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Synthesis, in vitro evaluation and molecular docking studies of new triazole derivatives as antifungal agents

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ABSTRACT

On the basis of the active site of lanosterol 14α-demethylase from *Candida albicans* (CACYP51), a series of 1-(2-(2,4-difluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl)-1*H*-1,2,4-triazol-5(4*H*)-one derivatives were synthesized as fluconazole analogs. Results of the preliminary antifungal tests against eight human pathogenic fungi in vitro showed that these compounds exhibited activities to some extent, and some displayed excellent antifungal activities against *C. albicans* than reference drug fluconazole. Flexible molecular docking was used to analyze the structure–activity relationships (SARs) of the target compounds. The designed compounds interact with CACYP51 through hydrophobic, van der Waals and hydrogen-bonding interactions.

During the past two decades, the life-threatening infections caused by pathogenic fungi have become increasingly common especially in the immuno-compromised hosts suffering from tuberculosis, cancer, AIDS and organ transplant cases.[1–3] The common antifungal agents (Fig. 1) used in clinic are azoles (such as fluconazole, itraconazole and voriconazole),[4] polyenes (such as amphotericin B[5,6] and nystatin),[7] echinocandins (such as caspofungin and micafungin)[8] and allylamines (such as naftifine and terbinafine).[9] Among those, azoles have fungistatic, orally active and broad-spectrum activities against most yeasts and filamentous fungi and were widely used in antifungal chemotherapy.[10] However, their clinical application value has been hampered by their relatively high risk of toxicity, the emergence of drug resistance, pharmacokinetic deficiencies and/or insufficiencies in their antifungal activities. So there is still an urgent need for novel broad-spectrum and low-toxicity antifungal agents.

Azole antifungals function by competitive inhibition of the lanosterol 14α-demethylase(CYP51), the enzyme that catalyzes the oxidative removal of the 14α-methyl group of lanosterol to give $\Delta^{14,15}$-desaturated intermediates in ergosterol biosynthesis. Selective inhibition of CYP51 would cause depletion of ergosterol and accumulation of lanosterol and other 14-methyl sterols leading to the growth inhibition of fungal cells.[11] Currently, the crystal structure of CYP51 of fungi has not been obtained, eukaryotic CYP51s are membrane-associated proteins, and solving their crystal structures remains a challenge. Thereby, we have constructed a 3D model of CYP51 from *Mycobacterium tuberculosis* (MTCYP51) through homology modeling on the basis of the crystal structure of MTCYP51 (*M. tuberculosis* 14α-demethylase) which is the only crystal structure for the CYP51 family reported by Podust et al. in 2001.[12] The active site of the model divided into four sections: a coordination bond with iron of the heme group, the hydrophilic H-bonding region, the hydrophobic region, and the narrow hydrophobic cleft formed by the residues in the helix B′-meander 1 loop and N-terminus of helix 1.[13]

Research indicated that the triazole ring in the scaffold of triazole antifungals was positioned perpendicularly to the porphyrin plane with a ring nitrogen atom coordinated to the heme iron of CYP51 and was of key importance for the antifungal activity. The halogenated phenyl group was deep in the same hydrophobic binding cleft in the active site of the enzyme CYP51 and long side chains surpassed the active site and interacted with residues in the substrate access channel.

Here we designed a series of 1-(2-(2,4-difluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl)-4-substituted phenyl-1*H*-1,2,4-triazol-5(4*H*)-one compounds, which containing a

