Antileishmanial activity of nano-amphotericin B deoxycholate

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Methods: We have formulated nanoparticles (10–20 nM) from amphotericin B deoxycholate (1–2 μM) by applying high-pressure (150 argon) milling homogenization and have tested their efficacy in a J774A cell line and in hamsters. Parasite survival and tissue burden in spleen were evaluated for nano-amphotericin B and conventional amphotericin B. Both nano-amphotericin B and conventional amphotericin B were injected intraperitoneally at 5 mg/kg per day for 5 days.

Results: The inhibition of amastigotes in the splenic tissue with nano-amphotericin B was significantly more than with conventional amphotericin B (92.18% versus 74.57%, P = 0.005). Similarly, the suppression of parasite replication in the spleen was also found to be significant (99.18% versus 97.17%, P = 0.05). In a cytotoxicity test, nano-amphotericin B against the J774A cell line had a CC50 of 12.67 mg/L in comparison with 10.61 mg/L for amphotericin B, far higher than the doses used for ED50.

Conclusions: Nanoparticles of amphotericin B had significantly greater efficacy than conventional amphotericin B. This formulation may have a favourable safety profile, and if production costs are low, it may prove to be a feasible alternative to conventional amphotericin B.

Keywords: visceral leishmaniasis, Leishmania donovani, nanoparticles, nanomedicine, in vitro, in vivo

Introduction

The treatment options for visceral leishmaniasis (VL), potentially caused by Leishmania donovani, are limited and unsatisfactory. The drugs available are mostly parenteral and have serious toxicity. This problem is further compounded in Bihar (India) where widespread resistance to pentavalent antimonials persists.1 Amphotericin B has been used as the first-line treatment in these regions because of its nearly 100% cure rates; however, adverse drug reactions (ADRs) are a major limiting factor. Amphotericin B still remains the most important drug. A total dose of 15–20 mg/kg has to be given as intravenous infusions either daily or on alternate days, necessitating prolonged hospitalization. In addition, ADRs are universal, which can occasionally be serious.1 The development of lipid formulations of amphotericin B, especially liposomal formulations, has alleviated this problem. This strategy targets intramacrophage organisms, increasing bioavailability and reducing toxicity.2 Nevertheless, the prohibitive cost of these formulations puts them beyond the reach of most of the patients in the endemic areas of VL, which represent the poorest areas of the world.2

A different approach to lipid formulations could be nanonization of the drug. The nanoparticles are recognized as foreign bodies and phagocytosed by the macrophages leading to target-specific delivery, as Leishmania harbours inside the macrophage phagocytic system.3 Furthermore, the solid nanoparticles are characterized by high weight per volume, which is an ideal situation for sustained drug release by gradual diffusion from the depot. Thus, the drug can be delivered in higher doses and over a shorter duration to achieve cure of the disease.

We have developed nanoparticles of amphotericin B deoxycholate using high-pressure milling homogenization. The present study is aimed at evaluating this formulation in L. donovani infection.
Nanom-amphotericin B in visceral leishmaniasis

Materials and methods

Host

*Mesocricetus auratus*, Syrian golden hamsters, were selected as the animal model. The model mimics several aspects of human VL and can withstand repeated spleen biopsies to check parasite load in addition to a high susceptibility to develop *L. donovani* infection.7 The J774A macrophage cell line (obtained from NCCS, Pune) was used as a cellular host for the *in vitro* intracellular test of antileishmanial activity against amastigotes.

Parasites

*L. donovani* parasites (MZP 301) drawn from splenic aspirates of VL patients from an endemic region (Muzaffarpur, Bihar, India) were grown in a biphasic medium and cultured in RPMI-1640 medium (HyClone) supplemented with 0.2% NaHCO3, 2.05 mM l-glutamine, 12 mM HEPES buffer (HiMedia, India), 15% (v/v) heat-inactivated fetal bovine serum (HIFBS; Gibco, Germany) and 50 mg/L gentamicin at 25 °C. The parasites were used in *in vitro* experiments. The WHO reference strain of *L. donovani* (MHOM/IN/80/DD8) conserved in hamsters was used for *in vivo* experiments.

