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Aminophthalocyanine-Mediated Photodynamic Inactivation of *Leishmania tropica*

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Photodynamic inactivation of *Leishmania* spp. requires the cellular uptake of photosensitizers, e.g., endocytosis of silicon(IV)-phthalocyanines (PC) axially substituted with bulky ligands. We report here that when substituted with amino-containing ligands, the PCs (PC1 and PC2) were endocytosed and displayed improved potency against *Leishmania tropica* promastigotes and axenic amastigotes *in vitro*. The uptake of these PCs by both *Leishmania* stages followed saturation kinetics, as expected. Sensitive assays were developed for assessing the photodynamic inactivation of *Leishmania* spp. by rendering them fluorescent in two ways: transfecting promastigotes to express green fluorescent protein (GFP) and loading them with carboxyfluorescein succinimidyl ester (CFSE). PC-sensitized *Leishmania tropica* strains were seen microscopically to lose their motility, structural integrity, and GFP/CFSE fluorescence after exposure to red light (wavelength, ~650 nm) at a fluence of 1 to 2 J cm^{-2}. Quantitative fluorescence assays based on the loss of GFP/CFSE from live *Leishmania tropica* showed that PC1 and PC2 dose dependently sensitized both stages for photoinactivation, consistent with the results of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. *Leishmania tropica* strains are >100 times more sensitive than their host cells or macrophages to PC1- and PC2-mediated photoinactivation, judging from the estimated 50% effective concentrations (EC_{50}s) of these cells. Axial substitution of the PC with amino groups instead of other ligands appears to increase its leishmanial photolytic activity by up to 40-fold. PC1 and PC2 are thus potentially useful for photodynamic therapy of leishmaniasis and for oxidative photoinactivation of *Leishmania* spp. for use as vaccines or vaccine carriers.

Photodynamic therapy (PDT) uses photosensitizers (PS) that are light excitable to produce cytotoxic reactive oxygen species (ROS) in the presence of atmospheric oxygen for the clinical treatment of tumors, skin cancer, and other cutaneous diseases (1). PS can be applied exogenously, as is the case with phthalocyanines. Alternatively, PS can be induced endogenously with delta-aminolevulinate (ALA) to upregulate heme biosynthesis of the target cells for overproducing photosensitive intermediates of this pathway, i.e., porphyrins (2). PDT has the potential to solve the significant problems of emerging drug resistance in infectious and malignant diseases, since it is not known to elicit such resistance (3, 4). Light and PS alone are noncytotoxic and thus do not select for a resistance phenotype. In combination, a burst of powerful cytotoxic ROS is rapidly generated, which simultaneously attack multiple cellular molecules, making it unlikely to induce the development of resistance. This assumption is supported experimentally by observations with porphyrinogenic *Leishmania* species (see below).

The potential of PDT has been explored for treating infectious diseases caused by different pathogens (5, 6), including trypanosomatid protozoa in the genus of *Leishmania* causing cutaneous leishmaniasis (CL). PDT with clinically acceptable photosensitizers showed promising results by using daylight for illumination in ongoing trials of CL patients (7) and for treating protracted infection in drug-resistant cases (8). Anti-*Leishmania* PDT has been assessed experimentally *in vivo* in different animal models of CL and *in vitro* in infected macrophages. More recent work includes the use of different photosensitizers, e.g., hypericin, phthalocyanines, phenothiazines, methylene blue, and zinc oxide, as such or after PEGylation or in liposome-/nanoparticle-encapsulated forms, to generate ROS by illumination using different light sources, e.g., light-emitting diodes (LED), lasers, and sunlight (9–17). The intrinsic susceptibilities of both stages of *Leishmania*, i.e., promastigotes and amastigotes, to PDT has been examined in that context but rarely has been explored at the cellular and molecular levels. Such mechanistic investigation is essential for improving the discriminatory efficacy of PDT and broadening the scope of its applications.

Previously, we made *Leishmania* spp. sensitive to PDT by targeting PS for intracellular accumulation. For cytosolic accumulation of PS, *Leishmania* spp. were first engineered transgenically to express the 2nd and 3rd enzymes in the heme biosynthesis pathway missing in these trypanosomatids (18, 19). Such transfectants

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were then exposed to ALA, a product of the 1st enzyme in this pathway, resulting in cytosolic accumulation of the uroporphyrin I (URO) in the absence of downstream enzymes. These porphyric *Leishmania* spp. are rapidly inactivated after brief exposure to light, which excites their cytosolic URO to generate singlet oxygen ($^1O_2$) (20), an extremely short-lived (half-life, 1 to 3 μs) but highly reactive ROS. Mechanisms for the detoxification of $^1O_2$ are not known to exist among nonphotosynthetic organisms, like *Leishmania* species. Indeed, no development of PDT resistance was noted after as many as six consecutive cycles of ALA-induced porphyrinogenesis, followed by light exposure for cytolysis. After each PDT cycle, very few cells survived and were invariably aporphyric, attributable to the efflux of URO. However, the survivors remained as sensitive to the PDT as the parental transfectants when recovered after each PDT cycle or cloned after the last cycle of the six treatments (20). Photodynamic vaccination of hamsters based on this principle produced cell-mediated immunity that is adaptively transferable to naive animals against visceral leishmaniasis (21). The porphyrinogenic mutants have the potential to serve as a universal platform for vaccine delivery, as shown by the findings that ALA-induced porphyrinogenesis, followed by photoinactivation of *Leishmania in situ*, reversed the immunosuppression of macrophages caused by the infection (22). For endosomal accumulation of PS in *Leishmania*, silicon(IV)-phthalocyanines (PC) attached axially with bulky ligands (PC14 and PC15) were identified as effective by screening a panel of ~17 novel PCs (23). Endocytosis of PC14 and PC15 by *Leishmania* spp. sensitizes them to photoinactivation. These PCs are more discriminatory than aluminum(III)-PC against *Leishmania* spp. versus their host cells or macrophages (24). When preoxidatively photoinactivated with PC15, *Leishmania* cells were shown to deliver transgenically expressed ovalbumin (OVA) more effectively than conventional means to antigen-presenting cells (APCs) for presentation to activate OVA epitope-specific T cells (23). Significantly, oxidatively photoinactivated *Leishmania* spp. after double sensitization with URO and PC produced no lesions in mice (25), indicative of their safety for applications: vaccination and immunotherapy.

