Short communication

Therapeutic evaluation of free and liposome-loaded furazolidone in experimental visceral leishmaniasis

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1. Introduction

Leishmaniasis is a parasitic disease that afflicts 12 million people worldwide and can be fatal if individuals are infected with the visceral species. Traditional therapy is out-dated and ineffective, often resulting in intense adverse effects, relapses and the need for hospitalisation. Study of drugs that are already in clinical use for the treatment of other diseases could represent a cost-effective alternative for the treatment of neglected diseases. This is exemplified by the recent discovery of miltefosine, an oral anticancer drug with promising in vivo antileishmanial activity[1]. Furazolidone (FZ) is a synthetic nitrofuran derivative that is clinically used as an antibacterial and antiprotozoal agent. Its antileishmanial activity was previously reported by Berman and Lee in 1983[2] and by Neal et al. in 1988[3]. FZ has in vivo antileishmanial activity was previously reported by Berman and clinically used as an antibacterial and antiprotozoal agent. Its

2. Material and methods

2.1. Drugs and chemicals

Hydrogenated phospholipids were kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol, MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue] and RPMI 1640 medium without phenol red (RPMI-PR-1640) were purchased from Sigma (St Louis, MO). DAPI (′,6-diamidino-2-phenylindole dihydrochloride) and fluorescein-5-isothiocyanate (FITC) were purchased from Molecular Probes (Eugene, OR). The pentavalent antimonial compound glucantime® was obtained from sanofi-aventis (São Paulo, Brazil) and pentamidine was from Sideron (São Paulo, Brazil).

2.2. Bioassay procedures

BALB/c mice and golden hamsters were supplied by the animal breeding facility at the Adolfo Lutz Institute (São Paulo, Brazil).
Animals were maintained in sterilised cages and were given water and food ad libitum. Animal procedures were performed with the approval of the Research Ethics Commission and in agreement with the Guidelines for the Care and Use of Laboratory Animals from the National Academy of Sciences.

2.3. Parasite maintenance

*Leishmania (L.) chagasi* amastigotes (MHOM/BR/1972/LD) were isolated from golden hamsters no later than 60–70 days post infection [8].

2.4. Mammalian cells

Peritoneal macrophages were collected from the peritoneal cavity of female BALB/c mice by washing with RPMI 1640 supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a 5% CO2 humidified incubator.

2.5. Determination of the 50% inhibitory concentration (IC50)

The activity of FZ treatment on *L. (L.) chagasi* intracellular amastigotes was determined using previously infected macrophages [9]. Pentavalent antimony (SbV) was used as a standard drug. Test compounds were incubated for 120 h under the same conditions. Parasite burden was defined as the mean number of infected macrophages out of 600 cells (triplicate recordings). Data obtained represent the mean of two independent assays.

2.6. Cytotoxicity against mammalian cells

Macrophages were obtained from the peritoneal cavity of BALB/c mice in RPMI-PR-1640 medium. Cells were incubated with FZ at various concentrations (2.3–300 µg/mL) for 48 h at 37 °C. Pentamidine was used as the reference drug. Viability of the macrophages was determined using the MTT assay [10]. Data obtained represent the mean of two independent assays.

2.7. Furazolidone entrapment in liposomes

Liposomes were prepared by the lipid hydration method followed by extrusion through polycarbonate membranes [11]. For lipidosome preparation, saturated egg phosphatidylcholine, phosphatidylserine and cholesterol were used at a 7:2:1 molar ratio. For preparation of fluorescent liposomes, 100 µg of long-chain dialkylcarboxyanine (DIL C18) was added to the lipid mixtures before drying. Untrapped material was separated from the liposomes by centrifugation (4000 × g for 15 min) and the final phospholipid concentration was determined by the Stewart assay [12]. The average diameter of the liposomes was determined by transmission electron microscopy. The concentration of encapsulated FZ was determined in a high-performance liquid chromatography (HPLC) binary system (Prominence LC-20; Shimadzu Corp., Kyoto, Japan) using an ultraviolet photodiode array detector SPD-M20A on a reverse phase ACE C18 column (4.6 mm × 250 mm, 5 µm particle size).

2.8. Biodistribution studies using laser scanning confocal microscopy

Golden hamsters (*Mesocricetus auratus*) were infected with 1 × 10^8 amastigotes of *L. (L.) chagasi* via the intraperitoneal (i.p.) route. Sixty days post infection, FZ-LP labelled with the fluorescent marker DIL C18 was injected (i.p., 300 µL/animal; n = 4) and animals were euthanised 7 h later. The spleen and liver were removed and fixed in 3.5% formaldehyde. After washing with phosphate-buffered saline (PBS), they were permeabilised with a PGN solution (PBS, 0.2% gelatin and 0.15% NaNO3) containing 0.1% saponin and submitted to labelling. Samples were then incubated for 1 h with a polyclonal anti- L. (L.) chagasi (dog serum) antibody (diluted 1:50 in PGN) and washed three times with PBS. This was followed by incubation for 1 h with anti-dog immunoglobulin G (IgG) conjugated antibodies and with 10 µM DAPI to label DNA-rich structures. Finally, the samples were imaged on a Bio-Rad 1024-UV confocal system (Bio-Rad Hercules, CA) using a 100× 1.4 NA oil immersion objective with phase contrast. Image J software (http://rsb.info.nih.gov/ij/) was used to analyse fluorescence intensity distributions, to adjust contrast or brightness of acquired images and to combine images. Sequential optical sections (z-series) were rendered using Voxx software (http://www.nephrology.iupui.edu/imaging/voxx/) or the surface-rendering module of Huygens Essential (http://www.svi.nl).

