Micelle/Monomer Control over the Membrane-Disrupting Properties of an Amphiphilic Antibiotic

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Abstract: A homologous series of amphotericin B-oligo(ethylene glycol) conjugates has been synthesized, via condensation of the carboxylic acid group of amphotericin B with oligo(ethylene glycol)-substituted amines [$NH_2(CH_2CH_2O)_nCH_3$, where n = 1, 6, 15], and examined with respect to their aggregation properties and their ability to disrupt the membrane integrity of *Candida albicans* and human red blood cells. Incremental increase in the size of the oligo(ethylene glycol) moiety leads to a significant increase in the critical micelle concentration of the macrolide and to a corresponding increase in the concentration that is required for hemolysis. In sharp contrast, the same increase in oligo(ethylene glycol) size affords only a modest reduction in antifungal activity; the net result is a separation of antifungal and hemolytic activity. These results clearly highlight the need for taking into account the aggregation properties of membrane-disrupting antibiotics with respect to target recognition and specificity and also with regard to rational molecular design.

Introduction

We have recently reported a mechanistic study of the disruption of lipid bilayers by two commonly used detergents, Triton X-100 and sodium deoxycholate.[1] Our principal finding was that surfactant monomer and micelles can disrupt compact bilayers (e.g., choleserol-rich phospholipid membranes) in fundamentally different ways. Specifically, we showed that attack by monomer resulted in a "leaky" bilayer but that attack by a corresponding micelle was much more catastrophic; i.e., membrane integrity was lost through a "rupture" process. In striking contrast, analogous targets that were loosely packed (e.g., fluid phospholipid bilayers that were devoid of sterol) were susceptible only toward leakage, regardless of whether the attack came in the form of micelles or monomer. Thus, we demonstrated that the combination of lipid packing and the aggregation state of the attacking surfactant controls the mechanism by which phospholipid membranes are disrupted. On the basis of these results, we hypothesized that rupture/leakage processes could have direct relevance to the biological action of those amphiphilic antibiotics that function by membrane disruption. Specifically, we proposed that micellar and monomeric forms of amphiphilic antibiotics could have different modes of action and very different selectivity features.

In order to test our hypothesis, we have chosen to examine amphotericin B (AmB) as a representative example. This polyene macrolide antibiotic, which is produced from soil bacterium, *Streptomycetes nodosus*, is known to function at the membrane level by "punching holes" in the plasma membrane of fungal cells.[2-5] The fact that AmB has a strong tendency to aggregate in solution (i.e., it has a very low critical micelle concentration) made it particularly attractive to us as a test case.[6-8] In principle, one may expect that a highly selective monomer attack could be hidden by a catastrophic and less discriminate attack by micelles.[1] Recent results by Bolard and co-workers, in fact, lend credence to such a proposal.[6,9]

Although AmB has been the subject of numerous studies, its supramolecular structure within lipid membranes remains to be defined. In the most commonly accepted model, cyclic arrays of the macrolide plus intercalated sterol create water-filled pores, i.e., the "barrel stave" model.[10] Subsequent alignment of two such pores across the plasma membrane, or a thinning of the membrane in the vicinity of a single pore, results in a channel through which ions and other vital cellular constituents are released. The slight preference for killing fungal cells is presumed to be due to stronger complexation with ergosterol (the predominant sterol found in fungal cell membranes) as compared with cholesterol (the predominant sterol found in mammalian cell membranes). Whether or not the monomeric form of the drug is intrinsically more selective in destroying fungi than the micellar form and whether such selectivity can be exploited by rational molecular design (i.e., monomer/micelle control via chemical modification) were the two key issues that we sought to clarify in the present study.