Isolation of metacyclic-stage parasites and raising in *in vitro* infection

Metacyclic-stage parasites were isolated from 6 day old parasite cultures that were centrifuged, washed twice with 0.02 M PBS (pH 7.2) and resuspended in complete RPMI. FBS (5%) was added to the parasite solution and incubated for 30 min at 37 °C for opsonization in order to obtain the infective metacyclic-stage parasites. Incubation was followed by washing with PBS twice before use in *in vitro* tests.

Antileishmanial drug

The amphotericin B deoxycholate (antifungal/leishmanicidal) drug marketed under the trade name of Funigzone was converted into a nano form and used as the test drug to be compared with Funigzone as the reference drug *in vitro* and *in vivo*.

Preparation of nano-amphotericin B drug (*synthesis and characterization of amphotericin B nanoparticles*)

The synthesis of amphotericin B nanoparticles was carried out employing a mechanical miller. It was then performed in a locally fabricated mechanical miller under the same conditions. The milling vial had a volume of 40 cm³, and the prongs by the mill could be varied to achieve a maximum speed of 300 rpm. Three steel balls of different sizes (two steel balls of 12.5 mm diameter and one steel ball of 5 mm diameter) were used. The optimum results with regard to the production of nanoparticles were achieved by milling for 30 min. The milled powder was taken out and sonicated for 10 min in benzene. The milled powder was characterized by scanning electron microscopy (SEM; Philips: XL 20) and transmission electron microscopy (TEM; Philips E M CM-12 and Tecnai 20G²). The microstructural details of milled particles were monitored through SEM under secondary electron imaging. For SEM studies, a thin film of silver (Ag) (≤50 Å) was deposited on amphotericin B particles to avoid electron beam charging effects. For TEM (Tecnai 20G²) studies of the amphotericin B particles, particles that floated on the surface of distilled water, and hence were expected to be thin, were picked up. These were mounted on a 300 mesh copper grid pre-coated with formvar. As amphotericin B is a low-strength atomic-bonded solid, in order to avoid electron-beam-induced dissociation, a low-dose mode of TEM operation was selected. In order to check the microstructure and crystalline nature of supplied and milled amphotericin B, TEM and SEM investigations were carried out.

Reconstitution of drug

Amphotericin B was reconstituted in sterile, chilled, triple-distilled water to obtain a stock solution of 5 mg/mL. Five percent dextrose was added and stored at 4 °C between the doses for a maximum of a week. Similarly, nano-amphotericin B was also reconstituted at the same concentration and stored. The drugs were further diluted in RPMI according to the concentrations required for *in vitro* tests.

*In vitro* assay on *L. donovani* promastigotes

Working solutions of both drugs, nano-amphotericin B and amphotericin B, at concentrations of 0.8 mg/L were prepared. Aliquots of 200 µL of the drugs in duplicate were dispensed in the first row of a 96-well plate (Corning Inc., COSTAR). In all the remaining wells of selected columns, 100 µL of medium was added. From the drug wells, 100 µL of drugs was aspirated and transferred to the successive well of the second row using a multi-channel pipette. The process was continued to obtain 2-fold dilutions, and the last row was left as the control row. The parasite culture in the stationary phase was washed with RPMI-1640 medium and resuspended to obtain 1×10⁴/mL in the same medium containing 15% HIFBS, pH 7.2. Aliquots of 100 µL of the parasite suspension were dispensed in all the medicated rows and the non-medicated row so as to obtain drug concentrations from 0.4 to 0.025 mg/L and the control in duplicate. The plate was incubated at 25 °C for 24 h in a cold incubator.

