Chemico-Biological Interactions 332 (2020) 109296

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Targeting intracellular *Leishmania (L.) infantum* with nitazoxanide entrapped into phosphatidylserine-nanoliposomes: An experimental study

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ARTICLE INFO

Keywords:
Leishmania
Therapy
Macrophage interaction
Liposomes
Nitazoxanide

ABSTRACT

Leishmaniasis is a parasitic neglected tropical disease and result in a broad spectrum of clinical manifestations, ranging from a single ulceration to a progressive and fatal visceral disease. Comprising a limited and highly toxic therapeutic arsenal, new treatments are urgently needed. Targeting delivery of drugs has been a promising approach for visceral leishmaniasis (VL). Phosphatidylserine-liposomes have demonstrated superior efficacy in VL, targeting intracellular parasites in host cells through macrophage scavenger receptors. In this work, we investigated the *in vitro* and *in vivo* efficacy of the antihelminthic drug nitazoxanide in a nanoliposomal formulation against *Leishmania* (*L.*) *infantum*. Physicochemical parameters of liposomes containing nitazoxanide (NTZ-LP) were determined by dynamic light scattering and small angle X-ray scattering. The efficacy of the formulation was verified in an intracellular amastigote model and in an experimental hamster model. Our findings showed that NTZ-LP was able to eliminate the amastigotes inside the host cell with an IC_{50} value of 16 μM. NTZ-LP was labelled a fluorescent probe and by spectrofluorimetry, we observed that the infected macrophages internalized similar levels of the drug to the uninfected cells. The confocal microscopy images confirmed the uptake and demonstrated a diffuse distribution of the NTZ-LP in the cytoplasm of *Leishmania*-infected macrophages, with the vesicles in a closer proximity to the parasites. For the *in vivo* efficacy, the liposomal NTZ-LP was administrated intraperitoneally to *Leishmania*-infected hamsters for 10 consecutive days at 2 mg/kg/day. By qPCR we demonstrated a reduction of the parasite burden by 82% and 50% in the liver ($p < 0.05$) and spleen ($p < 0.05$), respectively. NTZ (non-liposomal) was administered at 100 mg/kg/day *per oral* (p.o.) for the same period, but demonstrated no efficacy. This liposomal formulation ensured a targeting delivery of NTZ to the intracellular parasites, resulting in an good efficacy at a low dose in animals, and it may represent a new candidate therapy for VL.

1. Introduction

The repositioning of drugs has been widely explored in the last years in all pharmaceutical fields. In the case of neglected tropical diseases (NTDs) that affect poor population in developing countries, it could be considered an excellent opportunity to reduce the costs and time for a new alternative therapy. Leishmaniasis is a protozoal parasitic disease caused by *Leishmania* spp. and is included among the six most important NTDs for the WHO [1]. The disease causes a spectrum of clinical manifestations depending of the parasite specie, ranging from a single cutaneous ulceration to a fatal and progressive visceral form. It affects 12 million people in 98 countries, with a global incidence estimated at approximately 0.9–1.6 million cases occurring each year [1]. Clinical therapy has many limitations, including restrict and old arsenal of drugs, causing serious adverse effects and relapses. The co-infections of visceral leishmaniasis (VL) with HIV induces a dramatic immunosuppression, resulting in less effective treatments; only co-administrations have been shown to improve treatment [2].

Nitazoxanide (NTZ) [2-(Acetyloxy)-N-(5-nitro-2-thiazolyl)benzamide], was originally discovered in the 1970s, first used as a veterinary anthelmintic agent for intestinal protozoa and later to treat human helminths [3,4]. Approved in 2002 by the United States Food and Drug Administration (FDA) against cryptosporidiosis (*Cryptosporidium* species) and giardiasis (*Giardia intestinalis*), it is commercialized as Alinia®

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https://doi.org/10.1016/j.cbi.2020.109296
Received 1 September 2020; Received in revised form 5 October 2020; Accepted 19 October 2020
Available online 20 October 2020

or Annita® as an oral suspension and/or tablets [5]. Nitazoxanide is a compound belonging to the thiazolides, composed by a nitrothiazole-ring and a salicylic acid moiety linked by an amide. This chemical group has been showing success against a broad variety of parasites, anaerobic bacteria and viruses. Recently, the drug was proposed as a candidate against coronavirus-2 (SAR CoV 2) in a combination therapy with hydroxychloroquine [6] and azithromycin [7]. Belardo and co-workers [8] showed the activity of NTZ against influenza A virus (H1N1, H3N2, H5N9, H7N1) [9]; NTZ has also shown anti-cancer activity [4], anti-coronavirus activity, anti-HIV activity [10] as well as anti-nematode activity [11]. The *in vivo* anti-*Leishmania donovani* activity of nitazoxanide had been described by Zhang and co-workers [12], which demonstrated the activity of NTZ when orally administrated at an elevated dose of 400 mg/kg in a murine experimental model (BALB/c). However, the bioavailability of nitazoxanide is limited due to its poor water solubility [13].