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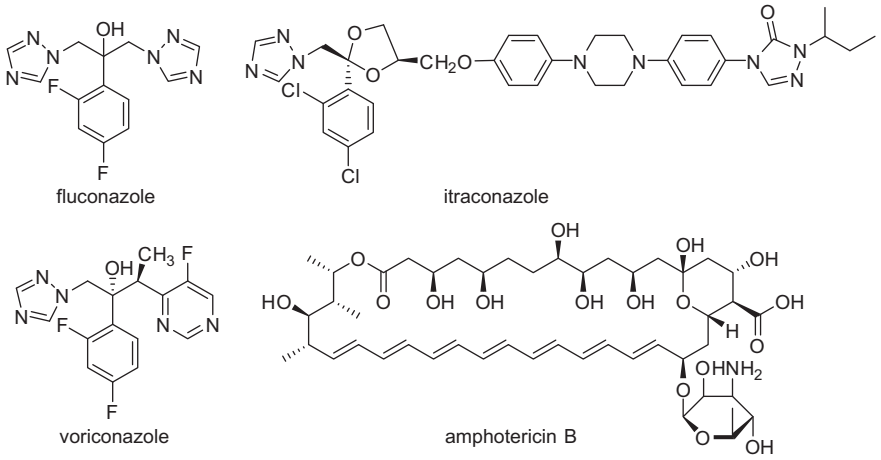
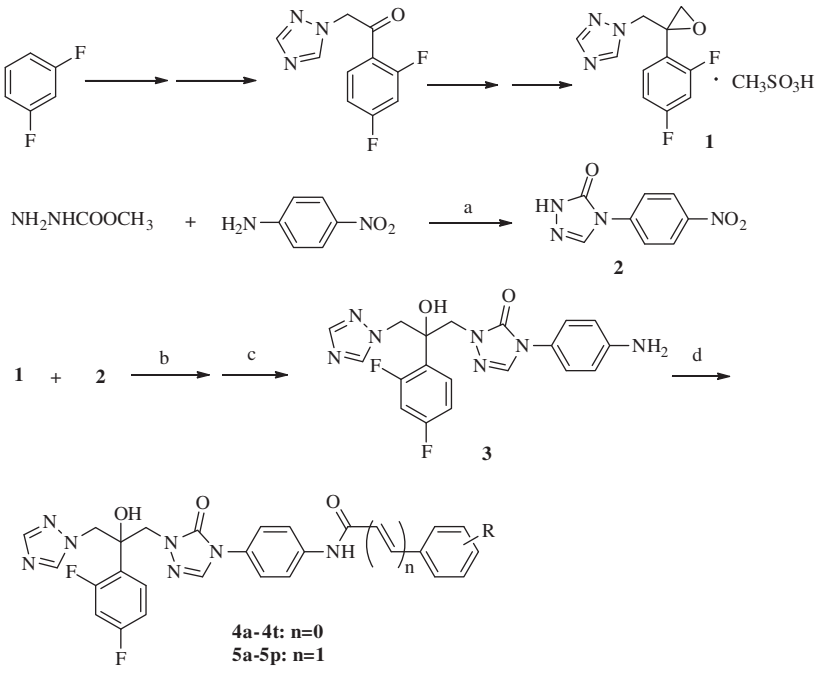


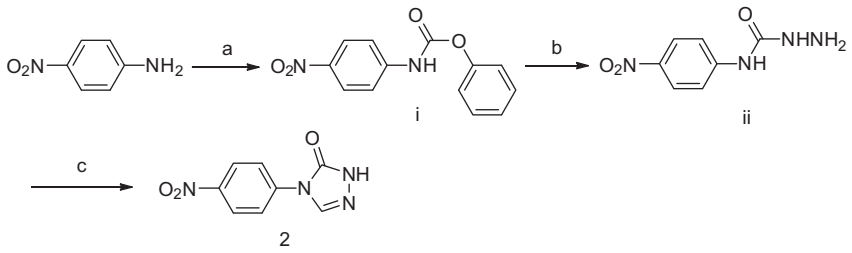
Figure 1. Antifungal agents used in clinical therapy.

triazole ring, a difluorophenyl group and a triazolone side chain, aiming to find potent systemic antifungal agents that have a broad antifungal spectrum.

Synthetic routes of the target compounds **4a–4t** and **5a–5p** were outlined in Scheme 1. According to a known procedure, the oxirane intermediate **1** was obtained by Corey–Chaykovsky expoxidation in the presence of trimethylsulfoxonium iodide and NaOH.[14] Intermediate **2** was originally synthesized by a three-step procedure outlined in Scheme 2. First, phenyl chloroformate was added dropwise to a stirred mixture of *p*-nitroaniline, pyridine and EtOAc at 0 °C. The mixture was stirred for 3 h at room temperature to give compound **i**. The yield was 97.7%. Second, hydrazine hydrate was added to a solution of compound **i** in dimethoxyethane, the resulting reaction mixture was stirred for 24 h at room temperature to give compound **ii**. The yield was 81.3%. Finally, treatment of compound **ii** with formamidine acetate in DMF at room temperature for 30 min gave the intermediate **2**.[15] The yield was 82.2%. In this Letter we applied a 'one-pot' method to synthesize the intermediate **2**. Here in our method, hydrazinocarboxylate and trimethyl orthoformate was added to a solution of *p*-nitroaniline in methanol. After the reaction mixture was refluxed for 2 h, 25% sodium methoxide was added dropwise to it which continued to reflux for another 3 h. The resulting mixture was concentrated under reduced pressure, then added 30 ml water and adjusted pH value to 1 with concentrated hydrochloric acid. Filtration of the latter mixture gave the intermediate **2**.[16] The yield was 76.4–85.6%. Apparently, the former method needs three steps, while ours is a one step procedure. It is more succinct and is of more efficiency. Treatment of **2** with excess **1** in the presence of potassium carbonate in DMF gave an important intermediate, which reduced with 10% Pd–C under H_2 atmosphere led to the synthesis of compound **3**. Target compounds **4a–4t** and **5a–5p** were obtained by using different aromatic acid or substituted cinnamic acid to react with **3** in the presence of *N*-(3-dimethylamino-propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl)



