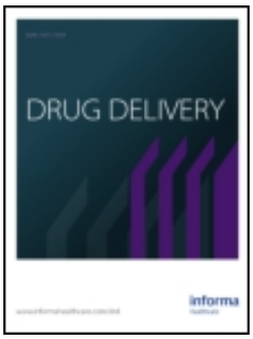

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Determination of the Relative Toxicity of Amphotericin B Formulations: A Red Blood Cell Potassium Release Assay

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NeXstar Pharmaceuticals, Inc., San Dimas, California, USA

Amphotericin B remains the drug of choice for the treatment of life-threatening, systemic fungal infections. Nevertheless, the use of amphotericin B in the traditional deoxycholate formulation (d-AmB) is limited by severe toxic side effects. Several relatively new formulations have recently received regulatory approval: amphotericin B colloidal dispersion (ABCD), amphotericin B lipid complex (ABLC), and liposomal amphotericin B (AmBisome). We quantitate the level of reduction of intrinsic toxicity of these formulations over d-AmB using a red blood cell potassium release assay, employing a variety of blood sources and incubation times. We also examine the practice of diluting d-AmB in the parenteral nutrition product Intralipid. Overall, the propensity in each formulation of amphotericin B to partition into the red cell membrane during incubation is measured by determining the concentration of amphotericin B required to achieve 50% potassium release. The concentrations leading to 50% potassium release were in the order AmBisome ≫ ABLC ≈ ABCD > d-AmB ≈ d-AmB/Intralipid and reflect inversely the degree to which each formulation makes amphotericin B available to mammalian, cholesterol-containing membranes. To evaluate intrinsic efficacy in an analogous way we investigated the availability of amphotericin B to partition into ergosterol-containing membranes by carrying out potassium release experiments using *Candida albicans* fungal cells instead of red blood cells. In contrast to the results obtained with red cells, the concentrations required to achieve potassium release from fungal cells were essentially identical for all formulations. It can be concluded that the intrinsic, physicochemical component of therapeutic index improvement in these formulations follows the order AmBisome ≫ ABLC ≈ ABCD > d-AmB ≈ d-AmB/Intralipid.

Keywords ABCD, ABLC, AmBisome, Amphotericin, *Candida albicans*, Erythrocyte, Liposome

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Address correspondence to Gerard M. Jensen, NeXstar Pharmaceuticals, Inc., 650 Cliffside Drive, San Dimas, CA 91773, USA. E-mail: gjensen@nexstar.com

Amphotericin B (Brajtburg et al. 1990; Warnock 1991) is a natural fermentation product from *Streptomyces nodosus* and has broad-spectrum antifungal activity (Steinberg et al. 1956). The amphotericin B molecule consists of a large, amphipathic macrolide lactone ring that presents seven conjugated double bonds on one side and seven polar hydroxyl groups on the other. The macrolide ring also contains a six-membered ketal ring, which is modified with a mycosamine sugar. Amphotericin B has activity against most of the principal fungal pathogens relevant to disease in humans, and exhibits a low propensity to be defeated by fungal resistance mechanisms. It therefore remains the antifungal of choice for the treatment of life-threatening infections, despite severe acute and chronic toxicity, including dose-limiting nephrotoxicity. The primary mechanism of action of both the toxic and antifungal activity of amphotericin B is thought to be a compromise of the barrier function of mammalian and fungal membranes, respectively.

Amphotericin B forms pores or channels in sterol-containing membranes, which cause leakage of protons, monovalent cations, and other cell constituents, leading to cell death. The structure of these channels is not firmly understood, but good evidence exists for structures containing eight molecules of amphotericin B aligned with the hydroxyl-bearing face of the molecule oriented toward the aqueous center of the pore, while the polyene backbone is associated with lipid components, possibly including eight sterol molecules (DeKruijff et al. 1974; Vertut-Croquin et al. 1983; Khutorsky 1992). This structure spans approximately half of the membrane bilayer, but nevertheless comprises a functioning pore in sterol-containing membranes effecting transport across the bilayer (Van Hoogevest and DeKruijff 1978).

That there is any usable window of therapeutic index at all in amphotericin B is due to the fact that the available concentration of amphotericin B needed to achieve pore formation in fungal membranes (where ergosterol is the primary membrane sterol) is significantly lower than that needed to achieve pore formation in mammalian membranes (where cholesterol is the primary sterol). This likely reflects the ~10-fold higher binding affinity of amphotericin B for ergosterol versus cholesterol: $K_a = 6.9 \times 10^5\ M^{-1}$ versus $5.2 \times 10^4\ M^{-1}$ (Readio and

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Bittman 1983). Chemical modification, combination therapy, and formulation efforts have all been attempted in an effort to improve the therapeutic index of amphotericin B. The latter strategy has proved most successful, leading recently to three commercial preparations of amphotericin B with improved toxicity profiles.

The relative toxicity of an amphotericin B formulation will depend on the propensity of the amphotericin B to partition into mammalian membranes in vivo, and on the biopharmaceutics of the formulation (defined as the combination of pharmacokinetics and biodistribution). The former is dependent on both the relative energetic stability of amphotericin B in the delivery vehicle and the ease of transfer to a target mammalian membrane (with or without involvement of plasma proteins). The biopharmaceutics, too, will be affected by the partitioning propensity to some degree.

