Targeted Delivery of a Heme Oxygenase Inhibitor with a Lyophilized Liposomal Tin Mesoporphyrin Formulation

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Tin mesoporphyrin (SnMP) is a competitive inhibitor of heme oxygenase being examined clinically for the treatment of hyperbilirubinemia. Since liposomes have been shown to target SnMP to the spleen and increase its efficacy (S. A. Landaw, G. S. Drummond, and A. Kappas, *Pediatrics* 84, 1091–1096, 1989), we began investigating the feasibility of the preparation and scaleup of a liposomal SnMP formulation for clinical use. SnMP liposomes were prepared by high-pressure homogenization of a suspension of SnMP and egg phosphatidylcholine (1:20, w/w) in lactose-phosphate buffer, resulting in SnMP liposomes that were less than 200 nm in diameter and had encapsulation efficiencies of up to 90% at pH 5. The SnMP liposomes could be sterile filtered and lyophilized in a 1-day cycle with retention of the encapsulation efficiency and particle size. Following injection into rats, the distribution of liposomal SnMP to spleen at 2 and 6 hr after dosing was 5–20 times higher than for aqueous SnMP. Lyophilized SnMP liposomes were also more effective than aqueous SnMP in decreasing bilirubin production in bile-cannulated rats. The results suggest the potential for producing a safe, sterile, and effective lyophilized formulation of SnMP liposomes for targeting of heme oxygenase inhibitors to the spleen.

KEY WORDS: heme oxygenase inhibitor: hyperbilirubinemia; tin mesoporphyrin; liposomes; lyophilization; targeted delivery to spleen.

INTRODUCTION

A number of metalloporphyrins, primarily tin and zinc porphyrins, have been investigated as potential drugs for the treatment of jaundice (hyperbilirubinemia) and related disorders (1–3). These compounds have been shown to be potent inhibitors of heme oxygenase, the enzyme which catalyzes the conversion of heme (iron protoporphyrin) to biliverdin, which then is reduced to bilirubin by biliverdin reductase. Heme oxygenase is the rate-limiting enzyme in the heme degradative pathway. The most success has been achieved with the tin compounds, tin protoporphyrin (SnPP) and tin mesoporphyrin (SnMP), whose structures are shown in Fig. 1; these are currently the only such compounds which have been approved for clinical trials.

Several clinical studies have shown SnPP to be effective in the treatment of various hyperbilirubinemia disorders, including neonatal jaundice (4). However, SnPP is a highly light-sensitive and unstable compound (5). SnMP, with ethyl groups substituted for vinyl groups, is more stable than SnPP (6) and is five times more potent *in vivo* in inhibiting bilirubin production than SnPP (7). Thus, from a commercial perspective, SnMP is a more viable drug than SnPP.

Spleen and liver are the major sites of heme oxygenase activity, accounting for ca. 75 and 25%, respectively, of the bilirubin produced. When injected as an aqueous solution, SnPP and SnMP are not distributed to an appreciable extent to the spleen; this may account for the fact that parenteral administration of the drugs leads to only a 25–35% reduction in bilirubin output. Recently, Landaw *et al.* have found that incorporation of SnPP and SnMP into sonicated liposomes significantly increases the delivery of the drugs to the spleen, thus increasing the efficacy of the drug (8). This takes advantage of the natural tendency of liposomes to be preferentially taken up by the reticuloendothelial system (RES) tissues such as the spleen and the Kupffer cells of the liver. The results demonstrated the concept that passively targeting a heme oxygenase inhibitor to the spleen by means of liposomes increases its ability to diminish bilirubin production. Therefore, testing a liposomal formulation of a heme oxygenase inhibitor in the clinic is of great interest. The work reported here was initiated to determine if a liposomal delivery system for SnMP is commercially feasible.

The commercial scaleup of liposomal products for clinical use has been difficult. For parenteral use, liposomes must generally be prepared such that they can be terminally sterile filtered, thus requiring their size to be <200 nm and therefore unilamellar or oligolamellar. Sonication of large multilamellar liposomes has been the classical method for accomplishing this (9), as done by Landaw *et al.* (8), but this has several drawbacks from a commercial perspective (10). Several newer methods which are more applicable to clinical and commercial situations include extrusion through 100- to 200-nm polycarbonate membranes (11), detergent dialysis (12), and high-pressure emulsification, e.g., by a device known as the Microfluidizer (Microfluidics Corp.), for which pilot- and production-scale versions are available (13).

