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Original article

Synthesis, *in vitro* antifungal activity and *in silico* study of 3-(1,2,4-triazol-1-yl)flavanones

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ABSTRACT

A series of novel 3-(1,2,4-triazol-1-yl)flavanones were synthesized based on the *N*-phenethylazole pharmacophore of azole antifungals. The results of antifungal assay revealed that 4′-fluoroflavanone derivative **4c** exhibited the best profile of activity against *Candida* and *Saccharomyces* strains. Compound **4c** was 4–16 times more potent than reference drug fluconazole against *Candida albicans* and *Saccharomyces cerevisiae*. The molecular docking study with lanosterol 14α-demethylase, *in silico* toxicity risks and drug-likeness predictions were used to better define of title compounds as antifungal agents. The favorable drug-like property of compound **4c** makes 3-(1,2,4-triazol-1-yl)flavanone prototype as a promising lead for the future development of azole antifungal agents.

1. Introduction

In recent decades, the rising in the number of immunocompromised hosts, such as patients suffering from AIDS, tuberculosis, diabetes, and undergoing organ transplantations and cancer chemotherapy has been complicated with opportunistic fungal infections and become a major cause of morbidity and mortality in these patients [1]. Although several antifungal agents have recently been introduced to the clinical chemotherapy, the number of available drugs to treat life-threatening fungal infections is still limited and more new alternative drugs with improved efficacy are needed [2,3]. The majority of available chemotherapeutic agents suffer from drug-related toxicity, hazardous drug–drug interactions and pharmacokinetics deficiencies. However, triazole antifungals including fluconazole, itraconazole, voriconazole and posaconazole (Fig. 1) represent an interesting class of azole antifungal agents. These compounds have become the most rapidly expanding group of antifungals with the advantages of low toxicity, high oral bioavailability, and broad spectrum of activity against several fungi [4]. Thus, the triazole class of antifungal agents currently plays a leading role in the field of drug development. These antifungal agents inhibit the cytochrome P450-dependent lanosterol 14α-demethylase (CYP51) by a mechanism in which the N-4 of triazole ring binds to the heme iron atom in the binding site of the enzyme [5,6].

As a part of our works on azole antifungal agents [7–10], recently we have designed conformationally constrained azole antifungals, namely 3-imidazolylflavanones (Fig. 2) [11]. Based on the *N*-phenethylazole pharmacophore of azole antifungals, these compounds possess pharmacophoric backbone from two sides. In this series, compounds containing a substituent on 2-phenyl ring showed significant antifungal activity. Moreover, modification of the 4-oxo group to oxime resulted in the changes of antifungal activity profile [11]. These encouraging results prompted us to prepare triazole analogs of 3-azolylflavanones and investigate their structure–activity relationships. Triazole antifungals have several

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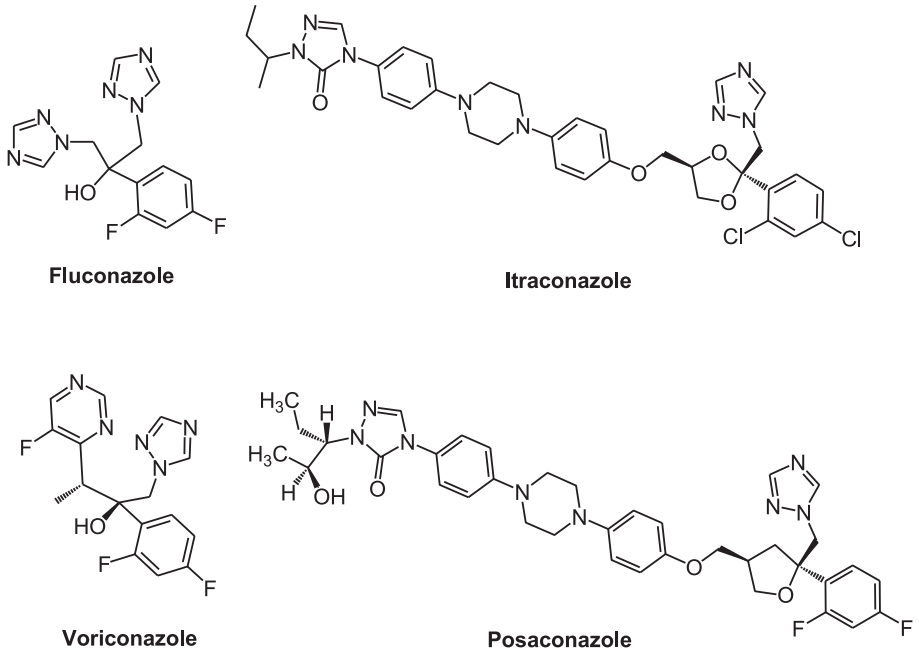


Fig. 1. Triazole antifungal agents.

advantages over imidazole agents. For example, they have good oral bioavailability and are metabolized more slowly, making them longer acting than imidazoles. Moreover, triazoles have higher selectivity for human CYP450 enzymes, resulting to lesser side effects and drug–drug interactions [12].

Considering the advantages of triazole-containing antifungal drugs, we describe herein, synthesis, antifungal activity and *in silico* study of 3-(1,2,4-triazol-1-yl)flavanones (Fig. 2).

2. Chemistry

As depicted in Scheme 1, the synthesis of compounds **4** and **5** was started from 2-hydroxyphenacyl bromide (**1**). The reaction of compound **1** with 4-amino-1,2,4-triazole in refluxing 2-propanol gave aminotriazolium bromide **2**. In this reaction, 2-hydroxyphenacyl substituent was regioselectively connected to N-1 of 1,2,4-triazole. Deamination of compound **2** was occurred in diluted hydrochloric acid in the presence of sodium nitrite to afford 1-(2-hydroxy phenacyl)-1,2,4-triazole (**3**) [13]. The reaction of compound **3** with appropriate benzaldehyde derivative produced *trans*-triazolylflavanones **4**. This reaction was occurred in 2-propanol, in the presence of piperidine as a catalyst and under reflux conditions. In the ^{1}H NMR spectra of triazolylflavanones **4**, two doublet signals related to the H-2 and H-3 of chroman ring appear at ca. 6.01–6.65 ppm with a coupling constant $J_{2,3} > 12$ Hz, which indicated that the relative configuration of these compounds is 2,3-*trans*.

To obtain triazolylflavanone oxime derivatives, we primarily used a mild reaction condition which has been reported for imidazolylflavanone oximes [11]. Accordingly, compound **4** was treated with hydroxylamine hydrochloride in the presence of potassium carbonate in methanol at room temperature. Surprisingly, using these very mild conditions the reaction resulted in a mixture of unknown degraded products. Thus, the reaction was attempted by using the excess of hydroxylamine hydrochloride in refluxing methanol in the absence of potassium carbonate. For completion of the reaction, it should be refluxed for long time. However, the corresponding oxime was produced in good yield.