In the present study, we screened additional PCs, leading to the identification of amino-PC conjugates (PC1 and PC2) that are significantly more effective and discriminating than the PCs previously identified for PDT of *Leishmania* species. This efficiency was demonstrated qualitatively in both stages of *L. tropica* by fluorescence microscopy and fluorometry for the uptake of PC1 and PC2, and quantitatively by fluorescence/metabolic cell viability assays after photoinactivation. The effectiveness and discriminatory potentials of PC1 and PC2 are predicted from the disparity between *Leishmania* and J774 macrophages in their 50% effective concentrations (EC_{50}s) for photoinactivation.

MATERIALS AND METHODS

Novel phthalocyanines. We examined 4 phthalocyanines (PC): the aminophthalocyanines PC1 and PC2, the triethylene glycol-substituted Zn(II)-phthalocyanine PC4 (26, 27), and PC3, which was prepared from silicon(IV) phthalocyanine-dichloride treated with triethylene glycol (see Fig. S1 legend in the supplemental material for chemical structures). All compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare 1 mM stock solutions, from which working solutions (0.01 to 10 μM) were prepared by serial 10-fold dilutions.

Cells. The *Leishmania* strains used included 2 virulent strains of *L. tropica*, EP41 (see Fig. S2 in the supplemental material) and CBU10. Promastigotes were grown to stationary phase at 25°C in HEPES-buffered medium 199 or Schneider's insect medium with 10% heat-inactivated fetal bovine serum (HIFBS) and as axenic amastigotes at 33°C in Schneider's insect medium with 10% HIFBS (pH 5.3) (23). Axenic amastigotes are slightly larger than the amastigotes in infected macrophages and are more infective than promastigotes *in vitro* and *in vivo* (see Fig. S2C and D in the supplemental material). Macrophages of the J774 cell line were cultured at 35°C without 5% (vol/vol) CO_2 atmosphere in RPMI 1640 medium HEPES buffered to pH 7.4 plus 10% (vol/vol) HIFBS (22–24).

Photosensitization of cells. *Leishmania* cells were suspended to 5×10^7 to 5×10^8 cells ml^{-1} in HBSS-BSA for exposure to serial dilutions of PC1 to PC4 in the dark overnight. The controls included cells exposed to diluent alone. In separate experiments, promastigotes were exposed to fluoroisothiocyanate (FITC)-dextran (molecular mass, ~4,000 Da; Sigma) for 2 days, washed three times with HBSS-BSA by centrifugation, and then exposed to 1 μM PC1 for 1 day in the dark. J774 macrophages were PC treated as a monolayer in Hanks' balanced salt solution (HBSS) HEPES buffered to pH 7.4 plus 0.01% bovine serum albumin (HBSS-BSA) or culture medium under otherwise identical conditions. All cultures were kept in the dark for various time periods of up to 24 h.

Exposure of photosensitized cells to red light. Control and PC-sensitized *Leishmania* cells were washed and suspended in HBSS-BSA. The cell suspensions in 24-well culture plates and monolayers of J774 macrophages in 25-cm^{-2} tissue culture (TC) flasks were exposed from the bottom to red light (wavelengths, >650 nm) via a red filter (product no. 650021; Smith-Victor Co., Bartlett, IL, USA) over a light box for 20 to 30 min at a fluence of 1 to 2 J cm^{-2} (22, 23).

Cell viability assay. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay was done, according to the manufacturer's protocol (Molecular Probe kits; Sigma) and as previously described (22, 23). A freshly prepared MTT stock solution (5 mg ml^{-1}) was added to promastigote and axenic amastigote cell suspensions at 0.5×10^8 to 1×10^8 ml^{-1}. Formed formazan was solubilized for reading in a BioTek Synergy HT plate reader (Texas Instruments) and analyzed using the GEN 5 version 5.11.1 software.

Quantitative fluorescence assay. *L. tropica* EP41 and CBU10 transfected by electroporation with *egfp*-p6.5 (28) were used to express green fluorescent protein (GFP), as described previously (24). Stable transfectants were obtained and grown in the presence of 10 μg ml^{-1} tunicamycin.

Also used were *Leishmania* cells exposed for 10 min at 37°C to 5 to 10 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) at 10^8 cells ml^{-1} in HBSS-BSA. CFSE is converted by intracellular nonspecific esterases into cell-impervious fluorescent products, rendering live *Leishmania* cells fluorescent (29).

GFP transfectants or CFSE-loaded *Leishmania* cells in HBSS-BSA were dispensed in triplicate 100-μl aliquots at 5×10^6 cells 100 μl^{-1} $well^{-1}$ to 96-well microtiter plates for detergent lysis with 0.5% (vol/vol) Nonidet P-40 to release cellular GFP/CFSE. The lysates were read for fluorescence at a sensitivity setting of 60 in a BioTek Synergy HT plate reader (excitation wavelength, 485/20 nm; emission wavelength, 528/20 nm). The data collected were analyzed using the GEN 5 version 5.11.1 software.

***Leishmania* uptake of PCs assessed by fluorometry.** Promastigotes were exposed in the dark to 0.1 μM PC1 and PC2 at 5×10^7 cells ml^{-1} under nongrowing conditions in HBSS-BSA. The controls were set up without cells under the same conditions. The samples were dispensed in 1-ml aliquots in the dark at 25°C and harvested at different intervals for up to 24 h. The cells were washed once, sedimented as pellets, and then stored at −20°C. At the completion of the experiments, PC was extracted from each frozen pellet with 2.5 ml of DMSO. The fluorescent intensities of the extracts were read (excitation wavelength, 608 nm; emission wavelength, 682 nm) in an LS-50B spectrofluorometer (PerkinElmer) using the FL WinLab software. Concentrations of the PC in the samples were expressed in nanomoles per 10^{-7} cells by interpolation of their fluorescent intensities versus the readings from a linear standard curve of PC concentrations from 0 to 20 μM.

