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Activity of a Heat-Induced Reformulation of Amphotericin B Deoxycholate (Fungizone) against *Leishmania donovani*

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The heat treatment of amphotericin B deoxycholate (Fungizone), which was previously shown to induce superaggregation and decrease the toxicity of the drug to mammalian cells, increased its activity against *Leishmania donovani* in BALB/c mice, whereas it reduced its toxicity. Heat treatment preserved the activity of Fungizone against *L. donovani* HU3-infected mouse peritoneal macrophages.

Amphotericin B (AmB) in its commercial formulation Fungizone is the "gold standard" treatment for systemic fungal infections and is the recommended second-line treatment for visceral leshmaniasis (VL) infections when conventional tetravalent antimony (SbV) therapy is inappropriate or ineffective (4). Unfortunately, AmB causes acute side effects following intravenous (i.v.) administration, and these limit its more extensive clinical use. Recently, lipid formulations of AmB have successfully been developed to greatly reduce the toxicity of AmB and so enable higher doses of the drug to be given (see reference 3 for a review). Dose for dose, Fungizone has greater activity than liposomal AmB against fungal infections (9); however, against experimental VL infections, lipid formulations are more active (1, 2, 5, 6, 8, 13, 14, 18, 20–22, 24, 28). Three lipid formulations have been approved for clinical use: AmBisome (NeXstar, Cambridge, United Kingdom), Abelcet (The Liposome Co., Princeton, N.J.), and Amphocil (Sequus Pharmaceuticals Inc., Menlo Park, Calif.). Despite their proven success against leishmaniasis (7, 10, 23), they are not frequently used against the disease due to their expense. The developing tropical and subtropical countries, where leishmaniasis affects 6 million individuals (27), cannot routinely afford expensive medication. Simple heating of Fungizone at 70°C for 20 min is an inexpensive procedure which could be used to improve the therapeutic index of AmB, as shown for the therapeutic index of AmB against candidiasis and cryptococcosis (19), and encourage its more widespread use.

In this study, the in vitro and in vivo antileishmanial activities of Pentostam (SbV), Fungizone, and heated Fungizone were compared. The differences in their relative toxicities to mammalian cells and mice were observed.

Leishmania donovani MHOM/ET/67/L82 amastigotes were maintained in golden hamsters (Charles Rivers, Margate, United Kingdom). The parasites were harvested from an infected spleen for in vitro and in vivo assays.

A stock solution of Fungizone (Bristol Myers Squibb, La Défense, France) was prepared from a marked bottle by the addition of 10 ml of sterile 5% dextrose (aqueous). Heated Fungizone was prepared as described previously (11). Pentostam (100 mg of SbV/ml) and powdered sodium stibogluconate (NaSbV) were provided by Glaxo Wellcome, London, United Kingdom. NaSbV powder was dissolved in 0.25% methylcellulose for in vivo administration. Drug dilutions were made daily in complete medium for in vitro tests and in 5% dextrose for in vivo tests.

For in vitro assays, peritoneal macrophages were harvested from female CD1 mice (Charles Rivers) 24 h after starch (Sigma) induction and were dispensed into 16-well Lab-tek slides (Nunc Ltd., Chicago, Ill.) at a concentration of 4×10^4/well (100 μl/well) in RPMI 1640 medium (Gibco BRL, Paisely, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (Sera-Lab, Oxon, United Kingdom). After 24 h, the macrophages were infected with *L. donovani* amastigotes at a ratio of 10 amastigotes to 1 cell. After 24 h, the infected cells were exposed to drug for 5 days (the cell overlay and drug were replaced on day 3). Prior to drug administration, Fungizone solutions were incubated for 15 min at 37°C to allow the AmB to bind to the proteins in the serum (26). The role of lipoproteins in the endocytosis of AmB in cells by specific receptors has already been shown (15, 25). Cells were treated with both formulations at concentrations ranging from 1 μM to 0.5 nM. The experiment was terminated on day 5 by methanol fixation and Giemsa staining. The percentage of infected macrophages was evaluated microscopically. The 50% effective doses (ED_{50}s) were determined by linear regression analysis (*xl*fit; Microsoft Excel) with 95% confidence limits. *P* values were calculated by Student's *t* test.