Development of drug delivery system is an essential practice to increase the safety and reduce the toxicity of drugs and has been a promising approach for cancer [14], but also for infectious diseases as leishmaniasis [15]. Due to a poor bioavailability and targeting, including other interferences (e.g. metabolism, degradation), many drugs lose activity after oral administration [16], but delivery systems like liposomes can improve the efficacy. Liposomal amphotericin B has been used for many years in the clinical treatment of leishmaniasis, reducing doses and toxicity of the drug [2]. The benefit of a liposomal formulation is mainly attributed to the targeting ability, consequently reducing the amount of free drug in the body and the toxicity [17,18]. The interaction of liposomes with the target cells is the key feature to consider when designing a controlled release system [19]. The targeting of nanoliposomes containing negatively charged phosphatidylserine (PS) to mononuclear phagocytes cells has been shown promising results [20]. This is due to a specific class of macrophage scavenger receptors (CD36 and SR-B) responsible for the rapid binding and uptake of these liposomes [21,22]. Based in this concept, the FDA-approved drug nitazoxanide was entrapped into negatively charged PS-nanoliposomes and evaluated against *Leishmania (L.) infantum* in *ex vivo* and *in vivo* models.

2. Material and methods

2.1. Drugs and chemicals

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Thiazol blue; MTT), cholesterol, M − 199 medium, miltefosine, nitazoxanide, RPMI-PR-1640 medium (without phenol red) and sodium dodecyl sulfate (SDS), were purchased from Sigma-Aldrich (St Louis, MO). Chloroform, DMSO, glycerol and methanol were purchased from Merck. DAPI (4_,6-diamidino-2-phenylindole dihydrochloride) and DIL (C18) (1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate) were purchased from Molecular Probes (Eugene, OR). Pentavalent antimony (Glucantime®) was obtained from Sanofi-Aventis (São Paulo, Brazil). The hydrogenated phospholipids were kindly donated by LIPOID GmbH (Ludwigshafen, Germany).

2.2. Bioassay procedures

Animals**.** Male golden hamsters (*Mesocricetus auratus*, 120 g) and female BALB/c mice (20 g) were obtained from the animal breeding facility at the Instituto Adolfo Lutz, Brazil. The animals were maintained in sterilized cages under a controlled environment, receiving water and food *ad libitum*. All procedures performed were previously approved by the Animal Care and Use Committee from Instituto Adolfo Lutz – Secretary of Health of Sao Paulo State (Project number CEUA 04/2016) in agreement with the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences.

2.3. Parasites and mammalian cell maintenance

Leishmania (*L.*) *infantum* (MHOM/BR/1972/LD) promastigotes were maintained in M − 199 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Gibco), 0.25% hemin (Sigma-Aldrich) and 5% human urine at 24 °C. Amastigotes were obtained from the spleen of golden hamsters previously infected and purified by differential centrifugation. Peritoneal macrophages were collected by washing the peritoneal cavity of BALB/c mice with RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS and were maintained at 37 °C in a 5% CO2 humidified incubator.

2.4. Evaluation of in vitro anti-L. (L.) infantum activity

Peritoneal macrophages (1×10^5 cell/well) in 16-well slide chambers (NUNC) were infected with amastigotes at a ratio of 10:1 (amastigotes/macrophage) and treated with NTZ-LP (60–1 μM) for 120 h. Stained slides (Giemsa) were counted using light microscopy and IC_{50} determined by the number of infected macrophages out of 400 cell [20]. Miltefosine was used as standard drug and untreated cells as a negative control.

2.5. Evaluation of in vitro mammalian toxicity

Peritoneal macrophage (6 × 104 cells/well), seed in 96-well plates were incubated with the compounds up to 200 μM in a 5% CO2 humidified incubator at 37 °C. The 50% Cytotoxic Concentration (CC_{50}) was determined by the colorimetric MTT method [23]. The selectivity index was determined using the following equation: CC_{50} against NCTC cells/IC_{50} against amastigotes.

2.6. Nitazoxanide entrapment in liposomes

Liposomes were prepared by the lipid hydration method followed by freeze-thawing [24]. For liposomes preparation, saturated egg phosphatidylcholine, phosphatidylserine and cholesterol were used at a 7:2:1 M ratio. The drug candidate was diluted in methanol and sonicated in an ultrasonic bath for 5 min at room temperature (solution A); the solution B consisted of saturated egg phosphatidylcholine, saturated egg phosphatidylserine and cholesterol was dissolved in chloroform. The mixture of solutions A and B was further sonicated for 10 min in a bath sonicator. The resultant solution was evaporated using a rotary evaporator at 55 °C at 40 rpm for 60 min. A pre-heated (55 °C) solution of 2.25% glycerol (v/v) 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 min without vacuum. To reduce the vesicle size, liposomes were sonicated under heating (55 °C) for 30 min. A freeze-thawing process was performed using three consecutive cycles of freezing with liquid nitrogen followed by thawing at room temperature. The untrapped drug was separated from the liposomes by centrifugation (2000g for 15 min). The concentration of the encapsulated compound was determined by RP-HPLC [25], using C18 column (ACE, 250 × 4.6 mm, 5 μm) in a Prominence UPLC apparatus equipped with a photodiode array detector (Shimadzu, Japan). For preparation of fluorescent liposomes, 2% of long-chain dialkylcarbocyanines (DIL C18) (related with phosphatidylcholine) was added to the lipid mixtures before drying and the unentrapped probe eliminated by centrifugation (2000g for 15 min).

2.7. In vitro uptake of NTZ-LP by infected macrophages

Macrophages, collected from the peritoneal cavity of BALB/c mice, were seeded at 1×10^5/well in a black 96-well microplate and incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h. *Leishmania (L.) infantum* amastigotes were previously isolated from the spleen of infected hamsters, separated by differential centrifugation and added to

the macrophages at a ratio of 1:10 (macrophage/amastigotes). Non-infected macrophages were used as control. After 24 h of incubation, liposomal nitazoxanide labelled with the fluorescent probe DIL C18 (NTZ-LP-DIL) and the uptake was monitored in a spectrofluorimeter (FilterMax 5 Molecular Devices) every 15 min over 360 min, using excitation and emission filters of 549 and 565 nm, respectively [26].

