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In vitro phototoxicity of ultradeformable liposomes containing chloroaluminum phthalocyanine against New World *Leishmania* species

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ABSTRACT

The use of photodynamic therapy (PDT) against cutaneous leishmaniasis (CL) based on chloroaluminum phthalocyanine (ClAlPc) is a promissory alternative therapy. The main purpose of this article was to assess the internalization and *in vitro* phototoxic activities of ClAlPc encapsulated in ultradeformable liposomes (UDL-ClAlPc) in *Leishmania* parasites and mammalian cells. Cell internalization was determined by fluorescence microscopy, cell and parasite damage by standard MTT or direct microscopic analysis and a phototoxic index (PI) was calculated as the compound activity (IC_{50}) at 0 J/cm^2/IC_{50} at 17 J/cm^2. Liposomal and free ClAlPc were internalized by infected and non-infected THP-1 cells and co-localized in the mitochondria. Treatment of UDL-ClAlPc was almost 10 times more photoactive than free ClAlPc on THP-1 cells and promastigotes and intracellular amastigotes of *Leishmania chagasi* and *Leishmania panamensis*. Liposomal compounds were active on non-irradiated and irradiated cells however PI higher than 50 were calculated. PI for amphotericin B referential drug were lower than 1.2. Empty liposomes tested at the same lipid concentration of active ClPcAl-liposomes were non-toxic. Upon photodynamic treatment a nonselective-parasite activity against intracellular amastigotes were observed and loss of membrane integrity resulting in a release of parasites was detected. Further studies oriented to evaluate both the state of infection after PDT and the effectiveness of UDL as delivery vehicles of ClAlPc in CL experimental models are required.

1. Introduction

Leishmania is an intracellular parasite belonging to the genus *Leishmania* that lives in phagosomal compartment of macrophages of vertebrate host and transmitted to humans via the bite of sandflies. Leishmaniasis is a severe public health problem globally where 350 million people are at risk of contracting the disease. Twelve million are currently infected and an estimated 2 million new cases occur annually [1]. The infected humans develop a wide range of clinical manifestations that include cutaneous, mucocutaneous and visceral forms [2]. Cutaneous leishmaniasis (CL) usually is caused by *Leishmania major* and *Leishmania tropica* in the Old World and *Leishmania* (*Viannia*) *braziliensis*, *Leishmania* (*V*) *panamensis* and *Leishmania mexicana* in the New World. Infected patients typically develop mild to severe ulcerative lesions limited to a single (localized leishmaniasis) or to various sides of the skin resulting in severe disfigurement, disability, and social psychological stigma [1,2].

Pentavalent antimonies (Sb^V) followed by amphotericin B (AmB), pentamidine isethionate, paromomycin and miltefosine constitute the major drug options for the disease. These drugs present significant side-effects, are limited, toxic, expensive and with variable effectiveness [3,4]. Efforts for developing antileishmanial drugs have been directed mainly towards the search of treatment against the visceral form of the disease. Such efforts include the use of AmB liposomal (AmBisome®), oral administration of miltefosine and clinical trials in phase III of parenteral paromomycin [5,6]. In the case of CL a new topical formulation of paromomycin (WR279, 963), for the treatment of localized CL is evaluated in multicenter phase III clinical trial in Tunisia [7], and the intravenously use of AmBisome in CL infected patients [8]. However, the need to evaluate different protocols of treatment, new formulations and other alternative therapies for CL is mandatory [9–11].

Photodynamic therapy (PDT) is a promissory treatment used in neoplastic and skin diseases [12,13]. In this therapy a photosensitiser (PS) in presence of molecular oxygen is excited with visible light inducing the formation of reactive oxygen species (ROS),

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which are highly toxic to the cells and targeted tissues. PDT has been evaluated against CL in both experimental models and clinical trials. Different PS such as porphyrins, benzophenoxazine derivate, phthalocyanines (Pc) and endogenous protoporphyrin induced by 5-delta aminolevulinic acid (PpIX-ALA) have been photoactive against different species of *Leishmania* [14–23]. ClAlPc is a second-generation of PS, chemically stable and exhibit suitable photo physical properties (such as high triplet quantum yields and long triplet lifetimes) for medical procedures. Although it has a high molecular weight, their hydrophobic nature allows easily the interaction with bilipid layers. It has been used successfully in cancer with a great margin of safety in clinical trials [24,25]. In leishmaniasis, ClAlPc have been active *in vitro* against New World *Leishmania* species after light irradiation [20,21].