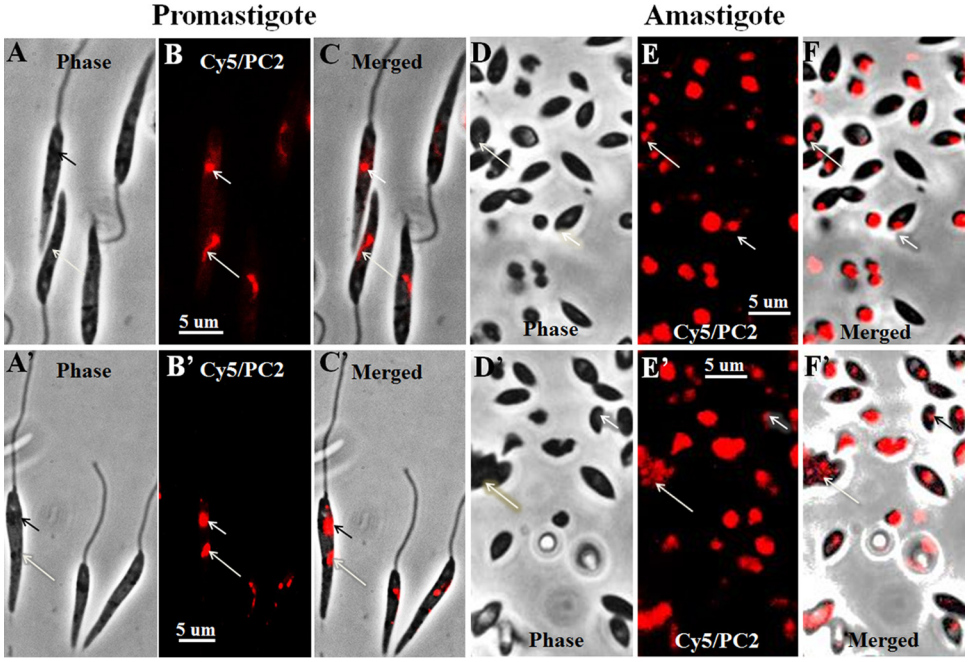

FIG 1 Fluorescence microscopy of *L. tropica*, showing uptake of aminophthalocyanine conjugate PC2 by both stages. Shown are promastigotes (A to C and A′ to C′): and axenic amastigotes (D to F and D′ to F′). (A, A′, D, and D′) Phase contrast. (B, B′, E, and E′) Cy5 fluorescence for phthalocyanine PC2. (C, C′, F, and F′) Phase/Cy5 merged images. Cells were loaded in the dark with 1 μM PC2 for ~16 h and washed once before imaging. See Materials and Methods for experimental details. Short arrow, PC-positive endosomes near flagellar pockets; long arrow, PC-positive filamentous multivesicular bodies and endosomes distant from the flagellar pocket.

Assays for dark-condition toxicity of PCs. GFP transfectants at 5×10^6 ml^{-1} in culture medium were divided into 12-ml aliquots for exposure to a mixture of PC1 and PC2 in 10× serial dilutions from 0 to 1 μM. Cultures were handled in two ways to minimize light exposure. Normally used conditions were referred to as "in the dark" in all experiments described as such. Twelve-milliliter aliquots of cell suspensions were each exposed to 0 to 1 μM PCs in separate culture vessels wrapped in aluminum foil. The culture vessels were unwrapped for daily sample collection under incipient or ambient-light conditions with the room light turned off. Under the more-stringent conditions, samples from different experiments to be collected daily were aliquoted (3 ml each) and wrapped together in aluminum foil to keep them in total darkness for the duration of the incubation. The samples under both conditions were processed daily for the collection of cell pellets, which were kept frozen until the completion of the experiments, when they were subjected to GFP fluorescence assays as described in quantitative fluorescence assay. The data are presented as % of the controls from untreated cells collected at each time point.

Fluorescence microscopy. Nikon Eclipse 80i and TE2000-S microscopes with CCD cameras were used with the Metamorphosis (version 6.1) or NIS Elements version AR 4.20.01 software for image capture and analysis (18, 22–24). The cells were examined under living conditions by placing concentrated cell suspensions in 3-μl aliquots each on a glass slide covered with an 18-mm^2 glass coverslip. The wet mounts were first scanned under phase contrast to localize the cells. Cells exposed to PCs were then imaged for subcellular localization of PC fluorescence using a Cy5 filter set. GFP/CFSE fluorescent *Leishmania* cells were examined using the FITC filter set. Images captured under different settings were merged by using the software programs mentioned above. The filter sets (Chroma Technology Co., Brattleboro, VT) used were (i) HQ620/60 (620-nm exciter), Q660LP (660-nm dichroic), and HQ700/80 (700-nm emitter) for phthalocyanines and (ii) HQ480/40 (480-nm exciter), Q505LP (505-nm dichroic), and HQ535/50 (535-nm emitter) for GFP and CFSE.

Data analysis/presentation. All experiments were repeated at least twice and, in most cases, three times, using mainly EP41 but also CBU10 for confirmation. The results obtained were comparable among repeat experiments. The data presented represent the means ± standard errors of the values obtained in triplicate for individual samples from representative experiments. Statistical analyses were performed for pairwise data comparison by two-tailed Student *t* tests in GraphPad Prism version 5. *P* values of ≤0.05 were considered significant.

RESULTS

***Leishmania* uptake of aminophthalocyanine conjugates (PC1 and PC2).** Of the 4 phthalocyanines (PC1 to PC4) examined (see Fig. S1 in the supplemental material), PC1 and PC2 were detected by fluorescence microscopy in *Leishmania* cells, which are axially substituted each with two symmetrical ligands, consisting of mono- and di-amino groups, respectively. No uptake of PC3 and PC4 by *Leishmania* cells was observed, irrespective of the concentrations and periods of incubation used (not shown).