In these assays, no toxicity to macrophages was seen with either formulation at the doses tested. ED_{50}s were found to be 0.035 μg/ml for heated AmB deoxycholate and 0.024 μg/ml for unheated AmB deoxycholate (Table 1). The difference in the activities of the two formulations was not significant ($P > 0.05$). Both Fungizone formulations were more active than sodium stibogluconate. In aqueous solution, AmB exists as a mixture of different species in equilibrium: monomers and soluble and unsoluble aggregates (17). Under the conditions of the in vitro experiments, for concentrations of AmB below 1 μM, both formulations were mainly in the monomeric form (12), and this could give an explanation for their similar activities.

For in vivo assays, 8- to 10-week-old female BALB/c mice (weight, 20 g) were infected i.v. with 1.5×10^7 *L. donovani* L82 amastigotes and were randomly sorted into groups of five mice. At 7 days postinfection, one mouse was killed to check for the patency of infection and drug administration commenced. Sodium stibogluconate was administered subcutaneously for 5

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TABLE 1. In vitro activities of Pentostam and unheated and heated Fungizone against *L. donovani* MHOM/ET/67/L82 in mouse peritoneal macrophages

Formulation	ED_{50} (μg/ml)[a]	ED_{90} (μg/ml)[a]
NaSbV	21.13 ± 1.93[b]	>30[b]
Fungizone	0.024 ± 0.0008[c]	0.067 ± 0.001[c]
Heated Fungizone	0.035 ± 0.0024[c]	0.07 ± 0.015[c]

[a] Values are means ± standard errors of the means.
[b] Concentrations represent micrograms of SbV per milliliter.
[c] No significant difference ($P > 0.05$).

consecutive days. Both Fungizone formulations were administered i.v. for 3 days (dosed on alternate days): 0.04 and 0.2 mg/kg of body weight for the unheated Fungizone formulation and 0.04, 0.2, and 1 mg/kg for the heated Fungizone formulation. At 1 mg/kg, unheated Fungizone was fatal to the mice, so a reduced dose of 0.5 mg/kg was administered daily. The maximum tolerated dosage of heated Fungizone was 2.5 mg/kg per day. All mice were killed on day 14 postinfection. Their livers were weighed, and impression smears were made, fixed with 100% methanol, and stained with Giemsa stain. Parasite numbers were determined by counting the number of amastigotes per 500 nuclei and multiplying that value by the weight of the liver (in milligrams). The ED_{50}s were also determined by linear regression analysis. In a second experiment a higher inoculum of amastigotes, 2×10^7/mouse, was used.

The acute toxicity of unheated Fungizone prevented a direct comparison with the heat-treated formulation. Unheated Fungizone was toxic at 1 mg/kg, while it was possible to inject safely 2.5 mg of the heated formulation per kg. This reduction in toxicity has already been demonstrated for healthy mice or for mice with candidiasis or cryptococcosis (19). Possible explanations for this are the physicochemical properties of the heat-induced superaggregates (11). Both experiments demonstrated that heat-treated Fungizone had an approximately twofold increased antileishmanial activity over that of the untreated formulation (Table 2). The elevated ED_{50}s in experiment 2 reflect the higher level of infection in the mice due to the larger parasite inoculum given in this assay. Both Fungizone formulations were 20- to 60-fold more active than sodium stibogluconate. These formulations were administered by the intravenous route and were then passively transferred to the liver. In general, relatively large (diameter, >0.1 μm) structures are cleared from the blood by the mononuclear phagocyte system (16). The large size of the heated Fungizone aggregates (600 nm) perhaps allows them to be efficiently captured by the mononuclear phagocyte system and to be transferred to the site of infection with amastigotes. This formulation could also act as a reservoir for monomeric AmB.

TABLE 2. In vivo activities of Pentostam and unheated and heated fungizone against *L. donovani* MHOM/ET/67/L82 in BALB/c mice

Expt no. and formulation	ED_{50} (mg/kg)[a]	ED_{90} (mg/kg)[a]
Expt 1		
NaSbV	8.52 ± 0.70	33.69 ± 5.62
Fungizone	0.361 ± 0.067	>0.5[b]
Heated Fungizone	0.144 ± 0.018	0.997 ± 0.012
Expt 2		
NaSbV	16.79 ± 7.32	45 ± 4.8
Fungizone	>0.5	>0.5[b]
Heated Fungizone	0.37 ± 0.14	>1

[a] Values are means ± standard errors of the means.
[b] ED_{90}s were not reached with Fungizone in these experiments.

The pharmacokinetics and activity of heated Fungizone remain to be elucidated, but this study suggests that the heat treatment of Fungizone could provide a simple and inexpensive way to increase the therapeutic index of this formulation for the treatment of visceral leishmaniasis.

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