Drug Delivery and Translational Research (2019) 9:76–84
https://doi.org/10.1007/s13346-018-00603-0

ORIGINAL ARTICLE

Amphotericin B-loaded nanoparticles for local treatment of cutaneous leishmaniasis

Aiman Abu Ammar[1,2] · Abed Nasereddin[3,4] · Suheir Ereqat[4,5] · Mary Dan-Goor[3] · Charles L. Jaffe[3] · Eyal Zussman[1] · Ziad Abdeen[4]

Published online: 27 November 2018
© Controlled Release Society 2018

Abstract

Cutaneous leishmaniasis (CL) is an infectious, parasitic disease caused by the protozoan *Leishmania.* Amphotericin B (AMB) is a macrolide polyene antibiotic presenting potent antifungal and antileishmanial activity, but due to poor water solubility at physiological pH, side effects, and toxicity, its therapeutic efficiency is limited. In the present study, poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) loaded with AMB were generated to reduce drug toxicity and facilitate localized delivery over a prolonged time. AMB NPs were characterized for particle size, zeta potential, polydispersity index, and degree of aggregation. In vitro assessments demonstrated its sustained activity against *Leishmania major* promastigotes and parasite-infected macrophages. A single intralesional administration to infected BALB/c mice revealed that AMB NPs were more effective than AMB deoxycholate in terms of reducing lesion area. Taken together, these findings suggest that AMB NPs improve AMB delivery and can be used for local treatment of CL.

Keywords Amphotericin B · Nanoparticles · Sustained release · Cutaneous leishmaniasis · Topical therapy

Introduction

Leishmaniasis is an infectious disease spread by the bite of *Leishmania*-infected female Phlebotomine sandflies while taking a blood meal [1]. This disease has several clinical manifestations which are typically categorized as cutaneous leishmaniasis (CL), the most common form typified by localized skin lesions at the site of infection; mucocutaneous leishmaniasis which causes extensive destruction of the nasopharynx region and fails to heal without treatment; and visceral leishmaniasis (VL), the fatal form of the disease where parasites reside in the spleen, liver, and bone marrow [2]. After deposition into the skin, *Leishmania* are taken up by macrophages and other phagocytes where they differentiate into the intracellular form, the amastigote [3]. Drugs currently administered to treat CL are constrained by treatment period, route of administration, suboptimal efficacy, toxicity, and cost [4].

Amphotericin B (AMB) is a macrolide polyene antibiotic presenting potent antifungal and antileishmanial activity, but its poor water solubility at physiological pH (< 1 mg/L), high cost, side effects, and toxicity limit the therapeutic efficiency and clinical applicability of this drug [5, 6]. AMB exerts its antileishmanial activity through a high-affinity interaction with ergosterol, the main membrane component of fungi and *Leishmania* [7]. Its binding induces the formation of membrane channels that enhance permeability to ions and small molecules, leading to parasite death [8]. AMB is active against both the extracellular promastigote and intracellular amastigote forms with ED_{50} values from 0.01 to 0.27 μM

Aiman Abu Ammar and Abed Nasereddin contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13346-018-00603-0) contains supplementary material, which is available to authorized users.

✉ Aiman Abu Ammar
aimanab@jce.ac.il

[1] NanoEngineering Group, Department of Mechanical Engineering, Technion, Israel Institute of Technology, 3200003 Haifa, Israel

[2] Present address: Department of Pharmaceutical Engineering, Azrieli College of Engineering, 9103501 Jerusalem, Israel

[3] Department of Microbiology and Molecular Genetics, IMRIC, Hebrew University-Hadassah Medical Center, Jerusalem, Israel

[4] Al-Quds Nutrition and Health Research Institute, Faculty of Medicine, Al-Quds University, East Jerusalem, Palestine

[5] Biochemistry and Molecular Biology Department, Faculty of Medicine, Al-Quds University, East Jerusalem, Palestine

[9]. The conventional AMB formulation is a micellar suspension of AMB with sodium deoxycholate (Fungizone®). However, this formulation may lead to rapid release of the drug that can induce surfactant-related toxicity [10, 11]. Hence, clinical use of the micellar AMB formulation is associated with frequent and severe side effects, including fever, chills, nausea, vomiting, anemia, and nephrotoxicity, which often result in treatment failures [12, 13]. Liposomal AMB (AmBisome®), a lipid-based formulation of AMB, bears a significantly improved toxicity profile compared with conventional amphotericin B deoxycholate [14]. AMB formulations are used to treat all forms of leishmaniasis, and as such a need exists for a drug formulation with reduced toxicity, which delivers the drug to its target as efficiently as possible [6].

Nanoparticles (NPs) are submicron-sized polymeric colloidal particles, which can be applied to entrap, dissolve, bind, or encapsulate a wide variety of drugs within their polymeric matrix [15, 16]. Polymeric NPs are generated from either natural or synthetic polymers [17]. Poly(D,L-lactic-co-glycolic acid) (PLGA) NPs are extensively used for controlled and targeted delivery of drugs [18–20] due to their biodegradability, biocompatibility, and FDA approval [21, 22]. Moreover, PLGA polymers exhibit a wide range of degradation rates depending on their composition and molecular weight [17, 23, 24]. PLGA and modified PLGA NPs [poly(ethylene glycol) (PEG), chitosan, and mannose] containing AMB have been produced and several tested in vitro for activity against *Leishmania* [25]. To date, almost all the studies have employed *Leishmania donovani* or *Leishmania infantum*, species which cause VL [1]. However, only one report tested these NPs in animal models demonstrating that parasite spleen burden was lower in *L. donovani*-infected hamsters treated with PLGA-PEG NPs containing AMB (93.2%, 5 mg/kg for 7 days) as compare to animals treated with a similar dose of the free drug (74.4%) [26].

Currently, the AMB formulations in clinical use for CL are intravenously administered. Topical administration of AMB is challenging, owing to its properties and inadequate skin permeability [27]. A nano-delivery system may be advantageous, by facilitating direct AMB delivery at the site of infection and release of the drug in a sustained manner. The purpose of this study was to design a PLGA-nanoparticulate drug system for controlled release of AMB that would support intralesional AMB delivery and antileishmanial activity against *Leishmania major* which causes CL.

