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EXPERIMENTAL THERAPEUTICS

Depot Subcutaneous Injection with Chalcone CH8-Loaded Poly(Lactic-Co-Glycolic Acid) Microspheres as a Single-Dose Treatment of Cutaneous Leishmaniasis

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ABSTRACT Conventional chemotherapy of cutaneous leishmaniasis (CL) is based on multiple parenteral or intralesional injections with systemically toxic drugs. Aiming at a single-dose localized therapy, biodegradable poly(lactic-co-glycolic acid) (PLGA) microparticles loaded with 7.8% of an antileishmanial nitrochalcone named CH8 (CH8/PLGA) were constructed to promote sustained subcutaneous release. *In vitro*, murine macrophages avidly phagocytosed CH8/PLGA smaller than 6 μm without triggering oxidative mechanisms. Upon 48 h of incubation, both CH8 and CH8/PLGA were 40 times more toxic to intracellular *Leishmania amazonensis* than to macrophages. *In vivo*, BALB/c were given one or three subcutaneous injections in the infected ear with 1.2 mg/kg of CH8 in free or CH8/PLGA forms, whereas controls received three CH8-equivalent doses of naked PLGA microparticles or meglumine antimoniate (Glucantime; Sanofi-Aventis). Although a single injection with CH8/PLGA reduced the parasite loads by 91%, triple injections with free CH8 or CH8/PLGA caused 80 and 97% reductions, respectively, in relation to saline controls. Meglumine antimoniate treatment was the least effective (only 36% reduction) and the most toxic, as indicated by elevated alanine aminotransferase serum levels. Together, these findings show that CH8/PLGA microparticles can be effectively and safely used for single-dose treatment of CL.

KEYWORDS cutaneous leishmaniasis, microparticles, chemotherapy, depot, PLGA, polymeric microparticle

Leishmaniasis is a collective term describing vector-borne diseases caused by different species of the protozoan parasite *Leishmania* where clinical manifestations vary from cutaneous leishmaniasis (CL) to life-threatening visceral leishmaniasis (VL). CL, the object of this study, is the most common form, affecting 1.2 million people annually in 98 countries where it constitutes a serious health problem (1). At the site of the sandfly bite, the dermotropic parasites infect local macrophages, causing primary skin lesions that typically evolve from papules to ulcers with a raised border and central depression which may reach many centimeters in diameter. Spontaneous healing is common in the Old World but rare in the New World (2, 3). Although in a few CL patients infected in the New World with *Leishmania amazonensis* and *L. braziliensis*, the initial lesion may spread to different parts of the skin (diffuse CL) or to the oronasal mucosa (mucosal leishmaniasis), respectively, in the great majority (>95%) of cases worldwide, the lesion remains localized (localized CL [LCL]) (3). Despite that, the mainstay LCL treatment is similar to other more serious CL and VL forms, advocating one or more series of parenteral injections with antimonials, pentamidine, or amphotericin B. The efficacy of oral drugs such as miltefosine, fluconazole, or ketoconazole is controversial (4). All

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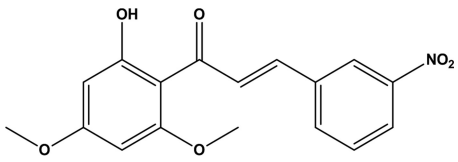

FIG 1 Chalcone CH8 (3-nitro-2′-hydroxy-4′,6′-dimetoxychalcone).

of these treatments produce systemic toxicity, ranging from arthralgia to kidney failure (5), which is unacceptable for patients with uncomplicated LCL.

Local therapies have also presented restrictions. Although cryotherapy and thermotherapy require specific equipment and trained personnel to avoid burnings (6), topical paromomycin (7) and amphotericin B (Drugs for Neglected Diseases Initiative [DNDi]) creams have shown partial or no efficacy. Failure to permeate skin and reach the deep dermis where infected macrophages reside may be due not only to the thickened LCL epidermis (8) but also to drug physical inappropriateness (e.g., molecular size and logP) (9), and the demanding strong permeation enhancers that are frequently irritating to the skin (10). Strategies aiming at bypassing skin permeation have included intralesional infiltrations with antimonials and amphotericin B (11–14). However, due to the rapid clearance to the circulation, repeated injections are needed, leading to discomfort, occasional systemic toxicity, and reduced patient compliance (15). Besides, repeated hospital visits are impracticable for patients with limited mobility, and those living in remote areas or in a conflict zone. Therefore, a local, effective, safe, and short therapy remains urgently needed for the most common and uncomplicated LCL, such as those with up to four lesions, each no more than 3 to 4 cm in diameter, afflicting more than 1 million people worldwide (6).

