Journal of Photochemistry and Photobiology B: Biology 117 (2012) 157–163

Contents lists available at SciVerse ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

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

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ARTICLE INFO

Article history:
Received 2 May 2012
Received in revised form 6 September 2012
Accepted 26 September 2012
Available online 12 October 2012

Keywords:
Ultradeformable liposomes
Photodynamic therapy
Chloroaluminum phthalocyanine
Leishmania panamensis
Leishmania chagasi

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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http://dx.doi.org/10.1016/j.jphotobiol.2012.09.018

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.