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Discovery of highly potent novel antifungal azoles by structure-based rational design

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ABSTRACT

On the basis of the active site of lanosterol 14α-demethylase from *Candida albicans* (CACYP51), a series of new azoles were designed and synthesized. All the new azoles show excellent in vitro activity against most of the tested pathogenic fungi, which represent a class of promising leads for the development of novel antifungal agents. The MIC_{80} value of compounds **8c**, **8i** and **8n** against *C. albicans* is 0.001 μg/mL, indicating that these compounds are more potent than fluconazole, itraconazole and voriconazole. Flexible molecular docking was used to analyze the structure–activity relationships (SARs) of the compounds. The designed compounds interact with CACYP51 through hydrophobic, *van der Waals* and hydrogen-bonding interactions.

The incidence of systemic fungal infections has been increasing dramatically due to an increase in the number of immunocompromised hosts, such as patients undergoing anticancer chemotherapy or organ transplants and patients with AIDS.[1] In contrast to the large number of antibacterial antibiotics, there are very few antifungal agents that can be used for life-threatening fungal infections. These drugs are amphotericin B,[2] 5-fluorocytosine, azoles (such as fluconazole and itraconazole),[3] and echinocandins (such as caspofungin and micafungin).[4] Because of their high therapeutic index, azoles are first-line drugs for the treatment of invasive fungal infections. Unfortunately, the broad use of azoles has led to development of severe resistance,[5,6] which significantly reduced the efficacy of them. This situation has led to an ongoing search for new azoles. Several novel triazole antifungal agents (Fig. 1), such as voriconazole,[7] posaconazole,[8] ravuconazole[9] and albaconazole,[10] are marketed or currently in the late stages of clinical trials.

Azole antifungals act by competitive inhibition of the lanosterol 14α-demethylase (CYP51),[11] which is the key enzyme in sterol biosynthesis of fungi. Selective inhibition of CYP51 would cause depletion of ergosterol and accumulation of lanosterol and other 14-methyl sterols resulting in the growth inhibition of fungal cells.[12] Because eukaryotic CYP51s are membrane associated proteins, solving their crystal structures remains a challenge. In our continual interest in rational antifungal drug design, three-dimensional (3D) models of CYP51 from *Candida albicans* (CACYP51) and *Aspergillus fumigatus* (AFCYP51) have been constructed through homology modeling.[13,14] The mechanism of azoles binding with CACYP51 has been investigated by flexible molecular docking[13,15] and site-directed mutagenesis.[16] On the basis of the results from molecular modeling, highly potent new azoles[15] and novel non-azole CACYP51 inhibitors[17] have been designed and synthesized, which demonstrates that the utilization of structural information of fungal CYP51s can accelerate the discovery of novel antifungal agents. In this Letter, structure-based rational drug design was successfully used to the discovery a series of new azoles with excellent in vitro antifungal activity and broad antifungal spectrum.

The chemical synthesis of compounds **8a–p** and **9a–c** is outlined in Schemes 1 and 2, respectively. As a key intermediate of our designed compounds, the oxirane compound **4** was synthesized by our reported procedure.[15] The *N*-methyl-substituted phenoxybutan-1-amine side chains **7a–p** were synthesized via two steps. Excess 1,4-dibromobutane were treated with various substituted phenols to give 1-(4-bromobutoxy)-substituted benzenes **6a–p**. Compounds **6a–p** were subsequently reacted with methylamine in EtOH at room temperature to afford side chains **7a–p.** The target compounds **8a–p** were obtained by treating epoxide **4** with side chains **7a–p** in the presence of triethylamine and EtOH at 80 °C with moderate to high yields. Compounds **9a**, **9b**

