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Repurposing azole antifungals into antileishmanials: Novel 3-triazolylflavanones with promising *in vitro* antileishmanial activity against *Leishmania major*

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ABSTRACT

Previously, we have described a series of azole antifungals namely 3-(1,2,4-triazol-1-yl)flavanones (TFs) containing an *N*-(phenethyl)azole framework required for sterol 14α-demethylase (CYP51) inhibitory activity. Similar mechanism of action of azoles in fungi and protozoan parasites prompted us to investigate the potential effects of TFs against promastigote and amastigote forms of *Leishmania major* (*L. major*), as well as their toxicity against macrophages, apoptosis induction and *in silico* interactions with the target enzyme. All compounds showed more potent anti-parasitic activity against *L. major* in comparison with reference azole drug fluconazole and standard antileishmanial agent glucantime. Among the tested compounds, the 4-chloro derivative (TF-2) was found to be the most potent one, being about 13 times more potent than fluconazole against promastigotes. TF-2 decreased both mean infection rate of macrophages (MIR) and mean number of amastigotes per macrophages (MNAPM), significantly more than fluconazole ($P < .001$). Furthermore, the cytotoxicity assay against J774.A.1 macrophages revealed that this compound displays high selectivity against amastigotes over macrophages (SI = 30.21). The *in silico* study showed that TF-2 can properly accommodated in the active site of parasitic CYP51 and coordinated to the heme. The SAR analysis showed that the introduction of 4-chloro on 2-phenyl moiety results in the best profile of activity and selectivity. Accordingly, the compound TF-2 prototype can be considered as promising candidate for development of new antileishmanial agents.

1. Introduction

Leishmaniasis is a group of neglected tropical diseases with a significant clinical and epidemiological diversity, and is caused by protozoan parasite genus *Leishmania* [1]. The clinical manifestations may be varied from cutaneous or mucocutaneous lesions to fatal visceral leishmaniasis [2]. Leishmaniasis is widespread in many parts of the world especially in Asia, Africa and Latin America. It is estimated that about 350 million people are at risk of infection worldwide [3]. Therefore, leishmaniasis has a serious impact on global public health. *Leishmania* parasites are transmitted to humans by the bite of phlebotomine sandflies, and essentially have two distinct forms in their life cycle; a motile flagellated promastigote form and a non-motile amastigote form [4]. The promastigote form lives in the alimentary canal of the sandfly vector. After transmitting to mammalian hosts, the parasite survives and multiplies as amastigote within vacuoles of macrophages [5].

Currently, there is no effective vaccine for leishmaniasis. Therefore, the treatment of leishmaniasis relies primarily on chemotherapy. A limited number of drugs including stibogluconate (Pentostam), meglumine antimonate (Glucantime), amphotericin B and miltefosine are available for control of leishmanial infections [6]. Among these drugs, pentavalent antimonials have been originally designed as antileishmanial agents, but amphotericin B and miltefosine have been primarily introduced as antifungal and anticancer agents, respectively. These drugs have various shortcomings like parenteral administration, long term of treatment, toxic side effects and high cost of treatment [7]. Miltefosine associates with teratogenic effect and cannot be used in

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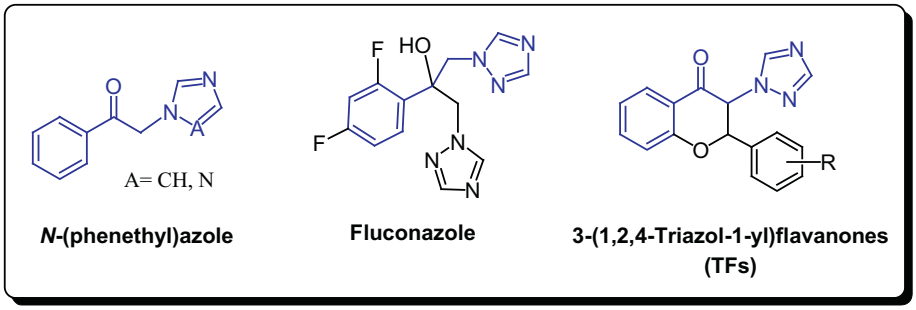


Fig. 1. Structures of fluconazole and 3-(1,2,4-triazol-1-yl)flavanones (TFs) containing *N*-(phenethyl)azole pharmacophore.

pregnancy [8]. Besides mentioned drugs, there are several potential antileishmanial agents at different stages of clinical development. In general, various approaches for exploring new effectual, safe, and low-cost drugs for treatment of leishmaniasis are in steps forward [9–13]. Azole antifungal agents such as miconazole, econazole, ketoconazole, fluconazole, and itraconazole are potentially active against *Leishmania*, but there are conflicting reports for their use in the treatment of cutaneous and visceral leishmaniasis [2,14–18].