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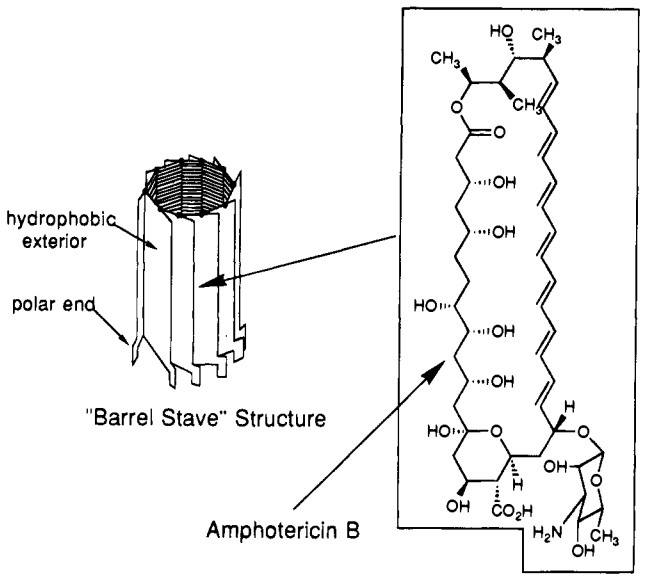

Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from commerical sources and used without further purification. House-deionized water was purified using a Millipore Milli-Q-filtering system containing one carbon and two ion-exchange stages. EM Science silica gel was generally used for chromatographic purification. Egg phosphatidylcholine (egg PC) was obtained from Avanti Polar Lipids (Birmingham, AL) as a chloroform solution. Vesicle extrusions were carried out using a Lipex Biomembrane apparatus (Vancouver, BC). Amphotericin B was purchased from Sigma Chem. Co. (St. Louis, MO) and used directly. All ^{1}H NMR spectra were recorded on a Bruker 360 MHz instrument; chemical shifts are reported in ppm and were referenced to residual solvents. UV spectra were recorded on a Milton Roy Spectronic 1201 spectrometer. Vesicles were prepared in phosphate-buffered saline (PBS, 155 mM NaCl, 7 mM Na_2HPO_4, and 3 mM KH_2PO_4 adjusted to pH 7.4) by extrusion using methods previously described.[1] The *cmc* of AmB and each of the conjugates were determined at 37 °C using PBS buffer. High resolution mass spectra were obtained at the Washington University Resource for Biomedical and Bio-organic Mass Spectrometry Center, St. Louis, MO. Antifungal activities (MIC values) were determined using three different strains of *Candida albicans*. All tests were done by broth microdilution in yeast nitrogen base-glucose. The initial inoculum size was 1×10^4 blastospores per mL. Trays were incubated at 37 °C for 24 h. Concentrations that were required for inducing 50% release of hemoglobin from 3×10^7 cells/mL, after incubation for 1 h at 37 °C are reported as K_{50} values. Specific procedures that were used for K_{50} and K^+ release determinations were similar to those previously described.[11]

Methoxyhexaethylene Glycol. To a suspension of NaH (60%) (0.72 g, 18 mmol) in 10 mL of dry tetrahydrofuran was added 10 mL of a solution of tetrahydrofuran containing hexaethylene glycol (4.24 g, 15 mmol) at 0 °C. Methyl iodide (1.12 mL, 18 mmol) was then added to the mixture in a dropwise fashion. The mixture was stirred at room temperature for 5 h and then quenched by addition of 0.5 mL of water. The product mixture was poured into 70 mL of water and extracted with CH_2Cl_2 (6×50 mL), and the combined extracts washed with 50 mL of a saturated NaCl solution and dried over anhydrous Na_2SO_4. The solvent was then removed under reduced pressure, and the residue was purified by column chromatography (silica gel) using $CHCl_3$/CH_3OH (9/1, v/v) as an eluent to give 1.72 g (39%) of methoxyhexaethylene glycol as an oily product, having ^{1}H NMR (360 MHz, $CDCl_3$) δ 2.8 (bs, 1 H), 3.38 (s, 3 H), 3.54−3.75 (m, 24 H).

Tosylate of Methoxyhexaethylene Glycol. To a solution that was made from 5 mmol of dry methoxyhexaethylene glycol and 5.6 mL of triethylamine (40 mmol) in 30 mL of dry tetrahydrofuran was added 10 mL of a tetrahydrofuran solution of *p*-toluenesulfonyl chloride (10 mmol). The mixture was stirred for 24 h at room temperature. The mixture was then filtered, and the solid was washed with 4×10 mL of tetrahydrofuran. The combined tetrahydrofuran solutions were then concentrated under reduced pressure. The residue was washed with 100 mL of *n*-hexane (three times for 30 minutes) in order to remove the excess of *p*-toluenesulfonyl chloride. The crude product was then purified by silica gel chromatography using CH_2Cl_2/CH_3OH (97/3, v/v) as eluent to give a 68% yield of the tosylate of methoxyhexaethylene glycol, having ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 3 H), 3.39 (s, 3 H), 3.67 (m, 22 H), 4.18 (t, 2 H), 7.36 (d, 2 H), 7.82 (d, 2 H).