The performance of a PS as an antileishmanial drug for topical application can be improved if loaded into a suitable carrier for transdermal delivery that can also change the way of its internalization by the infected macrophage population. Liposomes are colloidal vesicles ranged in size from ~50 to 1000 nm in diameter as delivery vehicles of some antileishmanial drugs such as AmB, Sb^V, miltefosine [26–28]. They are composed by one or more bilayers of lipids with neutral, positive, or negative charge and are able to transport hydrophilic or lipophilic molecules into the tissues and cells. They are distinguished on the basis of their size and the number and arrangement of their lipid bilayers. Ultradeformable liposomes (UDL) [29], are unilamellar liposomes composed by phospholipids and an edge activator that can improve the transcutaneous delivery of active principles. The unique ability of these specially designed liposomes relies on their lower elastic modulus in comparison to conventional liposomes [29], which allows the UDL to be capable of penetrate through the *stratum corneum* (SC) driven by the transepithelial humidity gradient [30], and shuttle their inner aqueous content to several tens of microns into the viable epidermis [31], instead of aggregate or coalesce on the skin surface as conventional liposomes do [32].

The use of ClAlPc loaded into UDL could facilitate its access to *Leishmania*-infected cells, increase its photo activity by conserving its monomeric structure and reduce toxicity in case of transdermal administration. This work reports the activity of UDL-PcAlCl against promastigotes and intracellular amastigotes of *Leishmania chagasi* and *L. panamensis*. Additionally, the internalization and co-localization of the compound at the mitochondria was evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Chloroaluminum phthalocyanine (CLAlPc), phorbol 12-miry-state 13- acetate (PMA), AmB, MTT (3-(4. 5-dimethylthiazolyl-2)-2. 5-diphenyltetrazolium bromide), HEPES buffer, adenosine and hemin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dimethyl sulphoxide (DMSO) was obtained from Carlo Erba Reagenti (Rodano, Italy). RPMI 1640 culture medium, fetal calf serum (FCS) and trypsin–EDTA were obtained from Gibco (Grand Island, NY, USA). The mitochondrial probe JC-1 was purchased from Molecular Probes Inc (Eugene, OR, USA). Soybean phosphatidylcholine (SPC, phospholipon 90 G, purity >90%) was a gift from Phospholipid/Natterman, Germany. Sodium cholate (NaChol) - edge activator- was purchased from Sigma–Aldrich, Argentina.

2.2. Liposome preparation

UDL-CLAlPc and empty liposomes (empty-UDL) were prepared according to Montanari et al. [33]. Briefly, UDL composed of SPC and NaChol at 6:1 (w/w) ratio, were prepared by mixing lipids from a $CHCl_3$:CH_3OH:DMSO (1:1:0.06, v/v) solution. ClAlPc (165 nmol/mL) was dissolved into the solution (except on the empty-UDL mixes). The mix was further rotary evaporated at 40 °C in round bottom flask until organic solvent elimination. The thin lipid film was flushed with N_2, and hydrated with 5 ml 10 mMTris–HCl buffer plus 0.9% (w/v) NaCl, pH 7.4, up to a final concentration of 43 mg SPC/ml. The suspension was sonicated (45 min with a bath type sonicator 80 W, 40 kHz) and extruded 12 times through two stacked 0.2 and 0.1 μm pore size polycarbonate filters using a 100 ml Thermobarrel extruder (Northern Lipids, Canada). Ultradeformability was tested allowing the suspension to pass through a 50 nm pore membrane under low pressure (less than 0.8 MPa) [34].

