Biopharmaceutics of Boronated Radiosensitizers: Liposomal Formulation of MnBOPP (Manganese Chelate of 2,4-(α,β-Dihydroxyethyl) Deuterioporphyrin IX) and Comparative Toxicity in Mice

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Abstract □ Binary treatment modalities such as photodynamic therapy (PDT) and neutron capture therapy (NCT) combine low-toxicity electromagnetic irradiation with an appropriate radiation sensitizer to enhance selectivity for tumor targets. The porphyrin derivative tetrakiscarborane carboxylate ester of 2,4-(α,β-dihydroxyethyl) deuterioporphyrin IX (BOPP) shows tumor-selective uptake and is active in both treatment modalities. BOPP also chelates paramagnetic ions such as Mn^{2+}, and therefore its tissue accumulation and selectivity can be detected noninvasively by using magnetic resonance imaging. However, local and systemic toxicity appears elevated for the Mn^{2+} chelate (MnBOPP), but is poorly characterized. Here we have developed a liposomal formulation of MnBOPP and compared its toxicity with that of MnBOPP administered to mice in saline. The optimal liposome composition and maximal capacity to accommodate MnBOPP were investigated by differential scanning calorimetry and by encapsulation efficiency. MnBOPP was encapsulated quantitatively at up to 12 mol % (drug:lipid) in liposomes of varying composition, and remained incorporated during extended dialysis. Phase separation of drug- and lipid-rich domains was observed above 12% drug. MnBOPP in buffered saline was lethal to animals at 90 μmol/kg, and caused severe necrosis at the injection site at dose levels of 60 μmol/kg or greater. In contrast, MnBOPP formulated in liposomes was well tolerated at the highest tested dose of 135 μmol/kg, with the elimination of local toxicity.

Introduction

The combination of treatment modalities to achieve greater therapeutic effect and lower side effects is a frequent objective in the development of new cancer therapies. In such approaches as photodynamic therapy (PDT)[1,2] or neutron capture therapy (NCT),[3,4] electromagnetic radiation of comparatively low toxicity interacts with a sensitizer compound of low toxicity, and their combination results in a high cell-kill effect. In both cases, confining the irradiation to the disease field and confining the sensitizer to the tumor cells are essential for maximal selectivity of effect.

In NCT, selective uptake of boronated radiosensitizer compounds into tumor tissues is an important factor that determines the effectiveness of therapy.[3,5] A boronated porphyrin, the tetrakiscarborane carboxylate ester of 2,4-bis-(α,β-dihydroxyethyl) deuterioporphyrin IX (BOPP),[6] has shown selective tumor deposition in mice bearing C-6 murine glioma, and the ratio of BOPP taken up in brain tumor versus normal brain was 400:1.[7] An additional advantage of metalloporphyrins such as BOPP is that the porphyrin nucleus can chelate paramagnetic ions, such as manganese, while retaining their tumor-localizing property, so that the pharmacokinetics and selectivity of uptake can be monitored by noninvasive methods such as magnetic resonance imaging (MRI).[8,9] Therefore, the manganese chelate of BOPP (MnBOPP) is an agent of dual functions: that of a radiation sensitizer in boron NCT and that of a contrast agent for enhancement of tumors in MRI. In this dual-functional agent, boron serves as the key element for therapy and Mn serves as the key element for MRI contrast enhancement. By combining an imaging function with a therapeutic function, therapy could be individualized both to patients and to the disease state, and irradiation could be applied at the time of peak tumor selectivity or effect.