Drug release depots comprised of biodegradable polymeric microparticles have appeared as an effective alternative to repeated drug intake in chronic therapy against cancer, diabetes, hormone, inflammatory diseases, and mental disorders (16–20). In this context, poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymer PLGA are appropriate polymers to prepare slow-release systems due to their favorable biocompatibility and biodegradability (21). Chalcones are a new class of potential antileishmanials (22), and their hydrophobic nature seems adequate for association with PLGA. In murine LCL caused by *L. amazonensis*, Piñero et al. used transchalcone-loaded PLGA film disks implanted at a site distant from the lesion for sustained systemic delivery (23). Aiming at a local intracellular delivery, we showed previously that PLA nanoparticles entrapped with a plant-derived chalcone (DMC) are effectively internalized by infected macrophages and more active than the naked drug when given by intralesional route (24). Due to the low plant extraction yield, DMC analogues were chemically synthesized, among them the nitrosylated chalcone CH8 (Fig. 1) was selected as a model drug for its high antileishmanial activity (50% inhibitory concentration [IC_{50}] = 0.7 μM) and selectivity index (SI; SI = 143). In addition, CH8 showed an intralesional efficacy that was 60-fold higher than the pentavalent antimonial pentostam in a murine model of LCL caused by *L. amazonensis* (25).

In the present study, we proposed to prepare and use CH8-loaded PLGA microparticles for a safe and effective treatment for LCL that bypassed skin permeation and promoted both the drug uptake by infected macrophages and local sustained release.

RESULTS

CH8/PLGA microparticles characteristics. Both naked PLGA and CH8/PLGA particles displayed unimodal size distribution (Fig. 2). The average mean sizes at day 4.3 were 5.3 and 6.2 μm, respectively, with 2.4 and 2.3 (span) values (Table 1). PLGA and CH8/PLGA zeta potentials were −11 and −12.5 mV, respectively, indicating a slight increase in size and negative surface charge due to loading with CH8.

The percentage of drug loading (calculated as the weight of drug in grams loaded in the microparticles/the weight of polymer and drug in grams initially added in the

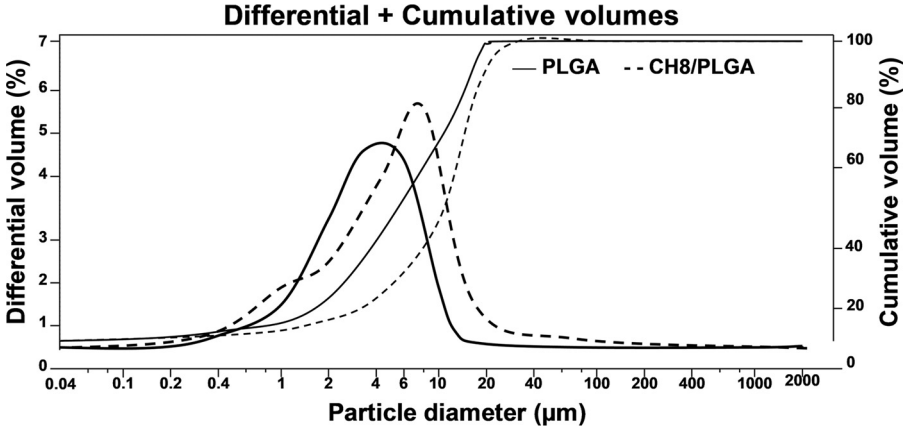

FIG 2 Microparticle size distributions. Naked PLGA (continuous lines) and drug-entrapped CH8/PLGA (dotted lines) microspheres were analyzed for the percentage of differential volume (Gaussian lines) and cumulative volume (increasing lines).

formulation × 100) was 7.8%, corresponding to an entrapment efficiency of 78% (that is, the percentage of drug that was successfully entrapped into microparticles). The scanning electron microscopic images in Fig. 3 show that particles were spherical and smooth, with no visible pores. Figure 3A and B (a higher magnification) show the CH8 crystals measuring, on average, 120 μm prior to CH8-loaded microparticles preparation. Figure 3C and D (higher magnification) show the obtained microparticles, which were about 20 times smaller than the drug crystals. The scarce CH8 crystal outside CH8/PLGA confirms the high drug entrapment efficiency.

Intracellular uptake and parasite killing. The capacity of macrophages to internalize CH8/PLGA microparticles was evaluated under optical microscopy (Fig. 4). After the addition of 50 microparticles/macrophage for 3 h, an average of 10.5 ± 1.2 particles smaller than 6 μm was seen inside the cells. No further increase in the numbers of internalized particles was seen after 6 h (not shown). Despite the efficient particle uptake by macrophages, entrapment in CH8/PLGA did not increase CH8 activity against intracellular amastigotes (IC_{50} = 10.7 and 7.2 μg/ml, respectively) as measured after 48 h of incubation (Table 2). However, entrapment in PLGA did reduce CH8 cytotoxicity to macrophages (50% cytotoxic concentrations [CC_{50}] = 290.2 and 429.7 μg/ml, respectively), resulting in similar selectivity index values.

