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Novel antifungal drug delivery: stable amphotericin B-cholesteryl sulfate discs

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Summary

To improve the clinical utility of a potent fungicidal agent, amphotericin B (AB), we developed a novel drug dosage form based on the specific interaction of AB with sterols. AB and sodium cholesteryl sulfate at an equimolar ratio form a thermodynamically stable discoidal complex of uniform size. The therapeutic index of the drug is significantly improved (4–6-fold) as a direct result of the unique physical properties of this colloidal system. This drug/lipid complex does not hemolyze red blood cells, exhibits only low binding to plasma lipoproteins in vitro, alters plasma kinetics and tissue distribution compared to a deoxycholate/AB micellar formulation, and is effective in eradicating fungal infections in vivo.

Introduction

The need for effective antifungal therapies to treat disseminated fungal infections in immunocompromised patients, such as acquired immune deficiency syndrome (AIDS) or cancer patients after intensive chemotherapy and thermotherapy, has significantly increased in recent years. Amphotericin B, a broad-spectrum fungicidal agent, is the drug of choice for these life-threatening fungal infections (Medoff et al., 1983; Hughes et al., 1990).

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Amphotericin B is an amphiphilic molecule that has a high affinity for membrane sterols. In model membrane systems, AB interacts preferentially with cholesterol but has little affinity for phospholipids (Norman et al., 1976; Medoff et al., 1983). The selectivity of AB is thought to be related to its approx. 10-fold higher binding affinity for ergosterol ($K_d = 6.9 \times 10^5$), the main sterol found in fungal cell membranes, than for cholesterol ($K_d = 5.2 \times 10^4$), the predominant sterol found in mammalian cells (Readio and Bittman, 1982). When introduced into a membrane, AB is believed to form 1:1 complexes with sterol(s), and eight such complexes aggregate to form a pore, which lyses the cell.

Because of the poor water solubility of AB

(Asher et al., 1977), the current clinical formulation (Amphotericin B for injection, USP) is solubilized in a bile salt, sodium deoxycholate, to form a micellar dispersion. The clinical utility of the drug in this system is limited, however, due to adverse effects, including fever, chills, nausea, vomiting, anemia, and nephrotoxicity. Nephrotoxicity is most severe and is often dose-limiting.

Drug delivery systems such as liposomes previously have been used in attempts to increase antifungal specificity and decrease the toxicity of AB (New et al., 1981; Graybill et al., 1982; Taylor et al., 1982). Some liposomal AB preparations have been evaluated clinically and showed promising results (Lopez-Berestein et al., 1985, 1987, 1989). The optimal formulation for clinical use, however, has not yet been established. Studies with liposomes prepared with various types of phospholipids, sterols or sterol derivatives, including cholesteryl hemisuccinate and cholesteryl sulfate, have indicated that both the efficacy and toxicity of the drug can be manipulated by lipid composition, size, and type of the lipid carriers (Juliano et al., 1987; Szoka et al., 1987; Jullien et al., 1989).

We describe here a novel formulation, amphotericin B colloidal dispersion (ABCD), based on the unique affinity of AB for sterols. The sodium salt of cholesteryl sulfate (CS), a naturally occurring metabolite of cholesterol, forms a stable colloidal complex with AB at a 1:1 drug to lipid molar ratio. ABCD differs from conventional phospholipid liposomes in its ultrastructure and has remarkably reduced toxicity in vivo compared to a micellar formulation of AB. We report here the structure of this novel complex and its ability to act as a drug delivery system for AB by measuring its safety, efficacy, and pharmacokinetics in animals.

Materials and Methods

Materials

Amphotericin B, USP, was obtained from Dumex (Copenhagen, Denmark). Sodium cholesteryl sulfate was a product of Genzyme Corp. (Framingham, MA). Amphotericin B for Injection, USP (Fungizone^{R}), was obtained from E.R. Squibb & Sons, Inc. (Princeton, NJ). All other chemicals were USP grade.