PC1 and PC2 were taken up at both *Leishmania* stages, as noted in the images captured using the Cy5 filter set (Fig. 1). The uptake of both PCs was time dependent. Cells examined immediately after exposure to 0.1 to 1 μM PC1 or PC2 showed no intracellular fluorescence. Fluorescence began to emerge in cells exposed to the PCs for ≥1 h and increased gradually in intensity upon further incubation. After ≥4 h, PC fluorescence was readily discernible in discrete cellular entities of promastigotes (Fig. 1, promastigote). PC fluorescence appeared in vacuoles in the proximity of the flagellar pocket (short arrow), in multivesicular tubular structures, and vacuoles (long arrow) distant from the flagellar pocket (Fig. 1A to C and A′ to C′ merged images). The axenic amastigotes also appeared to have PC fluorescence in the similar intracellular structures (Fig. 1D to F, arrows), although these small cells have

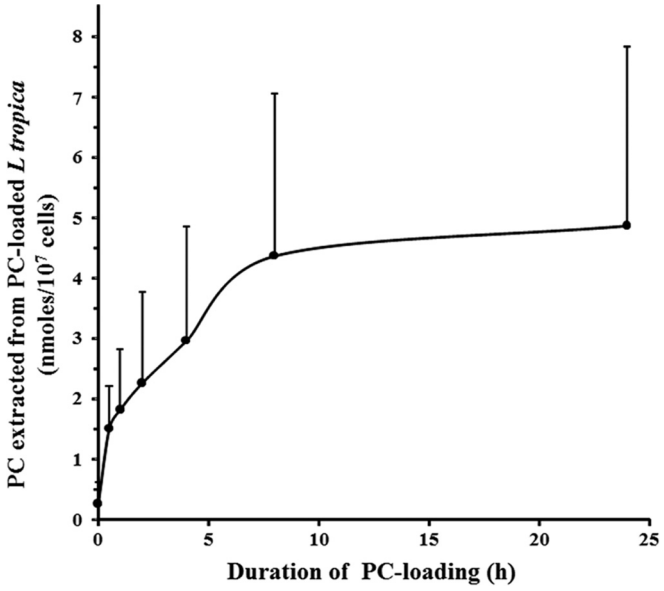

FIG 2 Saturation kinetics of aminophthalocyanine uptake by *L. tropica*. *x* axis, time periods in hours for sample collections after incubation of promastigotes with a mixture of 1 μM PC1 and 1 μM PC2 in the dark for ~24 h; *y* axis, cellular concentrations of PCs in nanomoles per 10^7 cells, as determined by fluorometry of the extracts from PC-loaded cells taken at different times after incubation. See Materials and Methods for experimental details for PC extraction with DMSO, fluorometric readings of the extracts for fluorescence intensities, and determination of PC concentrations by intrapolation against the values from a standard curve of graded PC concentrations. Note that the fluorescence readings of cell-associated PCs have saturation kinetics with times of incubation within each experimental set but vary considerably with different experiments, accounting for the large standard errors seen.

no external flagella for orientation. PC fluorescence varied with individual amastigotes (Fig. 1D to F) but was present in all amastigotes when the fluorescence intensity was digitally optimized (Fig. 1D′ to F′). Fluorescence intensity appeared higher in both stages after exposure to PC2 than to PC1. PC1 was seen to colocalize with FITC-dextran in promastigotes (see Fig. S3 in the supplemental material).

The uptake of PC1 and PC2 followed saturation kinetics, as determined by fluorometric quantitation of cell-associated PCs (Fig. 2). Since the cells were washed before PC extraction, cell surface-associated PC was excluded from the obtained readings. Spontaneous precipitation of PCs and cell proliferation did not contribute to the readings, since both were found to be minimal within the time frame under the conditions of incubation. Thus, the rise of cell-associated PCs with time was reflective of its uptake, reaching a plateau in ~7 h. The kinetics of the uptake was comparable among different experiments, although the readings varied significantly from one experiment to another, as reflected in the large standard errors (Fig. 2). We attribute this to the inherent variations of different lots of cells used and/or different efficiencies of PC extraction from different sample sets.

Microscopy of *Leishmania* photolysis mediated by PC1 and PC2. When sensitized with PC1 and PC2 and then exposed to red light, promastigotes were found to lose their motility (not shown) and structural integrity (Fig. 3, A3 and A5). In contrast, they remained motile and intact in the groups of no treatment or those treated with PC1 and PC2 alone without red light exposure (Fig. 3, A1, A2, and A4). This finding was further indicated by the loss of cellular fluorescence when using GFP/CFSE-fluorescent cells (see Fig. S3 in the supplemental material). After red light exposure of PC1- or PC2-loaded GFP transfectants, the GFP fluorescence was reduced, especially with PC2 (Fig. 3, B3, B5, D3, and D5), but not in the control groups of no treatment and PC treatment alone (Fig. 3, B1, B2, B4, D1, D2, and D4). The images taken under the Cy5 filter showed that PC1 and PC2 fluorescence was absent in untreated cells (Fig. 3, C1 and D1), as expected, and differed little among PC1- and PC2-treated cells with or without red light exposure (Fig. 3, C2 to C5). In the merged images, PC-GFP colocalization was evident in cells treated with PC1 or PC2 alone (Fig. 3, D2 and D4) but not at all or to a lesser extent after exposure to red light (Fig. 3, D3 and D5).

Similar results were obtained by using other fluorescent cells of both EP41 and CBU10: CFSE-loaded promastigotes and axenic amastigotes and GFP-transfected axenic amastigotes (not shown, but see quantitative data presented in Fig. 4).

In the presence of PC3 and PC4, *Leishmania* cells showed no cellular fluorescence and retained their motility and cellular integrity with or without red light exposure (not shown).

PC dose-dependent sensitization of *Leishmania* for photoinactivation. MTT reduction and loss-of-cellular-GFP/CFSE fluorescence assays (see Materials and Methods) showed that both promastigotes (Fig. 4A to C) and axenic amastigotes (Fig. 4D to F) progressively lost their viability with exposure to increasing concentrations from 0 to 1 μM of the PCs (Fig. 4, C, PC1, and PC2 at bottom). The loss of fluorescence was significantly higher among red light-exposed samples (all white bars) than that in samples not exposed to red light (all gray bars) (Fig. 4, *** to *, $P < 0.001$ to 0.05). Promastigotes (Fig. 4A to C, white versus gray bars) were seen to respond more consistently to both PC1 and PC2 than axenic amastigotes (Fig. 4D to F, white versus gray bars). The differences between red light and no light for each PC concentration used were significantly greater for promastigotes (Fig. 4A to C, white versus gray bars; ***, $P < 0.001$) than for axenic amastigotes (Fig. 4D to F, white versus gray bars; *** to *, $P < 0.001$ to 0.05), indicating that the promastigotes are more susceptible to PC1- and PC2-mediated photoinactivation. This conclusion is also evident in a comparison of each set of the values under the light condition at each PC concentration for both stages, especially at the highest concentration of PCs (Fig. 4A to C versus D to F, white bars at 1 μM PC; ***, $P < 0.001$). At the highest PC concentration of 1 μM used, PC2 appeared to be more effective than PC1 against promastigotes (Fig. 4A to C, PC1 versus PC2 white bars at 1 μM; *** to **, $P < 0.001$ to 0.01). This difference was less pronounced for axenic amastigotes (Fig. 4D to F, PC1 and PC2 white bars at 1 μM), especially PC1-treated GFP transfectants in response to red light (Fig. 4E, gray versus white bars at PC1 0.01 to 1 μM).