Azole antifungals interfere with ergosterol biosynthesis by inhibiting the C-14 demethylation of sterols in *Leishmania*. A cytochrome P450-dependent enzyme namely sterol 14α-demethylase (CYP51) catalyzes the oxidative removal of the 14α-methyl group of sterols to give 14,15-unsaturated intermediates [19,20]. Therefore, the inhibition of CYP51 by azoles would cause depletion of ergosterol and accumulation of 14-methyl sterols resulting in the growth inhibition of protozoan parasites [21]. Previously, we have described a series of azole antifungals namely 3-(1,2,4-triazol-1-yl)flavanones (TFs) based on the *N*-(phenethyl)azole structure (Fig. 1) [22]. The common pharmacophoric structure for the most of azole drugs is *N*-(phenethyl)-imidazole or 1,2,4-triazole. Since the azole compounds which have been well-known for the control of fungal infections can be considered as a lead structures for antileishmanial drug discovery, we investigated the potential antileishmanial activity of TFs. Thus, we report here the *in vitro* antiparasitic activity of TFs against promastigote and amastigote forms of *Leishmania major* (*L. major*) and their structure-activity relationships, toxicity in host cells and *in silico* molecular modeling.

2. Materials and methods

2.1. Preparation of test compounds

The target compounds 3-(1,2,4-triazol-1-yl)flavanones (TFs) were synthesized as described previously [22]. The structures and chemical names of tested compounds (TF-1–TF-11) are listed in Table 1. Fluconazole and Glucantime® (Aventis Single source) were purchased from commercial sources. All compounds were dissolved in dimethyl sulfoxide (DMSO) and then serially diluted with culture medium to obtain a concentration range of 1.5–500 μg/mL. The highest final concentration of DMSO was < 1%, thus the medium containing 1% DMSO was used as control.

2.2. Parasites and culture

The Iranian reference strain of *L. major* (MRHO/IR/75/ER) was obtained from Pasteur Institute (Tehran, Iran). The parasites were passaged in susceptible BALB/c mice for maintenance of infectivity. For experiments, the promastigotes were grown in blood agar and Novy-MacNeal-Nicolle medium (NNN) at 25 °C. The parasites in stationary phase were washed with phosphate buffered saline and re-cultured in Roswell Park Memorial Institute medium (RPMI-1640, Sigma) at 2×10^6 cells/mL density. The RPMI-1640 medium was supplemented with 10% of heat-inactivated Fetal Bovine Serum (FBS), HEPES (25 mM, pH 7.2), glutamine, pH ~7.2, penicillin (100 IU/mL) and streptomycin (100 μg/mL). Promastigotes in late logarithmic or stationary phase were utilized for assessing drug susceptibility at various concentrations of compounds in 96-well plates. Positive control (parasites with no drug) and negative control (medium without parasite) were used side by side. All experiments were performed in triplicate.

2.3. Treatment of amastigotes with compounds

In this study, J774A.1 cell line (ATCC number TIB-67) was received from Pasteur Institute, Tehran, Iran. The macrophage cells were preserved in RPMI-1640 and then diluted in RPMI-1640 medium. A cell survival test was done by using trypan blue solution (0.2% in saline). Then after 1–2 min macrophage cells were counted using a Neubauer hemocytometer light microscope. In brief, 200 μL of the macrophage cells (10^6 cells/mL) was added into SPL's Cell Culture Slides (8-chamber slide, Korea) and incubated at 37 °C with 5% CO_2 for 3–4 h. The non-adherent macrophage cells were washed with phosphate buffered saline and then plates were incubated at 37 °C under 5% CO_2 atmosphere for 16 h. After that, macrophage cells were infected with *L. major* promastigotes with a parasite/macrophage ratio of 20:1 (10^7 cell/mL). Following 3–5 h incubation, free promastigotes were detached by washing with PBS. After 24 h incubation at 37 °C and 5% CO_2, finally the test compounds were added to the plate wells of SPL's Cell Culture Slides in the concentrations of 1.5–500 μg/mL. The media were renewed after 48 h. After 72 h incubation, the plates were washed with PBS, fixed in methanol, stained with Wright-Giemsa solution, and examined with a light microscope. The mean infection rate of macrophages (MIR) and also mean number of amastigotes per macrophages (MNAPM) were recorded. Macrophages containing amastigotes with no drugs and macrophages alone were considered as positive and negative controls, respectively [12]. All experiments were done in triplicate.

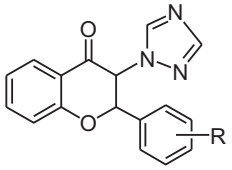
2.4. Cell toxicity assay

The macrophages J774A.1 (2×10^5 cells/well) were seeded in 96-well microplates and incubated at 37 °C under 5% CO_2 atmosphere for 24 h. Then, the medium was removed and replaced with fresh RPMI-1640 containing different concentrations of each compound (1.5–500 μg/mL), and incubation was continued for 72 h. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [13].

