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Amphotericin B induced interdigitation of apolipoprotein stabilized nanodisk bilayers

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Amphotericin B nanodisks (AMB-ND) are ternary complexes of AMB, phospholipid (PL) and apolipoprotein organized as discrete nanometer scale disk-shaped bilayers. In gel filtration chromatography experiments, empty ND lacking AMB elute as a single population of particles with a molecular weight in the range of 200 kDa. AMB-ND formulated at a 4:1 PL:AMB weight ratio, separated into two peaks. Peak 1 eluted at the position of control ND lacking AMB while the second peak, containing all of the AMB present in the original sample, eluted in the void volume. When ND prepared with increased AMB (1:1 phospholipid:AMB molar ratio) were subjected to gel filtration chromatography, an increased proportion of phospholipid and apolipoprotein were recovered in the void volume with the AMB. Prior to gel filtration the AMB-ND sample could be passed through a 0.22 µm filter without loss of AMB while the voided material was lost. Native gel electrophoresis studies corroborated the gel permeation chromatography data. Far UV circular dichroism analyses revealed that apoA-I associated with AMB-ND denatures at a lower guanidine HCl concentration than apoA-I associated with ND lacking AMB. Atomic force microscopy revealed that AMB induces compression of the ND bilayer thickness consistent with bilayer interdigitation, a phenomenon that is likely related to the ability of AMB to induce pore formation in susceptible membranes.

Amphotericin B (AMB) is a potent antifungal agent that is widely used in the treatment of systemic fungal infections. Recently, we reported a novel lipid formulation of AMB wherein the antibiotic integrates into nanometer scale, disk-shaped, apolipoprotein stabilized particles (1), referred to as nanodisks (ND). *In vitro* studies of AMB-ND revealed potent antifungal activity together with decreased toxicity toward erythrocytes and cultured hepatoma cells compared to AMB deoxycholate micelles (1). Furthermore, *in vivo* efficacy studies in mice

infected with either *Candida albicans* (1) or the protozoal parasite, *Leishmania major* (2) revealed that AMB-ND elicit strong antibiotic effects. Recent studies investigating the unique spectroscopic properties of this heptaene macrolide antibiotic in ND revealed AMB concentration and temperature dependent changes in spectral properties consistent with AMB self association in the ND lipid milieu (3).

A question that arises relates to whether AMB distributes among ND particles in a uniform manner or selectively partitions to create a population of AMB rich particles that co-exist with particles lacking AMB. At ratios of phospholipid:AMB used in the study by Hargreaves et al. (2006) (4:1, 40:1 or 400:1 w/w), a uniform distribution model would require that AMB molecules be individually surrounded by phospholipid. Given the amphoteric nature of AMB and its propensity to self-associate (4), such a model seems unlikely.

In an effort to better define the molecular organization of AMB, phospholipid and apolipoprotein in AMB-ND, gel permeation chromatography, electrophoresis and atomic force microscopy studies have been conducted. The data obtained indicate that, at high phospholipid:AMB ratios, AMB does not partition uniformly among ND particles but, rather, combines with phospholipid at an optimal ratio to create a subpopulation of apolipoprotein stabilized particles that possess a ~1:1 phospholipid:AMB molar ratio. Increasing the starting AMB content to achieve a 1:1 phospholipid:AMB molar ratio results in generation of a uniform population of larger diameter ND in which the bilayer is interdigitated.

EXPERIMENTAL PROCEDURES

Materials. AMB (USP grade) was obtained from Research Organics Inc. Dimyristoylphosphatidylcholine (DMPC) was from Avanti Polar Lipids Inc. Recombinant apolipoprotein A-I (apoA-I) was produced as previously described (5).

AMB-ND preparation. Phospholipid vesicle dispersions were prepared as described earlier (1). To the dispersed lipid a given amount of AMB from a stock solution (30 mg/ml in dimethylsulfoxide; DMSO) was added in a subdued light environment. Subsequent addition of apoA-I in buffer leads to a time-dependent decrease in sample turbidity with full sample clarity achieved by bath sonication with the sample temperature maintained below 25 °C. AMB-ND preparations contained 0, 2.5 or 12 mg AMB per 10 mg DMPC and 4 mg apolipoprotein. In the latter case, this corresponds to slightly higher than a 1:1 phospholipid:AMB molar ratio. When higher amounts of AMB were introduced to the reaction mix, precipitation occurred with loss of AMB. All preparations were dialyzed overnight at 4 °C and filter sterilized (0.22 μm) before use.