*In vitro* assay on *L. donovani* intramacrophage amastigotes

The J774-A macrophage cell line was used for the *in vitro* intracellular drug efficacy test. The macrophages were resuspended at 2.5×10⁵ cells/mL in serum-free RPMI-1640. Two hundred microlitre cell suspensions per well were plated on 8 chamber Lab-Tek tissue culture slides (NUNC, USA) and were allowed to adhere for 2 h in a CO2 incubator with a supply of 5% CO2 at 37 °C. The wells were washed twice with serum-free medium, and the adherent macrophages were infected with metacyclic-stage parasites (0.1 mL of resuspended solution) of *L. donovani* maintaining a Leishmanial macrophage ratio of 10:1 in a 200 µL final solution of a complete medium and incubated overnight. Free promastigotes were washed with serum-free medium, and the cultures were incubated in the complete medium supplemented with 15% (v/v) HIFBS (Gibco) and 50 mg/L gentamicin overnight. After incubation, different concentrations of amphotericin B (0.02–0.005 mg/L) and nano-amphotericin B (0.005–0.0006 mg/L) were dispensed, except in the control well (without drug). The slides were then incubated for 72 h in a CO2 incubator with a supply of 5% CO2 at 37 °C, followed by methanol fixing for 1 min and staining with Giemsa (Qualigens, Mumbai, India). The slides were observed for infected macrophages and the numbers of amastigotes within cells.

Calculation of percentage of inhibition and ED₅₀

The extracellular parasites from drug-treated and control wells were counted after treatment for 24 h using a Neubauer chamber.

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Microscopic enumeration was done for each of the duplicate wells for different concentrations. Parasites with degraded body surface and without movement were not counted. Taking the mean of the control wells to equate to 100% survival, all the counts at different concentrations were converted into percentages. Giemsa-stained slides of the infected macrophage cell line were observed using a light microscope (Zeiss, AXIO, Imager A1), and the numbers of amastigotes and infected macrophages per 100 cells were counted. Percentage inhibition was calculated by using the following formula:

\[ PI = \frac{(PC-PT)}{PC} \times 100 \]

where \( PI \) is the percentage of inhibition, PC the number of amastigotes/infected macrophages per 100 cells in the control well and PT the number of amastigotes/infected macrophages per 100 cells in the drug-treated well.

The ED50 (concentration of drug that inhibits 50% of the parasites) was obtained by plotting a graph of the percentage of inhibition at different concentrations of all the observations using Origin 6.1 version software.

**Cytotoxicity against the J774A cell line**

The J774A cell line was incubated in a 96-well plate containing 2.5 × 10^4 cells/well. The plates were incubated overnight in a CO2 incubator, with a supply of 5% CO2 at 37°C. The test and reference drugs at different concentrations (12.8–0.0025 mg/L) were dispensed in triplicate, and three wells were left as control wells. The plates were incubated for 72 h, and an MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed to assess cell proliferation or viability. Briefly, 50 μL of 0.5% MTT was added to all wells and incubated for 4 h. The plate was spun down at 3000 rpm for 20 min at 4°C, and the supernatant was aspirated. The yellow tetrazolium salt reduced to blue formazan within cells was left to dissolve in 100 μL of dimethyl sulfoxide, and the OD at 540 nm was determined. Results were expressed as percentage reductions in cell viability, compared with untreated control wells. The cytotoxic concentration required to kill 50% of the cells (CC50) was obtained from a graph of the ODs plotted against drug concentration taking the OD of the control well as 100% survival.

**Hamster studies**

Two sets of experiments comprising 14 male hamsters (35–45 g), 4–5 in each subgroup, were carried out. They were infected intracardially with 10^7 amastigotes of Ddb L. donovani isolated from infected hamster spleen. Potency of infection was checked by conducting spleen biopsies of three to four randomly selected hamsters from each set on day 30 post-inoculation. Hamsters were anaesthetized and operated on according to the Committee for the Purpose of Control and Supervision of Experiments on Animals, Delhi, India by injecting thiopentone (Thiosole sodium; Neon Laboratories Limited, India) at 80 mg/kg intraperitoneally. Spleen smears from specimens obtained by biopsies were prepared and stained with Giemsa to confirm infection. The infected animals from each set were randomly divided into three groups, nano-amphotericin B (group A), amphotericin B (group B) and control (group C), each comprising four to five animals and included in the chemotherapy trials the next day. These animals were treated intraperitoneally with amphotericin B and nano-amphotericin at a dose of 5 mg/kg body weight for 5 consecutive days, whereas an equal volume of PBS was given to the control group. Autopsies were conducted on day 7 post-treatment (pt). The weight of the spleen was measured immediately after autopsy, and dabbed imprints on glass slides were prepared. Visceral infection was monitored microscopically by using Giemsa-stained imprints, in which parasite burdens were measured by counting of the number of amastigotes per 500 nuclei. The size of commercial amphotericin B by SEM and TEM was 10–20 nm. The SAD pattern from the particles showed that the size was in the nano region. TEM explorations of the milled particles confirmed the nano sizes of the milled amphotericin B (10–20 nm). The SAD pattern from the particles showed that even after milling, they retained a crystalline nature.