Preparation of ABCD

ABCD was prepared by isothermally injecting a solution (400 ml) of equimolar ratio of AB and CS in dimethyl sulfoxide into a 5 mM Tris, 0.1 mM EDTA buffer, pH 7.0 (7 l), at 50–55°C. Concentrations of AB in the dimethyl sulfoxide and the final aqueous phase after the injection were 25 and 1.35 mg/ml, respectively. Our preliminary experiments showed that size and shape of the colloidal particles are affected by the temperature and the rate of injection and the ratio of AB and CS used. After being cooled to room temperature, the aqueous phase was concentrated about 4-fold and diafiltered to remove residual dimethyl sulfoxide by using a hollow fiber membrane cartridge (Amicon, Danvers, MA). Lactose at 95 mg/ml was added to bring the osmolarity to 290 mOsmol/kg, and the final solution was filter sterilized through a 0.22 μm membrane filter (Millipore, Bedford, MA).

Electron microscopy

Negative staining of ABCD was performed with 2% potassium phosphotungstate at pH 6.45–6.5 on carbon-coated grids by the drop procedure (Hamilton et al., 1980). Briefly, a drop of ABCD at 1 mg/ml was placed on the grid. Excessive fluid was removed from the grid surface with a Whatman no. 1 filter paper and a drop of 2% phosphotungstate was immediately added. After 0.5–1 min of the contact time, the excessive fluid was again removed and the preparation was allowed to air dry. Grids were examined and photographed at $\times$20 000 magnification and 80 kV in a Siemens 101 electron microscope. For the particle size measurement, electron microscopic images taken at $\times$20 000 magnification were enlarged precisely 3 times. Particle diameters were determined directly from the enlarged prints ($\times$60 000) by using a magnetic digitizer (Hipad digitizer, Houston Instruments, Houston, TX) as previously described (Chen et al., 1984). Mean diameter and standard deviation were calculated from 100 randomly selected particles.

Electron spin resonance (ESR) spin-labeling

ESR spin-labeling, using a stearic acid spin label (5-doxylstearic acid with the 4,4-dimethyl-3-oxazolidinyl group attached to C-atom 5; Lasic and Hauser, 1985), was performed to evaluate the shape of the colloidal particles. The spin label was added to ABCD in a molar ratio of 1:100 (spin label/AB). An ethanol solution of the spin label was dried in a tube and an appropriate amount of ABCD was added. The mixture was left at room temperature for several hours with occasional mixing. ESR spectra were measured with a Varian E9 ESR X band spectrometer (modulation frequency 100 kHz, receiver gain 10^4-fold).

Gel permeation chromatography

A Sephacryl S-1000 column (Pharmacia, 1.5 × 65 cm), in 5 mM Tris, 0.1 mM EDTA, 0.02% NaN_3, pH 7.4 was used to investigate the size homogeneity of ABCD. A 2–4 ml sample (5 mg/ml) was applied to the column and fractions of 2.3 ml were collected. Each column fraction was analyzed for content of AB and CS. Mean particle diameter in selected fractions was also measured. In some column runs, fractions containing AB were pooled and amounts of AB in the pooled samples were determined. Recovery of AB mass from the column was about 95%.

Incubation of human blood or plasma with ABCD

The hemolytic effect of ABCD was compared with deoxycholate/AB micelles (Fungizone) in vitro. Fresh human blood was collected in vacutainers containing EDTA to give a final concentration of 0.1%. The blood was mixed with ABCD or deoxycholate/AB micelles to give a final AB concentration of 28 μg/ml. Samples were incubated at 37°C for 30 min, then centrifuged at 3000 rpm. Hemolysis was indicated by the presence of red color in the plasma supernatant.

To investigate dissociation of ABCD in plasma, human plasma was mixed with ABCD or deoxycholate/AB micelles at a final AB concentration of approx. 0.3 mg/ml and incubated at 37°C for 30 min. Plasma lipoproteins were separated by sequential preparative ultracentrifugation (Havel et al., 1955) and concentrations of AB associated with the lipoprotein fractions were determined.

Acute intravenous toxicity of ABCD in mice

60 male and 60 female Swiss Webster mice, weighing 18–22 g each, were used to determine the intravenous toxicity of ABCD at doses of 20, 30, 40, 50, 60, and 70 mg AB/kg of body weight. For the deoxycholate/AB micellar formulation, 50 male and 50 female mice received doses of 2, 3, 4, 5, and 7 mg AB/kg. Mortality of the animals was monitored for 7 days, and the LD_{50} was determined by the Litchfield-Wilcoxon method (Litchfield and Wilcoxon, 1949).