Gel filtration chromatography. Samples were applied to a 2.5 x 40 cm column of Sepharose 6B equilibrated in phosphate buffered saline (0.1 M sodium phosphate, pH 7.0, 150 mM sodium chloride) and eluted in 2 ml fractions.

Far UV circular dischroism spectroscopy. Far UV circular dichroism (CD) measurements were performed on a Jasco 810 spectropolarimeter. Protein samples were dissolved in 50 mM sodium phosphate, pH 7.0. For guanidine HCl denaturation experiments, samples (0.2 mg/mL) were incubated overnight at a given denaturant concentration in order to attain equilibrium, and ellipticity was measured at 222 nm (0.1 cm path length). Ellipticity values were converted into molar ellipticity (millidegrees cm^2 $decimole^{-1}$) using a mean residue weight value of 113.4 for wild type and 114.3 for the deletion mutant. Protein secondary structure content was calculated using the self-consisted method with Dicroprot version 2.6 (6).

Analytical procedures. Protein concentrations were determined by Bradford assay with bovine serum albumin as standard. Non denaturing PAGE was performed on 4-20 % acrylamide slab gels and stained with Gel Code Blue (Pierce Chemical Co.). The AMB content of samples was determined by transferring an aliquot to a solution of DMSO and measuring absorbance at 416 nm using an AMB extinction coefficient at 416 nm = 1.214 x 10^5 M^{-1} cm^{-1} in DMSO (1). Choline containing phospholipids were quantified by enzyme based colorimetric assay (Wako) and phospholipid phosphorus content was quantitated as described by Bartlett (7).

Atomic force microscopy. Atomic force microscopy images were obtained with an Asylum Research MFP instrument. All images were acquired in aqueous tapping mode using silicon nitride cantilevers (0.1 N/m). Levers with blunt tips were discarded. All images were obtained in room temperature (~21ºC) aqueous solution. Samples were prepared by immobilizing ~2 mL of ND particles in imaging buffer (10 mM Tris-HCl (pH8.0), 150 mM NaCl, and 10 mM $MgCl_2$) onto a freshly cleaved mica surface and allowed to incubate for several minutes. Several rinse steps with imaging buffer followed. The topographies were post-processed with IgorPro software (WaveMetrics, Oregon) to remove first order slope in the line scans. Height and area histograms were calculated with custom software analysis and the data are given as mean +/- SD. Images were false colored and sectioned to illustrate ND size.

RESULTS

Gel filtration chromatography studies. ND provide a lipid milieu capable of incorporating significant quantities of the water insoluble polyene antibiotic, AMB (1). The product particles are disk shaped phospholipid - apolipoprotein complexes. The AMB component has been proposed to intercalate between phospholipid molecules in the disk bilayer. To gain insight into the structural organization of the AMB, phospholipid and apolipoprotein components of ND particles, gel filtration chromatography experiments were performed. ND lacking AMB eluted from a Sepharose 6B column as a single symmetrical peak of phospholipid and protein (data not shown). When ND formulated with 2.5 mg AMB or 12 mg AMB / 10 mg DMPC and 4 mg apoA-I were applied to the column, essentially 100 % of the AMB eluted in the void volume (**Figure 1, panel A**). When the phospholipid content of the fractions was examined, in the case of ND generated with a 4:1 DMPC:AMB ratio, approximately 25 % of the phospholipid mass eluted in the void volume, with the remainder recovered in fractions corresponding to empty ND (fractions 15 - 25). When the molar ratio of DMPC:AMB was changed to 1:1, nearly all of the phospholipid eluted in the void volume (**Figure 1, panel B**). When the distribution of apoA-I was examined as a function of ND particle AMB content, a small amount of apoA-I eluted in the void volume when ND were prepared at a 4:1 DMPC:AMB weight ratio, with the bulk of the protein eluting in a peak centered around fraction 20. On the other hand, when the ratio of DMPC to AMB was changed to 1:1, a larger proportion of the apoA-I eluted in the void volume with a substantial amount also eluting in a region of the profile corresponding to lipid free apoA-I (**Figure 1, panel C**).

