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Promising antileishmanial activity of novel imidazole antifungal drug Luliconazole against *Leishmania major*: *in vitro* and *in silico* studies

Azar Shokri*a, Mahdi Abastabarb, Masoud Keighobadici, Saeed Emamid, Mahdi Fakhar **a, Saeed Hosseini Tesnizi, Koichi Makimura, Ali Rezaei-Matekolaie, Hassan Mirzaeic

*a Molecular and Cell Biology Research Center, Department of Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
b Invasive Fungi Research Center (IFRC), Department of Mycology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
c Pharmaceutical Sciences Research Center, Student Research Committee, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran
d Department of Medicinal Chemistry and Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran
e Department of Medical Mycology, School of Medicine, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
f Laboratory of Space and Environmental Medicine, Graduate School of Medicine, Teikyo University, Tokyo, Japan
g Infectious and Tropical Diseases Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

**Corresponding author: M. Fakhar, Molecular and Cell Biology Research Center, Department of Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Farah Abad, Sari 48471-91971, Iran. Tel/fax: +981133543248. Email: mahdif53@yahoo.com

*These authors contributed equally to the work. (M.Keighobadi as co-first author contributed equally with A. Shokri as first author).
Graphical abstract

A novel candidate for treatment of leishmaniasis in the near future

Luliconazole  Ketoconazole
Highlights

- We investigated in-vitro anti-leishmanial activities of Luliconazole as a novel imidazole antifungal drug on *Leishmania major*.
- In amastigote stage, Luliconazole at the dose of 0.07 µM decreased the number of amastigotes significantly more than both Ketoconazole (p = 0.043) and meglumine antimoniate (p<0.004).
- Docking study revealed that Luliconazole can properly interact with the target enzyme in *Leishmania* mainly via coordination with the heme and multiple hydrophobic interactions.
- According to the potent efficacy of Luliconazole in very low concentration against *L. major*, it could be considered as a novel candidate for treatment of leishmaniasis.

Abstract

**Objectives:** Pentavalent antimonials have been used for the treatment of leishmaniasis for more than 70 years; however their toxicity limits their benefits. Unfortunately, the efficacy of the first line drugs has been decreased and resistance seems to be noticeable. Luliconazole is a new azole with unique effects on fungi which has not been tested on *Leishmania* parasites yet.

**Methods:** In the present study, we evaluated cytotoxicity and anti-leishmanial activities of Luliconazole on promastigotes and intracellular amastigotes of *Leishmania major* (*L. major*) in vitro. In addition, the 3D structure of Luliconazole was prepared by ACD ChemSketch 11 Software.

**Results:** Our results on promastigotes revealed that Luliconazole with IC₅₀ value of 0.19 µM has greatly higher potency than Ketoconazole (KCZ), Meglumine Antimoniate (MA) and Amphotericin B (AmB) (IC₅₀= 135 µM, 538 µM and 2.52 µM). In amastigote stage, at the dose of 0.07 µM, Luliconazole decreased the mean infection rate (MIR) and the mean number of amastigotes per macrophages (MNAPM) more effectively than MA (p<0.004) and KCZ (p<0.043) but there was no different association with AmB (p>0.05). Docking study of
Luliconazole with cytochrome P450 sterol 14α-demethylase (PDB code: 3L4D) revealed that this azole drug can properly interact with the target enzyme in *Leishmania* mainly via coordination with the heme and multiple hydrophobic interactions.

**Conclusion:** These results showed the potent activity of Luliconazole in extremely low concentration against *L. major*; therefore, it could be considered as a new candidate for treatment of leishmaniasis in the close future.