Materials and methods

Materials

Poly(D,L-lactide-co-glycolide) (PLGA, lactide: glycolide (50:50) RESOMER® RG 504 H, MW 38,000–54,000), amphotericin B deoxycholate, and TWEEN® 80 were purchased from Sigma-Aldrich (St Louis, MO, USA). Amphotericin B (BIA0103) was acquired from Apollo Scientific (Cheshire, UK). Macrogol 15 hydroxystearate (Solutol® HS 15) was donated by BASF (Ludwigshafen, Germany).

Preparation of AMB NPs

AMB-loaded nanoparticles were prepared using a modified nanoprecipitation method [28]. Briefly, the organic phase, consisting of 6 mg AMB in 200 μL DMSO and 15 mg PLGA in 2.5 mL acetone, was added to 5 mL aqueous solution containing 0.1% *w*/*v* Solutol® HS 15. The suspension was stirred at 900 rpm over 15 min, and then concentrated by solvent evaporation, followed by centrifugation for 5 min at 4000 rpm. NPs were washed with water, to remove residual DMSO, and filtered through a 0.45 μ membrane filter. Blank NPs were prepared using the same method, but without adding AMB at any stage of the preparation.

Physicochemical characterization of NPs

Particle size distribution and zeta potential of the NPs were characterized by dynamic light scattering (DLS) using a Malvern's Zetasizer (Nano series, Nanos-ZS, UK). Samples were prepared in water.

Determination of drug-loading efficiency

AMB NPs were dissolved in 5% DMSO in methanol (*v*/*v*) and the drug concentration was determined using a Genesys-10 UV-Vis spectrophotometer (Thermo Spectronic, Rochester, USA). AMB was detected at a wavelength of 407 nm.

Morphological evaluation of AMB NPs

Morphological and size evaluation of AMB NPs was carried out using a high-resolution scanning-electron microscope (HR-SEM, Carl Zeiss Ultra-Plus). The samples were fixed on a silicon SEM-stub.

Determination of AMB aggregation state in AMB NPs

AMB (dissolved in DMSO) and AMB NPs were diluted in PBS (pH 7.4) or methanol, to a final concentration of 4 μg/mL and 0.1% (*v*/*v*) DMSO. The UV-Vis spectra of the solutions were then recorded from 300 to 450 nm (Genesys-10 UV-Vis spectrophotometer) at 25 °C. The aggregation ratio was calculated by dividing the maximal absorption of peak I (325–340 nm) by peak IV (406–409 nm).

In vitro release kinetics

AMB NPs containing 0.4 mg AMB were placed in dialysis bags with a molecular cut-off of 12–14 kDa. The dialysis bags were suspended in 50 mL PBS (containing 1% *w*/*v* Tween 80, pH 7.4) and incubated at 37 °C in an orbital shaking bath (50 rpm). At predetermined time intervals, 2 mL of the release medium were discarded and replaced by an equal volume of fresh medium, in order to maintain sink conditions. After the 2-h sampling time point, the entire volume of medium (50 mL) was replaced with fresh medium, and the medium refreshment procedure was resumed. The concentration of AMB was determined by UV-Vis (Genesys-10 UV-Vis spectrophotometer) at a wavelength of 407 nm. The cumulative percentage of drug released was plotted against time and fitted into Korsmeyer–Peppas model.

In vitro assessments

Parasite culture

Leishmania major promastigotes MHOM/IL/2010/LRC-L1412 (P2407) were maintained at 26 °C in RPMI-1640 medium supplemented with 10% fetal calf serum. Intracellular amastigotes were kept at 37 °C in a 5% CO_2 incubator, in complete RPMI 1640 containing 10% fetal calf serum.

Cytotoxicity assay on THP1 cells

To evaluate the cytotoxic effect of AMB NPs, the human leukemia monocyte cell line THP1 was used. THP1 cells in RPMI with 10% fetal calf serum were aliquoted in triplicates (5.0×10^5 cells/mL; 125 μL per well) into 96-well plates (Nunc, Roskilde, Denmark). AMB NPs and blank NPs were then diluted with the same medium (0.01–6.7 μg/mL), added to the cells, and incubated for 24 h. 2-anilino paullone; a cytotoxic compound (1 μM) was used as a positive control as previously described [29]. The AlamarBlue viability indicator (AbD Serotec, Oxford, UK) was added [25 μL (10%) per well] and plates were incubated for an additional 24 h. Fluorescence (ex: 544 nm; em: 590 nm) was measured by a microplate reader (Fluoroskan Ascent FL, Finland). Percent of THP1 inhibition was then calculated form cell viability plot.

Evaluation of AMB NP antileishmanial activity on *L. major* promastigotes

The leishmanicidal activity of AMB NPs on *L. major* promastigotes was carried out using the AlamarBlue assay, as previously described [30]. Briefly, 5×10^6 parasites/mL (125 μL, *L. major* promastigotes) were seeded into each well of 96-well plates (Nunc, Roskilde, Denmark). Serial dilutions (0.001 to 2.5 μg/mL) of AMB, AMB NPs, and blank NPs were made in complete promastigote medium containing 1% DMSO, aliquoted in triplicates (125 μL per well), and incubated for 24 h. The AlamarBlue viability indicator was added (25 μL per well) and plates were incubated for an additional 24 h. Fluorescence (ex: 544 nm; em: 590 nm) was measured by a microplate reader (Fluoroskan Ascent FL, Finland). Complete medium, both with and without DMSO, served as negative controls (0% inhibition of promastigote growth). IC_{50} was determined using serial threefold dilutions of the test compounds from 0.01 to 20 μg/mL.