The phosphorus in the phospholipid content of liposomes was determined after perchloric acid digestion according to Böttcher et al. [35]. Briefly, 1 mL of phosphorus standard solutions (from 80 μmol/mL) or an appropriate amount of the liposome samples was added in 15 mL glass tubes to 0.2 ml of 70% perchloric acid and incubate for 30 min at 180 °C. The tubes were cooled to ambient temperature and 2 mL of ammonium molybdate solution and 0.25 mL of ascorbic acid were added. The samples were mixed and place the tubes in boiling water for 10 min. After cooled, the absorbance of the standards and samples were determined at 830 nm. The concentration of the phospholipids in the liposome sample was determined from the absorbance by reference to the standard curve;

The final concentration of ClAlPc in liposomes was determined using a Hewlett Packard/Agilent 8453 UV–Vis spectrophotometer at 672 nm wavelengths. A standard curve of ClAlPc (0.1–10 μM) was prepared in DMSO. Mean particle size and Z potential of each liposomal preparation were determined by dynamic light scattering with a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern. Worcestershire, UK) at 25 °C. Stock solutions of ClAlPc and AmB were prepared in DMSO (final concentration 0.1%. v/v). Work solutions were prepared in RPMI 1640 culture medium immediately before assays. DMSO was not toxic for the cells at working concentrations.

2.3. Parasites and cells

Two strains of *Leishmania*, each from a different species, were used in this study: *L. panamensis* (MHOM/PA/71/LS94) and *L. chagasi* (MHOM/BR/74/PP75). Promastigotes were grown in Schneider culture medium supplemented with 10% of inactivated fetal bovine serum (hiFCS) at 28 °C. Human leukemic cell line (THP-1, ATCC TIB 202) was used as parasite-host cells maintained by continue culture using RPMI 1640 medium supplemented with 10% hiFCS at 37 °C in humidified atmosphere of 5% CO_2. THP-1 cells were differentiated to its adherent phenotype with 10 ng/mL PMA by 72 h. Intracellular amastigotes were obtained after THP-1 cell infection with promastigotes in stationary-phase of growth in a cell:parasite ratio of 1:10 for 48 h at 37 °C, 5% CO_2. The percent of infected cells before the experiment was determined by counting infected and non-infected cells on methanol fixed and Giemsa-stained slides. Cells with one or more amastigotes inside them were considered positives for infection. The percent of THP-1 cells infected with *L. chagasi* parasites was 83.6 ± 5% and with *L. panamensis* was 55.3 ± 5%.

2.4. System of irradiation

A biological photoreactor equipped with four lamps (50 W, 120 V), 0.33 mV power and 4 infrared filters (Edmund Optics) was used as source of irradiation with a spectral range of 597–752 nm.

2.5. Phototoxic assays

THP-1 cells, promastigotes and intracellular amastigotes of *L. chagasi* and *L. panamensis* were incubated with serial dilutions of free ClAlPc and UDL-ClAlPc (0.0282–1666 nM), empty-UDL (0.0004–2.83 mM of phospholipids), AmB (120–30000 nM) or medium alone for 24 h at 37 °C in humidified atmosphere of 5% CO_2. The compounds were retired by washing twice the cell monolayers with culture medium, or by two cycles of centrifugation (3 min at 3830 × g) of promastigotes followed by resuspension in culture medium. Cells and parasites were irradiated with a fluency of 17 J/cm^2. Control cells were maintained without irradiation. Cell viability or parasite inhibition was determined as following: For THP-1 cells, the viability was determined 24 h post-irradiation using MTT (3-(4.5-dimethylthiazol-2-yl)-2.5 diphenyl-tetrazoliumbromide) colorimetric method [36]. The percentage of cytotoxicity was calculated by the equation: cytotoxicity (%) = (OD control group-OD treatment group)/OD control group × 100. For promastigotes, the inhibition of parasite growth was microscopically determined 24 h post-irradiation by counting parasite number in a haemocytometer. For intracellular amastigotes the inhibition of parasite growth was microscopically determined 72 h post-irradiation by counting infected and non-infected cells on methanol fixed and Giemsa-stained slides.