2.8. Confocal microscopy studies (in vitro)

Peritoneal macrophages were applied (1×10^5/well) to 16-well slide chambers (Nunc®) and infected with *L. (L.) infantum* amastigotes at a ratio 1:10 (macrophage/amastigotes) for 24 h at 37 °C, as previously described. Liposomal nitazoxanide DIL (NTZ-LP-DIL) was prepared in the presence of the fluorescent marker DIL (C18) and incubated with infected macrophages for 3 h at 37 °C. The cells were washed with PBS, fixed with 4% formaldehyde, stained with DAPI (1 mM) and processed for confocal microscopy. A giemsa-stained slide chambers was prepared to confirm the parasite burden. 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) [27].

2.9. Experimental studies (in vivo)

The efficacy of free and liposomal NTZ treatment was determined using young male golden hamsters previously infected (i.p. route) with *L. (L.) infantum* amastigotes. Forty days post infection, hamsters were treated intraperitoneally for ten consecutives days with free and liposomal NTZ (n = 5/group). The control group was treated with the vehicle (2.25% glycerol v/v). The animals were euthanized 50 days post-infection and the parasite burden was evaluated by real time PCR using RNA samples obtained from the spleen and liver fragments [28].

2.10. Dynamic light scattering

DLS is an interesting tool to investigate liposomes hydrodynamic diameter, D_h, both in the absence and presence of NTZ, using the translational diffusion coefficient anf the fluctuation of the scattered light at a fixed angle, which in the present study was set to 90°. DLS was performed in a ZetaSizer ZS90 equipment (Malvern, UK) using a He–Ne laser, λ = 632.8 nm (being λ the radiation wavelength) as a light source. Measurements were performed at 25 °C. Samples were diluted 20-fold (or more) to ensure transparent solutions, in this case avoiding the multiple scattering inside the cuvette. In all studied samples, results show a unimodal distribution and represent the average of, at least, four experiments.

D_h is related to the translational diffusional dynamics of the vesicle, and can be written using the Stokes-Einstein relationships, as follows: where k_B is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the solvent, D_h and D_{app} are the hydrodynamic diameter and the apparent translational diffusion coefficient, respectively [26].

2.11. ζ-Potential

ζ-Potential measurements of liposomes in the absence and presence of NTZ were carried out to check the possible effect of the drug on the liposome surface charge. The ζ-potential was measured in a ZetaSizer ZS90 equipment (Malvern, UK), a He–Ne laser, λ = 632.8 nm as a light source. The detector (which uses the LDV (Laser Doppler Velocity) approach to calculate the electrophoretic mobility) was positioned at 17°. The data was acquired using also the PALS (phase analysis light scattering) methodology.

It is well-known that ζ-potential value could indicate the colloidal system stability, regarding the electrostatic repulsion since it gives information on the liposome net change at the surface. The ζ-potential is the electrical potential (in units of mV) at the so-called double layer, or the slipping plane at the vesicle surface. In this study, the ζ-potential was calculated using the Helmholtz-Smoluchowski equation. Such approach can describe quite precisely water water-based systems in the presence of high ionic strength (I) > 1 mM, and particle diameter (D_H), >100 nm, which is exactly our case herein. The measurement for each sample was repeated for at least three times. In order to avoid multiple scattering, similar to DLS measurements, samples were diluted at least 20-fold, in the appropriate buffer before the measurements [26].

2.12. Small angle X-ray scattering measurements (SAXS)

SAXS measurements were performed in the SAXS1 beam line in the National Synchrotron Light Laboratory, LNLS, Campinas, SP, Brazil. The X-ray wavelength used was λ = 0.1488 nm, and the sample-to-detector distance was ~1000 mm, providing a q-range of $0.1 < q < 4$ nm-1, where q is the scattering vector, which is $q = \frac{4\pi}{\lambda}\sin(\theta)$, being$2\theta$ the scattering angle. A bidimensional PILATUS 300k detector was used. Samples were set between two flat mica walls with a 1 mm spacer and a thermal bath was used for temperature control, set to 22 °C. Scattering data were normalized for the acquisition time (100 s). Samples' attenuation as well as the befferr contribution were considered in the data reduction process.

SAXS data analysis for liposome-like structures can be theoretically evaluated supposing that the liposome diameter is much larger size then the X-ray wavelength, λ. Under such assumption, the scattering curve can be described by an infinite plane with a non-homogeneous cross section in the z direction and across the membrane bilayer [29,30].The scattering intensity can be written as:

$$I(q) = kn_p\frac{P_t(q)}{q^2}$$

Where k is a normalization constant, n_p is the lipid concentration, q is the scattering vector and $P_t(q)$ is the bilayer cross-section form factor. In this study, $P_t(q)$ was calculated supposing the three-step model [30]. In this case, each leaf let of the bilayer is composed by three different electron density profiles: the inner methyl group, or CH_3 region, the subsequent paraffinic chains (or alkyl chains) and the outer polar head group. Moreover, each region has it on electron density value and thickness: ρ_{pol} is the electron density for the polar head group, whereas ρ_{CH2} and ρ_{CH3} are the electron density of the alkyl chain and the CH_3 regions, respectively. The thicknesses are: R_{pol}, R_{CH2} and R_{CH3} for the polar head group, the paraffinic chains and for the CH_3 region, respectively. The theoretical Scattering curves were fitted against the experimental SAXS curves using GENFIT software. The software also allows the combination of different theoretical models, in order to better describe the studied system. According to the literature [29,30] the fitting parameters were set to vary in a specific range, in order to void non-physical results, i.e., R_{CH3} was set to vary in the (0.15 nm $< R_{CH3} <$ 0.35 nm) range, ρ_{CH3}(150 e nm^{-3} $<\rho_{CH3} <$ 200 e nm^{-3}) andρ_{par}(250 e nm^{-3} $<\rho_{par} <$ 330 e nm^{-3}). It is kwon that in liposomes composed of a mixture of lipids, generally cannot be described. The remaining fitting parameters were allowed to vary in a broader range, in order to ensure a reasonable description of the systems. As it will be shown latter in the text, in the presence of BPQ two distinct fitting models were used to adjust the theoretical scattering function to the experimental data. A more detailed explanation of this model can be found elsewhere [29,30].

2.13. Statistical analysis

The determination of the CC50 and IC50 values was obtained from

Table 1
Antileishmanial and mammalian cytotoxicity of NTZ-LP.

Drugs	*Leishmania (L.) infatum* amastigotes IC_{50} (±SD)	Mammalian Cytotoxicity CC_{50} (±SD)
NTZ-LP	15.9 ± 1.0	32.9 ± 7.2
NTZ	27.8 ± 3.1	68.1 ± 21.0
miltefosine	6.5 ± 3.0	119.7 ± 4.2

IC_{50}: 50% inhibitory concentration; CC_{50}: 50% cytotoxic concentration; ±SD standard deviation.

sigmoid dose-response curves. The statistical significance (p value) between the samples was evaluated through the One-way ANOVA method using the Tukey's Multiple Comparison test. All analyses were performed using Graph Pad Prism 5.0 software. The samples were tested in duplicate or triplicate and the assays were repeated at least twice.

3. Results

3.1. Antileishmanial activity and mammalian cytotoxicity

The *in vitro* antileishmanial activity of liposomal NTZ (NTZ-LP) was evaluated against intracellular amastigotes of *L. (L.) infantum* (Table 1). The NTZ-LP eliminated 100% of amastigotes after 120 h incubation, without affecting the morphology of the host cell (Fig. 1). The nanoliposomal NTZ showed an IC_{50} value of 15.9 μM and a 50% cytotoxic concentration (CC_{50}) value of 32.9 μM. Free drug (NTZ) was used as internal control and resulted in an IC_{50} value of 27.8 μM against amastigotes and CC_{50} value of 68.1 μM. Miltefosine was used as standard drug and showed an IC_{50} value of 6.5 μM against amastigotes and a CC_{50} value of 119.7 μM.

3.2. Uptake of NTZ-LP-DIL by infected and uninfected macrophages (in vitro)

To compare the uptake capability of nanoliposomes containing nitazoxanide by infected macrophages with uninfected macrophages, cells were incubated (up to 360 min) with the formulation labelled with the fluorescent probe DIL C18. The results represented in Fig. 2, showed a similar uptake of vesicles in both groups, confirming the internalization of the liposomes. The presence of intracellular amastigotes in infected macrophages was confirmed by light microscopy after Giemsa staining (Fig. 2C).

3.3. In vitro distribution of NTZ-LP-DIL by confocal studies

To investigate the delivery of nitazoxanide liposomes to the parasitophorous vacuole of *Leishmania*-infected macrophages, the liposomal formulation was prepared in the presence of the lipophilic fluorescent marker DIL (C18) and then analysed by a confocal microscope. After 3 h of liposome incubation, we could detect co-localization of liposomes (red label, Fig. 2B) with amastigotes (small blue label, Fig. 2B) suggesting the delivery inside the macrophages near the parasites. A Giemsa-stained slide chamber was prepared to demonstrate parasite burden (Fig. 2C).

3.4. Experimental evaluation of nanoliposomes containing nitazoxanide (NTZ-LP)

After 40 days of infection, *L. (L.) infantum* infected hamsters were treated with nitazoxanide at 2 mg/kg/day for ten consecutives days and the parasite burden was evaluated in the spleen and liver by real-time PCR (qPCR). The drug formulation was able to reduce the parasite burden by 82% ($p < 0.05$) and 50% ($p < 0.05$) in the liver and spleen, respectively. Free drug (NTZ) was used as control and administered for the same period at 100 mg/kg/day. The qPCR data demonstrated a similar parasite burden to untreated control group, demonstrating no efficacy of the free drug (non-liposomal). The untreated group was used for normalization (100% infection) of the parasite burden in spleen and liver (Fig. 3).