Evaluation of AMB NP antileishmanial activity on intracellular amastigotes

Luciferase-expressing Lm:pSSU-int/LUC promastigotes were used to infect macrophages, as previously described [31, 32]. The infected THP1 cells (10^5 cells per well) were treated with AMB and AMB NPs (0.001–2 μg/mL). Luminescence (intracellular amastigote viability) was measured by a Fluoroskan microplate reader after adding Steady-Glo® (Promega). Calculation of the IC_{50} values and statistical analysis were carried out using GraphPad Prism Version 6.0b (GraphPad Software, Inc. San Diego, CA).

In vitro release and sustained antileishmanial activity of AMB NPs

AMB NPs, AMB, and blank NPs were diluted (20, 2, and 0.2 μg/mL) in RPMI 1640 medium supplemented with 10% FCS. At various time points after diluting the NPs, 1 mL medium was taken and added, in triplicates (125 μL/well), to 5×10^6 *L. major* promastigotes per milliliter. After 24 h, 10% Alamar Blue was added and incubated for an additional 24 h. The fluorescence output, as a result of AlmarBlue reduction by viable parasites, was read using a Fluoroskan plate reader (ex: 544 nm, em: 590 nm). The percent growth inhibition of the *L. major* promastigotes was calculated as follows:

$$Y = \left[\frac{k-y}{k}\right] * 100 \quad (1)$$

where k is the negative control (untreated parasites) and y is the readings of AMB/AMB NP-treated parasites.

In vivo study

Animal experiments were conducted in accordance with the Institutional Animal Ethical Committee guidelines which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Eighth edition 2011, ethical number: MD-14-13923-3).

BALB/c mice (5 to 6 weeks old) were subcutaneously inoculated with 10^7 *L. major* stationary phase promastigotes (20 μL) at the base of the tail. The lesions were measured once a week. The lesion size was determined by measuring the maximal diameter of the outer edges perpendicularly and parallel to the dorsal line of the lesion using a mechanical caliper. Lesion area was calculated as π (length/2 × width/2)^2. After appearance of lesions on day 73, and prior to treatment, the mice were redistributed among the cages so each group contained animals with equal-sized lesions. Four treatment groups ($n = 6$–7) were established: control—PBS, blank NPs, 1 mg/kg AMB deoxycholate (intralesional), and 1 mg/kg AMB NPs (intralesional). A single intralesional dose (100 μL) of each treatment was then administered to the mice and lesion area determined at subsequent intervals.

Statistical analysis

To determine statistical significance in the in vivo study, Kruskal-Wallis test and Dunn's multiple comparisons test were performed. Differences were considered significant if $P < 0.05$.

Results

Characterization of AMB NPs

AMB NPs were prepared using the solvent deposition technique [33]. A solvent mixture was used to ensure a low mean particle size of 90 nm, with a satisfactory polydispersity index (PDI) of 0.27, various aggregation states of AMB [34], and negative zeta potential value of approximately − 27 mV. SEM analysis of AMB NPs was in good agreement with results obtained from DLS (Fig. S1).

The amount of AMB incorporated in PLGA NPs was determined by UV spectroscopy, and the efficiency of AMB loading was approximately 80%, with a drug content of 24% (*w*/*w*).

Determination of AMB aggregation state in AMB NPs

The peak I to peak IV intensity ratio (I/IV ratio) is a quantitative means of determining the aggregation state of AMB. At 4 μg/mL, AMB dissolved in methanol showed the least aggregation, I/IV ratio (0.37), compared to that of AMB in PBS (1.5) and AMB NPs (3.0). Moreover, a 20-min incubation at 37 °C led to a decrease in the AMB NP aggregation state (I/IV ratio = 1.97; Fig. 1a), while at 1 μg/mL the I/IV ratios for AMB in methanol, AMB NPs, and AMB NPs after 20 min at 37 °C were 0.34, 1.46, and 0.78, respectively (Fig. 1b). Additionally, after dilution of AMB NPs, the I/IV ratio was altered by increased absorbance at 407 nm, accompanied by decreased absorbance at 327 nm; this ratio decreased further following a 20-min incubation at 37 °C.

In vitro release of AMB

AMB release from AMB NPs exhibited a biphasic pattern over a period of 7 days, with an initial burst release accompanied by a sustained release phase (Fig. 2). In the present case, the data were fitted to the Korsmeyer–Peppas model, resulting in a release exponent (*n*) of 0.22 (Fig. S2) indicating Fickian release, in which drug diffusion is the primary factor in drug release [35].

Cytotoxicity assay on THP1 cells

The cytotoxicity of blank NPs and AMB NPs was investigated using the THP1 human macrophage cell line. A negligible cytotoxic effect of blank NPs and AMB NPs on THP1 cells was observed up to a concentration of 0.08 μg/mL, demonstrating their potential as nano-carriers for therapeutic applications. At 0.2 μg/mL, AMB NPs and blank NPs inhibited cell viability by 16 and 4%, respectively. At higher concentrations, both blank NPs and AMB NPs were relatively more toxic (Fig. 3).

In vitro antileishmanial activity of AMB NPs

The antileishmanial activity of free AMB and AMB NPs was determined 48 h following exposure of *L. major* promastigotes and infected macrophages to the loaded particles. The half maximal inhibitory concentration (IC_{50}) values on *L. major* extracellular promastigotes were determined in three independent experiments and show that there is no significant difference in IC_{50} between AMB and AMB NPs (0.079 ± 0.004 and 0.083 ± 0.005 μg/mL, respectively). In comparison, no inhibition was observed using the blank NPs (Fig. S3).

Viability of luciferase-expressing *L. major* amastigotes in infected macrophages was evaluated following treatment with AMB and AMB NPs. It is important to point out that macrophages are the primary host cells for the intracellular stage of the parasite which is responsible for human and animal disease. A significant difference was observed ($P < 0.01$) between the IC_{50} of AMB versus AMB NPs (0.071 ± 0.004 and 0.035 ± 0.001 μg/mL, respectively), i.e., AMB NPs were two times more effective against amastigotes. At a concentration of 0.2 μg/mL, AMB NPs and AMB induced 100 and 90% inhibition of intracellular parasites, respectively (Fig. S4). However, at lower concentrations (< 0.1 μg/mL), differences between the effects of AMB versus AMB NPs on infected macrophages were more pronounced.

