Original Communication

The photodynamic effects of protoporphyrin IX, protoporphyrin IX dimethyl ester or metallated protoporphyrin IX on *Leishmania tarentolae* in culture

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ABSTRACT

Leishmania are parasites that cause leishmaniasis for which there are currently few treatments. Protoporphyrin IX (PIX), PIX-ester, PIX-Zn, and PIX-Sn were used at various concentrations to test their ability to inhibit growth of axenic *Leishmania tarentolae*. The compounds (in dimethyl sulfoxide, DMSO) were added at different concentrations to the cell cultures and control cells were exposed to DMSO only. Cells were exposed to 2 hours of direct fluorescent light (350-750 nm, 750 Lux) or kept in the dark. The cells were evaluated by microscopy, the MTT viability assay, and confocal microscopy. After 48 hours in culture, some compounds were observed to have negative effects on *Leishmania* promastigote growth when compared to DMSO controls depending on the concentration and light exposure. The amastigote form was more negatively affected by PIX-Sn relative to PIX. A MitoSOX™ probe was used to identify the presence of superoxide, suggesting a mechanism for cell inhibition. This research suggests that PIX especially with metallation shows potential to treat leishmaniasis.

KEYWORDS: *Leishmania*, porphyrins, photodynamic effect, metal ions

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INTRODUCTION

Leishmania are trypanosomatid digenetic parasitic protozoans capable of infecting humans and other animals. The sand fly is the major vector and is critical in spreading these parasites in more than 88 countries worldwide putting millions of people at risk [1]. The World Health Organization (WHO) estimates that annually 1.5-2 million new cases develop [2]. The major forms of the diseases are cutaneous, mucocutaneous, and visceral. While most cases of leishmaniasis are cutaneous and not life threatening, they do cause much hardship both economically and socially. The WHO considers leishmaniasis to be a poverty-related disease which is endemic in 98 countries located in tropical and subtropical regions. In addition, the parasites compromise the immune system, since the major infected cells are macrophages; therefore resistance to HIV and other serious problems is reduced [3]. Major treatments involve antimony drugs such as meglumine antimoniate and sodium stibogluconate as well as amphotericin B, ketoconazole, and miltefosine [3]. However, these drugs can be expensive and may require trained medical assistance during treatment. In addition, drug resistance has been reported as well as unpleasant side effects such as joint pain, fatigue, gastrointestinal upset, and anemia [1]. Thus new treatments are needed. Since developing countries suffer the most from leishmaniasis, these new

treatments should be low cost, with easily transported drugs that require no refrigeration and little medical expertise to dispense and monitor. One strategy for new drugs involves the fact that *Leishmania* are unable to make their own porphyrin and thereby require exogenous sources [4, 5]. Therefore, porphyrin analog testing has been previously considered [6, 7]. In addition, photodynamic therapy can be used because porphyrins and analogs can absorb visible light [7]. As porphyrin derivatives have the ability to localize in cancer cells and induce oxidative stress [8], the same mechanism could be used to treat leishmaniasis with potentially less harmful effects for the host compared to current treatments.

Photodynamic therapy, involving treatment of patients with 5-aminolevulinic acid (ALA) before light exposure, has been successful in treating patients with cutaneous leishmaniasis during clinical tests [8, 9]. The effects of such PDT are generally credited to endogenously produced porphyrins, particularly protoporphyrin IX (PIX). However, *Leishmania* are reported to lack key enzymes required to produce PIX [10], thus it is likely that the beneficial effects of PIX would be the result of production or accumulation of PIX in host cells followed by the intake and accumulation of PIX into the *Leishmania* cells which have invaded the host cells. Zinc (II) protoporphyrin-IX (PIX-Zn) and tin (IV) protoporphyrin-IX (PIX-Sn) are of particular interest because any phototoxic effects of these compounds could have additional potential benefits since PIX-Zn and PIX-Sn are reported to be heme oxygenase (HO) inhibitors [11]. This enzyme is the major regulatory enzyme involved in the heme degradation pathway. Pham *et al.* revealed that infections of macrophages by *Leishmania* amastigotes induce an increase in HO-1 in macrophages [11]. This increase in HO-1 prevented assembly of NADPH oxidase thus decreasing superoxide production. Treatment of amastigote infected macrophages with a HO inhibiting metalloporphyrin, tin (IV) mesoporphyrin, increased superoxide levels significantly [11].