Another concern with a liposome product is stability. The stability of a ready-to-use liposomal product could be limited due to leakage of the drug from the liposome, change in liposome size on storage, and chemical stability of the drug and lipid components. The shelf life required for a commercial liposomal product is frequently 2 years; preparation of clinical material requiring multiple injections over several weeks can be problematic. There are several approaches to overcoming stability problems with liposomes wherein the final dosage form of a liposomal drug is constituted from a powder just before use. These include a "proliposome" technique (14), a "remote loading" technique (15), and lyophilization. A number of workers have shown that inclusion of a disaccharide such as trehalose, sucrose, or lactose allows a liposomal formulation to be lyophilized without loss of drug encapsulation or change in size (16,17), thereby allowing a long shelf life. Furthermore, lyophilization of liposomes using a "dehydration-rehydration vesicle" (DRV) technique has been described (18), whereby lyophilized liposomes are reconstituted with a fraction of the original vol-

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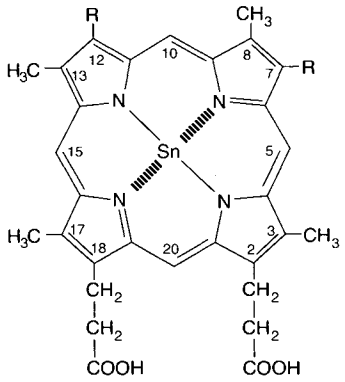

Sn PP; R = $-CH=CH_2$

Sn MP; R = $-CH_2-CH_3$

Fig. 1. Structures of tin protoporphyrin (SnPP) and tin mesoporphyrin (SnMP).

ume of water, which can lead to a higher encapsulation efficiency. Lyophilization is most useful to prepare liposomal formulations of lipophilic drugs, but the correct choice of pH at which the drug is most lipophilic allows lyophilization of water soluble drugs. We report here our results using SnMP liposomes of a defined size distribution prepared using the Microfluidizer and lyophilization of the liposomes to provide a formulation which is adaptable for use in a clinical situation.

MATERIALS AND METHODS

Materials. Tin mesoporphyrin dichloride was obtained from Abbott Laboratories; egg phosphatidylcholine, 99% (Type V-EA; 100 mg/mL in chloroform/methanol, 9:1, with 0.1% butylated hydroxytoluene), and D-lactose monohydrate (ACS reagent) were from Sigma Chemical Company; Bio-Gel P-100, 50–100 mesh, was from Bio-Rad Laboratories. For studies involving liposomes with a 1:10 (w/w) SnMP/lipid ratio, egg phosphatidylcholine, 99%, from Nippon Fine Chemical Co. (Type PCE; 10% solution in chloroform) was used. All other materials were reagent or HPLC grade.

Methods. All solutions and formulations containing SnMP were stored in the dark and exposure to light was minimized. Typically, 120 mg of tin mesoporphyrin dichloride was dissolved in 50 mL of methanol; to it was added 24 mL (2.4 g) of egg phosphatidylcholine (99%) in chloroform/methanol (9:1). The solvent was rotary evaporated in subdued light and the flask was evacuated under high vacuum for an additional hour to remove traces of organic solvent. The lipid film was taken up in three 100-mL portions of 0.05 *M* lactose/0.004 *M* sodium phosphate buffer, pH 6. The mixture was stirred for 2 hr, its pH was adjusted to 5.0, and then it was homogenized with a Microfluidizer (Model M-110, equipped with an interaction chamber and a back pressure module; Microfluidics Corporation, Newton, MA) for the desired number of passes at an air pressure of 60–80 psi. The volume was brought up to 400 mL with buffer and the pH readjusted to 5.0 if necessary.

For lyophilization, the liposomal SnMP solution was filtered through a 0.45-μm filter, and 5-mL aliquots (1.5 mg SnMP) were filled into 12-mL serum vials. The material was then lyophilized (Hull Lyophilizer Model 8FS12, Hull Corporation, Hatboro, PA) using the following cycle: final shelf temperature, −40°C; product temperature, −30°C; hold time, 2 hr; primary drying shelf set point, +10°C; vacuum set point, 200 μm; product temperature, 0°C; hold time, 2 hr; secondary drying shelf set point, +40°C; vacuum set point, 100 μm; product temperature, +30°C; hold time, 8 hr; and final shelf temperature, +25°C. Lyophilization was usually complete within 1 day. Vials were reconstituted with 1 mL sterile water or 0.02 *M* sodium phosphate; where indicated, the pH was adjusted to 7 with 1 *N* HCl after reconstitution.

For HPLC analysis, SnMP samples (either 1 mL of solution or the contents of 1 vial of lyophilized SnMP liposomes containing 1–2 mg of SnMP) were diluted with 0.05 *M* tetra-*N*-butyl ammonium phosphate buffer (pH 12) to 25 mL. To 2 mL of this solution, 0.117 g sodium chloride and 10 mL hexane were added. After shaking 24 hr, the sample was centrifuged and the hexane layer was discarded. To 1 mL of the aqueous layer, 24 mL of the pH 12 buffer was added, and the solution was analyzed by HPLC for drug potency (19) using a Waters WISP automatic sample injector, an Applied Biosystems Spectraflow 400 pump, an Applied Biosystems 783 detector at 405 nm, a Spectra-Physics 4270 integrator, and a Hamilton PRP-1 column (22 cm × 4.6 mm, 5 μm). Mobile phase was 32:68 acetonitrile/0.05 *M* tetra-*N*-butyl ammonium phosphate, pH 12 (flow rate, 1.0 mL/min; injection volume, 25 μL). Recovery of SnMP from extracted standards was 97.4 ± 6.4%; sensitivity was in the range reported for SnPP (19).

