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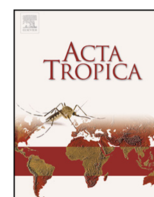

In vitro antileishmanial activity of novel azoles (3-imidazolylflavanones) against promastigote and amastigote stages of *Leishmania major*

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ABSTRACT

Leishmaniasis is a protozoan infectious disease widely distributed all around the world. First line drugs including antimoniales are insufficient due to resistance in endemic areas and high toxicity. Azole antifungals like ketoconazole (KCZ) are also used as antileishmanial agents for several decades. In the present study, we evaluated in vitro antileishmanial effects of new azole antifungals namely 3-imidazolylflavanones (IFs) and their oximes (IFOs) against *Leishmania major (L. major)* parasites. The obtained results showed remarkable effect of our compounds on promastigote and amastigote stages of *L. major*. In particular, the 4-chloro analog of flavanone (IF-2) and 3-chloro substituted flavanone oxime (IFO-3) with IC_{50} values ≤8.9 μg/mL were 8-fold more potent than KCZ (IC_{50} = 72 μg/mL) against promastigote form of *L. major*. In amastigote stage, the compounds IF-2 and IFO-2 decreased the mean number of infected macrophages (MIR) more than KCZ ($p < 0.005$). In addition, compounds IF-1, IF-2, IF-4, IFO-2, IFO-3 and IFO-5 decreased the mean number of amastigotes per macrophages (MNAPM) significantly more than KCZ ($p < 0.005$). All compounds decreased both MIR and MNAPM significantly more than control ($p < 0.001$). Compounds IF-2 and IFO-2 with parasite survival of 7.70% and 20% had the highest inhibition on intracellular amastigotes. Although most of compounds displayed acceptable selectivity index, compound IF-2 had the highest CC_{50} value (115.4 μg/mL) and SI (383.3). We concluded that our new synthetic azoles displaying potent activity against *L. major* could be considered as new hits for drug development in the field of antileishmanial therapy.

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1. Introduction

Leishmaniasis as a neglected parasitic disease is caused by *Leishmania* genus and transmitted by bite of infected female sand fly (Valdivia et al., 2015). There is a wide spectrum of disease from mild self healing cutaneous to visceral clinical forms which could be fatal in untreated cases (Valdivia et al., 2015). Leishmaniasis threatens about 350 million people all around the world. World Health Organization (WHO) estimates that 12 million people are affected by the *Leishmania spp.* in Africa, Asia, South America and Europe (Kumar Haldar et al., 2011).

Cutaneous leishmaniasis (CL) is common form of leishmaniasis and widely distributed all around the world. Ninety percent of CL cases occur in Iran, Afghanistan, Brazil, Saudi Arabia, Peru, and Syria (Jeddi et al., 2011; WHO 2016). Current treatments of leishmaniasis including pentavalent antimonials are insufficient due to their toxicity, side effects and drug resistance (Roberts et al., 2003). Usage of other available drugs such as pentamidine and miltefosine has been restricted by their high cost, incidence of resistance and insufficient effect (Jeddi et al., 2011).

Several studies have demonstrated the antileishmanial effects of azole antifungals (Marrapu et al., 2011; Tahghighi et al., 2012).

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Azole antifungals like ketoconazole (KCZ) have been used in the treatment of leishmaniasis for more than two decades (Saenz et al., 1990). The mechanism of action of azoles in fungi is inhibition of cytochrome P450-dependent lanosterol 14α-demethylase enzyme, leading to blockage of ergosterol biosynthesis. Therefore, azoles can upset the biosynthesis of fungi cell membranes (Monzote 2009; Babazadeh-Qazijahani et al., 2014). The enzyme is present in *Leishmania* parasites and azoles are effective against them as well as fungi.

A structural survey of imidazole-containing antifungal drugs such as KCZ reveals the presence of one common pharmacophoric backbone namely *N*-(phenethyl)imidazole. This backbone structure is characterized by an aryl moiety attached to the nitrogen of imidazole ring with a two carbons linker (Ji et al., 2000). In the search for finding new and more effective azoles, we have previously designed and synthesized different series of chroman-based azole antifungals bearing a conformationally constrained backbone (Emami and Shafiee, 2001; Emami et al., 2002; Emami et al., 2004a,b; Ayati et al., 2012). Based on *N*-(phenethyl)imidazole scaffold which is proposed to be essential in the interaction with enzyme, we have particularly designed and synthesized 3-imidazolylflavanones (IFs) and their related oximes (IFOs). These compounds also possess the pharmacophoric backbone from two sides (Emami et al., 2009; Emami and Foroumadi, 2009). On the other hand, the flavanone scaffold can be found in some flavonoids that are natural products with various biological properties including antifungal and antileishmanial activities (Pretorius 2003; Weidenbçrner and Jha 1997). For example, 5,6,7-trihydroxy-4′-methoxyflavanone and sakuranetin (4′,5-dihydroxy-7-methoxyflavanone) were isolated from *Baccharis retusa* and showed antileishmanial activity against various species such as *L. amazonensis*, *L. braziliensis*, *L. major*, and *L. chagasi* (Grecco et al., 2010; Grecco et al., 2012).

