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Research Article

Drug Targeting to Macrophages With Solid Lipid Nanoparticles Harboring Paromomycin: an *In Vitro* Evaluation Against *L. major* and *L. tropica*

Maryam Heidari Kharaji,[1] Delaram Doroud,[2] Tahereh Taheri,[1] and Sima Rafati[1,3]

Abstract. Leishmaniasis is a worldwide disease that leads to high mortality and morbidity in human populations. Today, leishmaniasis is managed via drug therapy. The drugs that are already in clinical use are limited to a number of toxic chemical compounds and their parasite drug resistance is increasing. It is therefore essential, in order to circumvent the current difficulties, to design a new anti-leishmanial drug treatment strategy. Besides producing new, active anti-leishmanial entities, another promising strategy could be developing novel delivery systems and formulations of the existing pharmaceutical ingredients to improve drug efficacy. In the present study, paromomycin sulfate (PM), as one of the promising anti-leishmanial drugs, was formulated in solid lipid nanoparticles (SLN), and its *in vitro* efficacy was investigated against different strains of *Leishmania* using a MTT test, Parasite-Rescue-Transformation-Assay, SYTO Green staining, and fluorescent microscope imaging. The results show that PM-loaded SLN is significantly more effective than PM in inhibiting parasite propagation ($P < 0.05$) and that cytotoxicity of PM-SLN formulations is size dependent. According to our results, delivery of the drugs to the macrophages via nanoparticle utilization seems to be an accessible and practical approach.

KEY WORDS: cutaneous leishmaniasis; drug delivery system; paromomycin sulfate; solid lipid nanoparticle.

INTRODUCTION

Leishmaniasis is one of the parasitic diseases caused by genus *Leishmania* (1). *Leishmania* parasites are dimorphic and change during their life cycle between two forms of promastigotes and amastigotes. Clinically, *Leishmania* parasites cause several forms of diseases in humans, including cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MCL) (1). As a neglected disease, the resources invested in the detection, treatment, and monitoring of leishmaniasis are extremely limited. Unlike VL, CL mortality is rare and is usually reported as being related to the co-infections with other diseases including malaria, or more recently, HIV, or the side effects of treatment (2). Cutaneous leishmaniasis is the most frequent form of leishmaniasis caused by species such as *Leishmania major*, *Leishmania tropica*, and *Leishmania aethiopica* in the old world and *Leishmania mexicana*, *Leishmania amazonensis*, and *Leishmania guyanensis* in the new world (3). CL is confined to the skin and is a self-healing disease with treatment duration depending on the infecting species. CL can, however, evolve into a more severe disease, such as MCL (2). Each

year, 1.5 million new cases of CL are reported (4). To date, there is no effective treatment against CL and current therapies often fail and have many restrictions. Currently, pentavalent antimonials are the first-line treatment for CL. Antimonials are contraindicated in older individuals, during pregnancy, and in the patients suffering from renal and cardiac diseases. Pentavalent antimonials require daily injections and treatment duration is rather long. Resistance to pentavalent antimonials is increasing (5,6). Due to the antimonial resistance issue, amphotericin B (AMB) has been introduced as a first-line treatment. Its toxicity along with its cost are the most highlighted limitations of its utilization (7,8). One of the most promising drugs for anti-leishmanial chemotherapy is paromomycin sulfate (PM). Considering toxicity and cost, PM could be a good choice for treatment. In 1960, the anti-*Leishmania* activity of PM was first reported (9). PM is an aminoglycoside antibiotic, effective against CL and VL. PM disrupts the mitochondrial membrane potential, inhibits protein synthesis, and its site of action is 16S ribosomal subunit (10). In addition, PM changes the lipid metabolism, the fluidity of the membrane, and the mitochondrial operation (11). Due to its chemical structure, PM is a hydrophilic compound with a high molecular weight. Its physicochemical properties contribute to insufficient concentration of PM at the sites of infection after topical administration (12). The lack of an effective drug against CL has recently prioritized the creation of new, alternative therapeutic drugs. To this end, various pharmaceutical formulations have been assessed for the treatment of CL (9,13–15). The effects of the topical form of PM in

[1] Department of Immunotherapy and Leishmania Vaccine Research, Pasteur Institute of Iran, Tehran, Iran.

[2] Regulatory Department, Production and Research Complex, Pasteur Institute of Iran, Tehran, Iran.

[3] To whom correspondence should be addressed. (e-mail: s_rafati@yahoo.com)

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clinical trials for the treatment of CL have been promising, but due to the powerful barrier nature of the skin, this form of PM is not always effective (16–18). Lipid-based formulation of PM, i.e., liposomal PM, was prepared and its topical effects on the treatment of leishmaniasis evaluated (9,14). Recently, solid lipid nanoparticles (SLN) have received much attention due to advantages, such as low cost and safety, compared to other colloidal nanoparticles (19). Solid lipid nanoparticles were introduced in 1990 (20). SLN is composed of a solid lipid core and emulsifiers with a size less than 1000 nm. The lipid core matrix has an important role in controlling drug release (21,22). The use of physiological lipids enhances biocompatibility and safety of the formulations based on SLN (23). Both hydrophilic and lipophilic pharmaceutical ingredients can be formulated into SLN (24). The pharmaceutical applications of SLN have been evaluated in different studies (25–29). The preparation of PM-loaded SLNs (PM-SLN) has been described previously (30). In the present study, SLN is used as a PM delivery system (Fig. 1), and the *in vitro* efficacy of the resulting formulation was evaluated against *L. major* and *L. tropica*. It is worth mentioning that both *L. major* and *L. tropica* are the most common strains in specific countries such as Iran, Iraq, and Afghanistan (31).

MATERIAL AND METHODS

Reagents

PM sulfate (P5057) was purchased from Sigma-Aldrich (USA). SYTO Green, sodium dodecyl sulfate (SDS), M199, RPMI-1640, dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA). RPMI-1640 and fetal calf serum (FCS) were obtained from Gibco (Germany).