2.5. MTT assay

To determine IC_{50} values (50% inhibitory concentrations) of TFs against *L. major*, MTT assay was used [13]. In brief, parasites were seeded in 96-well plates containing serial dilutions of test compounds in RPMI-1640 medium and incubated at 25 ± 1 °C for 72 h. Next the media was renewed with 0.5 mg/mL solution of MTT and the plates were further incubated at 37 °C for 4 h. The plates were centrifuged (500 × *g* for 5 min) and the resulting pellets were dissolved in isopropyl

Table 1
Structures, chemical names, and *in vitro* biological activities of TFs against amastigotes of *L. major* and macrophages.



Compound	R	Chemical name	Amastigote IC_{50} (μg/mL)	Cytotoxicity IC_{50} (μg/mL)	SI[a]
TF-1	H	*trans*-2,3-Dihydro-3-(1*H*-triazol-1-yl)-2-phenyl-4*H*-1-benzopyran-4-one	5.63 ± 0.20	17.16 ± 0.14	3.04
TF-2	4-Cl	*trans*-2-(4-Chlorophenyl)-2,3-dihydro-3-(1*H*-triazol-1-yl)-4*H*-1-benzopyran-4-one	1.57 ± 0.20	47.43 ± 0.44	30.21
TF-3	4-F	*trans*-2-(4-Fluorophenyl)-2,3-dihydro-3-(1*H*-triazol-1-yl)-4*H*-1-benzopyran-4-one	11.53 ± 2.11	31.84 ± 0.25	2.76
TF-4	4-Me	*trans*-2,3-Dihydro-3-(1*H*-triazol-1-yl)-2-(4-methylphenyl)-4*H*-1-benzopyran-4-one	20.28 ± 0.33	41.51 ± 0.56	2.04
TF-5	4-OMe	*trans*-2,3-Dihydro-3-(1*H*-triazol-1-yl)-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one	15.27 ± 0.22	32.16 ± 0.26	2.10
TF-6	3-Cl	*trans*-2,3-Dihydro-2-(3-chlorophenyl)-3-(1*H*-triazol-1-yl)-4*H*-1-benzopyran-4-one	6.87 ± 0.21	35.66 ± 0.27	5.19
TF-7	3-F	*trans*-2,3-Dihydro-2-(3-fluorophenyl)-3-(1*H*-triazol-1-yl)-4*H*-1-benzopyran-4-one	10.22 ± 0.31	18.43 ± 0.17	1.80
TF-8	3-OMe	*trans*-2,3-Dihydro-3-(1*H*-triazol-1-yl)-2-(3-methoxyphenyl)-4*H*-1-benzopyran-4-one	8.21 ± 0.23	34.36 ± 0.52	4.18
TF-9	2-Cl	*trans*-2-(2-Chlorophenyl)-2,3-dihydro-3-(1*H*-triazol-1-yl)-4*H*-1-benzopyran-4-one	8.66 ± 0.31	30.54 ± 0.24	3.52
TF-10	2-OMe	*trans*-2,3-Dihydro-3-(1*H*-triazol-1-yl)-2-(2-methoxyphenyl)-4*H*-1-benzopyran-4-one	5.23 ± 0.26	46.62 ± 0.58	8.91
TF-11	2,4-Cl_2	*trans*-2-(2,4-Dichlorophenyl)-2,3-dihydro-3-(1*H*-triazol-1-yl)-4*H*-1-benzopyran-4-one	2.43 ± 0.36	42.59 ± 0.59	17.52
Flu[b]			24.04 ± 0.32	42.59 ± 0.18	1.77
Glu[c]			85.07 ± 3.92	42.67 ± 0.53	0.50

[a] Selectivity index.
[b] Fluconazole.
[c] Glucantime.

alcohol. The absorbance of samples was measured using an ELISA plate reader (Synergy H1Hybrid Reader, BioTek) at 492 nm [12]. The linear regression analysis of concentration-inhibition curves was used to obtain IC_{50} values.

2.6. Flow cytometric analysis

The effect of selected compound TF-2 on promastigotes of *L. major* was assessed by flow cytometry. The stationary phase of parasites was obtained in the RPMI-1640 medium for flow cytometric assessment. Then, parasites were cultured in 24-well plates (6×10^5 promastigotes per well) in the presence of 6 μg/mL of compound TF-2, as well as in the absence of TF-2 (negative control). The promastigotes (6×10^5 per well) were stained with Annexin V-FITC and propidium iodide using an Annexin V-PI apoptosis detection kit (eBioscience). According to the kit instructions, the promastigotes (6×10^5) were washed three times with PBS and centrifuged at 3200 rpm for 5 min. Afterwards, promastigotes pellet was re-suspended in 5 μL Annexin V solution and then incubated for 15 min at room temperature and centrifuged at 3200 rpm for 5 min. Finally, 100 μL bindding buffer was added to the cell pellet and examined with the flow cytometer (Becton Dickinson) and were analyzed by FlowJo Software [23].

