The Effect of Serum Albumin on Amphotericin B Aggregate Structure and Activity

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Purpose. Mild heat treatment of Fungizone® (FZ, an amphotericin B:deoxycholate preparation) leads to a new self-associated form (HFZ) that demonstrates improved therapeutic index *in vivo*. The origin of the improvement may lie in the differential stability in the presence of serum proteins. The purpose of this study is to assess the effect of human serum albumin (HSA) on the structure and stability and *in vitro* channel forming ability of these two preparations against model fungal and mammalian membrane vesicles.

Methods. Kinetic absorption and CD spectroscopy were used to assess the kinetic and equilibrium stability of the characteristic amphotericin B complexes in the presence of HSA. Kinetic fluorescence spectroscopy of pyranine entrapped in model fungal and mammalian membrane vesicles was used to measure the cation-selective channel forming ability of HZ and HFZ delivered from HSA.

Results. It is shown that FZ is rapidly converted from its aggregated form to a protein-bound monomer in the presence of HSA, whereas HFZ demonstrates greater stability by persisting as a stable inactive aggregate. Fluorescence measurements of ion currents show that HSA attenuates the membrane-activity of both preparations. However, the activity of both HFZ and FZ remains significant against ergosterol-containing membranes. This is the first direct measurement of the intrinsic channel forming abilities of these amphotericin B preparations in the presence of serum proteins.

Conclusion. These data provide a mechanistic rationale for the similar efficacy and lower toxicity of HFZ.

KEY WORDS: serum albumin; polyene; antifungal; liposome; drug delivery; polyene macrolide.

INTRODUCTION

Amphotericin B (AmB) is a heptaene macrolide antifungal agent produced by *Streptomyces nodosus.* The conventional AmB-deoxycholate formulation, Fungizone® (FZ) has very high toxicity yet it is still used after 40 years because it is very effective at clearing deep fungal infections. Despite its nephrotoxicity and acute toxicity, it remains the most widely used drug for most systemic fungal infections (1–3).

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ABBREVIATIONS: AmB, Amphotericin B; FZ, Fungizone; HFZ, Heat-treated Fungizone; LUV, large unilamellar vesicle(s); FCCP, carbonyl cyanide *p*- (trifluoromethoxy) phenylhydrazone; EPC, egg phosphatidylcholine; HDL, high density lipoproteins; LDL, low density lipoproteins; LPDP, lipoprotein depleted serum fraction; CD, circular dichroism; HSA, human serum albumin; SVD, singular value decomposition.

Less nephrotoxic liposomal and lipid-associated AmB formulations have been successfully developed (*e.g.,* AmBisome, Abelcet, Amphocil), but their use may be limited by high cost (4). A proposed mechanism by which liposomal and other novel AmB formulae reduce toxicity involves complexing AmB to release monomers below a critical aggregation concentration. Because a specific soluble aggregate is a nonselective channel-former but monomers are selectively damaging toward fungi, any method that reduces free AmB concentration is expected to reduce direct toxicity (5,6). In addition, liposomal systems may modify AmB toxicity by binding differentially to serum lipoproteins (7–9).

As a potential simple and inexpensive alternative to liposomes, simple AmB or Fungizone (FZ) solutions may be treated with moderate heat (70°C for 20 min) to produce a new self-associated state of AmB, the "superaggregate" referred to as heated Fungizone, HFZ (10–13). HFZ has been shown to demonstrate reduced toxicity while still remaining effective against model fungal infections and therefore to possess a greater therapeutic index (10,12,13). In our previous work on this system, we demonstrated greater kinetic and thermodynamic stability of HFZ aggregates as well as intrinsically less channel-forming activity against model mammalian membranes (7). In addition, it was shown that HFZ binds less to LDL than FZ as well as demonstrating reduced TNF-a stimulating activity in human monocytes (7).

Serum albumin is also an important modulator of drug activity. We previously showed that the majority of AmB from both FZ and HFZ remains in the lipoprotein-depleted fraction (LPDP) of whole human serum (which is mostly albumin) in *in vitro* experiments (7). We postulate that HFZ owes at least part of its improved therapeutic index to greater stability of the nontoxic HFZ aggregate in the presence of serum and serum albumin. Association with albumin may reduce the hemolytic activity of both preparations as reported for FZ (14), but at the same time, we expect that relative selectivity will be maintained toward model fungal membranes (ergosterol containing). What is currently unknown is the effect of serum albumin on the mechanism of AmB action. This study represents the first direct test of AmB membrane channel forming activity in the presence of serum albumin.

