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Combinational Sensitization of *Leishmania* with Uroporphyrin and Aluminum Phthalocyanine Synergistically Enhances their Photodynamic Inactivation *in vitro* and *in vivo*[†]

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Abstract

Leishmania were previously shown to undergo photolysis when their transgenic mutants were induced endogenously to accumulate cytoplasmic uroporphyrin or when loaded exogenously with aluminum phthalocyanine chloride. A combinational use of both is reported here, which renders *Leishmania* far more susceptible to photolysis. Fluorescence microscopy of cells loaded with the two photosensitizers localized them to different subcellular sites. Pre-exposure of *Leishmania* to both synergistically sensitized them for photolysis as extracellular promastigotes and as intracellular amastigotes in infected macrophages *in vitro* when illuminated at specific wavelengths to excite the respective photosensitizers for production of reactive oxygen species. Both *Leishmania* stages lost their viability completely when doubly photosensitized optimally and illuminated at low intensity, the host cells being left unscathed. Inoculation of mice with photo-inactivated *Leishmania* produced no lesions, which invariably developed in the control groups during a period of observations for eight weeks. Pre-treatment of *Leishmania* with both photosensitizers rendered these cells susceptible to clearance from the ear dermis by white light illumination. The results suggest that double photosensitization for synergistic activity enhances the efficacy and safety of photodynamic therapy in general and for *Leishmania* in particular.

Introduction

Photodynamic therapy (PDT) uses photosensitizers (PS) to generate cytolytic reactive oxygen species (ROS) in the presence of atmospheric oxygen for clinical treatment of skin tumors and other cutaneous diseases (1). PS can be delivered exogenously, such is the case with phthalocyanines or produced endogenously, for example, with delta-aminolevulinate (ALA) for over-production of porphyrins in the target cells (2). Illumination of PS-loaded diseased cells/tissues results in their destruction via the generation of powerful cytolytic ROS, which attack multiple cellular targets, making it unlikely to select for resistance – a universal problem in radio- and chemotherapy of all diseases (3). PDT is potentially usable to treat all infectious and non-infectious diseases, provided that the PS can be targeted specifically to the causative agents or diseased cells, thereby minimizing collateral damage to the surrounding healthy cells/tissues (4, 5).

We have begun to explore a novel strategy of targeting PS by exploiting the unusual mechanism of *Leishmania* parasitism. In mammalian hosts, these trypanosomatid protozoa find their way to parasitize specifically the phagolysosomes of macrophages and other

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antigen-presenting cells (APC), i. e. the dendritic cells (6, 7). *Leishmania* spp. are genetically deficient in heme biosynthesis, making it possible to produce transgenic mutants, which are inducible with ALA for accumulation of uroporphyrin I (URO) as a PS (8–10). Selective photolysis of these uroporphyrin mutants (DT) in the APC phagolysosomes was found to ensue by illumination of infected APCs with dim light. This outcome not only indicates the susceptibility of *Leishmania* to PDT but also suggests the potential use of such photo-inactivated mutants to release drugs/vaccines in the phagolysosomes for activation/presentation in chemo-/immuno-therapy and – prophylaxis (8–10, 14). Similarly, pre-loading of *Leishmania* with externally supplied ALPhCl or other phthalocyanines has the potential to achieve the same aims (11–12).

In the present study, we explore a combinational approach by loading the *Leishmania* DT mutants both endogenously with URO via the use of ALA and exogenously with ALPhCl. This approach is expected to enhance photodynamic inactivation of these mutants, thereby improving their utility after such modification as an efficient and safe vehicle for drug/vaccine delivery in future application. We report here a synergistic activity of the URO/ALPhCl combination in photosensitization of *Leishmania* for photolysis to completion under both the *in vitro* and *in vivo* conditions used.

Materials and Methods

Cells and mice

Uroporphyrinogenic mutants of *Leishmania amazonensis* (RAT/BA/74/LV78) clone 12-1, doubly transfected with pX-*alad* and p6.5-*pbgd* (DT) (8–10), were routinely grown as promastigotes at 25° C in Medium 199 (Sigma) buffered with 25 mM HEPES to pH 7.4 and supplemented with 10% heat inactivated fetal bovine serum (HIFBS). Mutants were placed under the selective pressures of tunicamycin and G418 to express ALAD and PBGD, the 2nd and 3rd enzymes in the heme biosynthetic pathway (13). Before exposure of these DT mutants to ALA for inducing cytoplasmic accumulation of uroporphyrin I (URO), they were briefly grown in drug-free medium to stationary phase to avoid the potential cytotoxicity of the drugs carried over to the host cells.