**Keywords:** *Leishmania major*, Luliconazole, anti-leishmanial activity, *in silico* study

1. **Introduction**

Leishmaniasis, as a neglected infectious disease, is caused by *Leishmania* spp. and it has wide range of spectrums including cutaneous, mucocutaneous and visceral forms. Cutaneous leishmaniasis (CL) is a mild form, mucocutaneous which involves mucosa and visceral form can be fatal if left untreated. The infection is transmitted by the bite of infected female sand fly. Some forms of the disease are self-limited and some cases need to be treated [1, 2]. The most common form of the disease is CL. Although this form is not fatal, it can leading to skin lesions, which leave permanent scars and in some cases cause serious disability [2]. Treatment of leishmaniasis is challenging and also no vaccine is available for prevention. For over seventy years antimonials has been used to treat the leishmaniasis [3]. Meglumin Antimoniate (MA) and sodium stibogluconate (SSG) are the first line drugs for the treatment of leishmaniasis, which are administered through intravenous or intramuscular injection, but they are toxic [1]. Their mechanism of action is not fully understood but possibly parasite or host infected cells are involved in biologically reducing Sb (V) to Sb III to perform antileishmanial activity.
Unfortunately, the efficacy of present drugs are reduced in some countries including Iran [4], and second line drugs like Amphotericin B and Miltefosine have serious toxic side effects [1]. There is an imperative need for the finding of new leishmanicidal drugs with less toxicity and more efficacies [5, 6]. Therefore, various strategies and approaches for finding new effective, safe, and inexpensive drugs for treatment of leishmaniasis are in progress [7-10]. Azoles (imidazoles and triazoles) are widely used in fungal infections and their mechanism of action is inhibition of ergosterol biosynthesis in the cell membranes [11]. Ergosterol is essential for membrane stability and integrity. Deficiency in ergosterol leads to membrane damage and cell death. Recent studies showed efficacy of azoles on *Leishmania* parasites [12]. A number of azole antifungal drugs such as Ketoconazole (KCZ) and Fluconazole have been evaluated in different studies. The efficacy of Ketoconazole varied with the parasite species and is not commonly used. Moreover, treatment with Fluconazole has revealed satisfactory healing trend of ulcer [13].

Luliconazole, \((2E)-[4R]-4-(2,4\text{-dichlorophenyl})-1,3\text{-dithiolan-2-ylidene}(1H\text{-imidazol-1-yl})\text{acetonitrile}\) is a new antifungal imidazole agent with unique structure and especially effective against dermatophytes and azole-resistant and susceptible *Aspergillus fumigatus* strains [14, 15]. Luliconazole is an *R*-enantiomer and is more effective than Lanoconazole as a racemic mixture. Efficient therapeutic effects of Luliconazole in topical formulation have been shown and in some reports it is more effective than terbinafine [14, 16].

Both allylamines and imidazoles inhibiting the ergosterol synthesis, which is essential component of cell membranes. Allylamines works by blocking the squalene conversion to squalene-2, 3-epoxide, which is the precursor of ergosterol formation, while imidazoles inhibit Cytochrome P450 (CYP450) 14α-demethylase enzyme specifically, leading to disruption of lanosterol to ergosterol conversion. Imidazole also interferes with triglycerides and phospholipids synthesis, which causes accumulation of toxic levels of hydrogen peroxide within cells. This accumulation
of hydrogen peroxide toxic level can destroy sub-cellular organelles and finally cell necrosis and death \[17,18\].

However, to date, no data is available on the susceptibility profile of *Leishmania* isolates against Luliconazole. Thus, the aim of the present study was to investigate the in vitro activity of Luliconazole against promastigote and intracellular amastigote of *L. major* and also prepare the three dimensional (3D) structure of Luliconazole using ACD ChemSketch 11 Software.

2. Materials and Methods

2.1. Drugs supply

Meglumine antimoniate (MA) (Glucantine®, Rhône–Poulenc, France), KCZ (Janssen Pharmaceutica, Beerse, Belgium), Luliconazole (Nihon Nohyaku Co, Osaka, Japan) (Fig. 1) and Amphotericin B (AmB) (Sigma, Saint-Quentin-Fallavier, France) were commercially available. The stock solutions of Luliconazol, KCZ, MA and AmB were prepared in Dimethyl sulfoxide (DMSO) (1 mg/mL).

2.2. Parasite culture

Promastigotes of *L. major* Iranian reference strain (MRHO/IR/75/ER) were grown in NNN medium and sub-cultured in RPMI-1640 medium (Gibco, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 μg/mL), and 25 mM HEPES, pH 7.2 at 26 °C. Cultures were monitored daily and sub-cultured regularly to obtain stationary phase of *Leishmania*.

2.3. Treatment of promastigotes with drugs

The susceptibility of promastigotes was carried out according to the method described by Shokri et al [12]. Serial dilutions of Luliconazole, KCZ, MA and Am B were prepared in RPMI-1640 (pH, 7.2). Final concentrations of Luliconazole, KCZ, MA and Am B were 0.003 to 8 μM, 2.5 to
300 µM, 5 to 600 µM and 0.002 to 10 µM, respectively. Promastigotes in stationary phase (10⁶/mL) in a volume of 100 µL were added to each well, then 10 µl of each concentrations were added and incubated at 25 ± 1 °C for 72 h. The medium with no drug was considered as positive control. All experiments were performed in triplicate. MTT assay was performed by preparing MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Aldrich, USA) in sterile PBS and 10 µL of MTT solution was added in each well, incubated at 25 ± 1 °C for 4 h. The reaction was stopped by using isopropyl alcohol and the optical density (OD) was read by ELISA reader (Synergy H1, BioTeck) at 490 nm. The IC₅₀ (the concentration of test compound that is require for 50% inhibition) values were determined by CalcuSynv2 Demo program (Table 1).