Pharmacokinetic studies of ABCD

Male Sprague-Dawley rats weighing 250–300 g were divided randomly into two groups of six animals each. They received either ABCD or deoxycholate/AB micelles by bolus injection in the tail vein at a daily dose of 1 mg AB/kg body weight for 15 consecutive days. At the end of the experiment, all animals were killed. Concentrations of AB in plasma and tissue samples were determined.

Analytical techniques

Hydrodynamic diameter of ABCD was measured at 20°C on a Nicomp Model 200 dynamic laser particle sizer. The concentration of AB in ABCD was determined spectrophotometrically at 405 nm in 50% methanol using a molar absorptivity of 1.715×10^5 l mol^{-1} cm^{-1} and by high-performance liquid chromatography (HPLC) at 405 nm using a reverse-phase column (Waters micro-Bondpack C-18, 1 ml, Waters Associates, Milford, MA) and a mobile phase of 45% acetonitrile in 2.5 mM Na_2EDTA. For plasma samples, AB was first extracted on a solid-phase cartridge (Bond-Elut C-18, Analytichem International, Harbor City, CA). The unretained plasma fraction was washed off the cartridge with methanol/10 mM phosphate buffer (35:65, v/v). AB retained on the cartridge was then eluted with 0.75 ml of acetonitrile/2.5 mM Na_2EDTA (60:40, v/v) and the eluant was injected onto the HPLC column for quantitation. The assay sensitivity was 5 ng/ml and the inter- and intra-day