Preparation of PM-SLN

A modified high shear homogenization (HSH) microemulsion technique was used to prepare PM-SLN as previously described (30,32). Briefly, lipid (stearic acid) was heated to 85°C. Tween 80 (0.75%) was dispersed in the melted

Fig. 1. Schematic diagram of solid lipid nanoparticles (SLN)

lipid until the dispersion appeared clear. The aqueous phase containing paromomycin (90 mg) was heated to 85°C and added to the melted lipid under stirring. The lipid/drug ratio was adjusted to 4. Afterwards, the resulted emulsion was subjected to high shear homogenizer (IKAUltra-Turrax, T25, Germany) at 18,000 rpm for 20 min. The resulted nanoemulsion was immediately dispersed in cold double distilled water (2–5°C) followed by stirring to solidify the nanoparticles. The properties of each PM-SLN formulation such as size, polydispersity index, % paromomycin loading, zeta potential, and entrapment efficiency were determined. The particle diameter of each sample was measured in triplicate by dynamic light scattering (Malvern, Nano-ZS, UK) as previously described (30,32).

Parasite

The *L. major* strain (MRHO/IR/75/ER) and *L. tropica* strain (MOHM/IR/09/Khamesipour-Mashhad) were used in this experiment. Promastigotes were grown at 26°C in M199, pH 7.4, supplemented with 5% FCS for *L. major* and 10% FCS for *L. tropica*.

Cell Culture

Human monocyte cell line THP1 (ATCC® TIB-202™) were grown at 37°C in the presence of 5% CO_2 in RPMI-1640 supplemented with 10% fetal calf serum. Cells were counted and then treated with 5 µg/ml phorbol 12-myristate 13-acetate (PMA) to form adherent cells. PMA-treated THP1 was used for further experiments.

Cytotoxicity Evaluations

The cytotoxicity was investigated by cultivating PMA-treated THP1 (5×10^6 cells) in the presence of different concentrations (25-50-100-200-400-800-1600-3200-6400 µg/mL) of PM-SLN formulation (PM-SLN 15% (120, 980, 1500 nm), PM-SLN 12.5% (240 nm)), PM, and blank SLN (as controls) in 96-well culture plates (Orange Scientific, E.U.) for 48 h at 37°C. Cell viability was assessed by colorimetric assays using MTT reagent (33,34). Absorbance rates were measured at optical density (OD) of 570 nm. Results were expressed as the mean percentage reduction of THP1 cells compared with non-treated control samples and shown by 50% inhibitory concentration THP-1 cells (CC_{50}).

Promastigote Drug Susceptibility Assay

The efficacy of the different PM-SLN formulation (PM-SLN 15% (120 nm), PM-SLN 12.5% (240 nm)) concentrations (25-50-100-200-400-800-1600-3200 µg/mL) on the stationary phase of *L. major* and *L. tropica* promastigote form (2×10^7 parasites) was assessed in a 48-h time period, at 26°C. Drug susceptibility was examined by MTT assay (35,36) and 50% concentration of the drug causing 50% inhibition in promastigotes growth (µg/ml) was calculated (IC_{50}).
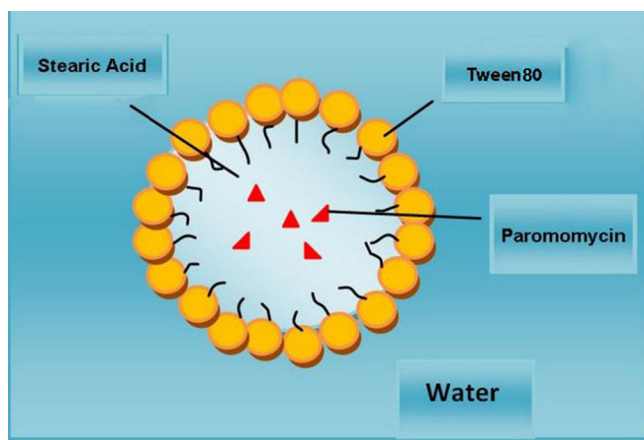
Infection of the THP-1 With *L. major* and *L. tropica* Promastigotes and EC$_{50}$ Determination

PMA-treated THP1 (5×10^5 cells) were plated in 96-well culture plate and chamber slide (Thermo Scientific, USA) in RPMI-1640 medium supplemented with 10% FCS. After 24 h of incubation at 37°C in 5% CO_2, stationary phase promastigotes of *L. major* and *L. tropica* were added to the wells (1:20 THP1 cell to parasite) and the cultures were incubated at 37°C in 5% CO_2 for 24 h allowing the parasites to infect the cells. Afterwards, free parasites were removed by washing with serum-free RPMI-1640 medium and the infected THP-1 were treated with different concentrations of drugs as mentioned previously for 48 h at 37°C in 5% CO_2.

Parasite-Rescue-Transformation-Assay

The infected PMA-treated THP1 in 96-well culture plate from the previous step was washed with serum-free RPMI-1640 medium. Then, RPMI-1640 with 0.05% SDS was added to each well for cell lysis. The plate was shaken for 30 s and complete Schneider culture medium (supplemented with 10% FBS) was added to each well. The plates were incubated at 26°C for 72 h in order to transform the rescued amastigotes to promastigote form. After 72 h incubation at 26°C, all the rescued live amastigotes were transformed into promastigote forms. The effect of the formulations was evaluated by MTT and EC$_{50}$ (µg/ml) (50% effective concentration) was determined [37].

