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Combination of antitumor ether lipid with lipids of complementary molecular shape reduces its hemolytic activity

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Abstract

Because the therapeutic use of the antitumor ether lipid 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine (ET-18-OCH_3) is restricted by its hemolytic activity we explored the use of lipid packing parameters to reduce this toxicity by creating structurally optimized ET-18-OCH_3 liposomes. We postulated that combination of ET-18-OCH_3, which is similar in structure to lysophosphatidylcholine, with lipid molecules of complementary molecular shape (opposite headgroup/chain volume) would likely yield a stable lamellar phase from which ET-18-OCH_3 exchange to red blood cell membranes would be curtailed. To quantitate the degree of shape complementarity, we used a Langmuir trough and measured the mean molecular area per molecule (MMAM) for monolayers comprised of ET-18-OCH_3, the host lipids, and binary mixtures of varying mole percentage ET-18-OCH_3. The degree of complementarity was taken as the reduction in MMAM from the value expected based on simple additivity of the individual components. The greatest degree of shape complementarity was observed with cholesterol: the order of complementarity for the ET-18-OCH_3-lipid mixtures examined was cholesterol $>>$ DOPE $>$ POPC $\approx$ DOPC. Phosphorus NMR and TLC analysis of aqueous suspensions of ET-18-OCH_3 (40 mol%) with the host lipids revealed them to all be lamellar phase. For ET-18-OCH_3 at 40 mol% in liposomes, the hemolytic activity followed the trend of the reduction in MMAM and was least for the ET-18-OCH_3/cholesterol system ($H_{50} = 661$ μM ET-18-OCH_3) followed by ET-18-OCH_3/DOPE ($H_{50} = 91$ μM) and mixtures with POPC and DOPC which were comparable at $H_{50} = 26$ μM and 38 μM, respectively: the H_{50} concentration for free ET-18-OCH_3 was 16 μM. This experimental strategy for designing optimized liposomes with a reduction in exchange, and hence toxicity, may be useful for other amphipathic/lipophilic drugs that are dimensionally compatible with lipid bilayers. © 1997 Elsevier Science B.V.

Keywords: Liposome; ET-18-OCH_3; Monolayer; Lipid toxicity

Abbreviations: ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine; PC, phosphatidylcholine; DOPC, dioleoyl-PC; POPC, 1-palmitoyl-2-oleoyl-PC; DOPE, dioleoyl-phosphatidylethanolamine; MMAM, mean molecular area per molecule; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; H_{50}, concentration at which 50% hemolysis occurs; mN/m, milli-Newton per meter; CMC, critical micellar concentration

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1. Introduction

The ether lipid 1-*O*-octadecyl-2-*O*-methyl-sn-glycero-3-phosphorylcholine (ET-18-OCH_3) has demonstrated antitumor activity in both in vitro and in vivo studies [1–3] and has undergone evaluation clinically [4]. Although the mechanisms (likely multifactorial) for the tumoricidal activity of ET-18-OCH_3 in vivo remain unclear it is well established that for i.v. administration hemolysis is a primary dose-limiting toxicity [4].

The disruptive effect of ET-18-OCH_3 upon red cells may be explained for the most part by its structural similarity to lysophosphatidylcholine (lysoPC). In solution ET-18-OCH_3 organizes into micelles above a critical concentration of 0.5 μM [5]. In these dynamic structures ET-18-OCH_3 is free to exchange with biological membranes where it likely disrupts lipid–lipid and lipid–protein packing.

One approach to reduce the availability, and hence toxicity, of amphipathic compounds has been to incorporate these compounds into lipid based carrier systems (i.e., liposomes [6], lipid complexes [7,8]). To successfully incorporate ET-18-OCH_3 into liposomal membranes, its detergent nature, derived from its molecular shape, must be considered in order to choose the appropriate carrier lipid(s). The molecular shape (i.e., volume and cross-sectional area of polar to nonpolar regions) of lipids dictates whether they form micelles, lamella, or inverted micellar (hexagonal I and II) structures [9]. Several investigators have shown that lysoPC, which adopts micellar structure, forms lamellar structures when combined with lipids of opposite polar/nonpolar shape due to complementary side-by-side packing [10–13].