Based on these data we hypothesized that, during formation of ND, AMB does not distribute uniformly among the bulk phospholipid present, but rather, it selectively associates with ND phospholipid in a fixed stoichiometry such that AMB molecules interact both with other AMB and DMPC to achieve a preferred organizational state. At 2.5 mg AMB / 10 mg DMPC the system contains phospholipids mass in excess of that

required to achieve the preferred AMB-DMPC molecular organization. In this case excess DMPC exists in complexes with apoA-I as empty ND. An apparent intrinsic difference in behavior of these co-existing populations of particles is revealed by gel permeation chromatography of the sample. A further observation is that, prior to gel permeation chromatography, AMB-ND preparations could be filtered through a 0.22 µm membrane without loss of AMB. By contrast, after gel permeation chromatography the voided material, which contains all of the AMB present in the original sample, could not be filtered through a 0.22 µm membrane. Taken together, these data suggest that AMB solubilized in ND aggregates upon gel permeation chromatography resulting in its separation from the bulk of the lipid and apolipoprotein, which remains as discrete ND particles. From the composition of the voided material, it appears that AMB selectively associates with itself in a preferred ratio with phospholipid and small amounts of apoA-I to generate large AMB - DMPC aggregates.

If AMB selectively interacts with a fixed amount of DMPC (generating particles with an approximate 1:1 DMPC:AMB molar ratio), then the maximum amount of AMB that can be solubilized by incorporation into ND should correspond to a 1:1 molar ratio of these components (AMB molecular weight = 924 g/mole; DMPC molecular weight = 678 g/mole). Consistent with this we found that approximately 12 mg AMB can be solubilized by 10 mg DMPC and 4 mg apoA-I, an amount far greater than the 2.5 mg AMB per 10 mg DMPC mass reported earlier (1). In the case of ND prepared with 12 mg AMB per 10 mg DMPC, gel filtration results in elution of all of the AMB and phospholipid present in the sample in the void volume with apoA-I distributed between the voided fraction and a later eluting fraction that corresponds to free apoA-I. Again, although AMB recovered from the gel filtration column following chromatography of these ND was soluble in buffer, it could not be filtered through a 0.22 µm filter. These data support the concept that AMB interacts with phospholipid in an optimal ratio to generate a population of ND particles that are enriched in AMB and are protected from aggregation by apoA-I. The reason why AMB-DMPC-apoA-I complexes, but not DMPC-apoA-I complexes (empty ND) are susceptible to fusion / aggregation upon gel filtration chromatography is unclear but appears to involve apoA-I dissociation from the ND surface.

Native PAGE studies. Native gradient PAGE of AMB-ND prepared at a 4:1 weight ratio of DMPC:AMB revealed a discrete population of particles with a diameter in the range of 8.5 nm (1). On the basis of the gel filtration results described above, however, it is conceivable that electrophoretic separation may induce a similar alteration in AMB containing particles. To determine if nondenaturing polyacrylamide gel electrophoresis induces AMB aggregation similar to that observed upon gel filtration, native PAGE analysis was performed on particles prepared with increasing amounts of AMB and a fixed amount of phospholipid and apolipoprotein (**Figure 2**). The results indicate an AMB concentration dependent decrease in the intensity of the ND band migrating at 8.5 nm together with an increase in the amount of the apparently aggregated material recovered at the top of the gel. Furthermore, the AMB concentration-dependent decline in 8.5 nm particle content was accompanied by the appearance of a band corresponding to free apoA-I and an apparent band of aggregated material that did not enter the gel. To determine if the aggregated material recovered at the top of the gel contained AMB, the gel was directly visualized without staining (Figure 2 lower panel). This region, and only this region, of the unstained gel showed a distinct yellow hue, consistent with the color of AMB. Thus,

we conclude that native gel electrophoresis induces a similar coalescence of AMB and DMPC giving rise to large aggregates that fail to enter the gel.