SYTO Green Staining and Fluorescent Microscope Imaging

After 48 h, each well of chamber slide containing the infected PMA-treated THP1 with prepared formulations was washed with serum-free RPMI-1640 medium. Afterwards, methanol was used to fix the cells. After drying, the slide was stained with SYTO Green staining solution (100 nM) in dark conditions at room temperature. After 15 min, the slide was washed with water and allowed to dry. The digital images of THP1 were taken with an Epi-fluorescence microscope (Nikon, E200, Japan) [37]. THP1 cells infection rate was calculated by the following equation [38]:

$$\% \text{ of cell infection} = \frac{\text{Number of infected cell}}{\text{Total of THP1 cell}} \times 100$$

Statistical Analysis

Statistical analysis and CC$_{50}$, ED$_{50}$, and IC$_{50}$ were calculated using GraphPad Prism (version 5.0; GraphPad Software, Inc 2007, San Diego, CA, USA). Student's *t* test was used for the comparison between groups. The *P* value <0.05 was considered significant.

RESULTS

PM-SLN Formulation

In our previous investigation, different criteria including the type of methods (microemulsion or solvent diffusion) and kind of lipid (cetyl palmitate or stearic acid) were comparatively studied. Their effects on average diameter, size distribution, and entrapment efficiency of the lipid nanoparticles for maximized entrapment efficiency, size particle reduction, and distribution were determined [32]. Three quantitative factors—paromomycin content, weight fraction of Tween 80, and drug-to-lipid ratio—were also investigated at two levels for SLN formulation in a fractional factorial design [32]. Briefly, the obtained results indicated that microemulsion was the most efficient method and stearic acid was the preferred lipid for SLN formulation [32]. The size, polydispersity index, the percent of paromomycin loading, and the zeta potential of the particles are shown in Table I. The average size of the particle was reported to be 299.08 nm. The drug released from the PM-SLN nanoparticulate formulation gradually and lasted 24 h in the aqueous media. The average entrapment efficiency of the prepared formulations was reported to be 42–46% [30,32].

Cytotoxicity Evaluation

The results of a MTT test indicated that blank SLN and PM are non-toxic for the cells, even at high concentrations (25–6400 µg/ml), as shown in Fig. 2a, b. The obtained CC$_{50}$ for both PM and blank SLN was 6400 µg/ml. The PM-SLN 15% with the size of 980 nm and the PM-SLN 15% with the size of 1500 nm were cytotoxic and therefore were excluded from further efficacy evaluation (Fig. 2c, d). CC$_{50}$ of the PM-SLN 15% with the size of 980 and 1500 nm was 80 and 70 µg/ml, respectively. PM-SLN 12.5% (240 nm) and PM-SLN 15% (120 nm) were only toxic for cells at higher concentrations (6000 µg/ml) as indicated in Fig. 2e, f. For PM-SLN 15% (120 nm) and PM-SLN 12.5% (240 nm), the CC$_{50}$ was 4000 and 4500 µg/ml, respectively.

Effects of Formulation on Promastigotes and IC$_{50}$ Evaluation

The IC$_{50}$ (µg/ml) of formulations for *L. major* and *L. tropica* are shown in Fig. 3, panels 1 and 2, respectively. Blank SLN has no effect on *L. major* and *L. tropica* promastigotes (Fig. 3, panels 1, 2: a and e). The results for *L. major* promastigote revealed that PM-SLN is more effective than PM (*P*<0.05). IC$_{50}$ of PM was 3100 µg/ml (Fig. 3, panel 1, b) and of both formulations of PM-SLN (15 and 12.5%) was 1600 µg/ml (Fig. 3, panel 1, c and d). The results for *L. tropica* promastigote were almost identical to those of

Table I. Properties of Various PM-SLN Formulations Utilized in This Study. The Size, Polydispersity Index, % Paromomycin Loading, and Zeta Potential Are Shown for Each Formulation

Formulations	Size (nm)	Zeta potential	Polydispersity index
PM-SLN 15%	120	532.43±164.40	0.67±0.05
PM-SLN 15%	900	572.43±120.40	0.57±0.05
PM-SLN 15%	1500	506.43±214.40	0.25±0.05
PM-SLN 12.5%	240	507.97±250.47	0.57±0.05

The values are means±standard deviations (*n*=3). The mean entrapment efficiency was found to be (42–46%) in all formulations
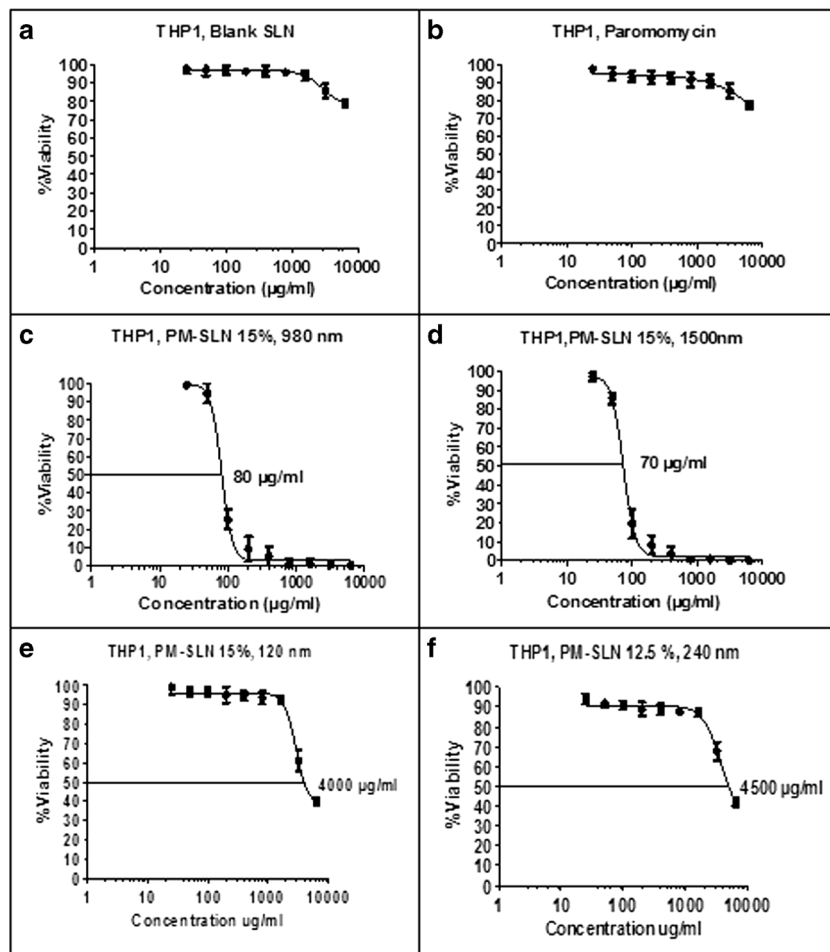
Fig. 2. Cytotoxicity evaluation using THP-1 cell line. Blank SLN and PM (**a**, **b**) are non-toxic for the cells (25–6400 µg/ml). PM-SLN 15% (980 nm) and PM-SLN 15% (1500 nm) (**c**, **d**) are cytotoxic. PM-SLN 15% (120 nm) and PM-SLN 12.5% (240 nm) (**e**, **f**) are only toxic for cells at higher concentrations (6400 µg/ml)