3.5. Dynamic light scattering (DLS) and zeta-potential (ZP) measurements (DLS)

Liposome hydrodynamic diameter, D_H, was measured by means of dynamic light scattering, in the absence and presence of entrapped NTZ. The results showed values of 183 and 169 nm for the systems in the absence and presence of NTZ. Such changes in D_H values, i.e., a decrease of ~8% in the presence of NTZ, are not so significant in terms of DLS taking into account the PDI variation. In all studied cases, the PDI values were <0.2, indicating quite monodisperse systems. Concerning the ZP values, a different behaviour was observed. In this case, the presence of NTZ induced a significant decrease in ZP value. In the absence and presence of NTZ, the liposome ZP were −31 ± 4 mV and −56 ± 2 mV, respectively. Interestingly, the presence of NTZ had a remarkable effect on the liposome surface. Therefore, according to these experiments one can conclude that NTZ relies on the liposome surface, without changing significantly if hydrodynamic diameter, but decreasing the liposome

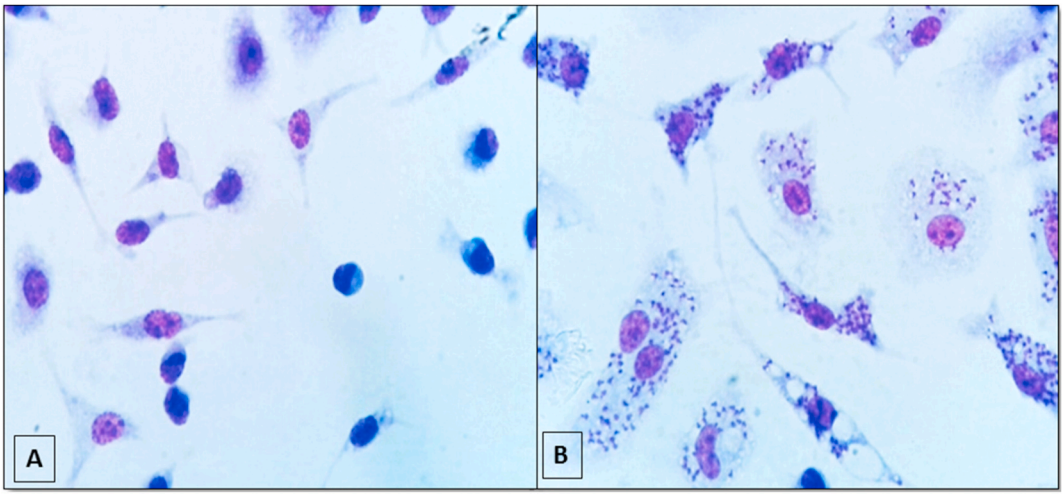

Fig. 1. Light microscopy image of *Leishmania*-infected macrophages treated with with nanoliposomal nitazoxanide and stained with Giemsa. Macrophages were treated for 120 h at 25 μM (A) and untreated macrophages were used as control (B). Magnification 1000X.

