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Topical treatment of *L. major* infected BALB/c mice with a novel diselenide chitosan hydrogel formulation

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ABSTRACT

Topical therapy is the ideal outpatient treatment of cutaneous leishmaniasis (CL) because of the ease of administration and lower cost. It could be suitable as monotherapy for localized cutaneous leishmaniasis (LCL) or in combination with systemic therapies for more severe forms of the disease. Although paromomycin (PM) ointment can be recommended for the treatment of LCL caused by *Leishmania major*, a more effective topical treatment should be achieved regarding the physicochemical properties of this aminoglucoside and its rather poor intrinsic antileishmanial activity, that hampers the accumulation of enough amount of drug in the dermis (where the infected macrophages home) to exert its activity. In this work, we determined a 50% effective dose of 5.6 μM for a novel compound, bis-4-aminophenyldiselenide, against *L. major* intracellular amastigotes. This compound and PM were formulated in chitosan hydrogels and *ex vivo* permeation and retention studies in the different skin layers were performed with pig ear skin in Franz diffusion cells. The results showed that less than 2–4% of the diselenide drug penetrated and permeated through the skin. In contrast, the percentage of PM penetration was about 25–60% without important retention in the skin. When topically applied to lesions of *L. major* infected BALB/c mice, the novel diselenide chitosan formulation was unable to slow lesion progression and reduce parasite burden. Considerations during the process of novel drug development and formulation discovery algorithm for CL are discussed.

1. Introduction

The term leishmaniasis describes a range of diseases caused by different species of protozoa of the genus *Leishmania* which are prevalent in tropical and subtropical regions with visceral and tegumentary forms. Cutaneous leishmaniasis (CL) is one of the major tropical dermatosis of immense public health significance. It is prevalent in 82 countries and it is estimated that 350 million people worldwide are at risk of infection, with an estimated prevalence of 12 million cases and an annual incidence of be 1.5 million cases per year (WHO, 2010).

The clinicopathological picture of CL is mainly divided in three phenotypes: localized cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL) whose manifestations range from small cutaneous nodules to gross mucosal tissue destruction. The nature, magnitude and spread of the lesions depend on the *Leishmania* species but also on endemic regions, host factors and immuno-inflammatory responses. In general terms, species prevalent in the Old World (Africa, Asia and Southern Europe) (OWCL) produce limited clinical manifestations compared with New World species (Latin America) (NWCL) that currently develop to MCL.

The severity of the lesions and the risk of dissemination determine the treatment of choice that can be parenteral or local. Parenteral treatments, such as antimonial derivatives or amphotericin B (AmB), have important limitations of toxicity, efficacy and cost and, in the case of antimonials, emergence of resistance (Monge-Maillo and Lopez-Velez, 2013).

Local therapies are attractive options offering reduced systemic toxicity and outpatient treatment. Moreover, although their use in the form of monotherapy is limited to the less severe forms of CL

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without risk of dissemination, the application of an effective local therapy in combination with the systemic treatment could also be useful to accelerate the healing of the lesions and reduce the scar formation in more severe tegumentary forms. In fact, topical therapies should be addressed not only to eliminate the parasite but also to promote wound-care and re-epithelization without scar formation as well as to prevent super-infection of the lesions. Local treatments evaluated in patients included physical therapies, such as cryotherapy and thermotherapy, the intralesional (i.l.) injection of antimonial derivatives, the topical administration of a paramomycin–methylbenzethonium chloride (PM-MBCL) ointment or the immunotherapy with imiquimod in a cream (Gonzalez et al., 2009, 2008). The advantages of topical against physical therapies or i.l. injections are fewer adverse effects, less pain, ease of administration and lower costs.

Currently, the ointment described already 50 years ago containing 15% of the aminoglucoside PM and the cationic surfactant MBCL is the common topical treatment (El-On et al., 1984). However, it is far to be satisfactory. It is irritant and it can be only considered for lesions produced by *Leishmania major* for which the efficacy ranges from 17–68% (Kim et al., 2009; Reithinger et al., 2007). Its poor efficacy could be ascribed to the amount of PM available in the dermis, insufficient for the eradication of the parasite that homes inside the macrophages deeply in the skin, or to an inadequate interval of time (Carneiro et al., 2012, 2010; Ferreira et al., 2004). Besides, IC_{50} values reported for PM *in vitro* are high (Seifert et al., 2010), indicating a rather poor intrinsic antileishmanial activity that further increases the amount of drug needed in the dermis to exert its activity. In fact, the location of the parasite in the dermis makes the topical treatment of CL particularly problematic: (i) the drug has difficulties to permeate through the different skin layers or (ii) it is rapidly eliminated by dermal blood supply. Although it was initially though that PM had poor capacity to permeate through the skin because of its inconvenient physicochemical properties (high molecular weight (MW) and solubility in water) (Carneiro et al., 2010), there are some works evidencing its rapid and high permeability through damaged skin (Bavarsad et al., 2012). Consequently, formulations such as liposomes, that increase the time of PM retention in the skin, have shown superior efficacy in mice infected with *L. major* (Bavarsad et al., 2012; Jaafari et al., 2009).

