IN VITRO AND IN VIVO ACTIVITY OF AMPHOTERICIN B-LIPID FORMULATIONS AGAINST EXPERIMENTAL TRYpanosoma CRUZI INFECTIONS

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Abstract. The activities of four amphotericin B formulations, Fungizone®, AmBisome®, Amphocil®, and Abelcet®, were compared in vitro and in vivo against Trypanosoma cruzi infections. In vitro, Fungizone and Amphocil were highly active against T. cruzi Y strain amastigotes in macrophages with 50% effective dose (ED₅₀) values of 0.027–0.028 µg/ml, which were 7-fold and 42-fold more active than AmBisome and Abelcet, respectively. In vitro activities of all formulations against T. cruzi amastigotes in Vero cells were similar, with ED₅₀ values in the range of 2.0–4.2 µg/ml. Acute infections of the T. cruzi Y strain in BALB/c mice were suppressed in all animals by a single 25 mg/kg dose of the liposomal formulation AmBisome. At the same dose, the two other lipid formulations, Amphocil and Abelcet, increased the survival rate but did not suppress infection in all animals.

South American trypanosomiasis, or Chagas disease, caused by the kinetoplastid parasite Trypanosoma cruzi, has a prevalence of 16–18 million and an estimated 90 million individuals are considered to be at risk of infection.¹ The drugs currently recommended for the treatment of Chagas disease are the 2-nitroimidazole benznidazole and the nitro-furan nifurtimox.² Both drugs have limitations: efficacy is restricted to the acute phase of the disease, there are serious toxic side effects, patients require long courses of treatment, and there is marked variation in the drug sensitivity of parasite populations.³ Many different classes of drugs have been studied in the search for alternative therapies,³ and much current interest has focused upon the potential of sterol biosynthesis inhibitors, for example, antifungal azoles.⁴–⁶

The polyene antibiotic amphotericin B has previously been shown to have activity in vitro against amastigotes, trypanomastigotes, and epimastigotes of T. cruzi,³ as well as against trypanomastigotes at 4°C in studies to find new drugs to prevent transmission of Chagas disease by blood transfusion.⁷,⁸ Amphotericin B also exhibited activity against T. cruzi in infected mice,⁹ although this was not confirmed in another study.¹⁰ However, there have been limited clinical studies on the therapeutic efficacy of amphotericin B in Chagas disease.³ Amphotericin B exerts its selective activity against fungi as well as protozoa Leishmania and T. cruzi through a higher affinity for ergosterol and ergosterol-like sterols, the predominant membrane sterol in these eukaryotic microorganisms, than for cholesterol, the predominant sterol in mammalian cells.¹¹–¹³ The use of amphotericin B has been limited by its high toxicity. Recently, lipid formulations that have lower toxicity have been developed for the treatment of systemic mycoses.¹²,¹⁴,¹⁵ Three commercially available lipid formulations, the liposome AmBisome® (NeXtar, San Dimas, CA), the colloidal dispersion Amphocil® (Liposome Technology, Inc., Menlo Park, CA), and the lipid complex Abelcet® (The Liposome Company, Ltd., London, United Kingdom), have also been shown to be effective against experimental¹⁶–¹⁹ and clinical visceral leishmaniasis,²⁰–²² as well as against experimental cutaneous leishmaniasis.²³ In this study, the experimental activities of these three commercial lipid formulations of amphotericin B were compared with the parent drug, amphotericin B deoxycholate (Fungizone®; E. R. Squibb and Sons, Hounslow, United Kingdom) and the standard drug benznidazole, against experimental infections of T. cruzi in vitro and in vivo.

MATERIALS AND METHODS

In vitro assays. Trypanomastigote test. Trypanosoma cruzi MHOM/BR/00/Y trypanomastigotes were harvested in vitro from Vero cell layers in Dulbecco’s minimal essential medium (Life Technologies, Paisley, United Kingdom) plus 10% heat-inactivated fetal calf serum (Harlan Sera-Lab, Loughborough, United Kingdom). A total of 10⁵ trypanomastigotes/100 µl were exposed to the drugs (30, 10, 3, 1, 0.3, and 0.1 µg/ml) and viability (motility) was assessed microscopically at 24, 48, and 72 hr at either 37°C or 4°C and a minimal inhibitory concentration (MIC) value determined by microscopic examination.

