Effectiveness of liposomal buparvaquone in an experimental hamster model of Leishmania (L.) infantum chagasi

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A R T I C L E   I N F O

Article history:
Received 2 December 2011
Received in revised form 10 January 2012
Accepted 11 January 2012
Available online 20 January 2012

Keywords:
Leishmaniasis
Buparvaquone
Leishmania (L.) infantum chagasi
Liposomes
Therapy

A B S T R A C T

The objective of this study was to develop a novel liposomal formulation, containing phosphatidylyserine (PS), of buparvaquone (BPQ) and to evaluate its in vivo effectiveness in Leishmania (L.) infantum chagasi-infected hamsters. The activity of BPQ was evaluated against both the promastigote forms of different Leishmania species and the intracellular amastigotes of L. (L.) infantum chagasi. Buparvaquone was entrapped in PS-liposomes (BPQ–PS-LP), and the drug was quantified by ultra-high-performance liquid chromatography. The treatment was quantified by detecting the RNA of the living amastigotes in the spleen and the liver by real-time PCR. In vitro assays with L. (L.) infantum chagasi intracellular amastigotes were performed in peritoneal macrophages for the evaluation of the 50% inhibitory concentration (IC50). BPQ–PS-LP at 0.33 mg/kg/day for eight consecutive days reduced the number of amastigotes by 89.4% (P < 0.05) in the spleen and by 67.2% (P > 0.05) in the liver, compared to 84.3% (P < 0.05) and 99.7% (P < 0.05), respectively, following Glucantime® treatment at 50 mg/kg/day. Free BPQ at 20 mg/kg/day failed to treat the hamsters when compared to the untreated group. BPQ was significantly (P < 0.05) selective against L. (L.) infantum chagasi intracellular amastigotes, with an IC50 value of 1.5 μM; no in vitro mammalian cytotoxicity could be detected. Other cutaneous species were also susceptible to BPQ, with IC50 values in the range 1–4 μM. BPQ–PS-LP caused a significant reduction in the parasite burden at a 60-fold lower dose than did the free BPQ. These results show the potential of PS-liposome formulations for the successful targeted delivery of BPQ in visceral leishmaniasis.

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1. Introduction

Leishmaniasis remains among the most important parasitic diseases in the developing world. Visceral leishmaniasis (VL) has an estimated incidence of 500,000 new cases per year and is the life-threatening form of leishmaniasis; it is the consequence of severe hepatosplenomegaly, which leads to death in untreated patients (Maltezou, 2008). Epidemiologic studies indicate that leishmaniasis is abundant in the Americas and is a great public health concern. In the New World, the disease is caused by a number of different parasite species that are capable of producing a wide variety of clinical manifestations: VL is usually caused by Leishmania (L.) infantum chagasi, and cutaneous leishmaniasis (CL) is usually caused by L. (V.) braziliensis or L. (L.) mexicana species (Grimaldi and Tesh, 1993).

There is a shortage of safe therapeutic options to treat VL, resulting in poor patience adherence and relapses due to the unfinished treatments and the severe adverse effects (den Boer et al., 2009; Murray, 2001). Outside of India, the main therapy remains the class of pentavalent antimonials, a highly toxic option for both CL and VL. In India, where there is resistance to antimonials, the oral drug miltefosine and the liposomal amphotericin B have provided a significant improvement in leishmaniasis therapy (Maltezou, 2008; Murray, 2001). In Brazil, antimonials are still effective for most infections despite their high toxicity, but limited data about the resistance to these drugs is available. All current treatments with these drugs have limitations; therefore, novel, safe and low-cost treatments are still required (Torres et al., 2010).

Buparvaquone (BPQ) (Fig. 1), a veterinary drug used to treat theileriosis, has shown promising activities against protozoan parasites, including Leishmania spp. (Croft et al., 1992), Plasmodium falciparum (Gokhale et al., 2006), and Cryptosporidium parvum (Müller and Jacobs, 2002). Despite its anti-leishmanial activity at nanomolar concentrations (Croft et al., 1992), in vivo experiments with L. (L.) donovani showed only a weak suppression of the parasite burden in BALB/c mice at 100 mg/kg/day. The poor distribution and low bioavailability of BPQ contributed to the limited in vivo efficacy in models of VL (Croft et al., 1992) and CL (Garnier et al., 2007). BPQ was also tested at 5 mg/kg/day for 12 days in dogs infected with L. (L.) infantum chagasi and with VL symptoms, but no effect was seen (Vexenat et al., 1998).