Activation of macrophage oxidative mechanisms. To verify whether PLGA uptake interfered with oxidative mechanisms important for leishmanial parasite killing by macrophages, the production of nitric oxide (NO) and reactive oxygen species (ROS) was compared in infected and uninfected macrophages after the addition of CH8, PLGA, and CH8/PLGA to cells. Figure 5A shows that *Leishmania*-infected macrophages were less responsive to lipopolysaccharide (LPS) for NO production than uninfected cells, in line with previous studies (26–28). CH8 inhibited LPS-stimulated NO response in infected cells, which is compatible with chalcone's antioxidant properties (29, 30). Despite the normal capacity of infected macrophages to respond to zymosan with ROS production, neither CH8 nor PLGA displayed ROS-activating properties (Fig. 5B). In all,

TABLE 1 Size, surface charge, and drug loading[a]

Sample	Distribution of particle diam (μm)					Zeta potential (mV)	Drug loading (%)
	d(4.3)	d10	d90	SD	Span[b]		
PLGA	5.3	1.3	10.4	2.3	2.4	−11.0 ± 2.6	NA
CH8/PLGA	6.2	0.6	13.4	4.9	2.3	−12.5 ± 2.8	7.8 ± 1.5

[a]Zeta potential and drug loading are expressed as means ± the standard errors of the mean (n = 4 to 10). NA, not applicable.
[b]Span = (d90 − d10)/d50.

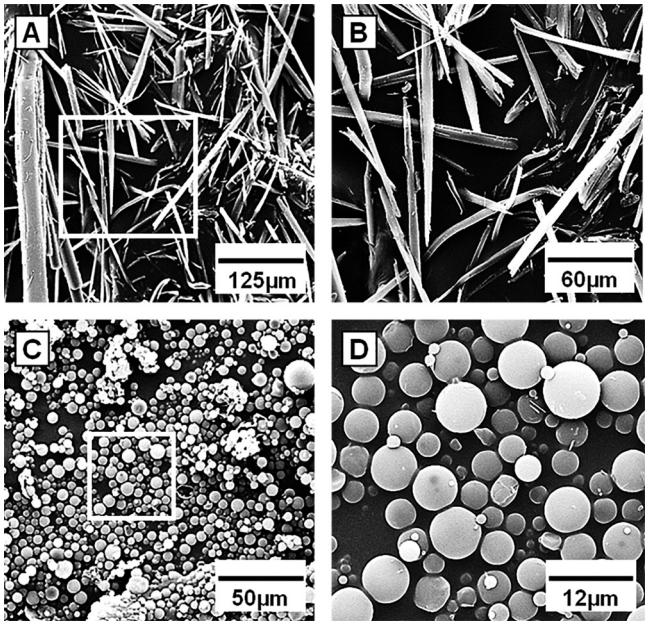

FIG 3 Scanning electron microscopy images of CH8 crystals (raw material) (A and B) and CH8-loaded PLGA microparticles (C and D). Inset squares are shown magnified on the right (B and D).

these results show that PLGA does not revert the antioxidative mechanisms of macrophages by CH8, which would otherwise contribute with the CH8 killing of intracellular parasites.

Efficacy of treatment of murine CL. The capacity of CH8/PLGA to reduce the necessary number of drug injections to one was evaluated in the mouse ear model of infection with *L. amazonensis*, where treatment started on day 9 postinfection to maximize the opportunity of demonstrating differences between the formulations. For that, the infected ears were injected subcutaneously with one or three doses of free CH8, CH8/PLGA, or the same dose (27 μg) of the reference drug meglumine antimoniate (Glucantime; Sanofi-Aventis). The curative parameters were lesion growth (Fig. 6A) and parasite loads measured on day 90 of infection (Fig. 6B). Although local inflammation caused by the subcutaneous injection may unrelatedly increase the lesion sizes, that was not the case here since untreated and phosphate-buffered saline (PBS)-injected ears showed similar sizes at most time points. With the three-dose schedule, meglumine antimoniate was less effective than free CH8 in controlling lesion growth and reducing the parasite loads (42% versus 78%) compared to animals given PBS alone (0%). It is noteworthy that entrapment of CH8 in CH8/PLGA further increased its efficacy to 97%. That was not due to an intrinsic PLGA effect, since naked microparticles

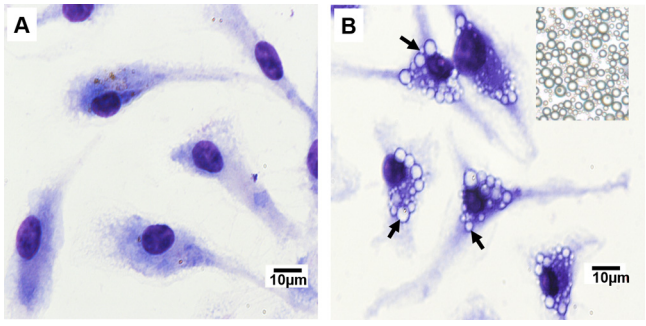

FIG 4 Microparticle uptake by macrophages. Untreated macrophages (A) and macrophages incubated for 3 h with CH8/PLGA microparticles (B). Cells and microparticles alone (inset image in panel B) were stained with Giemsa prior to imaging by optical microscopy.