The viable cells was typically used for determine apoptosis. There is Phosphatidylserine (PS) in the inner of live membrane cell. In the early step of apoptosis, PS translocated to extracellular. PS identified by Annexin V. Therefore necrotic cells were Annexin V negative/PI positive (Q1), early apoptosis; Annexin V positive and PI negative (Q2), live cells; Annexin V negative and PI negative (Q3) and finally late apoptosis; Annexin V positive and PI positive (Q4).

2.7. Data analysis

The obtained data were analyzed by ANOVA, multiple comparison and Kruskal-Walis using SPSS 16 software. The IC_{50} values were estimated using GraphPad Prism 6.0 software. Dunn's test was used for comparison the values of amastigote form. Furthermore, $P < .05$ was assumed as a significant difference.

2.8. Molecular docking

Molecular docking studies were performed using the AutoDock 4.2 software [24]. The protein structure of cytochrome P450 sterol 14α-demethylase of *Leishmania infantum* (PDB: 3L4D) was provided from RCSB protein data bank. The selected compounds were docked into the active sites of CYP51 *L. infantum*. The Lamarckian genetic algorithm (LGA) was used for local search ligand conformational. Moreover, docking runs were performed using 100 GA runs; initial population of 150 individuals; a maximum of 27,000 generations; a mutation rate of 0.02; and a crossover rate of 0.8. The best binding pose of compounds was selected based on the lowest binding free energy and the coordination of nitrogen atom of triazole ring with iron atom heme group. Root mean square deviation (RMSD) was used for validation of the method. Cognate ligand extracted from the original protein structure and then re-docked in the active site of this protein. The obtained RMSD value (0.92 Å) was significantly < 3.0 Å that could be considered as a threshold.

3. Results

The antileishmanial activity of TFs against *L. major* was primarily screened for finding new potential triazole compounds and establishing their structure-activity relationships. The test compounds were evaluated for anti-promastigote and anti-amastigote activity in comparison with fluconazole as a triazole reference drug, using MTT assay and counting of amastigotes in macrophages. The obtained results against promastigotes and amastigotes are presented in Figs. 2 and 3, respectively.

3.1. Anti-promastigote activity

As evidenced from data (Fig. 2), the standard azole drug fluconazole showed marginal activity (IC_{50} = 75.57 μg/mL) while all compounds showed significant leishmanicidal activity with IC_{50} values between 5.65 and 40.55 μg/mL. The highest activity was observed with 4-chloro analog TF-2 (IC_{50} = 5.65 μg/mL), being about 13-times more active than fluconazole (Fig. 4). Notably, all compounds displayed IC_{50} values less than that of fluconazole ($p = .001$). The less potent compound TF-4

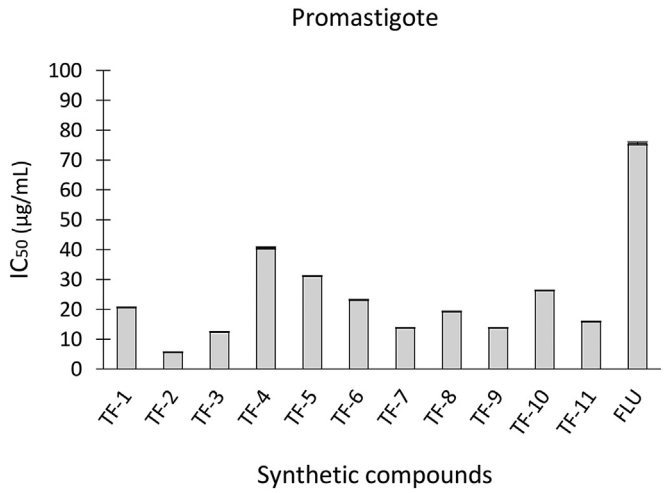


Fig. 2. The IC_{50} values of TFs and fluconazole (FLU) against promastigotes of *L. major*.

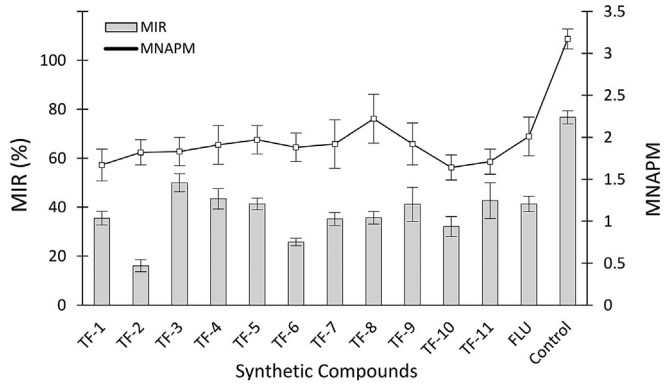


Fig. 3. Comparing mean number of amastigotes per macrophages (MNAPM) and mean infection rate of macrophages (MIR) for TFs, fluconazole (FLU) and control.

was approximately 2-fold more active than fluconazole.