The water solubility of BPQ is very low (<1 mg/L); consequently, it is poorly soluble in biological media, such as gastric fluids.
supplemented with 10% fetal calf serum and were maintained at 37 °C and 5% CO₂ in a humidified incubator.

2.4. Determination of the in vitro anti-leishmanial activity

To determine the 50% inhibitory concentration (IC₅₀) against *Leishmania* promastigotes, the drugs were dissolved in dimethyl sulfoxide (DMSO) and diluted with M-199 medium in 96-well microplates. The initial concentrations used were 3.06 µM buparvaquone and 3.21 µM pentamidine. Each drug was tested twice at eight range concentrations prepared at two-fold dilution steps in triplicate. The promastigotes were counted in a Neubauer hemocytometer and seeded at 1 × 10⁵/well at a final volume of 150 µL. Controls with DMSO and without drugs were performed. Pentamidine was used as a standard drug. The plate was incubated for 24 h at 24 °C, and the viability of the promastigotes was verified by the diphenyltetrazolium assay (MTT) (Tada et al., 1986). Briefly, MTT (5 mg/mL) was dissolved in PBS and sterilized through 0.22 µm membranes; 20 µL/well was added and incubated for 4 h at 24 °C. Promastigotes incubated without compounds were used as the viability control. The formazan extraction was performed using 10% SDS for 18 h (80 µL/well) at 24 °C, and the optical density (OD) was determined using a plate reader (Multiskan MS – UNICIENCE) at 550 nm. The data analysis was performed using GraphPad Prism 5.0 software. A viability of 100% was defined based on the optical density of the control promastigotes after normalization. To determine the IC₅₀ value for BPQ against *L. (L.) infantum chagasi* intracellular amastigotes, peritoneal macrophages (5 × 10⁴ per well) were collected from the peritoneal cavity of BALB/c mice as described above, added to 16-well chamber slides (NUNC®), and incubated for 24 h at 5% CO₂ and 37 °C. *L. (L.) infantum chagasi* amastigotes were isolated from the spleens of previously infected hamsters, separated by differential centrifugation and added to macrophages at a ratio of 1:10 (amastigotes:macrophage). The plate was further incubated for 24 h prior to drug incubation. Glucantime was used as the standard drug, and the test was performed for 120 h at 37 °C. The initial concentrations used were 3.06 µM buparvaquone and 100 µg/mL Glucantime (shown in µg/mL because the molecular weight of Glucantime is unknown). Each drug was tested twice at eight concentrations prepared as two-fold dilution steps in duplicate. At the end of the assay, the slides were fixed with methanol and stained with Giemsa prior to counting under a light microscope. The IC₅₀ was determined by counting 500 macrophages per well and assessing the number of infected macrophages (Reimão et al., 2010).

2.5. Cytotoxicity against mammalian cells

Peritoneal macrophages, kidney cells from *Rhesus* monkeys (LLC-MK-2) and tumor-human monocyte cells (THP-1) were incubated with BPQ up to a concentration of 229.75 µM for 48 h at 37 °C. Pentamidine was used as the reference drug. The viability of the mammalian cells was determined using the MTT assay (Tada et al., 1986).

2.6. BPQ entrapment in liposomes

For the liposome preparation, BPQ (6 mg) was diluted in methanol (1575 µL) and sonicated in a bath sonicator for 10 minutes at 25 °C (solution A). Solution B consisted of saturated egg phosphatidylcholine, saturated egg phosphatidylserine and cholesterol (7:2:1 molar ratio) dissolved in chloroform (3150 µL). The mixture of solutions A and B was further sonicated for 10 minutes. The mixture was evaporated in a rotary evaporator at 55 °C at 60 rpm for 40 minutes in a vacuum and protected from light. A pre-heated (55 °C) solution of 2.25% glycerol (9 mL) was added to the lipid film.
using glass beads. The swelling process of the pre-formed liposomes was performed in a rotary evaporator at 55 °C at 80 rpm for 60 minutes without a vacuum. To reduce the size of the vesicles, the liposomes were sonicated in a bath sonicator under heating (55 °C) for 30 minutes. A freeze-thaw process was performed using three consecutive cycles of freezing with liquid nitrogen followed by thawing at room temperature. The untrapped BPQ was separated from the liposomes by centrifugation (4000g for 15 minutes). The average diameter of the liposomes was determined by transmission electron microscopy using phosphotungstic acid staining (New, 1992).