2.4. Treatment of intracellular amastigote with drugs

To determine the susceptibility of intracellular amastigotes, all the drugs were tested in several concentrations as used against promastigotes. Each experiment was carried out in triplicate. Macrophage cell line J774.A.1 (ECACC number 91051511) was obtained from Iranian National Cell Bank (Pasteur Institute, Tehran, Iran). Macrophage cells were grown in RPMI-1640 medium. The cells were diluted in medium and viability test was performed by adding 90 µL of trypan blue solution (0.2%) in normal saline containing 0.01% sodium azide to 10 µL of the cell suspension (10⁶ cells/mL). After 2 min, the cells were counted under light microscope, and viability was calculated as follows:

\[
\% \text{Viability} = \left( \frac{\% \text{ of live cells}}{\text{all counted cells}} \right) \times 100
\]

In brief, 200 µL of the cells (10⁶ cells/mL) was added into 8-chamber slide (SPL. Korea) and incubated at 37 °C with 5% CO₂ for 3 h. Then, cultured promastigotes (10⁶ cells/mL) were added to the macrophage cells and incubated at 37 °C with 5% CO₂ for 24 h. Then medium containing non-infecting promastigotes was removed, plates were washed with 1x phosphate buffered saline (1x PBS), and new RPMI-1640 medium and appropriate solutions (10 µL) of Luliconazole or KCZ in concentrations explained for promastigotes was added and incubated at 37 °C for 72 h. In
addition, MA, KCZ, and Am B were used as a reference drugs. Dried slides were fixed with ethanol, stained by Wright-Giemsa and studied under light microscope. Macrophages containing amastigotes with no drugs and macrophages alone were considered as positive and negative controls, respectively. Drug activity was evaluated by counting the number of amastigotes inside macrophages by examining 100 macrophages [10].

2.5. Cytotoxicity assessment (CC\(_{50}\)) and Selectivity Index (SI):

Cytotoxicity assay was achieved with J774.A.1 macrophages cells coated in 96-well plates at 2×10\(^5\) cells/well. The plates were incubated for 72 h at 37 °C in a humidified incubator with 5% CO\(_2\). Control cells were incubated with culture medium plus DMSO. Cell viability was verified using MTT colorimetric assay [19]. Moreover, selectivity index (SI) was achieved by dividing the CC\(_{50}\) to IC\(_{50}\) of amastigotes for all investigated drugs.

2.6. In silico study

The 3D structure of Luliconazole used in this study was prepared by ACD ChemSketch 11 Software. Also, the structure of cytochrome P450 sterol 14α-demethylase (PDB code: 3L4D) was obtained from Protein Data Bank. For preparation of protein structure, water molecules and cognate ligand were removed. Furthermore, non-polar hydrogens were merged and Kollman charges were assigned to the protein structure. Non-polar hydrogens were added to the ligand; Gasteiger charges were assigned; then, suitable Lamarckian Genetic Algorithm (LGA) parameters were determined as described: 100 GA runs; 27000 maximum generations.

The validity of the method was taken in terms of root mean square deviation (RMSD) of the cognate ligand atoms in the re-docked and crystallographic conformations (RMSD = 0.39 Å). In this study, all the steps were performed by AutoDock 4.2 program [20]. The best pose with the highest score was chosen for evaluation the interactions and estimating the free energy of binding [21].

2.7. Data analysis
The data were analyzed using SPSS version 16.0 software (SPSS Inc., Chicago, IL). ANOVA test, multiple comparison test and t-test were used. The IC₅₀ values of MA, Luliconazole, KCZ for both promastigote and amastigote stages were compared using t-test, and P<0.05 was considered as a significant difference.

3. Results

3.1. Anti-promastigote activity

The azole antifungal drug Luliconazole was initially tested in vitro on cultured promastigotes. All tests were carried out in triplicate. MA was used as reference drug and also KCZ as a well-known azole used in leishmaniasis. Within promastigote viability assay, several concentrations of Luliconazole showed significant decrease in OD as measured by MTT method (P<0.05). Luliconazole could significantly reduce viability of promastigotes at various concentrations displaying IC₅₀ value of 0.19 µM (Table 1). The IC₅₀ value of MA, KCZ and AmB were 538 µM, 135 µM and 2.52 µM, respectively, being significantly higher than that of Luliconazole 0.19 µM (p<0.001).