Previously we demonstrated that MnBOPP is selectively localized in intracranial 9L brain tumors in rats,[10] a tumor model that bears many similarities to *Glioblastoma multiforme*, a lethal human brain tumor.[11] In MRIs, the uptake of MnBOPP significantly enhanced the contrast between tumor and normal brain. Preliminary results in our laboratory also suggested that high doses of MnBOPP administered in saline had acute systemic toxicity and delayed chronic toxicity, and that drug encapsulated in liposomes was better tolerated by animals at high doses (≥100 μmol/kg, unpublished data). Moreover, the magnitude of brain tumor contrast enhancement in MRIs suggested that there was comparable tumor-selective uptake of MnBOPP for both the saline and liposomal forms. Peak contrast enhancement was observed at ~24 and 70 h after administration of free and liposomal MnBOPP, respectively.[12] Because liposomes offer the possibility for high-dose administration of MnBOPP with reduced toxicity, we have investigated the optimal conditions for liposomal encapsulation of MnBOPP and compared the in vivo toxic effects of liposomal MnBOPP with that of drug administered in buffered saline.

Experimental Procedures

Materials—Egg phosphatidylcholine (EPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and poly(ethylene glycol) (PEG) 1900 conjugated to distearoylphos-

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phatidylethanolamine (DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (CHOL) was from Sigma Chemicals (St. Louis, MO) and was recrystallized three times from methanol prior to use. MnBOPP (molecular weight 1485) was synthesized as described previously.[6] Female BALB/C mice (6 weeks, 20−25 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN).

Preparation of Liposomes—Liposomes were prepared by a reversed-phase evaporation (REV) procedure.[13] Typically, 10 μmol of phospholipid in chloroform were mixed at defined ratios with MnBOPP dissolved in methanol (4−5 mM), and the solvents were removed with a rotary evaporator. The dried film of drug and lipid was then resuspended in 0.5−1.0 mL of isopropyl ether, emulsified by brief (15 s) sonication after the addition of 350 μL of saline/HEPES buffer (140 mM NaCl, 20 mM *N*-2-hydroxyethyl-piperazine-*N*′-[2 ethanesulfonic acid], pH 7.4, osmolality 290 mOsm/kg), and converted into a liposome suspension by removal of ether under reduced pressure. Liposome preparations were extruded to a final diameter of ~0.08−0.10 μM by passing three times each under high pressure (~1250 kPa or 185 psi) through double-stacked polycarbonate membranes of decreasing pore size (1, 0.4, 0.2, and 0.08 μm) with a gas extruder (Mico Instrument, Middleton, WI). Extruded liposomes were then dialyzed free of residual drug with a membrane tubing having a molecular weight cutoff of 12 000−14 000 Da (Spectrapor Medical Industries, Los Angeles, CA). Dialysis was performed overnight at 4 °C against three changes of a 400-fold excess of saline/HEPES buffer.

Analytical Methods—The drug concentration in liposome samples before and after processing was determined spectrophotometrically after extraction from liposomes using methanol. The visible absorption spectrum of MnBOPP dissolved in methanol has peaks at 367 and 460 nm. A calibration curve was constructed for optical density at 460 nm as a function of MnBOPP concentration in methanol. Phospholipid concentrations were determined by phosphorus assay.[14] The results were expressed as "encapsulation efficiency", which is defined as the drug:lipid ratio after dialysis divided by the initial drug:lipid ratio (i.e., before extrusion or other processing).

Differential scanning calorimetry (DSC) was performed on a Perkin-Elmer DSC-2C instrument with samples sealed in aluminum pans. Thermograms were acquired and recorded on chart paper using a scan speed of 2.5 K/min, a sensitivity setting of 1 mCal/s, a temperature range of 20−60 °C, and a chart speed of 40 mm/min. After acquisition, the data were input into a computer (Macintosh, Apple Inc., Cupertino, CA) using a digital scanner and DataThief (National Institute for Nuclear Physics and High Energy Physics (NIKHEF), Amsterdam, The Netherlands), a program that extracts scanned data into a digital form. The DSC instrument was calibrated over a wide range of temperatures using various standard samples provided by the manufacturer. Each sample contained 14 μL of liposomes at a phospholipid concentration of 55 mM, and the drug:lipid molar ratios investigated were 0, 3, 7, 11, and 15% (moles of drug per 100 mol phospholipid). Thermograms were also obtained for MnBOPP dissolved in saline/HEPES buffer. All samples were maintained at the initial temperature (295 K) for 15 min to ensure temperature equilibration.