TABLE 2 *In vitro* antiamastigote and antimacrophage cytotoxicity

Sample	Mean concn (μg/ml) ± SD[a]		SI[b]
	Amastigotes (IC_{50})	Macrophages (CC_{50})	
CH8	7.2 ± 1.9	290.2 ± 46.7	40.3
CH8/PLGA	10.7 ± 2.2	429.7 ± 60.2*	40.2
PLGA	>1,000†	>1,000†	ND
Glucantime	45.5 ± 1.6	175.5 ± 2.6	3.9

[a]Mean values ± SD (n = 3). The IC_{50} and CC_{50} values were determined by logarithmic regression analysis.
[b]The selectivity index (SI) was calculated as CC_{50}/IC_{50}. *, P <0.01 in relation to free CH8. †, value extrapolated from curve fitting. ND, not determined.

failed to control lesion and parasite growth. To verify the sustained release effect, mice were given a single dose of CH8/PLGA on day 9. After 81 days, the parasite loads were significantly lower than in animals given three weekly doses of PBS, CH8, or meglumine antimoniate (91, 50, and 82%, respectively) (Fig. 6B).

Treatment toxicity. Although there were no visible signs of local toxicity such as redness or edema, confirmed by the observation that ears were never thicker than untreated controls even during treatment (days 9 to 23, Fig. 6), it was interesting to evaluate the possible systemic effects of drugs leaking into the circulation. Apart from

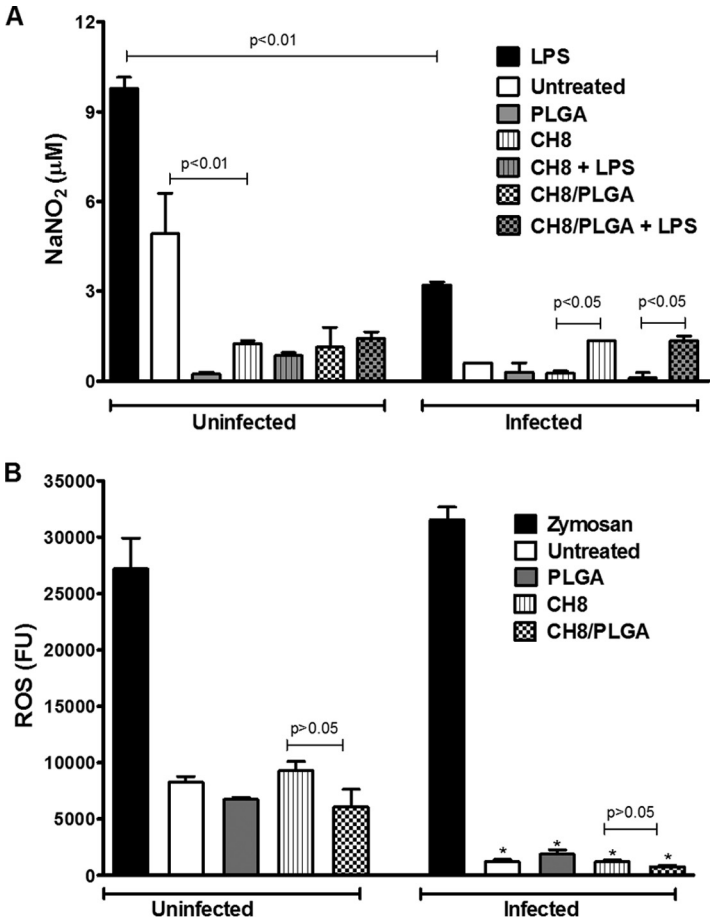

FIG 5 NO and ROS production by macrophages. *L. amazonensis*-infected and uninfected peritoneal macrophages were treated with CH8/PLGA, PLGA, or free CH8 (15 μg of CH8/ml and equivalent amounts of PLGA) or left untreated. (A) For NO production, LPS (1 μg/ml) was added just prior to CH8 or CH8/PLGA, as indicated. Cells were incubated for 48 h, when the $NaNO_2$ content was determined in the culture supernatants. (B) For ROS production, Zymosan (250 μg/ml) was added as a control. H_2DCFDA (10 μM) was immediately added after the indicated compounds, and after 20 min of incubation, ROS production was evaluated by fluorescence emission. FU, fluorescence units. Means ± the standard deviations are presented (n = 3). *, P < 0.05 in relation to the same additive in uninfected cells.