Growth of promastigotes in the presence of PC1 and PC2. Room light exposure was minimized under normally used and more-stringent conditions, as defined in Materials and Methods. Daily collected samples included small aliquots from all for brief microscopic observations, which showed no gross differences in motility, integrity, or density of the promastigotes exposed to all PC concentrations (0 to 1 μM) under both conditions throughout the incubation period of 4 days. GFP fluorescence assays of the samples showed differences in PC-mediated growth inhibition between the two conditions. Under normally used conditions,

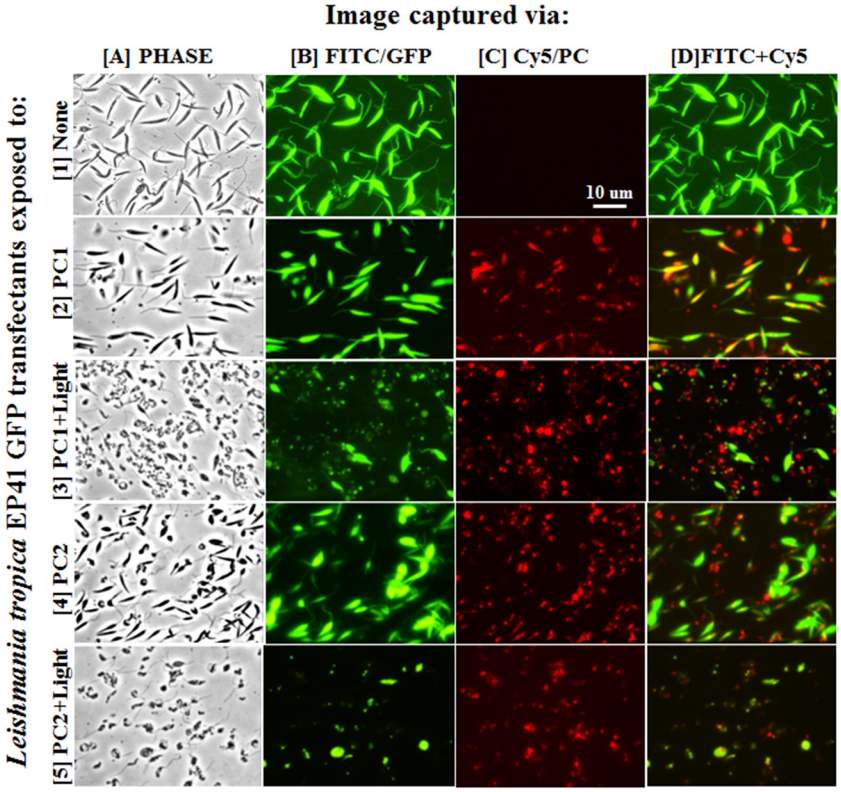

FIG 3 Phase contrast and fluorescence microscopy of GFP-transfected *L. tropica* promastigotes, showing PC-mediated photoinactivation. The columns represent the settings used to capture the images of GFP-transfected promastigotes. Column A, phase contrast for assessing cellular integrity; column B, FITC filter set for GFP fluorescence; column C, Cy5 filter set for PC fluorescence; and column D, merged images of columns B and C. The rows represent the treatments of the cells. Row 1, no treatment; row 2, exposure to 1 μM PC1 for 1 day in the dark; row 3, sample in row 2 further exposed to red light for 30 min, followed by 1 day of incubation; row 4, exposure to 1 μM PC2 for 1 day in the dark; and row 5, sample in row 4 further exposed to red light for 30 min, followed by 1 day of incubation. See Materials and Methods for experimental details. Note that cellular disintegration (A3 and A5) and loss of GFP fluorescence (B3, D3, B5, and D5) of the promastigotes occurred only after photoinactivation.

GFP fluorescence was reduced in samples exposed to all PC concentrations used by up to 25% of that of the PC-untreated controls on day 4 (Fig. 5A). The loss of fluorescence increased progressively with time at PC concentrations from 0.1 to 1 μM but was not evident until day 2 at 0.01 μM. These data are consistent with those for photoinactivation under the dark conditions presented in Fig. 4. Under dark conditions controlled more stringently, there was also a loss of GFP fluorescence at all PC concentrations used but to a much smaller extent, being especially notable at 0.01 to 0.1 μM (Fig. 5B).

Differential sensitivities of *Leishmania tropica* versus J774 macrophages to PC1- and PC2-mediated photoinactivation. *Leishmania* cells are far more sensitive than their host cells or J774 macrophages to PC-mediated photoinactivation. The J774 cells actively endocytosed PC1 and PC2 into their endosomes, which accumulated in the perinuclear region (see Fig. S4 in the supplemental material). The PC-loaded macrophages remained unchanged morphologically (see Fig. S4 in the supplemental material, 3rd row) and metabolically at all PC concentrations used up to 10 μM, irrespective of exposure to red light (Fig. 6A and B, open and filled squares). The EC_{50} of both PCs for these cells is thus >10 μM. Under the same conditions, both *Leishmania* stages progressively lost their viability to photoinactivation with increasing concentrations of PCs up to 1 μM (cf. Fig. 4A and D). Their loss of cell viability was complete at 10 μM with or without red light exposure (Fig. 6A and B, circles and triangles at 10 μM). The EC_{50}s of PC1 and PC2 for reducing *Leishmania* viability were estimated from the graphs presented in Fig. 6. Under the dark conditions, the EC_{50}s of both PCs appeared to fall between 1 and 10 μM, being comparable for the two *Leishmania* stages (Fig. 6A and B, filled triangles and circles). The EC_{50}s were significantly lowered by red light exposure, varying with PCs and *Leishmania* stages. The estimated EC_{50}s of PC1 are ~0.3 μM and ~0.1 μM, and those of PC2 are 0.2 μM and 0.07 μM for axenic amastigotes and promastigotes, respectively. Thus, PC2 is 1.5 times more effective than PC1 against both *Leishmania* stages, while axenic amastigotes are ~3 times more resistant than promastigotes to both PCs. All EC_{50}s were estimated from data obtained by the MTT assay, the only method usable to assess the viability of both *Leishmania* and macrophages. These EC_{50}s of PC1 and PC2 for *Leishmania* are consistent with the data presented in Fig. 4A and D under the light conditions but appeared higher than those from the fluorescence assays, judging from the data presented in Fig. 4C to E.