Amastigote test. Murine (CD1; Charles Rivers, Ltd., Margate, United Kingdom) peritoneal macrophages were harvested 24 hr after starch (Life Technologies) induction (2% aqueous intraperitoneally) and 100 µl was dispensed into 16-well Lab-tek® (Life Technologies) tissue culture slides at a concentration of 5 × 10⁵/ml. After 24 hr, the cells were infected with T. cruzi strain Y trypanomastigotes, which were harvested from Vero cell feeder layer cultures, at a ratio of 5 parasites:1 macrophage. After 24 hr, the level of infection was checked by microscopy, and the infected cells were exposed to drug (10, 3, 1, 0.3, 0.1, and 0.03 µg/ml) in quadruplicate. All tissue culture slides were maintained at 37°C in a 5% CO₂/air mixture. After three days, the cultures were fixed with methanol, stained with Giemsa, and examined microscopically to determine the percentage of cells infected in treated and untreated controls. The 50% effective dose (ED₅₀) values (MSxfit®; ID Business Solutions, Guildford, United Kingdom) were calculated. This experiment was repeated in infected Vero cells.

In vivo assay. Groups of 5 female BALB/c mice (Charles Rivers, Ltd.) were inoculated with 2.5 × 10⁹ trypanomastigotes of either the T. cruzi MHOM/BR/00/Y or T. cruzi MHOM/CL/00/Tulahuan strains harvested from infected murine cardiac blood. After 5 days, tail blood was examined for the presence of parasites. Mice were treated in all in vivo experiments five days postinfection when positive parasitemia was detectable by examining tail blood microscopically. Only mice with positive parasitemias were included in the experiments. In a first series of experiments, mice were given multiple doses of amphotericin B or a lipid-amphotericin B formulation. Mice were given an intravenous bolus on alternate days for a total of 6 doses. Benznidazole was given
by the oral route for 5 consecutive days. The groups were checked daily and tail blood parasitemias were monitored weekly (number of parasites per 10 microscope fields, × 40 magnification) to give an indication of the level of infection up to 60 days postinfection. In a second series of experiments, mice were given a single dose of amphotericin B or a lipid formulation; one group of control mice were treated orally with 5 consecutive doses of benznidazole.

All experiments were conducted, under license, according to United Kingdom Home Office regulations.

**Drugs.** Benznidazole was a gift from Hoffman-La Roche (Basil, Switzerland) and nifurtimox was a gift from Bayer Plc. (Newbury, United Kingdom). AmBisome® was kindly provided by Dr. R Profitt (NeXstar) and Abelcet® was provided by G. McGettigan (The Liposome Company, Ltd.). Fungizone® (E. R. Squibb and Sons) and Amphocil® (Zeneca, Ltd., Macclesfield, United Kingdom) were obtained commercially. All formulations were prepared immediately prior to each experiment and were stored at 4°C, between doses, for a maximum of 1 week.

**RESULTS**

**In vitro assays.** In the assay against trypomastigotes, Fungizone was the most active of the four amphotericin B formulations tested, followed in decreasing order of activity by Amphocil, Abelcet, and AmBisome. Fungizone had an MIC of 0.3 μg/ml after 24 hr at 37°C and less than 0.1 μg/ml at 4°C. At this time point, Amphocil was the most active of the lipid formulations, showing MIC values of 1.0 and 0.5 μg/ml at 37°C and 4°C, respectively. AmBisome and Abelcet were inactive at a concentration of 30 μg/ml after 24 hr at 37°C and 4°C. Benznidazole was inactive at 30 μg/ml, the highest concentration tested, after 24 hr at both 37°C and 4°C. After 48 hr, there was no change in the activity of benznidazole and AmBisome, which remained inactive, or Fungizone and Amphocil, which remained active. The activity of Abelcet increased and showed MIC values of 0.3 μg/ml at 37°C and 10 μg/ml at 4°C.

All formulations were highly active against amastigotes in both macrophage and Vero cells. Fungizone and Amphocil showed significantly higher levels of activity (P < 0.05) against *T. cruzi* amastigotes in infected macrophages than AmBisome and Abelcet; Fungizone was approximately 42 times more active than Abelcet (Table 1.) Infected Vero cells required a significantly (P > 0.05) higher concentration of amphotericin B than macrophages to clear the parasites. Fungizone and Amphocil again displayed higher anti-trypanosomal activity than AmBisome and Abelcet in this model.

Microscopic examination of the macrophages gave an indication of the toxicity of the amphotericin B formulations; AmBisome caused no damage to cells at a concentration of 3 μg/ml, whereas other formulations at this concentration obliterated the cells.