Amphotericin B is virtually insoluble in aqueous media. Therefore, conventional amphotericin B (d-AmB) is provided as a mixed micelle with deoxycholate as the surfactant. This formulation provides relatively little energetic stability to the amphotericin B molecule outside of the conferral of water solubility. Improved formulations that have reached regulatory approval include a colloidal dispersion that complexes amphotericin B in a 1:1 mole ratio with the sterol cholesteryl sulfate (ABCD) (Guo et al. 1991); a lipid complex that is an association of dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), and amphotericin B in a 10:7:3 mole ratio (ABLC) (Janoff et al. 1988; Perkins et al. 1992; Bhamra et al. 1997); and a liposomal formulation of amphotericin B, with a composition of hydrogenated soy phosphatidylcholine (HSPC), cholesterol, disteroylphosphatidylglycerol (DSPG), and amphotericin B in a 2:1:0.8:0.4 mole ratio (AmBisome) (Adler-Moore and Proffitt 1993; Fujii 1996). ABCD is reported to exist as disk-like particles of diameter > 100 nm and thickness < 10 nm. ABLC is thought to exist as micron-sized ribbon-like structures, and AmBisome consists of small unilamellar vesicles < 100 nm in diameter. All three of these formulations have markedly altered biopharmaceutics relative to d-AmB (Fujii 1996; Janknegt et al. 1992).

In this article we examine the relative toxicity of these formulations as measured by potassium release from red blood cells during incubation at 37°C. These data directly measure the availability of amphotericin B to mammalian membranes at physiological temperatures and are further shown to reflect the high-dose acute toxicity manifested in murine in vivo models. In addition to d-AmB, ABCD, ABLC, and AmBisome, we examine the practice of diluting d-AmB in the 20% fat emulsion Intralipid, which has been claimed (Kirsh et al. 1988; Moreau et al. 1992; Joly et al. 1996) to be a method of reducing toxicity, despite contrary information (Sievers et al. 1996). Intralipid is an emulsion of soybean oil, egg yolk phospholipids, and glycerin, used for parenteral nutrition. Finally, to directly measure the availability (and thence the intrinsic efficacy) of amphotericin B to ergosterol-containing, fungal membranes, potassium release experiments are performed using *Candida albicans* fungal cells in place of the red blood cells.

METHODS

Fresh (< 2 days) Sprague-Dawley rat whole blood was obtained from Lampire Biological Laboratories (Pipersville, PA) or Harlan Bioproducts for Sciences (Indianapolis, IN). Blood was preserved in sodium ethylenediamine tetraacetate (EDTA). Just prior to use, cells were washed 3 times in a buffer consisting of 147 mM NaCl, 3 mM KCl, and 10 mM dibasic sodium phosphate (adjusted to pH 7.4 with HCl). Centrifugation was at 3000 × *g* for 12 min at 4°C. Serial dilutions of AmBisome (NeXstar Pharmaceuticals Inc.), Abelcet (ABLC, The Liposome Company, Inc.), Amphotec (ABCD, Sequus Pharmaceuticals, Inc.), or Fungizone (d-AmB, Bristol-Myers Squibb Company) (the latter three obtained from Priority Health Care Corporation, Altamonte Springs, FL) were prepared in D5W, the recommended diluent specified in each product package insert. A deoxycholate solution (10 mM) was also serially diluted as a control and showed no effect on potassium values except at the highest concentrations (see later discussion). Incubates were prepared with 450 μl washed blood and 50 μl diluted amphotericin B product. Amphotericin B concentrations in the incubates spanned a range from 0.5 mg/ml to 0.02 μg/ml. The method was implemented on a BioMek 2000 biorobotic workstation (Beckman Instruments, Fullerton, CA). Incubates were prepared in duplicate in 96-well "deep-well" plates (1 ml/well). Sample plates were incubated at 37°C for 0–12 h, with constant, gentle agitation achieved by erecting each plate on its side on an orbital rotator, allowing the incubate to mix along the long axis of the assay well. After incubation, the plates were centrifuged at 1000 × *g* for 10 min at 4°C. The supernatant was evaluated for $[K^+]$ using a Beckman EL/ISE ion-selective electrode clinical electrolyte analyzer (Beckman Instruments, Brea, CA). Porcine blood was obtained from Lampire Biological Laboratories and was preserved in CPDA. Human blood was collected from healthy donors, preserved in sodium EDTA, and pooled. Intralipid (20%; Pharmacia, Inc., Clayton, NC) was obtained from Priority Health Care Corporation (Altamonte Springs, FL).

Analogous amphotericin B titrations were performed for each formulation in *Candida albicans* cells as follows. Yeast cells were grown in a primary culture of Sabourad dextrose broth and grown overnight. Yeast were harvested by pelleting the cells in a GSA rotor at 1000 × *g* at 4°C. The cells were washed three times in the same buffer used to wash the rat red cells. The pellet was reconstituted in the buffer to give a ~ 50% cell suspension. Titrations, incubation, and analysis for external potassium were carried out in a manner analogous to the red cell titrations, except that the incubates were permitted to vent during incubation.