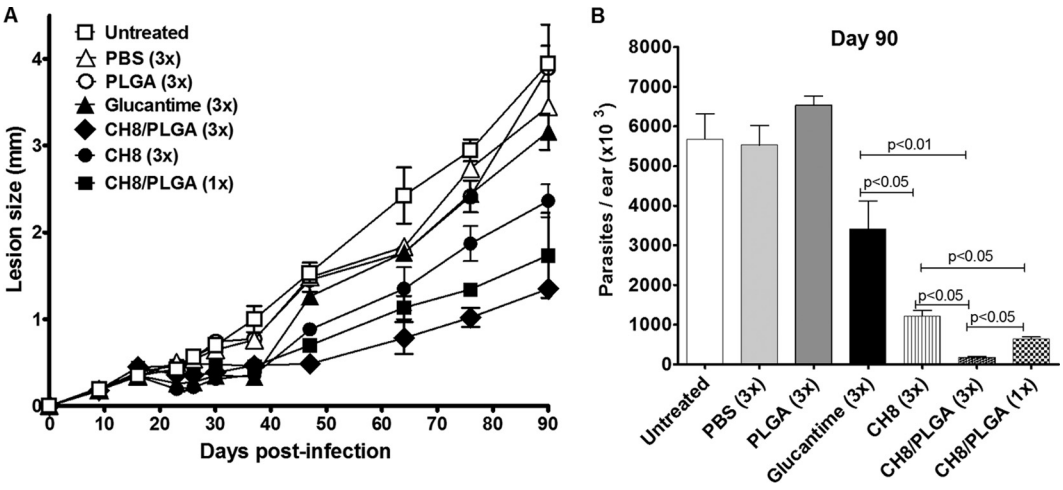

FIG 6 Effect of CH8/PLGA microparticle depot in murine CL. Mice were infected in the ear with *L. amazonensis* and given intralesional subcutaneous injections on days 9, 16, and 23 postinfection with meglumine antimoniate, CH8, or CH8/PLGA, with all drugs administered at 1.2 mg/kg/dose. The controls were vehicle (PBS) or PLGA given on the same days. CH8/PLGA was also given as a single injection on day 9. (A) Lesion sizes were measured on the indicated days. (B) Parasite loads in the ear, measured on day 90 by a limiting dilution assay. Means ± the standard errors of the mean (SEM) are presented ($n = 5$ to 8).

the slight ($P < 0.05$) weight loss of meglumine antimoniate-treated mice detected on days 16 to 23, no other group presented weight variations significantly different from PBS controls (Fig. 7).

As more sensitive parameters, biochemical markers of hepatic (aspartate transaminase [AST] and alanine transaminase [ALT]), and kidneys (creatinine) toxicity were measured in the sera after termination of the three-dose schedule with meglumine antimoniate, CH8, and CH8/PLGA. As seen in Fig. 8, with the exception of meglumine antimoniate, which increased ($P < 0.05$) ALT levels, no other treatment affected AST, ALT, or creatinine levels. These findings indicate that, unlike treatment with meglumine antimoniate, which led to a transient weight loss and hepatic toxicity, treatment with CH8 and PLGA led to no detectable toxicity.

DISCUSSION

The great majority of LC cases are uncomplicated, presenting up to four localized lesions. Still, due to difficult skin permeation after topical application or rapid clearance to the circulation after intralesional injections, available drugs are often given by systemic routes unacceptably exposing patients to systemic toxicity, besides demand-

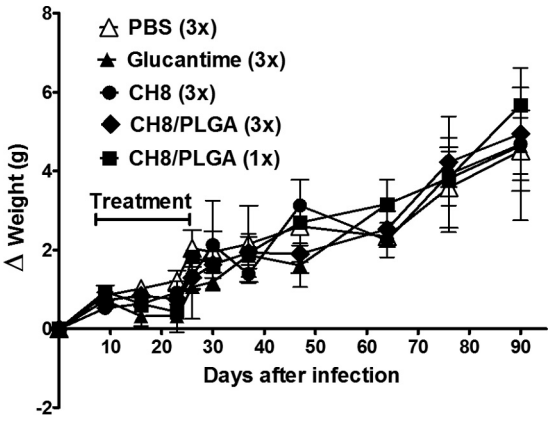

FIG 7 Body weight gain. Mice were infected and treated on days 9, 16, and 23 (three times) or day 9 only (once) as described in Fig. 6 and periodically weighed on the indicated days. Means ± the SEM are presented ($n = 5$ to 8).

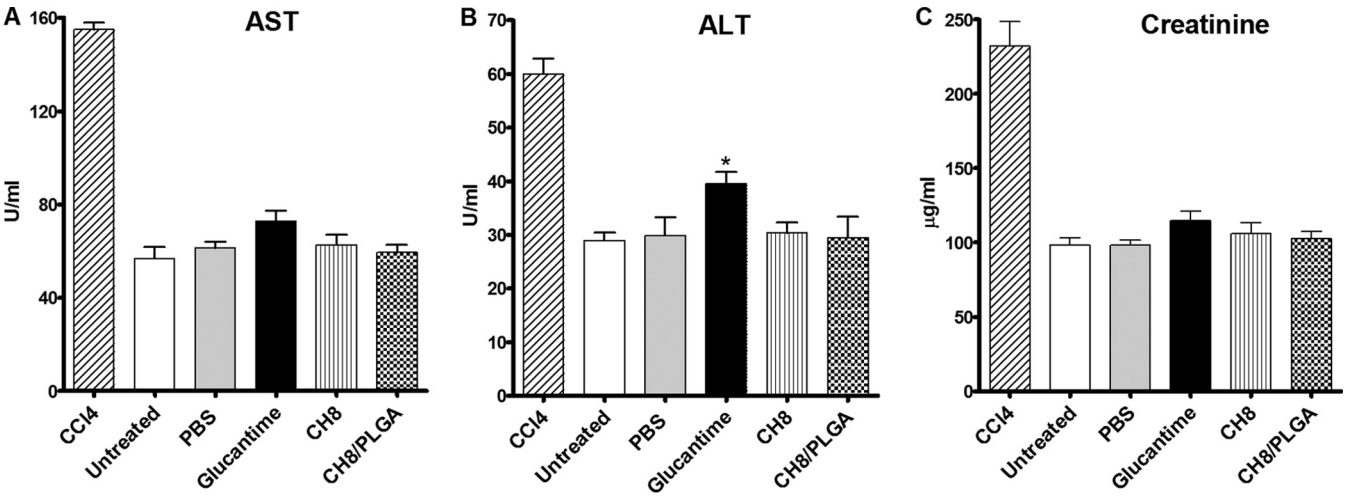

FIG 8 Evaluation of systemic toxicity. Mice were infected and treated with the indicated compounds on days 9, 16, and 23 as described in Fig. 6. Positive control was 1% CCl_4 given by intraperitoneal injection on day 23. Three days after the last dose, the sera were collected and individually assayed for AST (A), ALT (B), and creatinine (C). Means ± the SEM are presented ($n = 3$). *, $P < 0.05$.