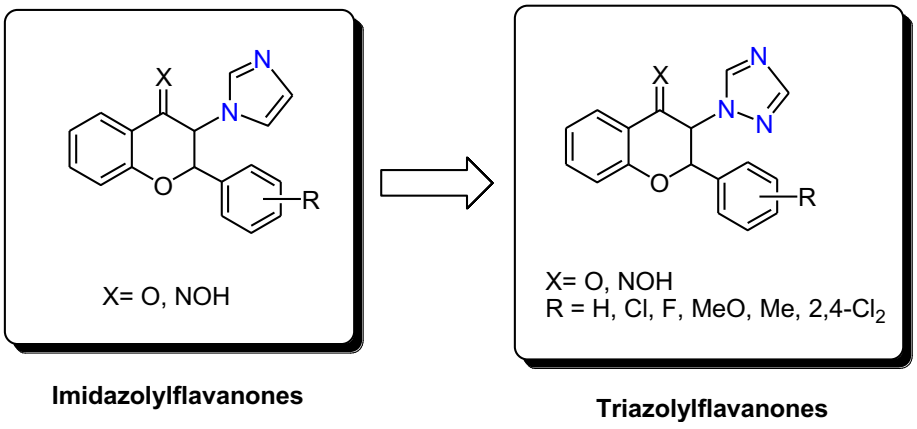
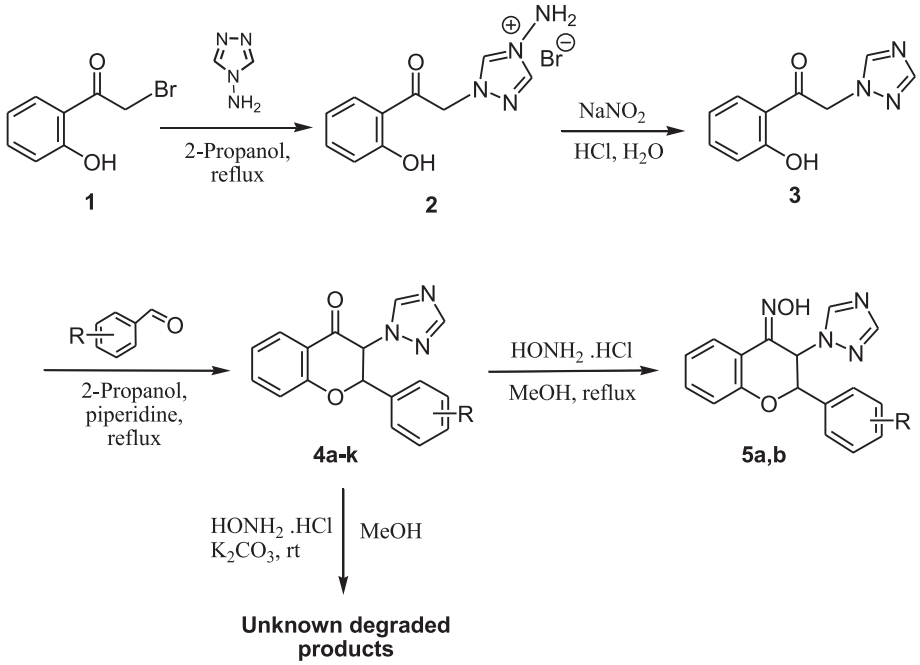


Fig. 2. Imidazolylflavanones possessing *N*-phenethylazole pharmacophore of azole antifungals and their triazole analogs as new antifungal agents.



Scheme 1. Synthesis of 3-(1,2,4-triazol-1-yl)flavanone derivatives.

3. Biology

The antifungal activity of synthesized compounds **4** and **5** was evaluated against *Candida albicans* ATCC 10231, *Candida glabrata* ATCC 90030, *Saccharomyces cerevisiae* NCYC 694, *S. cerevisiae* ATCC 9763 and *Aspergillus niger* ATCC 16404. The minimum inhibitory concentrations (MICs) of compounds were determined by microdilution method [14]. A stock solution of compounds was transferred into the plates with 96 U-shaped wells and serially diluted with Sabouraud dextrose broth in subsequent wells. Then, fungal suspension was added to each well to reach the final inoculum size of 0.5×10^3 CFU/mL. After incubation, the plates were observed for the absence or presence of visible growth in comparison with that of the drug-free control well. The endpoint MIC was the lowest concentration of the compound at which the test strain does not demonstrate visible growth. The MICs of tested compounds in comparison with fluconazole as a standard azole antifungal agent were listed in Table 1.

4. Results and discussion

4.1. Antifungal activity

The MIC values of target compounds against *C. albicans* revealed that 4′-halogenated flavanones **4b** and **4c**, and 3′-halogenated flavanones **4f** and **4g** showed the highest inhibitory activity against this fungi (MICs = 3.9–15.6 μg/ml). Their activities were 2–8 times more than that of fluconazole as standard drug. In addition, the antifungal activity of compound **4a** against *C. albicans* was comparable to fluconazole. The 4′-fluoroflavanone derivative **4c** exhibited remarkable activity against *C. glabrata* as potent as fluconazole (MIC = 31.3 μg/ml). However, the rest of compounds showed no activity against this microorganism. In the case of *S. cerevisiae* (NCYC 694), compounds **4c** and **4f** with MIC values of 0.98–1.95 μg/ml were more potent than fluconazole. Their activities were 8–16 times better than that of fluconazole. Moreover, compound **4k** had inhibitory activity at the concentration of 31.3 μg/ml against *S. cerevisiae* (NCYC 694). Most of compounds showed significant growth inhibitory activity against *S. cerevisiae* ATCC 9763. Among them, 4′-halogenated flavanones **4b** and **4c** with MIC values of 3.9 μg/ml were the most potent compounds. Also, compounds **4g** and **4k** showed noticeable activity against former strain of *S. cerevisiae*. All triazole compounds **4a**–**k** and **5a**,**b** as well as fluconazole were inactive against *A. niger* (MICs > 62.5 μg/ml). However, the MIC of ketoconazole (an imidazole antifungal) against *A. niger* was 15.6 μg/ml.