2.6. Morphologic changes

Morphological changes induced by treatment were analyzed on infected and non-infected cells 24–72 h post-irradiation. Giemsa-stained slides were examined under light microscopy. The images were recorded using a digital camera Nikon Coolpix 5000 and processed with Adobe Photoshop CS4 software.

2.7. Mitochondrial localization

L. chagasi-infected and non-infected THP-1 cells were incubated with UDL-ClAlPc (500 nM) and free ClAlPc (15000 nM) at 37 °C, 5% CO_2. After 24 h the cells were incubated with 10 μg/mL JC-1 probe for 10 min and were examined with an Olympus FV100 confocal microscope using a multi-line Argon laser (457, 488 and 515 nm, 40 mW) and Alexa Fluor® 488 filter for ClAlPc and JC-1. The presence of ClAlPc and JC-1 was discriminated by adding a red and green pseudocolor respectively. The merge of ClAlPc (pseudocolored red) and JC-1 (pseudocolored green) was observed yellow-orange. Records of at least three different fields were made. Images were analyzed using FV1000 Viewer software and Adobe Photoshop CS4.

2.8. Index calculations and statistical analysis

The activities against parasites and cells were expressed as the compound concentration able to inhibit (IC_{50}) or kill (CC) 50% of parasites and cells. The data were calculated using the software Msxlfit; GO (Business Solution, Guildford, UK).

In order to determine if the activities against parasites or cells after treatment were specifically due by the stimulation of the compound by visible light and not by the compound or formulation *per se*, a phototoxic index (PI) values were determined. They were calculated by dividing the compound activity (IC_{50}) at 0 J/cm^2 by the IC_{50} at17 J/cm^2. Specific PI values > or = 2 were arbitrarily defined.

In order to compare the amount of compound that causes cell toxicity with the amount that causes the antileishmanial effect, the Selective Index (SI) were determined. They were calculated using the ratio: SI = CC_{50} in mammalian THP-1 cells/IC_{50} in parasites. SI values > or = 3 were arbitrarily defined as parasite-selective compounds. A higher therapeutic index is preferable to a lower one: Lower SI values indicate a narrow therapeutic range.

For comparison a Student's *t* test was used and values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Liposomal characterization

The size of UDL-ClAlPc was 109 nm ± 0.8, Zeta potential of −12.0 ± 1.21 and polidispersion index (Pdi) of 0.15. The concentration of phospholipids and ClAlPc was 35.35 mg/mL and 72.7 nmol/mL of suspension respectively (compound/lipid ratio: 1.48 nmol ClAlPc/1 μmol of phospholipids).

3.2. Internalization and mitochondrial co-localization

As observed in Fig. 1 (red[1] colour) infected THP-1 cells internalized both UDL-ClAlPc and free ClAlPc. Co-localization with the mitochondria probe was observed in both cases, but in a more evident way after treatment with free-ClAlPc (Fig. 1, yellow colour).

3.3. Phthalocyanine activities

Treatment with UDL-ClAlPc was more phototoxic than free ClAlPc on THP-1 cells (CC_{50} 1.35 versus 22.50 nM), *L. chagasi* (IC_{50} 0.03 versus 2.73 nM) and *L. panamensis* (IC_{50} 0.31 versus 4.00 nM) respectively (Table 1, see fluency 17 J/cm^2). In addition, it was also more phototoxic on *L. chagasi* (IC_{50} 0.69 versus 14.24 nM) and *L. panamensis* (IC_{50} 1.49 versus 31.19 nM) intracellular amastigotes (Table 1, see fluency 17 J/cm^2).