Circular dichroism spectroscopy studies. The gel filtration and native PAGE data presented above suggest that AMB-ND are intrinsically less stable than control ND lacking AMB. To examine this, the effect of guanidine HCl on the secondary structure of the apoA-I component of ND was evaluated as a function of AMB content (**Figure 3**). Consistent with previous studies, association of apoA-I with DMPC to create ND particles confers considerable stability to this protein (8). Whereas lipid free apoA-I gave rise to a denaturation transition midpoint of 1 M guanidine HCl, the corresponding value for apoA-I associated with empty ND was ~ 3 M. Incorporation of small amounts of AMB (0.25 mg AMB per 10 mg DMPC) had no discernable effect on apoA-I stability while increasing the AMB content to 20 % of the phospholipid mass (2.5 mg AMB per 10 mg DMPC) induced a shift in apoA-I denaturation profile, yielding a transition midpoint of 2.3 M guanidine HCl. These data show that inclusion of AMB in ND affects the ability of apoA-I resist denaturation, a result that correlates with the apparent dissociation of apoA-I during gel filtration or electrophoresis.

Atomic force microscopy of ND. To further examine the effect of AMB on ND structure, AFM experiments were conducted. AFM images like scanning electron microscopy except that it scans a tip over the surface and monitors surface topography in a physiological environment (**Figure 4, panel A**). Images of empty ND at high concentration revealed close packing of the disks that gave rise to a measured diameter of the disks is 11.1 ± 2.1 nm (**Figure 5, panel A and B**). This value is in general agreement with values obtained by non denaturing gradient gel electrophoresis. At lower ND concentrations, individual particles were visualized (**Figure 4, panel C**). The height of the disks was observed to be 5.21± 0.37 nm (**Figure 4, panel F**), consistent with the thickness of a DMPC bilayer (9). Introduction of a maximal concentration of AMB (12 mg AMB per 10 mg DMPC) has two major effects on this system (**Figure 4, panels D and G**). The diameter of the particles increased to ~35 +/-15 nm and very few particles were observed with a diameter similar to those in the empty ND sample. At the same time, the height of the disks was reduced. The largest reduction in height was observed in the center of the particles (2.45± 0.35 nm) while the perimeter of the particles were ~3.43± 0.62 nm in height. These height values were force independent, that is, changing the force of imaging had little effect on the absolute particle height values. These data indicate that AMB incorporation caused major effects on ND structure. To further investigate the effects of AMB in ND particle size and structure, particles were prepared that contained an intermediate AMB content (2.5 mg AMB per 10 mg DMPC). Under this condition evidence for the co-existence of two particle populations was obtained (**Figure 4, panels E and H**). One population displayed the particle diameter and height characteristics similar to that of empty ND while the second population was similar in size and height to ND prepared with the maximal AMB content.

DISCUSSION

We recently reported the generation of apolipoprotein stabilized nanoscale sized bilayer disks that possess significant quantities of the ampiphilic macrolide polyene antibiotic, AMB (1). Interest in this formulation has been generated by the observation that AMB-ND show efficacy in a mouse model of candidiasis with low toxicity toward red blood cells and cultured hepatoma cells. Recently, AMB-ND have been shown to be highly effective in the treatment of

cutaneous *Leishmania major* infection in BALB/c mice (2). In considering the molecular basis for the apparent enhanced efficacy of AMB-ND, questions exist about the distribution of AMB among ND particles and the nature of the interaction of AMB with other ND particle components.