**Statistical analysis**

GraphPad Prism5 version was used for statistical analysis. An unpaired t-test (two-tailed) was applied to determine the significance of the ED50 of nano-amphotericin B over amphotericin B in extracellular promastigotes and intracellular amastigotes and inhibition of infected macrophages. The same test was used for the statistical analysis of inhibition of amastigotes in vivo (spleen) and suppression of parasite replication. The Mann–Whitney test (two-tailed) was used for significance testing of mean amastigotes per 500 nuclei and parasitic burden after the treatment.

**Results**

**Structure/size of the nano formulation**

The size of commercial amphotericin B by SEM and TEM was found to be 1–2 μm. The particles were composites of grains that were found to be separated by grain boundaries. The representative selected area electron diffraction (SAD) pattern from a grain showed its crystalline character. Thus, the important relevant result from the SEM and TEM studies illustrated that the supplied amphotericin B particles are quite large (≥1 μm), which may produce toxicity.

On high-pressure milling, the amphotericin B polygonal particles got divided into smaller particles. The average particle size of the milled particles was between 10–20 nm. This particle size was in the nano region. TEM explorations of the milled particles confirmed the nano sizes of the milled amphotericin B (10–20 nm). The SAD pattern from the particles showed that even after milling, they retained a crystalline nature.

**Nano-amphotericin B against extracellular promastigotes**

The in vitro antileishmanial activity of nano-amphotericin B against promastigotes of L. donovani was more effective than the reference drug amphotericin B. The mean (± SD) ED50 of amphotericin B (0.1125 ± 0.0148 mg/L) was significantly lower than that of the nano formulation (0.0148 mg/L) as 100% survival.
Nano-amphotericin B in visceral leishmaniasis

In vitro efficacy of nano-amphotericin B against amastigotes

The intracellular amastigotes of *L. donovani* parasite (MZP 301 strain) were inhibited by nano-amphotericin B. The ED$_{50}$ of nano-amphotericin B was 3.69-fold lower than that of amphotericin B for the reduction of infected macrophages ([0.012 (SD ± 0.004) versus 0.003 (SD ± 0.001)] ($P = 0.0009$)) (Table 1).

Cytotoxicity of test drugs against the J774A cell line

The nano-amphotericin B and amphotericin B drugs were checked against the J774-A cell line to determine whether the doses used for ED$_{50}$ on intramacrophage amastigotes were toxic to the cell itself; experiments revealed that CC$_{50}$ values were far higher (12.67 and 10.61 mg/L) than the ED$_{50}$ doses (0.004 and 0.012 mg/L, respectively) for intracellular amastigotes.

Effect of test drug on in vivo intracellular amastigotes

In the hamsters that were infected and treated, the results of both experiment sets were merged and mean values were taken for further analysis. The load of the parasites (pt) was found to be significantly less for group A (92.2 ± 6.7%) compared with group B (74.6 ± 14.8%) ($P = 0.0045$) (Table 2).

Applying the formula mentioned earlier, there was a highly significant reduction in the total parasite burden in spleen in the treated groups, especially in the group treated with nano-amphotericin B. Compared with the control group (179.95 × 10$^4$ parasites), the reduction in the parasitic burden in spleen was very high ($P = 0.0002$); 121.6-fold (1.48 × 10$^4$) in group A and 35.35-fold (5.09 × 10$^3$) in group B. Between groups A and B, the total amastigote counts were significantly different after treatment ($P = 0.05$). The percentage of suppression in spleen parasite replication was 99.18% (SD ± 0.78) and 97.17% (SD ± 2.62) in groups A and B, respectively ($P = 0.05$).