The encapsulation efficiency of the SnMP was measured by gel permeation chromatography, using a Bio-Gel P-100 column, 1.5 × 25 cm, and elution with lactose/phosphate buffer of the same pH and osmolarity as the liposome preparation. Fractions were collected by a fraction collector and analyzed for SnMP content by HPLC or spectrophotometrically (Hewlett-Packard Model 8452A diode array spectrophotometer). Encapsulation efficiency (%) was calculated by dividing the amount of SnMP in the liposome fraction by the total amount of SnMP applied.

Particle size analysis was performed with a Nicomp Model 270 laser submicron particle size analyzer (Pacific Scientific HIAC/Royco Instrument Division). The mean particle diameter was determined from the Gaussian analysis with volume weighting.

Animal Studies. For the tissue distribution study in rats, SnMP liposomes were prepared and lyophilized as described above at pH 5, 1.5 mg SnMP in 5 mL/vial. After reconstitution with 1 mL sterile water/vial, one portion (24 vials) was used as is (pH 5.0), and another portion (24 vials) was adjusted to pH 7.0 with 1 *N* NaOH. A solution of SnMP in saline was prepared by dissolving SnMP in 0.9% saline at pH 12 (1.5 mg/mL); when dissolved, the pH was adjusted to 7 with 1 *N* HCl. Rats weighing 250–280 g were injected intravenously with 10 mg/kg body weight (13.3 μmol/kg) SnMP in liposomes (pH 5 or 7) or in saline, and sacrificed at 2, 6, or 24 hr after dosing. The organs were removed (kidney, lung, liver, and spleen), homogenized by digestion in Soluene 100 (Packard Radiomatic, Tampa, FL), and analyzed for tin content by atomic absorption spectroscopy (Perkin-Elmer 5000 spectrophotometer with HGA-500 heated graphite atomizer).

The efficacy of lyophilized SnMP liposomes was tested

in bile-cannulated rats. SnMP liposomes were prepared in 0.0625 *M* lactose/phosphate buffer (0.36 mg/mL SnMP), lyophilized at pH 5 (4 mL/vial), and reconstituted with 1 mL sterile water per vial and the pH adjusted as necessary (final pH 5 or 7). Bile-cannulated rats were injected at a dose of 1.6 or 4 μmol/kg body weight, and bilirubin excretion in bile was monitored (7). Animals were sacrificed 16 hr postinjection and the liver and spleen removed. SnMP tissue concentration (fluorometric) and heme oxygenase activity were measured in these organs (7).

RESULTS AND DISCUSSION

Preparation and Characterization of Tin Mesoporphyrin Liposomes. SnMP is most soluble at pH >7. Its solubility properties are affected not only by the ionization of its propionic acid side chains, but also by the ligation at the tin atom. When SnMP dichloride is dissolved at high pH, the chlorides eventually exchange with hydroxide ions. Thus, at high pH, SnMP exists primarily as a dihydroxide complex and has a high aqueous solubility, whereas at acid pH the predominant species is the diaquo complex, which has a low aqueous solubility (5). At neutral pH, a monoaquo–monohydroxide complex exists in equilibrium with the other two species, and SnMP has an intermediate aqueous solubility at this pH. Using these considerations, preparation of SnMP liposomes was studied as a function of pH. Initial studies examined SnMP liposomes composed of egg phosphatidylcholine at a lipid-to-drug ratio (w/w) of 20, because of the favorable encapsulation efficiencies previously found under these conditions (8). Following rotary evaporation of the solvent from a drug–lipid solution in chloroform/methanol, the drug–lipid film could be dissolved in buffer containing sodium phosphate and lactose. Lactose was included because of its cryoprotective properties for liposomes (16,17) as well as its acceptability for parenteral formulations. The suspension was then homogenized by passage through the Microfluidizer and filtered through a 0.45-μm filter. SnMP liposomes prepared by this procedure were physically stable by visual inspection for several months at 4°C, with no visible sign of precipitation.

The size distribution of SnMP liposomes were measured by a laser light-scattering technique. The mean size was found to be dependent on the number of passes through the Microfluidizer. For four passes, the mean size was generally 100–150 nm and could, therefore, potentially be sterilized by filtration through a 0.22-μm filter. Indeed, some liposomal SnMP preparations were found to pass easily through a 0.22-μm filter. Given this size range and preparation method, it is probable that the liposomes are unilamellar or oligolamellar, with one to five lamellae (11,13).