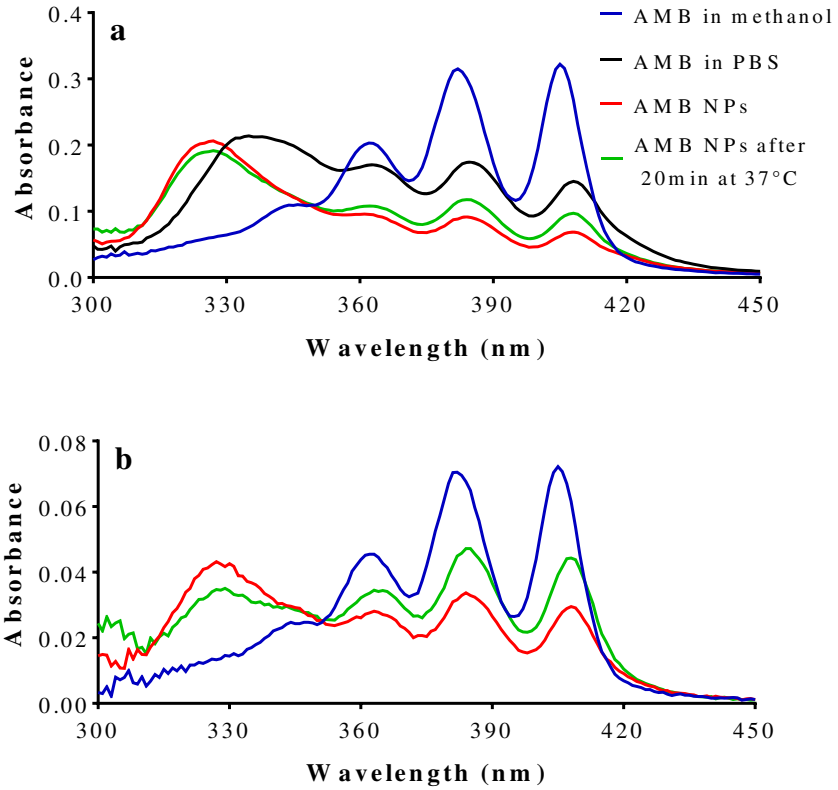

Fig. 1 UV-Vis absorbance spectral properties of AMB NPs. **a** Spectra of AMB (4 μg/mL) dissolved in PBS or methanol, AMB NPs, and AMB NPs after incubation at 37 °C for 20 min. **b** Spectra of AMB (1 μg/mL) dissolved in methanol, AMB NPs, and AMB NPs after incubation at 37 °C for 20 min

In vitro release and sustained antileishmanial activity of AMB NPs

Prior to determining the in vitro release profile of drug from AMB NPs, *L. major* promastigotes were incubated with increasing concentrations of AMB NPs (20, 2, 0.2 μg/mL) for 48 h and the percent growth inhibition assessed. At concentrations of 20 and 2 μg/mL, both AMB and AMB NPs inhibited growth completely, whereas at the lower concentration (0.2 μg/mL), AMB induced 39% growth inhibition, while the efficacy of AMB NPs remained stable (100% inhibition). Therefore, a concentration of 0.2 μg/mL AMB NPs was used

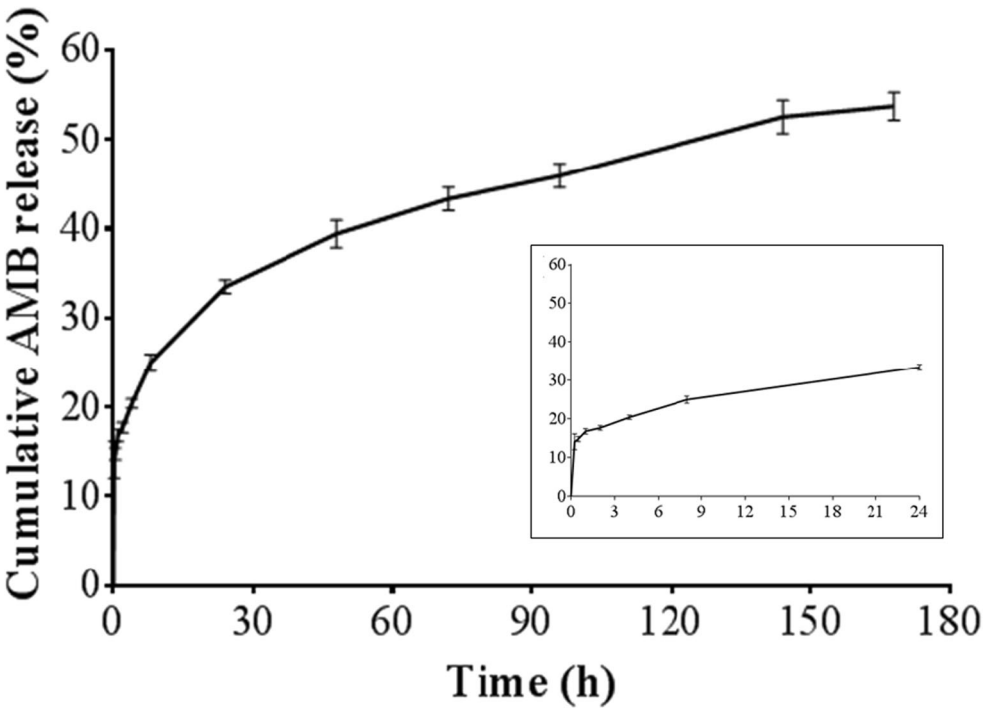

Fig. 2 In vitro drug release of AMB from AMB NPs in PBS with 1% Tween® 80 (pH 7.4, 37 °C) at different time intervals. Insert shows the drug release profile over the initial 24 h. Values are mean ± s.d. of three experiments