3.2. Anti-amastigote activity

The *in vitro* activity of TFs against the amastigote stage was evaluated by defining MNAPM and MIR (Fig. 3). All of TFs significantly inhibited the growth rate of amastigotes in macrophages as compared with control. Also, most of compounds reduced the MNAPM and MIR more than fluconazole ($p = .001$). In particular, compound TF-2 effectively reduced MIR to 16.08%. On the other hand, a survey on the obtained results against amastigotes (Table 1) reveled that all compounds had potent activity, displaying IC_{50} values in the range of 1.57–20.28 µg/mL. All compounds were more potent than fluconazole (IC_{50} = 24.04 µg/mL). Notably, the activity of the most potent compound TF-2 (IC_{50} = 1.57 µg/mL) was 15-fold superior to that of fluconazole (Fig. 4). Moreover, compound TF-11 with IC_{50} value of 2.43 µg/mL showed promising activity against amastigotes.

3.3. Cytotoxic activity against macrophages

The cytotoxic activity of test compounds TFs in comparison with fluconazole and glucantime was evaluated against macrophages (cell line J774A.1), and the obtained IC_{50} values were listed in Table 1. Furthermore, selectivity index (SI) was calculated for each compound and shown in Table 1. All compounds showed cytotoxicity against macrophages at higher concentrations respect to the intracellular

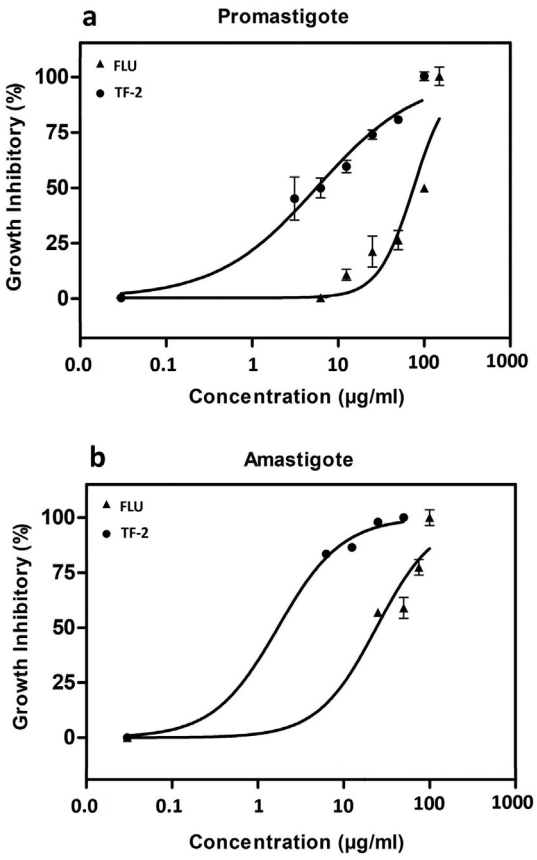


Fig. 4. Dose-response curve of TF-2 and fluconazole (FLU) against promastigote and amastigote forms of *L. major*.

amastigotes. The 4-chloro and 2,4-dichloro analogs (TF-2 and TF-11) with SI of 30.21 and 17.52 displayed the best profile of activity against amastigotes.

3.4. Induction of apoptosis by compound TF-2

The promising compound TF-2 was subjected to flow cytometric assay to evaluate its potential for induction of apoptosis in *Leishmania* parasites. Accordingly, the laboratory strain of *L. major* was treated with TF-2 and fluconazole at the concentrations of 6 µg/mL and 80 µg/mL, respectively for 24 and 48 h and then analyzed by flow cytometry. The promastigotes that treated with compound TF-2 or fluconazole indicated 32.02 and 12.63% of apoptosis after 24 h respectively. The percent of apoptosis in the control (untreated cells) was 0.37%. While only 4.36% of apoptosis was detected in the control, TF-2 and fluconazole showed 39.7 and 17.8% of apoptosis after 48 h incubation, respectively (Fig. 5).

3.5. Molecular docking

In order to explain interactions of selected compound TF-2 with the target enzyme, molecular docking study was carried out. Cytochrome P450 sterol 14α-demethylase (CYP51) enzyme is a potential target for azoles in *Leishmaina* spp. As seen in Fig. 6, the N-4 atom of the triazole ring in the TF-2 structure is located over the porphyrin prosthetic group and coordinated to the Fe atom of heme. The distance between Fe atom of heme group and the N-4 atom of triazole ring is 2.82 Å that being adequate for appropriate coordination. Moreover, the free energy of binding for the best docked pose was −7.63 kJ/mol. In this study, we used Schrodinger program to show hydrophobic interactions. As shown in Fig. 7, there are several hydrophobic interactions between the best