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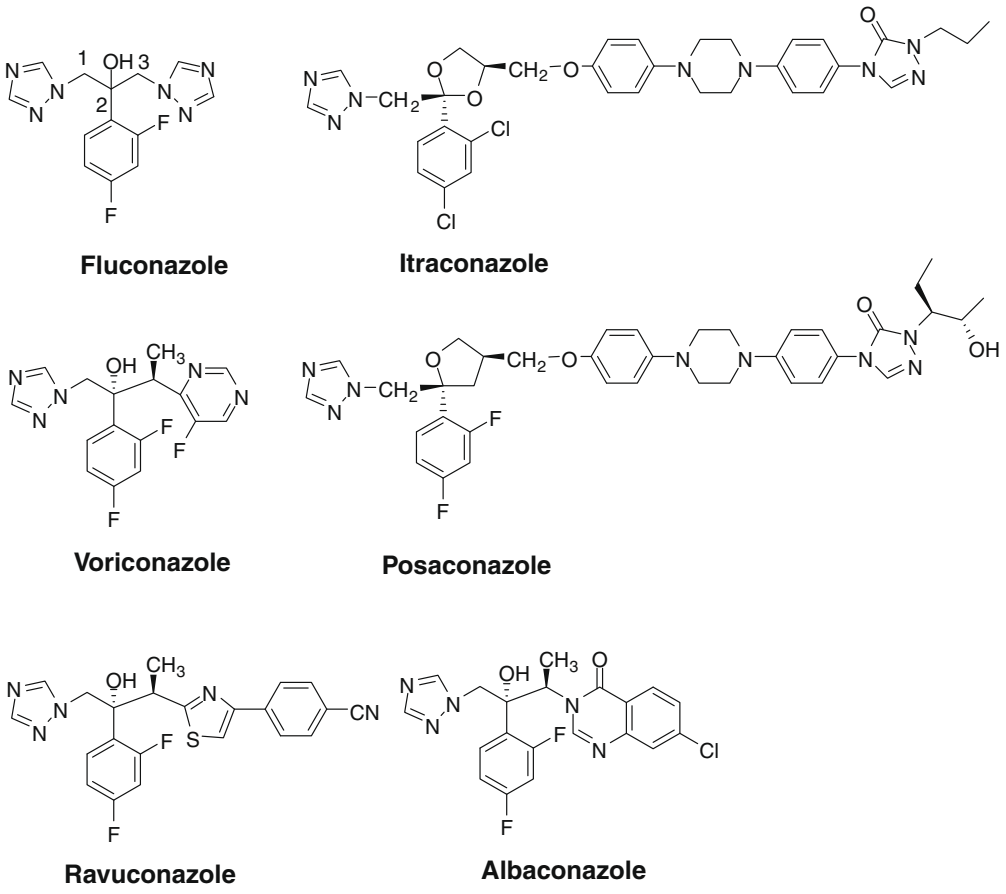
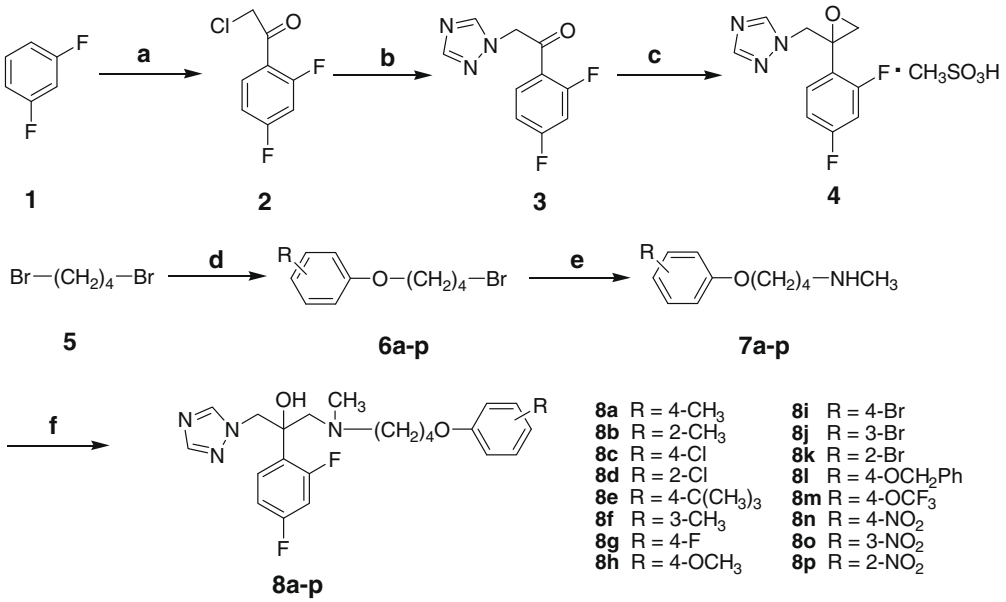