Experiments In Vivo—MnBOPP was administered to mice in saline/HEPES buffer at doses of 45, 60, 75, and 90 μmol drug per kilogram of body weight. The dose range for liposomal drug was 75, 90, 110, and 135 μmol/kg. Six mice per group were treated at each dose level, and the drug was injected via tail vein. The injection volume was <0.3 mL/20 gm body weight. After injection, the mice were observed for mortality and weighed regularly for 6 weeks. The maximum tolerated dose (MTD) is defined as the dose at which animals survived but had ~10% weight loss.

Results

Optimization of the Liposome Formulation—Preliminary experiments raised concern over potential local and systemic toxicity of the Mn^{2+} chelate of BOPP (data not shown), and liposomes were investigated for mitigation of the toxicity. Although MnBOPP is moderately water soluble, it appeared also to be incorporated quantitatively in liposomes, which suggested localization of the drug in the liposome membrane phase (data not shown). Therefore, the interactions between MnBOPP and liposome membrane constituents were investigated in greater detail. The drug:lipid ratio was varied, and the effect on membrane structure was investigated by DSC. DPPC was used as the predominant phospholipid because of its highly cooperative and well-defined phase transition at 41.3 °C. DPPC liposomes without drug showed a large, sharp transition at ~41 °C (Figure 1). MnBOPP alone did not undergo a distinct phase transition, as indicated in Figure 1 by the thermogram for 100% MnBOPP.

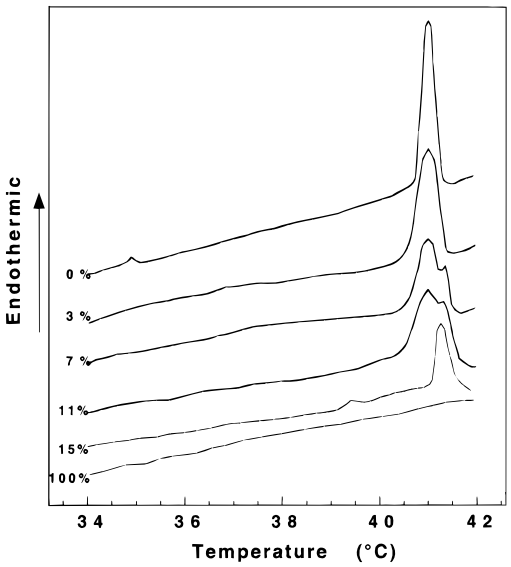

Figure 1—Incorporation of MnBOPP in lipid bilayers. DSC thermograms of DPPC liposomes containing various molar ratios of MnBOPP. Liposomes composed of DPPC and the indicated amount of drug were made, processed, and analyzed by DSC as described in Experimental Procedures. The drug:lipid ratios varied from 0% (no drug) to 100% (no phospholipids). The ratio is calculated as the number of moles of drug per 100 mol of phospholipid; the phospholipid concentration was not adjusted, i.e., the total moles of drug and phospholipid do not sum to 100%.

Inclusion of 3 mol % MnBOPP in DPPC bilayers (3 mol drug in 100 mol phospholipid) resulted in broadening of the main transition. The transition width at half-height increased from 0.3 to 0.46 °C. At 7 mol % MnBOPP, the main transition peak was further broadened and a shoulder appeared on the high-temperature side, suggesting the formation of a new phase that was possibly enriched in MnBOPP. Because this phase melts at higher temperature, lipid packing in the new phase may be different from, and tighter than, the lipid-rich domain. This new phase became more prominent at 11 mol % MnBOPP, and at 15 mol % MnBOPP, the predominant transition occurred at the temperature characteristic of the new MnBOPP-rich phase. Concomitantly, the transition endotherm characteristic of pure DPPC disappeared. The new phase apparently resulted from interactions between MnBOPP and DPPC; MnBOPP itself does not undergo a thermal transition in this temperature range, and DPPC undergoes a thermal transition at a lower temperature. Based on the DSC results, 10−12 mol % was hypothesized as the maximum capacity for DPPC bilayers to accommodate MnBOPP.