In this study, we examined ET-18-OCH_3 in combination with lipids expected to complement its molecular shape in order to design a stable liposome system from which the exchange of ET-18-OCH_3 would be curtailed. To assess side-by-side packing we used a Langmuir trough to determine the mean molecular area per molecule (MMAM) for mixtures of ET-18-OCH_3 with other lipids in monolayer films. Considering the reduction in MMAM (degree of shape complementarity), we formulated liposomes with ET-18-OCH_3 and host lipid (40 mol% ET-18-OCH_3) and found a correlation between the shape complementarity of the monolayer films and the hemolytic activity of the corresponding liposome systems. Importantly, the monolayer work indicated that ET-18-OCH_3 experienced significantly greater shape complementarity, concomitant with a much reduced hemolytic activity, when combined with cholesterol than when combined with dioleoylphosphatidylethanolamine (DOPE), a scenario not expected from previous literature as both lipids form lamellar structures with lysoPC [9–13]. This strategy to measure shape complementarity may be useful for the incorporation of other amphipathic/lipophilic compounds into liposome/lipid-based carrier systems, especially where reasonably high drug to lipid ratios are desired in order to reduce lipid dosing.

2. Materials and methods

2.1. Materials

All phospholipids were purchased from Avanti Polar Lipids (Alabaster, Alabama). ET-18-OCH_3 was purchased from either Avanti Polar Lipids or from Alexis Corporation of San Diego, California (used only for the NMR studies). Our own testing indicated that both materials were $>$ 99% pure (HPLC, data not shown). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO). All lipids and reagents were of the highest purity available.

2.2. Monolayer studies

The mean molecular area per molecule (MMAM) of ET-18-OCH_3 formulations was determined using a Langmuir-Blodgett mini-trough equipped with a dual barrier mechanism and a Wilhelmy balance, all from KSV Instruments (Trumbull, Connecticut). ET-18-OCH_3 formulations were prepared from lipid powders that were dissolved in a solvent system of hexane:ethanol 9:1, assayed and then combined. Single lipid solutions and mixtures were made to contain an overall lipid concentration of approximately 1.5 mM which was quantitated precisely by phosphate [14] and cholesterol assays [15]. Monolayers were formed by drop-wise addition of sample on to the aqueous surface (10 mM Hepes, 150 mM NaCl buffer, pH 7.2) at the center of the trough. Sample was added until the surface pressure just started to rise

above zero, at which time the exact volume of sample added was recorded. After waiting 3 min for solvent evaporation and the monolayer to stabilize, the surface area was reduced at a rate of 15 cm^2/min and surface pressure recorded. The solution level was maintained 1 mm below the top edge of the trough to avoid leakage; contact was maintained with the barriers (composed of Delrin) due to their hydrophilic nature. The MMAM obtained by extrapolating the steepest portion of the pressure-area curve to the value at zero surface pressure represented the value A_o. The highest collapse pressure measured for any lipid in this study ($\leq$ 47 mN/m) was well below the values we were able to attain with disaturated PC molecules ($>$ 62 mN/m), indicating that it was not an artifactual consequence of a technical limitation. All experiments were done at 23°C.

2.3. Liposome preparation

Liposomes were prepared by first mixing the appropriate lipids in solvent, drying the lipid to a thin film using vacuum rotary evaporation, and hydrating the resulting film with buffer solution (10 mM Hepes, 150 mM NaCl, pH 7.4). The resulting dispersions were used without further processing for NMR studies. For hemolysis studies, the lipid dispersions were then repeatedly extruded through polycarbonate Nuclepore membranes (Nuclepore Corp., Pleasanton, CA) of defined pore size. Liposomes were first passed 10 times through a membrane of 0.2 μm pore size and then passed 10 times through a membrane of 0.1 μm pore size. For ET-18-OCH_3/cholesterol, the mixtures were first passed through a 0.4 μm pore size filter 10 times before proceeding to the 0.2 μm pore size filter and finally the 0.1 μm pore size filter. Liposome size was determined by quasi-elastic light scattering using a Nicomp Model 270/370 Submicron Particle Sizer from Pacific Scientific (Menlo Park, CA); mean diameters via a number weighted distribution are reported.

