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Antiproliferative and ultrastructural effects of BPQ-OH, a specific inhibitor of squalene synthase, on *Leishmania amazonensis*

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Abstract

Parasites of the *Leishmania* genus require for the growth and viability the de novo synthesis of specific sterols as such as episterol and 5-dehydroepisterol because cholesterol, which is abundant in their mammalian hosts, does not fulfill the parasite sterol requirements. Squalene synthase catalyzes the first committed step in the sterol biosynthesis and has been studied as a possible target for the treatment of high cholesterol levels in humans. In this work we investigated the antiproliferative and ultrastructural effects induced by 3-(biphenyl-4-yl)-3-hydroxyquinuclidine (BPQ-OH), a specific inhibitor of squalene synthase, on promastigote and amastigote forms of *Leishmania amazonensis*. BPQ-OH had a potent dose-dependent growth inhibitory effect against promastigotes and amastigotes, with IC_{50} values 0.85 and 0.11 μM, respectively. Ultrastructural analysis of the treated parasites revealed several changes in the morphology of promastigote forms. The main ultrastructural change was found in the plasma membrane, which showed signs of disorganization, with the concomitant formation of elaborated structures. We also observed alterations in the mitochondrion–kinetoplast complex such as mitochondrial swelling, rupture of its internal membrane and an abnormal compaction of the kinetoplast. Other alterations included the appearance of multivesicular bodies, myelin-like figures, alterations of the flagellar membrane and presence of parasites with two or more nuclei and kinetoplasts. We conclude that the BPQ-OH was a potent growth inhibitor of *L. amazonensis*, which led to profound changes of the parasite's ultrastructure and might be a valuable lead compound for the development of novel anti-*Leishmania* agents.

Index Descriptors and Abbreviations: *Leishmania*; Squalene synthase; Sterol biosynthesis inhibitors; Ergosterol; Chemotherapy; Ultrastructure

1. Introduction

Parasites of the genus *Leishmania* belong to the Trypanosomatidae family and are responsible for the different forms of leishmaniasis, which are present in many countries and affect millions of people around the world (Hirst and Stapley, 2000; Morel, 2000; WHO, 1990, 1997). Leishmaniasis existes in three clinical forms: visceral, cutaneous and mucocutaneous. The specie *Leishmania amazonensis* is responsible in Brazil for the cases of cutaneous disease, where lesions are confined to skin. In some infections with *L. amazonensis*, where the patients are resistant to treatment and the immune system fails to react against the parasite, the disease can develop into diffuse leishmaniasis. In these cases the lesions cover a large part of the body and never heal without treatment (Marsden and Jones, 1985). Another important feature of this species is its capacity to cause visceral leishmaniasis and post-kalazar dermal leishmaniasis (Barral et al., 1991).

The drugs currently available for the treatment of leishmaniasis are unsatisfactory, due to limited efficacy, frequent toxic effects and increasing drug resistance (Croft, 2001; Croft et al., 1997; WHO, 1997). Thus, there is a pressing need for new therapeutic approaches, with improved efficacy and safety. The primary treatment for leishmaniasis is still

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based on pentavalent antimonials such as Pentostam and Glucantime. The second-line compounds used to resistant cases generally include pentamidine and amphotericin B (Croft and Yardley, 2002). One increasingly interesting approach for development of new anti-*Leishmania* are ergosterol biosynthesis inhibitors. Ergosterol is the main sterol of trypanosomatids and fungi and is not present in the mammalian cells, where the major sterol is cholesterol (Barrett-Bee and Ryder, 1992; Oehlschlager and Czyzwska, 1992; Urbina, 1997). Various studies have shown the effect of ergosterol biosynthesis inhibitors in *Leishmania*. Some enzymes, such as HMG-CoA reductase (Urbina et al., 1993), farnesyl-pyrophosphate synthase (Docampo and Moreno, 2001), squalene synthase (Bergstrom et al., 1995; Braga et al., 2004; Urbina et al., 2002, 2004), squalene epoxidase (Urbina et al., 1988; Vannier-Santos et al., 1995), C14α-demethylase (Lazardi et al., 1990, 1991; Urbina et al., 1988, 1998, 2003; Vannier-Santos et al., 1995) and $\Delta^{24(25)}$-sterol methyltransferase (Vivas et al., 1996; Magaraci et al., 2003; Rodrigues et al., 2002; Urbina et al., 1996; Lorente et al., 2004) which participate in this biosynthetic pathway, have been studied in *Leishmania* and *Trypanosoma*.