**In vivo assays.** In a first series of experiments, the activity of multiple doses of AmBisome was evaluated against acute infections of the *T. cruzi* Y and Tulahuan strains in BALB/c mice to establish the comparable activity of Fungizone and AmBisome. Untreated controls of Y strain-infected mice died, on average, 18 days postinfection. The administration of 6 doses of Fungizone at 0.5 mg/kg (maximum tolerated dose) or AmBisome at 12.5 mg/kg enabled 4 of 5 and 5 of 5 mice to survive until the end of the experiment 60 days postinfection. The effect of AmBisome was not curative since trypomastigotes were observed in the tail blood of treated mice for 3 weeks post-treatment. In studies on *T. cruzi* Tulahuan strain–infected mice, untreated controls survived until 13 days postinfection. Mice treated with the lowest dose of AmBisome (6.25 mg/kg) survived until the end of the experiment 60 days postinfection. However, when multiple doses were used, no difference in activity was observed between dose levels on survival time.

Therefore, in a second series of *in vivo* studies the activities of single doses of the amphotericin B formulations were compared; benznidazole was included as the positive control drug (5 days, given orally). In the first experiment, all mice in the untreated control group died by day 11 postinfection. Mice treated with the control drug (benznidazole, 5 and 15 mg/kg) died and only 3 of 5 mice from the group receiving the highest dose (45 mg/kg of benznidazole) survived until the end of the experiment (60 days postinfection). Only the highest dose of AmBisome (25 mg/kg) suppressed the infection sufficiently for all mice (5 of 5) to survive until the end of the experiment. At this highest dose, only 3 of 5 mice survived in the Abelcet- and Amphocil-treated groups. At 5 mg/kg, 1 of 5 mice survived in the AmBisome-treated and Amphocil-treated groups and 0 of 5 survived in the Abelcet-treated group. At lower doses (1 and 0.2 mg/kg), only treatment with AmBisome prolonged survival.

The experiment was repeated with a lower parasite inoculum (1.75 × 10⁵) to prolong survival time and accentuate drug effects. Figures 1 and 2 show that 25 and 5 mg/kg of AmBisome suppressed the infection to enable all mice (5 of 5 in each group) to survive until day 60, 2 of 5 mice to survive at a dose of 1 mg/kg, and 1/5 mice to survive at a dose of 0.2 mg/kg. At the highest dose of 25 mg/kg, 3 of 5 mice per group treated with Abelcet or Amphocil survived (Figures 3 and 4, respectively) and a reduction in survival times accompanied the decreasing doses.

The *in vivo* experiments demonstrate that a single, high dose of amphotericin B-lipid formulation can suppress the acute stage of infection and that AmBisome was the most effective formulation with the lowest host toxicity. Peripheral blood parasitemia was monitored weekly throughout the experiments. In the high-dose groups, the positive control drugs (nifurtimox or benznidazole) cleared parasites from the blood more rapidly than amphotericin B; clearance was achieved after 1 week whereas in the amphotericin B-treated mice it was more than 3 weeks postinfection before trypomastigotes disappeared from the blood.
AMPHOTERICIN B-LIPID FORMULATIONS AGAINST T. CRUZI

FIGURE 1. In vitro activity of benznidazole and Fungizone® against Trypanosoma cruzi. iv = intravenous.

FIGURE 2. In vitro activity of AmBisome® against Trypanosoma cruzi. iv = intravenous.

FIGURE 3. In vitro activity of Abelcet® against Trypanosoma cruzi. iv = intravenous.


DISCUSSION

Although amphotericin B is an established antifungal agent with confirmed activity against T. cruzi in vitro and in vivo, it is not used in the treatment of Chagas because of its major drawback of toxicity. This mammalian toxicity appears especially evident in T. cruzi-infected BALB/c mice, where the maximum tolerated dose in vivo was 0.5 mg/kg, which is lower than the 1.0 mg/kg tolerated dose observed for Leishmania-infected BALB/c mice. The activity of amphotericin B lipid formulations, which were developed to overcome the problem of toxicity and used clinically against both systemic fungal infections and visceral leishmaniasis, offers a new opportunity to explore the potential of amphotericin B for the treatment of Chagas disease.

There was a difference in vitro between the activities of the amphotericin B formulations against both the extracellular trypomastigotes and intracellular amastigotes in either macrophages or Vero cells. A rank order of activity of Fungizone > Amphocil > AmBisome > Abelcet was observed in all in vitro experiments at 37°C for amastigotes and at both 37°C and 4°C for trypomastigotes (Table 1). This variation in activity between the formulations could be due to a number of reasons. Two main differences between murine peritoneal macrophages and Vero cells could influence drug uptake and intracellular concentration and thus activity values. 1) Peritoneal macrophages are fully differentiated and nondividing cells whereas Vero cells are an immortal dividing cell line. Therefore, the number of cells/culture increases and total amount of drug available/Vero cell decreases in comparison with a more stable macrophage assay. 2) Macrophages are highly phagocytic and actively take up molecules, for example, lipoproteins via specific receptors such as low-density lipoprotein (LDL)/high-density lipoprotein (HDL), which are present on all cells but at higher levels in comparison to the non-phagocytic Vero cell.