Mouse macrophages of the J774 line were grown at 35° C in RPMI 1640 supplemented with 10% HIFBS. Male BALB/c X C57BL/6 mice (~ 25 gm, 8–12 weeks old) were used for the *in vivo* studies.

Leishmania infection of macrophages—J774 cells were infected with *Leishmania* by mixing a suspension of these cells and that of the DTs in RPMI 1640+20% HIFBS at a host-parasite ratio of 1:10, i. e. 10^6 host cells and 10^7 DTs/ml. The mixture was plated at 4 ml/25 cm² TC flask or 1 ml/well in a 12 well plate. The host cell-parasite mixtures were incubated for 16–24 hrs at 35° C for adhesion of the J774 cells to the substratum and their infection by the DTs. Infected cultures were subsequently maintained under the same conditions with daily medium renewal. To estimate the infection quantitatively, cells were flattened under a glass coverslip and examined under phase contrast with a 100X oil immersion lens for tallying infected and non-infected cells, and the total number of intracellular amastigotes in up to 100 infected cells. The values so obtained were used to estimate the total parasite load per culture, i. e. [total number of macrophages per flask] × [% infected cells] × [average number of *Leishmania*/host cell] (12).

Leishmania infection of mice—All mice were housed in the animal facility of Rosalind Franklin University and handled per IACUC-approved protocol (no. # 05-02). Mice were anaesthetized under isoflurane vapor and each inoculated subcutaneously with 10^6 – 10^7 stationary phase promastigotes in 20–100 ul of phosphate buffered saline. Mice were

grouped at 4–6/cage according to the sites of inoculation, i. e. tailbase, footpad and ear dermis. Mice were monitored twice weekly for development of lesions, which were measured, if present, with a Vernier caliper. At the conclusion of the experiments, i. e. 8 weeks, lesions or tissues at the injection sites were excised sterilely and emulsified in a dounce homogenizer. The homogenates were cleared of tissue debris by passing through a nylon mesh. Parasite loads in the homogenates were assessed first by microscopic counting of amastigotes in a hemocytometer and then by their growth for 2 weeks after limiting dilutions of the homogenates in the complete medium.

Phototsensitization of Leishmania with URO and AlPhCl

In Vitro: For cytoplasmic URO accumulation, DT mutants at 50×10^6 cells/ml were exposed to 1 mM ALA in Hank's Balanced Salt Solution (pH 7.4) + 0.01% BSA (HBSS +BSA) for ~48 hrs at 25° C in the dark (8–10). Control DT mutants were simultaneously prepared under the same conditions without ALA (Sigma). Aluminum phthalocyanine chloride (AlPhCl) (Sigma) was added to both uroporphyrin and non-uroporphyrin DT preparations in 10X serial dilutions of 0.01–1 µg/ml to produce URO+AlPhCl double and URO or AlPhCl single photosensitization. Double photosensitization of uroporphyrin DTs by exposing them to 0.01 and 0.1 µg AlPhCl/ml was referred to heretofore as Protocols 1 and 2, respectively. Uroporphyrin DTs without further exposure to AlPhCl served as the URO single photosensitization. All cells were treated overnight in the dark and washed by centrifugation at $3,500 \times g$ for 5 min at 4° C thrice in HBSS to remove extracellular ALA and/or AlPhCl. Since the exposure of these cells to PS in the dark *per se* produced no adverse effect, they were incubated overnight to optimize the loading efficiency. Non-photosensitized cells without any treatment or exposed to light alone were prepared similarly in HBSS+BSA. All cells were finally resuspended in HBSS+BSA at a cell density of 10^8 cells/ml before use.

Photosensitivity of the doubly sensitized DTs was further studied as intracellular amastigotes in macrophages. J774 macrophages were infected with URO+AlPhCl-loaded DTs (as described above). The infected cells were further exposed to 1 mM ALA for 24 hrs to boost the URO loading of intracellular DTs. Infected cultures were washed and incubated in ALA-free complete media for 1–2 days. All photosensitized cultures were incubated in the dark and handled when necessary under minimal light exposure until completion of the experiment.