We wanted to extend these studies by testing the *in vitro* effects of protoporphyrin IX, PIX-dimethyl ester, and metallated PIX with axenic promastigotes and amastigotes with and without light treatment. We also demonstrate the phototoxic effects of exogenously supplied PIX, PIX-ester and metallated

PIX on *Leishmania tarentolae* cells in culture and the involvement of intracellular superoxide production correlated to the presence of PIX.

MATERIALS AND METHODS

Experiments using the promastigote form involved either of two strains of *Leishmania tarentolae* cell cultures (30143 from the ATCC or LEM-125 from Dr. Larry Simpson's lab at the University of California, Los Angeles) grown in heat sterilized Brain and Heart Infusion (BHI) broth and 100 U/mL penicillin and 0.1 mg/mL streptomycin (Sigma-Aldrich) supplemented with hemin (20 μM) in 25 cm^2 sterile flasks (Corning). This *Leishmania* species was selected due to the ease and safety of culturing and no known ability to infect humans and having physiological similarity to species which can infect humans [10]. In some experiments, cells were placed in 24-well sterile culture trays (from Becton Dickinson) prior to evaluation. The different test compounds, dissolved in DMSO, were added to the cells, as a single dose, to give a final concentration of 1, 5, or 10 μM. The control cells received only DMSO (final concentration of 0.15%). Protoporphyrin IX disodium salt, protoporphyrin IX dimethyl ester, (both from Aldrich Chemical Company), Zn (II) protoporphyrin IX, and Sn (IV) protoporphyrin IX dichloride, (both from Frontier Scientific Porphyrin Products) were used without further purification. Trays were set up in duplicate to assess the effects of light exposure. One tray was incubated under a fluorescent lamp (350-750 nm, 750 Lux) for two hours after the compounds were added while the other tray was incubated in the dark and had no additional light exposure other than ambient light exposure during handling. Light microscopy and cell viability assays were performed each day. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoium bromide (MTT) viability colorimetric assay (using the method of Morgenthaler, *et al.* 2008 [6]), and data are reported as the mean $\pm$SD for n = 3 determinations. All data were corrected by subtracting the absorbance readings from samples containing only medium (+ DMSO) from the absorbance readings obtained from cell cultures. Test cells (+ compounds) were compared to DMSO control cells (+ DMSO) and results are reported as percent of same day control cells.

The amastigote form of *L. tarentolae* was obtained following method of Taylor *et al.* [12] using the LEM-125 strain. The promastigote stage was cultured at 26°C in Schneider medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 IU/mL penicillin/0.1 mg/mL streptomycin, and 2 mM L-glutamine at pH 6.9. The promastigotes were cultured for 3 days, and then the cells were transferred to Scheider medium supplemented with 10% FBS and 2 mM L-glutamine at pH 5.0 and incubated at 32°C. The cell density was adjusted to 2 x 10^5 parasites/ml. Once the amastigote form was established, cell cultures containing amastigotes in Schneider medium were transferred in a 24-well sterile culture tray (1 mL of culture per well). The same method described for the evaluation of LEM-125 promastigotes was used to evaluate amastigotes.

Using *Leishmania panamensis* axenic amastigotes or intracellular amastigotes, the PIX and metalled PIX were incubated (in the dark or with light treatment) then cell viability was measured using the MTT assay (for axenic cells) or using flow cytometry with GFP-clones (for intracellular amastigotes) using the methods reported by Taylor *et al.* [7, 13].

The induced production of superoxide was tested using the MitoSOX™ fluorescent imaging probe (Invitrogen) according to the manufacturer's recommendations. Confocal fluorescence imaging of the probe was carried out using a Leica confocal microscope DMIRE2 with an argon ion laser set at 458 nm for excitation and the emission was detected in the 500-660 nm range.

RESULTS AND DISCUSSION

When evaluated by light microscopy, the control promastigote cells, with a final concentration of 0.15% DMSO, appeared in general to have the typical oval shape, while cells exposed to the compounds had a more spherical shape. Both control and experimental promastigote cells had flagella and were motile. The amastigote cells with or without exposure to compounds had a similar appearance.