2.4. Hemolysis

Hemolysis was assessed as previously described [16]. Each sample was serially diluted with buffer solution and 0.3 ml of each diluted solution was mixed with 0.3 ml of washed human red blood cells [4% in PBS (v/v)]. For controls, 0.3 ml of the red blood cell suspension was mixed with either 0.3 ml of buffer solution (negative hemolysis control) or 0.3 ml of distilled water (positive hemolysis control). Samples and standards were placed in a 37°C incubator and agitated for 20 h. Tubes were centrifuged at low speed ($2000 \times g$) for 10 min to pellet RBCs. Two hundred μl of the supernatant was removed and mixed with 1 ml of water. Hemolysis was quantitated by absorbance at 550 nm using a Shimadzu 2101 UV-Vis Scanning Spectrophotometer (Shimadzu Corp., Kyoto, Japan). One hundred percent hemolysis was defined as the maximum amount of hemolysis obtained from free ET-18-OCH_3.

2.5. Phosphorus nuclear magnetic resonance (NMR) spectroscopy

Spectra were obtained at 121.51 MHz on a Bruker AC300 NMR Spectrometer (Bruker Instruments, Billerica, MA) at either 23°C or 37°C. 16K points were acquired with a sweep width of 42 kHz using a 6 μs 90 degree pulse. The average of 3500 acquisitions was processed with 50 Hz line broadening. Samples contained a total of 38 mM ET-18-OCH_3 (approximately 60 mg/ml total lipid) except for the ET-18-OCH_3/cholesterol mixtures which contained 76 mM ET-18-OCH_3 (60–90 mg/ml total lipid).

2.6. Thin-layer chromatography (TLC)

Aqueous samples were first mixed with methanol and chloroform in the ratio 0.8:2:1 to form a single monophase. An aliquot of this was then applied to a silica gel plate which was run in chloroform/methanol/water (65:25:4) and then developed with iodine vapor. Standards of each lipid were also run.

3. Results and discussion

To design a liposomal membrane that will accommodate ET-18-OCH_3 and minimize its exchange to other membranes, the molecular shape of ET-18-OCH_3 and the host lipid must complement one another or other than ideal side-by-side packing will occur (see Fig. 1). As discussed by Kumar [9],

molecular shape can be described theoretically based upon geometry, hydration, molecular motion, and other considerations. A dimensionless packing parameter ($S = V/al$) has been described which defines lipid molecules in terms of headgroup area at the interface (a) and the volume (V) and length (l) of the hydrocarbon region [9,17]. Because it has a phosphocholine headgroup and a single chain, ET-18-OCH_3 would be characterized by this analysis as having an inverted cone shape, like lysoPC ($S < 0.74$), and is therefore expected to form micelles, which it does (CMC = 0.5 μM, [5]). Phosphatidylcholine (PC), for example, is considered to be cylindrical ($S \approx 0.75–1$), consistent with its ability to form lamella, and DOPE is considered cone-shaped ($S > 1$), thus explaining why it forms inverted micellar structures (hexagonal II phase) [18].

To determine which lipids would combine best with ET-18-OCH_3, and in what molar ratio, we employed a Langmuir trough to measure mean molecular area per molecule for lipids deposited as a monolayer film. As illustrated in Fig. 1, for those lipids that would complement the shape of ET-18-OCH_3 (cone), the MMAM for the mixture would be less than that expected from simple addition of the values for the individual components. That is, the expected MMAM (A_0) based on additivity can be expressed for a two component system as

$$A_0 = X_1 \cdot A_1 + X_2 \cdot A_2 \quad (1)$$

where X is the mol fraction of each component and A_1 and A_2 are the MMAMs measured for the individual lipids [19]. The magnitude of the deviation from this value then reflects the degree to which shape complementarity occurs (inclusive would be motional restrictions, changes in hydration, etc.).