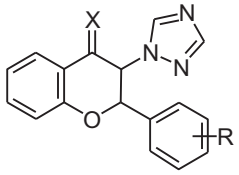
From the *in vitro* antifungal activity data of unsubstituted triazolylflavanone **4a** and halo-substituted compounds **4b**, **4c**, **4f** and **4g** against *C. albicans*, it is revealed that the presence of halogen on the 3- or 4-position of 2-phenyl ring is beneficial for activity. In contrast, the introduction of chlorine atom at the position 2 diminished the growth inhibitory activity against *C. albicans* (compound **4i** vs. compound **4a**). Also, the observed MIC values of 2,4-dichloro derivative **4k** and 4-chloro compound **4b** against *C. albicans* demonstrated the negative effect of chlorine substitution on the 2-position. Although, 4-chloro analog **4b** and 4-fluoro derivative **4c** showed same activity against *C. albicans*, but 3-chloro-substituted compound **4f** was more potent than 3-fluoro analog **4g**. The results of antifungal bioassay against other yeasts *C. glabrata* and *S. cerevisiae* revealed that the type and the position of the substitutions may have different effects, related to the type of strains. For example, the introduction of second chlorine atom in the 4-chlorophenyl derivative decreased the activity against *C. albicans* and *S. cerevisiae* ATCC 9763 (compound **4k** vs. **4b**), while this modification enhanced the potency against *S. cerevisiae* NCYC 694.

In general, 4′-fluoroflavanone derivative **4c** exhibited the best profile of activity against yeasts. The conversion of ketone to oxime (compounds **5** vs. compounds **4**) could not improve antifungal activity. Among the ketones, compounds containing methyl or methoxy substituents were inactive while 3- or 4-chloro and 3- or 4-fluoro derivatives showed antifungal activity.

4.2. Docking study

The molecular basis of the antifungal activity of fluconazole and related azole antifungals is the inhibition of ergosterol biosynthesis,

Table 1
The minimum inhibitory concentrations (MICs, μg/ml) of compounds **4a**–**k** and **5a**,**b**.



Compound	X	R	*C. albicans* ATCC 10231	*C. glabrata* ATCC 90030	*S. cerevisiae* NCYC 694	*S. cerevisiae* ATCC 9763	*A. niger*[a] ATCC 16404
4a	O	H	**31.3**	>62.5	>125	**125**	>62.5
4b	O	4-Cl	**3.9**	>62.5	>125	**3.9**	>62.5
4c	O	4-F	**3.9**	**31.3**	**0.98**	**3.9**	>62.5
4d	O	4-Me	>125	>62.5	>125	>125	>62.5
4e	O	4-OMe	>125	>62.5	>125	**125**	>62.5
4f	O	3-Cl	**3.9**	**62.5**	**1.95**	>125	>62.5
4g	O	3-F	**15.6**	>62.5	>125	**15.6**	>62.5
4h	O	3-OMe	>125	>62.5	>125	>125	>62.5
4i[b]	O	2-Cl	>125	>62.5	>125	**125**	>62.5
4j[b]	O	2-OMe	>125	>62.5	>125	>125	>62.5
4k	O	2,4-Cl_2	**125**	>62.5	**31.3**	**31.3**	>62.5
5a	NOH	H	>125	>62.5	>125	**125**	>62.5
5b	NOH	2,4-Cl_2	>125	>62.5	>125	**62.5**	>62.5
Fluconazole			**31.3**	**31.3**	**15.6**	**15.6**	>125

The significance of bold values in comparison with control group was less than 0.05 ($P < 0.05$).
[a] The MIC value of ketoconazole against *A. niger* was 15.6 μg/ml.
[b] Nitrate salt.

which is necessary to maintain fungal cell membrane integrity and permeability. Indeed, azole antifungals inhibit the cytochrome P-450 lanosterol 14α-demethylase (CYP51) which is responsible for the oxidative removal of the 14α-methyl group of lanosterol [15]. Accordingly, the preliminary molecular docking calculations of the representative compound **4c** were performed to provide more insight into the interactions as well as to rationalize the observed antifungal activity of title compound as lanosterol 14α-demethylase inhibitor. Because no experimental data are available for the target enzyme *Candida* P450 DM in the Protein Data Bank, the crystallographic structure of Cytochrome P450 14α-sterol demethylase from *Mycobacterium tuberculosis* (MTCYP51) was used for the docking study. The docking and subsequent scoring were performed using the default parameters of the FlexX program implanted in LeadIT 2.0.2. The active site of the enzyme was defined to include residues within 6.5 Å radius around bound inhibitor (fluconazole). Fluconazole was re-docked into the MTCYP51 active site in order to check and validate the accuracy of our docking protocol (Fig. 3).

As discussed in chemistry section, *trans*-triazolylflavanones **4** have two stereogenic centers C2 and C3. The method for obtaining *trans*-triazolylflavanones **4** was non-enantioselective. Thus, the isolated compound **4c** might be mixture of (*RR*)- and (*SS*)-enantiomers. Docking experiments on both the (*RR*)- and (*SS*)-enantiomers of the target compound **4c** indicated that *p*-fluorophenyl ring of both enantiomers has been situated in a very lipophilic pocket made by hydrophobic amino acids and interacts with aromatic rings or aliphatic side chains of amino acids Leu321, His259, Phe255, Val434, Met79 and Tyr76 through hydrophobic interactions (Figs. 4 and 5). Rotation of the *p*-fluorophenyl ring of (*SS*)-isomer in the docked conformation makes an additional hydrophobic interaction with aromatic ring of Phe78. The aromatic part of chromanone ring in both enantiomers constitutes hydrophobic interactions with aromatic or aliphatic parts of Phe83, Leu100 and Met99 (Figs. 4 and 5). Meanwhile, the N-4 atom of the triazole ring in both enantiomers is positioned above the porphyrin plane and coordinated to the heme iron.

Generally, in the binding model of azole antifungals with CYP51, the maximal effective distance between the N-4 atom of the triazole ring and the Fe atom of heme was 3 Å [16]. Based on our docking experiment, distance between the triazole nitrogen of (*RR*)-enantiomer and Fe atom of heme (1.9 Å) is less than that of (*SS*)-enantiomer (2.3 Å), indicating stronger coordination bond. Moreover, the free energy of binding (Δ*G*) for the best docking pose with the highest score indicated that (*RR*)-enantiomer with $\Delta G = -38$ kJ/mol should have a better interaction than the (*SS*)-enantiomer ($\Delta G = -17$ kJ/mol). It should be noted that many of marketed azole antifungals such as miconazole, bifonazole, sertaconazole, ketoconazole, itraconazole and terconazole are racemic

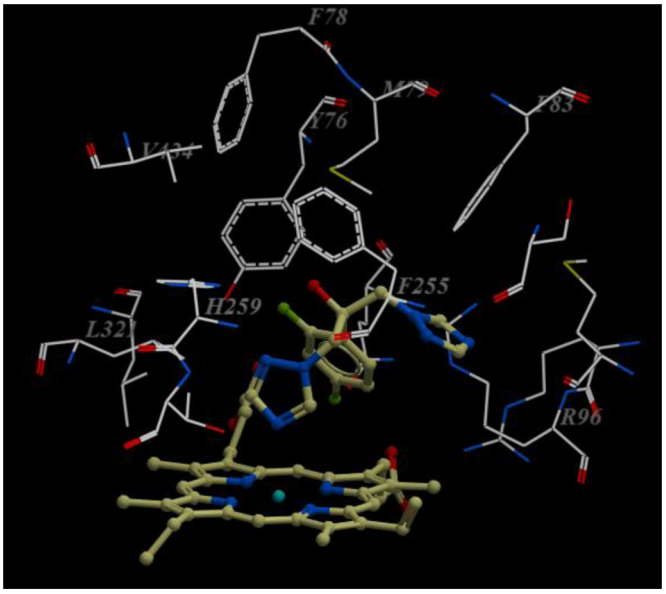


Fig. 3. Conformation of fluconazole in the active site of Cytochrome P450 14α-sterol demethylase after docking calculations. For clarity only amino acids within 7 Å distant from the docked ligand are shown.