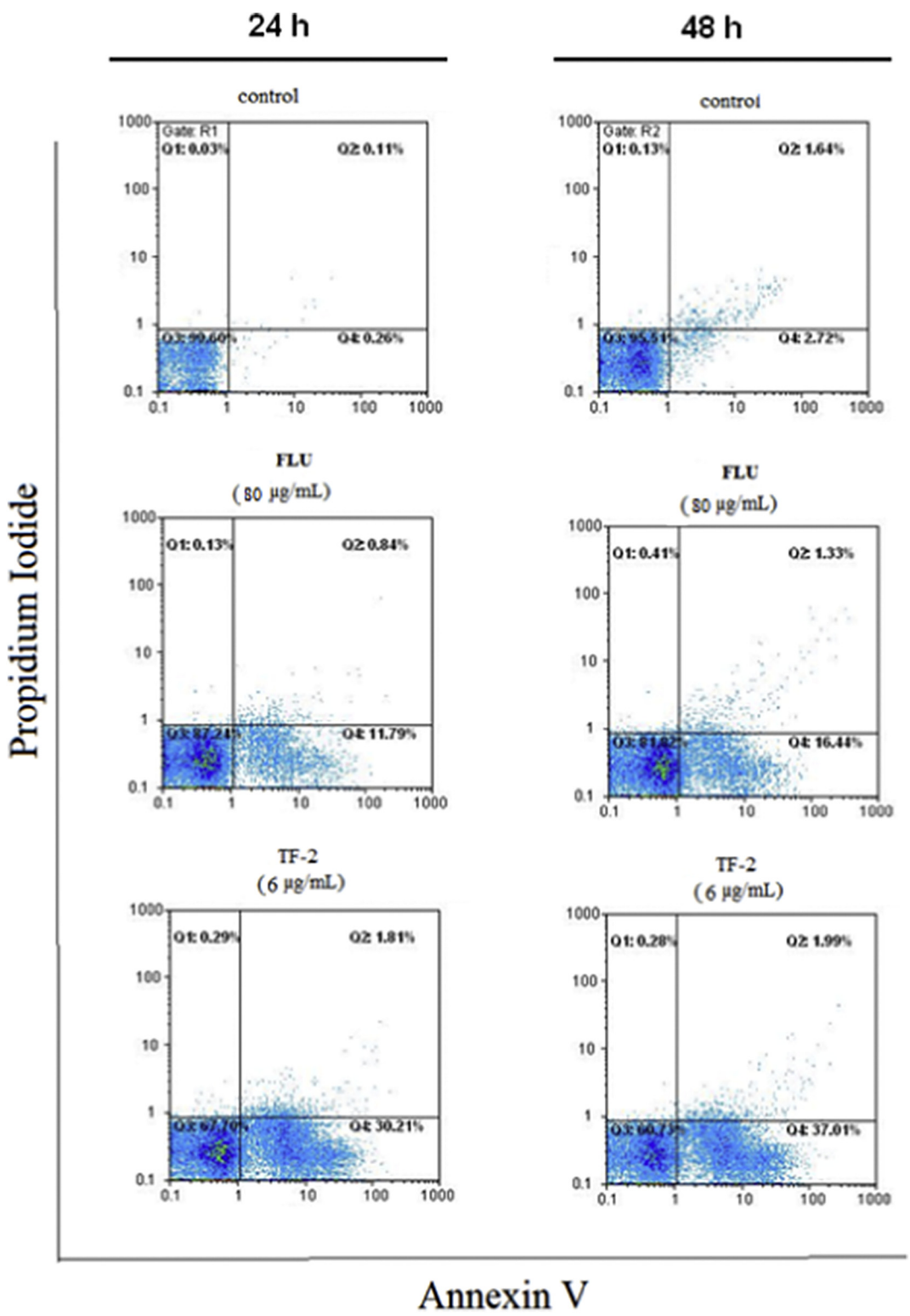


Fig. 5. Flow cytometric analysis of *L. major* after 24 and 48 h treatment with TF-2 (6 μg/mL) and fluconazole (80 μg/mL).

docked compound (TF-2) and amino acids residues of target enzyme. The 4-chlorophenyl moiety of compound TF-2 involved in the hydrophobic interactions with Tyr74, Leu327, Leu330 and Met329. Moreover, the amino acid residue of Tyr87 interacted with chroman ring as pi-pi stacking. In addition, other amino acids residues including Met77, Phe261, Ala259, Ala262, Leu101, Ala258, Leu98, Met255 and Ala86 were in the contact with rest of the target enzyme (Fig. 7).

4. Discussion

Several investigations demonstrated that azole antifungals are potentially active against *Leishmania* spp. [14–18,21]. The azole antifungal agents inhibit sterol 14α-demethylase (CYP51) by a mechanism in which the nitrogen atom of azole ring binds to the heme iron atom in the binding pocket of the sterol 14α-demethylase [25,26]. Since the sterols biosynthesis is critical for normal viability, proliferation and

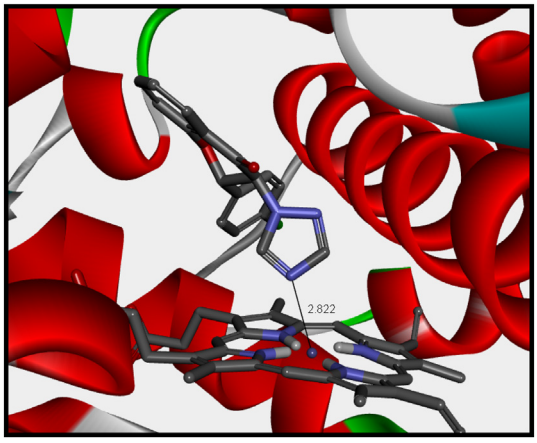


Fig. 6. 3D-model representation of compound TF-2 in the active site of CYP51 (PDB code: 3L4D). N-4 atom of the triazole ring is located over the porphyrin and coordinated to the Fe atom of heme.