MnBOPP incorporation into DPPC membranes was investigated quantitatively as a function of the initial drug:lipid ratio. Ratios were included that were above and below the optimal suggested by DSC experiments (Figure 1). Following preparation of the liposomes, extrusion through controlled-pore membranes was performed to remove unincorporated drug that had precipitated.[15] Liposomes pass through the membrane pores, and are extruded to a diameter that approximately equals the pore size of the filter.[16] Because extrusion would not remove unencapsu-

Table 1—Encapsulation Efficiency of MnBOPP in DPPC Liposomes[a]

initial drug:lipid ratio, %[b]	drug:lipid ratio after extrusion, %[b]	drug:lipid ratio after dialysis, %[b]	encapsulation efficiency, %[c]
4	3.8 ± 0.46	3.6 ± 0.47	91 ± 11
8	7.6 ± 0.25	7.1 ± 1.5	89 ± 2
12	11.6 ± 0.36	11.0 ± 0.25	91 ± 3
20	11.6 ± 0.46	11.5 ± 0.68	57 ± 5

[a] Data are expressed as mean ± standard deviation from at least 3 experiments. [b] Drug:lipid ratio is the molar ratio between MnBOPP and DPPC phospholipid. [c] Encapsulation efficiency is defined as the ratio between the final (after dialysis) and initial drug/lipid ratio.

lated drug molecules that are in soluble monomer or micellar form, dialysis was performed after extrusion, and the drug:lipid ratio was determined before and after each processing step.

At drug:lipid ratios of ≤10 mol % (MnBOPP:DPPC), incorporation of drug was quantitative (Table 1), which is a characteristic of lipophilic agents located within the membrane bilayer. Neither extrusion nor dialysis reduced the drug:lipid ratio significantly (Table 1), suggesting a lack of substantial precipitated MnBOPP or unincorporated drug in monomer or micellar form. Dialysis was prolonged, and MnBOPP retention in the liposome further suggested stable incorporation of the drug in the bilayer.

For drug:lipid ratios ≥10 mol %, a plateau value of ~12 mol % (MnBOPP:DPPC) incorporation was observed after extrusion. This ratio was determined to be the maximal drug:lipid ratio achievable in DPPC membranes.

The membrane physical state can have major impact on both liposome performance in vivo and on the incorporation of lipophilic molecules. Therefore, encapsulation efficiency was investigated for a selection of liposome compositions that represent a broad range of physical or in vivo performance properties. This selection of compositions (Table 2) included low- and high-transition temperature phospholipids, charged and neutral (zwitterionic) compositions, cholesterol-containing formulations, and polymer-coated liposomes with extended circulation times in vivo.[17] Table 2 shows the effect of lipid composition on the incorporation of MnBOPP. Three phosphatidylcholines (EPC, DPPC, and DSPC) were tested individually, with or without cholesterol, at initial drug:lipid ratios of ~8%. For EPC liposomes, in which the bilayer is in a fluid state, the drug:lipid ratio achieved was ~7% following extrusion, and subsequent dialysis had little effect on the incorporation of MnBOPP. The addition of CHOL had little effect on the encapsulation efficiency in EPC liposomes.

For DPPC liposomes, in which the bilayer exists in the gel phase, encapsulation efficiency was similarly high. The addition of CHOL to DPPC liposomes did not alter MnBOPP incorporation.

In contrast, the drug:lipid ratio achieved for DSPC liposomes prepared with an 8 mol % drug:lipid ratio, the MnBOPP incorporation was ~3.4 mol % after extrusion; overall, only 40% of the initial drug was incorporated. Like DPPC liposomes, DSPC liposomes exist in the gel phase at room temperature. However, the DSPC acyl chain length is greater. The addition of CHOL, which broadens the phase transition and imparts a fluidizing effect, did not increase MnBOPP incorporation markedly.