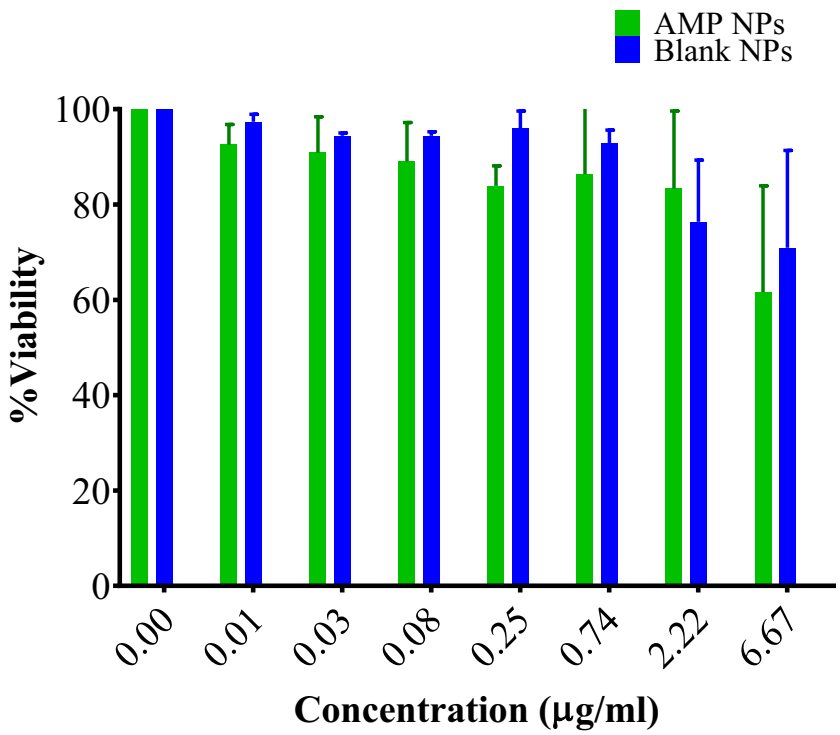

Fig. 3 Evaluation of AMB NP cytotoxicity. The effect of different concentrations of empty PLGA NPs and AMB NPs on the viability of THP1 cells. 2-Anilino paullone was used as a positive control (1 μM) and showed significant toxicity (0% viability, data not shown). Percent THP1 viability was calculated using the AlamarBlue assay. Results are presented as mean ± SD ($n = 3$)

for the in vitro release experiment. AMB NPs fully inhibited promastigotes growth over 15 days, indicating slow AMB release and subsequent long-term exposure (Fig. 4). Statistical analysis using two-tailed t test revealed that the differences between AMB and AMB NPs were significant ($P < 0.01$) over the time course of the experiment, and proved the crucial role of AMB encapsulation in enhancing parasite inhibition for extended time period.

In vivo study

We sought to compare the therapeutic efficacy of intralesional administration of AMB deoxycholate and AMB NPs. As depicted in Fig. 5, while no significant difference was noted between control, blank NPs, and AMB deoxycholate 1 mg/kg, AMB NPs elicited a significantly greater lesion-reducing effect as compared to controls ($P < 0.01$).

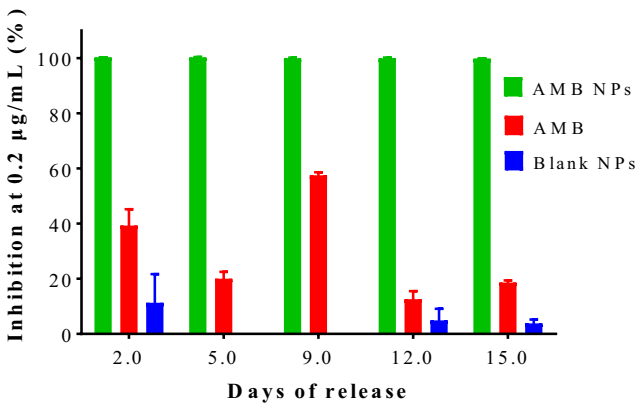

Fig. 4 In vitro release and long-term antileishmanial activity of free AMB (red), AMB NPs (green), and blank NPs (blue) over time at a concentration of 0.2 μg/mL AMB. The percent inhibition of *L. major* promastigotes was assessed daily using the AlamarBlue assay

Discussion

AMB is a macrolide polyene antibiotic possessing potent antifungal and antileishmanial activity. However, it has several drawbacks, such as high toxicity and poor water solubility which limit its efficacy. PLGA NPs loaded with AMB were produced to minimize AMB toxicity and boost its therapeutic outcome. AMB NPs were prepared by the nanoprecipitation approach. While preparation procedure including organic solvents, stabilizers, and surfactants is known to affect the AMB NPs properties, the conditions utilized in this study produced a homogeneous population of NPs (size 90 nm, PDI 0.27) with negative zeta potential value, and high efficiency of AMB loading (Fig. S1) similar to those described in previous publications using different solvents, stabilizers, and/or surfactants [25, 36]. The particle size was smaller than that seen using the emulsion-solvent-evaporation procedure [37, 38].

The toxicity of AMB has been associated with its aggregate form [39], where the selectivity of AMB between parasitic and mammalian cells depends on its aggregation state and the nature of membrane sterol [40]. AMB molecules tend to remain in their monomeric form in organic solvents such as methanol, and display four peaks at 346, 362, 382, and 407 nm. These results depicted in Fig. 1 indicate that AMB