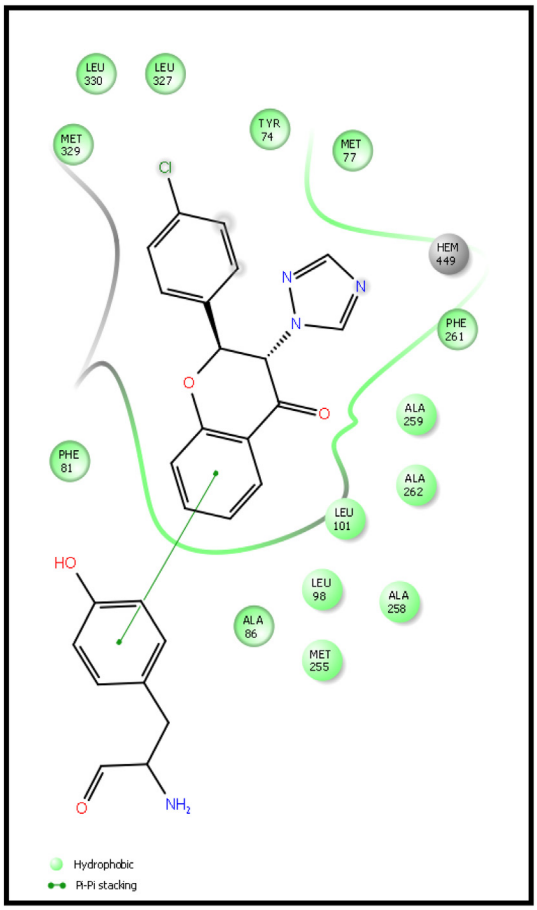


Fig. 7. 2D-model representation of hydrophobic interactions for compound TF-2 in the active site pocket of CYP51 (PDB code: 3L4D). Only important amino acids for interaction are shown.

infectivity of *Leishmania* parasites, the sterol 14α-demethylase inhibitors obtained from azole antifungal drug discovery programs can be investigated in parallel for treatment of leishmaniasis [21].

Previously we have designed and synthesized a series of new azole antifungals namely 3-triazolylflavanones (TFs), bearing *N*-(phenethyl)-azole backbone in their core structure. The *N*-(phenethyl)-azole scaffold is common pharmacophore for the most of azole drugs (Fig. 1). Most of azole drugs have flexible *N*-(phenethyl)-azole scaffold, but our compounds possess conformationally constrained framework. In this study, we screened the anti-parasitic potential of TFs (11 compounds) against promastigote and amastigote forms of *L. major* in comparison with fluconazole as a standard triazole drug. Since there are more frequent studies on *in vitro*, *in vivo* and clinical trials of fluconazole for treatment of leishmaniasis [21], thus we used this drug as a reference. All compounds showed significant activity against both forms more potent than fluconazole. Among them, 4-chlorophenyl derivative (TF-2) exhibited the best profile of activity. Compound TF-2 displayed IC_{50} values of 5.65 and 1.57 μg/mL against promastigotes and amastigotes, being at least 13 times more potent than fluconazole. Moreover, the cytotoxicity assay against macrophages revealed that this compound had highest selectivity against amastigotes (SI = 30.21). Compound TF-2 effectively inhibited the growth rate and infectivity of amastigotes in macrophages as evaluated by determining MNAPM and MIR. Molecular docking studies of selected compound (TF-2) with sterol 14α-demethylase (CYP51) enzyme indicated that this compound can properly fit in the active site and coordinated to the heme *via* N-4 of triazole ring. Furthermore, hydrophobic interactions play important role in the inhibition of the target enzyme.

From a structural point of view, compounds TF2-11 are distinguished by the type and position of substituent on 2-phenyl group of flavanone. The introduction of methyl or methoxy groups at the *para*-position of TF-1 results in compounds TF-4 and TF-5 with diminished activity against promastigotes and amastigotes. In contrast, the 4-chloro analog (TF-2) was more potent than unsubstituted compound (TF-1). As evidenced from data, the IC_{50} values of 3-chloro and 2-chloro derivatives (TF-6 and TF-9) were higher than that of 4-chloro regio-isomer (TF-2), thus the displacement of chloro- substituent from *para* to *meta* or *ortho* positions cannot improve the anti-parasitic activity. Interestingly, compound TF-11 with 2,4-dichloro substituent was more potent than 2-chloro congener (TF-9) and less potent than 4-chloro analog (TF-2) against amastigotes. The comparison of fluoro derivatives TF-3 and TF-7 with their corresponding chloro analogs (TF-2 and TF-6) indicated that the chloro group is more favorable than fluoro group. The obtained IC_{50} values of methoxy derivatives revealed that the *ortho* position is more favorable for MeO group (compare TF-10 with TF-5 and TF-8).