The encapsulation efficiency, defined as the ratio of drug encapsulated inside liposomes to the total amount of drug in the formulation, was found to be dependent on pH for SnMP liposomes. When prepared at pH 5, encapsulation approximated 90%, while at pH 7, encapsulation was usually low (≤10%). These results are not surprising based on the solubility properties and lipophilicity of SnMP at these pH's (5).

Lyophilization of Tin Mesoporphyrin Liposomes. Lyophilization of liposomes has the potential for extending the shelf life of liposomal formulations to 2 or more years, the standard usually required for parenteral drug products. To investigate lyophilization of SnMP liposomes, the latter were prepared and microfluidized at pH 5 using 0.05 *M* lactose/0.002 *M* sodium phosphate buffer, at a drug concentration of 0.3 mg/mL. After filtration through a 0.45-μm filter, the mixture was filled into 12-mL vials (5 mL each, 1.5 mg SnMP), and lyophilized in a 1-day cycle. Each vial was reconstituted with 1 mL sterile water so that the final SnMP concentration was 1.5 mg/mL, the final pH was 5, and the solution was isotonic. No significant change in particle size was observed after lyophilization, and the encapsulation efficiency after reconstitution remained ca. 90%. When liposomes were reconstituted into buffer yielding a final pH of 7, the encapsulation efficiency ranged from 45 to 60%. These results are summarized in Table I.

The differences in encapsulation efficiency of SnMP liposomes as a function of pH and lyophilization probably can be accounted for by the location and orientation of the drug within the liposomes. At pH 5, SnMP appears to partition into the lipid bilayer in a similar manner to heme, *viz.*, with the carboxylate groups protruding into the polar headgroup region of the bilayer and the remainder of the molecule partially embedded in the hydrophobic region (20). This also means that there would be two orientations of SnMP within the bilayer: one in the inner monolayer and one in the outer monolayer. However, when prepared at pH 7, the majority of the drug appears to localize in the aqueous phase, either outside of the liposome or in the aqueous core of the liposome, leading to a low encapsulation efficiency. If prepared and lyophilized at pH 5 and reconstituted to a final pH of 7, drug will tend to leave the bilayer and enter the aqueous phase. However, only drug in the outer monolayer will become unencapsulated; drug located in the inner monolayers (or in the inner lamellae of oligolamellar liposomes) will

Table I. Encapsulation Efficiency of Tin Mesoporphyrin Liposomes

Sample	Buffer	Encapsulation efficiency[a]
pH 5, not lyophilized	pH 5, 0.05 *M* lactose, 0.004 *M* phosphate	90% (0.045)
pH 5, lyophilized, reconstituted	pH 5, 0.25 *M* lactose, 0.02 *M* phosphate	80–90% (0.040–0.045)
pH 7, not lyophilized	pH 7, 0.05 *M* lactose, 0.004 *M* phosphate	5–10% (0.0025–0.0050)
Prepared at pH 5, lyophilized, reconstituted in pH 7 buffer	pH 7, 0.25 *M* lactose, 0.02 *M* phosphate	45–60% (0.023–0.030)

[a] Encapsulation efficiency is given as percentage of drug bound to liposomes relative to amount of total drug; values in parentheses are absolute encapsulation efficiencies (moles SnMP/mole lipid).

probably remain entrapped in the aqueous regions of the liposomes. Thus, a moderate (45–60%) encapsulation efficiency is attained under these conditions. This would require that diffusion of SnMP across the bilayer and its flip-flop from the inner to the outer bilayer is slow relative to the time scale of the experiments (i.e., several hours). Another contributing factor could be that the lyophilization and reconstitution processes as carried out led to a higher than expected encapsulation efficiency [*viz.*, 45–60 vs 5–10% (Table I) for reconstitution at pH 7], as has been observed with DRV liposomes (18).

The actual ligation of SnMP under these conditions, however, is uncertain. When prepared by the method used for these studies, the initial species associated with the phospholipid is the dichloride complex. The kinetics of the conversion of this complex to the aquo species in aqueous media have not been determined. It is possible that this reaction is slow enough so that the dichloride complex is the species actually present in liposomes at pH 5. The difference in behavior of SnMP liposomes at pH 7 could then be due partly to a more rapid conversion of the dichloride species to diaquo– and monoaquo–monohydroxide complexes.

The effect of lipid/drug ratio on the properties of lyophilized SnMP liposomes was also examined. SnMP liposomes with 1.25 to 2.5 mg/mL drug were prepared in 0.0625 *M* lactose/phosphate buffer, pH 5, and lyophilized (4 mL/vial). When the 2.5 mg/mL formulation was reconstituted with 1 mL of sterile water, a gel formed. Similarly, a 1.88 mg/mL formulation gave a viscous suspension on reconstitution. However, a 1.25 mg/mL preparation was easily reconstituted to a final concentration of 5 mg/mL, giving a free-flowing liposomal formulation that could be filtered through a 0.45-μm filter. Thus it appears that at a lipid/drug ratio of 10:1 (w/w), lyophilized SnMP liposomes cannot be prepared above initial concentrations of 1.25 mg/mL (or 5 mg/mL final concentration). For 20:1 (w/w) lipid/drug ratios (and therefore higher lipid concentrations), it is likely that final SnMP concentrations of 2.5 mg/mL or less will be required for efficient reconstitution.