2.7. Size determination of the liposomes and the quantification of BPQ
The concentration of the encapsulated BPQ was determined using ultra-high-performance liquid chromatography (UPLC) with a binary AT system (Prominence LC-20: Shimadzu Corp., Kyoto, Japan) and an ultraviolet photodiode detector array (PDA) SPD-M20A on a reverse phase ACE C18 column (4.6 × 250 mm, 5 μm particle size). The wavelength was set at 251 nm. The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The wavelength was set at 251 nm. The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA). The flow rate was 1 mL/minute using an isocratic method with acetonitrile:methanol (7:3 v/v) and 0.1% trifluoroacetic acid (TFA).

2.8. Experimental studies with L. (L.) infantum chagasi-infected hamsters
Young male Golden hamsters (110 g) were infected intraperitoneally (i.p.) with L. (L.) infantum chagasi amastigotes (1 × 10^6/animal). Forty-three days after the infection, the hamsters were treated for eight consecutive days as follows: BPQ at 20 mg/kg/day, administered i.p. in two doses of 10 mg/kg at an interval of 8 h (n = 5/group); BPQ entrapped in PS-liposomes (BPQ–PS-LP) at 0.33 mg/kg/day (n = 5/group) and the pentavalent antimonial (Glucantime®) at 50 mg/kg/day (n = 5/group). The control group (n = 5/group) received the BPQ vehicle (10% ethanol, 15% oil-based Cremophor® EL solution, and 75% PBS). The animals were euthanized 51 days post-infection. A tissue sample of the spleen and liver (approximately 200 mg) was removed, weighed and used for RNA extraction (Reimão et al., 2011).

2.9. Real-time PCR analysis
RNA samples were obtained from the spleen and liver fragments collected from the studied hamsters, and one sample was collected from a healthy animal and used as a negative control. The RNA extraction from the spleen and liver samples and from parasite cultures was carried out as previously described (Reimão et al., 2011). All TaqMan primers and probes were selected as previously reported (Reimão et al., 2011) and supplied by Applied Biosystems. The amplification runs contained two negative controls (pure water and a negative DNA sample collected from a healthy hamster) and one positive control (DNA sample extracted from a culture of promastigotes of a standard strain of L. (L.) infantum chagasi) (Reimão et al., 2011).

2.10. Statistical analysis
The data obtained were reported as the mean and standard deviation of duplicate samples from two independent assays. The IC_{50} values were calculated using sigmoid dose-response curves generated by Graph Pad Prism 5.0 software, and the 95% confidence intervals were included. The Mann–Whitney test (unpaired two-tailed) was used for significance testing.

3. Results
3.1. In vitro anti-leishmanial activity and mammalian cytotoxicity
Different concentrations of BPQ were incubated with Leishmania spp. promastigotes, and the parasite viability was determined by the colorimetric MTT method. All Leishmania species were susceptible to BPQ in the rank order (from low to high IC_{50} values) of L. (L.) braziliensis, L. (L.) infantum chagasi, L. (L.) major, and L. (L.) amazonensis, with IC_{50} values in the range of 1–4 μM (Table 1). The IC_{50} of pentamidine was determined for each species and was as follows: L. (L.) infantum chagasi (IC_{50} = 0.32 μM; 95% C.I = 0.23–0.44 μM); L. (L.) amazonensis (IC_{50} = 0.47 μM; 95% C.I = 0.38–0.58 μM); L. (L.) major (IC_{50} = 0.56 μM; 95% C.I = 0.35–0.85 μM); and L. (L.) braziliensis (IC_{50} = 0.09 μM; 95% CI = 0.03–0.15 μM). BPQ was also active against the intracellular amastigotes of L. (L.) infantum chagasi with an IC_{50} value of 1.5 μM after 120 h incubation. No toxicity to peritoneal macrophages was observed by light microscopy. Glucantime was used as the standard drug and had an IC_{50} of 13.06 μg/mL (95% C.I = 5.15–32.73 μg/mL).