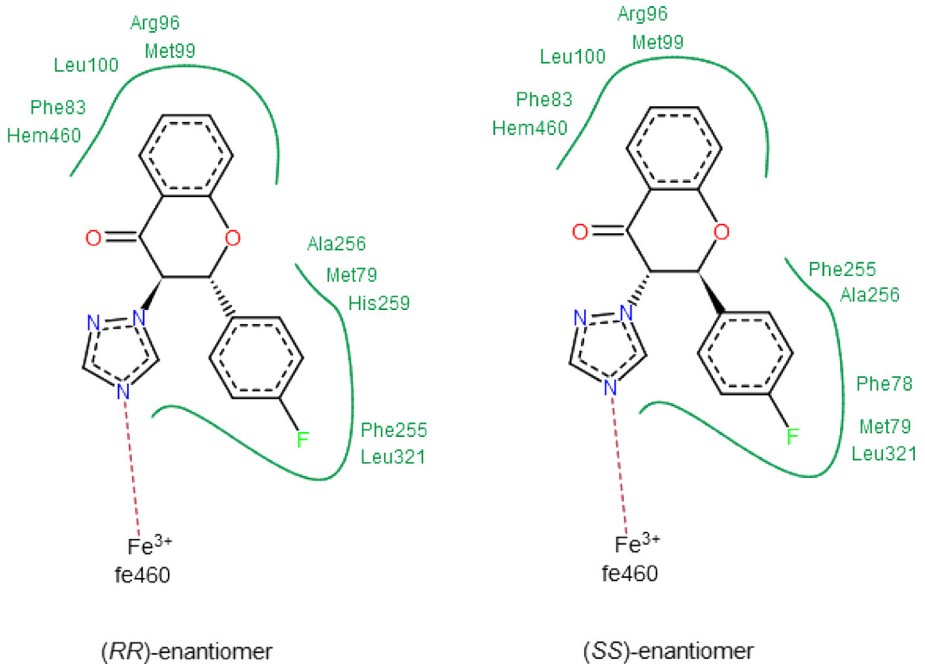


Fig. 4. Docking pose view (2D map) for (*RR*)- and (*SS*)-enantiomers of **4c**. Only important amino acids for interactions are shown.

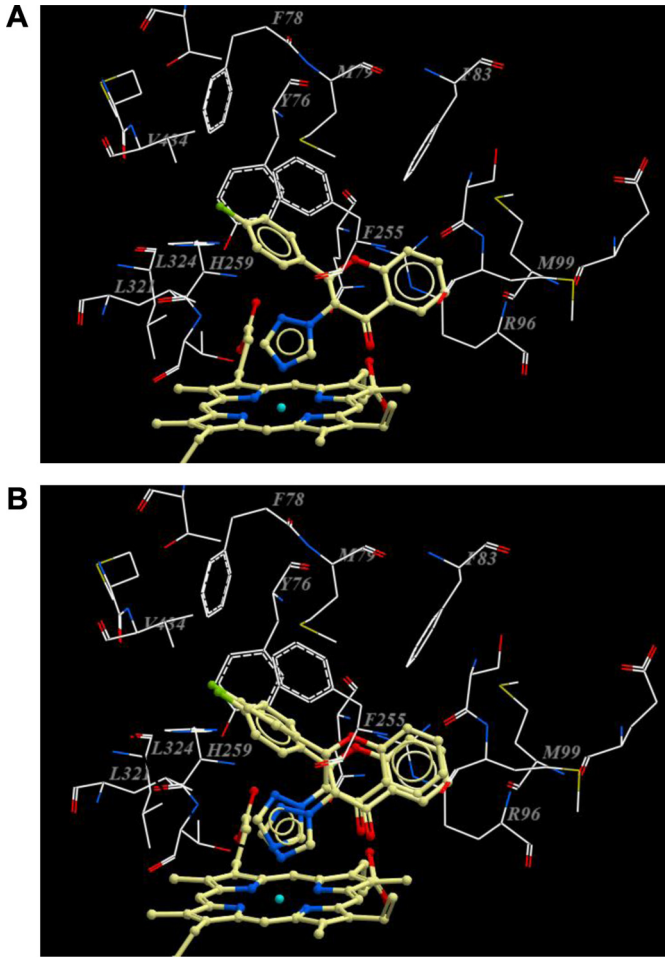


Fig. 5. (A) (*RR*)-Enantiomer of **4c** in the active site of Cytochrome P450 14α-sterol demethylase. (B) Binding mode of both (*RR*)- and (*SS*)-enantiomers of compound **4c** in the active site of enzyme. For clarity only amino acids within 7 Å distant from the docked ligand are shown.

mixtures. However, our docking study is promising to separate the enantiomers of **4c** which might have different antifungal activity.

Although, azoles are known to inhibit mainly CYP51 enzymes, and also our *in silico* study on the target compound **4c**, indicated its interactions with MTCYP51, but we cannot rebut other mechanisms which are responsible for antifungal activity. Only experiments on the isolated CYP51 could confirm our theoretical study.

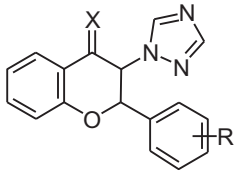
4.3. Drug-like properties

The concept of drug-likeness helps us to optimize pharmacokinetics and physicochemical properties, and to predict the overall potential of designed compounds to be qualified as a drug [17]. In the present work, OSIRIS Property Explorer program (http://www.organic-chemistry.org/prog/peo/) was used to predict the fragment-based drug-likeness and drug-score of title compounds in comparison with commercial triazole antifungals fluconazole and voriconazole. As summarized in Table 2, the analysis of theoretical toxicity risks (mutagenic, tumorigenic, irritant and reproductive effects) for these series of compounds using Osiris program showed that all title compounds have any potential of mutagenic, tumorigenic and irritant effects. 3-Methoxy analog **4h** showed high risk of reproductive effect, while remaining compounds as well as standard drugs fluconazole and voriconazole showed medium risk of reproductive effect. The estimated drug-likeness values of compounds revealed that all compounds with the exception of **4g**, had higher values compared to those of fluconazole and voriconazole. As depicted in Fig. 6, the drug-likeness and drug score values of compound **4c** (the most potent compound) were significantly higher than those of fluconazole and voriconazole. These results could be a measure of compound's potential to meet the criteria of a possible drug candidate.