DISCUSSION

The major contribution of the work presented here is the discovery of new PCs that are more effective than their congeners found previously against *Leishmania*. Evidence is provided from *in vitro* observations at the cellular level based on the previously estab-

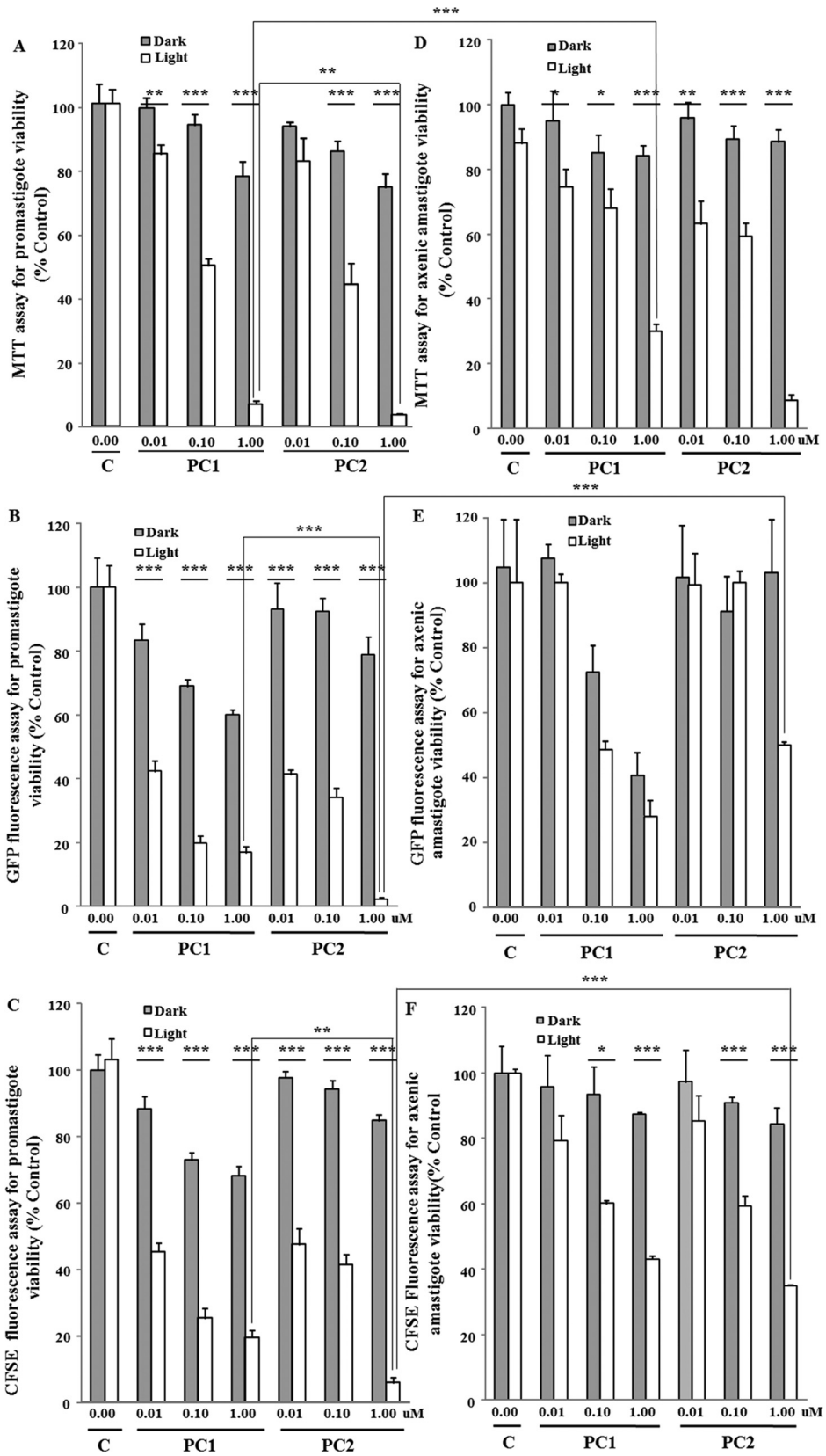

FIG 4 PC dose-dependent photoinactivation of both *L. tropica* stages, as determined by MTT reduction and fluorescence assays. Shown are promastigotes (A to C), axenic amastigotes (D to F), relative MTT reduction activities (A and D), relative intensities of GFP fluorescence detergent released from GFP transfectants (B and E), and relative intensities of FITC fluorescence detergent released for CFSE-loaded cells (C and F). *x* axis, controls (C) without treatments; PC1 and PC2, cells exposed to 0.01, 0.1, and 1 μM PC1 or PC2 overnight; gray and white bars, samples with and without red light treatment, respectively; *y* axis, percentage of control for MTT reduction activities and fluorescence intensities by normalizing the values of the experimental groups against those of no-treatment controls. *P* values (*, 0.01 to 0.05; **, <0.01 to 0.001; ***, <0.001) were calculated by paired Student *t* tests for the data between dark (gray bars) and light (white bars), and for paired data connected by brackets. The data presented represent the means ± standard errors of the values obtained in triplicate for individual samples from representative experiments.