Amphotericin B titrations were fit to the expression:

$$[K^+] = \frac{A[\text{ampho B}]^B[K^+]_{max} + [K^+]_{min}}{A[\text{ampho B}]^B + 1}$$

This expression is an adaptation of the IC50 type of expression that is generally applicable to sigmoidal, biological response curves. The fit parameters are then used to calculate the value K50, which is defined as the concentration of

amphotericin B leading to 50% leakage of intracellular potassium. A lower value of K50 indicates a higher availability of the total pool of amphotericin B in the given formulation and therefore a higher toxicity as measured by potassium release. Total potassium release was compared to that achieved by incubation after exposure to 10 μM valinomycin (CalBiochem, La Jolla, CA) for 1 h (conditions sufficient to equilibrate intra- and extracellular potassium based on comparison to detergent lysis).

K50 values obtained with this assay have an intra-assay precision of ~5%, and an interassay precision of ~10%.

LD50 values were determined for variable-dose amphotericin B tail vein injections using female C57Bl/6 mice, 17–23 g in weight, with 14-day observation of mortality. These studies were carried out in adherence to the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23, revised 1985).

After one of the 4-h *Candida albicans* incubations described earlier, the plate contents were sampled and diluted 10-fold in the wash buffer. Material was then further diluted by serial 10-fold dilutions, reaching a concentration of 10^{-8} of the original culture concentration. Then 100 μl of each appropriate dilution was plated on Sabourad 4% dextrose agar plates and incubated at 37°C for > 24 h. These plates were then counted to determine CFUs (colony-forming units) per milliliter of incubate. CFUs were determined for the culture dilutions that had the largest dilution (to minimize the amount of residual amphotericin B), gave a reasonable number of colonies, and where dilution normalized CFUs no longer changed with dilution.

RESULTS

There are several methods available for the testing of amphotericin B formulations for toxicity. Two methods commonly used are acute, high-dose lethality testing in sensitive mouse strains (e.g., C57Bl/6 or Swiss Webster) and in vitro incubations with erythrocytes (from a variety of sources). We chose the latter over the former because of greatly improved assay precision, and because the assay allows one to examine the intrinsic toxicity of the formulation itself (i.e., the physicochemical propensity of amphotericin B to become available to the red cell membrane) uncoupled from the pharmacokinetics or biodistribution. Erythrocyte potassium leakage was chosen over other markers of red-cell toxicity (e.g., hemolysis, Na^+/K^+-ATPase inhibition, oxidative damage, etc.) because these other markers occur at higher concentrations of amphotericin B (and/or longer times), and are most probably second-order effects. Incubations were performed at 37°C with incubation times from 0 to 12 h to better assess the kinetic components of formulation toxicity. This type of red blood cell assay is based on work by Butler and Cotlove (1971). Subsequent literature exists documenting the sensitivity of rodent erythrocytes to amphotericin B (Kotler-Brajtburg et al. 1979; Anhert-Hilger et al. 1982; Gruda et al. 1988, 1991), and we have chosen blood from Sprague-Dawley rats. Some published erythrocyte assays use temperatures significantly lower than 37°C; d-AmB and ABCD both react nearly identically at 4°C and 37°C in rat red blood cells, but ABLC has very slow

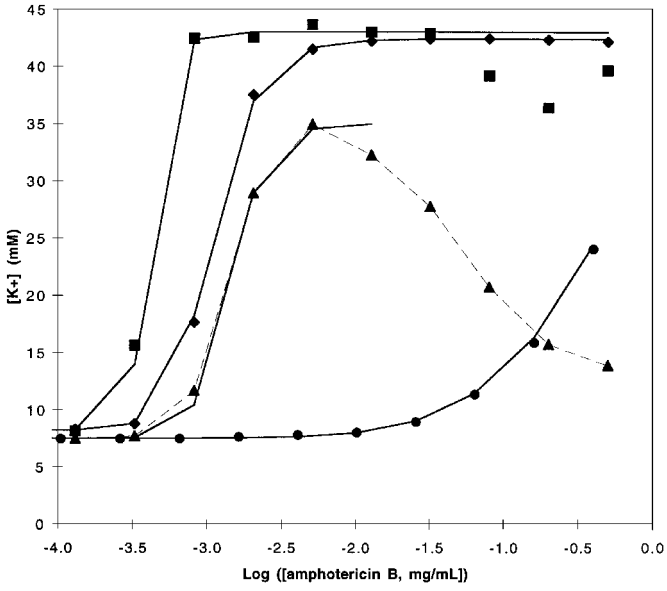

FIG. 1. Titration of amphotericin B formulations in washed rat blood. Incubation time and temperature were 4 h and 37°C, respectively. (●) AmBisome, (■) d-AmB, (◆) ABCD, (▲) ABLC. Solid curves are theoretical fits to the data generated as described in the text. The dashed curve follows the data for ABLC.

reactivity and AmBisome essentially none at the lower temperature (data not shown), possibly because of the temperature dependence of phase transitions in the lipid formulations. Therefore, meaningful testing with these formulations must be carried out at physiological temperatures.