First we obtained the area versus surface pressure profiles for ET-18-OCH_3 and several lipids we wished to test [cholesterol, 1-palmitoyl-2-oleoyl-PC (POPC), dioleoyl-PC (DOPC), and DOPE]. Cholesterol and DOPE were chosen because both have an inverted cone-shape and it had been shown previously from NMR and X-ray studies that those lipids form lamellar phase structures when mixed with lysoPC [10–12]. The area-pressure diagrams are shown in Fig. 2. As the surface area was reduced surface pressure increased as the lipids packed into an organized oriented monolayer, each characteristic for that lipid. At high surface pressure, collapse of monolayers occurred where material was lost into the aqueous subphase. As expected, the profile for ET-18-OCH_3 was similar to that observed for lysoPC [20] with a collapse pressure (37 mN/m) lower than that observed for the other lipids. MMAMs (A_0) were determined for each lipid by extrapolation of the steepest portion of the curves to the value at zero surface pressure. These values are listed in the legend to Fig. 2.

We next measured MMAMs for ET-18-OCH_3 mixed at various mole ratios with each of the other

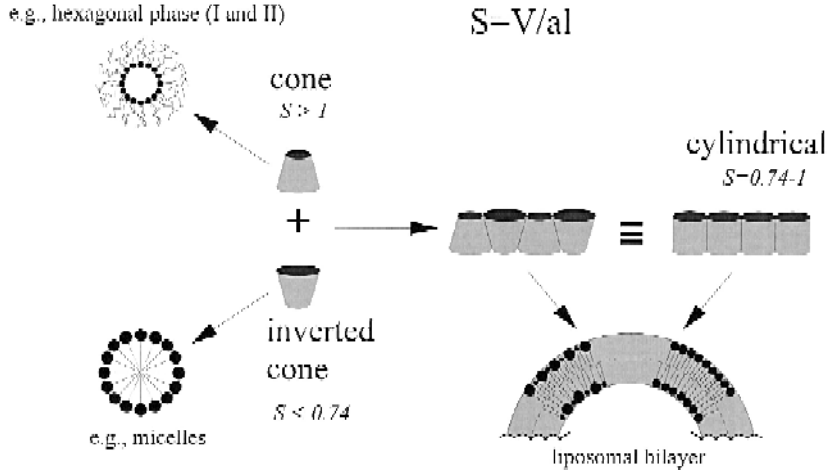

Fig. 1. Schematic representation of how molecular shape affects collective organization. Dark regions symbolize polar domains. The packing parameter (S) relates the area of the polar headgroup (a) and the volume (V) and length (l) of the hydrocarbon region (see Kumar [9] for further details).

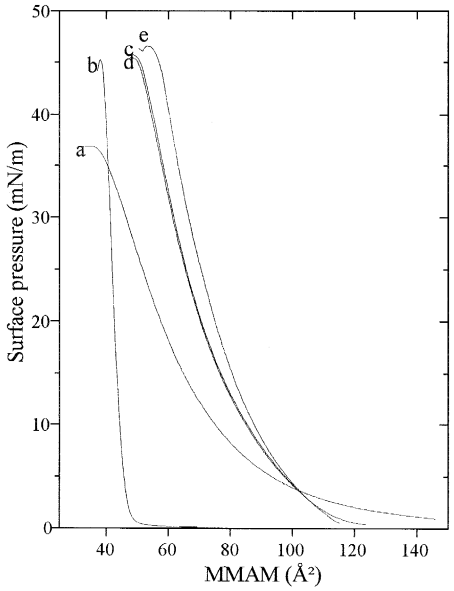

Fig. 2. Surface pressure versus mean molecular area per molecule for monolayer films of individual lipids. Lipids are ET-18-OCH_3 (a), cholesterol (b), POPC (c), DOPC (d), and DOPE (e). Films were compressed at 15 cm^2/min. Each curve is the average of three compressions. A_0 values (determined as described in Section 2.2) were 77.8 $Å^2$ for ET-18-OCH_3, 44.8 $Å^2$ cholesterol, 81.7 $Å^2$ for POPC, 82.0 $Å^2$ for DOPC, and 82.3 $Å^2$ for DOPE.