L. major promastigote (Fig. 3, panel 2). For *L. tropica* promastigote, IC_{50} of PM was 3200 µg/ml (Fig. 3, panel 2, f) and IC_{50} of PM-SLN 15 and 12.5% was 1600 and 1700 µg/ml, respectively (Fig. 3, panel 2, g and h). By comparing the formulation IC_{50}, the effectiveness of the PM-SLN formulations is evident ($P<0.05$) (Fig. 3). The inhibitory effect of PM and PM-SLN formulations is significantly different ($P<0.05$).

Parasite-Rescue-Transformation-Assay and EC_{50} Determination

The EC_{50} (µg/ml) of formulation for *L. major* and *L. tropica* amastigote forms are shown in the Fig. 4, panels 1 and 2, respectively. It shows that for *L. major* amastigote, the EC_{50} of PM was 1600 µg/ml (Fig. 4, panel 1, b) and for PM-SLN 15 and 12.5% was 390 and 800 µg/ml, respectively (Fig. 4, panel 1, c and d). For *L. tropica* amastigote, the EC_{50} of PM was 1600 µg/ml (Fig. 4, panel 2, f), and for PM-SLN 15 and 12.5% was 400 and 750 µg/ml, respectively (Fig. 4, panel 2, g and h). There were significant differences in the inhibitory effect of the PM and PM-SLN formulations against *L. major* and *L. tropica* amastigotes ($P<0.05$) (Fig. 4). As the cytotoxicity results showed, the CC_{50} of PM-SLN 12.5% (240 nm) and

PM-SLN 15% (120 nm) was 4500 and 4000 µg/ml (Fig. 2e, f). Considering the EC_{50} of the formulations, this means 390 µg/ml of PM-SLN 15% (120 nm) and 800 µg/ml of PM-SLN 12.5% (Fig. 4, panel 1, c and d) are toxic to cells, but also inhibit the propagation of *L. major* amastigotes. Correspondingly, the results for *L. tropica* showed 400 and 750 µg/ml of PM loaded in SLN (15 and 12.5%) (Fig. 4, panel 2, g and h) can also inhibit *L. tropica* amastigotes while being non-toxic to the cells.