Liposomes bearing hydrophilic polymer headgroups, such as PEG, and consisting of high-transition temperature phospholipids show greatly extended circulation times in vivo[17,18] as well as intratumor deposition.[18,19] MnBOPP incorporation into PEG-bearing liposomes was investigated for both fluid (EPC) and gel-phase (DPPC) lipids. MnBOPP was incorporated nearly quantitatively in either composition, and the maximal mole ratio achieved was 12% (Table 2).

Comparative Toxicity of MnBOPP Formulations—Free or liposomal MnBOPP was administered to healthy mice at various dose levels to determine the effect of formulation on the maximum tolerated dose (MTD) of the drug. Body weight and vital signs (respiration, heart rate) of the animals were monitored after bolus injection. Preliminary experiments using 2 mice per group showed that 100% (2 of 2) of the animals died following administration of free MnBOPP at a dose of 100 μmol/kg, whereas all survived at 80 μmol/kg (data not shown). A more comprehensive experiment with 6 mice per group showed that 90 μmol/kg free MnBOPP was lethal, resulting in 80% death (5 of 6 animals) within 48 h after injection. For all other doses tested, all animals survived the treatment (data not shown).

Figure 2 shows the percent body weight change following injection of free MnBOPP. MnBOPP at 75 μmol/kg mediated 11% weight loss, which did not recover until 30 days after injection. Local necrosis was observed at the injection site 24 h after injection in animals treated with ≥60 μmol/kg of free drug. Doses of 45 μmol/kg caused ~4% weight loss that recovered within 10 days after injection. Based on body weight changes, the MTD of free MnBOPP appeared to be ~75 μmol/kg.

Two liposome compositions incorporating MnBOPP, PEG−DSPE:EPC:CHOL (1:10:5) and PEG−DSPE:DPPC:CHOL (1:10:5), were tested in preliminary dose-ranging experiments using small numbers of animals. The two liposome formulations appeared to cause similar weight change profiles in mice (data not shown); therefore, only the PEG−DSPE:DPPC:CHOL (1:10:5) liposome formulation was selected for investigation with a larger number of animals. All animals survived injection of MnBOPP at doses up to 135 μmol/kg, and no necrosis was observed at the injection site. Observation of the animals was continued for 3 months. The maximal weight loss (~8%) occurred at the 135 μmol/kg dose level, and the body weight did not recover until day 40 after injection (Figure 3). However,

Table 2—Effect of Lipid Composition on Drug:Lipid Molar Ratios Achieved[a]

lipid composition	initial drug:lipid ratio, %[b]	drug:lipid ratio after extrusion, %[b]	drug:lipid ratio after dialysis, %[b]	encapsulation efficiency, %[c]
EPC	8.0 ± 0.3	7.0 ± 0.4	6.9 ± 0.2	87 ± 5
EPC/CHOL (2:1)	8.0 ± 0.5	6.4 ± 0.9	6.4 ± 1.2	80 ± 15
DPPC	8.0 ± 0.5	7.6 ± 0.5	7.3 ± 0.2	92 ± 4
DPPC/CHOL (2:1)	8.0 ± 0.2	7.4 ± 0.2	7.5 ± 0.5	94 ± 5
DSPC	8.0 ± 1.7	3.4 ± 0.8	3.1 ± 0.1	40 ± 9
DSPC/CHOL (2:1)	8.0 ± 1.0	4.3 ± 0.3	3.9 ± 0.7	50 ± 6
PEG–DSPE/EPC/CHOL (1:10:5)	12.3 ± 0.6	11.8 ± 1.1	11.0 ± 1.8	89 ± 11
PEG–DSPE/DPPC/CHOL (1:10:5)	12.3 ± 0.8	11.9 ± 1.3	10.5 ± 0.9	94 ± 3

[a] Data are expressed as mean ± standard deviation from at least 3 experiments. [b] Drug:lipid ratio is the molar ratio between MnBOPP and DPPC phospholipid. The ratio is calculated as the number of moles of drug per 100 mol of phospholipid. [c] Encapsulation efficiency is defined as the ratio between the final (after dialysis) and initial drug/lipid ratio.