5. Conclusion

Considering the leading role of triazole antifungals in the field of drug development, a series of novel 3-(1,2,4-triazol-1-yl)flavanone derivatives were synthesized based on the *N*-phenethylazole

Table 2
Drug-likeness properties of compounds **4a**–**k** and **5a**,**b** predicted by Osiris Property Explorer tool.



Compound	X	R	Log *S*	CLog *P*	MW	Toxicity risks[a]				Drug-likeness	Drug-score
						M[b]	T[c]	I[d]	R[e]		
4a	O	H	−2.95	2.66	291	(−)	(−)	(−)	(±)	3.15	0.68
4b	O	4-Cl	−3.69	3.27	325	(−)	(−)	(−)	(±)	5.21	0.62
4c	O	4-F	−3.27	2.72	309	(−)	(−)	(−)	(±)	2.57	0.62
4d	O	4-Me	−3.3	2.97	305	(−)	(−)	(−)	(±)	1.62	0.61
4e	O	4-OMe	−2.97	2.55	321	(−)	(−)	(−)	(±)	0.4	0.55
4f	O	3-Cl	−3.69	3.27	325	(−)	(−)	(−)	(±)	2.38	0.60
4g	O	3-F	−3.27	2.72	309	(−)	(−)	(−)	(±)	−0.26	0.48
4h	O	3-OMe	−2.97	2.55	321	(−)	(−)	(−)	(+)	2.33	0.49
4i	O	2-Cl	−3.69	3.27	325	(−)	(−)	(−)	(±)	2.96	0.61
4j	O	2-OMe	−2.97	2.55	321	(−)	(−)	(−)	(±)	2.41	0.66
4k	O	2,4-Cl_2	−4.43	3.89	359	(−)	(−)	(−)	(±)	5.29	0.53
5a	NOH	H	−3.33	2.89	306	(−)	(−)	(−)	(±)	2.06	0.63
5b	NOH	2,4-Cl_2	−4.80	4.12	374	(−)	(−)	(−)	(±)	4.35	0.48
Fluconazole			−2.18	−0.21	306	(−)	(−)	(−)	(±)	−1.13	0.46
Voriconazole			−3.23	1.11	349	(−)	(−)	(−)	(±)	0.42	0.55

[a] Ranked according to: (−) no bad effect, (±) medium bad effect, (+) bad effect.
[b] M: mutagenic effect.
[c] T: tumorigenic effect.
[d] I: irritating effect.
[e] R: reproductive effect.

pharmacophore of azole antifungals. The *trans*-isomer of target compounds was selectively prepared from the reaction of 1-(2-hydroxyphenacyl)-1,2,4-triazole with appropriate benzaldehyde in the presence of piperidine as a catalyst. The results of antifungal evaluation tests revealed that 4′-fluoroflavanone derivative **4c** exhibited the best profile of activity against *Candida* and *Saccharomyces* strains. Compound **4c** was 4–16 times more potent than standard drug fluconazole against *C. albicans* and *S. cerevisiae*. Finally, compound **4c** prototype with promising activity and useful properties can be considered as lead compound in the design and development of new azole antifungal agents.

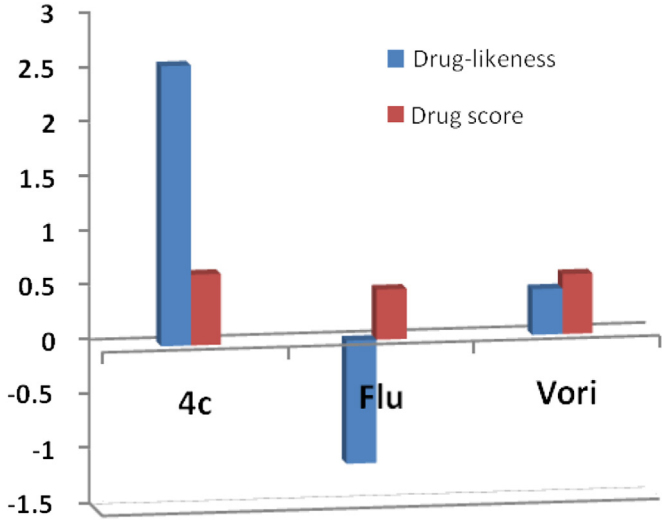


Fig. 6. Drug-likeness and drug score values of the most active compound **4c** compared to fluconazole and voriconazole as standard azole antifungals.

6. Experimental protocols

6.1. Chemistry

The 1-(2-hydroxyphenyl)ethanone derivatives **1**–**3** were prepared using the literature methods [13]. The purity of synthesized compounds were checked by thin-layer chromatography using silica gel 60 F254 plastic sheets (Merck), and UV light (254 nm) was used for visualization. Yields are based on isolated material and were not optimized. Melting points were determined in open glass capillaries using Bibby Stuart Scientific SMP3 apparatus (Stuart Scientific, Stone, UK) and are uncorrected. The IR spectra were obtained on a Perkin–Elmer FT-IR spectrophotometer using KBr disks. The NMR spectra were recorded using a Bruker 500 spectrometer and chemical shifts are expressed as δ (ppm) with tetramethylsilane (TMS) as internal standard. The mass spectra were obtained using a HP 5937 Mass Selective Detector (Agilent technologies).

6.1.1. General procedure for the synthesis of trans-3-(1H-1,2,4-triazol-1-yl)-flavanones *4a*–*k*

A solution of 1-(2-hydroxyphenyl)-2-(1*H*-1,2,4-triazol-1-yl) ethanone (**3**, 609 mg, 3.0 mmol) and appropriate benzaldehyde (3.15 mmol) in 2-propanol (5 mL) containing piperidine (1.05 mmol) was heated under reflux for 4–6 h. The excess of solvent was evaporated under reduced pressure. After cooling, the mixture was left in the fridge overnight. The precipitated product **4** was collected by filtration and washed with cooled 2-propanol and diethyl ether, respectively. In the cases of 2′-substituted flavanone derivatives **4i** and **4j**, after concentration of the reaction mixture, water was added. Then, the aqueous phase was decanted and the oily residue was dissolved in diethyl ether (5 mL), and treated with 65% HNO_3 (0.2 mL). The mixture was cooled at a fridge to complete

the precipitation of the nitrate salts **4i** or **4j**, which were collected by filtration and washed with diethyl ether.