This scenario justifies the necessity to investigate new formulations and drugs for the topical treatment of CL. We have previously reported the activity of novel selenium compounds *in vitro* against *Leishmania infantum*. Encouraged by the low IC_{50} values obtained against this strain by these compounds and due to their physicochemical characteristics (MW and log*P*), theoretically well- suitable for topical application, the aim of this work was to explore the potential of these novel derivatives for the topical treatment of CL in *L. major* infected BALB/c mice.

For this purpose, drugs were formulated in chitosan hydrogels. Chitosan is an attractive excipient biomaterial widely studied for wound care. It can be of interest in the topical treatment of CL because of its biocompatibility, its intrinsic antimicrobial activity and penetration enhancer properties (Dai et al., 2011). Moreover, its viscosity and bioadhesive capabilities could offer a sort of sustained release and prolong the exposition of the drug to the parasite.

2. Materials and methods

2.1. Materials

Chitosan 95/200 (Q 95/200) was supplied by Heppe Medical Chitosan GmbH (Halle, Germany). 2-hydroxipropyl-β-cyclodextrin (HP-β-CD, MW = 1310) and β-glycerolphosphate (β-GP, MW = 216) were obtained from Sigma–Aldrich (St. Louis, MO, Canada). Transcutol (TC) was obtained from Gattefossé (Saint Priest, Cedex, France). Lactic acid (MW = 90.08) was supplied by Sharlau (Barcelona, España). Paramomycin sulphate (PM, MW = 713.71) was obtained from Fluka (St. Louis, MO, Canada). 4-bis-aminophenyldiselenide (2a, MW = 342.2) and bis-benzyldiselenide (2o, MW = 340.2) were synthesized by the Organic and Pharmaceutical Chemistry Department of the University of Navarra as previously described (Plano et al., 2011). Ethanol absolute and nitric acid were supplied by Panreac (Barcelona, Spain). All other reagents were of analytical grade and were used without further purification.

2.2. Parasites

L. major promastigotes (clone VI, MHOM/IL/80/Friendlin) were maintained at 26 °C in continuous stirred Schneideŕs modified medium (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) and 40 mg/mL of gentamicin (Sigma, St Louis, MO, USA) in flasks. Procyclics were obtained after 1–2 days culture and metacyclics were purified from 5 to 6 days stationary cultures by treatment with peanut agglutinin (PNA) (Sigma, St Louis, MO, USA). Briefly, stationary promastigote cultures were washed twice in phosphate buffered saline (PBS, pH = 7.4, Gibco, Gaithersburg, MD, USA), resuspended to 2 mL of RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) and incubated with 20 μg/mL of PNA (5 mg/mL in PBS) to purify metacyclics from *L. major*. After 20 min of incubation, 10 mL of RPMI 1640 were added carefully and the suspension was centrifuged (Allegra X-30 centrifuge, Beckman Coulter, Fullerton, CA, USA) at 500 rpm for 5 min. The non-agglutinated promastigotes were collected, washed two times in PBS and used throughout the work.

2.3. In vitro cytotoxicity assay

To estimate the toxicity of selected compounds (2a and 2o), an *in vitro* cytotoxicity assay was performed in mouse peritoneal macrophages using Alamar Blue (AB). Briefly, BALB/c mice were inoculated with 1 mL of 3% (w/v) thioglycolate (Sigma–Aldrich, St Louis, MO, USA). After 3 days, animals were euthanized by cervical dislocation and 5 mL of cold RPMI 1640 medium were injected into the peritoneal cavity and then recovered with a syringe. Cells were collected from the peritoneal fluid by centrifugation (Allegra X-30 centrifuge, Beckman Coulter, Fullerton, CA, USA) at 1500 rpm for 10 min. Then, the pellet was resuspended in RPMI 1640 supplemented with 10% FBS, 1% of a 100 U/mL penicillin and 100 μg/mL of streptomycin solution (Sigma, St Louis, MO, USA). Macrophages were adjusted to 2.5×10^4 cells/well (final volume per well = 200 μL) and cultured in supplemented RPMI 1640 in 96-well plates at 37 °C under 5% CO_2 for 24 h. Then, different concentrations of 2a or 2o (0.25, 0.5, 1.25, 2.5, 5, 10, 20, 40, 60, 80, 120 and 150 μg/mL) were added to wells and plates were again incubated for 48 h. Previously, compounds were solved in DMSO (Sigma–Aldrich, St Louis, MO, USA). Cells without drug were used as control and cells treated with AmB (Sigma, St Louis, MO, USA) were used as positive control. After 48 h incubation, 20 μL of the AB reagent solution (final concentration of AB = 10% (v/v)) (Invitrogen, Carlsbad, CA, USA) were added to wells and plates were incubated for 4 h. Finally, sample fluorescences were measured using a microplate fluorimeter (λ excitation 560 nm; emission 590 nm; Polar Star Galaxy, BMG Labtechnologies, Offenburg, Germany). Results are expressed as mean ± SD for at least two independent experiments in quadruplicate.