Scheme 1. Synthetic routes of the target compounds. Reagents and conditions: (a) $HC(OCH_3)_3$, CH_3ONa, CH_3OH, *p*-toluenesulfonic acid, reflux, 5 h; (b) DMF, K_2CO_3, 80 °C, 6 h; (c) Pd/C, H_2, 4 h, rt; (d) RCOOH, EDC·HCl/DMAP, CH_2Cl_2, rt, 6 h.



Scheme 2. Original synthesis route of intermediate **2**. Reagents and conditions: (a) phenyl chloroformate, pyridine, EtOAc, rt, 3 h; (b) Hydrazine hydrate, dimethoxyethane, rt, 24 h; (c) formamidine acetate, DMF, rt, 30 min.

Table 1
In vitro antifungal activities of the target compounds (MIC_{80} μg ml^{-1})[a]

Compound no.	R	*C. alb*		*C. tro*	*C. par*	*C. kef*	*C. neo* BLS108	*T. nru*	A. fumi
		SC5314	Y0109						
4a	4-F	0.0625	1	0.0625	0.0625	4	64	16	>64
4b	2-F	0.0625	4	0.0625	0.0156	1	64	16	>64
4c	4-Cl	1	1	1	1	1	0.0625	>64	>64
4d	3-Cl	0.00024	0.00024	1	0.00024	4	16	>64	>64
4e	2,4-Cl	0.0625	0.0625	0.25	0.0156	4	>64	>64	>64
4f	3-Br	4	16	16	16	64	>64	>64	>64
4g	2-Br	1	4	4	1	>64	16	>64	>64
4h	4-OCH_3	1	1	4	1	16	>64	>64	>64
4i	3-OCH_3	0.0625	0.0625	1	0.0625	16	>64	>64	>64
4j	4-OCF_3	0.25	1	64	1	>64	>64	4	>64
4k	4-CH_3	0.0625	0.0625	1	0.25	16	>64	>64	>64
4l	3-CH_3	0.0625	0.0625	1	0.0625	>64	>64	>64	>64
4m	2-CH_3	0.25	0.0625	1	0.0156	>64	>64	>64	>64
4n	3,4-CH_3	0.25	0.25	0.25	0.25	4	64	>64	>64
4o	2,4-CH_3	0.0625	0.0625	1	0.0625	64	>64	>64	>64
4p	4-$CH_2CH_2CH_3$	0.0039	1	1	4	>64	>64	>64	>64
4q	4-$(CH_2)_3CH_3$	0.0156	0.0156	0.25	0.0625	>64	>64	>64	>64
4r	4-$(CH_2)_4CH_3$	4	>64	>64	>64	>64	>64	>64	>64
4s	4-C $(CH_3)_3$	0.0625	1	0.25	0.0625	>64	64	4	>64
4t	4-CH $(CH_3)_2$	4	4	4	4	>64	>64	>64	>64
5a	4-F	>64	>64	>64	>64	>64	>64	>64	>64
5b	3-F	16	16	>64	16	>64	>64	>64	>64
5c	2-F	1	1	4	4	>64	>64	>64	>64
5d	4-Cl	4	16	>64	>64	>64	>64	>64	>64
5e	3,4-Cl	4	4	4	64	>64	>64	>64	>64
5f	4-Br	1	4	>64	1	>64	>64	>64	>64
5g	3-Br	1	1	1	16	>64	16	>64	>64
5h	4-CH_3	1	4	4	>64	>64	>64	>64	>64
5i	2,3-CH_3	1	4	16	>64	>64	>64	>64	>64
5j	4-OCH_3	4	>64	>64	>64	>64	>64	>64	>64
5k	3,4-OCH_3	1	1	1	4	>64	>64	>64	>64
5l	2,5-OCH_3	1	16	>64	1	>64	>64	>64	>64
5m	4-NO_2	>64	>64	>64	1	>64	>64	>64	>64
5n	3-NO_2	0.00024	0.00024	0.25	0.0039	16	>64	64	>64
5o	2-NO_2	1	1	1	1	>64	>64	>64	>64
5p	3-CN	16	16	64	16	>64	>64	>64	>64
ICZ	—	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625
VCZ	—	0.0039	0.0039	0.0039	0.0039	0.0039	0.0039	0.0156	0.0039
FCZ	—	0.25	0.25	1	0.25	4	1	1	>64
AMB	—	0.25	0.25	1	0.25	0.25	1	4	1