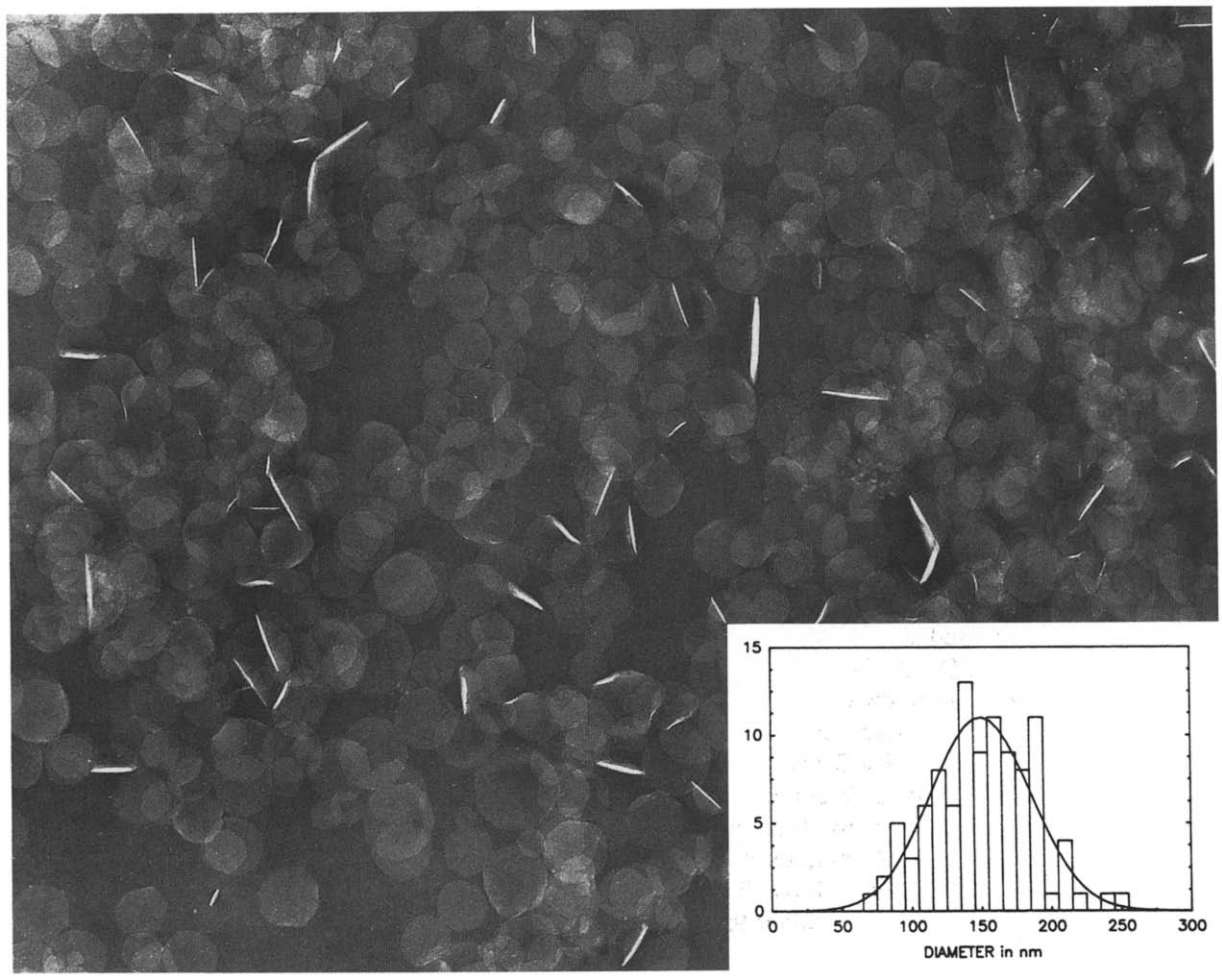

Fig. 1. Electron microscopic image of negatively stained ABCD (×45 000). The colloidal particles appeared as discs of uniform size. Needle structures represent edges of the discs. Some edges are thicker due to folding. (Inset) Histogram showing the distribution of particle diameters of ABCD by measuring 100 random particles from photographs.

coefficients of variation were 5% or less. Tissue samples were homogenized in methanol and then extracted and separated as described for the plasma samples. Concentration of CS in ABCD was determined by HPLC at 207 nm with a reverse-phase C18 column (Spherisorb S5 ODS-1, 4.6 × 250 mm) and a mobile phase of methanol/0.5 M aqueous dodecyltriethylammonium phosphate. The assay sensitivity was 17 μg/ml and the inter- and intra-day coefficients of variation were less than 5%.

Results and Discussion

Structure of ABCD

The aqueous dispersion of the colloid produced was slightly hazy to opalescent. Negative staining of the complex by electron microscopy revealed the presence of uniquely thin discs of highly uniform diameter (Fig. 1). This structure is different from the conventional micelles, liposomes, or ribbon-like phospholipid/AB aggregates reported recently (Janoff et al., 1988). Mi-

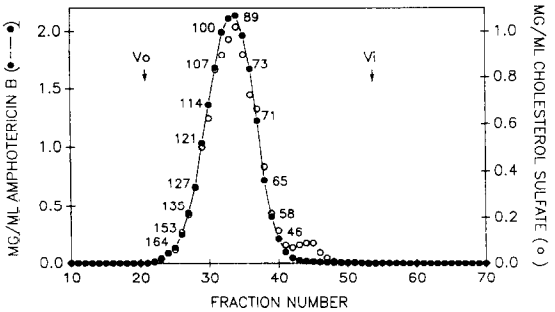

Fig. 2. Elution profile of ABCD from a Sephacryl S-1000 column (1.5×65 cm). Numbers along the elution profile are mean hydrodynamic diameters of ABCD in each fraction and were determined by dynamic light scattering. Arrows show the calibrated void volume (V_0) and included volume (V_i), respectively.

cellar systems are generally too small and unstable to be detected by electron microscopy, whereas the ribbon-like lipid aggregates are several micrometers in diameter. Phospholipid liposomes are typically closed bilayer structures as determined by electron microscopy of thin sectioning or negative staining techniques (Hamilton et al., 1980). The nonliposomal structure of ABCD was confirmed by radioactive sucrose entrapment studies, which showed that these disc-shaped particles do not have an entrapped aqueous volume (data not shown). The mean diameter of the discoidal particles, obtained directly from electron photomicrographs, was 143 ± 36 nm (Fig. 1, inset). Estimates of their edge thickness by this technique indicate that they are extremely thin (less than 5 nm). A hydrodynamic diameter of 100 ± 22 nm was obtained by dynamic light scattering using a laser particle sizer. This value corresponded to the theoretical hydrodynamic diameter of a thin disc with the diameter of 141 nm (Tanford, 1961).

To define further the nature and size homogeneity of the colloidal particles, we performed gel permeation chromatography of ABCD using a calibrated Sephacryl S-1000 column. Fractions eluted from the column were analyzed for AB, CS, and particle size. Greater than 98% of AB and 95% of CS mass from ABCD co-eluted in a single peak in similar proportion at a 1:1 drug to lipid molar ratio (Fig. 2). Particle size analysis of the individual fractions by dynamic laser particle sizer clearly indicates that the separation was a function of size, with mean hydrodynamic diameter ranging from 40 to 170 nm. These results strongly suggest that ABCD is an equal molar complex of AB and CS regardless the size of the particles.