In a similar experiment, SnMP liposomes (lipid/drug = 10:1, w/w) were prepared at an initial concentration of 0.33 mg/mL and a pH of 5, then lyophilized. On reconstitution with water (final drug concentration of 1.33 mg/mL), the encapsulation efficiency was found to be only 20–40%, in contrast to 90% for SnMP liposomes having 20:1 (w/w) lipid/drug ratios (Table I). Furthermore, it was found that lipid–drug ratios of 5 and below lead to destabilization of the liposome structure. These observations are similar to those reported for hemin, in which lipid/hemin ratios <5 lead to a population of hemin-micelle species smaller in size than liposomes (20). It is possible that a similar phenomenon could be occurring for SnMP, leading to lower apparent encapsulation efficiencies at low lipid/drug ratios.

Tissue Distribution in Animals. The distribution of drug to the kidney, lung, liver and spleen of rats was determined after intravenous administration of 10 mg/kg body weight (13.3 μmol/kg) SnMP in the following forms: (a) SnMP liposomes prepared, lyophilized, and reconstituted at pH 5; (b) SnMP liposomes prepared and lyophilized at pH 5 but reconstituted at pH 7; and (c) aqueous SnMP (in saline). Animals were sacrificed at 2, 6, or 24 hr after dosing. The organs were homogenized, digested, and analyzed for Sn content by atomic absorption (detection limit, 3.7 μg Sn/g tissue). The results of this study are shown in Fig. 2, with the data expressed in terms of total amount of drug taken up by the various organs. With all SnMP formulations, the liver was found to take up the highest amount of drug. Figure 3 shows the drug uptake on a per gram of tissue basis. Drug could not be detected in lung for any formulation; distribution to kidney and liver was similar for all three dosage forms. However, distribution of SnMP to the spleen was 5 to 20 times higher with the liposomes than with the aqueous formulation, the levels being below the detection limit in the spleen for the aqueous formulation. This effect was most

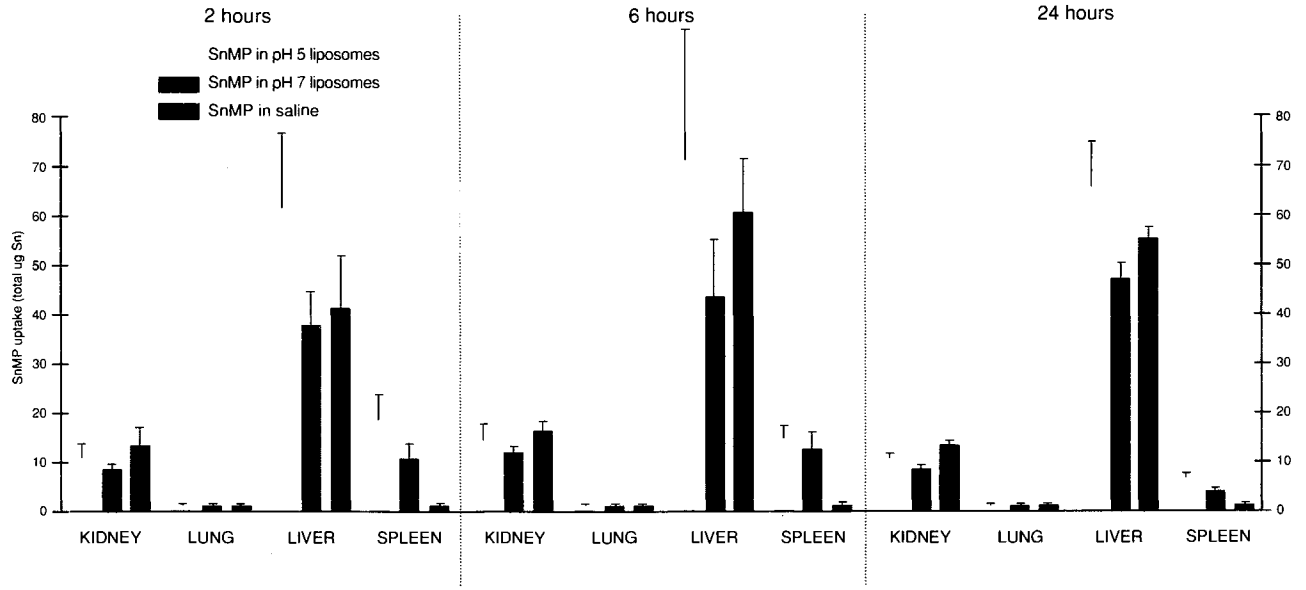

Fig. 2. Tissue distribution of tin mesoporphyrin administered to rats (10 mg/kg body weight dose) in liposomes or saline, showing total amount of drug in the organs. Results are mean ± SE.