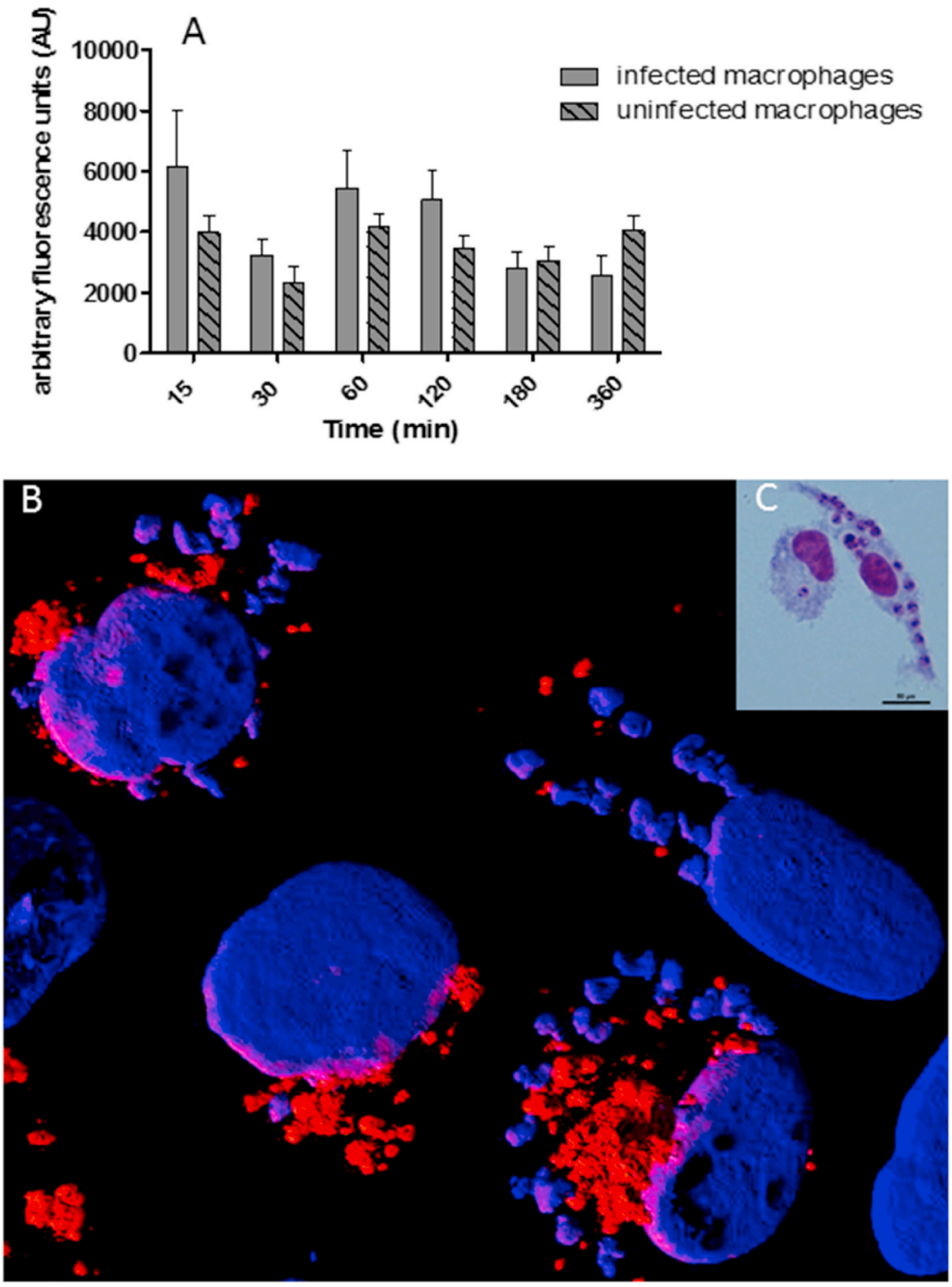

Fig. 2. (**A**) Uptake of nanoliposomal nitazoxanide by *Leishmania*-infected and uninfected macrophages. Cells were incubated with the formulation labelled with DIL C18 and monitored using spectrofluorimetry, with 15 min intervals. Excitation and emission lengths were 549 and 565 nm, respectively. (**B**) Confocal image of *Leishmania*-infected macrophages after incubation with nanoliposomes containing nitazoxanide. (**C**) Giemsa-stained macrophages with amastigotes by light microscopy (1000X magnification, bar = 50 μm). Blue fluorescence represents DAPI-stained nucleus of host cells and amastigotes and the red fluorescence corresponds to DIL C18 labelled nanoliposomes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

surface charge, as evidenced by the decrease in ZP value. In order to check if NTZ was inserted into the liposome bilayer small angle X-ray scattering experiments were carried out as follows.

3.6. Small angle X-ray scattering measurements (SAXS)

SAXS measurements were performed in the absence and presence of NTZ. The unilamellar vesicles were prepared with approximately 69% phosphatidylcoline, 21% phosphatidylserine and 10% cholesterol. Fig. 4 depicts the SAXS curves, along with the theoretical model used to analyse the data, es explained in the materials and methods section. The results showed that under the studied conditions, there is no specific influence of NTZ on the SAXS curve of PS-containing liposome (inset Fig. 4).This fact suggests that the drug does not affect the structure of the bilayer. Furthermore, there are no narrow or intense peaks in the SAXS curves, i.e., there is no indication of multi-lamellar systems. Moreover,

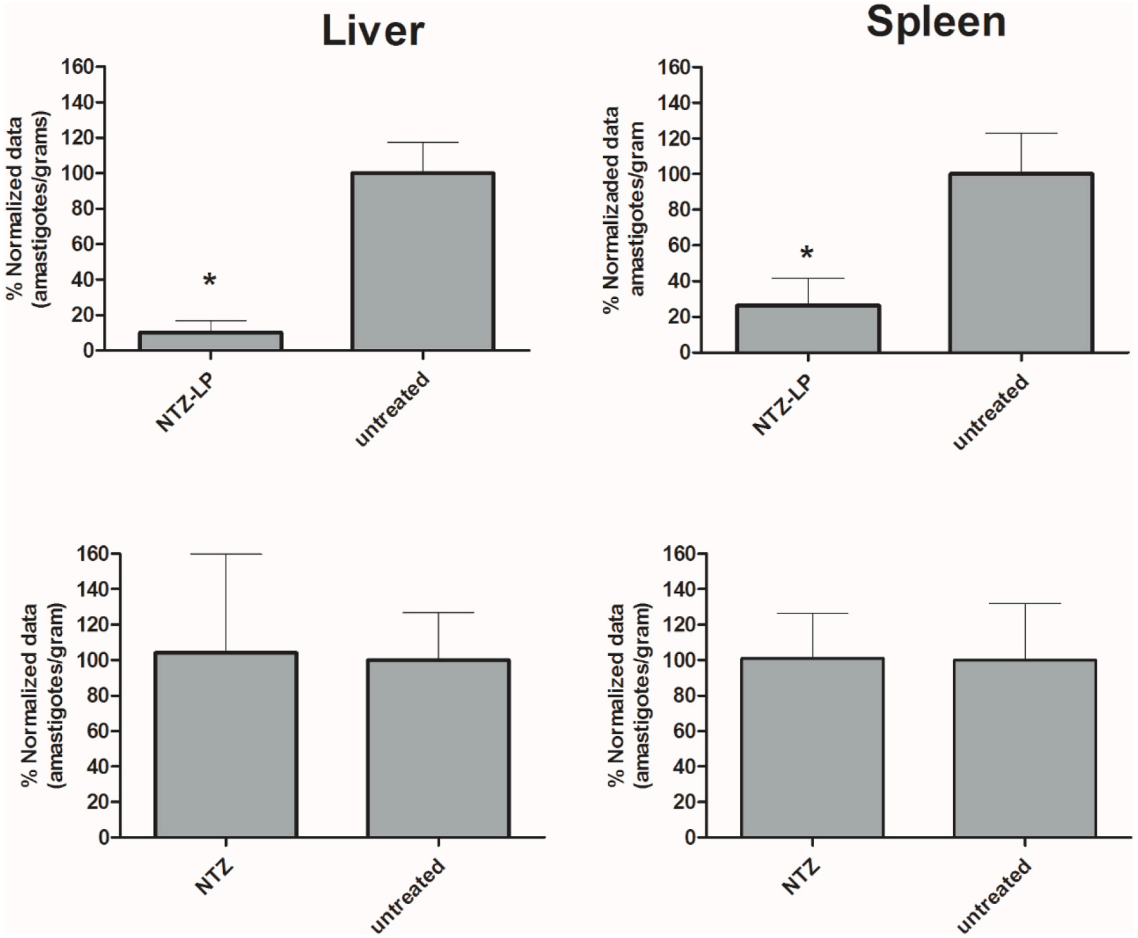

Fig. 3. *In vivo* efficacy of free and liposomal nitazoxanide (NTZ-LP) in *Leishmania infantum*-infected hamsters. The nanoliposomal drug and the free drug (NTZ) were i.p. administered for ten consecutives days at 2 and 100 mg/kg/day, respectively. Real-time PCR was used for quantification of the parasite burden in spleen and liver and the data was normalized based in untreated groups (control groups). *p < 0.05.

two lamellar systems were used to describe the SAXS curves, the best one was showed in Fig. 4 and the parameters summarized in Table 2.