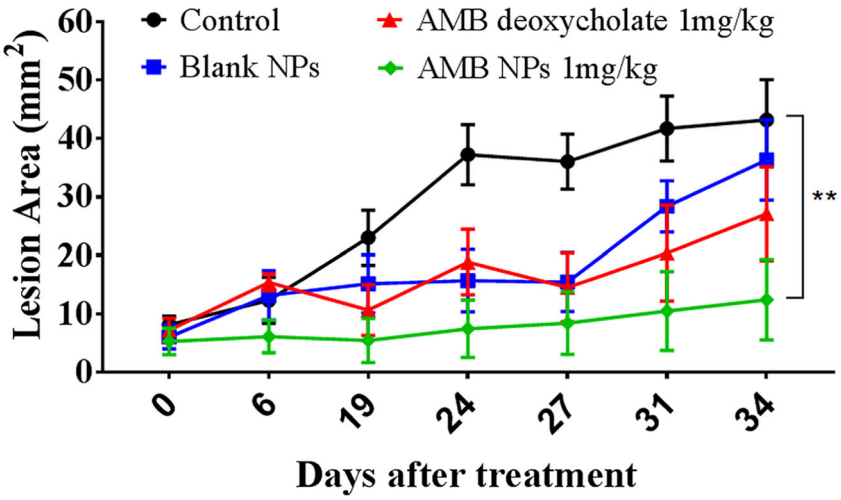

Fig. 5 Antileishmanial activity of AMB deoxycholate and AMB NPs in BALB/c mice infected with *L. major*. Following lesion appearance, each group mice were treated by one intralesional injection of the respective compounds. Control mice received no treatment. Lesion area = π (length/2 × width/2)^2. Values are means ± s.e. $^{**}P < 0.01$

incorporated in PLGA NPs exists in both aggregated and unaggregated forms. Furthermore, a hypsochromic shift from 335 to 327 nm was observed for AMB NPs dissolved in PBS. This shift could be explained by the time- and concentration-dependent alteration in the aggregation state of AMB (Fig. 1b), similar to what has been seen in other studies [36]. Taken together, AMB NPs may act as a reservoir of AMB monomers that are primarily released over an extended time as monomeric AMB species into the aqueous medium.

AMB NPs were associated with an extended release profile (Fig. 2). Numerous factors can affect the rate of drug diffusion, including polymer–drug interactions, drug–drug interactions, and water absorption, as well as PLGA degradation kinetics [41]. As shown in Fig. S2, fitting to Korsmeyer–Peppas model revealed that drug diffusion is the primary factor in drug release.

The human THP1 macrophage-like cell line was employed as they can be used in vitro to examine drug activity against the intracellular amastigote stage of the parasite, which is responsible for human disease [42], as well as the toxicity of NPs [43]. As depicted in Fig. 3, the cytotoxic effect of PLGA NPs was negligible at therapeutic concentrations of AMB, similar to a previous report demonstrating that various PLGA NP formulations had negligible cytotoxicity on MRC-5 fibroblasts as compared to free AMB or Fungizone® [36]. Moreover, AMB NPs were more effective against *L. major* amastigotes in infected macrophages at low concentrations. These findings agree with the results depicted in Fig. 3, which indicate a negligible toxic effect of AMB NPs on host macrophages in the therapeutic range. The IC_{50} (0.035 μg/mL) for the AMB NPs on intracellular *L. major* amastigotes was half that found for the free drug (0.071 μg/mL), and similar to IC_{50}'s reported for AMB-loaded PLGA NPs on intracellular *L. infantum* amastigotes (D12 = 0.03 μg/mL, DA12, D24, and DA24 = 0.08, 0.10, and 0.10 μg/mL, respectively). The lower IC_{50} of AMB NPs may be the result of encapsulation in PLGA NPs which increases its uptake into macrophages, similar to reports for bovine serum albumin [44]. Alternatively, the size of the NPs [45], may be responsible for the enhanced accumulation of AMB molecules in macrophages, consequently improving antileishmanial activity. Interestingly, AMB PLGA NPs prepared by the emulsion-solvent-evaporation method were also shown to be effective against *L. donovani*-infected mouse macrophages (J774.1), but no significant difference in efficacy between the NPs, AmBisome, and free drug was demonstrated at the concentration (1 μM) utilized [38].

The in vitro release experiment for evaluation of sustained activity of AMB NPs revealed that AMB NPs inhibited promastigote growth over 15 days (Fig. 4). These results indicate that AMB is released in a controlled manner, achieving antileishmanial activity for prolonged time. This profile is expected to minimize drug toxicity associated with exposure to high drug concentrations caused by rapid release, and to enhance efficacy. Sustained release of the drug and the protective role of PLGA NPs is important in preventing red blood cell lysis upon exposure to free AMB and surfactant [46, 47]. Free AMB showed fluctuating effects, which may be due to degradation of the compound (Fig. 4).

Although AMB deoxycholate (Fungizone®) is effective in treating visceral leishmaniasis, when administered intravenously, it is ineffective against CL caused by *L. major* [48, 49]. This study and previous reports demonstrate that PLGA NPs containing AMB demonstrated a significantly lower toxic effect both in vitro and in animal models when compared to AMB deoxycholate, and equal of better activity against intracellular amastigotes in vitro [25, 36, 38]. However, studies in mouse models for leishmaniasis are few, and all involve either intravenous or intraperitoneal injection of the PLGA NPs [25, 38, 50]. Therefore, we decided to compare the efficacy of AMB NPs and AMB deoxycholate after single intralesional injection. This localized method of treatment is frequently employed in treating human CL with other drugs [51]. AMB

NPs exhibited significant reduction in the lesion size compared to control, while the remaining groups showed inadequate efficacy (Fig. 5). So far, only a few reports of AMB-loaded PLGA NPs have been reported [25]. Among them, to the best of our knowledge, there are no reports describing intralesional injection. In conclusion, these findings suggest that intralesional AMB-loaded PLGA NPs improve AMB delivery and can be used for local treatment of CL, allowing controlled drug release and circumvention of systemic administration, as demonstrated in *L. major*-infected mice. However, further preclinical and clinical trials are needed to establish safety and efficacy of this kind of administration.