The LEM-125 promastigote cells exposed to 10 µM PIX or metalled PIX showed variable inhibitory effects 48 hours after the various compounds were added to the cell culture (Figure 1). PIX exposure resulted in a modest inhibitory effect (by about 35-50% at the two higher concentrations)
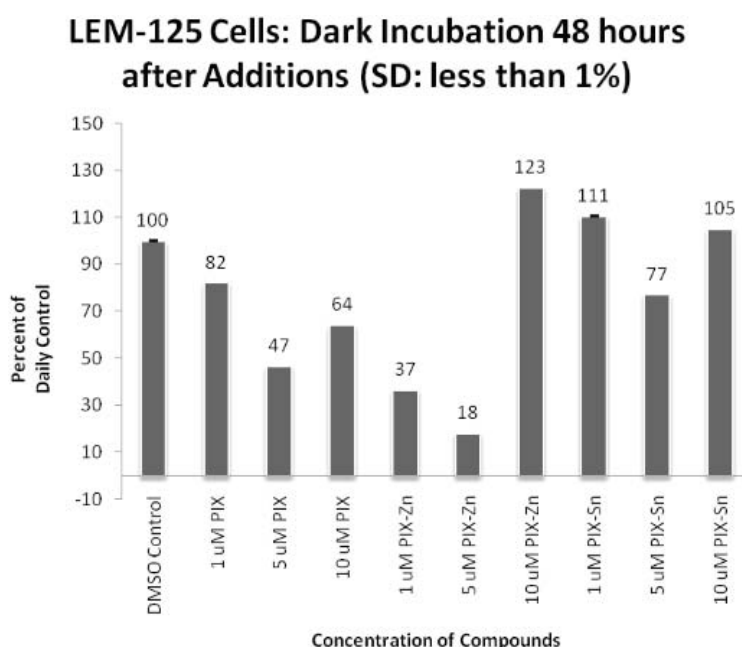
Figure 1. LEM-125 promastigotes were incubated with 1, 5 or 10 µM of the compounds (in DMSO) for 48 hours in the dark before MTT viability was evaluated. Data are reported as % of the DMSO only control cells. The mean values (n = 3) are indicated over the appropriate columns.
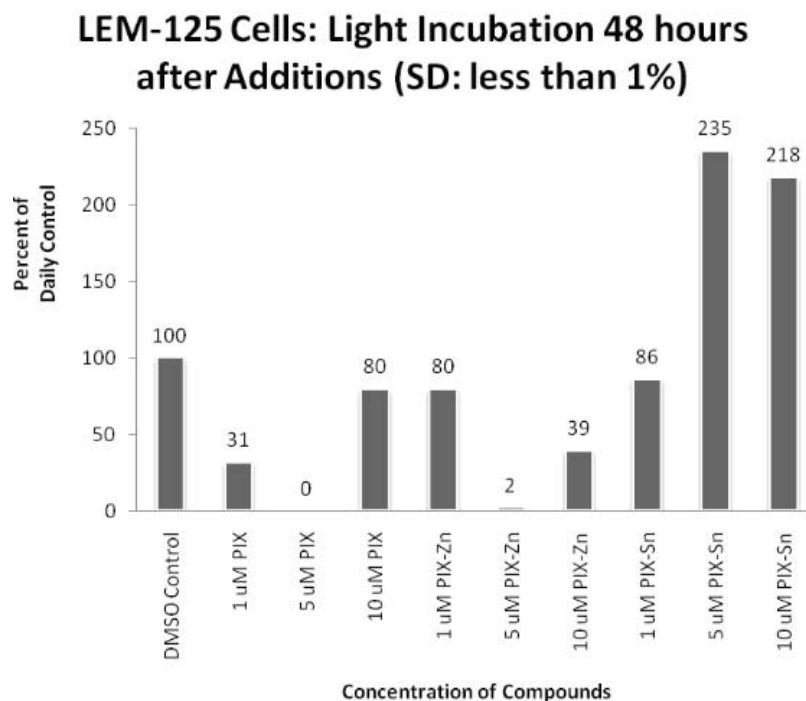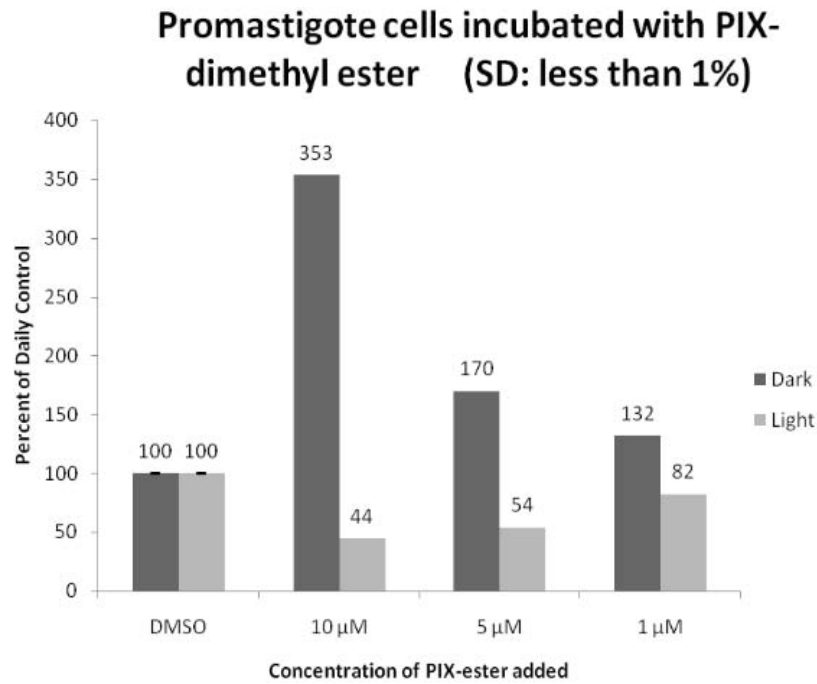
Figure 2. LEM-125 promastigotes were incubated with 1, 5 or 10 µM of the compounds (in DMSO) for 48 hours in the dark, following the 2 hours to light treatment, before MTT viability was evaluated. Data are reported as % of the DMSO only control cells. The mean values (n = 3) are indicated over the appropriate columns.