MATERIALS AND METHODS

Reagents

AmB as Fungizone (Bristol-Myers Squibb Pharmaceuticals, New York, N.Y.) was purchased from a commercial supplier. Whole human serum and human serum albumin (HSA) was purchased from Sigma (>99%, fatty acid and globulin free). Heat-treated Fungizone (HFZ) was prepared by heating Fungizone solutions for 20 min in a stable 70.0°C water bath as previously described (10).

Fluorescence Detected Membrane Ion Currents

Ion channel activity experiments were carried out as previously described (15,16). Briefly, large unilamellar egg phosphatidylcholine (EPC) vesicles containing 2 mM pyranine, 3mM FCCP, and 10 mole percent of either ergosterol or cho-

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lesterol were prepared by extrusion. The lipids were dispersed in a 15 mM K_2HPO_4, 2mM pyranine buffer with 200 mM KCl at pH 7.20 for ion permeability studies or in PBS for kinetic dilution and distribution studies. The lipid dispersions were then freeze-thawed and extruded ten times through 200 nm (with Lipex unit only) and 100 nm Nucleopore filters using a pressure (Lipex, Vancouver, BC) or manual (Avanti Polar Lipids, Alabaster, AL) extruder to make large unilamellar vesicles (LUV) of ca. 100 nm diameter. The external pyranine solution was removed and exchanged with a 15 mM K_2HPO_4 osmotically balanced (with sucrose) buffer through gel filtration with Sephadex G-25, thus forming a $[K^+]$ gradient of 6.7 K^+_{in}/K^+_{out} across the membrane. 3×10^{-6} M FCCP was added to pyranine-containing vesicles to allow for free equilibration of H^+ ions. The pyranine molecules entrapped in the target LUV provide a sensitive fluorescence assay for detecting this resulting interior vesicular pH change. The fluorescence intensity of the pyranine is linear between about 7.8 and 6.4 and so fluorescence intensity may be converted directly to internal pH. Hence, these AmB-induced ion currents can be observed as a dynamic change in pH and AmB membrane activity is reported as ΔpH/sec. In the present experimental configuration, a fluorescence decrease indicates $K^+ > Cl^-$ selectivity. According to the Nernst equation, ideal K^+ selectivity at low buffer capacity should produce a final ΔpH of –0.82. Valinomycin, an ideally K^+ selective ionophore, was used as a control. For the experiments in which AmB was introduced from an HSA solution, FZ or HFZ at 2 × the final concentration was incubated for >15 min at 37°C in a 30 mg/mL solution of HSA in the dilution buffer. The experiment was initiated by a stopped flow pulse mixing pyranine containing vesicles at a dilution of 1:1. The data reflect the average of three separate traces.

Circular Dichroism and Absorption Studies

Kinetic photodiode array spectral series were taken using a micro-volume stopped-flow reaction analyzer (Applied Photophysics Ltd., U.K., and SX.18MV). Spectral series were measured at 37°C from 300 to 450 nm using a diode array detector with an integration time of 10.080 ms. The mixing chamber had a 1.0 or 0.20 cm pathlength and a monochrometer slit width was generally fixed at 0.5 mm entry and 0.2 mm exit. Oversampling was employed with a 500-s log time base. All samples were protected from light to prevent photooxidation of AmB. SVD (singular value decomposition) and global analysis software from Applied Photophysics was used for data analysis. SVD analysis was used to reduce spectral noise and suggest a minimum number of species contributing to a spectral family (15).

Steady-state CD spectra were taken using the Applied Photophysics CD attachment. Fungizone or HFZ was incubated with buffer or human serum (diluted 1:1 with buffer due to high absorbance in the UV) or PBS buffer with 15 mg/mL HSA at 37°C for >15 min, and scanned using an Applied Photophysics CD attachment.