BPQ was also incubated with mammalian cells for toxicity studies, LLC-MK2 cells, THP-1 monocytes and BALB/c peritoneal macrophages did not show cytotoxicity at the highest concentration after 48 h (Table 1). Pentamidine was used as a reference and had an IC_{50} of 11.69 μM (95% C.I = 3.02–42.95 μM) against LLC-MK2; an IC_{50} of 8.22 μM (95% C.I = 4.55–14.92 μM) against THP-1 cells; and an IC_{50} of 11.43 μM (95% C.I = 3.02–14.95 μM) against peritoneal macrophages.

3.2. In vivo evaluation of free and liposomal BPQ in L. (L.) infantum chagasi-infected hamsters
L. (L.) infantum chagasi-infected hamsters were treated i.p. for eight consecutive days with free BPQ and BPQ entrapped in PS-liposomes (BPQ–PS-LP). The parasite burden was further evaluated and quantified by real-time PCR. The untreated group was used as a control and resulted in an average of 1141 (standard error of the mean, SEM = 259) amastigotes per gram in the spleen and 7234 (SEM = 4089) in the liver (Fig. 2). Free BPQ at 20 mg/kg/day did not result in therapeutic improvement, with a mean number

Table 1
<table>
<thead>
<tr>
<th>L. (L) infantum chagasi promastigotes</th>
<th>L. (L) infantum chagasi amastigotes</th>
<th>L. (L) amazonensis promastigotes</th>
<th>L. (L) major promastigotes</th>
<th>L. (V) braziliensis promastigotes</th>
<th>MK2</th>
<th>THP-1</th>
<th>Peritoneal macrophages</th>
</tr>
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<tbody>
<tr>
<td>BPQ IC_{50} μM (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>229.75</td>
<td>229.75</td>
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</tr>
<tr>
<td>2.24 (2.05–2.45)</td>
<td>1.50 (1.41–1.62)</td>
<td>4.19 (3.61–4.87)</td>
<td>3.28 (2.85–3.77)</td>
<td>1.50 (1.16–1.90)</td>
<td>&gt;229.75</td>
<td>&gt;229.75</td>
<td>&gt;229.75</td>
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Abbreviations: BPQ: buparvaquone; IC_{50}: inhibitory concentration 50%; 95% CI: 95% confidence interval.
of amastigotes per gram in the spleen and liver of 1944 (SEM = 770) and 8532 (SEM = 3424), respectively. BPQ–PS-LP reduced the number of amastigotes per gram in the spleen and liver of 1944 (SEM = 770) compared to the untreated group.

4. Discussion

BPQ has been found to be a promising lead hydroxynaphthoquinone compound against Leishmania spp. Despite its high in vitro efficacy, with IC₅₀ values between 0.005 and 0.12 μM (Venkatesh et al., 2008), it showed limited efficacy in L. (L.) donovani-infected mice when tested at 100 mg/kg/day (Croft et al., 1992). Since its discovery as an anti-leishmanial agent (Croft et al., 1992), several BPQ derivatives have been proposed to overcome the elevated lipophilicity (log P = 5.3). As an alternative, water-soluble phosphate prodrugs have been synthesized and were tested in L. (L.) donovani-infected mice. Buparvaquone-3-phosphate was shown to be the best oral formulation, but only a slight reduction in the liver parasite burden (34%) could be observed at 50 mg/kg/day (Garnier et al., 2007).

In the present work, BPQ exhibited in vitro activity against Leishmania spp. when tested against the promastigote forms. The Brazilian cutaneous species were susceptible to BPQ, which had the lowest IC₅₀ value in L. (V.) braziliensis. BPQ was also effective against other species, such as L. (L.) amazonensis, L. (L.) major and L. (L.) infantum chagasi, with a similar IC₅₀ value to the standard drug pentamidine. L. (L.) infantum chagasi promastigotes were also incubated with BPQ for 72 h, but no change in the IC₅₀ values was detected (data not shown). BPQ could also eliminate L. (L.) infantum chagasi intracellular amastigotes with a remarkable IC₅₀ value approximately 27-fold lower than the standard drug Glucantime®. Furthermore, no in vitro toxicity to mammalian cells was detected, resulting in a selectivity index higher than 150 using the L. (L.) infantum chagasi amastigotes. This is the first report of the in vitro anti-leishmanial activity of BPQ against L. (L.) infantum chagasi and L. (V.) braziliensis. Considering the different susceptibilities of the Leishmania species to BPQ, the observed IC₅₀ value against the intracellular amastigotes of L. (L.) infantum chagasi was consistent with that reported for L. (L.) donovani HU3 amastigotes (IC₅₀ = 0.48 μM) (Mäntylä et al., 2004).