*6.1.1.1. trans-2,3-Dihydro-2-phenyl-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one (**4a**).* Yield 60%; mp 120–121 °C; IR (KBr, cm^{-1}) ν_{max}: 1707 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 6.07 (d, 1H, J = 12.00 Hz, H-3), 6.45 (d, 1H, J = 12.5 Hz, H-2), 7.19 (d, 1H, J = 8.50 Hz, H-8), 7.22 (dt, 1H, J = 7.75 and 0.50 Hz, H-6), 7.32–7.37 (m, 3H, H-2′, H-4′ and H-6′), 7.43–7.47 (m, 2H, H-3′, H-5′), 7.71 (dt, 1H, J = 8.00 and 2.00 Hz, H-7), 7.88 (dd, 1H, J = 8.00 and 2.00 Hz, H-5), 7.92 (s, 1H, triazole H-5), 8.40 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 65.50, 81.83, 118.67, 120.12, 122.96, 127.56, 128.28, 128.90, 129.85, 135.87, 137.31, 146.10, 153.34, 161.33, 188.00. MS (*m*/*z*, %): 291 (M^+, 8), 256 (17), 222 (100), 194 (10), 170 (51), 145 (29), 120 (37), 92 (33), 63 (16). Anal. Calcd for $C_{17}H_{13}N_3O_2$: C, 70.09; H, 4.50; N, 14.42. Found: C, 69.88; H, 4.46; N, 14.66.

*6.1.1.2. trans-2-(4-Chlorophenyl)-2,3-dihydro-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one (**4b**).* Yield 71%; mp 156–157 °C; IR (KBr, cm^{-1}) ν_{max}: 1709 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 6.12 (d, 1H, J = 12.40 Hz, H-3), 6.45 (d, 1H, J = 12.41 Hz, H-2), 7.20 (d, 1H, J = 8.34 Hz, H-8), 7.23 (t, 1H, J = 7.60 Hz, H-6), 7.42 (d, 2H, J = 8.48 Hz, H-2′ and H-6′), 7.48 (d, 2H, J = 8.48 Hz, H-3′ and H-5′), 7.72 (dt, 1H, J = 7.76 and 1.61 Hz, H-7), 7.89 (dd, 1H, J = 7.86 and 1.49 Hz, H-5), 7.95 (s, 1H, triazole H-5), 8.43 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 65.80, 81.39, 119.06, 120.42, 123.43, 128.00, 129.43, 130.47, 134.86, 135.22, 138.18, 146.46, 152.73, 161.82, 188.12. MS (*m*/*z*, %): 325 (M^+, 6), 277 (5), 256 (100), 228 (10), 205 (38), 179 (22), 151 (14), 120 (45), 92 (32), 63 (14). Anal. Calcd for $C_{17}H_{12}ClN_3O_2$: C, 62.68; H, 3.71; N, 12.90. Found: C, 62.61; H, 3.60; N, 13.15.

*6.1.1.3. trans-2,3-Dihydro-2-(4-fluorophenyl)-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one (**4c**).* Yield 38%; mp 120–121 °C; IR (KBr, cm^{-1}) ν_{max}: 1709 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 6.11 (d, 1H, J = 12.44 Hz, H-3), 6.45 (d, 1H, J = 12.42 Hz, H-2), 7.15–7.20 (m, 3H, H-2′, H-6′ and H-8), 7.23 (t, 1H, J = 7.25 Hz, H-6), 7.50–7.54 (m, 2H, H-3′ and H-5′), 7.72 (dt, 1H, J = 7.79 and 1.70 Hz, H-7), 7.89 (dd, 1H, J = 7.85 and 1.63 Hz, H-5), 7.94 (s, 1H, triazole H-5), 8.42 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 65.47, 81.03, 115.88 (d, $J_{C,F}$ = 21.25 Hz), 118.66, 120.00, 122.99, 127.59, 130.54 (d, $J_{C,F}$ = 8.75 Hz), 132.14, 137.78, 146.01, 152.30, 161.27, 162.86 (d, $J_{C,F}$ = 243.75 Hz), 187.88. Anal. Calcd for $C_{17}H_{12}FN_3O_2$: C, 66.02; H, 3.91; N, 13.59. Found: C, 66.13; H, 3.78; N, 13.60.

*6.1.1.4. trans-2,3-Dihydro-2-(4-methylphenyl)-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one (**4d**).* Yield 69%; mp 166–167 °C; IR (KBr, cm^{-1}) ν_{max}: 1709 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 2.27 (s, 3H, Methyl), 6.03 (d, 1H, J = 12.43 Hz, H-3), 6.42 (d, 1H, J = 12.42 Hz, H-2), 7.14 (d, 2H, J = 7.97 Hz, H-2′ and H-6′), 7.17 (d, 1H, J = 8.38 Hz, H-8), 7.22 (dt, 1H, J = 7.52 and 0.75 Hz, H-6), 7.35 (d, 2H, J = 8.03 Hz, H-3′ and H-5′), 7.70 (dt, 1H, J = 7.78 and 1.69 Hz, H-7), 7.89 (dd, 1H, J = 7.87 and 1.57 Hz, H-5), 7.92 (s, 1H, triazole H-5), 8.41 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 21.65, 65.84, 82.13, 119.06, 120.41, 123.26, 127.98, 128.58, 129.86, 133.26, 138.11, 139.65, 146.39, 152.60, 161.79, 188.45. Anal. Calcd for $C_{18}H_{15}N_3O_2$: C, 70.81; H, 4.95; N, 13.76. Found: C, 70.73; H, 5.08; N, 14.00.

*6.1.1.5. trans-2,3-Dihydro-2-(4-methoxyphenyl)-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one (**4e**).* Yield 74%; mp 104–105 °C; IR (KBr, cm^{-1}) ν_{max}: 1710 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 3.72 (s, 3H, OCH_3), 6.01 (d, 1H, J = 12.69 Hz, H-3), 6.43 (d, 1H, J = 12.44 Hz, H-2), 6.88 (d, 2H, J = 8.65 Hz, H-3′ and H-5′), 7.17 (d, 1H, J = 8.34 Hz, H-8), 7.21 (t, 1H, J = 7.52 Hz, H-6), 7.40 (d, 2H, J = 8.65 Hz, H-2′ and H-6′), 7.70 (dt, 1H, J = 7.72 and 1.36 Hz, H-7), 7.88 (dd, 1H, J = 7.84 and 1.50 Hz, H-5), 7.93 (s, 1H, triazole H-5), 8.41 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 55.97, 65.87, 81.96, 114.64, 119.06, 120.40, 123.22, 127.97, 128.15, 130.12, 138.09, 146.36, 152.59, 160.69, 161.83, 188.55. Anal. Calcd for $C_{18}H_{15}N_3O_3$: C, 67.28; H, 4.71; N, 13.08. Found: C, 67.31; H, 4.79; N, 12.91.