lipids (Fig. 3). From the collapse pressures and pressure-area curves there appeared to be complete miscibility for these binary systems. For ET-18-OCH_3/cholesterol mixtures, the deviation from A_0 (represented as the dashed line) was significant with a MMAM that was ~ 25 $Å^2$ less than that expected for ET-18-OCH_3 and cholesterol at a 1:1 molar ratio (Fig. 3, top). Shown in Fig. 3 (bottom) are the differences between experimentally derived MMAM (A_{exp}) and A_0 expressed as a percentage of A_0:

$$\%\text{reduction} = \left(\frac{A_0 - A_{exp}}{A_0} \right) \cdot 100 \qquad (2)$$

The order for the percent reductions in MMAM for lipids mixed with ET-18-OCH_3 was cholesterol > DOPE > POPC > DOPC. These results were not surprising given that both DOPE and cholesterol possess an inverted cone shape [10,11]. However, what is surprising is the much larger reduction afforded the ET-18-OCH_3/cholesterol mixture as compared to ET-18-OCH_3/DOPE. Interestingly, for DOPE, DOPC, and POPC, the maximum reduction was noted at 40 mol% ET-18-OCH_3, while for cholesterol the profile was roughly symmetric about its maximum at 50 mol% ET-18-OCH_3.

With the degree of shape complementarity established, we next prepared liposome suspensions for assessment of hemolytic activity. First, we examined the phosphorus NMR signal of hydrated handshaken dispersions for the ET-18-OCH_3/lipid systems to confirm the existence of lamellar phase (see Fig. 4). The phosphorus NMR spectra for ET-18-OCH_3 at 40 mol% with both cholesterol and DOPE at 23°C and 37°C were typical of lamellar phase [21]. For DOPC and POPC mixtures with ET-18-OCH_3, there was an additional isotropic component on top of the broader lamellar signature. The spectrum resembled that for mixtures of small and large liposomes [22] and the

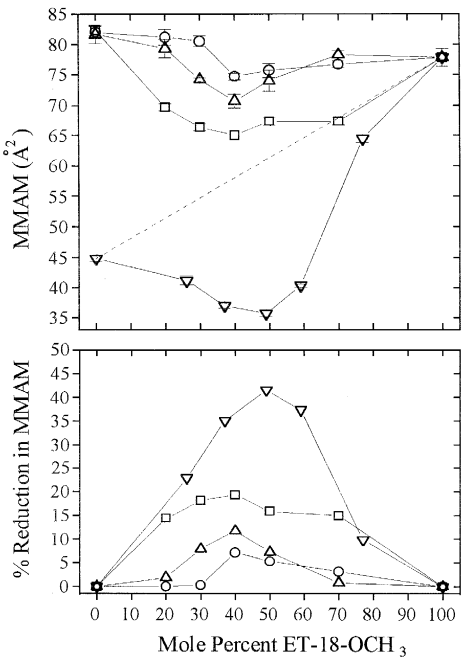

Fig. 3. Top panel: Mean molecular area per molecule as a function of ET-18-OCH_3 content where monolayers were formed from ET-18-OCH_3 mixed with DOPC (circles), POPC (up triangles), DOPE (squares), or cholesterol (inverted triangles). Data are the average of 3 experiments ± S.D. The dashed line represents the expected values (A_0) for cholesterol/ET-18-OCH_3 mixtures based on simple additivity of the individual values of MMAM (see text). The magnitude of the deviation from this curve indicates the degree of shape complementarity. A_0 curves for the other lipids were omitted for clarity. Bottom panel: Percent reduction in MMAM as a function of ET-18-OCH_3 (see Eq. (2)).