We also report the activity of the BPQ entrapped in PS-liposomes against L. (L.) infantum chagasi, the etiologic agent of VL in Brazil. In this study, BPQ–PS-LP was injected i.p. for eight consecutive days, resulting in a significant (P < 0.05) reduction of the number of amastigotes in the spleen (89.4%) when compared to the untreated group. A slight increase in efficacy (approximately 6%) was observed for the BPQ–PS-LP when compared to the standard drug Glucantime (P > 0.05). It should also be noted that all hamsters were shown to be highly infected, as confirmed by real-time PCR, therefore preventing the possibility of any false-positive results of the drug efficacy.

Despite the lack of a significant (P > 0.05) reduction of the liver parasite burden, the liposomal drug (BPQ–PS-LP) decreased the number of amastigotes by 67% when compared to the untreated group. It should also be noted that when the absolute number of parasites is considered, 80% of animals had fewer than 1000 amastigotes per gram. The treatment differences found in the spleen and liver of the BPQ–PS-LP-treated group could be attributed to the huge differences in the parasite burden of both organs. To achieve similar levels of spleen treatment, a higher amount of a drug must be delivered in a prolonged treatment, as a consequence of the 6-fold higher number of amastigotes in the liver when compared to the spleen. Furthermore, the higher accumulation of PS-liposomes in the spleen (Tempone et al., 2010) might have contributed to the differences found in our assays using the BPQ–PS-LP.

Considering that BPQ–PS-LP was administered at a 60-fold smaller dose than the free drug, these data demonstrate a considerable improvement in the activity of BPQ. This could be a result of the specific targeting delivery of BPQ to the Leishmania amastigotes in the infected organs through the interaction of the PS-liposomes with the macrophage scavenger receptors (Tempone et al., 2004). In a previous work (Tempone et al., 2010), furazolidone, a synthetic nitrofuran, when entrapped in a similar formulation of PS-liposomes, showed an enhanced activity when compared to the free drug against L. (L.) infantum chagasi experimental model. Furthermore, targeting to spleen and liver was observed, with an in vivo co-localization of liposomes within the parasites inside the macrophages (Tempone et al., 2010).

Free BPQ failed to reduce the liver and spleen parasite burden in L. (L.) infantum chagasi-infected hamsters. Despite the slight increase in the number of amastigotes in both organs, the free BPQ-treated group was not significantly (P > 0.05) different than the untreated group (control). The present data support the results obtained from the L. (L.) infantum chagasi naturally infected dogs because no in vivo efficacy could be detected after 12 days of treatment at 5 mg/kg (Vexenat et al., 1998). In contrast, free BPQ reduced the parasite burden of mice liver (Croft et al., 1992) infected with L. (L.) donovani by approximately 60% but at an oral daily dose of 100 mg/kg. These differences in efficacy could be explained by the different doses, route of administration, the Leishmania species and the different animal models used.

The use of real-time PCR has been a promising tool for the rapid evaluation of drug efficacy in animal models (Manna et al., 2008; Reimão et al., 2011) and of parasite kinetics in human blood (Sudarshan et al., 2011). Furthermore, the specific use of RNA allowed the detection of living amastigotes instead of dead parasites or residual DNA (Colombo et al., 2011), which could be found when DNA is analyzed. Thus, the efficacy of BPQ in the hamster model could be precisely quantified in the present work.

5. Conclusions

BPQ was effective in vitro against Leishmania spp., with an elevated selectivity index. Free BPQ failed to treat L. (L.) infantum chagasi-infected hamsters, but its inclusion into PS-liposomes is a promising anti-leishmanial formulation with activity at very low dose. Further studies, including understanding the different routes
of administration and determining the 50% effective dose in single-dose and multiple dose regimes, are now planned.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 08/09260-7). The authors thank the CNPq scientific research award given to AGT and the FAPESP scholarship given to JQR (08/11434-3) and to FAC (08/57245-7). We also thank Prof. Dr. Simon L. Croft (London School of Hygiene and Tropical Medicine) for criticism during the preparation of the manuscript and to provide the BPQ.

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