SYTO Green Staining and Fluorescent Microscope Imaging

The digital images of infected THP1 cells with *L. major* and *L. tropica* were taken with a fluorescent microscope (Fig. 5a, b). As mentioned previously, the infection percent of the THP1 cells with parasite (*L. major* and *L. tropica*) after treatment with PM-SLN (15%), PM-SLN (12.5%), and PM were determined with the equation that was previously mentioned and the results are summarized in the Table II. The percentage of infections for the *L. major*-infected THP1 cells after treatment with 400 µg/ml PM-SLN (15%), PM-SLN (12.5%), and PM was 48.5, 62.5, and 68.0, respectively. With 800 µg/ml of each drug, the percentage was 37.0, 53.0, and 61.5, and after treatment with 1600 µg/ml, it
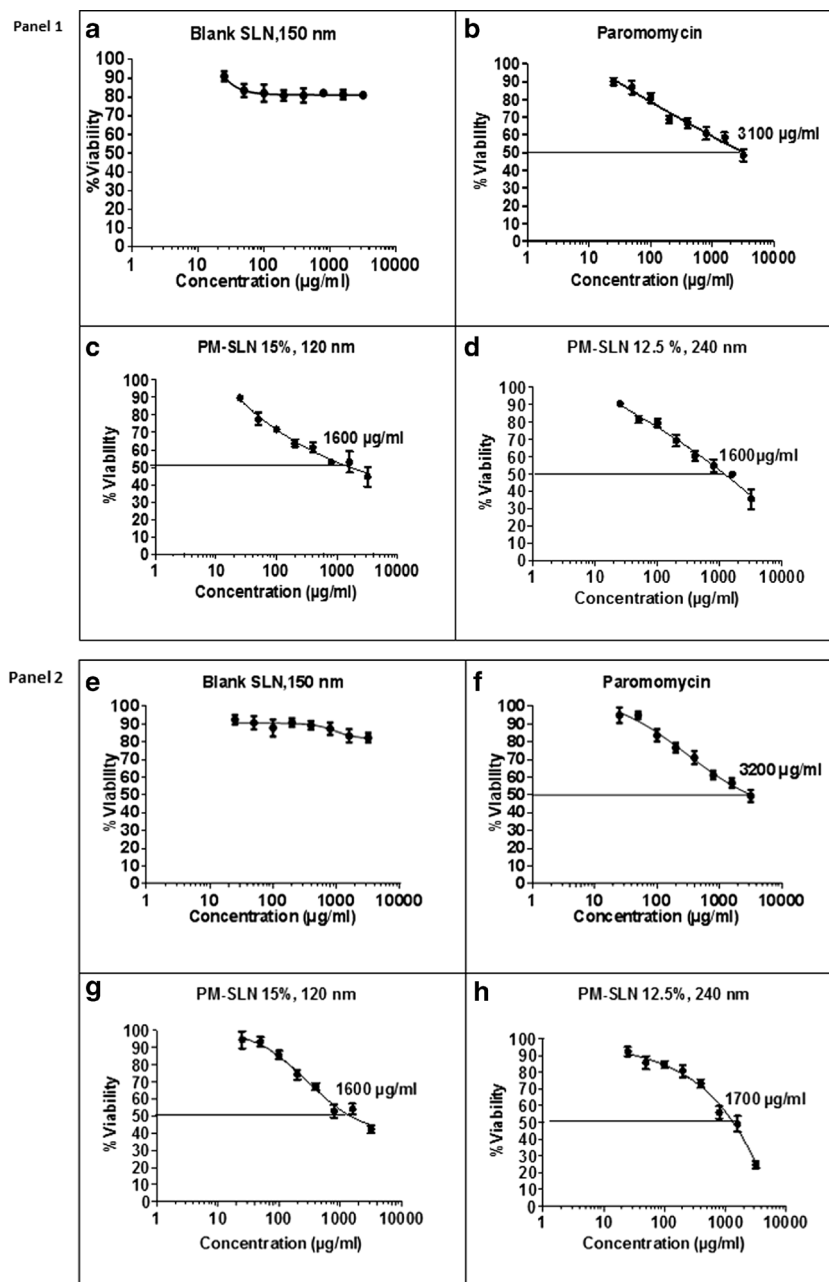
Fig. 3. The inhibitory effect of drugs on *L. major* (panel 1) and *L. tropica* (panel 2) promastigotes. The effect of drugs on *L. tropica* and *L. major* promastigotes is the same. Blank SLN has no effect on the *L. major* and *L. tropica* promastigotes (panel 1 (*a*), panel 2 (*e*)). For *L.major* promastigotes, the IC_{50} of PM-SLN formulations (panel 1 (*c* and *d*)) are lower than that of PM (panel 1 (*b*)) and this means the PM-SLN formulations are more effective. Similarly, for *L. tropica* promastigotes, the evaluation of IC_{50} shows that the PM-SLN formulations (panel 2 (*g* and *h*)) are more effective than PM (panel 2 (*f*)) for the inhibition of the parasite

was 23.5, 33.0, and 48.5, respectively (Table II). The infection percentage for the *L. tropica*-infected THP1 cells after treatment with 400 µg/ml PM-SLN (15%) was 48.0, with PM-SLN (12.5%) was 67.0, and with PM was 69.0. Using 800 µg/ml, it was 29.5, 47.0, and 61.5, respectively. The percentage of infections after treatment with 1600 µg/ml PM-SLN (15%) was 13.0, with PM-SLN (12.5%) was 22.5, and with PM was 48.5 (Table II). The percentage of infections in infected cells treated with the

PM-SLN formulation is significantly lower than infected cells treated with PM ($P<0.05$). As shown in Table III, EC_{50}/CC_{50} of PM-SLN 15% (120 nm) is lower than PM-SLN 12.5% (240 nm), which indicates that the efficacy of PM-SLN 15% (120 nm) is more than PM-SLN 12.5% (240 nm). As shown in Table IV, considering the size of the nanoparticle, the PM-SLN 15% (120 nm) is more effective and less toxic compared to PM-SLN 12.5% (240 nm).
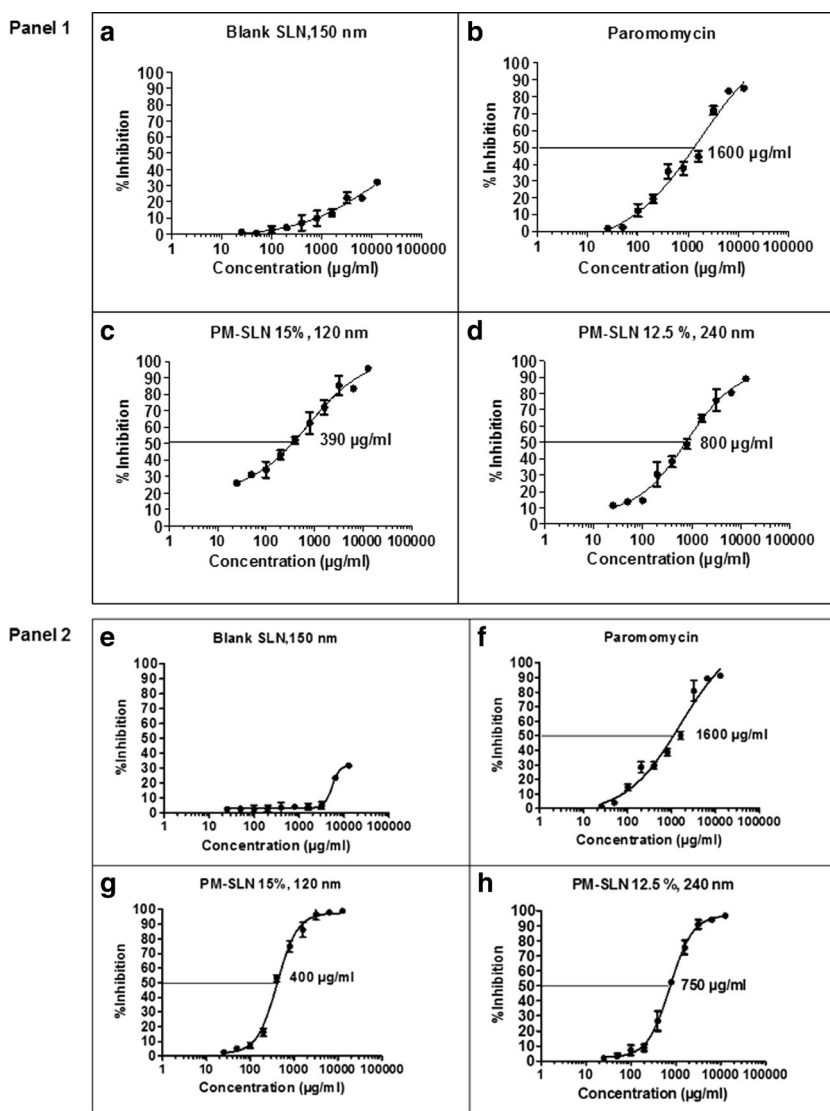
Fig. 4. Parasite-Rescue-Transformation-Assay for the amastigote form of *L. major* and *L. tropica*. The EC_{50} (μg/ml) of the drug was computed and shown in the figure. EC_{50} of the PM and PM loaded in SLN (15, 12.5%) for the *L. major* amastigote were 1600, 390, and 800 μg/ml (panel 1 (*c* and *d*)), respectively, and for the *L. tropica* amastigote were 1600 μg/ml (panel 2 (*f*)), 750 μg/ml, and 400 μg/ml (panel 2 (*g* and *h*)). Considering the drug's EC_{50}, PM-SLN formulations, as in the previous tests, were determined to have a greater inhibitory effect and a significant inhibition of amastigote propagation was observed when the SLN-PM formulation was used compared to PM