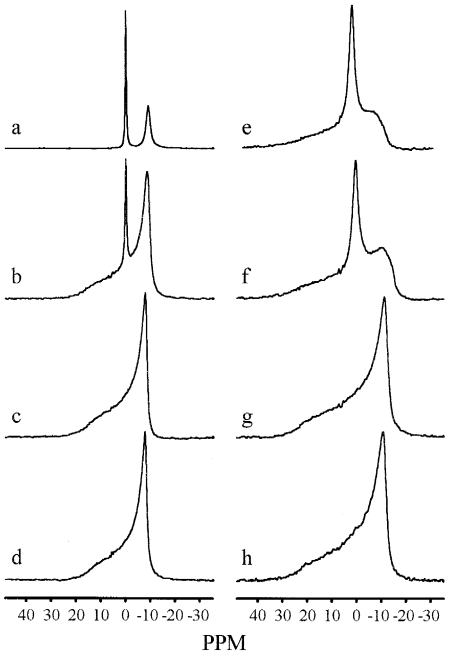

Fig. 4. Phosphorus NMR of ET-18-OCH_3 mixtures at various mol ratios with other lipids: (a) ET-18-OCH_3/cholesterol (60:40 mol ratio) at 23°C; (b) ET-18-OCH_3/cholesterol (50:50) at 23°C; (c) ET-18-OCH_3/cholesterol (40:60) at 23°C; (d) ET-18-OCH_3/cholesterol (40:60) at 37°C; (e) ET-18-OCH_3/POPC (40:60) at 23°C; (f) ET-18-OCH_3/DOPC (40:60) at 23°C; (g) ET-18-OCH_3/DOPE (40:60) at 23°C; and (h) ET-18-OCH_3/DOPE (40:60) at 37°C. The isotropic peak of (a) was assigned the value of 0 ppm.

line width appeared to be too broad (3.6–3.9 ppm at half height) to be ET-18-OCH_3 micelles [23]. However, we could not rule out large mixed micellar structures. To determine whether this component was due to mixed micellar structures or small vesicular structures, we collected the fraction that would not form a plug on top of the solution (the mixtures floated) upon centrifugation and examined the ratio of ET-18-OCH_3 to lipid by TLC (see Section 2 for details). The ratios (determined by TLC) were essentially the same as that for the bulk sample, indicating that the structures were small liposomes and not ET-18-OCH_3-enriched micellar structures (data not shown).

While the MMAM was comparable for ET-18-OCH_3 mixtures with cholesterol at 40, 50, and 60 mol% (Fig. 3B), NMR revealed only the 40 mol% ET-18-OCH_3 mixture to be completely lamellar (Fig. 4c). As the ET-18-OCH_3/cholesterol ratio was increased a narrow isotropic signal became more prominent. Given its narrow line width (0.48–0.95 ppm at half height) this signal most likely arose from micellar structures. In fact, previous work had indicated that the limit for lysoPC in lysoPC/cholesterol liposomes was near 50 mol% [23].

We next proceeded to make liposomes containing 40 mol% ET-18-OCH_3 for hemolysis testing. All liposomes were prepared by extrusion to assure uniform size distribution. While extrusion was more difficult (higher pressure required) for the ET-18-OCH_3/cholesterol mixture, which had a final size distribution (77 nm by light scattering – number weighting) slightly larger than that for the other samples (40 nm), the ratio of lipids in the final preparation was not different from that found prior to extrusion. Samples were next incubated with washed human red blood cells and hemolysis quantitated as described in Section 2. Shown in Fig. 5 are the hemolysis profiles for the four ET-18-OCH_3 liposome preparations, as well as free ET-18-OCH_3. Hemolysis was least for the ET-18-OCH_3/cholesterol formulation with the concentration of ET-18-OCH_3 that yielded 50% hemolysis (H_{50}) at 661 μM ET-18-OCH_3. This formulation was over 7 times less hemolytic than the next closest formulation which

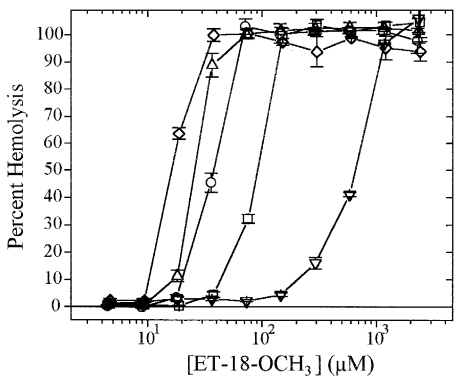

Fig. 5. Hemolytic activity of ET-18-OCH_3 incorporated in liposomes formed by extrusion through a 100 nm pore size filter. Symbols represent free ET-18-OCH_3 (diamonds), and ET-18-OCH_3 incorporated at 40 mol% into liposomes with the co-lipids POPC (circles), DOPC (up triangles), DOPE (squares), and cholesterol (inverted triangles). Each preparation was run in triplicate and the error bars represent S.D. Liposome size was measured by dynamic light scattering and found to be approximately 40 nm for samples except ET-18-OCH_3/cholesterol which had a mean diameter of 77 nm.

