Caelyx appearing to have an advantage over Myocet (25).
We and others have observed that alterations in the composition of liposomal formulations of doxorubicin can alter the LD_{50} of the formulations in mice (9,10). Mayer et al. were the first to observe that the stability of liposomal doxorubicin in the circulation is an important factor in the toxicity of this drug in liposomal form. Although they did not report the half-lives for release of doxorubicin in their formulations, a correlation existed between the percentage retention of doxorubicin after 24 hours and the LD_{50} of the formulations; formulations with more rapid release rates were more toxic. In these experiments, the formulations with the most rapid release rate, composed of egg phosphatidylcholine, was also the most toxic.

In our experiments, the rapid weight loss in the affected mice and the rapid rise course of the toxicity suggest that the toxicity rather than cardiotoxicity is likely gastrointestinal. Indeed, the heart AUCs for doxorubicin (Table 1) and the doxorubicin levels as a function of time in heart (Fig. 4) do not show high heart doxorubicin levels and do not show substantial differences between the different liposomal formulations. From Table 2, it is clear that the toxicity is not mediated by the lipids in the formulations, because empty liposomes (no drug) had no toxicity in the mice. We can speculate that the intermediate doxorubicin release rates for DPPC and POPC lead to delivery to doxorubicin to gastrointestinal cells at a rate, and over a period of time, that corresponds, for example, to the turnover time for these cells. Further experimentation, focusing on determining bioavailable drug concentrations, will be needed to establish the exact mechanism for toxicity for the DPPC and POPC formulations.

Anti-CD19-targeted liposomal doxorubicin binds rapidly to target cells in vitro and this triggers receptor-mediated internalization of the liposome-drug package into the B cells (14). Therefore, in vitro cytotoxicity of the targeted liposomes should be somewhat independent of the drug release rate. The in vivo situation is more complicated. At the time of treatment of the mice in our in vivo experiments (i.e., 24 hours postinoculation), most of the B cells have left the circulation (11), so several hours will be required for the anti-CD19 liposomal doxorubicin to find and bind to the target cells. During this time, the more rapid release formulations will have lost some of their contents and drug-depleted liposomes will be internalized into the target cells after binding, which would, we hypothesize, lower the therapeutic effect for these liposomes. This hypothesis is supported by the in vivo survival data in Table 2. HSPC-containing liposomes, which have the slowest doxorubicin release rates in vivo (315 hours) resulted in the greatest ILS (88% ILS), with one long-term survivor. The more leaky the formulation, the less the ILS relative to HSPC-containing liposomes. Targeted DOPC-containing liposomes, which have the fastest drug release rates in vivo (~2 hours), showed very little increase in ILS (8%) compared with nontargeted DOPC-containing liposomes.

The in vivo release rates varied over 2 orders of magnitude (1.9-315 hours), and although there was a clear correlation between release rate of the liposomes and degree of increased survival in the mice receiving the anti-CD19 formulations, at the extremes of the release rates (DOPC versus HSPC) only a 16-day increase in survival (69%) resulted. Clearly, the relationships that exist between leakage rates and survival times are very complex. Factors, such as the rate of redistribution of the released drug away from the target cells, the rate and extent of binding of drug-containing versus drug-depleted liposomes to the target cells, and the rate of bioavailability of the drug at its intracellular site of action (i.e., the rate of intracellular drug release from endosomes), must all be considered. Also to be considered is the rate and degree of accessibility of the liposomes to the target cells. We know from previous experiments that most of the cancer cells have left the central compartment by 24 hours, when the mice received treatment (11). Therefore, if the rate of binding of the anti-CD19-targeted liposomes to the cells is slow relative to the rate at which the cell become inaccessible to the liposomes, then the therapeutic effect will be reduced. Recently, an attempt has been made to mathematically analyze these complex relationships in vitro for free doxorubicin compared with anti-CD44-targeted liposomal doxorubicin in B16F10 melanoma cells (26). For cells that are freely accessible in culture, Elia et al. have reported that encapsulated liposomal doxorubicin was 5- to 6-fold more efficient in killing the target cells than the free drug. Studies such as this, if extended to in vivo analyses, and studies such as the in vivo tumor bioavailability studies currently being done in our laboratory will help to shed more light on the complex relationships among binding, internalization, release, and therapeutic efficacy of liposomal anticancer drug formulations.

Anti-CD19-mediated targeting of liposomes, having different doxorubicin release rates, to B lymphoma cells seems to significantly modify the toxicity levels of the intermediate-releasing formulations, particularly for DPPC- and POPC-containing formulations. The toxicity of the PMPC-containing liposomes was only modestly ameliorated in two of three replicate experiments where the anti-CD19 mAb concentration was ~75 μg/μmol phospholipid. However, in one experiment, where the mAb concentration was 158 μg/μmol phospholipid, the ILS for the targeted liposomes was 82% compared with ~78% for the nontargeted liposomes (data not shown), which suggests that high mAb concentrations, possibly leading to more avid or faster uptake, may play a role in alleviating toxicity. The most dramatic effect was observed in DPPC-containing liposomes, where anti-CD19-mediated targeting (68 μg mAb/μmol phospholipid) completely eliminated the mortality seen in the nontargeted formulations. It is possible that the relatively long in vivo release rates for doxorubicin from this formulation (125 hours) resulted in most of the drug being retained in the liposomes at the time they bound to and were internalized by the B cells.

This study shows that liposome composition, mechanism of drug uptake, and drug release rates are all important for the resulting toxicities and therapeutic effects of liposomal drugs. A wide range of therapeutic indices could be seen for various phosphatidylcholine/cholesterol liposomal formulations of doxorubicin depending on the drug release rates and whether the liposomes were targeted to internalizing antigens. Studies such as these can aid in the formulation of less toxic and more effective drug carriers.

**Acknowledgments**

We thank Elaine Moase for editorial assistance and helpful discussions.
Immunoliposomal Doxorubicin: Toxicity and Efficacy

References


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