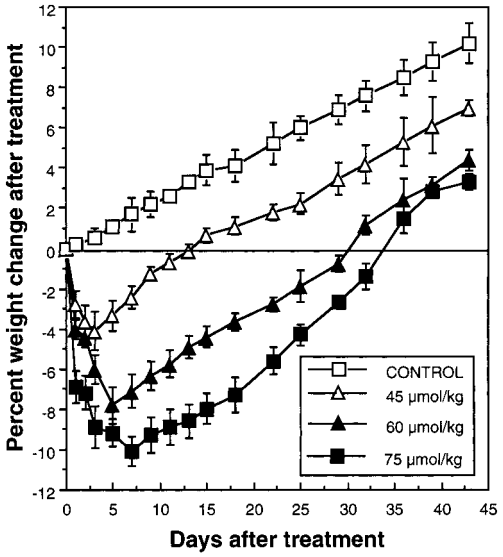

Figure 2—Toxicity of MnBOPP in saline. Body weight changes of mice were recorded after injection of MnBOPP dissolved in buffered saline. The concentration of MnBOPP was 10 mM in saline/HEPES buffer (140 mM, 20 mM, pH 7.4, osmolality 290 mOsm), and the volume of one injection was <0.3 mL/20 gm body weight. The weight change of each animal was normalized to its weight before injection, and each point represents the mean ± standard deviation for each group ($n = 6$).

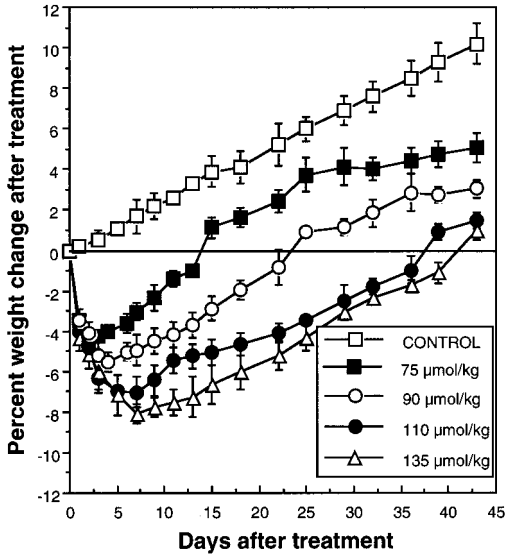

Figure 3—Toxicity of liposomal MnBOPP. Body weight changes of mice were recorded after injection of MnBOPP encapsulated in liposomes. MnBOPP was incorporated at a molar ratio of 12% (with respect to phospholipid) in liposomes composed of PEG–DSPE:DPPC:CHOL (1:10:5). The drug concentration was ~8 mM. If the volume required for a specific dose was >0.3 mL/20 gm body weight, it was split into two injections with an interval of ~10 min. The weight change of each animal was normalized to its weight before injection, and each point represents the mean ± standard deviation for each group ($n = 6$).

the MTD, defined as a 10% loss in body weight, was not observed in the dose range tested for either PEG−DSPE:EPC:CHOL (1:10:5) or PEG−DSPE:DPPC:CHOL (1:10:5) liposomes containing MnBOPP, even though the highest dose tested (135 μmol/kg) was nearly twice the tolerated dose of free drug (75 μmol/kg).