In vivo: Mice (4–6 per group) were inoculated with photosensitized and control DTs as described above. One day after inoculation, ALA was injected at the same site to foster uroporphyrinogenesis of the intracellular DTs.

Illumination of photosensitized Leishmania—Suspensions of photosensitized and control cells were placed at 10^8 cells/ml/well in 12 well tissue culture plates and illuminated in two different ways: [1] sequentially with a long wave UV lamp (366 nm) from the top (with the lid of the culture plates removed) for 20 min (9, 10) and red light (>650 nm) (2.5 mW/cm^2) from the bottom for 5 min (fluence = 0.75 J/cm^2) (12) to photodynamically excite URO and AlPhCl, respectively. A fluorescent light box covered with a red filter (11) was used as the source of the red light; and [2] cells were also illuminated with white light at a distance of 5 cm from the bottom of the well by using an optic cable, consisting of 75 end-emitting fibers, connected to a 250 W metal halide lamp as the light source (Eclipse II; Supervision) (10 J/cm^2) (12). This light source was also used to spot-illuminate the site that was inoculated with photosensitized and control DTs in mice. The fiber optic cable was placed 5 cm away from the skin to spot-illuminate the injection sites each for 20 min to deliver a fluence of 50 J/cm^2 . Under all conditions of illumination used, samples were not

subjected to heating above the ambient temperature, as determined by reading a thermometer placed in the same location for the same duration.

Cell viability assay—The viability of cells treated *in vitro* was assessed in 2 different ways: [1] microscopic assessment for the loss of cell integrity based on their permeability to propidium iodide. Cells were stained with propidium iodide (50 ug/ml) for 5 min on ice and washed thrice by centrifugation. Aliquots were mounted on glass slides, covered with cover slips and observed first under phase contrast and then epifluorescence using specific filter sets for propidium iodide (9). >500 cells were examined in randomly chosen fields. DTs were considered as dead when permeable to propidium iodide, as indicated by the fluorescence of their nuclei. These dead cells were counted to estimate their proportion against the total number of cells examined. [2] aliquots of treated samples were each inoculated at a cell density of 10^6 cells/ml in complete culture media for growth as promastigotes. Cultures were incubated for up to 5 days and viability assessed by MTT assay (9, 11).

The viability of treated *Leishmania* in mice was assessed by their ability to produce a lesion during an 8-week period. Parasite loads were estimated at the end point, as already described in “*Leishmania* infection of mice.”

Estimation of cell-associated URO and AIPhCl—Both URO and AIPhCl were extracted after optimal cell-loading conditions from the pellets of known cell numbers for fluorimetric evaluation of their amounts per cell population. URO was extracted with a mixture of perchloric acid/methanol and quantitatively assessed as described (8, 10). AIPhCl was extracted by vigorous vortexing of the cell pellets with a mixture of chloroform and methanol (ratio 2:1 v/v) (200 ul). Samples were cleared by centrifugation at 13,000 g for 15 min. AIPhCl in the clear supernatants was estimated by comparison to a standard curve of chemically pure AIPhCl in serial dilutions versus their fluorescence intensities (640 nm excitation/660 nm emission wavelength).

Fluorescence microscopy of URO- and AIPhCl-loaded cells—Aliquots of photosensitized and control cells were observed under living conditions in a Nikon Eclipse 80i microscope fitted with appropriate filter sets: D405/10 (405 nm exciter), Q485DCXR (485 nm dichroic) and RG610LP (610 nm emitter) (Chroma Tech Co., Brattleboro, VT) for URO; and HQ620/60(620-nm exciter), Q660LP (660-nm dichroic) and HQ700/80 (700-nmemitter) for AIPhCl. Images were captured with a Cool Snap ES camera in conjunction with Metamorphosis image acquisition software (version 6.2r6).

Statistical analysis—All experiments were repeated at least twice with triplicate samples for each treatment. Student t-test and one-way ANOVA were used to calculate statistical significance of the data where appropriate.