3.2. Anti-amastigote activity

Luliconazole decreased both the mean number of infected macrophages (MIR) and the mean number of amastigotes per macrophages (MNAPM) significantly more than control test (p<0.001). Luliconazole decreased MIR significantly more than MA at the doses of 0.07 µM (p = 0.002). Luliconazole at the dose of 0.07 µM decreased the number of amastigotes significantly more than both KCZ (p = 0.043) and MA (p<0.004) but there was no different association with AmB (p>0.05). MA, KCZ and AmB significantly decreased the MIR and amastigotes per macrophages more than control test (p<0.001).

3.3 Cytotoxicity (CC₅₀) and Selectivity Index (SI) assessment
The cytotoxicity Luliconazole, MA and KCZ of was determined at several concentrations of each compound. The cytotoxicity of Luliconazole (0.79 μM) was lower than other investigated drugs. In addition, the SI of AmB, Luliconazole, MA and KCZ was 24.90, 11.28, 5.43 and 1.79 respectively (Table 1).

3.4. Docking simulation

Azole drugs such as Luliconazole inhibit the Cytochrome P450 sterol 14α-demethylase (CYP51) enzyme that results in restriction of ergosterol biosynthesis in fungal and protozoan cells [22]. In order to clarify all interactions of Luliconazole with the target enzyme, docking study was performed. As shown in Fig. 2, the N-3 atom of the imidazole ring in the Luliconazole structure is located over the porphyrin prosthetic group and coordinated to the heme iron. Based on the obtained results, distance between the N-3 atom of imidazole ring and Fe atom of heme is 2.53 Å that being suitable for effective coordination. In addition, the free energy of binding for the best docked pose was -8.05 kJ/mol. As seen in Fig. 3, there is a carbon hydrogen bond between Ala262 with the C4-H of imidazole ring. In this study, we used Discovery Studio program (version16) to show hydrophobic interactions. There are several hydrophobic interactions between docked inhibitor (Luliconazole) and amino acids of target enzyme (CYP51). As depicted in Fig. 3, the 4-chloro group on the phenyl ring of Luliconazole contributed in the hydrophobic interactions with Val184, Pro181, and Phe76. Moreover, Met431 and Leu327 involved in the hydrophobic interactions with 2-chloro of phenyl moiety. The lipophilic center of Luliconazole (dithiolane ring) interacted with Tyr74. In general, other amino acids with hydrophobic residues including Val73, Met331, Met77, Phe81, Phe261, Val432, Thr266, Leu330 and Met329 were in the contact with rest of the Luliconazole molecule (Fig. 3).

4. Discussion
Current established therapy for leishmaniasis is pentavalent antimonials including SSG and MA administered both intravenously and intramuscularly. Despite the good responses in general, their efficacy has been decreased and resistance occurs in endemic regions of the disease. Moreover, second and third line drugs like Amphotericin B has serious limitations due to their toxic side effects [1]. Azoles are another choice for leishmaniasis treatment as they (imidazoles and triazoles) are widely used in fungal infections. Azoles mechanism of action is inhibition of ergosterol biosynthesis in the cell membranes [11].

There are several studies about inhibitory effect of azoles on Leishmania parasites [23]. Some of azoles inhibit 14α-demethylation which participates to the synthesis of membrane ergostrol mediated by cytochrome P450 [24]. An important point about azoles is oral administration of them. Ketoconazole have been used since 1980 with good effect on parasites but not commonly used for CL [13, 25]. Alrajhi et al [26] reported that the triazole drug fluconazole is effective for the treatment of CL caused by L. major. The most recent triazole drug Voriconazole has proven to have greater effect on Leishmania parasites than Fluconazole [27]. Itraconazole and Posaconazole were evaluated in vitro on Leishmania amazonensis and the results showed satisfactory effect on promastigotes with IC$_{50}$ values of 2.74 µM and 0.44 µM for Posaconazole and Itraconazole, respectively [23]. Itraconazole (100–400 mg/d) was evaluated by several clinical trials on small series of dermal lesions in India, Italy, Brazil, United Kingdom and Argentina and good efficacy revealed [13].

It seems that the azole drugs have same mechanism of action in fungi and Leishmania parasites. In order to find new and more efficient agents with lower toxicity and higher potency, researchers are trying to design and synthesize new azoles. In particular, we have recently synthesized and evaluated 3-imidazolylflavanone derivatives containing pharmacophoric backbone of azole antifungals against both promastigote and amastigote of L.major [12]. Evaluation of synthetic
novel aryloxy tetrahydronaphtyl azoles and related cyclohexyl azoles as anti-leishmanial agents revealed that some of them could strongly inhibit growth of *Leishmania* parasites [7].

