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Sunlight triggered photodynamic ultradeformable liposomes against *Leishmania braziliensis* are also leishmanicidal in the dark

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ABSTRACT

Being independent of artificial power sources, self administered sunlight triggered photodynamic therapy could be a suitable alternative treatment for cutaneous leishmaniasis, that avoids the need for injectables and the toxic side effects of pentavalent antimonials. In this work we have determined the *in vitro* leishmanicidal activity of sunlight triggered photodynamic ultradeformable liposomes (UDL). ZnPc is a hydrophobic Zn phthalocyanine that showed 20% anti-promastigote activity (APA) and 20% anti-amastigote activity (AA) against *Leishmania braziliensis* (strain 2903) after 15 min sunlight irradiation (15 J/cm^2). However, when loaded in UDL as UDL-ZnPc (1.25 μM ZnPc–1 mM phospholipids) it elicited 100% APA and 80% AA at the same light dose. In the absence of host cell toxicity, UDL and UDL-ZnPc also showed non-photodynamic leishmanicidal activity. Confocal laser scanning microscopy of cryosectioned human skin mounted in non-occlusive Saarbrücken Penetration Model, showed that upon transcutaneous administration ZnPc penetrated nearly 10 folds deeper as UDL-ZnPc than if loaded in conventional liposomes (L-ZnPc). Quantitative determination of ZnPc confirmed that UDL-ZnPc penetrated homogeneously in the *stratum corneum*, carrying 7 folds higher amount of ZnPc 8 folds deeper than L-ZnPc. It is envisioned that the multiple leishmanicidal effects of UDL-ZnPc could play a synergistic role in prophylaxis or therapeutic at early stages of the infection.

1. Introduction

Cutaneous (CL) and mucocutaneous leishmaniasis (MCL) are clinical manifestations of a group of diseases caused by dimorphic protozoa that belong to different species of the *Leishmania genus*, [1] which are transmitted to humans by sandfly bites. Infective parasites are hosted in skin macrophages and produce ulcerative lesions [2] as well as destructive mucosa inflammation in MCL [3]. 1.5 million new cases of CL arise worldwide each year [4], presenting a complex epidemiology that depends on intra and inter species variations [5]. The CL's geographic incidence is heterogeneous, including densely affected foci and dissemination areas in constant change [6] due to emigrations, tourism [7,8], urbanization [9] and the expansion of suitable ecosystems for the vector due to climatic changes [10]. A marked increase of cases in Europe and America has been recorded in the last decades, and new important epidemic foci have emerged [4,11].

Standard treatments are based on systemic or intralesional administration of pentavalent antimonials according to the leishmania specie and the clinical symptoms (intravenous or intramuscular 20–50 mg Sb(v)/kg weight/day for 30 days, or 1–3 ml under the edge of lesion and entire lesion every 5–7 days for a total of 2–5 times [12]), systemic amphotericin B or pentamidine isothionate [13,14]. The response to the treatment is slow and even inefficacious according to the species, with incomplete cure and relapse occurring within 6 months [13]. Treatments are linked to side effects such as hepatic alterations, biochemical pancreatitis, flattening of T waves in ECG, myalgia, arthralgia, thrombocytopenia, transient suppression of bone marrow and reversible renal insufficiency [15].

Thus, the search for an effective, simple, and low-cost treatment for CL that can be conveniently administered is still an active topic. In this scenario, topical treatment is preferable to systemic interventions [16]. The ointment of the highly hydrophilic antibiotic paromomycin (15%) associated to the permeation enhancer methyl benzethonium chloride (12%) (MBC), is relatively effective for CL treatment (*L. major*, *L. tropica*, *L. mexicana and L. panamensis*), but local side effects are frequently observed due to MBC [17]. On the other hand, topical amphotericin B (Amphocil in 5% ethanol) has been successful in treatment of *L. major* infected patients in Israel [18,19], but the high cost of Amphocil restricts their use and more extensive studies are needed.

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Photodynamic therapy (PDT) is a potentially applicable, safe and affordable technology that is currently in use for the treatment of cancer and age-related macular degeneration. PDT is based on the concept that a photoactivatable compound, called a photosensitizer, can be excited by light of the appropriate wavelength to generate cytotoxic singlet oxygen and free radicals [20]. PDT is an attractive option to conventional antimicrobial chemotherapy, since it does not induce resistant strains neither upon multiple treatments [21,22]. Although PDT has rendered several cases of cure with good cosmetic results [23,24], the lack of standarized data and the need for special medical equipment (lamps), have hampered the use of PDT against CL [25]. The use of daylight to PDT can be an alternative to this last drawback. Recently a Phase II clinical trial in Israel has been started to determine the efficiency of methyl aminolevulinate (MAL)-PDT daylight triggered for the treatment of CL (*L. major* and *L. tropica*) [26].

In the present work, we have determined the *in vitro* leishmanicidal activity of the hydrophobic photosensitizer Zn phthalocyanine (ZnPc) loaded in ultradeformable liposomes (UDL-ZnPc) both in the darkness and upon sunlight irradiation and screened the ability of UDL-ZnPc to penetrate intact skin.

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (SPC) (phospholipon 90 G, purity >90%) was a gift from Phospholipid/Natterman, Germany. Sodium cholate (NaChol), 1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(Lissamine™ rhodamine B sulfonyl) (Rh-PE), and Sephadex G-50 were purchased from Sigma-Aldrich, Argentina. The fluorophore 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was from Molecular Probes (Eugene, OR, USA). Q-tracker non-targeted Quantum Dots 655, with a *core/shell* of CdSe/ZnS covered by PEG (QD) was from Invitrogen (Hayward, CA). The hydrophobic ([tetrakis(2,4-dimetil-3-pentyloxi)-phthalocyaninate]zinc(II)) Zn phthalocyanine (ZnPc) was synthesized as described in Montanari et al. [27]. Other reagents were analytic grade from Anedra, Argentina.