was ET-18-OCH_3/DOPE with a H_{50} of 91 μM. The DOPC and POPC ET-18-OCH_3 formulations were similar (H_{50} of 38 and 26 μM, respectively) and not far above the H_{50} value for free ET-18-OCH_3 (H_{50} = 16 μM).

We subsequently compared the hemolytic activity of ET-18-OCH_3 small unilamellar vesicles formulated at 40 or 60 mol% with cholesterol and found that the 60 mol% material was as hemolytic as free ET-18-OCH_3 (data not shown). This confirmed our suspicion that ET-18-OCH_3, like lysoPC [9,23], does not form stable liposome structures above a 1:1 stoichiometry. This also indicated that the degree of shape complementarity in planar monolayers in no way predicts how molecules organize in solution. For the 40 mol% ET-18-OCH_3 formulations examined here, however, lamellar structure was confirmed by ^{31}P-NMR.

While it was not surprising that the degree of shape complementarity, and hemolysis, for ET-18-OCH_3 with cholesterol and DOPE was better than that for ET-18-OCH_3 with POPC and DOPC, it was surprising that the cholesterol system was so significantly better than the DOPE system. Considering their similar theoretical packing parameters (S = 1.2–1.3 [9]), one might have expected both lipids to pack equally well with ET-18-OCH_3. However, one must keep in mind that the theoretical packing parameter does not consider the favorable interactive effects of mixing two different lipids. That is, it does not predict alterations in hydration, chain motion, etc., that one lipid might invoke in another or mutual alterations as a consequence of mixing. The contributions of these 'shape'-altering effects would, however, be measured experimentally, as we have demonstrated here. Because lamellar phase was observed at both 23° and 37°C for both cholesterol and DOPE formulations, we do not believe the differences between the two systems can be attributed to non-ideal mixing in the ET-18-OCH_3/DOPE system, as micellar (ET-18-OCH_3) or hexagonal II (DOPE) phase signatures would have been apparent.

In conclusion, because hemolysis is a significant toxicity for i.v. administration of ET-18-OCH_3 [4], liposomal incorporation would seem to be an ideal solution. However, without consideration of lipid packing the result might be a formulation with little or no gain over free drug (e.g., the POPC and DOPC systems examined here). Additionally, incorporation of drug into liposomes at relatively low mole percentages (e.g., 1–5 mol%) may present a problem later when total lipid dosing may become an issue. Here, we assessed side-by-side packing of ET-18-OCH_3 with lipids expected to complement its molecular shape using monolayers spread onto a Langmuir trough. For the systems studied, ideal packing was observed at relatively high mol percentages of ET-18-OCH_3 (40 mol%). We then compared the order of shape complementarity of the monolayer films, the reduction in MMAM, to the hemolytic activity of the corresponding ET-18-OCH_3 (40 mol%) liposomes and found a correlation between the two. Importantly, we identified a mixture with a significantly reduced hemolytic activity (ET-18-OCH_3/cholesterol, H_{50} 661 μM) as compared to free ET-18-OCH_3 (H_{50} 16 μM). Presumably this reduction in hemolysis reflects the ability of the carrier system to accommodate ET-18-OCH_3 thus curtailing its exchange to other membranes. Using shape complementarity, it may be possible to identify other lipid candidates for combination with ET-18-OCH_3 or incorporate other amphipathic/lipophilic drugs (e.g., ET-18-OCH_3 analogs [24]) into liposome/lipid based carrier systems with similar reductions in exchange (toxicity) for these agents.

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