[a] Abbreviations: *C. alb. Candida albicans; C. tro. Candida tropicalis; C. par. Candida parasilosis; C. kef. Candida kefyr; C. neo. Cryptococcus neoformans; T. nru. Trichophyton rubrum; A. fumi. Aspergillus fumigates*. FCZ: fluconazole; VCZ: voriconazole; ICZ: itraconazole; AMB: amphotericin B.

and 4-dimethyl-aminopyridine (DMAP) in CH_2Cl_2 at ambient temperature.

The in vitro minimal inhibitory concentrations (MIC_{80}) of the compounds were determined by the micro-broth dilution method in 96-well microtest plates according to the methods defined by the National Committee for Clinical Laboratory Standards (NCCLS).[17] The tested fungi species included eight pathogenic fungi, which were found in dermatomycoses (*Trichophyton rubrum*) and systemic mycoses (*Candida albicans (*SC5314 and Y0109*)*, *Candida tropicalis*, *Candida parapsilosis*, *Candida kefyr*, *Cryptococcus neoformans BLS108*, and *Aspergillus fumigatus*). *C. albicans* and *C. neoformans* were provided by Shanghai Changzheng Hospital; *C. tropicalis*, *C. parapsilosis*, *C. kefyr*, *C. neoformans*, *T. rubrum* and *A. fumigatus* were provided by Shanghai Changhai Hospital. *C. albicans* and *C. neoformans* were purchased from ATCC, and other strains were clinic isolates. *C. albicans (*SC5314 and Y0109*)* and *C. neoformans* (BLS108) were used as the quality-controlled strains, and tested in each assay. *C. kefyr* was flucnazole resistant strain. Fluconazole, itraconazole, voriconazole and amphortericin B which served as positive controls were obtained from their respective manufacturers. The in vitro

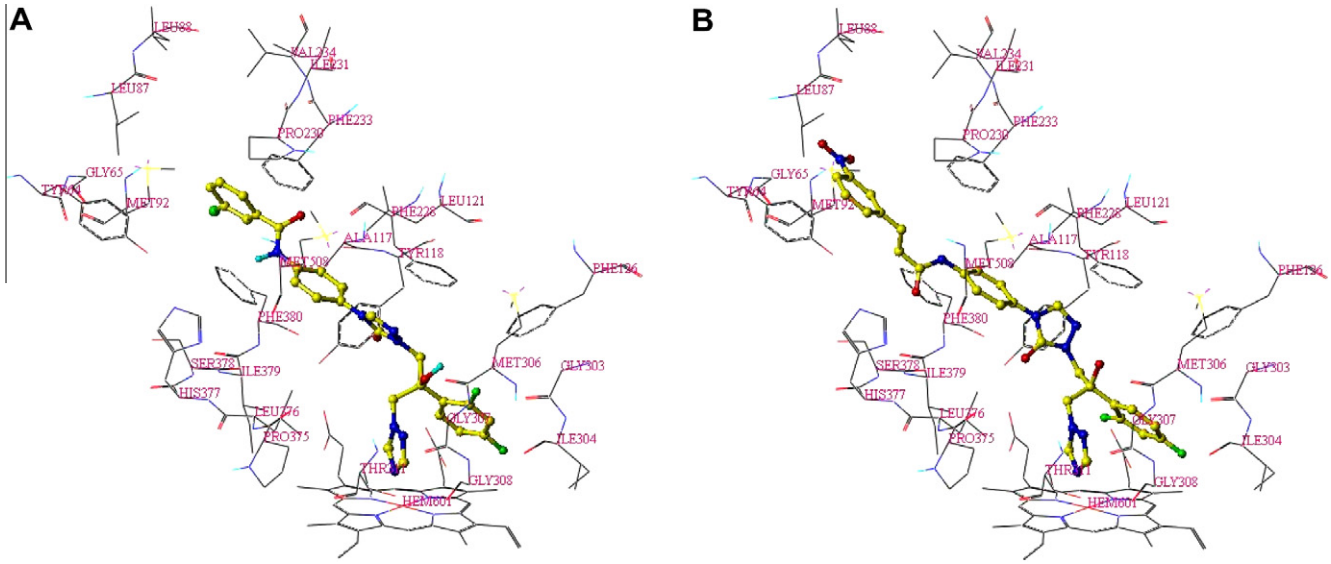


Figure 2. The docking conformation of compound **4d** (A) and **5n** (B) in the active site of CACYP51.

antifungal activities of all the target compounds were listed in Table 1. These data are the mean of three replicate tests performed with each antifungal compound.