Results

AIPhCl and URO photosensitize *Leishmania* at different subcellular sites

Loading of live *Leishmania* with externally supplied AIPhCl produced a distinct pattern of cellular fluorescence, which differed from what was produced by ALA-induced accumulation of URO in the DTs (Fig. 1A versus B). Incubation of these cells with AIPhCl alone resulted in cellular fluorescence that outlined the cell body, flagellar reservoir, nucleus and other unidentified intracellular cellular structures, but not the flagella (Fig. 1A phase contrast versus fluorescence). The cellular structures were clearly, albeit incompletely, delineated by fluorescence of intermittent intensity. Treated cells retained this pattern of

cellular fluorescence when exposed to increasing concentrations of AlPhCl up to 1 $\mu\text{g/ml}$. AlPhCl is hydrophobic, suggesting that it is associated with cellular membranes, accounting for the fluorescent image observed. In contrast, the endogenously produced URO gave a diffused pattern of cellular fluorescence, consistent with the previous findings (9, 10) in that it is present in the cytosol of these cells, their flagella, and in their endocytic vacuoles (Fig 1B). URO is distributed unequally in these cellular compartments and is apparently absent in some small vesicles, accounting for the patchy pattern of cytoplasmic fluorescence. These two PS's thus localize at two distinctly different subcellular compartments.

Sensitization of *Leishmania* with AlPhCl and URO for their photolysis is more effective when used in combination than individually in vitro

Double photosensitization of *Leishmania* DTs with AlPhCl and URO makes it possible to photolyze them in both stages to completion by illumination under the conditions used. No cytotoxicity was noted after loading of these cells with either or both of the 2 PS's in the dark. DT promastigotes so treated remained microscopically as intact and motile as untreated controls, barring photo-inactivation by prolonged exposure to microscope illumination. These cells were doubly photosensitized under the conditions, which were previously optimized for single pre-sensitization of promastigotes with the respective PS's, i. e. AlPhCl (12) or URO (9, 10). The viability of these sensitized cells was initially assessed 2 hrs post illumination on the basis of their permeability to propidium iodide for nuclear fluorescence (Fig. 2A [I], [II]). Sequential exposure of the singly sensitized cells to longwave UV and then red light under the described conditions did not result in their complete loss of viability, but reduced it to ~17% and ~3% of the control with the higher concentration (0.1 $\mu\text{g/ml}$ overnight) of AlPhCl used for loading (Fig. 2A [II] [b] versus [a]) and the URO accumulated by exposure to ALA (Fig. 2A [c] versus [a]), respectively. This differential outcome is not unexpected, considering the difference in the loading efficiencies calculated for the 2 PS's, i. e. 500 versus 28 pmoles/ 10^8 cells for URO and AlPhCl, respectively. Notably, pre-sensitization of cells with AlPhCl alone with increasing concentrations from 0.01 to 0.1 $\mu\text{g/ml}$ did not proportionally increase their photolysis; namely, a ~10-fold increase in the cellular loads of AlPhCl from ~2.4 to 28 pmoles/ 10^8 cells gave only a 5-fold decrease of their viability from ~87% to ~17% of the control (Fig 2A [I] [b] vs [II] [b]). Significantly, a complete loss of cell viability after illumination, not attainable by any of the single pre-sensitization conditions used, was achieved by double pre-sensitization according to protocol 2, i. e. a combination of URO and the higher concentration (0.1 $\mu\text{g/ml}$) of AlPhCl used for loading (Fig. 2A [II] [d]). The profiles of cell viability under the various conditions described were generally consistent with the patterns of cell growth after inoculation of treated cells into complete culture media (Fig. 2B [I], [II]). Cell viability assessed by MTT assay after incubation for 1 day also showed no survivors after Protocol 2 double photosensitization (Fig. 2B [II] [d] protocol 2 versus the rest). This negative outcome was further verified after prolonged incubation of these cultures for up to 5 days. Similar results of synergistic sensitization for photolysis were also obtained when the sequential illumination regimen of longwave UV and red light was replaced with white light at a fluence of 10 J/cm^2 (not shown).