In absence of light irradiation, treatments with UDL-ClAlPc or free ClAlPc were not able to destroy 50% of THP-1 cells or intracellular amastigotes at the maximum concentration used (Table 1, see >1666 nM). In contrast, mild toxicities were observed on *L. chagasi* (IC_{50} 26.39 nM) and *L. panamensis* (IC_{50} 50.62 nM) promastigotes (Table 1, see fluency 0 J/cm^2).

Phototoxic index (PI) higher than 50 was observed after liposomal and free ClAlPc treatment in parasites and THP-1 cells (Table 1). In contrast, PI of almost 1 or lower were showed after AmB treatment used in this study as antileishmanial referential drug (Table 1).

3.4. Phospholipids activities

The phospholipids presented in empty-UDL did not induce toxicity on THP-1 or intracellular amastigotes at a maximum concentration tested of 2.83 mM. However, some toxicity was observed on *L. chagasi* and *L. panamensis* promastigotes with IC_{50} values of 0.146 mM and 0.525 mM phospholipids respectively without irradiation (Table 2). PI values of 1 for *L. chagasi* and 4 for *L. panamensis* promastigotes were obtained indicating an increased effect of the phospholipids after irradiation against *L. panamensis* promastigotes.

The phospholipid concentration in the UDL-ClAlPc active liposomes after irradiation (see Table 2, 17 J/cm^2) was very low (in the range of 0.00005–0.001 mM of phospholipids) on parasites and cells (Table 2).

3.5. Parasite selectivity

A parasite selective activity for UDL-ClAlPc and free ClAlPc treatment after irradiation was observed in promastigotes of *L. chagasi*

[1] For interpretation of color in Fig. 1, the reader is referred to the web version of this article.

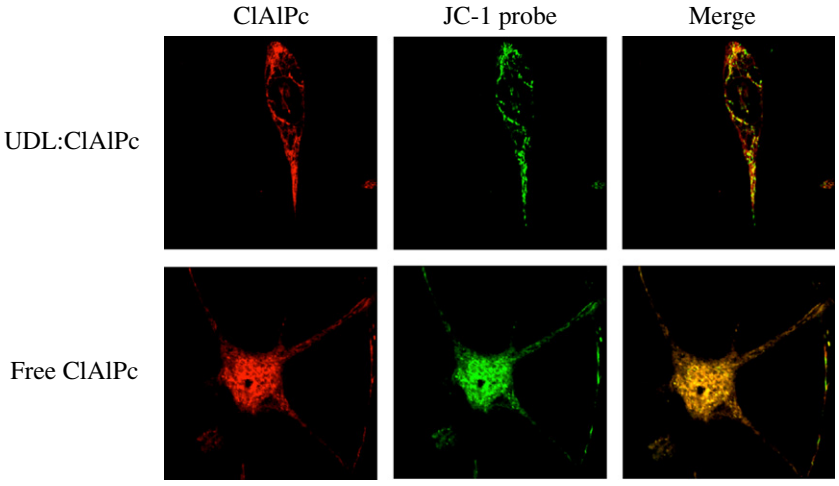

Fig. 1. Co-localization of ClAlPc with mitochondria. Confocal microphotographs showed *Leishmania*-infected THP-1 cells after incubation with ClAlPc both liposomal (500 nM) and free (15000 nM) (See Column ClAlPc, red colour) and with JC-1 mitochondrial probe (Column JC-1 green colour). The merge of both colours is seen in yellow. Different cell morphologies and mitochondrial probe distribution pattern were observed in THP-1 cells after PMA transformation and parasite infection.

Table 1
Toxicity of free and liposomal ClAlPc on parasites and mammalian cells. Compound toxicities against parasites and cells were evaluated in non-irradiated (0 J/cm^2) and irradiated (17 J/cm^2) cells. The activities of free and liposomal ClAlPc were expressed in nM ± SD of ClAlPc.