The aim of present study was to evaluate the in vitro activity of synthesized 3-imidazolylflavanones (IFs) and their related oximes (IFOs) on promastigote and amastigote stages of *L. major* as their potential action has been evaluated previously on different fungi strains.

2. Methods

2.1. Investigated drugs

KCZ was obtained from commercial source. The 3-imidazolylflavanones IFs and their oximes IFOs were synthesized according to the previously reported methods. The structures of all compounds were characterized by ^{1}H NMR and ^{13}C NMR spectroscopy and elemental analyses (Emami et al., 2009; Emami and Foroumadi, 2009). The structures and chemical names of tested compounds were listed in Table 1. All these compounds which possess two chiral centers at the C-2 and C-3 positions of chroman ring are racemic mixtures. The synthesized compounds including IFs and IFOs were dissolved in DMSO (Dimethyl sulfoxide) (1 mg/mL) and serial dilutions were prepared from 500 μg/mL to 0.97 μg/mL. Serial dilutions of KCZ were prepared as well.

2.2. Promastigote culture and treatment

In this study we used *L. major* vaccine strain (MRHO/IR/75/ER) cultured in NNN medium. For mass culture, 2×10^6 promastigotes/mL were transferred to RPMI-1640 supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), 10% heat-inactivated fetal calf serum (FCS), HEPES (25 mM, pH 7.2) and incubated in 25 ± 1 °C. Logarithmic and stationary phases appeared after 48 and 96 h, respectively. Parasites in stationary phase were used for evaluating drug susceptibility. Serial dilutions of compounds and KCZ as reference drug were prepared in 96-well micro titer plates. Promastigotes (10^5 cell/mL) were added to each well and incubated in 25 ±1 °C for 72 h. Promastigotes with no drug and medium without parasite were considered as positive and negative controls. Also, the medium containing 2% DMSO was used as control. All trials were done in triplicate (Shokri et al., 2011).

2.2.1. MTT assay

After incubation, 10 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-aldrich, USA) solution prepared in sterile PBS, was added to each well and incubated at 25 ± 1 °C for 4 h. The live cells produced formazan, a dark black product but dead cells did not produce such stains. The reaction was stopped by adding 100 μL acidified isopropyl alcohol and optical density was read by ELISA reader (Synergy H1Hybrid Reader, BioTek) at 570 nm. The IC_{50} values were calculated with CalcuSynv2 demo program.

2.2.2. Treatments on cell line and amastigotes

Macrophage line J774A.1 (ATCC number TIB-67) was obtained from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Macrophages were kept in RPMI medium. Cells were diluted in medium then viability test was performed by adding 90 μL of trypan blue solution (0.2% in saline) containing 0.01% sodium azide to 10 μL of cell suspension (10^6 cells/mL). After 2 min, cells were counted under light microscope, and viability was calculated as follows: %Viability = (% of live cells/all counted cells) × 100

Briefly, 200 μL of the cells (10^6 cells/mL) was added into 8-chamber slide (SPL, Korea) and incubated at 37 °C with 5% CO_2 for 4 h. After that, non-adherent cells were removed and plate was incubated at 37 °C and 5% CO_2 for 24 h. Then, macrophages were infected with *L. major* promastigotes with a parasite/macrophage ratio of 10: 1(10^7 cell/mL), following 4 h incubation, free promastigotes were separated by washing with PBS. After overnight incubation at 37 °C and 5% CO_2, the compounds and KCZ as a reference drug were added to the plate wells with concentrations 500–0.97 μg/mL (20 μL in medium). The media containing drugs were renewed after 48 h. After 60 h incubation, the plates were washed with PBS, fixed in methanol, stained with 10% Giemsa solution, and examined with a light microscope. The mean number of infected macrophages and also mean number of amastigotes in 100 macrophages were counted and the results presented as mean infection rate (MIR) and mean number of amastigotes per macrophage (MNAPM) and parasite survival (PS) (Ngure et al., 2009).

$$PS(\%) = \frac{\left(\text{No. of amastigotes in experimental culture}/100\text{ macrophages}\right) \times 100}{\text{No. of amastigotes in control culture}/100\text{ macrophages}}$$

Inhibition of intracellular amastigote growth achieved by 100-PS as well. Macrophages containing amastigotes with no drugs and macrophages alone were considered as positive and negative controls, respectively. All trials were done in triplicate (Shokri et al., 2011).

2.3. Cytotoxicity test

Toxicity of compounds against J774.A.1 macrophages was evaluated with cells plated in 96-well plates at 2×10^5 cells/well. After 2 h and cell adherence, the medium was removed, replaced by the fresh media containing several concentrations (500–0.97 μg/mL) of each compound, and incubated at 37 °C with 5% CO_2 for 60 h. Culture plus DMSO was incubated as control cells. All trials were done in triplicate and MTT colorimetric assay was used for determining

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