2.2. Preparation and characterization of ultradeformable liposomes

UDL and UDL-ZnPc were prepared as stated in Montanari et al. [27]. Briefly, UDL composed of SPC and NaChol at 6:1 (w/w) ratio, were prepared by mixing lipids from $CHCl_3$ and $CHCl_3$:CH_3OH (1:1, v/v) solutions, respectively, that were 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 in 10 mM Tris–HCl buffer plus 0.9% (w/v) NaCl, pH 7.4 (Tris buffer), 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 15 times through two stacked 0.2 and 0.1 μm pore size polycarbonate filters using a 100 ml Thermobarrel extruder (Northern Lipids, Canada). ZnPc was co-solubilized in the organic solution with lipids (2 mg ZnPc/g SPC) to prepare UDL-ZnPc.

Conventional – non ultradeformable, without NaChol – liposomes (L) were prepared by the same procedure.

Liposomal phospholipids were quantified by a colorimetric phosphate micro assay [28]. Mean particle size and Z potential of each liposomal preparation were determined by dynamic light scattering with a Nanozetasizer (Malvern).

2.3. Cytotoxicity on mammal cells

2.3.1. Lactate dehydrogenase (LDH) assay

J774 and Vero cells were maintained at 37 °C with 5% CO_2, in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin (PE/ST) and amphotericin (all from Invitrogen Corporation). Culture medium of nearly confluent cell layers was replaced by 100 μl of medium containing UDL (1 and 10 mM phospholipids). Upon 1 h incubation at 37 °C, suspensions were removed; cells were washed with PBS (140 mM NaCl, 8.7 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, pH 7.4) replaced by fresh RPMI medium and cells were incubated for 24 h at 37 °C. Upon incubation, supernatants were transferred to fresh tubes; centrifuged at 250 ×*g* for 4 min and LDH content was measured using lactate dehydrogenase CytoTox Kit (Promega) [29]. LDH concentration was expressed as percentage LDH release relative to treatment with the detergent Triton X-100 and then percentage of viability was calculated considering the LDH leakage of cells grown in medium.

2.3.2. Glutathione assay (GSH)

Total cellular glutathione of J774 cells was measured using the Tietze method [30]. Culture medium of nearly confluent J774 cells was replaced by 100 μl of medium containing free ZnPc (1.25 and 12.5 μM), UDL (1 and 10 mM) or UDL-ZnPc (1.25 μM ZnPc–1 mM phospholipids and 12.5 μM ZnPc–10 mM phospholipids). Upon 24 h incubation at 37 °C, suspensions were removed, replaced by fresh RPMI medium and one plate was exposed to direct sunlight along 15 min (light dose of 15 J/cm^2 at λ = 600–650 nm measured by Radiometer Laser Mate Q, Coherent), meanwhile other plate was kept in the dark. After treatments, cell were incubated for 24 h at 37 °C, then media were removed and cells were washed with PBS and collected into eppendorf tubes by trypsin treatment. Then trypsin was inactivated, cells were twice washed with PBS by centrifugation and finally suspended in 100 μl of 1 mM EDTA. Cells were lysed by sonication (tip sonicator 10 s) and cellular debris were removed by centrifugation (10000 ×*g* for 15 min at 4 °C). 20 μl aliquots of each supernatant were transferred to 96 wells plates for glutathione determination. The reaction was started by adding 180 μl of reaction mixture [60 μM 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB), 1.5 mM NADPH, 0.1 mM EDTA, and 2.4 U/ml GSH reductase in $NaHCO_3$ 0.1% (all from Sigma-Aldrich, St. Louis, MO, USA)]. Absorbance at 412 nm was monitored after 15 min with a microplate reader and the glutathione concentration was determined by comparing the rate of colour change with that of a GSH standard curve.

2.4. UDL-ZnPc internalization by promastigotes

Leishmania braziliensis promastigotes (STRAIN 2903) were cultured at 25 °C in Novy–McNeal–Nicolle biphasic medium [31] and RPMI 1640 supplemented with 10% FCS and PE/ST. Before treatments, promastigotes were taken from liquid phase and transferred to RPMI medium.

L. braziliensis promastigotes were incubated with UDL-ZnPc (1.25 μM ZnPc-1 mM phospholipids) for 15 min at 4 °C and 25 °C. Upon incubation, parasites were washed by centrifugation (3830 ×*g* for 3 min) and fixed in 2% v/v formaldehyde in PBS. The emission of ZnPc was monitored with a confocal laser scanning microscope (CLSM) Olympus FV300 equipped with a He–Ne 633 nm laser.

2.5. Anti-promastigote activity

Promastigotes were incubated for 5 min at 25 °C with empty L and UDL (1 and 0.1 mM phospholipids) (100 μl RPMI with 10% FCS and PE/ST). Upon incubation, samples were centrifuged (3800 ×*g* for 10 min at 20 °C), supernatants were removed and replaced by fresh RPMI medium. Parasites were further incubated for 3 h at 25 °C and mobility was evaluated microscopically.

Promastigotes (5×10^5) were incubated for 30 min at 25 °C with empty UDL (1 mM phospholipids), free ZnPc (1.25 μM), UDL-ZnPc and L-ZnPc (both 1.25 μM ZnPc-1 mM phospholipids). Upon incubation, samples were centrifuged (3800 ×*g* for 10 min at 20 °C),