2.9. Experimental studies

The efficacy of FZ and FZ-LP treatment was determined using young male golden hamsters (ca. 140 g) previously infected (i.p. route) with *L. (L.) chagasi* amastigotes (1 × 10^8/animal). Forty-five days after infection, the hamsters were treated intraperitoneally for 12 consecutive days with FZ, FZ-LP and SbV administered at 50 mg/kg, 0.5 mg/kg and 50 mg/kg, respectively (n = 6/group). The control group was treated with empty liposomes (without drug). FZ was diluted in Cremophor® EL containing 5% ethanol (no interference with the treatment could be detected in previous experimental assays). Animals were euthanised (58 days post infection) and spleen/liver infections were analysed by light microscopy using Giemsa-stained smears. The removed organs were weighed and compared with the untreated group. The number of amastigotes per 500 cell nuclei (liver and spleen) was determined and multiplied by the organ weight (mg) to obtain Leishman–Donovan units [13]. Two independent assays were performed to confirm the in vivo efficacy of FZ.

2.10. Statistical analysis

Data obtained represent the mean and standard deviation of duplicate or triplicate samples from two independent assays. IC50 values were calculated using sigmoid dose–response curves in GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA) and the 95% confidence intervals were included in parentheses. The Mann–Whitney test (unpaired two-tailed) was used for significance testing (P < 0.05) of parasitic burden and treatment.

3. Results

3.1. Antileishmanial activity and cytotoxicity assay of furazolidone

FZ treatment inhibited intracellular amastigotes of *L. (L.) chagasi* with an IC50 value of 1.58 µg/mL. Glucantime was used as a reference drug and resulted in an IC50 of 27.28 µg/mL. The cytotoxicity assay demonstrated that high concentrations of FZ were toxic to peritoneal macrophages after a 48 h period of incubation, resulting in an IC50 value of 13.45 µg/mL (Table 1).

3.2. Biodistribution of liposomal furazolidone in infected hamsters

FZ was loaded into phosphatidylserine liposomes and labelled with the fluorescent marker DIL C18. FZ-LP was found in the spleen (Fig. 1A) and liver (Fig. 1C) of *L. (L.) chagasi*-infected hamsters 7 h post infection. The in vivo co-localisation of FZ-LP and *L. (L.) chagasi*.
Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50} (95% CI) (µg/mL)</th>
<th>L. (L.) chagasi</th>
<th>THP-1 toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FZ</td>
<td>1.58 (1.51–1.66)</td>
<td>13.45 (11.49–15.75)</td>
<td></td>
</tr>
<tr>
<td>Pentamidine</td>
<td>N/D</td>
<td>8.72 (7.24–10.52)</td>
<td></td>
</tr>
<tr>
<td>Sbv</td>
<td>27.28 (25.7–28.8)</td>
<td>&gt;500</td>
<td></td>
</tr>
</tbody>
</table>

IC_{50}, 50% inhibitory concentration; CI, confidence interval; N/D, not determined; Sbv, pentavalent antimony.

Viability of cells was determined by the colorimetric MTT assay measured at 550 nm.

In vitro antileishmanial activity and cytotoxicity of furazolidone (FZ)\textsuperscript{a}.

3.3. Experimental in vivo treatment

Free FZ was effective against *L. (L.) chagasi* (Table 2), reducing the parasite burden by 82.5% in the spleen (*P* < 0.05) and 85% in the liver (*P* < 0.05) at a dose of 50 mg/kg. The reference drug Sbv\textsuperscript{a} was also tested in a treated control group, which resulted in a reduction in parasite burden by 95% (*P* < 0.05) and 92% (*P* < 0.05) in the spleen and liver, respectively. FZ-LP eliminated 74% of spleen (*P* < 0.05) and 32% of liver (*P* > 0.05) parasite burden at 0.5 mg/kg.

3.4. Encapsulation efficiency, size and lipid quantification

Physicochemical parameters of liposomes were evaluated following drug entrapment. FZ was entrapped in liposomes and resulted in a considerable encapsulation efficiency of 84.9%. As determined by HPLC analysis, the maximum FZ concentration in liposomes was 250 µg/mL. The lipid content was determined by the colorimetric Stewart assay; the formulation had a mean of 15.5 mg/mL phospholipids. Finally, FZ-LP was negatively stained and the mean diameter was analysed by transmission electron microscopy. It was determined that FZ-LP had an internal mean diameter of 149 nm with a multilamellar aspect (data not shown).