Double pre-sensitization of *Leishmania* DTs with AlPhCl+URO also synergized their photolysis in macrophages by illumination of the infected cultures (Fig. 3A solid circle vs rest of the groups for all the other experimental conditions). DT promastigotes subjected to Protocol 2 double photosensitization (Fig 2 [II] [d]) were found to infect J774 macrophages as well as the control groups, all giving a total number of intracellular parasites of $\sim 10^7$ per culture 3 days after infection (Fig. 3A, Day 3). After incubation for two days, the infected macrophages were pulsed for 24 hrs with another dose of ALA (1 mM) (Fig. 3, arrow) to further boost the URO loading of the uroporphyrin parasites (Fig. 3A). These cultures were

incubated for 1 day in ALA-free media to clear the small amounts of porphyrins in the host cells, resulting from their ALA-induced porphyrinogenesis. After illumination on day 4 with white light at 10 J/cm^2 (Fig. 3, arrow head), the parasite loads of infected cultures were assessed every 3–4 days up to day 12. The results showed that illumination cleared the parasite loads progressively to completion from the cultures with the doubly pre-sensitized DTs (Fig. 3A, solid circle), but not from those with singly pre-sensitized DTs (Fig. 3A, open circle and open triangle). Parasite loads remained essentially unchanged or marginally decreased in all the other control groups, including singly and doubly pre-sensitized DTs without exposure to light (Fig. 3A, open square and solid triangle). These observations were verified by the results from the growth of *Leishmania* in suitable media. Promastigotes grew from all samples, except those which were infected with protocol 2 doubly pre-photosensitized DTs. Throughout the 12 day period of observation, the host cells were not significantly affected by any of the treatments used (Fig. 3B). Double photosensitization of *Leishmania* thus synergized their photolysis not only as extracellular promastigotes but also as intracellular amastigotes in infected macrophages.

Doubly photosensitized *Leishmania* were susceptible to white light-mediated lysis in vivo

—During a period of observations for 8 weeks, DTs doubly pre-photosensitized according to protocol 2 produced no visible lesion in mice after inoculation into their tailbase followed by an ALA boost (Fig. 4A, long arrow) and spot-illumination (short arrow) at the site of injection (Fig. 4A, open triangle). Lesions invariably developed during the same period of observation among all the control groups included. The onset of their lesion development varied with the treatments, starting on week 2 with the untreated samples or light alone (Fig. 4A, solid square) and +URO-Light (open square), on week 3 with those of +AlPhCl-URO+Light (open circle), on week 4 with those of +URO+Light (solid square) and on week 6 with those of the protocol 1 AlPhCl+URO+Light (solid triangle). In all control groups for the duration of this study, lesions increased in size progressively, reaching 6–7 mm in diameter, except those produced by DTs doubly pre-sensitized by Protocol 1, in which case a much smaller lesion of ~2 mm in diameter was observed (Fig. 4A solid triangle). The differences in the lesion size were already notable on week 6 among the major groups, i. e. +URO-Light > +URO+Light > +AlPhCl (1)+URO+Light > +AlPhCl (2)+URO+Light (Fig. 4B, arrow head pointed to lesions). The results obtained demonstrated the synergistic activity of AlPhCl+ URO to enhance the photosensitivity of *Leishmania in vivo*, as indicated by suppressing their ability to produce lesions in mice.

After the same 8 week period, no parasite loads were detectable after injection of mice with DTs, which were doubly pre-sensitized and spot-illuminated *in situ* under the same regimen, but only at a dose of 10^6 DTs/site in the ear dermis (Fig. 5 [II] Ear). When inoculated with the doubly pre-sensitized DTs at 10^7 /site, subsequent spot-illumination of the injection sites decreased the parasite loads in the order of ~1 log in all sites examined, i. e. tailbase, footpad and ear (Fig. 5 [I] blank vs black bars). The reduction in the parasite loads to this extent is apparently sufficient to account for the difference that was observed in the development of a small lesion or no lesion by the doubly sensitized DTs (Fig. 4A–B). When the inoculum was reduced by 10-fold to 10^6 doubly pre-sensitized DTs/site, subsequent spot-illumination dropped the parasite loads dramatically at an order of 2 logs in the tailbase and footpad, and to an undetectable level in the ear (Fig. 5 [II] solid vs blank bars). Cultivation of the ear tissue homogenates yielded no promastigote growth, confirming the absence of viable DTs at this inoculation site under the conditions described. The synergistic activity of URO +AlPhCl thus sensitized DTs *in situ* for photo-inactivation not only to prevent them from producing lesions but also their elimination, even though this occurred at the specific site of ear dermis inoculated with a smaller cell number.