DISCUSSION

One of the most commonly used drugs for the treatment of cutaneous leishmaniasis is PM (39). Commercially available forms of PM are PM capsules (oral form), PM cream (topical form), and PM for injection (intramuscularly for VL) (39–41). PM formulations have some disadvantages, including a short half-life in plasma and rapid renal excretion (being a hydrophilic molecule with high molecular weight). All these factors decrease the concentration of PM at the sites of action, e.g., the reduction of PM oral absorption, thus making the oral administration of PM inefficient (8,9,12,14). According to the data obtained from clinical trials, the effect of the topical form of PM for the treatment of cutaneous leishmaniasis has been promising, but due to the

powerful barrier nature of the skin, it is not effective (16–18). One way to increase PM efficacy is to utilize a delivery system. In recent years, colloidal carriers, such as liposomes, emulsions, and polymeric nanoparticles, have been very promising in improving the bioavailability of the active pharmaceutical ingredients (9,42,43). In one recent study, PM-loaded liposomes were developed and investigated as a drug delivery system (8,9,12,14). The effect of PM formulated with stearylamine-bearing liposome on visceral leishmaniasis was investigated by Banerjee et al. (8). Jaafari et al. (2009) evaluated the effect of PM sulfate formulated with liposomes in the treatment of *L. major*-infected mice (9). It was reported that PM formulated with liposomes can help in controlling topical drug delivery (14). For the past decade, the use of SLN as an alternative carrier for colloidal drug delivery
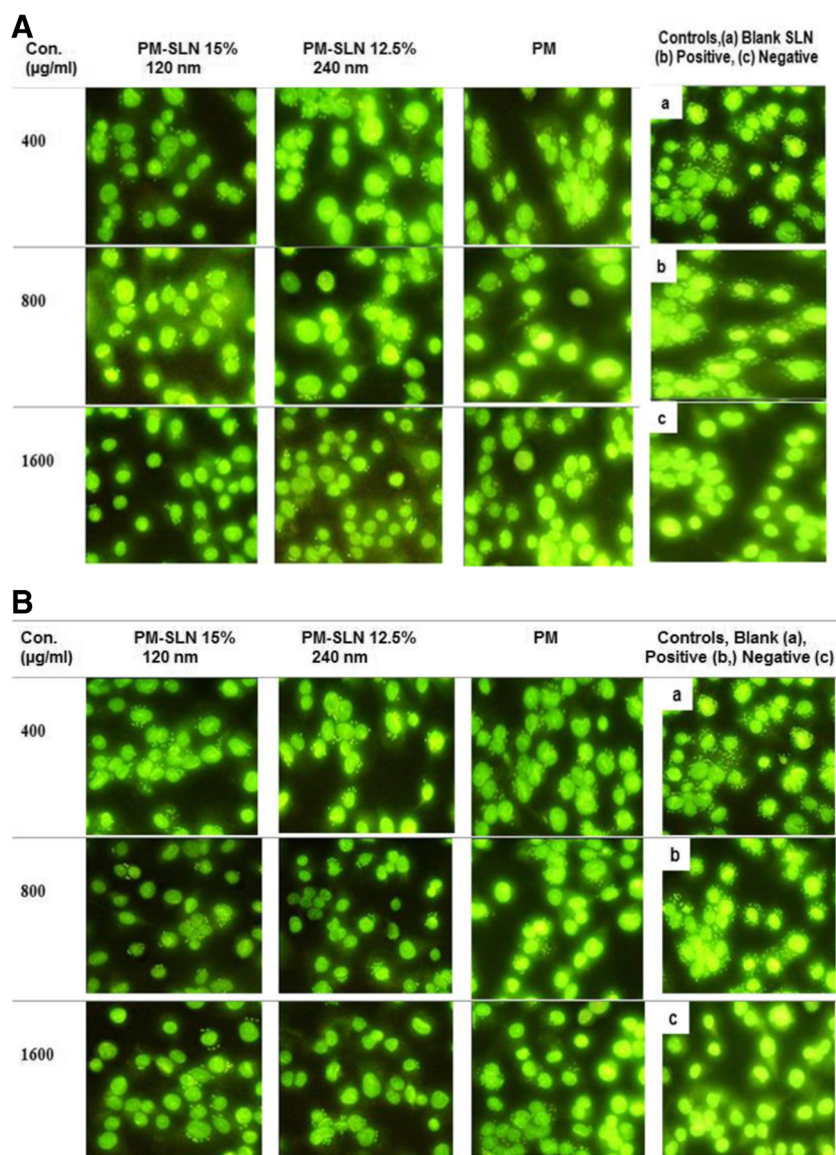
Fig. 5. SYTO Green staining and fluorescent microscope imaging of *L. major* (**A**) and *L. tropica* (**B**) infected cells after treatment with different concentrations of the drugs