RESULTS

Stability of Fungizone vs. HFZ in Human Serum Albumin Solution by Kinetic Absorption and CD

On self-association, the characteristic polyene vibronic structure of the AmB monomer absorption collapses to an intense blue shifted band characteristic of some type of stacked array of interacting absorbers. For FZ the principal transition shifts from 409 nm (in water) to ~332 nm when self-associated and HFZ shifts to 320 nm (11). Optical activity of these oligomers, measured by circular dichroism (CD) spectroscopy, is one of the most sensitive methods for monitoring subtle changes in the self-association of strongly absorbing molecules (17). The bilobed CD band observed in Figs. 1A and B almost certainly arises from excitonic coupling of the transition moments of a regular (or at least dominant) array of AmB molecules (5). According to exciton chirality theory an arrangement of AmB monomers showing positive helical arrangement (right hand screw-sense) gives rise to a bilobed CD spectrum with a short wavelength (high energy) band with a negative sign, whereas the arrangement of AmB monomers with negative arrangement of transition dipoles (left hand screw sense) would give rise to a bilobed CD spectrum with a short wavelength (high energy) band of positive sign (17). Thus, at a fundamental level the screw-sense of HFZ has not changed and the monomers must be arranged with a negative twist (Fig. 1).

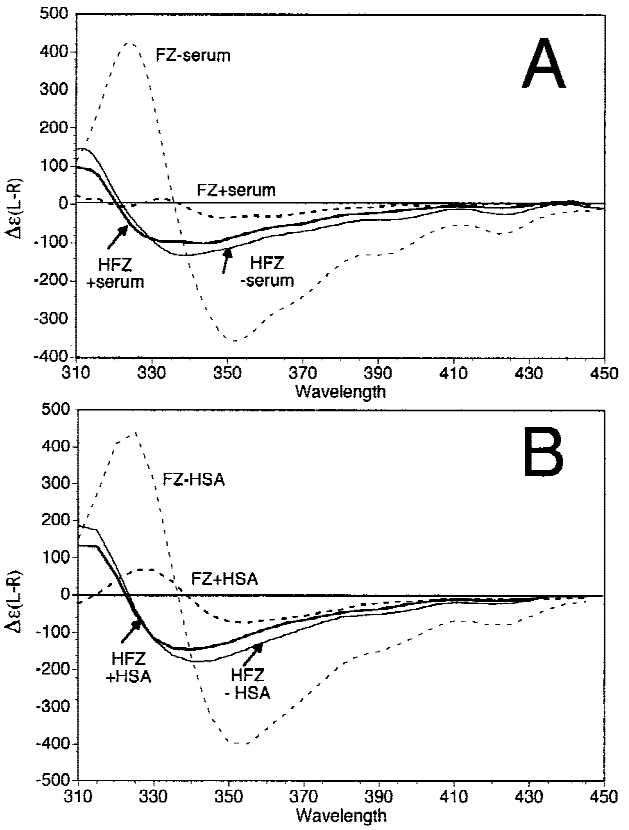

Fig. 1. (**A**) CD Spectra of HFZ and FZ in the presence of human whole serum and human serum albumin. The CD doublet is indicative of self-associated AmB; the monomer (in solution or protein bound) is essentially optically inactive and thus vanishes in the CD. Note the greater stability of HFZ aggregate in the presence of serum and albumin (indicated by doublet persistence). (**B**) CD spectra in HSA solution. These spectra show that most of the destabilization of FZ and HFZ can be attributed to serum albumin alone in agreement with our previous distribution studies in whole human serum (7,15). Whole serum was diluted 1:1 with dilution buffer, HSA was present at 15 mg/mL (0.23 mM), 37°C, >15 minutes incubation. CD units are in molar ellipticity.

Steady CD spectra of FZ in the presence of whole human serum at 1.0×10^{-5} M (after >15 minutes at 37°C) shows a precipitous reduction of the excitonic CD peak-to-trough band intensity by 93% (Fig. 1A), whereas HFZ shows a more modest reduction of 31%. To assess the effect of serum albumin alone, identical studies were carried out 15 mg/mL (0.23 mM) fatty acid free HSA. FZ still shows a significant dismantling of the aggregated state indicated by an 82% decline, whereas HFZ shows just a 23% loss of CD intensity. This level of HSA corresponds approximately to the HSA concentration in a 1:1 whole serum dilution so that the results are comparable to the serum study.