In the present study we took advantage of the phospholipid solubilization properties of recombinant human apoA-I to form discrete ternary complexes of AMB, phospholipid and apolipoprotein. Given the high content of AMB that can be fully solubilized in these particles (up to a 1:1 molar ratio with DMPC), it is likely that a repeating AMB-DMPC structural organization exists. As with non-AMB containing bilayer disks, it is envisioned that apoA-I molecules circumscribe the perimeter of the complexes in such a manner that the hydrophobic faces of its amphipathic alpha helices contact the fatty acyl chains of the phospholipid (or the AMB molecules). In the case of non-AMB containing ND such a model is supported by a large body of evidence including infra-red spectroscopy, fluorescence resonance energy transfer and electron paramagnetic resonance spectroscopy (10-12). Normally such ND complexes are quite stable and can be sized by gel filtration chromatography or non-denaturing PAGE. By contrast, introduction of AMB into ND particles has a major effect on the behavior of the particles. Gel filtration chromatography studies reveal a propensity of AMB to self associate / aggregate resulting in antibiotic elution in the column void volume along with a fixed amount of phospholipid and a lesser amount of protein. Two important conclusions can be drawn from these results. First, when prepared at a > 1:1 molar ratio of phospholipid:AMB, AMB does not partition equally into ND particles. Instead, AMB associates with DMPC to generate a repeating structural organization that possesses a 1:1 phospholipid:AMB molar ratio. This property was observed earlier by Janoff et al. (13), who found that mixture of AMB and DMPC and dimyristoylphosphatidylglycerol form extended ribbon-like structures that upon sucrose density gradient centrifugation results in freezing out of AMB from the bulk of the phospholipid. Second, apoA-I associated with ND particles that contain AMB and DMPC in this organizational state is less stably associated with the particle than apoA-I associated with ND that lack AMB. Based on this behavior of AMB, we conclude that, when prepared from a starting DMPC:AMB weight ratio of 4:1, two populations of ND are generated, those that contain AMB and DMPC in a 1:1 molar ratio and those that contain the remainder of the DMPC without AMB. This conclusion is supported by the results of native gel electrophoresis, which show that the more AMB present, the lower the amount of ~8.5 nm ND band, characteristic of DMPC-apoA-I complexes, observed.

Questions that arise from these observations relate to the precise morphology of AMB containing particles. Whereas negative stain electron microscopy studies reported earlier (1) suggest AMB containing ND are similar to “empty” ND, it must be recognized that, given the present interpretation, the major component in this sample corresponds to empty ND, with at most 25 % of the ND containing AMB. Support for this concept was obtained from experiments showing that up to 12 mg AMB can be fully solubilized upon formulation with 10 mg DMPC (1:1 molar ratio) and 4 mg apoA-I. Since this preparation does not contain empty ND (as seen by gel filtration and electrophoresis), they offer a useful system to characterize the structure and morphology of AMB containing ND.

Focusing on the curious finding that, upon gel filtration chromatography of AMB-ND, apoA-I dissociates from the particle, we evaluated the stability of ND associated apoA-I. Guanidine HCl denaturation studies revealed that the presence of AMB in ND

particles causes a decrease in apoA-I stability consistent with the hypothesis that apoA-I dissociates at lower guanidine HCl than when associated with empty ND. Subsequent atomic force microscopy experiments provided a plausible explanation for this result. Introduction of AMB at a ~1:1 molar ratio with DMPC generated a population of ND particles that were of larger diameter and reduced height than empty ND. The larger diameter of the AMB-containing ND suggests more than the two apoA-I molecules normally associated with an empty ND. The nature of this association is unknown but likely involves an extended "belt" conformation for apoA-I (14). Data obtained on empty ND corresponded to a bilayer thickness of about 5 nm in width, consistent with values reported by others (9). By contrast, AMB-ND gave rise to particles whose height was 2.5 nm in the center of the particle and between 3.0 and 4.0 nm at the edge of the particle. Given that the phospholipid component of empty ND is generally considered to exist as a bilayer, the present data suggest that AMB intercalation produces a perturbation in the bilayer that induces its phospholipid fatty acyl chains to interdigitate. In an interdigitated bilayer the hydrocarbon chains of phospholipids in opposing leaflets interpenetrate (15). In general, slippage of fatty acyl chains of a given phospholipid against corresponding phospholipid fatty acyl chains in the opposite leaflet of the bilayer can be induced by perturbation of the bilayer by such factors as pressure or small amphiphilic molecules (16). Among these are short chain alcohols such as ethanol or benzyl alcohol. In addition, amphiphilic agents such as atropine have been shown to induce bilayer interdigitation (17). In the case of ND membranes, bilayer interdigitation would be expected to have an effect on the association of apolipoprotein around the perimeter of the disk. Compression of the bilayer thickness may be anticipated to hinder apolipoprotein interaction such that dissociation occurs more readily than in a non-interdigitated bilayer containing ND (e.g. empty ND). The observation that the edge of AMB containing disks is ~ 1nm thicker than the central region of the disk may indicate that the perimeter of the disk is circumscribed by multiple apoA-I molecules that align in tandem. In this manner the edge of the disk would resist compression upon AMB induced phospholipid interdigitation although the stability of its association with the particle may be decreased. Thus, AMB dependent bilayer interdigitation can explain the loss of apoA-I from the ND particles upon gel filtration or electrophoresis and this occurrence would undoubtedly promote fusion of residual AMB-DMPC, creating the large aggregates that elute in the void volume following gel filtration chromatography.