while PIX-Sn had little effect on LEM-125 promastigote cells. The PIX-Zn, PIX-Sn, and PIX exposure resulted in an apparent inverse concentration dependency with the two lower concentrations being most inhibitory. There appears to be a threshold concentration at which the compounds become more nutritious rather than toxic. The 30143 strain exhibited similar effects on viability (data not shown) suggesting a general trend. Figure 2 shows cells exposed to light treatment. The 10 µM PIX exposed cells were inhibited approximately 25% more than the cells without light exposure while the cells exposed to 1 or 5 µM PIX were much more negatively affected. The cells exposed to 10 µM PIX-Zn with light treatment were much more negatively affected (39% viability relative to control cells) than cells not exposed to light. The PIX-Sn exposed cells exhibited little effect following light exposure and even increased cell viability at 5 or 10 µM levels. The LEM-125 promastigote cells that were incubated with PIX-ester also exhibited greater inhibitory effects with

light treatment (Figure 3) relative to dark exposed only. The PIX-ester at 10 µM had the greatest negative effect on the cells; with only a 44% of control response at 48 hours post exposure to compound and light treatment. Thus with the promastigotes, there are variable results as a function of both dose of the test compound and of light exposed with these four compounds.

The amastigote form appeared to be much more negatively affected than the promastigote form since the inhibitory effect was greater for PIX-Sn and PIX-Zn than PIX (Figure 4). After 48 hours, the amastigotes cells that were exposed to light and 5 or 10 µM of PIX-Sn exhibited less viability (0% vs control) relative to PIX and PIX-Zn at the same concentration of compound (Figure 5). These data indicate a substantial photodynamic effect. We do see a variable response to the added compounds implying effects on several cellular systems such as the heme oxygenase system along with a more non-specific photodynamic effect. These results appear to be due to a combination of type of protoporphyrin IX used and dose and the

Promastigote cells incubated with PIX-dimethyl ester (SD: less than 1%)

Figure 3. LEM-125 promastigotes were incubated with 1, 5 or 10 µM of the PIX-dimethyl ester (in DMSO) for 48 hours in the dark, with or without light treatment, before MTT viability was evaluated. Data are reported as % of the DMSO only control cells. The mean values (n = 3) are indicated over the appropriate columns.