Results for 4-h incubations of serial dilutions of d-AmB, ABCD, ABLC, and AmBisome are shown in Figure 1. Clearly, the AmBisome liposomal formulation of amphotericin B has a much lower ability to induce potassium leakage in rat red blood cells. The onset of significant potassium leakage occurs at an amphotericin B concentration nearly 300 times higher than for ABLC and nearly 1000 times higher than for d-AmB. Some data at the higher concentrations for d-AmB reveal the effects of the deoxycholate itself, which can cause the generation of solid material in the incubates, which spins down, decreasing the potassium values. The ABLC curve exhibits a "rollover" at higher concentrations, a phenomenon attributed to a slower reaction rate of the higher concentrations of ABLC in the washed rat red-cell incubation (discussed later), possibly due to a change in aggregation state going to higher concentrations. Overall, the red blood cell sensitivity is of the order d-AmB > ABCD > ABLC ≫ AmBisome. The calculated K50 values for d-AmB, ABCD, ABLC, and AmBisome are 0.4 μg/ml, 1.1 μg/ml, 1.4 μg/ml, and 400 μg/ml, respectively (Table 1). These are shown in Figure 2 plotted against the determined murine LD50 values for each product (cf. Guo et al. 1991; Janoff et al. 1988; Fujii 1996). Also shown in Figure 2 are data for other liposomal preparations of amphotericin B (L-AmB) having the same composition as AmBisome. These formulations were prepared during process development, scale-up, and

TABLE 1
Calculated K50 values for amphotericin B, and ratio of K50 for amphotericin B to K50 for d-AmB

Incubate	Calculated K50 (μg/ml amphotericin B) and in parentheses ratio to K50 for d-AmB			
	d-AmB	ABCD	ABLC	AmBisome
Washed rat blood, 4 h	0.42 (1)	1.10 (2.6)	1.43 (3.4)	400 (950)
Washed rat blood, 12 h	0.35 (1)	0.27 (0.8)	0.33 (0.9)	6.7 (19.1)
Whole rat blood, 4 h	0.91 (1)	4.94 (5.4)	3.59 (4.0)	500 (550)
Whole porcine blood, 12 h	1.61 (1)	5.95 (3.7)	3.25 (2.0)	219 (136)
Whole human blood, 12 h	2.54 (1)	14.66 (5.8)	10.44 (4.1)	> 3000 (1200)
Washed *C. albicans*, 4 h	29.1 (1)	30.9 (1.1)	34.7 (1.2)	30.7 (1.1)

validation studies for AmBisome, and they have significantly worse acute murine toxicity profiles. In C57Bl/6 mice, a murine strain very sensitive to amphotericin B, the LD50 of d-AmB is approximately 2.5 mg/kg. The AmBisome tested had an LD50 calculated to be ~ 160 mg/kg. ABCD and ABLC have LD50 values determined to be 32 (26–37) and 30 (27–34) mg/kg, respectively. The process/scale-up liposomal amphotericin B samples all had LD50 values determined to be < 100 mg/kg. The plot

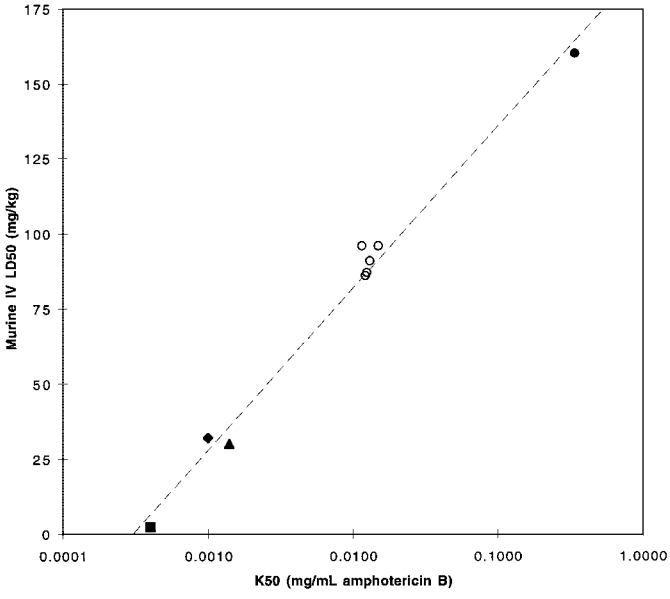

FIG. 2. Plot of murine LD50 versus K50 (see text and Table 1) for titrations of amphotericin B formulations in washed rat blood. Incubation time and temperature were 4 h and 37°C, respectively. (●) AmBisome, (O) process validation/scale-up preparations of liposomal amphotericin B exhibiting worse murine in vivo toxicity (see text), (■) d-AmB, (◆) ABCD, (▲) ABLC. The curve is a linear regression line for the data [log(K50) vs. LD50].