Discussion

Selective delivery of a sufficient quantity of boron (^{10}B) compound to the tumor site via the systemic route is a key requirement for the success of boron NCT as a tumoricidal modality.[3,4] In addition, the selective deposition of MnBOPP in tumor tissues may facilitate its application in diagnosis as an MRI contrast-enhancing agent.[10]

Here we established that the MTD of MnBOPP in saline was ~75 μmol/kg, as judged by body weight loss of animals. However, even at that dose, considerable injection-site toxicity was noted. The MTD determined here for the manganese chelate of BOPP is significantly lower than the MTD of ~200 mg/kg (147 μmol/kg) determined previously for the nonmetalated compound in mice.[7] Thus, free MnBOPP is roughly twice as toxic as BOPP. From the standpoint of dose-limiting systemic toxicity and local injection-site toxicity, mitigation of these effects using liposomes may aid in clinical application of the promising Mn-containing compound.

Liposomes have been shown to reduce the systemic toxicity of encapsulated drugs, and liposomes of small size bearing PEG moieties on the surface have shown enhanced tumor uptake.[17,18,20] Because of the beneficial effects of encapsulation on toxicity and selectivity of deposition, liposomes hold considerable promise for the delivery of boronated compounds in NCT.[21−24] With the reduction in toxicity observed here, dose escalation and possibly increased tumor uptake of MnBOPP may be achievable via liposome encapsulation. However, liposomal formulation of lipophilic compounds involves optimization of the large number of parameters that affect stability;[25−27] these parameters include the preferred membrane physical state, determined by the phase transition temperature (T_m) of the predominant lipid constituent, the lipid acyl chain composition, and the CHOL content, which both modifies T_m and has the effect of stabilizing liposomes in vivo. Additional parameters having impact on drug incorporation include the liposome membrane charge and the presence of surface-localized hydrophilic polymers.

To approach this problem of multiparameter optimization, a prototype liposome formulation was chosen; DPPC, a phospholipid with a defined phase transition, was used as a probe for membrane interaction of the drug. A maximal drug:lipid ratio was determined qualitatively by DSC. In the case of MnBOPP, exceeding the maximal capacity of the bilayer to accommodate the drug was manifested by the appearance of a new, higher-melting drug:lipid phase. This behavior contrasts with other cases,[15,28] in which exceeding the maximum drug:lipid ratio may be marked by an abrupt reappearance of the original T_m of the pure phospholipid component when catastrophic destabilization results in precipitation of drug outside of the membrane environment. An additional advantage of the use of DSC is that the progressive, MnBOPP-dependent changes observed in the T_m confirmed interaction of the drug with the bilayer of the membrane as well as suggested the existence of an unusual, higher-melting drug:lipid complex involving a specific lamellar phase state of the lipid. This unusual phase may be worthy of future study because it suggests the possibility for developing drug-rich particles that may be structurally distinct from liposomes (cf. ref 29).

The qualitative overview of bilayer maximal capacity for drug provided by DSC was investigated in greater detail using simple methodology to quantify the drug:lipid ratio; this investigation involved manipulations to resolve precipitated and weakly associated drug. Extrusion of liposomes through controlled-pore filters[16] not only redefines the mean diameter to a size that circulates more readily in the blood, but also allows the separation of liposomes from precipitated drug.[15] The subsequent dialysis step was performed to eliminate micellar or monomer drug because it would not be removed from liposomes during the extrusion step. Overnight dialysis had little effect on MnBOPP

incorporation, suggesting reasonably stable incorporation of the drug in the membrane.

Once the approximate bilayer capacity for MnBOPP was established using a prototype phospholipid, a small number of liposome compositions were chosen for further investigation, and these represented broad differences in membrane properties and in vivo performance. We observed quantitative incorporation of MnBOPP in most of these formulations, suggesting considerable latitude in selecting liposome constituents. A lack of such latitude could limit the ability to overcome possible future challenges posed as specific in vivo performance objectives are pursued.