Amastigote: Dark Incubation 48 hours after Addition (SD: less than 1 %)
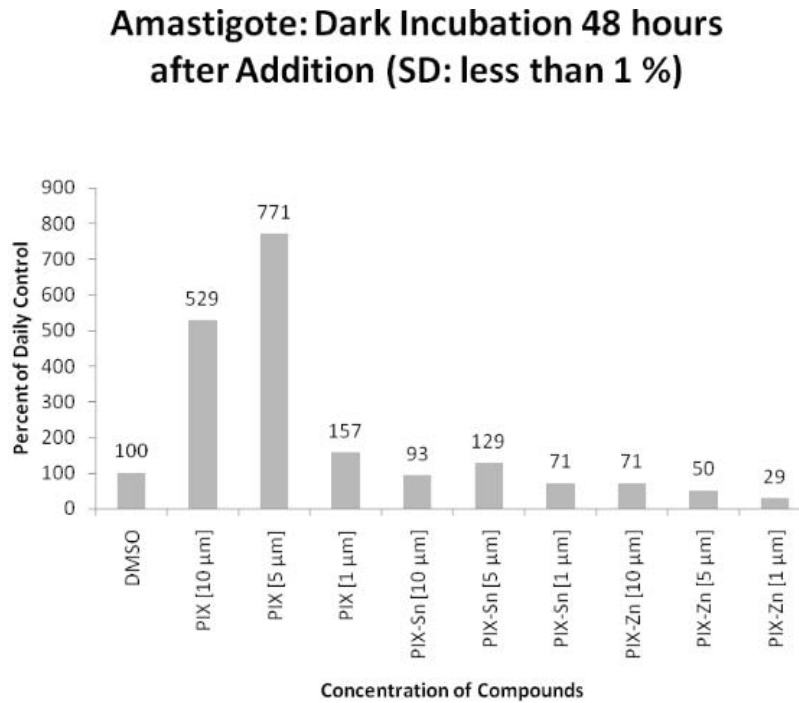
Figure 4. LEM-125 amastigotes were incubated with 1, 5 or 10 µM of the compounds (in DMSO) for 48 hours in the dark before MTT viability was evaluated. Data are reported as % of the DMSO only control cells. The mean values (n = 3) are indicated over the appropriate columns.

Amastigote: Light Incubation 48 hours after Additions (SD: less than 1 %)
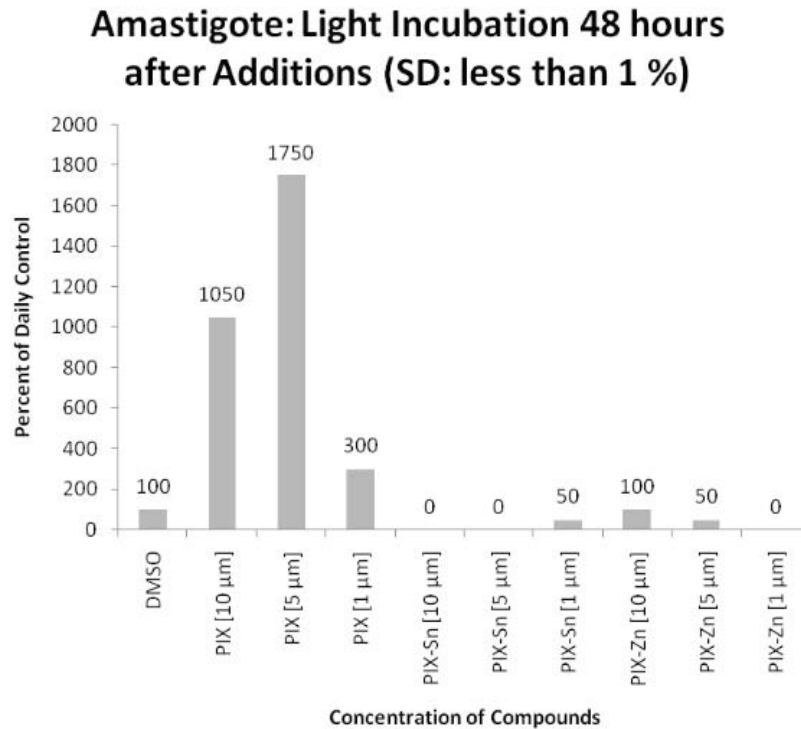
Figure 5. LEM-125 amastigotes were incubated with 1, 5 or 10 µM of the compounds (in DMSO) for 48 hours in the dark, following the 2 hours to light treatment, before MTT viability was evaluated. Data are reported as % of the DMSO only control cells. The mean values (n = 3) are indicated over the appropriate columns.

Table 1. Effectiveness (EC$_{50}$, µM) of compounds against axenic and intracellular amastigotes of *L. panamensis*.