Tosylate of Methoxypoly(ethylene glycol) (550). The tosylate of methoxypoly(ethylene glycol) 550 was prepared, directly, from commercial methoxypoly(ethylene glycol) (500) (Aldrich) using procedures similar to those used for the preparation of the corresponding hexaethylene glycol derivative, in 78% yield having ^{1}H NMR ($CDCl_3$) δ 2.46 (s, 3 H), 3.38 (s, 3 H), 3.65 (s, 47 H), 4.17 (t, 2 H), 7.35 (d, 2 H), 7.80 (d, 2 H).

ω-Aminohexaethyleneglycol Methyl Ether. The tosylate of methoxyhexaethylene glycol (5 mmol) and 1.85 g (10 mmol) of potassium phthalimidate were mixed with 20 mL of dry *N,N*-dimethylformamide and stirred for 3 h at 120 °C under a nitrogen atmosphere. The resulting mixture was diluted with 50 mL of CH_2Cl_2, and a white precipitate that was removed by filtration was washed with 50 mL of CH_2Cl_2. The combined organic solutions were washed sequentially, with 100 mL of 0.1 N NaOH and 100 mL of saturated NaCl and dried over anhydrous Na_2SO_4. Removal of solvent under reduced pressure afforded 1-phthalimidohexaethylene glycol methyl ether as an oily product that was used directly in the next step without further purification. Thus, after ca. 5 mmol of the crude 1-phthalimidohexaethylene glycol monomethyl ether was dissolved in 30 mL of ethanol and heated to the reflux temperature, 0.32 mL (10 mmol) of hydrazine was added. The mixture was then refluxed for 1.5 h under a nitrogen atmosphere, cooled to room temperature, and acidified by addition of a few drops of 6 N-HCl to pH 4. After additional refluxing (1.5 h), the product mixture was cooled to room temperature, and the phthalhydrazide that precipitated was removed by filtration and washed with 2×15 mL of ethanol. The combined organic solution was evaporated at 40 °C under reduced pressure, followed by addition of 40 mL of an ethanol solution of KOH (0.55 g, 10 mmol). The precipitated potassium tosylate was then removed by filtration and washed with ethanol (2×15 mL). The combined ethanolic solution was evaporated, followed by addition of 80 mL of $CHCl_3$. The yellowish solid that precipitated was removed by filtration and washed with $CHCl_3$ (2×20 mL). The combined chloroform solution was then extracted with 150 mL of 0.1 N HCl and 100 mL of water. The aqueous solution was then made basic with a 1 N NaOH solution (to pH 11) and extracted with methylene chloride (3×100 mL). Removal of solvent under reduced pressure, followed by purification by column chromatography [silica gel, CH_3OH/NH_4OH (30%) (9/1, v/v)], afforded a 54% yield (0.80 g) of ω-aminohexaethyleneglycol methyl ether having ^{1}H NMR ($CDCl_3$) δ 1.75 (s, 2 H), 2.84 (t, 2 H), 3.40 (s, 3 H), 3.47−3.54 (m, 4 H), 3.63 (m, 18 H).

ω-Aminopoly(ethyleneglycol) Methyl Ether (550). ω-Aminopoly(ethyleneglycol) methyl ether (550) was prepared, using procedures similar to those used for the synthesis of the corresponding hexaethylene glycol derivative, in 63% yield (2.06 g) having ^{1}H NMR ($CDCl_3$) δ 2.20 (s, 2 H), 2.90 (t, 2 H), 3.40 (s, 3 H), 3.65 (m, 47 H).