In sharp contrast, chromatography of the deoxycholate/AB micelles under the same experimental conditions resulted in micelle dissociation and drug precipitation within the column bed with no detectable amount of drug eluted. These results demonstrate one of the key differences between ABCD and conventional micelles. The micellar system is at thermodynamic equilibrium and, because this equilibrium depends largely on the critical micellar concentrations of the constituents, rapid dilution leads to instant instability. The data also suggest that ABCD is a thermodynamically stable complex that remains intact during passage through Sephacryl columns in which the sample is diluted approx. 50-fold.

ESR labeling, using 5-doxylstearic acid, was used to help elucidate the shape of the colloidal particles. It has been shown that this technique can distinguish between spherical, cylindrical (rod-like), and lamellar (smectic or vesicular) symmetries because of the different mobilities of the spin labels in these structures (Lasic and Hauser, 1985). The ESR spectra of 5-doxylstearic acid spin label incorporated in the colloidal particles show a typical lineshape of label molecules embedded in an anisotropic environment with lamellar symmetry (Fig. 3). Because the same

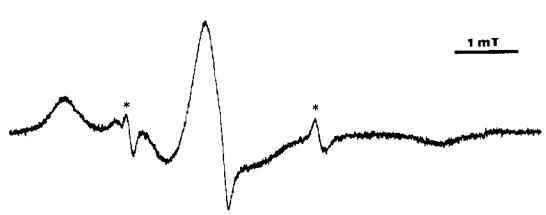

Fig. 3. The X band ESR spectrum of the ABCD labeled with 5-doxylstearic acid at 20°C. A typical lineshape of label molecules embedded in an anisotropic environment with lamellar symmetry is observed. A weak isotropic signal (*) due to the nonincorporated spin probe molecules is superposed on the spectrum. Bar indicates 1 mT (millitesla) of magnetic density.

sample did not show any encapsulated volume, the only possible structure that can be assigned is a disc. The splitting of the ESR spectrum gives a value of the order parameter $S_{33} = 0.79$, which is higher than S_{33} normally observed in phospholipid vesicles (approx. 0.60). This indicates tight, well-ordered molecular packing in the discoidal complex.

CS is a normal membrane constituent of many mammalian cells and is distributed widely in human tissues and fluids (Iwamori et al., 1981). X-ray analysis of the purified sodium salt of CS revealed that the molecules crystallize as a dihydrate and are arranged 'tail to tail' in a double layer (Pascher and Sundell, 1977). In fact, liposomes have been prepared by sonicating mixtures of CS and cholesterol (Brockerhoff and Ramsammy, 1982). Interaction of AB with CS may form rigid and tightly packed lipid layers that are unable to bend sufficiently to form closed vesicular structures (Lasic, 1988). Because of the presence of a polar hydroxyl region, AB probably forms a shield at the disc edges and is oriented in such a way that the seven hydroxyl groups along its side are in contact with the polar aqueous environment.

Stability in blood or plasma

After intravenous administration, the deoxycholate/AB micelles are believed to dissociate rapidly (Edmonds et al., 1989), resulting in a binding of AB to cholesterol-containing plasma membranes of cells and to cholesterol on the surfaces of plasma lipoproteins (Brajtburg et al., 1984). Plasma lipoprotein-bound AB is more toxic than AB alone in vivo (Koldin, et al., 1985). Moreover, exposure of the erythrocytes to drug can lead to hemolysis (Kinsky, et al., 1962).

We tested the hypothesis that ABCD forms a tight complex that does not readily dissociate after intravenous injection. When whole human blood was incubated with micellar AB, hemoglobin was rapidly released, indicating erythrocyte lysis, but this was not observed in human blood incubated with ABCD (Fig. 4, top). The hemolytic effect of the micellar formulation is likely due to the combined effects of AB and the deoxycholate carrier. When human blood plasma