Acknowledgments This research was funded by the GIP program of the Deutsche Forschungsgemeinschaft (DFG) German Research Foundation. EZ wish to acknowledge the financial support of the RBNI-The Russell Berrie Nanotechnology Institute at the Technion. CLJ holds the Michael and Penny Feiwel Chair of Dermatology.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Gutierrez V, Seabra AB, Reguera RM, Khandare J, Calderon M. New approaches from nanomedicine for treating leishmaniasis. Chem Soc Rev. 2016;45(1):152–68. https://doi.org/10.1039/c5cs00674k.
2. Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. Nat Rev Microbiol. 2011;9(8):604–15. https://doi.org/10.1038/nrmicro2608.
3. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science. 2008;321(5891):970–4. https://doi.org/10.1126/science.1159194.
4. Handler MZ, Patel PA, Kapila R, Al-Qubati Y, Schwartz RA. Cutaneous and mucocutaneous leishmaniasis: differential diagnosis, diagnosis, histopathology, and management. J Am Acad Dermatol. 2015;73(6):911–26; 27-8. https://doi.org/10.1016/j.jaad.2014.09.014.
5. Lemke A, Kiderlen AF, Kayser O. Amphotericin B. Appl Microbiol Biotechnol. 2005;68(2):151–62. https://doi.org/10.1007/s00253-005-1955-9.
6. Torrado JJ, Espada R, Ballesteros MP, Torrado-Santiago S. Amphotericin B formulations and drug targeting. J Pharm Sci. 2008;97(7):2405–25. https://doi.org/10.1002/jps.21179.
7. Mbongo N, Loiseau PM. Billion MA, Robert-Gero M. mechanism of amphotericin B resistance in Leishmania donovani promastigotes. Antimicrob Agents Chemother. 1998;42(2):352–7.
8. Paila YD, Saha B, Chattopadhyay A. Amphotericin B inhibits entry of Leishmania donovani into primary macrophages. Biochem Biophys Res Commun. 2010;399(3):429–33. https://doi.org/10.1016/j.bbrc.2010.07.099.
9. Escobar P, Matu S, Marques C, Croft SL. Sensitivities of Leishmania species to hexadecylphosphocholine (miltefosine), ET-18-OCH(3) (edelfosine) and amphotericin B. Acta Trop. 2002;81(2):151–7.
10. Yu BG, Okano T, Kataoka K, Sardari S, Kwon GS. In vitro dissociation of antifungal efficacy and toxicity for amphotericin B-loaded poly(ethylene oxide)-block-poly(beta benzyl L aspartate) micelles. J Control Release. 1998;56(1–3):285–91.
11. Wong-Beringer A, Jacobs RA, Guglielmo BJ. Lipid formulations of amphotericin B: clinical efficacy and toxicities. Clin Infect Dis. 1998;27(3):603–18.
12. Espuelas MS, Legrand P, Irache JM, Gamazo C, Orecchioni AM, Devissaguet JP, et al. Poly(e-caprolacton) nanospheres as an alternative way to reduce amphotericin B toxicity. Int J Pharm. 1997;158(1):19–27.
13. Alvarez C, Shin DH, Kwon GS. Reformulation of Fungizone by PEG-DSPE micelles: deaggregation and detoxification of amphotericin B. Pharm Res. 2016;33(9):2098–106. https://doi.org/10.1007/s11095-016-1948-7.
14. Stone NR, Bicanic T, Salim R, Hope W. Liposomal amphotericin B (AmBisome((R))): a review of the pharmacokinetics, pharmacodynamics, clinical experience and future directions. Drugs. 2016;76(4):485–500. https://doi.org/10.1007/s40265-016-0538-7.
15. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Preat V. PLGA-based nanoparticles: an overview of biomedical applications. J Control Release. 2012;161(2):505–22. https://doi.org/10.1016/j.jconrel.2012.01.043.
16. Reis CP, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation I. methods for preparation of drug-loaded polymeric nanoparticles. Nanomedicine. 2006;2(1):8–21.
17. Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev. 2003;55(3):329–47.
18. Kumari A, Yadav SK, Yadav SC. Biodegradable polymeric nanoparticles based drug delivery systems. Colloids Surf B: Biointerfaces. 2010;75(1):1–18. https://doi.org/10.1016/j.colsurfb.2009.09.001.
19. Zhou Z, Badkas A, Stevenson M, Lee JY, Leung YK. Herceptin conjugated PLGA-PHis-PEG pH sensitive nanoparticles for targeted and controlled drug delivery. Int J Pharm. 2015;487(1–2):81–90. https://doi.org/10.1016/j.ijpharm.2015.03.081.
20. Hudlikar MS, Li X, Gagarinov IA, Kolishetti N, Wolfert MA, Boons GJ. Controlled multi-functionalization facilitates targeted delivery of nanoparticles to cancer cells. Chemistry. 2016;22(4):1415–23. https://doi.org/10.1002/chem.201503999.
21. Kapoor DN, Bhatia A, Kaur R, Sharma R, Kaur G, Dhawan S. PLGA: a unique polymer for drug delivery. Ther Deliv. 2015;6(1):41–58.
22. Makadia HK, Siegel SJ. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. Polymers. 2011;3(3):1377–97.
23. Uhrich KE, Cannizzaro SM, Langer RS, Shakesheff KM. Polymeric systems for controlled drug release. Chem Rev. 1999;99(11):3181–98.
24. von Burkersroda F, Schedl L, Gopferich A. Why degradable polymers undergo surface erosion or bulk erosion. Biomaterials. 2002;23(21):4221–31.
25. Palma E, Pasqua A, Gagliardi A, Britti D, Fresta M, Cosco D. Antileishmanial activity of amphotericin B-loaded-PLGA nanoparticles: an overview. Materials 2018;11(7). doi:https://doi.org/10.3390/ma11071167.
26. Kumar R, Sahoo GC, Pandey K, Das V, Das P. Study the effects of PLGA-PEG encapsulated amphotericin B nanoparticle drug delivery system against Leishmania donovani. Drug Deliv. 2015;22(3):383–8. https://doi.org/10.3109/10717544.2014.891271.
27. Butani D, Yewale C, Misra A. Amphotericin B topical microemulsion: formulation, characterization and evaluation. Colloids Surf B: Biointerfaces. 2014;116:351–8. https://doi.org/10.1016/j.colsurfb.2014.01.014.