4. Discussion

In this study, we demonstrated that a novel liposomal formulation of furazolidone (FZ-LP) targets the intracellular amastigotes of *L. (L.) chagasi*. Preliminary data demonstrated that FZ has in vitro activity against intracellular amastigotes and is 17-fold more effective than treatment with Sbv\textsuperscript{a}, suggesting a potential use for this drug against *L. (L.) chagasi*. The first description of the anti-*Leishmania tropica* activity of FZ (IC_{50} = 0.53 µg/mL) was reported in 1983 by Berman and Lee [2]. Neal et al. [3] reported that FZ was not selective against the intracellular amastigotes of *Leishmania* (L.) donovani, *Leishmania* (L.) enrietti and *Leishmania* (L.) major, as the potential antiparasitic effect was observed at a concentration that was highly toxic to host cells. In our assay, the in vitro
correlation between antileishmanial activity and mammalian toxicity [Selectivity Index (SI)] was 8.5 for FZ based on the activity against *L. (L.) chagasi* amastigotes. These differences in drug effectiveness may be explained by the variability in drug susceptibility among *Leishmania* species, which would alter the SI of a particular drug.

Owing to the promising in vitro activity against *L. (L.) chagasi*, FZ was entrapped in liposomes containing phosphatidylserine and was labelled with the hydrophobic fluorescent marker DIL C18, a high-affinity probe for liposome membranes. When administered to *L. (L.) chagasi*-infected hamsters, FZ-LP was distributed to primary target tissues such as the liver and spleen. Using laser scanning confocal microscopy, co-localisation of FZ-LP with *L. (L.) chagasi* amastigotes within macrophages was demonstrated, suggesting that this formulation of liposomes could be a promising drug delivery system. This is the first description of a liposomal formulation of FZ and its in vivo delivery to intracellular amastigotes of *Leishmania* in mammalian tissues. The small size of the liposomes and the inclusion of the negatively charged phosphatidylserine might influence the targetability of FZ-LP, possibly resulting from the ligand-binding properties of macrophage scavenger receptors (SRs). Our previous in vitro work demonstrated that phosphatidylserine-based liposomes targeted intracellular *L. (L.) chagasi* amastigotes via SRs in macrophages to deliver high amounts of Sb[α][15]. SRs are abundantly expressed in macrophages and liver endothelial cells and represent attractive candidates for mediating the binding and intracellular uptake of liposomes containing negatively charged phospholipids owing to their broad ligand specificity for polyatomic compounds [14]. It has been shown that *Leishmania*-infected macrophages upregulate the SRs CD36, SRB-1 and MARCO and, consequently, could represent a promising target for phosphatidylserine liposomes [7]. It has also been reported that the reticuloendothelial system, similar to the liver, may act as a macrophage depot system for nanoparticles and may slowly release nanoparticle-incorporated drugs into the systemic circulation [15]. Owing to the nanoscale diameter of FZ-LP, this may be advantageous for treatment.

Based on the biodistribution of FZ-LP and the potential in vitro activity of the free drug, an experimental study using a hamster model was performed. The in vivo data demonstrated for the first time that free FZ was effective against *L. (L.) chagasi* at treatment levels comparable with Sb[α]. Furthermore, treatment with FZ-LP resulted in a considerable reduction of the spleen parasite burden and, to a lesser extent, the liver burden when administered at a 100-fold lower dose than free FZ. The efficacy is comparable with the effects of free FZ on the spleen; no significant difference between the two treatments was observed (P > 0.05). This result corroborates the observed targetability of the liposomal formulation, which improves the efficacy of FZ. The inability of FZ-LP to eliminate parasites in the liver may be a result of insufficient drug accumulation in this organ, which was confirmed by confocal microscopy. Despite the considerable FZ entrapment in liposomes, a limit of 250 μg/mL was achieved for each batch using the lipid hydration method, thus limiting the amount of drug administered to animals (maximum of 300 μL/animal). Future experimental assays should test higher doses of FZ-LP (>0.5 mg/kg), other dose regimens (>12 days) and different administration routes (intramuscular, intravenous, subcutaneous).

In conclusion, these data suggest that FZ-LP may be an effective drug candidate for inhibition of *L. (L.) chagasi*. Despite the extensive clinical use of FZ to treat other pathologies, toxicological studies are mandatory to assess the safety of this novel formulation of liposomes. Additionally, FZ may represent a cost-effective alternative for the chemotherapy of visceral leishmaniasis.

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**Competing interests:** None declared.

**Ethical approval:** All animal experiments were conducted under the license of and in accordance with the Instituto Adolfo Lutz (São Paulo, Brazil).

**Appendix A. Supplementary data**


**References**