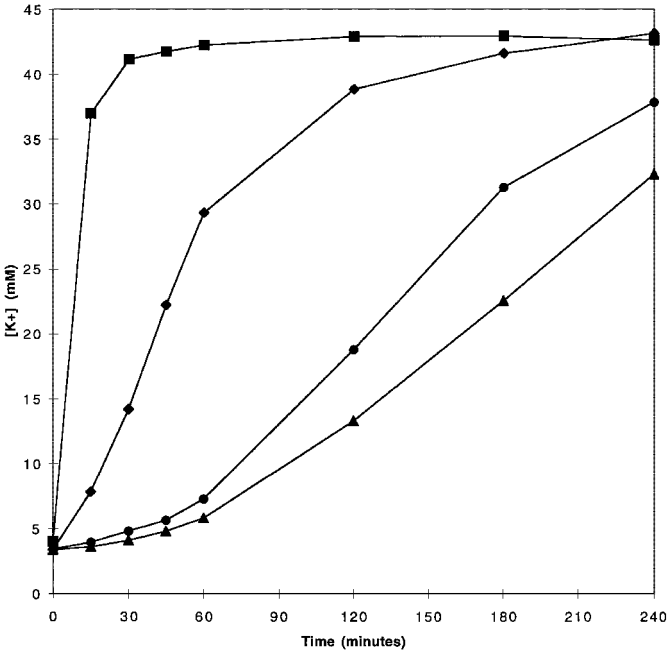

FIG. 3. Kinetics of potassium release during incubation of amphotericin B formulations in washed rat blood at 37°C. (●) AmBisome at 0.4 mg/ml, (■) d-AmB at 0.002 mg/ml, (◆) ABCD at 0.005 mg/ml, (▲) ABLC at 0.005 mg/ml.

of log(K50) versus LD50 shows that the K50 value is directly related to the determined LD50, suggesting that the acute lethal toxicity is related to the availability of amphotericin B to partition into mammalian membranes and that relative toxicity is well defined by the rat red blood cell assay.

Titration curves for ABLC and AmBisome after 4-h incubation yield data consistent with a slow equilibration of the drug-bearing moieties with the red blood cells, whereas for ABCD and d-AmB the amphotericin B is essentially immediately available. The time dependence over a 4-h period of incubation at 37°C is shown in Figure 3 for d-AmB, ABCD, ABLC, and AmBisome at concentrations of each product giving significant potassium release after 4 h. Clearly, d-AmB and ABCD rapidly equilibrate with the red blood cells with concomitant release of intracellular potassium. Both AmBisome and ABLC interact much more slowly and exhibit similar curves. (Note that the concentration of AmBisome is 80 times the concentration of ABLC in this plot.) The AmBisome curve is sigmoidal, and incubation beyond 4 h is predicted to lead to titration curves that reflect a state much closer to equilibrium. (It should be noted that this system will never come to true equilibrium, as both drug and red cell have finite lifetimes under the experimental conditions employed; the system will simply move to a temporal region of slower change). Incubation of the 4 formulations for 12 h leads to the curves shown in Figure 4. All curves now appear relatively similar in shape. d-AmB, ABCD, and ABLC are all now essentially superposable, indicating that virtually all of the amphotericin B present in these formulations is available to the red blood cells after 12 h. The K50 value for AmBisome lies

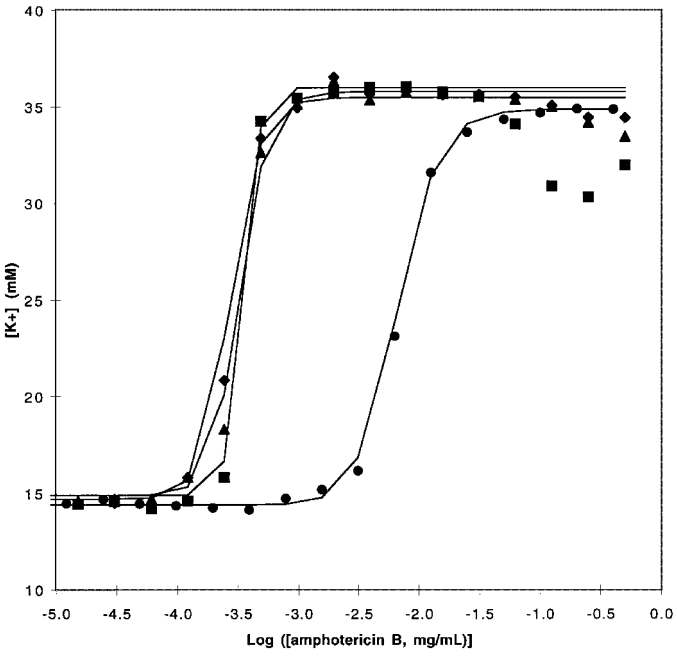

FIG. 4. Titration of amphotericin B formulations in washed rat blood. Incubation time and temperature were 12 h and 37°C, respectively. (●) AmBisome, (■) d-AmB, (◆) ABCD, (▲) ABLC. Curves are theoretical fits to the data generated as described in the text.