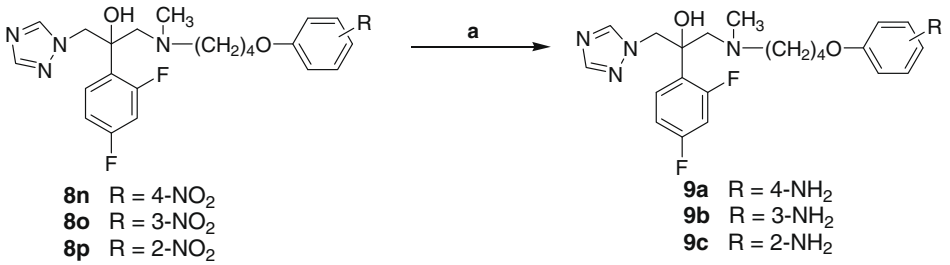
Figure 1. Chemical structures of azole antifungal agents.



Scheme 1. Synthesis of the target compounds **8a**–**p**. Reagents and conditions: (a) $ClCH_2COCl$, $AlCl_3$, CH_2Cl_2, 40 °C, 3 h, 50%; (b) triazole, K_2CO_3, CH_2Cl_2, rt, 24 h, 70.0%; (c) $(CH_3)_3SOI$, NaOH, toluene, 60 °C, 3 h, 62.3%; (d) phenol, K_2CO_3, DMF, 70 °C, 2 h, 91.8–92.3%; (e) methylamine alcohol solution, rt, 12 h, 97–98%; (f) **4**, Et_3N, EtOH, reflux, 9 h, 51.2–60.3%.

and **9c** were synthesized by the reduction of the nitro groups of compounds **8n**, **8o** and **8p** in the presence of Raney Ni and hydrazine hydrate (Scheme 2). All the target compounds were obtained as racemates.

In our previous studies, the active site of CACYP51 has been well characterized by multiple copy simultaneous search (MCSS).[17] Moreover, the binding mode of azole antifungal agents with CACYP51 can provide useful information for the rational design of



Scheme 2. Synthesis of the target compounds **9a**–**c**. Reagents and conditions: (a) Raney Ni, NH_2NH_2, EtOH, rt, 5 h, 94.5–96.4%.

new antifungal azoles. As discussed earlier,[17] the active site of CACYP51 can be divided into four pockets. The S1 pocket represents the hydrophilic hydrogen bond binding site, which is defined by Gln309, His310, and Ser312. Our docking studies indicate that the C2 hydroxyl group of azole antifungal agents interacts with the S1 pocket, possibly through the hydrogen-bonding interaction with His310 mediated by crystal waters.[13,15] The S2 pocket is above the heme ring representing the core hydrophobic area. The triazole ring of azole antifungal agents binds to the S2 pocket through the formation of a coordination bond with the iron atom of the heme group. The S3 pocket represents a narrow and hydrophobic cleft (facing BC loop). The difluorophenyl or dichlorophenyl group of the azole antifungal agents is located in the S3 pocket and forms hydrophobic interaction with Ala114, Phe126, Leu139, Ile304 and Met306. Because the triazole ring, diflurophenyl group and C2 hydroxyl group are regarded as essential pharmacophoric elements of azole antifungal agents,[13] they were retained in the present study. The difference between azole antifungal agents mainly lies in the side chains attached to C3 (Fig. 1), and most of the recent efforts aim to optimize them.[15,18–20] The C3 side chains of the antifungal azoles are found to be located in the S4 pocket, which represents a hydrophobic hydrogen bond binding site (facing FG loop). The fundamental purpose of the present drug design is to find a new type of side chain, which can be well accommodated in the S4 pocket. On the basis of the properties of the S4 pocket, a satisfied side chain should meet the following criteria. (1) Considering the hydrophobic nature of the S4 pocket, a hydrophobic side chain is necessary; (2) The side chain should have suitable length. Although a long side chain can form stronger hydrophobic and *van der Waals* interactions with CACYP51, it could also result in poor water solubility and low bioavailability. Therefore, a balance of affinity and drug-like properties should be achieved; (3) A hydrogen bond donor or acceptor should be introduced into the side chain, because an additional hydrogen-bonding interaction will further improve the affinity and specificity of the inhibitors. Ser378 is a well validated residue for the hydrogen-bonding interactions.[15,17]