Table II. %Infection of *L. major*-Infected THP1 Cells in Panel a and *L. tropica*-Infected THP1 Cells in Panel b of Fig. 5 After Drug Treatment. For Both Parasites, the Percentage of Infection in Infected Cells Treated With the PM-SLN Formulation Is Significantly Lower Than Infected Cells Treated With PM (for *L. major*: a(1), b(1)=*P<0.04; a(1), c(1)=*P<0.02; a(2), b(2)=*P<0.02; a(2), c(2)=**P<0.001; a(3), b(3)=**P<0.002; a(3), c(3)=**P<0.002; b(3), c(3)=*P<0.05). (For *L. tropica*: a(1), b(1)=*P<0.01; a(1), c(1)=*P<0.02; a(2), b(2)=*P<0.01; a(2), c(2)=**P<0.002; a(3), b(3)=**P<0.005; a(3), c(3)=**P<0.001; b(3), c(3)=*P<0.03)

Drugs	PM-SLN 15%[a]			PM-SLN 12.5%[b]			PM[c]			Blank SLN	No treatment
Concentration (µg/ml)	400 (1)	800 (2)	1600 (3)	400 (1)	800 (2)	1600 (3)	400 (1)	800 (2)	1600 (3)	400	–
% Infection for *L. major*	48.5±1.5	37.0±2.0	23.5±1.5	62.5±3.5	53.0±2.8	33.0±4.2	68.0±4.2	61.5±2.1	48.5±4.9	90.5±1.0	98.5±1.5
% Infection for *L. tropica*	48.0±1.0	29.5±0.5	13.0±1.0	67.0±3.5	47.0±2.8	22.5±3.5	69.0±4.2	61.5±2.1	48.5±4.9	91.0±1.5	98.0±1.5

Different numbers in parentheses [(1), (2), (3)] represent different concentration: (1) 400, (2) 800, (3) 1600
[a] PM-SLN 15%
[b] PM-SLN 12.5%
[c] PM

Table III. Ratio Comparison of EC_{50} to CC_{50} for PM-SLN 15% (120 nm) and PM-SLN 12.5% (240 nm). The EC_{50}/CC_{50} of PM-SLN 15% (120 nm) Is Lower Than PM-SLN 12.5% (240 nm)

Formulations	CC_{50} (μg/ml)	EC_{50} (μg/ml)		EC_{50}/CC_{50}	
		L. major			
L. tropica	*L. major*				
L. tropica					
PM-SLN 15% (120 nm)	4000	390	400	0.097	0.100
PM-SLN 12.5% (240 nm)	4500	800	750	0.177	0.166

systems like liposomes, fat emulsions, and polymeric nanoparticles has been considered due to their advantages over other colloidal carriers (21,23,44). Previously, Ghadiri et al. (2012) reported that loading PM into SLN formulation can control the release profile of the drug (30). In the present study, we assessed the efficacy and safety of the PM-SLN formulations in *in vitro* conditions for the first time. PM-SLN formulations with 15% PM and 12.5% PM were prepared and evaluated against *L. major* and *L. tropica*. (30). Jaafari et al. (2009) used liposomes containing 10 or 15% PM and evaluated the topical effect of the formulations on CL caused by *L. major*. El-On et al. (1984) revealed that 15% PM with 12% methyl benzethonium chloride (MBCl) ointment is effective in the treatment of CL in BALB/c mice. Effectiveness of topical formulations of 15% PM and 10% urea was reported (9,45,46). Here, we evaluated the *in vitro* anti-leishmanial activity of PM-SLN formulation against two *Leishmania* species. The cytotoxicity results on the THP-1 cell line using the MTT test showed that PM alone and free solid lipid nanoparticles are not toxic for mammalian cells (20). It was reported that PM is not cytotoxic on HCT-8 cell (33). Schöler et al. (2002) investigated the cytotoxicity of SLN using the MTT test on murine peritoneal macrophages (34). Müller et al. (1997) found that SLN showed lower toxicity compared to polyalkylcyanoacrylate and PLA/GA (47). Another study evaluated the cytotoxicity of SLN using human granulocytes and reported that SLN was the least cytotoxic formulation (48). In the Weyenberg et al. (2007) study, they assessed the effect of SLN on mouse 3T3 fibroblasts, J774 macrophages, and HaCaT keratinocytes and evaluated cell viability utilizing the MTT test (49). Our results further suggest that the toxicity of PM-SLN formulations depends on the size and dose.