### Discussion

Due to the particulate form of liposomes, the drug is preferentially engulfed by the macrophages, leading to targeted drug delivery. The effect of converting amphotericin B into nanoparticles that are considerably smaller (10–20 nm) and that do not lose their original character could be similar, leading to engulfment of these particles preferentially by the cells of the macrophage phagocytic system. This function may further improve as nanosizing increases the adhesion properties of cells to tissues.

When evaluated in *in vitro* studies, nano-amphotericin B retained its antileishmanial activity against both promastigotes and amastigotes. However, its efficacy was significantly more against the intracellular amastigotes than the promastigotes. The effectiveness of nano-amphotericin B was 1.4-fold better for extracellular parasites, whereas it was 3.7-fold better for the amastigotes, the stage responsible for VL, suggestive of a potent antileishmanial drug. Similar to the ED$_{50}$ of our results, Kayser reported a comparatively higher ED$_{50}$ for promastigotes than for amastigotes.$^6$ These results supported the output from golden hamsters in which the inhibition of *L. donovani* infection was significantly greater with nano-amphotericin B. Furthermore, the test of cytotoxicity against the J774-A cell line clearly demonstrated its comparative non-toxicity (CC$_{50}$ 12.6 versus 10.61 mg/L) towards the macrophage cells.

The reason behind the encouraging results of these nanoparticles of amphotericin B, especially with regard to intracellular

### Table 1. Drug concentrations (mg/L) for evaluation of efficacy in *in vitro* tests

<table>
<thead>
<tr>
<th>Parasites/macrophages</th>
<th>nano amphotericin B (± SD)</th>
<th>amphotericin B (± SD)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular promastigotes</td>
<td>0.0798 ± 0.0248</td>
<td>0.1125 ± 0.0148</td>
<td>0.0064</td>
</tr>
<tr>
<td>Intracellular amastigotes</td>
<td>0.0045 ± 0.0026</td>
<td>0.0166 ± 0.0079</td>
<td>0.0122</td>
</tr>
<tr>
<td>Infected macrophages</td>
<td>0.0031 ± 0.0013</td>
<td>0.0124 ± 0.0038</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

### Table 2. Responses to drugs of the amastigotes in the spleen in *in vivo* tests

<table>
<thead>
<tr>
<th>Group (no. of animals)</th>
<th>before treatment (7)</th>
<th>nano AMB (group A) (9)</th>
<th>AMB (group B) (10)</th>
<th>control (group C) (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen weight (g)</td>
<td>1.016</td>
<td>0.4891</td>
<td>0.6855</td>
<td>1.5571</td>
</tr>
<tr>
<td>Amastigotes/500 nuclei</td>
<td>373.84 ± 191.79</td>
<td>29.23 ± 25</td>
<td>95.09 ± 55.4</td>
<td>2506.71 ± 1465</td>
</tr>
<tr>
<td>Percentage inhibition</td>
<td>92.18 ± 6.69</td>
<td>74.56 ± 14.83</td>
<td>179.95 ± 278.74</td>
<td>0.0045</td>
</tr>
<tr>
<td>Parasite burden (LDU)</td>
<td>1.48 ± 1.4</td>
<td>5.1 ± 4.71</td>
<td>179.95 ± 278.74</td>
<td>0.05</td>
</tr>
<tr>
<td>Parasite replication</td>
<td>99.18 ± 0.78</td>
<td>97.17 ± 2.62</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

AMB, amphotericin B.
parasites, is probably due to the targeted delivery to tissues due to nanosizing. We also believe that the increased contact surface of the drug because of the uniform and significantly reduced size (1–2 μm to 10–20 nm) has improved its efficacy. Thus, smaller doses of nano-amphotericin B may be required to achieve the high cure rate currently observed. Hence, nanoparticles of amphotericin B if given intravenously are likely to be taken up by the macrophage phagocytic system, thereby considerably reducing the systemic side effects of amphotericin B akin to that seen with liposomal amphotericin B. If the cost of the production of nanoparticles of amphotericin B works out to be considerably less than that for liposomal amphotericin B, it will be worthwhile to instigate a large-scale study to look at the efficacy and toxicity of nanoparticles of amphotericin B. Animal survival and toxicity data will have to be generated for evaluating the efficacy and safety of such a preparation before clinical studies are contemplated.

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Transparency declarations

None to declare.

References