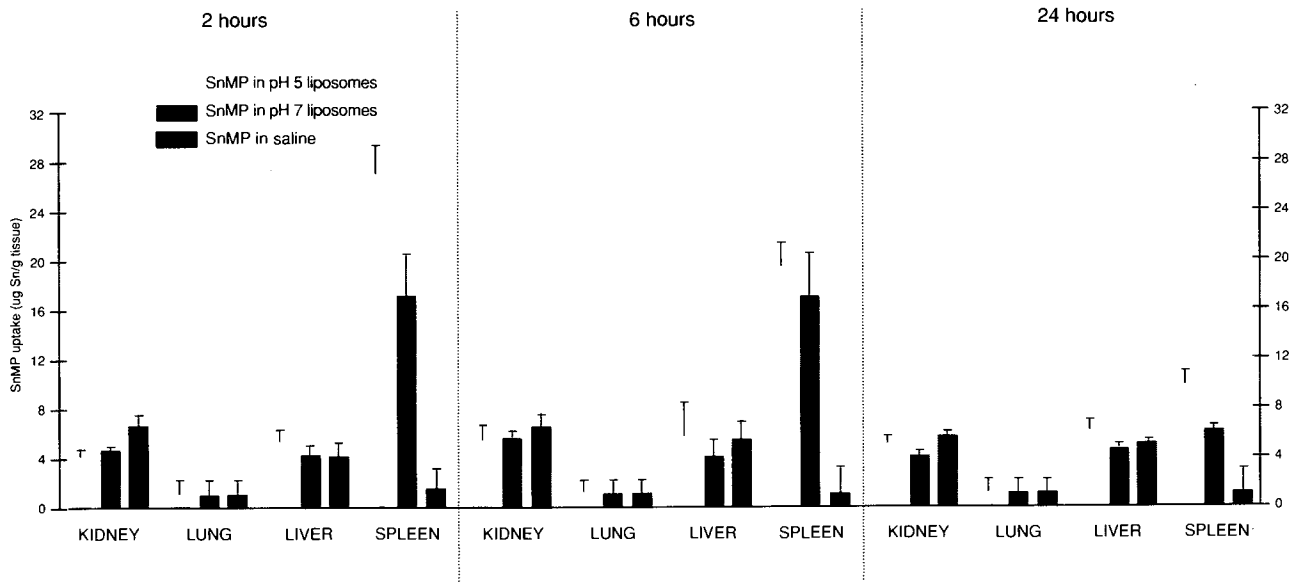

Fig. 3. Tissue distribution of tin mesoporphyrin administered to rats (10 mg/kg body weight dose) in liposomes or saline, showing drug concentration in tissue (μg Sn/g tissue). Results are mean ± SE.

pronounced around 2–6 hr, but was still apparent at 24 hr. The fact that the liposomes reconstituted at pH 5 appeared to be more efficient in distributing drug to the spleen than those reconstituted at pH 7 (Fig. 3) is consistent with the respective encapsulation efficiencies and reflects the fact that the pH 7 formulation could have contained 50% unencapsulated drug. A mass balance was also estimated from the data; total recovery of drug in the four organs examined ranged as follows: pH 5 liposomes, 20–24%; pH 7 liposomes, 15–17%; and SnMP in saline, 14–19%.

Bile-Cannulated Rat Experiments. To confirm the possibility that an altered distribution to the spleen would lead to a greater reduction of bilirubin output, samples of lyophilized SnMP liposomes were examined for their effect on heme oxygenase activity and on bilirubin production in bile-cannulated rats. The results for liposomes reconstituted at pH 5 and pH 7 are shown in Table II. Previous results with a nonlyophilized liposomal formulation prepared by sonication (8) are also shown for comparison. Drug uptake by tissues was measured by a fluorometric technique (8). Uptake by spleen 16 hr after administration of liposomal SnMP was four to five times higher than that with aqueous SnMP, confirming the tissue distribution results based on the atomic absorption method of analysis described above. Liposomal SnMP was significantly more efficient than aqueous SnMP in decreasing bilirubin production. For example, administration of 1.6 μmol/kg body weight liposomal SnMP resulted in about a 35% reduction in bilirubin output, while a dose of 10 μmol/kg body weight aqueous SnMP is required for the same effect (7). Both pH 5 and pH 7 liposomes were efficacious in inhibiting spleen and liver heme oxygenase and in decreasing bilirubin production 16 hr after administration, although the

Table II. Efficacy of Tin Mesoporphyrin Liposomes in Bile-Cannulated Rats[a]