PM-SLN formulations with a smaller size and lower dose showed slight symptoms of toxicity, but PM-SLN formulations with a larger size and higher dose were toxic for the THP1 cell line. As indicated in our results (Table IV), the efficacy of the PM-SLN formulations is size dependent. PM-SLN formulations with a size of 120 nm showed better efficacy than PM-SLN formulations with a size of 240 nm. PM-SLN formulations with a size of 980 and 1500 nm were toxic for the THP1 cell line in low concentrations (CC_{50}, 80 and 70 μg/ml); therefore, their effect on the parasite was not pursued. Indeed, due to their large size, PM-SLN formulations 15% (980 and 1500 nm) compared to PM-SLN formulations with 120 nm, with the same percentage of PM (15%), were more toxic. Also, toxicity of PM-SLN formulations 15% (980 and 1500 nm) is more than PM-SLN formulations with a lower percentage of PM (12.5%) and a smaller size (240 nm). These results suggest that reducing the percentage of PM in the formulation has no effect on PM-SLN formulation toxicity. Efficacy of PM-SLN 15% (120 nm) and PM-SLN 12.5% (240 nm) for two types of parasite (*L. major* and *L. tropica*) were compared. In summary, efficacy of PM-SLN 15% (120 nm) is higher than PM-SLN 12.5% (240 nm). Nanoparticle size effect on cytotoxicity and efficacy has already been evaluated (50–54). Lin et al. (2006) showed that larger SiO_2 nanoparticles have a greater cytotoxicity than smaller Min-U-Sil quartz particles on human lung cancer cells (55). In another study by Carlson et al. (2008), it was reported that cytotoxicity of silver nanoparticles is size dependent (56). In the Lee et al. (2007) study, paclitaxel-loaded TM-SLNs showed dose-dependent cytotoxicity against both OVCAR-3 and MCF-7 cells (57). It was reported that reaction of cells to nanoparticles depends on the size of the nanoparticles (58). The inhibitory effect of formulations on promastigotes was assessed using the MTT test and IC_{50} was analyzed. (35). According to our results, PM loaded in SLNs as delivery system can be more effective than PM for parasite inhibition. The results for *L. major* and *L.tropica* promastigote revealed that PM-SLN is more effective than PM ($P<0.05$). For *L. major* promastigotes, the IC_{50} of PM was 3100 μg/ml and of both formulations of PM-SLN (15 and 12.5%) was 1600 μg/ml. The results for *L. tropica* promastigote were practically the same and IC_{50} of PM was 3200 μg/ml and IC_{50} of PM-SLN 15 and 12.5% was 1600 and 1700 μg/ml, respectively. As the results show, IC_{50} of PM-SLN formulations is lower than PM; this suggests that PM in formulation can inhibit the parasites propagation at lower concentrations. Khosravi et al. (2011) also used the MTT test for evaluation of the effect of various concentrations of nanosilver particle solutions on *L. tropica* promastigotes and determined a 50% inhibitory concentration (36). We used parasite rescue and transformation assay to evaluate the effect of PM-SLN formulations on the amastigote form of

Table IV. Effect of size on PM-SLN formulations efficacy. EC_{50} of PM-SLN 15% (120nm) for *L. major* and *L. tropica* is almost identical. Results of EC_{50} for PM-SLN 12.5% (240nm) are the same but the ratio of EC_{50} to CC_{50} for PM-SLN 15% (120nm) is lower than PM-SLN 12.5% (240nm) and it means size of PM-SLN formulations could affect the drug efficacy

Drugs: %PM and size of SLNs (nm)		Toxicity on THP1 cell line (CC_{50}, μg/ml)	Effect on *L. major* amastigote (EC_{50}, μg/ml)	Effect on *L. tropica* amastigote (EC_{50}, μg/ml)	EC_{50}/CC_{50} for *L. major*	EC_{50}/CC_{50} for *L. tropica*
15%	120	4000	390	400	0.097	0.100
	980	80	–	–	–	–
	1500	70	–	–	–	–
12.5%	240	4500	800	750	0.177	0.166

the *L. major* and *L. tropica* and the results showed that PM-loaded SLN is more effective than PM. The results show that for *L. major* amastigote, the EC_{50} of PM was 1600 μg/ml and for PM-SLN 15 and 12.5% was 390 and 800 μg/ml, respectively. For *L. tropica* amastigote, the EC_{50} of PM was 1600 μg/ml and for PM-SLN 15 and 12.5% was 400 and 750 μg/ml, respectively. According to the presented data, the EC_{50} of PM-SLN (for both parasites) is reduced to 1:4 for the 15% and 1:2 for the 12.5% formulations when compare to the non-formulated PM. According to the EC_{50} results, SLN can increase the effectiveness of the PM. Direct counting methods were applied for the assessment of drug efficacy against the intracellular amastigotes following the staining with Giemsa under microscopic investigation (59–63). Counting cells takes a lot of time and counting the living parasites and defining the parasite viability through this assay is very difficult and, as a result, it may not be an accurate estimation of the EC_{50}. Parasite rescue and transformation assay can be a good alternative assay to previous methods to evaluate the effect of drugs on the amastigote form of the parasite (37). In this study, SYTO Green staining and fluorescent microscope imaging were used for investigating the level of infection of treated cells. The SYTO Green staining technique stained the intracellular form of the parasite (37). It should be noted that there was a contrast between the color of the amastigote nucleus and the cytoplasm of the cell and the amastigote was easily diagnosed. SYTO Green staining and fluorescent microscope imaging results confirmed and indicated that infected cells treated with PM-SLN formulation had the lowest level of infection and this formulation had the better inhibitory effect. According to the biocompatibility of the SLN as a delivery system and the data from *in vitro* analysis of IC_{50} and EC_{50} PM-SLN formulation, it can be proposed that the presented formulation might be efficiently utilized in the treatment of the cutaneous leishmaniasis, if the *in vivo* and other ongoing clinical studies results satisfactory.

CONCLUSION

Leishmaniasis is a serious health problem in many countries. Despite of all efforts, there is no definite treatment against leishmaniasis. It seems necessary to develop new treatment strategies for leishmaniasis. The solid lipid nanoparticles utilized in the formulations of present study have effective roll as drug delivery systems for the treatment of leishmaniasis. SLN as delivery system can enhance the capability of PM to penetrate into the macrophage. Our results show that the PM-SLN formulations were able to increase the efficacy of PM. Also, the efficacy of the PM-SLN formulations is size dependent. In addition, blank SLN is safe without any cytotoxicity. Therefore, PM-SLN formulation opens a new hope as antileishmanial compound against *L. major* and *L. tropica* infection. These promising findings could be completed by further *in vivo* investigation which is in progress. However, according to our results, delivery of the drugs to the macrophages via nanoparticle utilization seems to be accessible and practical, but requires further research in order to develop new cost-effective strategies for the treatment of the leishmaniasis.

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