*6.1.1.6. trans-2-(3-Chlorophenyl)-2,3-dihydro-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one (**4f**).* Yield 68%; mp 126–127 °C; IR (KBr, cm^{-1}) ν_{max}: 1699 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 6.14 (d, 1H, J = 12.39 Hz, H-3), 6.47 (d, 1H, J = 12.39 Hz, H-2), 7.21 (d, 1H, J = 8.08 Hz, H-8), 7.24 (t, 1H, J = 7.36 Hz, H-6), 7.34–7.37 (m, 2H, H-4′ and H-6′), 7.42 (m, 1H, H-5′), 7.59 (br s, 1H, H-2′), 7.72 (dt, 1H, J = 7.77 and 1.66 Hz, H-7), 7.90 (dd, 1H, J = 7.83 and 1.53 Hz, H-5), 7.97 (s, 1H, triazole H-5), 8.46 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 65.27, 80.91, 118.69, 119.96, 123.09, 126.94, 127.60, 128.01, 129.85, 130.87, 133.55, 137.84, 138.14, 146.11, 152.36, 161.16, 187.67. Anal. Calcd for $C_{17}H_{12}ClN_3O_2$: C, 62.68; H, 3.71; N, 12.90. Found: C, 62.61; H, 3.99; N, 12.86.

*6.1.1.7. trans-2,3-Dihydro-2-(3-fluorophenyl)-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one (**4g**).* Yield 54%; mp 122–123 °C; IR (KBr, cm^{-1}) ν_{max}: 1707 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 6.14 (d, 1H, J = 12.39 Hz, H-3), 6.45 (d, 1H, J = 12.37 Hz, H-2), 7.16–7.27 (m, 4H, H-5′, H-6′, H-6 and H-8), 7.34–7.41 (m, 2H, H-2′, H-4′), 7.72 (dt, 1H, J = 7.80 and 1.72 Hz, H-7), 7.90 (dd, 1H, J = 7.91 and 1.67 Hz, H-5), 7.96 (s, 1H, triazole H-5), 8.45 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 65.75, 81.37, 115.32 (d, $J_{C,F}$ = 22.25 Hz), 117.18 (d, $J_{C,F}$ = 20.75 Hz), 119.09, 120.38, 123.48, 124.83 (d, $J_{C,F}$ = 2.50 Hz), 128.00, 131.45 (d, $J_{C,F}$ = 8.13 Hz), 138.22, 138.85 (d, $J_{C,F}$ = 7.38 Hz), 146.50, 152.74, 161.56, 161.74, 188.04. Anal. Calcd for $C_{17}H_{12}FN_3O_2$: C, 66.02; H, 3.91; N, 13.59. Found: C, 65.94; H, 3.90; N, 14.07.

*6.1.1.8. trans-2,3-Dihydro-2-(3-methoxyphenyl)-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one (**4h**).* Yield 79%; mp 163–164 °C; IR (KBr, cm^{-1}) ν_{max}: 1711 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 3.72 (s, 3H, OCH_3), 6.04 (d, 1H, J = 12.37 Hz, H-3), 6.43 (d, 1H, J = 12.39 Hz, H-2), 6.90 (dd, 1H, J = 8.26 and 2.43 Hz, H-4′), 6.99 (d, 1H, J = 7.59 Hz, H-6′), 7.05 (br s, 1H, H-2′), 7.20 (d, 1H, J = 8.52 Hz, H-8), 7.21–7.26 (m, 2H, H-5′ and H-6), 7.71 (dt, 1H, J = 7.76 and 1.53 Hz, H-7), 7.89 (dd, 1H, J = 7.82 and 1.25 Hz, H-5), 7.95 (s, 1H, triazole H-5), 8.41 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 55.58, 65.42, 81.69, 113.69, 115.21, 118.70, 119.98, 120.36, 122.95, 127.58, 130.05, 137.30, 137.78, 146.07, 152.25, 159.55, 161.30, 187.94. MS (*m*/*z*, %): 321 (M^+, 9), 252 (100), 221 (9), 200 (40), 175 (13), 132 (36), 92 (33), 63 (10). Anal. Calcd for $C_{18}H_{15}N_3O_3$: C, 67.28; H, 4.71; N, 13.08. Found: C, 67.50; H, 4.63; N, 12.79.

*6.1.1.9. trans-2-(2-Chlorophenyl)-2,3-dihydro-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one nitrate (**4i**).* Yield 55%; mp 124–126 °C; IR (KBr, cm^{-1}) ν_{max}: 1714 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 6.48 (d, 1H, J = 12.55 Hz, H-3), 6.65 (d, 1H, J = 12.58 Hz, H-2), 7.19 (d, 1H, J = 8.34 Hz, H-8), 7.25 (t, 1H, J = 7.64 Hz, H-6), 7.36–7.45 (m, 3H, H-4′, H-5′ and H-6′), 7.72 (dt, 1H, J = 7.75 and 0.94 Hz, H-7), 7.88–7.95 (m, 3H, H-3′, H-5 and triazole H-5), 8.59 (br s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 64.43, 77.82, 118.53, 119.99, 123.24, 127.70, 128.14, 130.15, 130.34, 131.34, 132.70, 133.94, 137.90, 146.08, 151.95, 160.93, 187.03. Anal. Calcd for $C_{17}H_{13}ClN_4O_5$: C, 52.52; H, 3.37; N, 14.41. Found: C, 52.79; H, 3.25; N, 14.40.

*6.1.1.10. trans-2,3-Dihydro-2-(2-methoxyphenyl)-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one nitrate (**4j**).* Yield 62%; mp 141–143 °C; IR (KBr, cm^{-1}) ν_{max}: 1713 (C=O); ^1H NMR (500 MHz, DMSO-d_6) δ 3.71 (s, 3H, OCH_3), 6.32 (d, 1H, J = 12.62 Hz, H-3), 6.58

(d, 1H, J = 12.61 Hz, H-2), 6.87–6.98 (m, 2H, H-3′ and H-5′), 7.16 (d, 1H, J = 8.29 Hz, H-8), 7.21 (t, 1H, J = 7.22 Hz, H-6), 7.32 (dt, 1H, J = 7.86 and 1.52 Hz, H-4′), 7.60 (dd, 1H, J = 7.45 and 1.33 Hz, H-6′), 7.69 (dt, 1H, J = 7.79 and 1.65 Hz, H-7), 7.89 (dd, 1H, J = 7.85 and 1.52 Hz, H-5), 7.94 (br s, 1H, triazole H-5), 8.59 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 56.55, 64.78, 77.17, 112.49, 119.01, 120.42, 121.27, 123.21, 123.33, 128.04, 130.23, 131.85, 138.02, 158.33, 161.98, 188.55. Anal. Calcd for $C_{18}H_{16}N_4O_6$: C, 56.25; H, 4.20; N, 14.58. Found: C, 56.11; H, 4.33; N, 14.46.