Formulation	Dose (μmol/kg)	Sn porphyrin (pmol/mg protein)		Heme oxygenase (nmol/mg/hr)		Bilirubin production (%)
		Spleen	Liver	Spleen	Liver	
Saline control	0	0	0	13.8 ± 2.6	7.1 ± 1.6	100
Aqueous SnPP	10	56	150	3.6	0.6	75
	50	93	280	5.3	0.4	
Aqueous SnMP	1.6	56 ± 12	48 ± 4	6.1 ± 0.7	1.4 ± 0.2	84
	4.0	142 ± 35	101 ± 13	5.7 ± 1.1	1.6 ± 0.3	66
	8.0	162 ± 29	129 ± 15	5.5 ± 0.7	1.2 ± 0.3	61
Lyophilized SnMP liposomes (pH 5)	1.6	296 ± 119	76 ± 16	5.4 ± 1.0	1.9 ± 0.6	66
	4.0	349 ± 34	75 ± 8	4.1 ± 0.9	1.0 ± 0.2	56
Lyophilized SnMP liposomes (pH 7)	1.6	198 ± 23	46 ± 6	6.4 ± 0.9	1.3 ± 0.3	61
	4.0	307 ± 47	111 ± 17	2.9 ± 0.5	1.3 ± 0.1	53
Liposomal SnPP (sonicated)	14	860	210	0.4	0.9	—
Liposomal SnMP (sonicated)	10	1200	230	<0.4	0.5	—
	8.0	—	126 ± 15	0.5 ± 0.3	0.6 ± 0.2	42

[a] Results are mean ± SE.

latter probably had 50% unencapsulated drug when injected. Because of the higher encapsulation of the liposomes reconstituted at pH 5, these would probably be more desirable for clinical use, depending on stability at this pH.

The tissue distribution and bile cannulation studies demonstrate that the liposomal formulations target SnMP to the spleen. The high concentration of RES cells in the spleen relative to other organs results in the spleen having the highest apparent drug uptake per gram of tissue. Since the liver is much larger in size, however, this organ has the highest total uptake of SnMP. The results are consistent with other reports on liposome distribution *in vivo* (21), and it is apparent that the liposomal delivery system controls the distribution of SnMP to tissues. Interestingly, the distribution of SnMP to the liver is not significantly altered by encapsulation in liposomes. The mechanism of uptake of aqueous SnMP by liver is unknown but may be mediated by porphyrin receptors or may involve delivery by albumin (22).

Liposomal delivery systems for several other porphyrins have been examined. Hemin, which is used for treatment of porphyrias, provides an interesting contrast to SnMP. Serum albumin has a high affinity for hemin ($K_D = 10^{-8}M$) and thus removes hemin from liposomes immediately upon injection, with the result that hemin distribution to tissues does not change when injected in liposomes, regardless of lipid composition (23). The affinity of SnMP for albumin is much lower ($K_D = 10^{-5}M$) (24), and hence the drug is retained in liposomes and targeted to the spleen and liver. Liposomal delivery systems have also been examined for porphyrins used for photodynamic therapy of tumors (e.g., hematoporphyrin and its derivatives), and it appears that liposomes increase their uptake by tumors (25). Liposomal targeting could also be extended to other heme oxygenase inhibitors.

In conclusion, the results of this study provide preliminary indications that targeting of heme oxygenase inhibitors such as SnMP to the spleen is commercially feasible. While further stability, sterility, and toxicity studies would be required for clinical development, there is promise that the procedures described herein could be used to prepare a safe and sterile formulation with a long shelf life (in the dry lyophilized state) for clinical use.