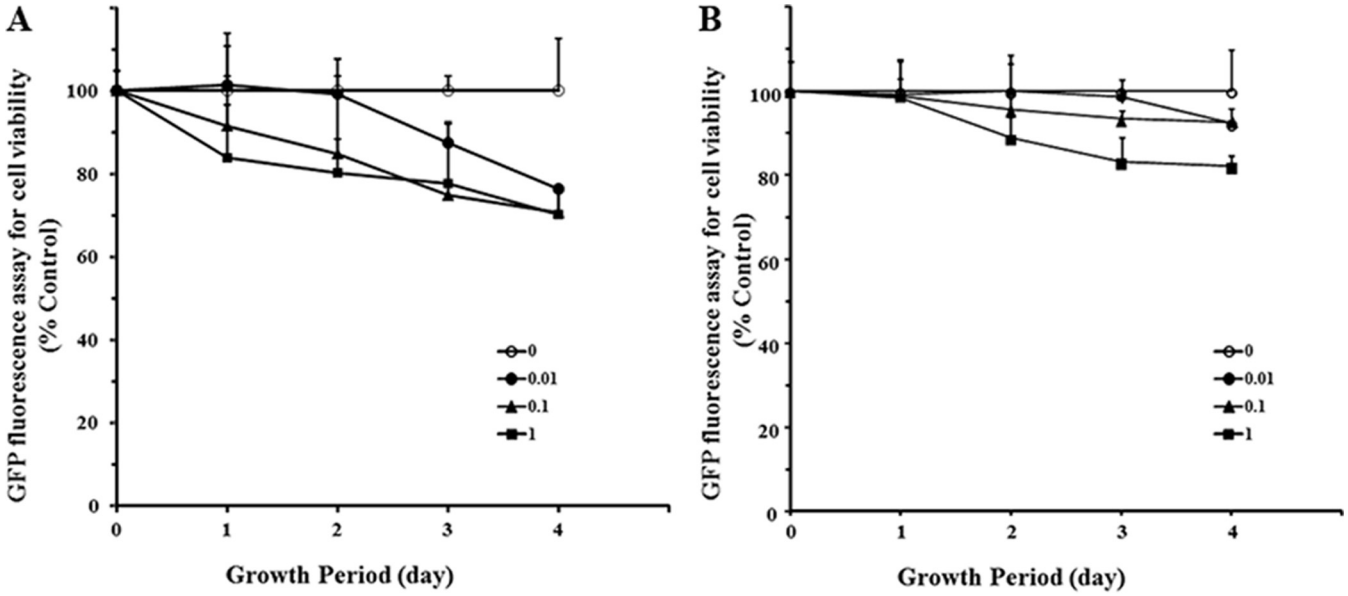

FIG 5 Effect of aminophthalocyanines on the growth of *L. tropica* promastigotes under dark conditions of different stringencies. (A and B) Cells grown under normally used and more-stringent dark conditions, respectively, as described in Materials and Methods. *x* axis, growth period in days after cultivation of GFP-promastigotes in the presence of 0 to 1 μM PCs; *y* axis, GFP fluorescence intensities in percentages of no-treatment controls, reflective of cell growth in graded PC concentrations. See Materials and Methods for the culture conditions used, daily collections of samples for storage as frozen pellets, and assay for cell growth based on fluorescence of detergent-released GFP. Note that cell growth is more affected under the normally used dark conditions (A) than under the more-stringent dark conditions (B). The data presented represent the means ± standard errors of the values obtained in triplicate for individual samples from representative experiments.

lished criteria for effective photodynamic inactivation of *Leishmania* species.

The uptake of PS by *Leishmania* is a prerequisite for its PDT effectiveness (18, 20, 23). PC1 and PC2 are clearly taken up by both stages of *L. tropica*, as evidenced by fluorescence microscopy (Fig. 1). The emergence of PC-positive vesicles near the flagellar pockets and lysosome-like multivesicular bodies and in distal vacuoles is a structural recapitulation of the endocytic pathway, as shown previously by using different PSs with similar properties, including PC14 and PC15 (23), and reconfirmed here by colocalization with FITC-dextran as an endosome marker (Fig. S3 in the supplemental material, colocalization). The uptake of all these PCs by *Leishmania* spp. is thus akin to the endocytosis of soluble molecules at the lining membrane of their flagellar pocket, where endosomes are formed for shuttling endocytosed cargo to lysosomes. The uptake of these PCs has saturation kinetics, consistent with endocytic activity (Fig. 2). The absence of PC fluorescence in the cytosol is indicative of very little, if any, uptake of PC1 and PC2 via transporters of the plasma membrane for soluble molecules, such as polyamines (30), consistent with the previous finding that spermidine did not compete with PC1 and PC2 for their uptake by mammalian cells (26).

PC1 and PC2 taken up by *L. tropica* effectively sensitize it to red light for photoinactivation. This is underscored by the facts that PC3 and PC4 are not taken up and have no photodynamic activity, consistent with previous observations using similarly ineffective PSs (18, 20, 23). Qualitative and quantitative data provide convincing evidence for PC1- and PC2-mediated photoinactivation of *Leishmania tropica*. This observation is indicated clearly by seeing red light-induced cellular changes of PC-loaded *Leishmania*, i.e., flagellar immobilization, cell disintegration, and loss of cellular GFP/CFSE fluorescence (Fig. 3). This loss of cell viability, as indicated in Fig. 3, is conclusively demonstrated quantitatively by three different assays, showing that it is PC dose dependent for both stages after red light exposure (Fig. 4). The use of three different methods lends credence to the obtained results. Some discordance of the data obtained from the 3 different methods is not unexpected, considering that they are based on different mechanisms, i.e., MTT reduction activity and detergent release of fluorescence from GFP/CFSE-labeled cells. While the MTT assay is a gold standard used extensively to assess cell viability (20, 23), it is less sensitive than the developed fluorescence assays (data not shown). The two fluorescence assays have relative merits for assessing anti-*Leishmania* PDT. GFP is sensitive as a protein to oxidative photodenaturation for a loss of fluorescence (20), but its use is limited by the necessity of producing transfectants for each species. In contrast, CFSE is universally applicable to any *Leishmania* species by one-step incubation, although the fluorescence is less detergent soluble, thereby reducing its sensitivity. Together, the qualitative and quantitative data presented leave no doubt that PC1 and PC2 are effective PSs for anti-*Leishmania* PDT.

The efficacy of PC1- and PC2-mediated photoinactivation of *Leishmania* is actually greater than what is reflected in the differences seen under the dark and light conditions used (Fig. 4). Unavoidable ambient light apparently contributes to photoinactivation of PC-loaded *Leishmania* spp. under the routine dark conditions used, since the growth inhibition under these conditions was reduced when dark conditions were controlled more stringently (Fig. 5). While dark-condition toxicity of PC1 and PC2 to *Leishmania* cannot be totally ruled out, it appears to be rather marginal in comparison to their anti-*Leishmania* photoinactivation.