ing frequent visits to a physician for medication. For this reason, formulations designed to reduce dosing frequency and potential drug toxicity, useful for patients who adhere poorly to frequent oral or injectable medication and have limited mobility, are urgently needed for CL. In the United States in 2016, there were eleven U.S. Food and Drug Administration-approved PLA/PLGA drug products available as microparticulate devices for subcutaneous or intramuscular administration to treat alcohol dependence, prostate cancer, hormonal disorders, schizophrenia, periodontitis, diabetes, and acromegaly (16). In CL, Piñero et al. surgically implanted 3-mm-diameter PLGA and PLA disks loaded with transchalcone in mouse ears to treat *L. amazonensis* infection in the footpads. These authors found that PLGA was superior to PLA (31 and 10% reductions in lesion sizes, respectively) (23), probably because of the faster PLGA degradation rate (31). However, all these devices are meant for sustained release in the circulation, not avoiding the systemic side effects. Local injections would therefore appear more appropriate for localized CL.

Although used for decades in Asia and Africa to treat CL (13), intralesional injections with meglumine antimoniate (≤5 ml/lesion every 2 weeks) has only recently been officially approved by the Ministry of Health in Brazil (15). Therefore, meglumine antimoniate would appear a first-choice drug for sustained intralesional release in microsphere depot. However, because it is a highly hydrophilic drug, a complex double-wall process is necessary to counteract the strong initial burst, short release period, and low encapsulation efficiency that normally accompanies encapsulation of hydrophilic drugs in PLA/PLGA particles (32, 33). Local injections with 130-nm liposomes loaded with meglumine antimoniate, miltefosine, and paromomycin have also been tested in *L. major*-infected mice, but only multiple doses of miltefosine-loaded formulations showed significant therapeutic effects (34). In this context, the nitrochalcone CH8 was used here as a model drug for its selective antileishmanial activity and lipophilicity, suitable for encapsulation in PLGA microparticles for sustained release in the infected tissue.

The CH8/PLGA microparticles were constructed with unimodal size distribution of 0.6 to 13 μm (days 10 to 90) to allow both rapid macrophage uptake of smaller particles (35) and extracellular depot with the larger ones (36). Indeed, the results showed rapid internalization of particles ≤6 μm within 3 h of incubation, with no further increase afterward. The negative surface charge (−12.5 mV) was sufficient to avoid cytotoxicity normally provoked by cationic particles (37), and yet it was mild enough not to produce repulsion and prevent uptake, as seen with PLGA microparticles of similar sizes but with

a stronger charge (−34.5 mV) that were only marginally internalized by J774 macrophages (38).

Our *in vitro* studies using a 48-h incubation with *L. amazonensis*-infected macrophages showed that entrapment in PLGA microparticles rendered CH8 more inert and less cytotoxic and yet maintained its high selectivity index at approximately 40 (Table 2). This may be either (i) due to a diminished phagocytic capacity of *Leishmania*-infected macrophages or (ii) an indication that more than 48 h is necessary for the particles to release additional drug in the intracellular milieu. Likewise, CH8 and CH8/PLGA did not activate macrophage microbicidal mechanisms (NO and ROS) (Fig. 5). This is consistent with the antioxidant (39) and anti-inflammatory (40) properties of chalcones and the lack of ROS activation by similar naked PLGA microparticles (41). Together, these results indicate that, *in vitro*, the CH8/PLGA antileishmanial activity was due to the direct action of the intracellularly released CH8 and not to macrophage activation.

In vivo, a lesion suppression model of infection with *L. amazonensis* was used to minimize the number of animals used and decrease their suffering. Although a triple CH8/PLGA intralesional injection was required to reduce the parasite loads by 97%, it is noteworthy that a single injection produced 91% reduction, whereas three injections with meglumine antimoniate, the gold standard for CL intralesional treatment, yielded only a 36% reduction. Despite the reported capacity of PLGA microparticles to activate macrophages for tumor necrosis factor alpha and interleukin-1β production *in vitro* (38), CH8/PLGA efficacy was not due to intrinsic PLGA activity since triple injection with naked microparticles had no effect on parasite growth (Fig. 6). None of the CH8 intralesional treatments affected body weight gain (Fig. 7) or induced detectable hepatic or nephrotoxicity, as measured by AST, ALT, and creatinine levels in the serum 3 days after the last of three doses (Fig. 8). Surprisingly, during the three-dose meglumine antimoniate treatment, a slight weight loss was verified, and significantly elevated ALT was observed at the end of treatment, a finding indicative of hepatotoxicity. This is in line with various studies showing that systemic absorption of meglumine antimoniate occurs after intralesional administration, leading to systemic adverse effects during and after intralesional meglumine antimoniate that include nausea, vomiting, dyspnea, dizziness, and anaphylactic shock (13), as well as altered hepatic function and white cell count changes (42).