4. Discussion

Nitazoxanide is an antiparasitic FDA-approved drug with a wide range of therapeutic applications, including antiviral [5,7–9] and antiprotozoal activity [12]. It demonstrated activity against *L.* (*L.*) *mexicana* promastigotes and *T. cruzi* epimastigotes with IC_{50} values in the range of 2 and 5 μM [31]. Our previous studies demonstrated that NTZ induced an oxidative stress in *Leishmania infantum*, causing depolarisation of the mitochondrial membrane and up-regulation of reactive oxygen species (ROS) [32]. Zhang and co-workers [12] demonstrated that NTZ can reduce the parasite burden of *Leishmania donovani*-infected BALB/c mice by 90% (spleen and liver) after a 10-days treatment at an elevated dose of 400 mg/kg/day. In our studies, NTZ as a free drug (non-liposomal) was administered to *Leishmania infantum*-infected hamsters and demonstrated no efficacy at the tested dose and regimen. The differences of treatment efficacy of NTZ could be ascribed to the different *Leishmania* species, dose and regimen of administration (400 mg/kg/day x 100 mg/kg/day), different drug presentation (tablets x free drug), but also to the animal model (mice x hamster). Additionally, it has also been shown that the bioavailability of nitazoxanide is limited due to its poor water solubility [13], limiting its efficacy in systemic diseases like VL.

Considering the need to reduce the dose and consequently, the toxicity, controlled and sustained drug delivery systems (e.g., liposomes) can play a major role. The benefit of a liposomal formulation is related to directing the drug to the target organ, reducing the amount of free drug in the body, thus decreasing its toxicity [17,18]. Studies published elsewhere have proved that liposomal amphotericin B (Ambisome®) shows higher clinical efficacy and safety than the free drug [33].

In this context, nanoliposomes entrapping NTZ were prepared with a negatively charged phospholipid (phosphatidylserine), aiming the targeting delivery of the drug to macrophages in the spleen and liver of *Leishmania*-infected hamsters. Our studies demonstrated a superior efficacy of NTZ when entrapped into PS-liposomes, since the free drug failed to reduce the parasite burden in the spleen and liver of animals. NTZ-LP was administered at a low dose (2 mg/kg/day) and resulted in a promising reduction of the parasite burden, especially in the liver of infected animals. This improved reduction of parasites could be ascribed to a superior uptake of the PS-liposomes by the liver. A biodistribution study of PS-liposomes containing pentavalent antimony in *Leishmania infantum*-infected mice, demonstrated a slow clearance of the drug in the liver, resulting in sustained levels of the drug after administration [34].

The physicochemical parameters of liposomes as size, surface charge, composition, determine the interaction with targeted cells. In our study, the liposomes vesicles were smaller than 200 nm and had a negative charge on a unilamellar membrane (SUV). Although the drug caused interference in the membrane load, it was not able to modify the membrane, which remained negative after NTZ encapsulation. Physicochemical alterations of liposomal vesicles are described in the literature when encapsulating liposoluble drugs. However, in the present study there were no significant differences in vesicle size when