Absorption spectroscopy can demonstrate whether the AmB monomers are associated with serum albumin hydrophobic binding sites of free in solution (7,14). In the kinetic studies in Fig. 2, AmB as FZ or HFZ at 5.5×10^{-5} M was rapidly diluted to 5.0×10^{-6} M (a reasonable therapeutic concentration on initial infusion) in the presence of 0.23 mM HSA. The dissociation and redistribution was followed by diode array spectrometry from 300–450 nm and fitted by singular value decomposition and global fitting. In general, as previously reported for whole bovine serum, the spectra were best fitted by two or three sequential first order reactions (15). This may reflect both heterogeneity of the FZ and HFZ structures (18) or multiple binding sites on HSA. Single kinetic traces of the peak monomeric absorbance at 415 nm are shown in Fig. 2. The rate of monomerization and association with HSA was significantly faster and more extensive ($t_{½}$ ~ 35 s) for FZ than HFZ ($t_{½}$ ~ 100 s). The absorption maxima of the putative albumin bound monomer for both FZ and HFZ were blue shifted (415, 390 nm) compared with the aqueous monomer (409, 385 nm) confirming that the monomeric AmB was bound to a more nonpolar site(s) on HSA. There was no evidence by SVD of an aqueous species of monomeric AmB.

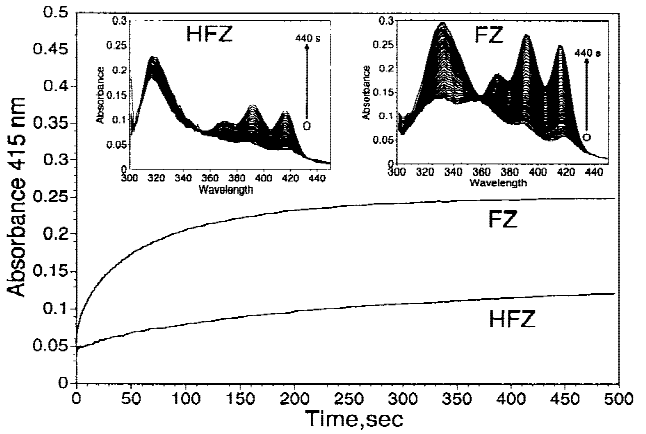

Fig. 2. Absorption kinetics at 415 nm of 5.0×10^{-6} M HFZ and FZ (final [AmB]) rapidly mixed with 15 mg/mL (0.23 mM) fat-free HSA from 0 to 500 seconds at 37°C. Note the greater stability of HFZ in the presence of serum albumin. At 500 s the majority of AmB as HFZ is still present as the benign self-associated form represented by a single blue-shifted band at ~320 nm as compared with the rapid loss of the aggregate at 330 nM in FZ (inset). The absorption max of the monomeric form of AmB (a typical polyene vibronic structure with $A_{max} = 415$ nm) clearly indicates protein-bound rather than aqueous AmB for both (characteristic $A_{max} = 409$ nm). The inset spectral families were collected on a log time scale from 0 to 440 s and processed by SVD. The FZ spectra were reconstituted with the first three SVD components and the HFZ spectra required only two.

Membrane Activity of Fungizone and HFZ in the Presence of Human Serum Albumin

The membrane ion channel forming activities of FZ and HFZ delivered form a pre-equilibrated (>15 min, 37°C, 30 mg/mL) HSA solution (final [HSA] = 0.23 mM) against 10 mole percent ergosterol and 10 mole percent cholesterol-containing egg PC membrane vesicles are shown in Figs. 3 and 4. Figure 3 shows a typical set of three averaged fluorescence traces converted to ΔpH for activity against ergosterol/EPC vesicles. The traces indicate a K^+ over Cl^- selectivity typical of AmB channels, and it is clear that the presence of HSA significantly reduces the activity of both FZ and HFZ at 3.0×10^{-6} M. Interestingly, both preparations have similar activity when delivered from HSA, albeit reduced, against these model fungal membranes.