The membrane physical state, governed largely by the T_m of the predominant lipid constituent, is a major parameter that affects the performance of liposomes in vivo.[30−32] In this study, liposomes encapsulating MnBOPP were formed from each of three phospatidylcholines (egg PC, DPPC, and DSPC) having increasing T_m. EPC (T_m, −5 to −17 °C) exists in the liquid crystalline state at room temperature, whereas DPPC (T_m, 41.3 °C) and DSPC (T_m, 55.8 °C) exist in the gel state. At room temperature or body temperature, membrane fluidity is decreased in the order EPC > DPPC > DSPC, as indicated by the T_m (EPC < DPPC < DSPC). Both fluid- (EPC) and gel-phase (DPPC) liposomes incorporated MnBOPP quantitatively. Interestingly, the nature of the gel-phase lipid had a major effect on drug incorporation; DSPC, which also forms gel-phase liposomes, had the lowest MnBOPP incorporation capacity (Table 2). This observation may be explained by the highly ordered, cooperative packing of lipid molecules in the gel phase. More fluid membranes, such as DPPC and EPC, may better accommodate bulky, lipid-soluble drugs such as MnBOPP within the bilayers because these lipid bilayers tend to have more disordered hydrocarbon domains.

Cholesterol was tested as a constituent in several formulations. Cholesterol increases the packing density of the phospholipid bilayer by inserting itself between the lipid molecules, resulting in decreased permeability of phospholipid bilayer to small polar molecules and preventing plasma proteins from penetrating.[26,33] Physical stability of liposomes often is enhanced by incorporation of CHOL in the bilayer.[26] Cholesterol also increases the disorder within bilayer, resulting in broadening or elimination of the T_m.[30−32] In this study, the addition of CHOL in EPC- or DPPC-containing liposomes did not change the drug: lipid ratio significantly (Table 2) at a molar ratio of 1:2 (CHOL:phospholipid), suggesting that CHOL did not alter significantly the interaction between EPC or DPPC and MnBOPP molecules. Interestingly, CHOL did not increase the incorporation of MnBOPP in DSPC liposomes, despite the broadening effect of CHOL on the T_m.[31,32]

The PEG headgroup of the PEG−DSPE lipid provides a hydrated coating on the surface of liposome and can hinder the access of plasma proteins to the liposome surface; such "sterically stabilized liposomes" (SSLs)[34] have shown a 5-fold increase in circulation time compared with liposomes without the surface coating.[18] The altered pharmacokinetics may allow increased extravasation of liposomes into tumors, resulting in the enhanced antitumor effect seen in various preclinical and clinical trials.[17] Here, PEG-containing liposomes of both fluid- and gel-phase lipids incorporated MnBOPP as efficiently as the optimal non-PEG formulations.

MnBOPP encapsulated in liposomes was much better tolerated than the free drug. The MTD of free MnBOPP in mice was ~75 μmol/kg, whereas that of the liposomal drug exceeded 135 μmol/kg for the PEG−DSPE-containing formulations that included either EPC or DPPC as the predominant lipid constituent. We observed previously[12] that tumor accumulation of MnBOPP administered in a prototype liposomal form was roughly comparable to that observed for free MnBOPP. The high incorporation of MnBOPP in the diverse liposome compositions defined here provides the opportunity to optimize further the tumor deposition in vivo and shows clearly the potential to increase the dose intensity of MnBOPP, with the aim of further enhancement in tumor delivery.

The amount of phospholipid required to administer the highest dose of MnBOPP (135 μmol/kg) was ~28 μmol lipid per animal. Although this concentration of lipid is high, we have previously administered comparable doses of liposomes to mice[35] up to 9 times in 3 weeks. Although those liposomes contained high concentrations of paclitaxel, a cytotoxic agent, all animals administered a dose of 30 μmol lipid per injection survived the entire 9-course treatment. Caution must be exercised in using these previous experiments to interpret the present results because different lipid compositions were used, and the liposomes contained a potent cytotoxic agent. However, the compositions used here would be cleared less rapidly to the lung, liver, and spleen than those used previously, and thus should have relatively less impact on the overall health of the animal. Thus, liposomal encapsulation appears to provide a means to administer higher doses of MnBOPP with reduced toxicity.

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