The results of preliminary antifungal activities indicated that most of the target compounds **4a–4t** showed higher activities against *C. albicans* (SC5314), *C. albicans* (Y0109), *Candida tropicalis* and *Candida parasilosis* than the standard reference drug FCZ and AMB. Compounds **4a**, **4b**, **4i**, **4l**, and **4o** exhibited equivalent antifungal activities of ICZ against *C. albicans* (SC5314), *C. albicans* (Y0109) and *C. parasilosis* with the MIC_{80} value of 0.0625 μg ml^{-1}. Additionally, compound **4d** exhibited excellent activities with an MIC_{80} value of 0.00024 μg ml^{-1} against *C. albicans* (SC5314), *C. albicans* (Y0109) and *C. parasilosis,* versus the MIC_{80} value of VCZ, which is 0.0039 μg ml^{-1}. Among compounds **4k** and **4p–4r**, which contain an alkyl substituent in the *para* position, **4q** exhibited higher antifungal activity with the minimal inhibitory concentrations (MIC_{80}) value in the range of 0.25–0.0156 μg ml^{-1}. It indicates that suitable length of the alkyl side chain is relative to its activity. As for cinnamic acid series, including **5a–5p,** their activities were not desirable against all the tested fungal pathogens except **5n**. The activities of **5n** were excellent with the minimal inhibitory concentrations (MIC_{80}) value in the range of 0.25–0.00024 μg ml^{-1}. Apparently, all the target compounds were negative against *C. neoformans*, *A. fumigates* and *T. rubrum* except **4c** with a MIC_{80} value of 0.0625 μg ml^{-1} against *C. neoformans*. The MIC_{80} values of compound **4b** and **4c** were four times lower than that of FCZ against *C. kefyr*.

The inactivity of these compounds against *A. fumigatus* was not a surprise because it has been known that this species possesses an intrinsic mechanism resistant to triazole antifungal,[18] but there are some papers in which compounds reported are active against *A. fumigatus*.[3] It gives us hopes to develop broad-spectrum and more affordable antifungal agents.

All the target compounds were obtained as racemates. However, in previous study, *R* isomers showed lower interaction energy with CYP51 than *S* isomers, which indicated that the *R* configuration might have better antifungal activity than the *S* isomers.[15] Hence, in the following discussion, all the docking conformations refer to *R* configuration of the compounds. To clarify the binding mode of our synthesized compounds, compounds **4d** and **5n** were docked in the active site of CACYP51 by the Builder module within Insight-ll2000 software package (Fig. 2). The triazole ring of the compounds binds to CACYP51 through the formation of a coordination bond with iron of heme group. The difluorophenyl group is located in a hydrophobic pocket and interacts with Phe126, Met306, Gly303, Ile304, Gly307, and Gly308. The terminal triazolone side chain was placed into a hydrophobic cleft formed by Gly65, Met92, Phe380, Met508, Tyr118, and Ala117.

In addition, all of the side chains were of the pharmcophores, and the spatial orientations of the pharmacophores oriented in the hydrophobic pocket. The side chains of inhibitors played an important role in adjusting the physic-chemical properties of the whole molecule. Comparing **4a–4t** with **5a–5p**, the overall in vitro antifungal activity of **4a–4t** is better than **5a–5p**, we assume that the longer side chain influence their spatial orientations in target enzyme which lead to their low antifungal activities. In cinnamic acid series, the activity of **5n** is an exception, we presume that the *meta*-nitro substituent enhance the interaction between three major active sites of CACYP51 with our inhibitor by interacting with the remote residues of target enzyme.

In summary, a series of novel triazole antifungal agents containing a triazolone side chain were successfully designed and synthesized. Wherein, we adopted a more convenient method to synthesize the intermediate **2** and acquired higher yield**.** The antifungal activities of target compounds were screened for eight human pathogenic fungi. In vitro antifungal activity assay indicated that most of these compounds showed higher antifungal activities against *C. albicans* than the reference drug FCZ and AMB. Compounds **4d** and **5n** possessed excellent activities against *C. albicans*, *C. tropicalis* and *C. parasilosis,* we also studied their structure–activity relationships (SARs) through molecular docking. All these observations will be helpful in designing newer antifungal agents for our further research.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.06.008.

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