Compound	Axenic Amastigotes		Intracellular Amastigotes	
	Dark	+ Light	Dark	+ Light
PIX	134 ± 6	71.2 ± 0.3	119.3 ± 0.03	36.0 ± 0.8
PIX-Sn	95.4 ± 1.5	56.9 ± 0.3	85.7 ± 1.5	30.8 ± 1.3
PIX-Zn	117 ± 10	45.8 ± 1.6	111 ± 5	16.5 ± 0.2

net results may be due to a combination of need of the organism for the uptake of the porphyrin required for cell growth and the accumulation which leads to negative effects so that there is a more complex response than a simple inhibitor mechanism.

Table 1 shows the effectiveness (reported as EC50, µM) of tested compounds against axenic and intracellular amastigotes of *L. panamensis*, a human parasitic species. Addition of PIX-Zn appeared to be the most inhibitory compound under these conditions. Intracellular amastigotes were the form most inhibited by any addition especially following light treatment. As shown in Table 1, the intracellular parasites were more sensitive to added porphyrin than the axenic form. In all cases, a photodynamic effect was indicated.

The accumulation of PIX, PIX-Sn and PIX-Zn in the cells was evaluated by confocal fluorescence microscopy to observe the uptake of the compounds by cells. The compounds were excited by an argon ion laser set at 458 nm and light emission was detected in the 500-660 nm range. Cultures that were exposed to light or grown in the dark were evaluated by confocal microscopy two hours after the addition of the compounds. Promastigote cells
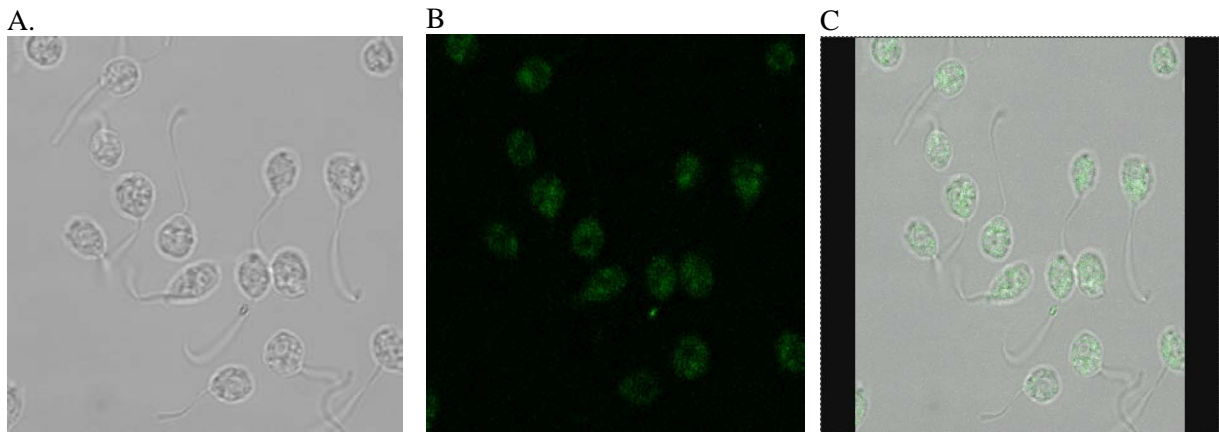
Figure 6. LEM-125 promastigotes evaluated by confocal microscopy. (A) cells incubated in the dark with 10 µM PIX for 2 hours (B) fluorescence image of the same cells (C) overlay of A and B indicating internal accumulation of the PIX by the cells.
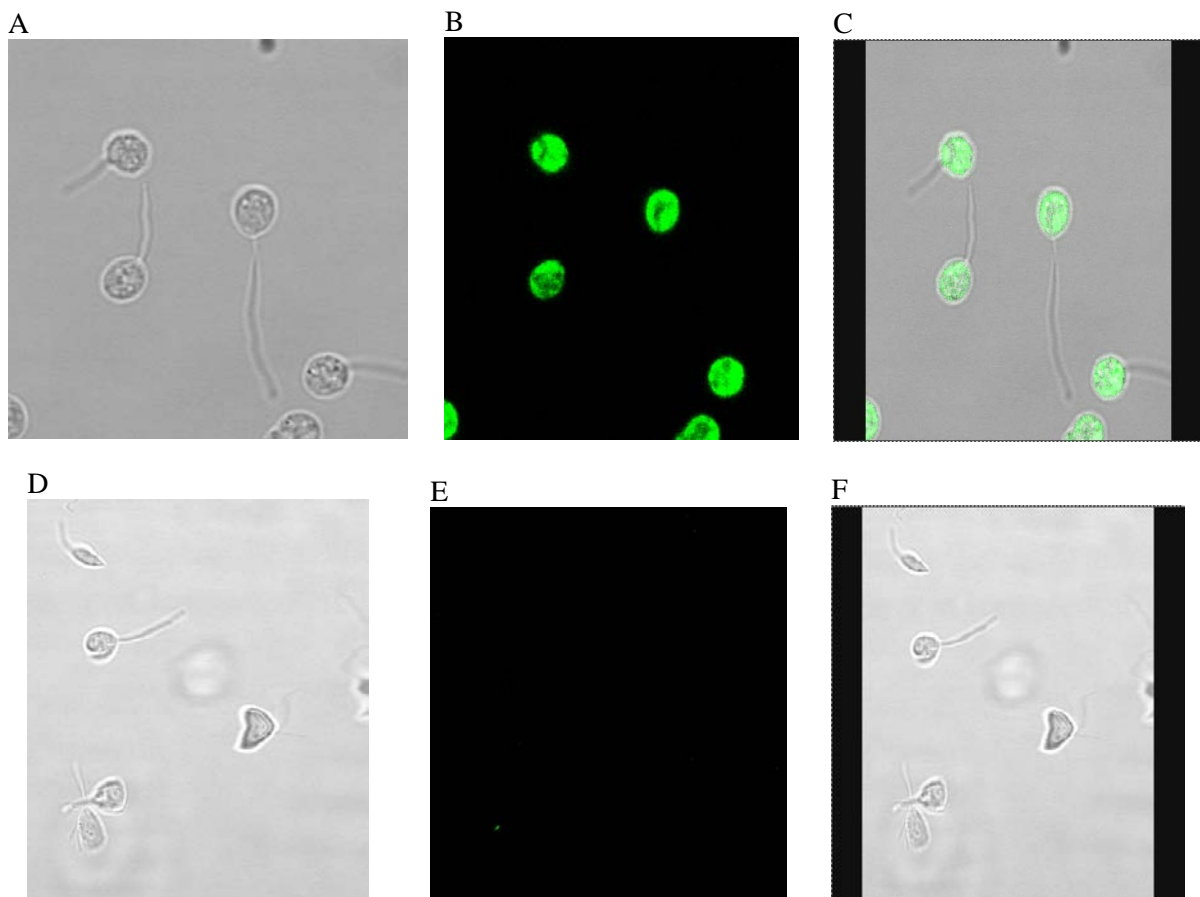
Figure 7. Confocal fluorescence images of superoxide probe MitoSOX™ in LEM-125 promastigotes. (A) Transmission image (B) in the presence of 10 µM PIX for 2 hours (C) overlay of A and B indicating superoxide production by these cells (D) transmission image in the presence of only DMSO (E) corresponding fluorescence image (F) overlay of D and E indicating no superoxide production in these cells.