As described previously, the title compounds TFs were primarily synthesized, and tested as antifungal agents against *Candida albicans*, *Candida glabrata*, *Saccharomyces cerevisiae* and *Aspergillus niger*. Although all compounds were inactive against *A. niger*, but halogenated compounds showed significant activity against other tested strains. In particular, the *para*-chloro and *para*-fluoro analogs (TF-2 and TF-3, respectively), and *meta*-chloro derivative (TF-6) showed the highest inhibitory activity against *Candida albicans* (MICs = 3.9 μg/mL), being 8-fold more active than fluconazole. In the case of *L. major*, all compounds showed more potent anti-parasitic activity in comparison with reference azole drug fluconazole. The *para*-chloro derivative (TF-2) was found to be the most potent one against *L. major*.

Based on the IC_{50} values of unsubstituted compound TF-1 and substituted compounds TF-2-11 against macrophages, it can be concluded that the introduction of both electron-donating and electron-withdrawing substituents on the 2-phenyl moiety results in decrease of cytotoxicity. Compound TF-2 with 4-Cl substituent was less cytotoxic against macrophages and showed the highest selectivity for amastigotes.

Recently, several azole compounds have been designed as conformationally constrained analogs of azole antifungals and screened for antileishmanial activity. For example, Marrapu et al. have been described a series of aryloxy tetrahydronaphthyl and cyclohexyl azoles as antileishmanial agents. The tetrahydronaphthyl imidazole analog containing 2,5-dichlorobenzyl ether was identified as the most potent compound against *Leishmania donovani* [27]. Furthermore, benzyl oxime derivatives of azolylmethyltetralones have been described by Verma et al. as azole antileishmanial agents [28]. The evaluation of the test compounds against *L. donovani* revealed that the compounds

comprising of imidazole ring were more active than corresponding triazole derivatives. This study showed that the substitution in the tetralin ring by different groups including methyl or halogens resulted in no appreciable change in the antileishmanial activity, whereas the substitution of chlorine in the benzyl group resulted in substantial enhancement of the activity. As mentioned above, our compounds contain *N*-(phenethyl)azole scaffold that frequently found in the structure of azole antifungals, while in the reported compounds by Marrapu et al. and Verma et al. this framework has been not preserved.

On the basis of molecular docking results, the N-4 atom of the triazole ring of compound TF-2 is coordinated to the Fe atom of heme, the aromatic part of chroman ring is formed π-π stacking and 4-chlorophenyl moiety contributes in the hydrophobic interactions with CYP51. These findings confirm the proposed phamacophoric role of *N*-(phenethyl)azole scaffold along with secondary aromatic moiety.

In several researches, flow cytometry has been used for evaluation of drugs against *Leishmania* parasites [23,29]. There is no report on flow cytometric analysis of azole action on *Leishmania* parasites. Doroodgar et al. examined the efficacy of tamoxifen on *L. major* and indicated that this drug can induce apoptosis (59.7%) in promastigotes after 48 h of drug treatment (50 μg/mL) [23]. Also, da Silva et al. have investigated the cell death mechanisms of aryl thiosemicarbazone derivatives in *L. amazonensis* by flow cytometry in comparison with amphotericin B. The results showed that the selected compound was able to induce apoptosis and necrosis on promastigotes after 24 h treatment. In our research, it was shown that compound TF-2 at the concentration of 6 μg/mL can result in 39.7% of apoptosis in promastigotes after 48 h of treatment.

5. Conclusion

According to the optimistic reports from the antileishmanial activities of azole antifungals and their repositioning for treatment of tropical protozoan infections, we have evaluated the *in vitro* antileshmanial potential of eleven 3-(1,2,4-triazol-1-yl)flavanones (TFs) as conformationally restricted analogs of azole antifungals. All compounds showed more potent anti-parasitic activity against amastigote and promastigote forms of *L. major* in comparison with reference azole drug fluconazole and standard antileishmanial agent glucantime. Particularly, the 4-chloro derivative (TF-2) was found to be more promising compound, being about 13 times more potent than fluconazole. Furthermore, the cytotoxicity assay revealed that this compound displays high selectivity against amastigotes over macrophages (SI = 30.21). The *in silico* study showed that TF-2 can properly accommodated in the active site of parasitic CYP51 and coordinated to the heme. These findings demonstrated that the compound TF-2 prototype can be considered as promising candidate for development of new antileishmanial agents.

Conflict of interest

Authors declare that have no competing interests.

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