*6.1.1.11. trans-2-(2,4-Dichlorophenyl)-2,3-dihydro-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one (**4k**).* Yield 70%; mp 155–157 °C; IR (KBr, cm^{-1}) ν_{max}: 1714 (C=O); ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.47 (d, 1H, J = 12.56 Hz, H-3), 6.64 (d, 1H, J = 12.54 Hz, H-2), 7.20 (d, 1H, J = 8.31 Hz, H-8), 7.25 (t, 1H, J = 7.41 Hz, H-6), 7.56 (dd, 1H, J = 8.39 and 1.62 Hz, H-5′), 7.60 (d, 1H, J = 1.72 Hz, H-3′), 7.72 (t, 1H, J = 7.14 Hz, H-7), 7.90–7.94 (d and s, 2H, H-5 and triazole H-5), 7.97 (d, 1H, J = 8.41 Hz, H-6′), 8.59 (s, 1H, triazole H-3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 64.79, 77.77, 118.96, 120.49, 123.61, 128.08, 128.83, 130.05, 132.16, 132.47, 135.29, 135.92, 138.20, 146.53, 152.57, 161.33, 187.79. Anal. Calcd for $C_{17}H_{11}Cl_2N_3O_2$: C, 56.69; H, 3.08; N, 11.67. Found: C, 56.73; H, 2.97; N, 11.70.

*6.1.2. Synthesis of trans-2,3-dihydro-2-phenyl-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one oxime (**5a**)*

A solution of compound **4a** (146 mg, 0.5 mmol) and hydroxylamine hydrochloride (104 mg, 1.5 mmol) in methanol (5 mL) was heated under reflux for 24 h. After completion of the reaction, the reaction mixture was concentrated under reduced pressure and mixed with water (15 mL). The mixture was left in the fridge overnight. The precipitated solid was collected by filtration and washed with water (3 × 5 mL). The crude product was recrystallized from methanol–water to give compound **5a**. Yield 79%; mp 242–244 °C; IR (KBr, cm^{-1}) ν_{max}: 3442 (OH), 1604 (C=NOH); ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.63 (d, 1H, J = 2.75 Hz, H-3), 6.18 (d, 1H, J = 2.83 Hz, H-2), 7.07–7.12 (m, 2H, H-6 and H-8), 7.13–7.18 (m, 2H, H-2′ and H-6′), 7.24–7.29 (m, 3H, H-3′, H-4′ and H-5′), 7.39 (dt, 1H, J = 7.77 and 1.57 Hz, H-7), 7.79 (s, 1H, triazole H-5), 7.93 (dd, 1H, J = 8.24 and 1.56 Hz, H-5), 7.96 (s, 1H, triazole H-3), 11.83 (s, 1H, NOH). ^{13}C NMR (125 MHz, DMSO-d_6) δ 53.76, 79.28, 118.78, 119.14, 122.90, 124.08, 126.85, 128.99, 129.05, 131.66, 136.59, 145.66, 145.99, 152.01, 156.37. MS (m/z, %): 306 (M$^+$, 15), 289 (11), 237 (100), 220 (15), 178 (31), 121 (11), 102 (13), 77 (22), 63 (10), 51 (11). Anal. Calcd for $C_{17}H_{14}N_4O_2$: C, 66.66; H, 4.61; N, 18.29. Found: C, 66.90; H, 4.45; N, 18.18.

*6.1.3. Synthesis of trans-2-(2,4-dichlorophenyl)-2,3-dihydro-3-(1H-1,2,4-triazol-1-yl)-4H-1-benzopyran-4-one oxime (**5b**)*

A solution of compound **4k** (180 mg, 0.5 mmol) and hydroxylamine hydrochloride (104 mg, 1.5 mmol) in methanol (5 mL) was heated under reflux for 68 h. The excess of methanol was evaporated under reduced pressure. The concentrated solution was mixed with water (15 mL) and the mixture was left in the fridge overnight. The precipitated solid was collected by filtration and washed with water (3 × 5 mL). The crude product was recrystallized from methanol–water to give compound **5b**. Yield 81%; mp 204–206 °C; IR (KBr, cm^{-1}) ν_{max}: 3436 (OH), 1591 (C=NOH); ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.78 (d, 1H, J = 2.62 Hz, H-3), 6.24 (d, 1H, 2.69 Hz, H-2), 6.85 (d, 1H, J = 8.48 Hz, H-8), 7.10–7.19 (m, 2H, H-6 and H-6′), 7.27 (dd, 1H, J = 8.48 and 1.98 Hz, H-5′), 7.41 (dt, 1H, J = 7.83 and 1.41 Hz, H-7), 7.71 (d, 1H, J = 2.06 Hz, H-3′), 7.82 (s, 1H, triazole H-5), 7.93 (d, 1H, J = 7.74 Hz, H-5), 8.17 (s, 1H, triazole H-3), 12.00 (s, 1H, NOH). Anal. Calcd for $C_{17}H_{12}Cl_2N_4O_2$: C, 54.42; H, 3.22; N, 14.93. Found: C, 54.41; H, 3.53; N, 15.01.

6.2. Micro-dilution method for MICs determination

The MICs of compounds were determined by micro-dilution method [14]. A stock solution of compounds was prepared in Sabouraud dextrose broth (SDB)/DMSO (9:1). Then, 200 μL of the stock solution was transferred into the plates with 96 U-shaped wells and serially diluted by mixing with 100 μL of SDB in subsequent wells. Then, aliquot of 100 μL of fungal suspension (1×10^3 CFU/mL) was added to each well to reach the final inoculum size of 0.5×10^3 CFU/mL. After 24, 48 and 72 h of incubation at 35 °C, the plates were observed for the absence or presence of visible growth in comparison with that of the drug-free control well. The endpoint MIC was the lowest concentration of the compound at which the test strain does not demonstrate visible growth.

6.3. Docking study

3D-structures of compounds were generated using HyperChem and geometrically optimized using semiempirical AM1 method in Gaussian 98. FlexX, a fully automated docking program available on LeadIT 2.0.2 package was used to dock compounds into the active site of the enzyme. FlexX considers ligand flexibility by changing the conformations of the ligand in the active site while making the protein rigid. The X-ray coordinate of MTCYP51 in complex with fluconazole (PDB id: 1EA1) was retrieved from Protein Data Bank (http://www.rcsb.org). All crystallographic water molecules were removed. The docking and subsequent scoring were performed using the default parameters of the FlexX program implanted in LeadIT 2.0.2. The active site of the enzyme was defined to include residues within 6.5 Å radius around bound inhibitor. Final scores for all FlexX solutions were calculated by a consensus scoring function (CScore) and used for database ranking. Finally the best pose with the highest score was selected for investigating the interactions, HYDE assessment and calculating the free energy of binding (ΔG) [18,19].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.06.008.

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