28. Abu Ammar A, Raveendran R, Gibson D, Nassar T, Benita S. A lipophilic Pt(IV) oxaliplatin derivative enhances antitumor activity. J Med Chem. 2016;59(19):9035–46. https://doi.org/10.1021/acs.jmedchem.6b00955.
29. Ryczak J, Papini M, Lader A, Nasereddin A, Kopelyanskiy D, Preu L, et al. 2-Arylpaullones are selective antitrypanosomal agents. Eur J Med Chem. 2013;64:396–400. https://doi.org/10.1016/j.ejmech.2013.03.065.
30. Shimony O, Jaffe CL. Rapid fluorescent assay for screening drugs on Leishmania amastigotes. J Microbiol Methods. 2008;75(2):196–200. https://doi.org/10.1016/j.mimet.2008.05.026.
31. Keurulainen L, Siiskonen A, Nasereddin A, Kopelyanskiy D, Sacerdoti-Sierra N, Leino TO, et al. Synthesis and biological evaluation of 2-arylbenzimidazoles targeting Leishmania donovani. Bioorg Med Chem Lett. 2015;25(9):1933–7. https://doi.org/10.1016/j.bmcl.2015.03.027.
32. Haavikko R, Nasereddin A, Sacerdoti-Sierra N, Kopelyanskiy D, Alakurtti S, Tikka M, et al. Heterocycle-fused lupane triterpenoids inhibit Leishmania donovani amastigotes. MedChemComm. 2014;5(4):445–51.
33. Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm. 1989;55(1):R1–4.
34. Van de Ven H, Paulussen C, Feijens PB, Matheeussen A, Rombaut P, Kayaert P, et al. PLGA nanoparticles and nanosuspensions with amphotericin B: potent in vitro and in vivo alternatives to Fungizone and AmBisome. J Control Release. 2012;161(3):795–803. https://doi.org/10.1016/j.jconrel.2012.05.037.
35. Peppas NA. Analysis of Fickian and non-Fickian drug release from polymers. Pharm Acta Helv. 1985;60(4):110–1.
36. Van de Ven H, Paulussen C, Feijens P, Matheeussen A, Rombaut P, Kayaert P, et al. PLGA nanoparticles and nanosuspensions with amphotericin B: potent in vitro and in vivo alternatives to Fungizone and AmBisome. J Control Release. 2012;161(3):795–803.
37. Carraro TCMM, Khalil NM, Mainardes RM. Amphotericin B-loaded polymeric nanoparticles: formulation optimization by factorial design. Pharm Dev Technol. 2016;21(2):140–6.
38. Nahar M, Mishra D, Dubey V, Jain N, editors. Development of amphotericin b loaded PLGA nanoparticles for effective treatment of visceral leishmaniasis. 13th International Conference on Biomedical Engineering; 2009: Springer
39. Barwicz J, Christian S, Gruda I. Effects of the aggregation state of amphotericin B on its toxicity to mice. Antimicrob Agents Chemother. 1992;36(10):2310–5.
40. Wang Y, Ke X, Voo ZX, Yap SS, Yang C, Gao S, et al. Biodegradable functional polycarbonate micelles for controlled release of amphotericin B. Acta Biomater. 2016;46:211–20. https://doi.org/10.1016/j.actbio.2016.09.036.
41. Fredenberg S, Wahlgren M, Reslow M, Axelsson A. The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems—a review. Int J Pharm. 2011;415(1–2):34–52. https://doi.org/10.1016/j.ijpharm.2011.05.049.
42. Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, Konno T, et al. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Cancer Res. 1982;42(4):1530–6.
43. Grabowski N, Hillaireau H, Vergnaud J, Tsapis N, Pallardy M, Kerdine-Romer S, et al. Surface coating mediates the toxicity of polymeric nanoparticles towards human-like macrophages. Int J Pharm. 2015;482(1–2):75–83. https://doi.org/10.1016/j.ijpharm.2014.11.042.
44. Guedj AS, Kell AJ, Barnes M, Stals S, Goncalves D, Girard D, et al. Preparation, characterization, and safety evaluation of poly(lactide-co-glycolide) nanoparticles for protein delivery into macrophages. Int J Nanomedicine. 2015;10:5965–79. https://doi.org/10.2147/IJN.S82205.
45. Abamor ES. Antileishmanial activities of caffeic acid phenethyl ester loaded PLGA nanoparticles against Leishmania infantum promastigotes and amastigotes in vitro. Asian Pac J Trop Med. 2017;10(1):25–34. https://doi.org/10.1016/j.apjtm.2016.12.006.
46. Italia JL, Yahya MM, Singh D, Ravi Kumar MN. Biodegradable nanoparticles improve oral bioavailability of amphotericin B and show reduced nephrotoxicity compared to intravenous Fungizone. Pharm Res. 2009;26(6):1324–31. https://doi.org/10.1007/s11095-009-9841-2.
47. Radwan MA, AlQuadeib BT, Siller L, Wright MC, Horrocks B. Oral administration of amphotericin B nanoparticles: antifungal activity, bioavailability and toxicity in rats. Drug Deliv. 2017;24(1):40–50. https://doi.org/10.1080/10717544.2016.1228715 .
48. Sundar S, Mehta H, Suresh AV, Singh SP, Rai M, Murray HW. Amphotericin B treatment for Indian visceral leishmaniasis: conventional versus lipid formulations. Clin Infect Dis. 2004;38(3):377–83. https://doi.org/10.1086/380971.
49. Yardley V, Croft SL. A comparison of the activities of three amphotericin B lipid formulations against experimental visceral and cutaneous leishmaniasis. Int J Antimicrob Agents. 2000;13(4):243–8.
50. de Carvalho RF, Ribeiro IF, Miranda-Vilela AL, de Souza Filho J, Martins OP, Cintra e Silva Dde O, et al. Leishmanicidal activity of amphotericin B encapsulated in PLGA–DMSA nanoparticles to treat cutaneous leishmaniasis in C57BL/6 mice. Exp Parasitol. 2013;135(2):217–22.
51. Burza S, Croft SL, Boelaert M. Leishmaniasis. Lancet. 2018;392(10151):951–70. https://doi.org/10.1016/S0140-6736(18)31204-2.