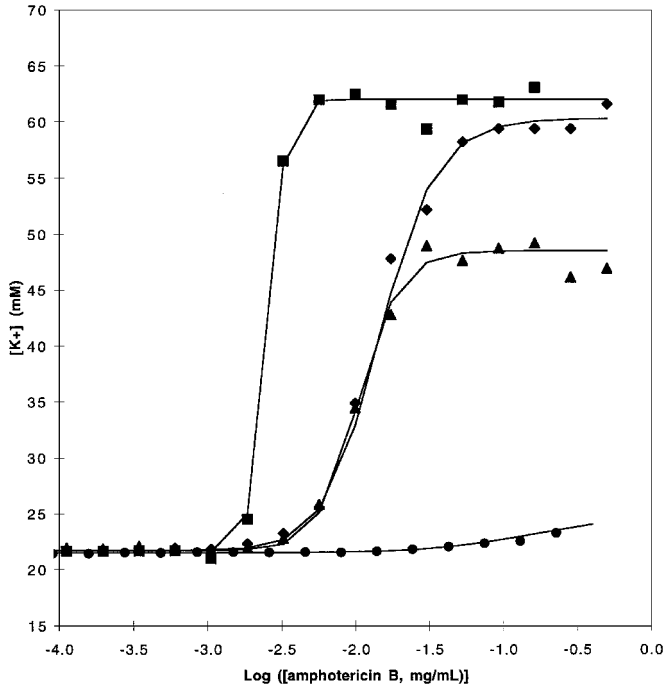

FIG. 5. Titration of amphotericin B formulations in whole, pooled human blood. Incubation time and temperature were 12 h and 37°C, respectively. (●) AmBisome, (■) d-AmB, (◆) ABCD, (▲) ABLC. Curves are theoretical fits to the data generated as described in the text.

at ~20-fold higher concentration than the other three formulations (Table 1). The narrowing of the (still large) gap between d-AmB (and the other formulations) and AmBisome is due to the aforementioned total availability of amphotericin B in these formulations, which causes a reduction in the dynamic range of the assay for these formulations.

To examine the species dependence of these results, and to assess the role of the wash butter or plasma proteins, experiments were carried out in whole rat blood, whole porcine blood, and whole human blood. K50 values from 4-h incubations in whole rat blood and 12-h incubations in whole porcine and whole human blood are shown in Table 1. Titration curves for human blood are shown in Figure 5. Results in all cases were qualitatively the same. In each case, d-AmB has the lowest calculated K50, ABCD and ABLC have K50 values somewhat higher, and AmBisome has a K50 at least 100-fold higher than ABCD and ABLC as detailed in Table 1. The 12-h whole porcine and whole human blood experiments still reflect differences between ABCD, ABLC, and AmBisome relative to d-AmB, reflecting an overall slower rate of interaction of all formulations with the red cells in the presence of the plasma proteins. Four-hour titration curves for whole rat blood and 12-h tiration curves for whole pig blood (not shown) and 12-h titration curves for whole human blood (Figure 5) exhibit a smaller degree of "rollover" for ABLC than washed rat blood. It is possible that the presence of plasma proteins alters the aggregation state change mentioned earlier, leading to relatively more rapid interaction of ABLC and the red cells at the higher concentrations. The kinetic behavior of these formulations is also somewhat similar in whole human blood to the behavior in washed

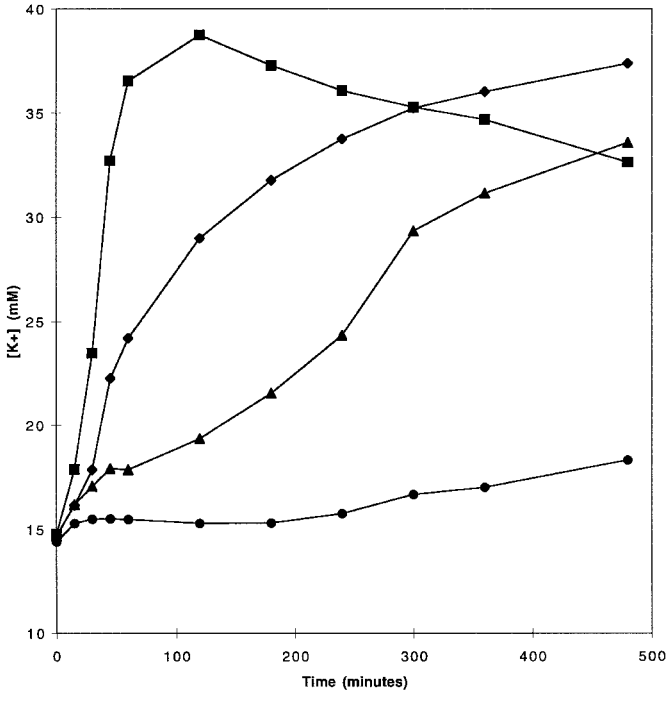

FIG. 6. Kinetics of potassium release during incubation of amphotericin B formulations in whole, pooled human blood at 37°C. (●) AmBisome at 0.8 mg/ml, (■) d-AmB at 0.01 mg/ml, (◆) ABCD at 0.1 mg/ml, (▲) ABLC at 0.1 mg/ml.

rat blood (Figure 6). d-AmB and ABCD both again react very rapidly, while AmBisome displays a curve with a somewhat longer reaction time than in washed rat blood. ABLC, however, reacts significantly more rapidly relative to AmBisome in whole human blood than in washed rat blood, probably reflecting the effects of plasma proteins. The evidence for the influence of plasma proteins on ABLC observed here is in accord with data presented by Bhamra et al. (1997). Overall, it can be concluded that the results determined for potassium release in washed rat blood are relevant to behavior in human whole blood, and that species differences and plasma proteins play a second-order role in the interaction of these formulations with mammalian membranes, with the exception of the relative rate of reaction of ABLC in whole blood.