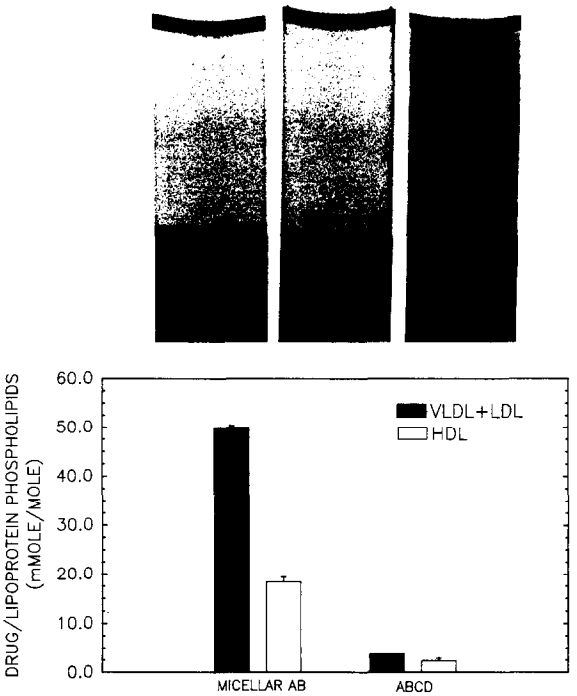

Fig. 4. (Top) Whole human blood (3.5 ml) centrifuged after incubating for 10 min at 37°C with 28 μg/ml deoxycholate/AB micelles (right tube), or 28 μg/ml ABCD (middle tube), vs drug free control (left tube). (Bottom) Incorporation of AB into plasma very low/low density lipoproteins (VLDL+LDL) and plasma high density lipoproteins (HDL) from deoxycholate/AB micelles or ABCD. Amounts of AB recovered in the combined lipoprotein fractions were 24 and 2% for micellar AB and ABCD, respectively.

was incubated with deoxycholate/AB or discoidal ABCD, the amount of AB that bound to subsequently isolated very low and low (isolated together as one fraction), and high density plasma lipoproteins was 14 and 8 times less, respectively, in the case of ABCD (Fig. 4, bottom). Together, these studies indicate that AB remains bound to its lipid carrier after ABCD administration, thus reducing the possibility of rapid distribution of the drug to cholesterol-containing red cells and plasma lipoproteins in the blood.

Acute toxicity in mice

Because the stable, tightly associated AB/CS complex may also minimize AB delivery to non-targeted host cells in vivo and thus improve the safety of the drug, acute intravenenous toxicities

of ABCD and deoxycholate/AB micelles, based upon AB equivalents, in mice were determined. The LD_{50} values for ABCD in male and female mice were 36 and 38 mg/kg, respectively, and the LD_{50} for deoxycholate/AB were 2.6 and 2.0 mg/kg, respectively (Table 1). Thus, ABCD showed a 13–19-fold increase in safety compared to the deoxycholate/AB micelles.

Pharmacokinetics of ABCD

Reduction of AB-related side effects depends on delivering less drug to specific non-target organs, especially the kidneys. We studied the tissue distribution of AB after administration of ABCD and deoxycholate/AB micelles in rats by bolus injection at a daily dose of 1 mg/kg for 15 consecutive days. Administration of ABCD showed a significantly less AB in the plasma and most other tissues after 2 weeks of daily administration (Fig. 5) as compared to the micellar formulation. Reduced concentrations in the kidneys correlated with decreased renal toxicity (Table 2), indicating that ABCD lowered drug-related adversities by reducing delivery to this organ.

Although ABCD led to significantly higher AB concentrations in the liver, no associated hepatotoxicity was observed in terminal gross pathological examinations. The similarity in hepatic enzyme levels found in serum of the ABCD-treated animals as compared to controls (Table 2) suggests that ABCD is probably taken up intact by reticuloendothelial system (RES) of the liver. One of the functions of the RES of the liver and other organs is to remove foreign particulates (including fungal cells) from the circulation. Although the metabolism of this pool of ABCD is presently unknown, as is the mechanism by which AB is delivered to fungi in vivo, animal efficacy studies (below) have shown that ABCD retains the spectrum and potency of AB as an antifungal agent, indicating that this colloidal system is capable of delivering drug to fungi even though it reduces the delivery of drug to many tissues in the host.