Amphotericin B Conjugate 1. Amphotericin B (Sigma) (69.3 mg, 60 μmol) was suspended in 2 mL of *N,N*-dimethylacetamide (DMA), stirred at room temperature, and then treated with 600 μmol of triethylamine, 600 μmol of diphenylphosphorylazide (DPPA), and 600 μmol of 2-methoxyethylamine (45.2 mg) in the dark under a nitrogen atmosphere. After 115 h, the reaction mixture was poured into 80 mL of ethyl ether. The crude product which precipitated was purified by solubilization and reprecipitation (four times) by use of CH_3OH/ethyl ether systems. The resulting pearl yellow solid (72.4 mg) was further purified by silica gel chromatography (two times) using CH_3OH/NH_4OH (30%) (9/1, v/v) as the eluent to give 37.1 mg (62%) of **1** having R_f 0.61 [EMS silica, CH_3OH/NH_4OH (30%) (95/5, v/v)]; UV−vis (CH_3OH) λ_{max} 406 (ϵ 0.99×10^5 M^{-1} cm^{-1}); ^{1}H NMR (360 MHz, CD_3OD) δ 0.98−1.90 (m, 25 H), 1.95−3.10 (m, 7 H), 3.37 (s, 3 H),

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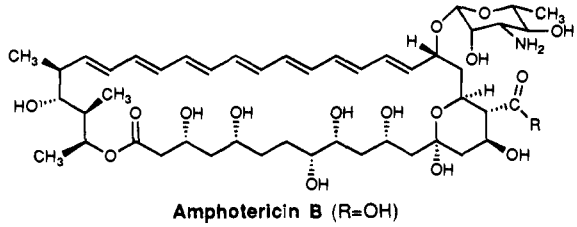
3.21−3.80 (m, 8 H), 3.80−4.42 (m, 8 H), 5.35 (m, 2H), 6.03−6.42 (m, 13 H); HRMS (FAB) calcd for $C_{50}H_{80}N_2O_{17}$ + Na, 1003.5354; found, 1003.5347 $(M + Na)^+$.

Amphotericin B Conjugate 2. Amphotericin B conjugate **2** was prepared, using procedures similar to those used for the preparation of **1**, in 33% yield having R_f 0.58 [EMS silica, CH_3OH/NH_4OH (30%) (9/1, v/v)]; UV−vis (CH_3OH) λ_{max} 406 (ϵ 1.29 × 10^5 M^{-1} cm^{-1}); IR (KBr) ν 1723 cm^{-1}(C=O, ester, lactone), 1665 cm^{-1} (C=O, amide); ^1H NMR (360 MHz, CD_3OD) δ 6.03−6.42 (m, 13 H), 5.35 (m, 2H), 3.80−4.42 (m, 8 H), 3.36−3.63 (m, 27 H), 3.34 (s, 3 H), 1.95−3.10 (m, 7 H), 0.98−1.70 (m, 25 H); HRMS (FAB) calcd for $C_{60}H_{100}N_2O_{22}$ + Na, 1223.6665; found, 1223.6681 $(M + Na)^+$. A large-scale synthesis of **2**, using similar procedures, afforded 11.0 g (61%) of the conjugate starting with 14.0 g of AmB.

Amphotericin B Conjugate 3. Amphotericin B conjugate **3** was prepared, using procedures similar to those that were used to prepare **1**, in 23% yield having R_f 0.32−0.56 [EMS silica, CH_3OH/NH_4OH (30%) (9/1, v/v); UV−vis (CH_3OH) λ_{max} 406 (ϵ 1.35 × 10^5 M^{-1} cm^{-1}). Based on the ratio of oligoether CH_2 protons (δ 3.65) to heptaene protons (δ 6.03−6.42), the average oligo(ethylene glycol) chain length is estimated to contain 15 ethylene glycol units.

Results and Discussion

Experimental Design. The experimental approach that we have taken in this study was to synthesize a homologous series of amphotericin B-oligo(ethylene glycol) conjugates (**1**−**3**) of varying critical micelle concentration (*cmc*) and to examine their ability to disrupt the integrity of representative fungal and mammalian cell membranes. Elongation of the oligo(ethylene glycol) segment was expected to increase the *cmc* of the macrolide by increasing its hydrophilicity, with minimal perturbation in overall molecular structure. Because these oligoether units are hydrophilic, we expected that they would simply extend the "head group" region of the molecule without altering the macrolide's ability to "recognize" ergosterol-rich bilayers. At submicellar concentrations, therefore, each conjugate was expected to destroy the integrity of fungal membranes via ergosterol-recognition and channel formation but not those of mammalian cells. In their micellar state, effective disruption of both fungal and mammalian membranes was anticipated. Thus, we hypothesized that *the antifungal activity of AmB should be separated from its activity toward mammalian cells by conjugation with oligo(ethylene glycol)s and that the magnitude of this separation would increase as the cmc of the conjugate increased.*