exhibited fluorescence after the addition of PIX (Figure 6), which indicates the cells' ability to take up the compound. This was not surprising since others have reported a porphyrin transport system in *Leishmania* [4]. Cells incubated with only DMSO did not exhibit fluorescence (data not shown).

In order to evaluate a possible mechanism of cell inhibition, the MitoSOX™ fluorescent probe (Invitrogen) was used to detect superoxide formation inside the cells (Figure 7). In Figure 7C, the promastigotes can be seen exhibiting a positive superoxide response induced by the presence of 10 µM PIX, while the cells that were incubated only with DMSO (Figure 7E) did not exhibit a positive superoxide response.Since superoxide production is generally considered one criterion for apoptosis [14, 15], these data suggest that *Leishmania* are undergoing apoptosis under these experimental conditions.

CONCLUSIONS

The results from this study indicate that PIX, PIX-dimethyl ester, PIX-Sn or PIX-Zn, individually or in combination, could be a promising treatment for leishmaniasis especially in the cutaneous form since the dermal infiltrate is initially made up primarily of amastigotes and their host macrophage cells [3]. These fairly stable compounds do not require refrigeration, can be easily transported, and then formulated into a topical cream which could be applied to the infected skin. The person thus treated could then sit out in the sunlight for a pre-determined time to promote a photodynamic effect. The data allow us to speculate that porphyric patients, who have defects in their heme biosynthesis, may be more resistant to infections by *Leishmania* than the general public. Future work by medical epidemiologists to collect data on the incidence of leishmaniasis in porphyric patients could help confirm this proposal.

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