	Fluency (J/cm^2)	*L. chagasi* parasites						*L. panamensis* parasites							
		Promastigotes			Intracelular amastigotes			Promastigotes			Intracelular amastigotes			THP-1 cells	
		IC_{50} (nM)[a]	PI[d]	SI[e]	IC_{50} (nM)	PI	SI	IC_{50} (nM)	PI	SI	IC_{50} (nM)	PI	SI	CC_{50} (nM)[b]	PI
Free-ClAlPc	0	>1666[c]	>610	1	>1666	>117	1	>1666	>417	1	>1666	>53	1	>1666	>74
	17	2.73 ± 0.51		8	14.24 ± 2.62		2	4.00 ± 0.098		6	31.19 ± 0.688		0.7	22.50 ± 0.33	
UDL-ClAlPc	0	26.39 ± 0.48	880	63	>1666	2414	1	59.62 ± 0.79	192	28	>1666	>1118	1	>1666	>1234
	17	0.03 ± 0.0003		45	0.69 ± 0.08		2	0.31 ± 0.02		4	1.49 ± 0.01		0.9	1.35 ± 0.01	
AmB[f]	0	21.69 ± 0.44	0.8	355	106.90 ± 2.34	1.05	72	148.62 ± 6.09	1.16	52	43.20 ± 0.32	1.13	178	7693 ± 324.0	0.86
	17	27.49 ± 0.67		326	102.00 ± 3.96		88	128.23 ± 5.02		70	38.20 ± 4.40		235	8966 ± 43.37	

[a] IC_{50}: Compound concentration able to inhibit 50% of parasites.
[b] CC_{50};Compound concentration able to kill 50% of THP-1 cells.
[c] >1666: It means that at the highest ClAlPc concentration evaluated in this study (1666 nM), the 50% of inhibition (or citotoxicity) could not be reached.
[d] PI: Phototoxic Index: ratio of IC_{50} value at 0 J/cm^2 and IC_{50} value after 17 J/cm^2.
[e] SI: Selective Index: ratio of CC_{50} in THP-cells and IC_{50} in parasites.
[f] AmB: amphotericin B used as antileishmanial reference drug. The results showed in the table correspond to one representative experiment from two.

Table 2
Toxicity mediated by the liposome-phospholipid composition. The table shows two different results: 1. The UDL-ClAlPc row indicates the phospholipid concentration present in the ultradeformable liposomes loaded with ClAlPc actives against parasites or mammalian cells with or without irradiation. The concentrations of ClAlPc loaded in those liposomes were not showed in this table but they corresponded to the ClAlPc concentration expressed in nM ± SD of ClAlPc in Table 1. 2. The row Empty-UDL showed the activity of empty-UDL against parasites and cells. The results were expressed as the phospholipid concentration able to be toxic for 50% of parasites (IC_{50}) and cells (CC_{50}) under non-irradiated (0 J/cm^2) or irradiated (17 J/cm^2) conditions.

Liposomes	Fluency (J/cm^2)	Phospholipids (mM ± SD)				
		L. chagasi		*L. panamensis*		THP-1 cells
		Promastigotes	Intracellular amastigotes	Promastigotes	Intracellular amastigotes	
UDL-ClAlPc	0	0.040 ±0.0008	>2.83	0.101 ±0.001	>2.83	>2.83
	17	0.00005 ±0.00000	0.001 ±0.0004	0.0005 0.00003	0.002 0.00001	0.002 ±0.00001
Empty-UDL	0	0.146 ±0.03	>2.83	0.525 ±0.01	>2.83	>2.83
	17	0.134 ±0.04	>2.83	0.133 ±0.011	>2.83	>2.83

(SI: 45 and 8) and *L. panamensis* (SI: 4 and 6); however no selectivity was observed against intracellular amastigotes (SI > 2) (Table 1). A parasite selective activities were observed after AmB treatment in promastigotes of *L. chagasi* (SI: 326) and *L. panamensis* (SI: 52) and intracellular amastigotes of *L. chagasi* (SI: 88) and *L. panamensis* (SI: 70). Despite the high toxicity displayed by the compounds against promastigotes in comparison with non-infected THP-1 cells, the compounds were not able to kill selectively intracellular parasites.