On the basis of the above hypothesis, compounds **8a–p** and **9a–c** were designed. The binding mode of the compounds was validated by flexible molecular docking (Affinity module within InsightII 2000 software package[21]). Figure 2 shows the docking conformation of compounds **8i** and **8n** in the active site of CACYP51. The C3 side chain of the compounds was oriented into the S4 pocket with an extended conformation. The *N*-methyl group formed hydrophobic and *van der Waals* interactions with Phe228. The butyl group mimics the alkyl chain of the natural substrate and interacted with the surrounding hydrophobic residues lined with Val509 and Val510. Hydrogen-bonding interaction between the oxygen atom of the side chain and Ser378 was observed. The substituted terminal phenyl group interacted with surrounding hydrophobic residues such as Leu376, Phe380, Leu461, Val404 and Met508. Moreover, the nitro group of compound **8n** formed

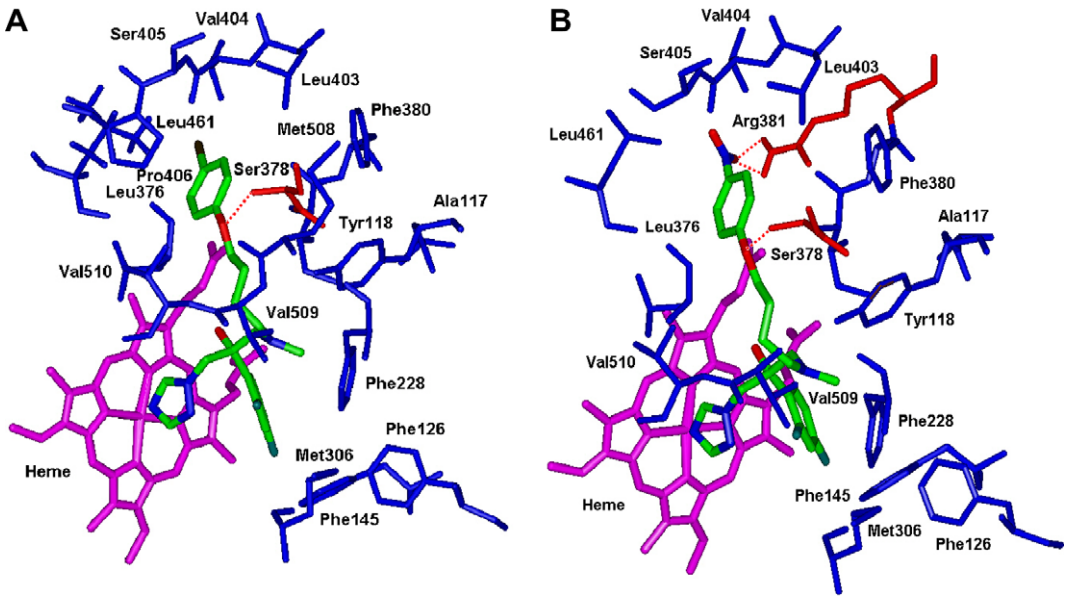


Figure 2. The docking conformation of compound **8i** (A) **and 8n** (B) in the active site of CACYP51. Important residues interacting with the compounds are shown and hydrogen bonds are displayed through red dotted lines. The images were generated using InsightII 2000 software package.

additional two hydrogen bonds with Arg381 (Fig. 2b). Compared to fluconazole, the designed compounds bound to CACYP51 with a lower value of interaction energy (Table 1).

In vitro antifungal activity of the synthesized compounds is listed in Table 2. In general, all the target compounds show excellent activity against most the tested fungal pathogens. The target compounds reveal the highest activity against *C. albicans* and *C. tropicalis*. Most of the compounds are more potent than fluconazole, itraconazole and voriconazole with their minimal inhibitory concentrations (MIC_{80}) values in the range of 0.0156–0.001 μg/mL. *Cryptococcus neoformans* has a worldwide distribution and is the most common cause of life-threatening fungal infections. All the target compounds show higher inhibitory activity against *C. neoformans* than fluconazole and itraconazole with their MIC_{80} values in the range of 0.25–0.004 μg/mL. Especially, the inhibitory activity of compounds **8i** and **8k** is fourfold higher than that of voriconazole. Fluconazole is not effective against *A. fumigatus*, while some of our compounds show moderate activity. For example, the MIC_{80} values of compounds **8c and 8i** against *A. fumigatus* are 4 μg/mL. However, they are less active than itraconazole and voriconazole. For the dermatophytes (i.e., *Trichophyton rubrum* and *Microsporum gypseum*), most of the compounds show higher activity than fluconazole and itraconazole with their MIC_{80} values in the range of 0.0625–0.0156 μg/mL. Compounds **8c**, **8i**, and **8n** exhibited strong in vitro antifungal activity with broad antifungal spectrum, which were worthy of further evaluation.