Due to the low solubility of CH8 in aqueous physiological solutions, it was not possible to measure the *in vitro* release rate from PLGA particles in sink conditions. In higher concentrations, the drug precipitated together with the microparticles and could not be independently measured. However, in the more relevant *in vivo* situation, a single dose of CH8/PLGA was found to be superior in controlling parasite growth than three doses of free drug, supporting the notion that PLGA effectively promoted *in situ* sustained CH8 release for at least 81 days. Future process adjustments to increase drug loading will likely improve its single-dose efficacy.

In conclusion, this study proposes a novel single-dose therapy for uncomplicated CL consisting of a delivery system that is stable under relatively high temperatures, suitable for field use in tropical countries. In addition to the greater patient compliance, this approach may help reduce hospital and personnel costs compared to the current treatments.

MATERIALS AND METHODS

Drugs. Chalcone CH8 was synthesized by aldol condensation as previously described (25). Glucantime (meglumine antimoniate, powder) was obtained from Sanofi-Aventis.

Animals and ethics statement. BALB/c mice (female, 8 weeks old, 23 g) used in the experiments were maintained under controlled temperature, filtered air, filtered water, autoclaved bedding, and using commercial food at our facilities at Federal University of Rio de Janeiro.

The animal protocols for this study were approved by the Federal University of Rio de Janeiro institutional Animal Care and Use Committee (protocol CAUAP118). The research was conducted in compliance with the principles stated in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (43).

CH8/PLGA microparticles preparation. Microparticles were prepared by multiple emulsion-solvent evaporation method (24). Briefly, 500 mg of PLGA (Purasorb PDLG 5004, 50:50, 0.83 dl/g; Corbion) was added to 10 ml of dichloromethane (DCM; 45% [wt/vol]; Vetec) containing prediluted chalcone CH8 (50 mg). The organic solution was agitated at 7,220 rpm for 30 s in an ice bath using a mechanic homogenizer (Heidolph DIAX 900). This first solution was gradually dropped into an aqueous solution of 3% (wt/vol) of polyvinyl alcohol (M_w = 13,000 to 17,000; Sigma-Aldrich) in ultrapure water under 7,220 rpm for 1 min. The final emulsion was further stirred at 400 rpm for 4 h at 40°C under vacuum in a rotary evaporator (Logen) to totally evaporate the DCM solvent. The microparticles were pelleted by centrifugation at 9,000 rpm for 10 min at 4°C (Hitachi centrifuge), washed three times in ultrapure water, and then vacuum filtered. The formed particles (CH8/PLGA) were allowed to dry overnight in an oven (Panasonic) at 40°C. Blank particles (PLGA) were prepared in the same way without CH8.

CH8/PLGA microparticles characterization. The particle size distribution was determined by laser scattering (Zetasizer 3000; Malvern Instruments). CH8/PLGA and PLGA were dispersed in 0.01% (wt/vol) Tween 80 solution in water under ultrasound for 20 s. To quantify entrapped drug, a dry powder sample of CH8/PLGA was dissolved in acetonitrile (Tedia) under bath sonication. The CH8 concentration was determined by UV/high-pressure liquid chromatography using a system consisting of a Shimadzu pump (LC-20AT), a reversed-phase C_{18} column (5 μm by 25 cm by 4.6 mm; Rexchrom ODS-S5-100), and a UV detector (SPDM20A). The parameters used were a mobile phase containing acetonitrile-aqueous 0.01% phosphoric acid (Tedia; 80:20 [vol/vol]), flow at 1 ml/min, and detection at a wavelength of 337 nm.

For morphological imaging, the dry microparticles were coated by sputtering with gold (JFC-1300; JEOL) and analyzed by using a scanning electron microscope (JSM-5600LV; JEOL) at an acceleration of 20 kV under a nitrogen atmosphere.

Microparticle uptake by macrophages. Mouse peritoneal macrophages (5×10^5 cells in 50 μl of RPMI medium) were allowed to adhere for 1 h at 37°C onto glass coverslips placed in triplicates in 24-well culture plates. The cells were washed twice with PBS and treated or not in triplicates with 2.5×10^7 PLGA-CH8 for 3 h at 37°C. At the end of treatment, the coverslips were dipped in prewarmed PBS to remove noninternalized microparticles. Dried cells were fixed in methanol and stained with Giemsa, and the internalized microparticles in ≥50 macrophages/coverslip were counted under an optical microscope equipped with a micro ruler (Nikon Ellipse Ti).