Amphotericin B (R=OH)

1, R= $NH(CH_2CH_2O)_1CH_3$
2, R= $NH(CH_2CH_2O)_6CH_3$
3, R= $NH(CH_2CH_2O)_{15}CH_3$

Target cells that were chosen for this study were human red blood cells (RBCs) and *Candida albicans*. Because of their lability, RBCs are routinely used to test the ability of a drug to damage the plasma membrane of mammalian cells.[11] Typically, such activity is measured as the concentration that is required to induce the release of 50% of the hemoglobin that is contained within a fixed concentration of cells, i.e., the drug's K_{50} value.[11] A common measure of antifungal activity is a compound's minimum inhibitory concentration, MIC, i.e., the minimum concentration of the drug that is needed to completely inhibit fungal growth over a fixed period of time.[11] In the work that is described herein, we have chosen *Candida albicans* as a representative fungal target because of its therapeutic importance.

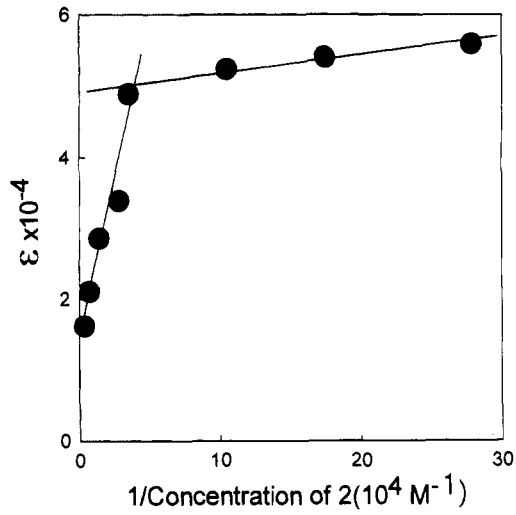


Figure 1. Plot of molar absorptivity (λ_{max} 409 nm) as a function of the reciprocal concentration of **2** in PBS at 37 °C.

Synthesis of Amphotericin B-Oligo(ethylene glycol) Conjugates. Condensation of AmB with ω-amino-hexaethyleneglycol methyl ether [$NH_2(CH_2CH_2O)_6CH_3$] in *N,N*-dimethylacetamide (DMA), using diphenylphosphorazidate (DPPA) as the coupling agent, afforded conjugate **2**; analogous coupling with a shorter and a longer amino-substituted ether afforded **1** and **3**, respectively.[12] The specific amino-oligoethers that were used to prepare **2** and **3** were synthesized by standard Gabriel methods, as described in the Experimental Section. One striking feature that distinguishes AmB and **1**, from **2** and **3**, is their water-solubility. In contrast to AmB and **1**, which have extremely low solubility in water (<10 μg/mL), conjugates **2** and **3** gave clear solutions at concentrations of 10 000 and >30 000 μg/mL, respectively.

Aggregation Properties of the Surfactants. In its monomeric state, AmB is known to have a characteristic absorption band at 409 nm.[6] Upon aggregation, the apparent molar absorptivity of this band decreases in magnitude.[6] If we let T, m, and P represent the total, the monomeric, and the micellar concentrations (i.e., the concentration of the drug that is present in micellar form) of the macrolide, respectively, and if ϵ represents the apparent molar absorptivity and ϵ_m and ϵ_p are the molar absorptivity for the monomeric and micellar components, respectively, then it can be readily shown that $\epsilon = \epsilon_p + (\epsilon_m - \epsilon_p)m/T$. At concentrations that are in excess of the cmc, m is constant, and ϵ is expected to be inversely proportional to T. Thus, by measuring apparent molar absorptivity as a function of the reciprocal of the macrolide concentration, one can estimate its *cmc* from the intercept of two straight lines. A typical plot that was used for the determination of the *cmc* of **2** is shown in Figure 1. By use of such methods, the *cmc* for values for AmB, **1**, **2**, and **3** are estimated to be 1.1, 4.4, 25, and 50 μM, respectively. Thus, as expected, elongation of the oligo(ethylene glycol) segment results in a continuous increase in the macrolide's *cmc*. It is noteworthy that the *cmc* for AmB is in reasonable agreement with previously reported values that have been estimated by light scattering (0.63 μM) and by circular dichroism (0.30 μM).[6,8]