Imidazoles selectively inhibit fungal Cytochrome P450 (CYP450) 14 \( \alpha \)-demethylase enzyme, which disrupts the conversion of lanosterol to ergosterol. Imidazoles also alter the synthesis of triglycerides and phospholipids, which leads to an accumulation of toxic levels of hydrogen peroxide within fungal cells. This leads to toxic concentrations of hydrogen peroxide which causes cell death.

Luliconazole an antifungal imidazole is well known for its specific effect on the variety of fungi including yeast and dermatophytes. In addition, it has significant fungicidal effect similar to Terbinafine against *Trichophyton spp*. Topical administration of Luliconazole 1% cream have shown to be effective even in a short period usage. Interestingly, in a recent study by Baghi et al [14], Luliconazole was more active against dermatophyte strains, compared to the common first-line drugs such as Itraconazole and Terbinafine.

To the extent of our knowledge, there is no study on the anti-leishmanial effects of Luliconazole and this is the first report of anti-leishmanial activity of this agent on *L. major*. Our study revealed that Luliconazole with low \( IC_{50} \) value of 0.19 \( \mu \)M can potently inhibit the growth of promastigotes (Table 1). In addition, in the concentration of 0.07 \( \mu \)M it can more effectively reduce the MIR and the number of amastigotes inside macrophages when compared to MA, KCZ and Am B.

MA is the first line drug for treatment of leishmaniasis and Ketoconazole is an imidazole used as anti-leishmanial agent. It should be noted that the obtained \( IC_{50} \) values for MA and Ketoconazole against promastigotes were 538 \( \mu \)M and 135 \( \mu \)M respectively, being significantly higher than that of Luliconazole. Our results indicated that Luliconazole can effectively inhibit growth of *L. major* at very low concentrations that is not cytotoxic for macrophages at this concentration.
As previously described, Luliconazole revealed acceptable SI (11.28) value with reference to other investigated drugs as control group. An important point for this finding is the toxicity of Luliconazole, but our experiment on macrophages cells (CC₅₀) indicated no toxicity in low concentrations. Among investigated drugs, Luliconazole had the lowest CC₅₀ (0.79 μM). Therefore we can consider it safe in these concentrations. The observed high potency of Luliconazole in our preliminary study makes it a good candidate for topical administration on dermal leishmanial lesions in animal model and in combination with MA for treatment of all clinical forms of leishmaniasis as well. As a whole, obtained results from molecular docking analysis indicated that Luliconazole interacted with essential amino acids residues of cytochrome P450 sterol 14α-demethylase enzyme in the active site and consequently inhibit effectively target enzyme and block sterol biosynthesis. Additionally, these results could confirm our experimental study.

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Declarations

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Competing Interests: None declared.

Ethical Approval: Not required.

References


Fig. 1 Chemical structures of Luliconazole and Ketoconazole.

Figure 2. 3D schematic representation of Luliconazole in the active site of Cytochrome P450 sterol 14α-demethylase (PDB code: 3L4D). N-3 atom of the imidazole ring in the Luliconazole structure coordinated to the heme iron. Luliconazole is shown as purple, heme ring colored in green and amino acid residues as gray.
Figure 3. 2D schematic representation of hydrophobic interactions for Luliconazole in the active site of Cytochrome P450 sterol 14α-demethylase (PDB code: 3L4D). Luliconazole contributed in the hydrophobic interactions. Only important amino acids for interaction are shown.
Table 1 Anti-leishmanial activity of Luliconazol, Meglumine antimoniate, Ketoconazole and Amphotericin B against promastigotes and amastigotes of *Leishmania major*.

<table>
<thead>
<tr>
<th>Investigated drugs</th>
<th>Cytotoxicity (CC₅₀) (μM)</th>
<th>SI</th>
<th>IC₅₀ (μM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Promastigotes</td>
<td>Amastigotes</td>
</tr>
<tr>
<td>Luliconazole</td>
<td>0.79</td>
<td>11.28</td>
<td>0.19</td>
<td>0.07</td>
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<tr>
<td>Meglumine antimoniate</td>
<td>491.84</td>
<td>5.43</td>
<td>538</td>
<td>90.44</td>
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<tr>
<td>Ketoconazole</td>
<td>43.27</td>
<td>1.79</td>
<td>135</td>
<td>24.04</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>18.43</td>
<td>24.90</td>
<td>2.52</td>
<td>0.74</td>
</tr>
</tbody>
</table>

SI: selectivity index = CC₅₀/IC₅₀ amastigote.