Squalene synthase (SQS) catalyzes an unusual head to head reductive dimerization of two molecules of farnesyl-pyrophosphate (FPP) in a two-step reaction to form squalene (Barrett-Bee and Ryder, 1992; Oehlschlager and Czyzwska, 1992; Urbina et al., 2002). This is the first committed step in the synthesis of sterols and is an attractive target for anti-parasitic chemotherapy because its inhibition does not affect the synthesis of other essential isoprenoids in the host (Gonzalez-Pacanowska et al., 1988). SQS has been under intense scrutiny with the aim of developing new cholesterol-lowering agents for humans. Several workers have described a potent effect of aryl-quinuclidine derivatives, such as 3-(biphenyl-4-yl)-3-hydroxyquinuclidine (BPQ-OH), in rat liver microsomes, where kinetic analysis showed that this drug is a selective inhibitor of squalene synthase (Bergstrom et al., 1995; Brown et al., 1996; McTaggart et al., 1996; Ward et al., 1996). The in vivo inhibition of squalene synthase by BPQ-OH in experimental animals has also been documented, with a significant reduction of cholesterol levels in treated animals (McTaggart et al., 1996). Other studies have shown that, in *Saccharmomyces cerevisiae* (Bammert and Fostel, 2000) and *Candida* albicans (De Backer et al., 2001),as well as in *Leishmania major* (Cotrim et al., 1999), the expression of SQS is strongly activated in the presence of sterol biosynthesis inhibitors, a fact which indicates that this enzyme may be an important regulator in ergosterol biosynthesis. A recent study showed the effect of BPQ-OH in *Leishmania mexicana* and *Trypanosoma cruzi*, with a potent inhibition of squalene synthase and a marked reduction of the parasites endogenous sterol, associated with an intense antiproliferative effect on the promastigote and epimastigote forms, respectively (Urbina et al., 2002). Other important study showed the effects of the BPQ-OH on the ultrastructure of epimastigote forms of *T. cruzi* (Braga et al., 2004). In the present study, we investigated the effects of BPQ-OH on the growth and cellular ultrastructure of both promastigote and amastigote forms of *L. amazonensis*.

2. Materials and methods

2.1. Parasites

The MHOM/BR/75/Josefa strain of *L. amazonensis* isolated from a patient with diffuse cutaneous leishmaniasis by Dr. C.A. Cuba-Cuba (Universidade de Brasília, Brasil) was used in the present study. It has been maintained by Balb/C footpad inoculation and, in the case of promastigotes, axenically cultured in Warren's medium (Warren, 1960) supplemented with 10% fetal bovine serum at 25 °C. Intracellular amastigotes of *L. amazonensis* were cultivated in peritoneal murine macrophages maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum in a atmosphere of 5% CO_2 at 37°.

2.2. In vitro activities of the SQS inhibitor

Growth experiments with *L. amazonensis* promastigotes were initiated with 2.0×10^6 parasite/mL and the drug was added at different concentrations, from stock solutions in dimethyl-sulphoxide (DMSO) after 24 h of growth. Cell densities were determined daily in a hemocytometer with a light microscope during five days. In addition, to observe the effects of BPQ-OH in the growth of intracellular amastigotes of *L. amazonensis*, peritoneal macrophages from Swiss mice were harvested by washing with DMEM (Gibco), plated in 24 well tissue culture chamber slides and allowed to adhere for 24 h at 37 °C in 5% CO_2. Adherent macrophages were infected with amastigotes isolated from murine lesions using a ratio of 1:10 at 37 °C for 2 h. After this time, the non-phagocytosed parasites were removed by washing and infected cultures were incubated for 24 h in DMEM medium without drugs. BPQ-OH in different concentrations was added after 24 h of interaction, when the number of amastigotes per macrophage was in the range of 2–5 amastigotes/cell, and the medium with drug was changed daily, for five days. The cultures were fixed in Bouin's solution and stained with Giemsa for 30 min. The percentage of infected cells was determined using light microscopy. The association indexes (mean number of parasites internalized multiplied by the percentage of infected macrophages divided by the total number of macrophages) were determined daily. The results are expressed as mean of three independent experiments.

2.3. Determination of IC_{50}

The concentration that inhibited the growth by 50% was determined using the expression (Martin et al., 2001)

$$I = \frac{I_{\max} C}{\mathrm{IC}_5 0 + C},$$