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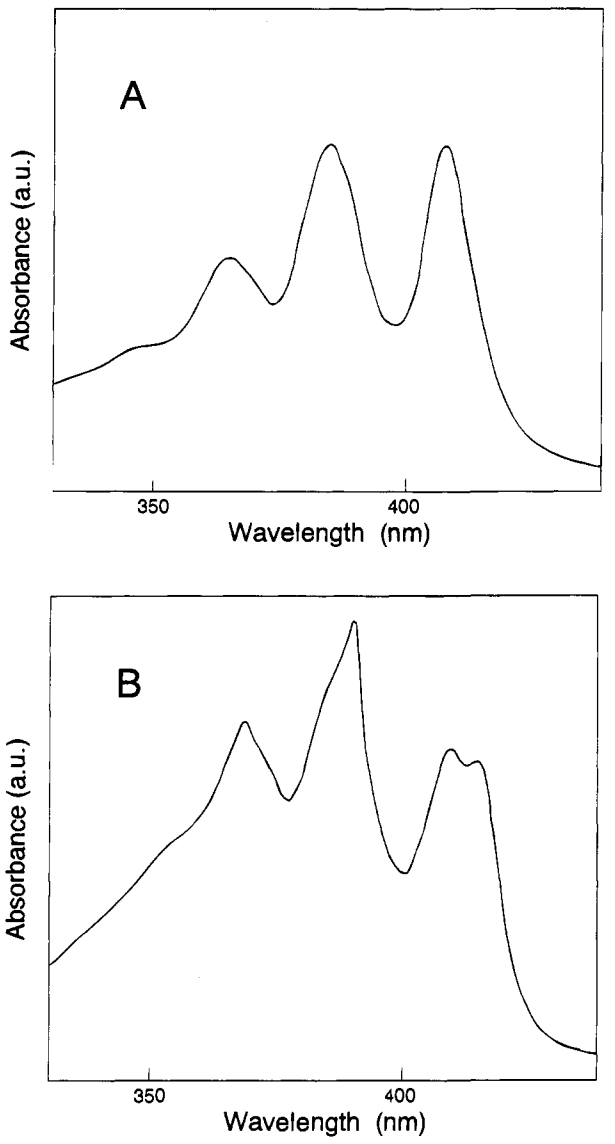

Figure 2. Absorption spectra for a 2.6 μM solution of **2** that has been incubated at 23 °C for 20 min with LUVs made (A) egg PC/cholesterol (2/1) and (B) egg PC/ergosterol (2/1). Spectra observed for **2** in pure PBS, and also in the presence of egg PC vesicles, were very similar to that of (A) (not shown).

Retention of Membrane Recognition Features. In order to confirm that these conjugates retain their ability to "recognize" ergosterol-rich bilayers, we have examined their interaction with sterol-containing large unilamellar vesicles (1000 Å diameter, LUVs) by UV−vis spectroscopy. Specific procedures that were used were similar to those previously described for AmB.[1,6] Incubation of a submicellar solution of **2** (2.6 μM) with a vesicular dispersion that was 1 mM in egg PC and 0.5 mM in cholesterol, for 20 min at 23 °C, showed an absorption spectrum that was essentially the same as that observed in the presence of egg PC vesicles (without sterol) and also the same as that found in buffer alone (Figure 2A). In striking contrast, similar incubation with a vesicular dispersion that was 1 mM in egg PC and 0.5 mM in ergosterol gave a spectrum that showed a strong diminution in the monomer band at 409 nm and new bands at 415, 390, and 375 nm (Figure 2B), a result which is indicative of self-association and binding with ergosterol.[6] Very similar results have been obtained with AmB and with conjugates **1** and **3** (not shown). Thus, these conjugates appear to recognize ergosterol-rich bilayers in a way that is similar to AmB, itself.[6]