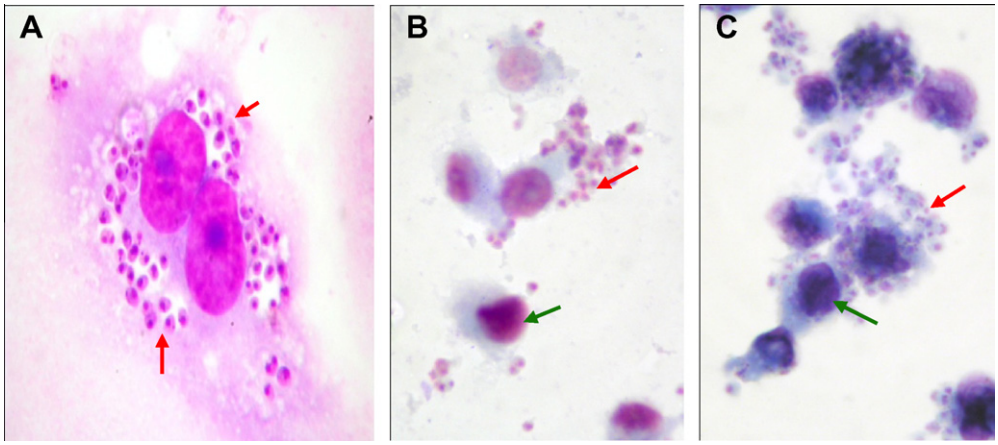

Fig. 2. Cell morphological alterations after irradiation. Microphotographs showed *L. chagasi*-infected THP-cells A. untreated; B. 1666 nM UDL-ClAlPc and C. 1666 nM free ClAlPc, 24 h post-irradiation. Red arrows point intracellular amastigotes and green arrows indicate the absence of nucleolus and chromatin condensation. The slides were coloured with Giemsa and observed at 1000X magnification.

3.6. Morphological changes after treatment

Upon photodynamic treatment loss of membrane integrity, hypervacuolization, chromatin condensation and absence of nucleolus were detected resulting in a release of parasites (Fig. 2). Few amastigotes were seen inside and outside the cell cytoplasm, and absence of parasite-kinetoplast was evident. Alterations in macrophages and parasites persisted still after 72 h post-irradiation. The viability of the released parasites was not tested in this study.

4. Discussion

Liposomes are the most widely investigated delivery system for phagocyte-targeted therapies. They reduce the toxicity and improve the effectiveness of the transported compounds providing advantages such as biocompatibility, low inflammatory response, cell specificity and compound stabilization [37,38]. Important features regarding to the *in vitro* antileishmanial activity of ClAlPc photosensitizer loaded in certain type of liposomes, the UDL, were demonstrated in this study.

UDL were prepared and characterized following a protocol used previously for encapsulation of hydrophobic and hydrophilic zinc phtahlocyanines [31]. As in that case, UDL-ClAlPc showed interesting physicochemical characteristics in size (~100 nm) and charge (−12.0 ± 1.21) and were internalized by macrophages. No qualitative differences were observed in the intensity of fluorescence after internalization of UDL and free ClAlPc in *Leishmania*-infected or non-infected cells; however quantitative measures were not done in this study. Although liposomes are the most widely studied carrier used for macrophage-specific drug delivery [37], there is not a consensus about right composition, type, size or charge of liposomes able to be internalized by macrophages [36–39]. Some studies showed that small and negatively charged liposomes are better internalized than large and positively charged liposomes but in contrast other studies have shown liposome uptake improved with increased size [40,41]. Optimal properties of liposomes as drug deliveries depend on multiple factors including the target cell and specific properties of the liposome formulation, for example, receptor mediated or no receptor mediated uptake [39].