More extensive data in terms of initial rates (I_o) in ΔpH sec^{-1} for activity against both cholesterol and ergosterol-containing vesicles in the presence of HSA are plotted in Fig. 4. The trend is similar to that observed in the absence of serum (15) but shifted toward higher concentrations. HFZ especially shows almost no activity in the presence of HSA even at 1.0×10^{-5} M. Single point comparisons with albumin-free AmB preparations were carried out at [AmB] = 5.0×10^{-6} M (Fig. 4; data not shown). FZ activity was −0.18 Δ pH/sec vs. ergosterol and -1.2×10^{-2} ΔpH/sec vs. cholesterol. HFZ activity was -5.7×10^{-2} ΔpH/sec vs. ergosterol and -1.3×10^{-3} ΔpH/sec vs. cholesterol. The results may expressed as relative percentage activity of AmB in the presence of HSA ($I_{o(+HSA)}/I_{o(-HSA)} \times 100\%$) as follows: FZ (cholesterol) = 36%; HFZ (cholesterol) = 25%; FZ (ergosterol) = 105%; and HFZ (ergosterol) = 44%. These data show that FZ's activity against ergosterol containing LUV is essentially unchanged by HSA whereas HFZ's activity is reduced by >50%. In the case of FZ at 5.0×10^{-6} M vs. ergosterol/PC LUV it is possible that we have reached the limit of our technique, which accounts for the nearly identical initial rates. However, in absolute terms there is still substantially more activity against ergosterol-containing membranes than cholesterol. The data would indicate that *selectivity* of AmB against

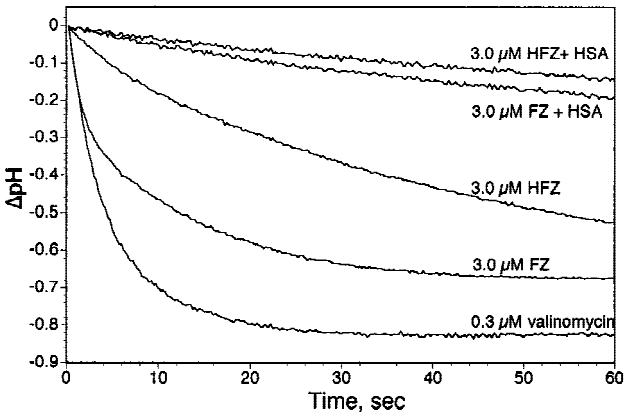

Fig. 3. Typical data in ΔpH versus time showing fluorescence detected ion currents on model fungal membranes (10 mol % ergosterol/EPC). For comparison, traces of 3.0×10^{-6} AmB as FZ or HFZ in the presence of 0.23 mM HSA or buffer alone are shown. Notice that at this concentration HSA seriously inhibits AmB induced cation channel formation as indicated by the slope of the traces. Typically, three shots were averaged for each trace.

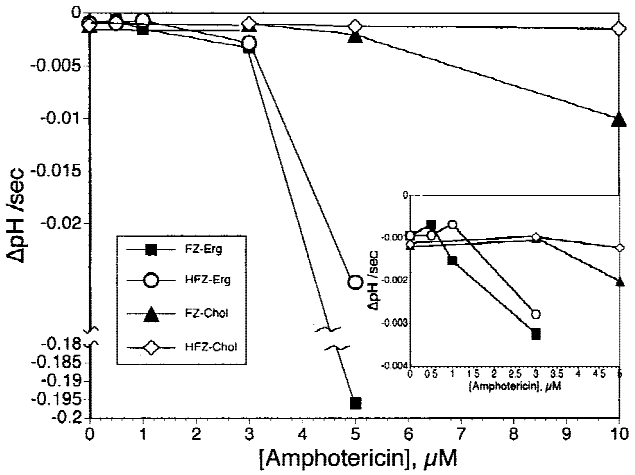

Fig. 4. Initial rates of ΔpH/s data as in Fig. 3 in the presence of 0.23 mM HSA (about 0.5 the actual concentration in serum; preequilibrated 15 min, 37°C). Notice the greater ability of FZ and HFZ to cause ion leakage in ergosterol containing membranes as expected. Against cholesterol-containing membranes, there is much less activity for both, but HFZ in particular shows negligible activity even at the highest [AmB]. These data suggest that FZ's activity against fungal membranes would be unchanged in serum whereas HFZ's activity would be reduced by >50%. Nonetheless, even this reduced activity exceeds that of uncomplexed FZ versus cholesterol by twofold, meaning that selectivity is preserved. Inset: An expansion of low [AmB] concentration to illustrate the break in activity in cholesterol vs. ergosterol vesicles occurring above 1×10^{-6} M.

fungal mimetic membranes is largely preserved in the presence of HSA and even relatively enhanced in the case of HFZ.