Antiamastigote cytotoxicity. Mouse peritoneal macrophages (5×10^5 cells in 50 μl of RPMI medium) were allowed to adhere for 1 h at 37°C onto glass coverslips placed in triplicates in 24-well culture plates. After the nonadherent cells were washed away using prewarmed PBS, the remaining cells were infected with 5×10^6 promastigotes of *L. amazonensis* (strain WHOM/BR/75/JOSEFA) for 4 h at 34°C. After removal of noninternalized parasites, the cells were cultured for a further 24 h at 37°C in RPMI supplemented with 5% heat-inactivated fetal bovine serum (Cultilab) to allow for intracellular amastigote growth. The infected macrophages were then treated for 48 h in triplicates with either CH8 (0.7, 1.5, 3, 6.1, 12.5, 25, 50, or 100 μg/ml) or PLGA and CH8/PLGA (CH8 equivalents). Meglumine antimoniate (6.1, 12.5, 25, 50, 100, and 200 μg/ml) was added as a positive control. The negative controls were incubated solely with the culture medium. At the end of the incubation period, the coverslips were washed in prewarmed PBS, and the cells were stained with Giemsa. The numbers of amastigotes (in total, 200 macrophages/coverslip) were counted under ×400 magnification microscopy, and the results were expressed as the drug concentration that inhibited parasite growth by 50% (i.e., the IC_{50}).

Anti-macrophage cytotoxicity. The leakage of cytoplasmic lactate dehydrogenase (LDH) into the culture supernatant was used as a measure of macrophage lysis, considering that leishmanial parasites do not produce LDH (44). Macrophages were infected and treated for 48 h with CH8, PLGA, CH8/PLGA, or meglumine antimoniate as described above for antiamastigote cytotoxicity, using higher drug concentration ranges. At the end of the incubation time, the culture plates were gently centrifuged (500 rpm for 5 min), the supernatants were collected, and LDH was quantified using a colorimetric assay kit according to the manufacturer's instructions (Doles, Brazil). The results were determined by using the following equation: % specific release = [(test release − spontaneous release)/(maximal release − spontaneous release)] × 100, where the maximum release refers to supernatants from cells treated with 1% Triton X-100, and spontaneous release cells were cultured without additives.

NO and ROS production. For NO production, macrophages were left uninfected or were infected with *L. amazonensis*, as described below, for antiamastigote activity. After 24 h of infection, the macrophages were incubated for 48 h with CH8 (15 μg/ml), CH8/PLGA (15 μg/ml of CH8/135 μg/ml of PLGA) or PLGA (135 μg/ml), and/or 1 μg/ml of LPS (*Escherichia coli* [Sigma-Aldrich]). The stable $NaNO_2$ product was measured in the culture supernatants using the Griess colorimetric method (45) and a $NaNO_2$ standard curve. For ROS, infected and noninfected macrophages were treated with CH8 (15 μg/ml), CH8/PLGA (15 μg/ml of CH8 per 135 μg/ml of PLGA), PLGA (135 μg/ml), or 250 μg/ml of Zymosan (*Saccharomyces cerevisiae* [Sigma-Aldrich]) for 24 h at 37°C. In the last 20 min of culture, a 10 μM concentration of the oxidation-sensitive fluorescent dye H_2DCFDA (Invitrogen) was added in the dark, and fluorescence was quantitated at 485-nm excitation and 528-nm emission.

Mouse infection and treatment. Mice (n = 5 to 8) were infected in the ear pinnae with 2×10^6 promastigotes of *L. amazonensis*. On days 9, 16, and 23 of infection (three doses) or on day 9 only (one dose), the ears were injected subcutaneously with CH8 or CH8/PLGA at a dose of 1.17 mg/kg (27 μg of CH8 equivalents in 10 μl of PBS). Controls received the same dose of naked PLGA, reference drug meglumine antimoniate, or PBS.

Efficacy parameters. Lesion sizes and parasite burdens were evaluated as infection parameters. Ear thicknesses were periodically measured during infection with a caliper gauge, and lesion sizes were expressed as the difference between infected and noninfected ears. On day 90 of infection, the parasite

burdens were measured by limiting dilution assay (46). The infected ears were individually minced and homogenized with 1 ml of medium M199 using a glass grinder. After sedimentation of large debris for 5 min, the tissue homogenates were serially diluted by three times in quadruplicates in M199 plus 20% HIFCS in 96-well microplates, followed by incubation at 26°C for 14 days. The original numbers of parasites in each ear were calculated from the last dilution at which motile promastigotes were seen, relative to a single amastigote at the start of incubation.

Systemic toxicity. Mice were individually weighted during treatment with CH8, CH8/PLGA, PLGA, and meglumine antimoniate on days 9, 16, and 23 of infection to assess weight loss. Animals were killed on day 26 of infection, the sera were collected and assayed for AST, ALT, and creatinine as parameters of liver and kidney toxicity, using a colorimetric commercial kit (Doles, Brazil) adapted for microvolumes (47). The positive-control sera were taken from mice which received 200 μl of 1% carbon tetrachloride (CCl_4) in soybean oil by the intraperitoneal route 3 days before serum collection (48).

Statistical analysis. One-way analysis of variance and the Bonferroni posttest were performed to demonstrate statistical differences ($P < 0.05$). To compare the difference between two groups, a nonparametric *t* test was used. The IC_{50}s were calculated from a curve of sigmoidal dose response. All analyses were conducted using GraphPad Prism 6 software.

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