Promastigotes of *L panamensis* and *L. chagasi* were more susceptible to ClAlPc loaded in liposomes than free compound. The activity of liposomes against *Leishmania* promastigotes (free live forms of parasites) probably depends of many factors such as the size, charge and lipid composition of the liposomal formulations [28,42]. The toxicity of UDL-ClAlPc induced in promastigotes after light irradiation was due to the photosensitizer and not the lipid matrix (the concentration of phospholipids in these liposomes −0.00005 mM- were almost 3000 times less than that required by the empty liposomes to eliminate the 50% of promastigotes (0.130 mM).

The mitochondrial localization of ClAlPc observed in this study after free administration, and less regarding to the liposomal compound, has been demonstrated for others PS such as ZnPc and protoporphyrin IX (PpIX) [43–45]. In contrast, hydrophilic cationic phthalocyanines with either Zn (II) or Si (IV) metal localized preferentially within the cell lysosomes in some cancer cells [46,47]. Mitochondrial localization of ClAlPc, as it has been demonstrate by ZnPc and PpIX, could promote the action of the PS on the host cell by apoptotic mechanism, but not on the parasite [43,48–50].

In this work uninfected macrophages were destroyed with similar (or quite lower) concentration of ClAlPc than intracellular amastigotes. Compared with the parasite selective activity showed after AmB treatment and/or the high susceptibility of promastigotes (free live forms of the parasites) to ClAlPc after irradiation, no selectivity in the activity of the compound was observed against intracellular forms of both species of parasites. This no selective activity against intracellular parasites also has been presented after treatment of others PS after irradiation [17,20]. *L major*-infected macrophages (J774.2 cells) treated with ALA did not show a decrease in the number of amastigotes after irradiation but, host-cell destruction accompanied by the release of live parasites was observed [17]. *L-major*-infect macrophages (J774.2 cells) treated in similar conditions with two phenothiazinium analogues showed similar or 1.6 times higher photosensitivity in J774.2 cells than intracellular parasites [18]. Photolysis of *L. amazonensis* intracellular parasites as well as host cells (J774 cells) were observed after infection with axenic amastigotes pre-treated with ClAlPc [20]. The fact that macrophages were more susceptible to photodynamic effects than *Leishmania* parasites could indicate, as it was demonstrated in *in vivo* experiments for the decrease of macrophages after PDT [17], that the death of parasites occurs by indirect mechanisms generated after the destruction of their host cells.

The higher antileishmanial activities showed by the UDL-ClAlPc after irradiation compared with free compound against *L. chagasi* and *L. panamensis* was expected. The use of liposomes for improving effectiveness of antileishmanial drug has been tested so far. Liposomes present some interesting characteristics on behalf of their useful application i.e. high capacity of encapsulation and

retention; ability to be phagocyted by macrophages, which are the *Leishmania* host cell; their relative safety and high versatility with respect to lipid composition, size and lamellarity [51]. One of the first examples of the use of an antileishmanial drug loaded in liposomal formulation was Sb^V encapsulated in multilamelar liposomes that was 600–700 times more active than the free compound in *L. donovani*-infected mice [52]. However, most of the liposomal formulations are designed to be used intravenously against visceral leishmaniasis. The treatment of visceral leishmaniasis with liposomal AmB is an example of how a nanovector improves its therapeutic index and reduces toxicity. In this case the liposomal formulation of AmB retains its monomeric state and modulates interaction with lipoproteins and prolonged the half-life of the compound in circulation [53].

5. Conclusion

Incorporation of ClAlPc in UDL increased its phototoxic effect against New World *Leishmania* species; however, non-selective parasite activity against intracellular amastigotes was observed. Further studies oriented to evaluate both the state of infection after PDT and the effectiveness of UDL used as delivery vehicles of ClAlPc in CL experimental models are required.

6. Abbreviations

CC_{50}	cytotoxic concentration 50
CL	cutaneous leishmaniasis
ClAlPc	chloraluminum phthalocyanine
Empty UDL	empty ultradeformable liposomes
IC_{50}	inhibitory concentration 50
PDT	photodynamic therapy
PI	phototoxic index
PS	photosensitizer
SI	Selective Index
UDL-ClAlPc	ultradeformable liposomes loaded with chloroaluminum phthalocyanine

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