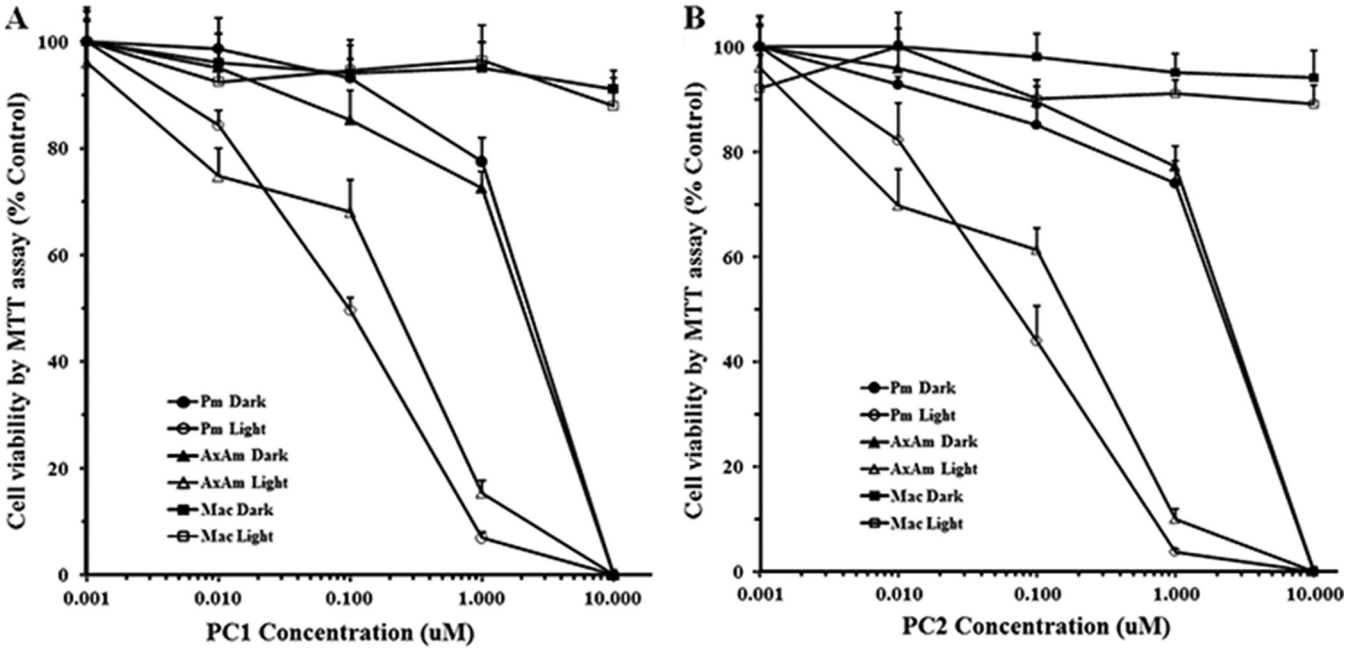

FIG 6 Disparity of *L. tropica* and macrophages in their susceptibility to PC-mediated photoinactivation. (A and B) Viability of J774 macrophages and both stages of *L. tropica* under light (open symbols) and dark (filled symbols) conditions after exposure to graded concentrations (0.001 to 10 μM) of PC1 and PC2, respectively. *x* axis, PC concentrations used from 0.001 to 10 μM; *y* axis, cell viability in percentage of untreated controls based on MTT reduction assays. AxAm light and dark (open and filled triangles, respectively), PC-treated axenic amastigotes with and without exposure to red light, respectively; Pm light and dark (open and filled circles, respectively), PC-treated promastigotes with and without exposure to red light, respectively; Mac light and dark (open and filled squares, respectively), PC-treated J774 macrophages with and without exposure to red light, respectively. See Materials and Methods for incubation conditions, MTT assay, and red light exposure for 20 min. The data presented represent the means ± standard errors of the values obtained in triplicate for individual samples from representative experiments.

For photoinactivation of *Leishmania*, the amino-PCs used here are 10 to 40 times more effective, depending on the *Leishmania* stage, than both the previously examined endosome-targeting pyridyloxy-PCs and mitochondrion-targeting anilinium PCs (23), as indicated by comparing the EC_{50}s of PC1 and PC2 (Fig. 6) versus those of PC14 and PC3.5 (23) (Fig. 2). Even more striking are the host-parasite differential sensitivities to these PCs for photoinactivation, as reflected in their EC_{50}s greatly in favor of anti-*Leishmania* PDT by >100 times for PC1 and PC2 (Fig. 6) but only ~3 times for PC14 and negative against *Leishmania* by 10- to 20-fold for PC3.5 (23). The differences of these PCs in mediating *Leishmania* photoinactivation are due neither to the use of different *Leishmania* spp. (data not shown) nor to their photodynamic properties *per se*, e.g., quantum yields (26), but apparently are due to their differences in the axial substitutions with different ligands. The axial substitution of the Si-PC with 2 symmetrical mono- or di-amino groups (see Fig. S1 in the supplemental material) instead of pyridyloxy groups (23) appears to significantly improve its endocytic bioavailability (cationicity and solubility) to *Leishmania* cells for photosensitization in an aqueous milieu. While the precise mechanisms remain unknown, empirical observations showed that PC1 and PC2 stayed soluble longer in the culture medium than the other PCs. Also unknown is the reason for the unexpected finding of the huge host-parasite disparity in susceptibility to PC1- and PC2-mediated photoinactivation under the experimental conditions used. At a greater fluence of illumination, PC1 and PC2 have been shown to inactivate mammalian cells other than macrophages (26). It awaits further study to determine if the macrophages are more PDT resistant because these phagocytes undergo respiratory burst and thus may be equipped with a higher capacity of antioxidant mechanisms than other mammalian cells.

In summary, the results obtained identify PC1 and PC2 as excellent PSs for oxidative photoinactivation of *Leishmania* for use as potential vehicles in drug/vaccine delivery, considering that they are up to 40 times more effective than those used previously (23). In addition, the finding that *Leishmania* spp. are >100 times more susceptible than macrophages to sensitization by PC1 and PC2 for photoinactivation suggests their potential utility for anti-*Leishmania* PDT, pending further evaluation in *in vitro* and *in vivo* models of experimental leishmaniasis.

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