DISCUSSION

Human serum albumin has at least 11 distinct binding sites for fatty acids and hydrophobic molecules as determined by recent X-ray structural studies (19). Two of these sites are thought to be of primary importance for AmB and other nonpolar drug molecule binding and transport (14,20). In agreement with these studies, it has been reported that AmB has two binding sites on albumin: a high-affinity (submicromolar) and low-affinity site (21). In our studies, [HSA] was always significantly greater than [AmB] and even at the highest [AmB], no more than 5% of albumin high affinity sites would be expected to be occupied. Nonetheless, *in vivo* it is possible that fatty acid or bile salt binding could interfere with AmB binding to albumin, especially postprandially. If this leads to more extensive binding of AmB to LDL due to displacement from albumin it might potentiate toxicity (22). It is interesting that both fasting (23) and feeding (24) have been associated with reduced AmB toxicity.

Aramwit *et al.* recently reported that bovine serum albumin reduces the hemolytic activity and concurrently increases the critical aggregation concentration of FZ and AmB solutions (14). It has also been reported that an albumin delivery vehicle reduced the toxicity of AmB in a rat infusion model (25). This corresponds nicely with the model for AmB activity, which proposes that there exists a toxic soluble oligomer, which causes hemolysis and damage to mammalian cells, whereas the monomer is primarily toxic toward fungi (5,6). Drug delivery vehicles that maintain a [AmB] lower than the CAC would be expected to reduce its toxicity by this mechanism.

Because both HFZ and FZ remain largely unbound to serum lipoproteins in whole serum *in vitro* there may be a difference in their interactions with serum albumin or lack of interaction that accounts for most of the observed toxicity differential *in vivo* (7,12,13,26). In the presence of HSA, we show that HFZ dissociates more slowly and to a lesser extent than FZ (Figs. 1 and 2). In both preparations, dissociated AmB associates with albumin in the monomeric state rather than appearing in the aqueous phase as judged by the absorbance. This is also consistent with a recent report demonstrating that there exist essentially no aqueous monomers of AmB in serum (27). Thus albumin likely acts as a reservoir for monomeric AmB *in vivo,* especially from FZ. If the core aggregate of HFZ is largely intact, it has a substantial size (reported to be a "web-like" structure ~300 nm in diameter (18)). This nontoxic core aggregate of HFZ is much more stable in the presence of HSA (Figs. 1 and 2) and would be more likely to be removed from the circulation by the reticuloendothelial system. This likely accounts for the higher persistent serum concentrations and slower clearance of AmB delivered as FZ as compared to some liposomal preparations and HFZ (28,29). The increased *in vivo* toxicity of FZ then may be due to maintenance of a potentially critical concentration of mobile AmB that is still able to cause cellular damage.

Although it is clear that albumin can attenuate the activity of AmB under some circumstances it is not clear whether increased albumin association directly affects its membrane channel forming ability or if it is sufficient in itself to predict lower toxicity. In the presence of 0.23 mM albumin AmB as FZ and HFZ have reduced channel-forming ability against cholesterol-containing membranes (Fig. 4). In fact, HFZ is essentially inactive against cholesterol-containing membranes even at 1.0×10^{-5} M, further emphasizing the importance of the thermodynamic and kinetic stability of the novel HFZ supramolecular structure in maintaining reduced levels of free AmB. However, both HFZ and FZ remain very potent above 3.0×10^{-6} M against ergosterol-containing membranes (model fungal membranes). In fact, the activity of FZ at 5.0×10^{-6} M is virtually unchanged by albumin solutions, suggesting direct and efficient transfer of monomeric AmB from albumin complexes and HFZ aggregates to fungal mimetic membranes. In all cases FZ remains more potent than HFZ in its channel forming activity, demonstrating that albumin association and monomerization is not sufficient to prevent channel formation in mammalian mimic systems, at least above 3.0×10^{-6} M. The roles of aggregate stability, rapid clearance from circulation, and other factors are clearly equally important.

We have proposed that essentially three intertwined factors influence the toxicity and efficacy of AmB preparations: 1) direct membrane toxicity via ion channel formation, 2) differences in distribution and delivery to tissues due to differences in serum lipoprotein and protein binding and stability, and 3) initiation of an inflammatory cytokine response (7). In this report we have shown that the presence of serum albumin reduces but does not eliminate direct membrane channel forming activity against mammalian model membranes, but this is less pronounced vs. model fungal mimetic

membranes. The albumin-bound monomeric form of AmB still retains much of its potency against ergosterol-containing membranes, suggesting direct transfer from protein to membrane. This again emphasizes the preeminence of the monomeric form, whether protein-bound or not, in antifungal efficacy.

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