The different physical properties of amphotericin B both as a free drug and in various lipid formulations, as well as interactions with the host milieu, have been reported. The importance of the aggregation state and particle size of amphotericin B under physiologic conditions and the effects on activity and toxicity have been established, as well as the...
interaction between amphotericin B and the biological milieu. By altering the formulation of amphotericin B, it is possible to manipulate these properties to reduce toxicity and improve uptake and efficacy. Fungizone and Amphocil are the more similar formulations; both are mixed micelles of amphotericin B and detergent. AmBisome consists of amphotericin B intercalated with phospholipid and sterol in a unilamellar liposome, whereas Abelcet is amphotericin B intercalated with sheets of phospholipid. It has been demonstrated in vitro that Fungizone rapidly dissociates, releasing amphotericin B that binds to very low density lipoprotein and LDL, whereas Abelcet releases the amphotericin B slowly, which then binds preferentially to HDL. Amphotericin B plus LDL is less toxic to cells than amphotericin B plus HDL. Although the association of amphotericin B with lipoprotein has been shown not to affect its antimicrobial activity in vitro, it may have some bearing in models of infection in vitro and in vivo. The difference in the rate of drug release from the matrix of the formulation could account for the higher anti-trypanosomal (trypanomastigotes and intracellular amastigotes) activity of Fungizone and Amphocil. At physiologic levels, amphotericin B in Fungizone has been shown to have higher, immediate, antifungal (Candida) activity in vitro than lipid-associated drug. AmBisome and Abelcet maintain their integrity longer, which would allow them to be endocytosed; thus, the drug would be more slowly accumulated within the host cell.

The pharmacokinetics of the four formulations in vivo have been established. All three amphotericin B-lipid formulations reach lower peak plasma levels in comparison with the conventional formulation of amphotericin B deoxycholate following intravenous administration. AmBisome has a prolonged circulation time and a higher peak plasma level in comparison with other lipid formulations. It is the smaller carrier of the three and is negatively charged; thus, it is not removed as quickly from the circulation by the reticulo-endothelial system as are Amphocil and Abelcet. All three formulations are concentrated in the liver, spleen, and lungs and have notably reduced nephrotoxicity compared with Fungizone. In T. cruzi-infected mice, we found that Fungizone followed by Amphocil were more toxic than AmBisome and Abelcet. Only 0.5 mg/kg of Fungizone could be tolerated by infected mice. Amphocil at a dose of 25 mg/kg was not well tolerated by mice, whereas AmBisome and Abelcet at this dose showed no signs of toxicity. The rapid dissociation of drug from carrier results in all tissues being exposed to high levels of drug immediately after administration.

All the formulations tested in vivo at high doses appeared to have a significant anti-trypanosomal activity. AmBisome was the most effective at lower single doses and was the least toxic to mice. However, none of the lipid-amphotericin B formulations cleared parasites from the blood as effectively as benznidazole. Further investigation with multiple doses is required to ascertain a curative dose for the treatment of chronic infection. Lipid-amphotericin B formulations have been successful against visceral leishmaniasis since they can be targeted to the site of infection, namely the macrophages of the reticulo-endothelial system, while reducing the toxicity of amphotericin B. On initial infection, T. cruzi trypomastigotes invade macrophages and multiply until the cell is ruptured, which releases infective trypomastigotes that invade macrophages and other host cells such as skeletal muscle and heart tissue. Over time, the number of trypomastigotes circulating in the blood decreases and amastigotes divide and form pseudocysts within deep tissue. Lipid formulations of amphotericin B must allow higher levels of this potent drug to be established in these tissues. AmBisome is most successful probably because it acts as a reservoir of drug, which increases the circulation time of amphotericin B and permits prolonged tissue exposure. The role of LDL/HDL and its interaction with lipid carriers containing cholesterol could be used in drug delivery; HDL directs cholesterol, a key component in lipid-amphotericin B formulations, towards the liver. Low-density lipoprotein directs them to the kidney, spleen, and importantly, the skeletal muscle, which would be of importance in chronic T. cruzi infections.

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