Discussion

In the present study, we demonstrate for the first time that loading of *Leishmania* with 2 different PS's (Fig. 1) synergistically sensitize them for photolysis to completion under both *in vitro* and *in vivo* conditions used (Figs. 2–5). Here, synergy is defined in the biological context; namely, the 2 PS's function together to produce specific photolysis of *Leishmania* that is not obtainable by using either PS alone. This definition is analogous to the synergistic effects, which were described for the finding that tumoricidal activities were enhanced by using two laser dyes with different intracellular targeting sites, i.e. rhodamine-123 and merocyanine-540 (15). In our case, *Leishmania* DTs were doubly loaded with URO that was induced to accumulate optimally via their exposure to 1 mM ALA (8–10) and with AlPhCl, which was also optimized previously to the loading concentrations of 0.1 µg/ml that is non-toxic to the host cells (12). The synergistic effect was clearly shown by the absence of any viable *Leishmania* after double photosensitization, but not single photosensitization under the same illumination conditions used, i. e. longwave UV+red light or white light. The simplest explanation for the observed synergism may lie in the differences in the photodynamic and other properties between URO and AlPhCl. Indeed, these 2 PS's sensitize *Leishmania* at different subcellular sites (Fig. 1). In addition, it is known that they are photo-excitabile at different wavelengths to produce different primary species of ROS. Thus, illumination of the doubly pre-sensitized cells may hypothetically generate more ROS both in quantity and quality, which attack more targets in these cells than those singly pre-sensitized. Moreover, the application of 2 different PS's is less likely than single photosensitization to leave cells in the population as a whole un-sensitized. Indeed, loading of *Leishmania* DTs via either ALA-induced uroporphyrinogenesis or exposure to externally supplied AlPhCl at the concentrations used is stochastic, always leaving a small percent of non-sensitized and thus photolytically insensitive cells in the population (9, 12). Double photosensitization of *Leishmania* must have eliminated such cells altogether or reduced them to a negligible number to account for the complete photolysis observed.

The *in vivo* results obtained in mice warrant further discussion beyond their provision of evidence for the synergism of double photosensitization. The doubly pre-sensitized *Leishmania* as promastigotes and as amastigotes in infected macrophages are evidently cleared completely *in vitro* by sequential illumination with longwave UV and red light or white light, as shown by microscopy and cultivation (Figs. 2–3). This is also the case *in vivo*, but only for those inoculated into the mouse ear dermis illuminated with white light (Fig. 5). Photolytic clearance of the parasites from this site, but not from the footpad and tailbase, is perhaps attributable to the translucency of ear dermis to illumination. However, since the parasite loads produced by a larger inoculum were reduced, but not cleared by illumination at this site, it still presents a barrier to white light, despite its spectrum includes both URO and AlPhCl-excitabile wavelengths. The results obtained provide information of relevance to further investigation in photodynamic vaccination. Of interest to determine is whether photolysis of uroporphyrin DTs after additional photosensitization with AlPhCl used here or other effective phthalocyanines (11) would trigger an immune clearance of parasites that may survive in the ear dermis or escape from that site to other places, e. g. lymph nodes after the 8 week experimental protocol used in our study. Previously, vaccination of hamsters with uroporphyrin DTs photodynamically lysed *in vivo* was shown to protect them from challenges with Indian *L. donovani* by eliciting immunity that is adoptively transferrable with splenic lymphocytes to naïve animals (14). It will be of interest to evaluate this by using doubly sensitized *Leishmania* in the mouse model, for which experimental conditions are expected to differ from those used for the *L. donovani*-hamster model.

In summary, results presented demonstrate that sensitization of *Leishmania* with 2 different PS's acts synergistically to produce an outcome of complete photolysis in contrast to single photosensitization both *in vitro* and *in vivo*. This approach will help us in future experimental designs for assessing the efficacy and safety of photo-inactivated *Leishmania* as a potential carrier for drug/vaccine delivery to the phagolysosomes of APC for eliciting effective photodynamic immune-therapy and –prophylaxis (8–12, 14).