The finding that AMB is capable of inducing ND bilayer interdigitation has important implications in terms of the mechanism of action of this potent antifungal agent. It is generally accepted that AMB interaction with itself and membrane sterols leads to pore formation that results in leakage of cell contents and cell death. The higher activity of AMB toward fungal cell membranes has been ascribed to a preferred interaction with ergosterol versus cholesterol present in mammalian cell membranes (18). While the precise organization of conducting AMB membrane pores is not known, two prevailing models exist. In one model AMB forms a "half-pore" in one leaflet of the bilayer, with a functional conducting pore occurring when two half-pores, in opposite leaflets of the bilayer, join one another to make a double pore that spans the bilayer width. In a second model, suggested to exist in egg phosphatidylcholine bilayers, AMB forms a functional half pore that results from a local compression of the bilayer width (19,20). This latter model may be explained on the basis of AMB induced membrane

interdigitation. Interdigitation of a target membrane will decrease the bilayer thickness such that a pore that is the length of an AMB molecule may span the membrane. Another key factor in the process of AMB induced membrane pore formation is its propensity to self-associate. It is postulated that AMB pores are comprised of 8 - 12 AMB molecules complexed with an equivalent number of membrane sterols, presumably via their hydrophobic polyene face such that the interior diameter of the resulting hydrophilic pore would allow unrestricted ion movement. Thus, AMB self association, interaction with sterols and ability to induce bilayer interdigitation may represent critical aspects of membrane pore formation that give rise to the beneficial biological and pharmacological activity of this, and possibly other, polyene antibiotics.

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Figure Legends

Figure 1. **Gel filtration chromatography of ND particles**. AMB-ND prepared at AMB:DMPC:apoA-I weight ratios of 2.5:10:4 (open triangles) and 12:10:4 (filled circles) were applied to a Sepharose 6B gel filtration column and eluted in 2 ml fractions. The AMB content (upper panel), DMPC content (middle panel) and apolipoportein content (lower panel) of each fraction was determined.

Figure 2. **Non-denaturing PAGE of ND**. Samples were applied to a 4-20% acrylamide Tris-glycine gel and electrophoresed for 24 h at 150 mV. The gel was stained with Coomassie Blue. Lane 1) Molecular weight standards; lane 2) lipid free A-I; lane 3) empty ND lane 4) AMB-ND (2.5 mg AMB / 10 mg DMPC); lane 5) AMB-ND (6 mg AMB per 10 mg DMPC); lane 6) AMB-ND (9 mg AMB per 10 mg DMPC) and lane 7) AMB-ND (12 mg AMB per 10 mg DMPC). Lower panel; same gel as above but not stained for protein.

Figure 3. **Effect of guanidine HCl on apoA-I secondary structure content**. ApoA-I in buffer (solid circles); apoA-I in empty ND (e.g. DMPC only; open circles); apoA-I in AMB-ND (0.25

mg AMB per 10 mg DMPC) (filled inverted triangles) and apoA-I in AMB-ND (2.5 mg AMB per 10 mg DMPC (open inverted triangles)

Figure 4. **Atomic Force Microscopy of ND**. Panel A upper portion) Diagram of tip surface probe; lower portion) Tip probe measurement of particle height (empty ND at concentration B) Image of empty ND at high concentration; Panels C-E) AFM images of ND at low conentration including empty ND (panel C); AMB-ND prepared at high AMB (12 mg AMB / 10 mg DMPC) (panel D) and AMB-ND prepared at low AMB concentration (2.5 mg AMB / 10 mg DMPC) (panel E). Panels F-H) Tip probe