Toxicity of d-AmB diluted in 20% Intralipid (and not processed further) was compared to d-AmB diluted in D5W at a variety of d-AmB/Intralipid ratios, as detailed in Table 2, in washed rat blood for 4- and 12-h incubations. In all cases the K50 value of the Intralipid dilution was lower than the corresponding D5W diluted d-AmB. This indicates that all of the amphotericin B becomes available to the red blood cell membrane at both incubation times. The lower (worse) K50 value likely represents red-cell lysis caused by the Intralipid itself. An examination of the kinetics of this interaction indicates only a very slight retardation of the rate of availability relative to d-AmB alone (Figure 7).

TABLE 2
Calculated K50 values for Fungizone (d-AmB) diluted in Intralipid or D5W

	Calculated K50 value (μg/ml amphotericin B)		
Incubate	Incubation time (h)	K50	K50 ratio, Intralipid/D5W
10% Fungizone in Intralipid	4	1.33	0.83
5% Fungizone in Intralipid	4	1.00	0.64
2.5% Fungizone in Intralipid	4	1.01	0.65
10% Fungizone in D5W	4	1.60	
5% Fungizone in D5W	4	1.56	
2.5% Fungizone in D5W	4	1.56	
10% Fungizone in Intralipid	12	0.98	0.82
5% Fungizone in Intralipid	12	1.13	0.88
2.5% Fungizone in Intralipid	12	0.91	0.63
10% Fungizone in D5W	12	1.19	
5% Fungizone in D5W	12	1.29	
2.5% Fungizone in D5W	12	1.44	

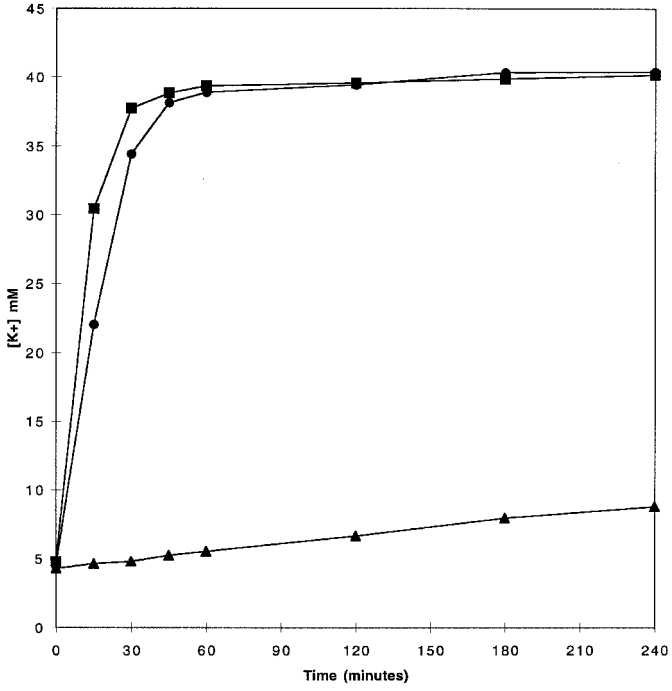

FIG. 7. Kinetics of release of potassium during incubation of d-AmB diluted in Intralipid or D5W in washed rat blood. Incubation temperature was 37°C. (●) d-AmB at 0.002 mg/ml in Intralipid, (■) d-AmB at 0.002 mg/ml in D5W, (▲) Intralipid alone.

DISCUSSION

The results presented here, taken together, provide evidence that the formulation of amphotericin B with the lowest intrinsic toxicity as assessed by potassium release is AmBisome. If one accepts hypothetically that the formulations d-AmB, ABCD, and ABLC represent optimized renderings of amphotericin B in surfactant, sterol without lipid, and lipid without sterol, respectively, these formulation types each result in different degrees of availability of amphotericin B to mammalian membranes with the toxicity of d-AmB > ABCD ≈ ABLC ≫ AmBisome. These results directly refute earlier suggestions that liposomal preparations of amphotericin B are intrinsically more toxic than extended membrane structures (Janoff et al. 1988; Perkins et al. 1992). The mixture of Intralipid and d-AmB (certainly an "unoptimized" lipid-based formulation) exhibits only very slight improvement in toxicity as demonstrated by reduced potassium release being observed only at the earliest time points. At longer time points (4 and 12 h) d-AmB/Intralipid appears to be slightly *more* toxic than d-AmB diluted in D5W. These results lend support to other published studies that find no justification for clinical use of this combination (Sievers et al. 1996).