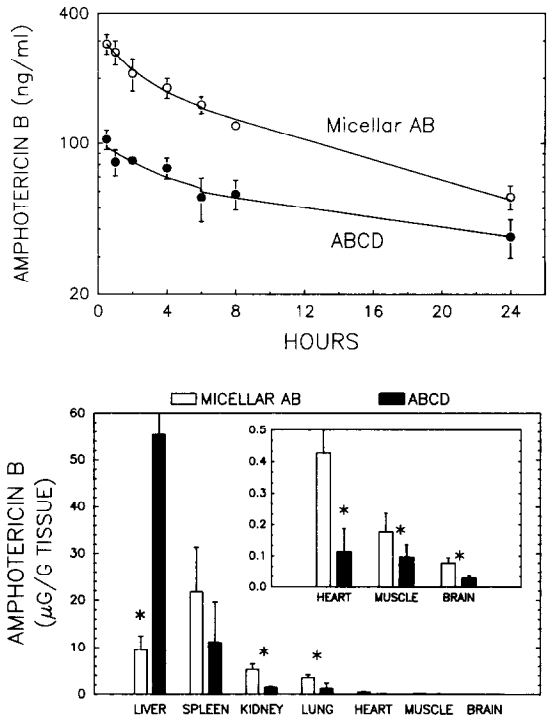

Fig. 5. (Top) Plasma AB concentrations in rats after a single intravenous dose (1 mg/kg) of deoxycholate/AB micelles or ABCD. (Bottom) Tissue distribution of AB in rats that received 14 daily doses (1 mg/kg/day) of deoxycholate/AB micelles or ABCD. $^*P < 0.05$.

TABLE 1

Acute intravenous toxicity of deoxycholate / AB micelles and ABCD in mice

Formulation	Male		Female	
	LD_{50}	95% confidence limits	LD_{50}	95% confidence limits
	(mg/kg)			
Deoxycholate/AB micelles	2.6	1.6–4.4	2.0	1.4–2.9
ABCD	36	29–45	38	31–47

Swiss Webster mice, weighing 18–22 g each, were used throughout the experiment. Death of the animals was monitored for 7 days following the intravenous injection.

TABLE 2

Serum urea nitrogen, alanine aminotransferase in rats after fourteen consecutive daily administrations (1 mg / kg per day) of ABCD or deoxycholate / AB micelles

Assay	Group		
	Undosed control	ABCD	Deoxycholate/AB micelles
Serum urea nitrogen (mg/dl)	12 ± 1.7	18 ± 4.5	34 ± 6.0 *
Serum alanine aminotransferase (IU/l)	43 ± 7.5	37 ± 3.6	33 ± 4.8

Values are means and standard deviations from six animals. * $P < 0.05$ between ABCD and micellar AB. Levels of serum urea nitrogen and alanine aminotransferase were used as nephrotoxicity and hepatoxicity indicators, respectively.

Efficacy in vivo

ABCD was effective in the treatment of murine systemic coccidioidomycosis. At a dose of 10 mg/kg, it eradicated *Coccidioides immitis* completely from all target organs of infection (liver, lungs, spleen) in all mice and there were no apparent signs of toxicity (Clemons et al., 1990). Based on the dose-response curves of survival data and reduction of fungal cells in organs vs dose of ABCD and deoxycholate/AB, we estimate that the effective dose of ABCD was about 3 times higher than that of the deoxycholate/AB. However, as reported above, the lethal dose of ABCD was 13–19 times higher than that of deoxycholate/AB micelles. The therapeutic index (toxic dose divided by effective dose) of ABCD relative to deoxycholate/AB was thus 4–6 times higher when administered to systemic *Coccidioides*-infected mice. In addition, deoxycholate/AB is often ineffective in treating fungal infections in neutropenic and immunodeficient patients (Bodey, 1977), but an AB/CS complex was shown to be more effective than deoxycholate/AB micelles in treating invasive aspergillosis in immunosuppressed rabbits (Patterson et al., 1989).

Conclusion

The current data provide evidence that the toxic effects of AB, a potent antifungal agent, can be modulated by formulating the drug into a stable lipid complex with sodium cholesteryl sulfate. The unique structure and tight association of the drug with this stable discoidal complex seem to prevent the incorporation of AB into the host tissues and cells. This may be responsible for the reduced AB distribution to tissues such as the kidney, where side effects most commonly occur, without the accompanying loss of antifungal activity. Because the therapeutic index of the drug is significantly improved in this dosage form, it should allow higher doses to be administered in humans and thus be more effective in treating disseminated fungal infections. The safety and efficacy of this novel drug dosage form are currently being evaluated in the clinical trials.

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