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Abbreviations

ALA	Delta-aminolevulinate
AlPhCl	Aluminum phthalocyanine chloride
DT	Uroporphyrinogenic <i>Leishmania</i>
PS	Photosensitizer
ROS	Reactive oxygen species
ST	Control transfectants
URO	Uroporphyrin I

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Fig. 1. Cellular localization of aluminum phthalocyanine chloride (AlPhCl) and uroporphyrin I (URO) in *Leishmania amazonensis* DT promastigotes

[A] DT promastigotes exposed in the dark to AlPhCl at 1 µg/ml for 16 hrs; and [B] DT promastigotes exposed in the dark to ALA (1 mM) for 48 hrs. Left panel, Phase contrast; Right panel, fluorescence microscopy. Note: membrane association of AlPhCl versus cytoplasmic accumulation of uroporphyrin I. Bar scale = 10 µm.

Fig. 2. Synergistic sensitization of *Leishmania amazonensis* DT promastigotes with ALPhCl and URO for their photolysis

[A] and [B]: Viability of uroporphyrinic *Leishmania* decreased by additional photosensitization with increasing concentrations of ALPhCl followed by illumination at wavelengths specific to the 2 photosensitizers. [a] Non-photosensitized control DT promastigotes; [b] DTs photosensitized with ALPhCl by incubation with 0.01 (Protocol 1) and 0.1 (protocol 2) $\mu\text{g/ml}$ in [I] and [II], respectively; [c] DTs exposed to 1 mM ALA for cytoplasmic URO accumulation; [d] DTs photosensitized with both PS's under the conditions as described. The values given at the bottom denote the cellular amounts of ALPhCl and URO at pmoles/ 10^8 cells calculated as described in the Materials and Methods.

All samples were illuminated sequentially with long wave UV (max 366 nm) and red light (>650 nm) for photodynamic excitation of URO and AlPhCl, respectively. The % cell viability was determined by: [A] propidium iodide permeability assays 2 hrs after illumination; and [B] MTT assay after culturing aliquots of the treated cells under promastigote culture conditions for 24 hrs. *p* values were calculated by paired Student-t tests.

Fig. 3. Selective and complete photolysis of intracellular DTs doubly photosensitized with URO and AIPhCl

Leishmania DT promastigotes pre-photosensitized under optimal conditions with URO and/or AIPhCl were used to infect J774 macrophages. About 2 days after infection, all cultures were again pulse-exposed to 1 mM ALA (arrow) for 24 hrs. 1 day after removal of ALA, infected cultures were exposed to white-light (10 J/cm^2) (arrow head) for 1 hr. Infected cultures not exposed to ALA (-URO) or light (-Light) served as controls. [A] The parasite loads and [B] the total number of the macrophages per culture were quantitatively estimated at the time points under various conditions ($\pm \text{URO} \pm \text{AIPhCl} \pm \text{light}$), as indicated; +AIPhCl(2) denotes conditions according to Protocol 2 in Fig. 2. p values were calculated by one-way ANOVA.

Fig. 4. Photodynamic inactivation of doubly photosensitized *Leishmania* in vivo.

[A] Mice in groups of 4–6 were each inoculated (10^7 parasites/tailbase) with control DTs and those photo-inactivated under conditions, as indicated in Fig. 4A ($\pm$ AIPhCl $\pm$ URO $\pm$ Light). Each site was injected with 100 μ l of 100 mM ALA 1 day after inoculation and spot-illuminated with white light (50 J/cm^2) 36 hrs thereafter. Lesions were measured weekly and the values of their diameter plotted with time for a period of 8 weeks, as shown. p values were calculated by one-way ANOVA. [B] Photographs of the lesions on week 6 produced by *Leishmania* treated under different conditions as indicated.

Fig. 5. Clearance of doubly photosensitized *Leishmania* in ear dermis by spot-illumination. Mice in another group of 4 were each inoculated with 10^7 (panel [I]) or 10^6 (panel [II]) of doubly and optimally pre-sensitized DTs (+AIPhCl(2)+URO) at different sites/group as indicated. Each site received another 10 μ l of 1 M ALA 2 days later and processed with (solid bar) and without (blank bar) spot-illumination ($\pm$ white Light at 50 J/cm²) after another 3 days. All groups of mice were humanely euthanized at week 8 in order to determine parasite loads (see Materials and Methods). *p* values were calculated by paired Student-t tests. #: $p < 0.05$ and *: $p < 0.01$.