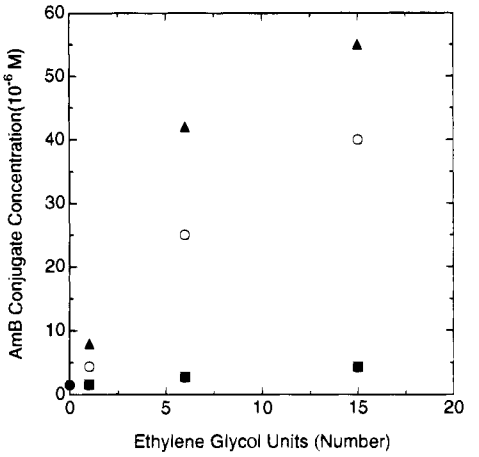

Figure 3. Plot of a number of ethylene glycol units in a given AmB-conjugate versus the conjugate's critical micelle concentration (○), concentration required for 50% hemolysis (▲), and minimum inhibitory concentration (■). An overlap region that includes the K_{50}, *cmc*, and MIC values for AmB, itself, is also indicated (●).

Separation of Antifungal and Hemolytic Activity. A comparison of the antifungal activity (MIC), hemolytic activity (K_{50}), and critical micelle concentration (*cmc*) for each of the AmB conjugates is presented in Figure 3. For purposes of clarity, both the aggregation properties and the biological activity of the conjugates are plotted as a function of the number of ethylene glycol units that are present. From this figure, it is readily apparent that an incremental increase in the length of the polyether moiety leads to a significant increase in the conjugate's critical micelle concentration and a significant loss in its hemolytic activity. It is also clear that this same increase in ethylene glycol content results in a relatively small decrease in antifungal activity; *the net result is a significant separation of antifungal and hemolytic activity, which increases as the cmc of the conjugate increases.* In order to put these results into perspective, it should be noted that the K_{50}, *cmc*, and MIC for the parent drug, AmB, are "bunched up" in the 1−2 μM range; i.e., the K_{50}, *cmc*, and MIC values are 1.8, 1.7, and 1.1 μM, respectively. Thus, the separation of antifungal and hemolytic activity that is depicted in Figure 3 is quite significant. Moreover, the fact that this separation increases as the length of the polyether moiety (and corresponding *cmc*) increases provides strong support for our rupture/leakage hypothesis.

Of special importance is the fact that AmB, **1**, **2**, and **3** all show significant hemolytic activity only when their concentration exceeds their *cmc*. With the exception of AmB, submicellar concentrations of each macrolide are sufficient for complete inhibition of fungal growth. That submicellar concentrations of AmB are, in fact, membrane-disrupting and cytotoxic toward *Candida albicans* was confirmed by K^+ release. Thus, a 0.1 μM solution of AmB was sufficient for releasing 50% of the K^+ that was present in 3×10^7 fungal cell/mL; for similar release by **1**, **2**, and **3**, submicellar concentrations of 0.8, 1.0 and 1.2 μM were required. In order to release 50% of the K^+ from RBCs (3×10^7 cells/mL), however, concentrations of AmB, **1**, **2**, and **3** had to be essentially at their *cmc*; i.e., 0.92, 4.3, 19, and 27 μM, respectively.

Conclusions

The results of this study clearly demonstrate that the membrane-disrupting properties of AmB toward fungal and mammalian cells can be controlled by adjusting the macrolide's critical micelle concentration through chemical modification. The feasibility of creating AmB conjugates that have activities

against *Candida albicans* that are similar to AmB itself, but which do not lyse RBCs at considerably higher concentrations, is particularly significant. It not only establishes the concept of drug design that takes into account supramolecular structure but also highlights these compounds as potential alternatives to AmB. In preliminary *in vivo* experiments in mice, **2** has been found to be an effective antifungal agent with respect to *Candida albicans*.[13]

A reasonable extrapolation of this work is that the membrane-disrupting properties of other amphiphilic agents (e.g., magainin, nystatin, etc.)[14] may be controllable by similar monomer/micelle adjustment. It is noteworthy, in this regard, that correlations between hydrophobicity and hemolytic activity for related classes of amphiphilic agents, which are beginning to appear in the literature, may be accounted for in terms of their aggregation properties in solution.[15]

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