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REFERENCES

1. G. S. Drummond and A. Kappas. Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxidation. *Proc. Natl. Acad. Sci. USA* 78:6466–6470 (1981).
2. A. Kappas, G. S. Drummond, C. S. Simionatto, and K. E. Anderson. Control of heme oxygenase and plasma levels of bilirubin by a synthetic heme analogue, tin-protoporphyrin. *Hepatology* 4:336–341 (1984).
3. A. Kappas and G. S. Drummond. Control of heme metabolism with synthetic metalloporphyrins. *J. Clin. Invest.* 77:335–339 (1986).
4. A. Kappas, G. S. Drummond, T. Manola, S. Petmezaki, and T. Valaes. Sn-Protoporphyrin use in the management of hyperbilirubinemia in term newborns with direct Coombs-positive ABO incompatibility. *Pediatrics* 81:485–497 (1988).
5. (a) W. R. Porter and K. Brandt. Kinetics of decomposition of tin protoporphyrin in aqueous solution at neutral pH (Abstract No. G04-X-05). *J. Pharm. Sci.* 76(11):S146 (1987). (b) W. R. Porter and K. Brandt. Chemistry of tin protoporphyrin in aqueous solution (Abstract No. G04-X-06) *J. Pharm. Sci.* 76(11):S146 (1987).
6. W. R. Porter, J. Bauer, and B. Norris. Unpublished results.
7. G. S. Drummond, R. A. Galbraith, M. K. Sardana, and A. Kappas. Reduction of the C_2 and C_4 vinyl groups of Sn-protoporphyrin to form Sn-mesoporphyrin markedly enhances the ability of the metalloporphyrin to inhibit in vivo heme catabolism. *Arch. Biochem. Biophys.* 255:64–74 (1987).
8. S. A. Landaw, G. S. Drummond, and A. Kappas. Targeting of heme oxygenase inhibitors to the spleen markedly enhances their ability to diminish bilirubin production. *Pediatrics* 84:1091–1096 (1989).
9. L. Saunders, J. Perrin, and D. B. Gammack. Ultrasonic irradiation of some phospholipid sols. *J. Pharm. Pharmacol.* 14:567–572 (1962).
10. A. L. Weiner, J. B. Cannon, and P. Tyle. Commercial approaches to the delivery of macromolecular drugs with liposomes. In M. Rossoff (ed.), *Controlled Release of Drugs: Polymers and Aggregate Systems*, VCH, New York, 1989, p. 233.
11. L. D. Mayer, M. J. Hope, and P. R. Cullis. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta* 858:161–168 (1986).
12. H. G. Weder and O. Zumbuehl. The preparation of variably sized homogenous liposomes for laboratory, clinical, and industrial use by controlled detergent dialysis. In G. Gregoriadis (ed.), *Liposome Technology, Vol. I. Preparation of Liposomes*, CRC Press, Boca Raton, FL, 1984, pp. 79–107.
13. (a). E. Mayhew, R. Lazo, W. J. Vail, J. King, and A. M. Green. Characterization of liposomes prepared using a microemulsifier. *Biochim. Biophys. Acta* 775:169–174 (1984). (b). S. Vemuri, C. Yu, V. Wangsatorntanakun, and N. Roosdorp. Large scale production of liposomes by a microfluidizer. *Drug Dev. Ind. Pharm.* 16:2243–2256 (1990).
14. N. I. Payne, P. Timmins, C. V. Ambrose, M. D. Ward, and F. Ridgway. Proliposomes: A novel solution to an old problem. *J. Pharm. Sci.* 75:325–329 (1986).
15. M. B. Bally, M. J. Hope, C. J. A. Van Echteld, and P. R. Cullis. Uptake of safranine and other lipophilic cations into vesicles in response to a membrane potential. *Biochim. Biophys. Acta* 812:66–76 (1985).
16. D. J. A. Crommelin and E. M. G. Van Bommel. Stability of liposomes on storage: Freeze dried, frozen or as an aqueous dispersion. *Pharm. Res.* 1:159–163 (1984).
17. L. M. Crowe, J. H. Crowe, A. Rudolph, C. Womersley, and L. Appel. Preservation of freeze-dried liposomes by trehalose. *Arch. Biochem. Biophys.* 242:240–247 (1985).
18. C. Kirby and G. Gregoriadis. Dehydration-rehydration vesicles: A simple method for high yield entrapment in liposomes. *Biotechnology* 2:979–984 (1984).
19. J. Bauer, C. Linton, and B. Norris. Stability-indicating high-performance liquid chromatographic analysis of tin protoporphyrin and other free acid metalloporphyrins. *J. Chromatogr.* 445:429–432 (1988).
20. J. B. Cannon, F.-S. Kuo, R. F. Pasternack, N. M. Wong, and U. Muller-Eberhard. Kinetics of the interaction of hemin liposomes with heme binding proteins. *Biochemistry* 23:3715–3721 (1984).
21. (a) T. M. Allen. Interactions of liposomes and other drug carriers with the mononuclear phagocyte system. In G. Gregoriadis (ed.), *Liposomes as Drug Carriers: Recent Trends and Prog-*

ress, John Wiley & Sons, Chichester UK, 1988, pp. 37–50. (b) J. H. Senior. Fate and behavior of liposomes in vivo: A review of controlling factors. *Crit. Rev. Ther. Drug. Carrier Syst.* 3:123–193 (1987).
22. U. Muller-Eberhard and H. Nikkila. Transport of tetrapyrroles by proteins. *Semin. Hematol.* 26:86–104 (1989).
23. J. B. Cannon, F.-S. Kuo, F. Vatandoust, H. H. Liem, and U. Muller-Eberhard. The effect of metalloporphyrins and heme liposomes on aminolevulinate synthase activity in rat liver. *Biochem. Biophys. Res. Commun.* 130:306–312 (1985).
24. E. Breslow, R. Chandra, and A. Kappas. Biochemical properties of the heme oxygenase inhibitor, Sn-protoporphyrin. *J. Biol. Chem.* 261:3135–3141 (1986).
25. G. Jori, E. Reddi, I. Cozzani, and L. Tomio. Controlled targeting of different subcellular sites by porphyrins in tumor-bearing mice. *Br. J. Cancer* 53:615–621 (1986).