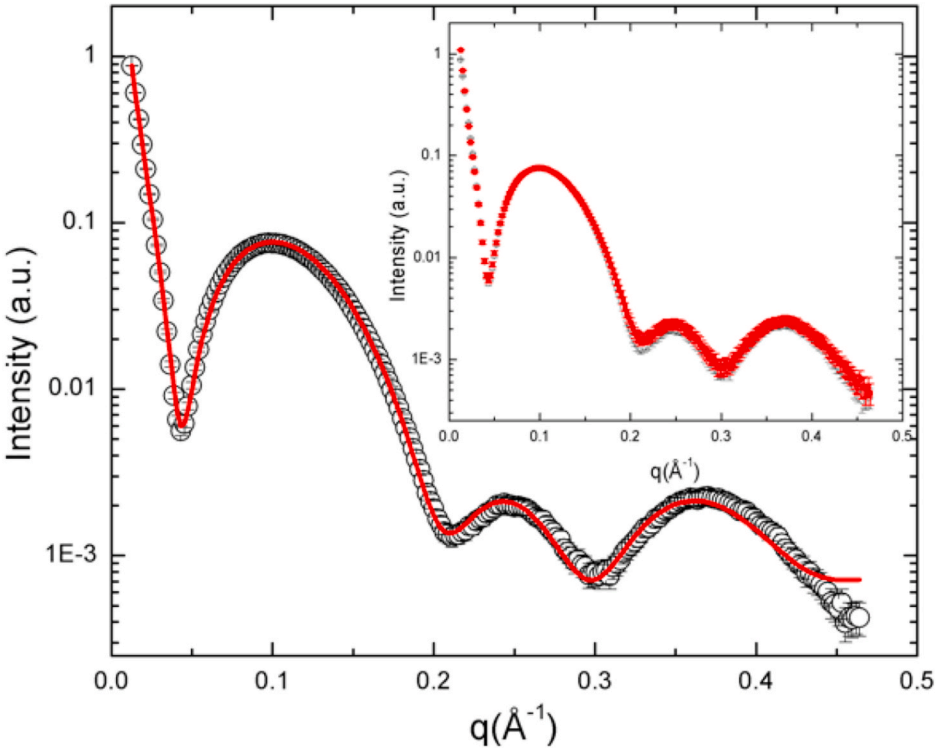

Fig. 4. SAXS curve of the lipid system in the presence of the drug (black line) along with the best fit obtained in the previously presented model (red line). In the figure, the scattering curves of the system in the absence (black) and presence (red) of the drug. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Setting parameters obtained by SAXS analyses curves.

	Lipid 1	Lipid 2
w (%)	85.0 ± 5.0	15.0 ± 3.0
R_{pol} (Å)	10.6 ± 0.5	5.7 ± 0.3
R_{par} (Å)	13.0 ± 0.6	20.5 ± 0.3
R_{CH3} (Å)	3.1 ± 0.2	1.8 ± 0.2
ρ_{pol} (e/Å3)	0.42 ± 0.01	0.43 ± 0.01
ρ_{par} (e/Å3)	0.32 ± 0.01	0.30 ± 0.01
ρ_{CH3} (e/Å3)	0.22 ± 0.01	0.22 ± 0.01

Where w is the weight of the model, R_{pol} and ρ_{pol} as well as R_{par}, ρ_{par}, R_{CH3} and ρ_{CH3} are the thicknesses and electronic densities of every single region of the bilayer.

compared to liposomes with and without NTZ, showing no drug interference in the formulation.

The number of lamellae is also a considerable factor within a liposomal system, since it can support the amount of encapsulated drug, which means, for fat-soluble drugs the greater number of lamellae, the more the amount of drug encapsulated, theoretically. The interaction of liposomes with target cells is one of the main characteristics to be studied when it is required to design a controlled drug delivery system [19]. The negative charge has been considered an important attribute to explain the efficiency of the formulation [20]. Targeting of phosphatidylserine-liposomes to the mononuclear phagocytic system has been shown to be a promising approach [20,35], since scavenger class B receptors (SR-B and CD36) have shown to be the main receptors responsible for their rapid elimination [20–22]. It should also be highlighted that the size of the liposomes has a strong influence on the uptake by macrophages. The present formulation was based on the preparation of SUVs liposomes, in an attempt to establish a selective and sustained targeting system, since liposomes with a diameter smaller than 200 nm have a lower risk of capillary obstruction when compared to large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs). Additionally, it has also been shown a superior targeting of SUVs to bone marrow, an important tissue affected by *Leishmania infantum* parasites [36].

The *in vitro* kinetics uptake of liposomal nitazoxanide was investigated in naive and *L. (L.) infantum*-infected macrophages using the fluorescent probe DIL C18. Despite the elevated number of intracellular amastigotes in *Leishmania*-infected macrophages, the levels of fluorescence in these cells were quite similar to the non-infected macrophages, suggesting an active uptake of the vesicles containing NTZ. Negatively charged liposomes containing phosphatidylserine are preferentially captured by macrophage scavenger receptors (ScavR) [37,38]. The abundance of ScavR in *Leishmania infantum*-infected macrophages [20] might be responsible for the sustained uptake of NTZ-LP observed in our studies.

To differentiate a surface association to an internalization of the liposomal vesicles into *L. (L.) infantum*-infected macrophages, a confocal microscopy study was performed using NTZ-LP labelled with the fluorescent probe DiL C18. The obtained images demonstrated the internalization of the liposomes with a homogeneous distribution across the cytoplasm, with the vesicles in a close proximity to the intracellular parasites. This data is in agreement to our previous study using an animal biodistribution model, where we demonstrated the co-localization of PS-liposomes containing furazolidone with the amastigotes in the liver and spleen of the infected animals [39].

5. Conclusions

Due to the poor bioavailability of nitazoxanide, leading to treatment failures in our experimental *Leishmania infantum* model, the entrapment of the drug into negatively charged nanolipossomes proved to be an important tool to improve the targeting delivery of the drug. The liposomal formulation of NTZ showed similar activity to the free drug in *in vitro* assays of intracellular amastigotes of *Leishmania*, and also

demonstrated co-localization of the vesicles within the parasites inside the host cells. Future pharmacokinetic studies using *Leishmania*-infected hamsters should be performed to evaluate the profile of the drug entrapped in PS-nanoliposomes.

CRediT authorship contribution statement

Erika Gracielle Pinto: Investigation, Writing - original draft, preparation, Formal analysis, Writing - review & editing. **Leandro R.S. Barbosa:** Formal analysis, Investigation, Writing - original draft, reparation. **Renato A. Mortara:** Investigation, Formal analysis. **Andre Gustavo Tempone:** Conceptualization, Methodology, Writing - review & editing, Data curation, Resources, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We also acknowledge Conselho Nacional de Pesquisa e Desenvolvimento (CNPq) for the scientific award to A.G.T and LIPOID GmbH (Ludwigshafen, Germany) for the phospholipids.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cbi.2020.109296.

Funding

This work was supported by grants from São Paulo State Research Foundation FAPESP (Project 2018/10279-6).

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