The origin of the stability of AmBisome in terms of the availability of the drug to red cells is due first to the fact that the liposomes are stable vesicles containing phospholipids and cholesterol, and there is no significant thermodynamic driving force to transfer amphotericin B from the liposomes to the red-cell membranes. Further, these particular vesicles are thermally stable at 37°C, as the membrane phase transition has a midpoint at ~ 55°C and this transition is only beginning to achieve onset at physiological temperatures. Additionally, the drug to lipid ratio in AmBisome is such that the amphotericin B molecules are arranged and stabilized in conventional pore structures as reflected by ion transport data and circular dichroism spectra reported for this formulation (Fujii et al. 1997). Finally, the presence of the anionic lipid DSPG may present the opportunity for favorable electrostatic stabilization of the amine-containing (albeit zwitterionic) amphotericin B molecule (as might also be the case for DMPG in ABLC and cholesteryl sulfate in ABCD).

The intrinsic toxicity to mammalian membranes demonstrated here by potassium release from erythrocytes is demonstrably reflective of the acute, high-dose toxicity seen in vivo in murine models. It is certainly reflective of overall toxicity as well, but this toxicity is a combination of intrinsic bioavailability to mammalian membranes, kinetics of this availability, and biopharmaceutics.

Increase in the therapeutic index for a drug can be achieved by reduction of toxicity and improvement or retention of efficacy. A large body of preclinical and clinical evidence for AmBisome (reviewed in Fujii 1996; Proffitt et al. 1991; Anaissie et al. 1991; Hiemenz and Walsh 1996) demonstrates that AmBisome retains almost all of the potency of d-AmB while reducing toxicity. This data includes comparable in vitro activity for AmBisome versus d-AmB, comparable efficacy for in vivo animal models, and comparable efficacy with lower toxicity in clinical studies. To assess this retention of efficacy in a manner analogous to the red cell toxicity assay we have incubated the different amphotericin B formulations in *Candida albicans* cells and measured potassium release (Gale 1974). The titration curves in this incubation are essentially superposable for all formulations for a 4-h incubation (Figure 8) and also for 2- and 12-h incubations (data not shown). K50 values (Table 1) are also very similar for all formulations. In order to demonstrate that potassium release observed here correlates with cell killing, we determined the number of CFUs per milliliter of cell culture after incubation for 4 h with AmBisome or d-AmB. Incubates with $[K^+]$ values at baseline exhibited 10^9 CFU/ml. As $[K^+]$ rises, CFUs per milliliter are observed to drop, reaching a level $< 10^4$ CFU/ml as $[K^+]$ reaches maximum values. Therefore, observation of potassium release in these incubates is reflective of events leading to cell death. While the yeast cell is a far more complicated system than the red cell, the results demonstrate conclusively that the intrinsic availability of amphotericin B in each formulation to partition into ergosterol-containing yeast cell membranes is retained.

Table 1 shows the ratio of red cell K50 values for each formulation relative to the K50 for d-AmB. ABLC and ABCD have

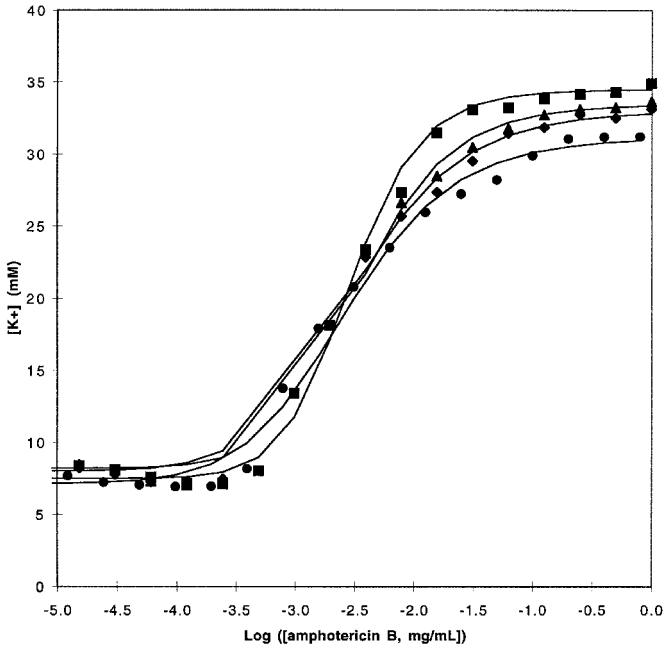

FIG. 8. Titration of amphotericin B formulations in *Candida albicans* cells. Incubation time and temperature were 4 h and 37°C, respectively. (●) AmBisome, (■) d-AmB, (◆) ABCD, (▲) ABLC. Curves are theoretical fits to the data generated as described in the text.

K50-based ratios in the range 1 to 6, while for AmBisome these are in the range 20 to > 1000. These ratios represent reduction in toxicity. The corresponding ratios for yeast cell K50 are near unity for all formulations (Table 1). Therefore, the improvements in intrinsic toxicity profile as measured by the change in red-cell K50 with formulation can likely be applied without attenuation to improvement of therapeutic index.

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