Table 1
Calculated interaction energies (kcal/mol) for the complexes of representative compounds with the active site of CACYP51

Compounds	E_{vdw}	E_{elect}	E_{total}
8a	−62.2	−3.4	−65.6
8b	−60.1	−1.2	−61.3
8c	−64.9	−5.5	−70.4
8i	−65.5	−5.2	−70.7
8n	−61.9	−9.6	−71.5
8o	−62.4	−2.1	−63.5
9a	−60.4	−2.3	−62.7
Fluconazole	−54.8	−3.2	−58.0

Table 2
In vitro antifungal activity of the target compounds (MIC_{80}, μg mL^{-1})[a]

Compd	*C. alb.*	*C. neo.*	*C. tro.*	*T. rub.*	*A. fum.*	*M. gyp.*
8a	0.0156	0.0625	0.004	0.0156	16	0.0625
8b	0.0625	0.0156	0.0156	0.0625	>64	0.0625
8c	0.001	0.0156	0.001	0.0625	4	0.0156
8d	0.0156	0.0156	0.0156	0.0625	>64	0.0625
8e	0.0625	0.25	0.0625	0.0625	>64	0.0625
8f	0.0156	0.0625	0.0156	0.0625	64	0.0625
8g	0.0156	0.0625	0.004	0.0156	16	0.0625
8h	0.0156	0.0625	0.004	0.0156	16	0.0625
8i	0.001	0.004	0.001	0.0156	4	0.0156
8j	0.0625	0.0625	0.001	0.0625	64	0.0156
8k	0.0156	0.004	0.0156	0.0625	>64	0.0625
8l	0.0156	0.0156	0.0156	0.0625	32	0.0156
8m	0.0156	0.0625	0.004	0.0156	>64	0.0625
8n	0.001	0.0156	0.004	0.0625	64	0.0156
8o	0.0156	0.0625	0.004	0.0625	64	0.0625
8p	0.0625	0.25	0.0625	0.0625	>64	0.0625
9a	0.0625	0.25	0.0156	1	>64	0.25
9b	0.0156	0.25	0.0156	0.25	>64	0.0625
9c	0.0625	1	0.0156	1	>64	0.25
FLZ	0.25	1	1	0.25	>64	1
ITZ	0.125	0.5	0.0156	0.125	2	0.125
VOR	0.0156	0.0156	0.001	0.0156	0.25	0.0156

[a] Abbreviations: *C. alb.*, *Candida albicans*; *C. neo.*, *Cryptococcus neoformans*; *C. tro.*, *Candida tropicalis*; *T. rub.*, *Trichophyton rubrum*; *A. fum.*, *Aspergillus fumigatus*; *M. gyp.*, *Microsporum gypseum*; FLZ: Fluconazole; ITZ: Itraconazole; VOR: Voriconazole.

The in vitro antifungal activity assay indicates that most of the 4-substituted and 3-substituted derivatives show higher antifungal activity than 2-substituted derivatives. The docking model shows that the active site of CACYP51 at position 2 of the bound compound is not large enough to accommodate an additional group. However, position 3 and 4 of the phenol group contains an extra small pocket defined by Leu461, Val404, Met508 and Leu403. Among the 4-substituted derivatives, their SARs are not so obvious. In general, nitro group, halogen and small alkyl substituted compounds (e.g., compounds **8a**, **8c**, **8h**, **8i and 8n**) are favorable for the antifungal activity. Compound **8n** has excellent antifungal activity because the 4-nitro group can form two hydrogen bonds with Arg381 (Fig. 2). The hydrogen bonds will be broken if the 4-nitro group of compound **8n** is moved to the position **3** and **2** (compounds **8o** and **8p**), or reduced to the amino group (compound **9a**), which results in the decrease of the antifungal activity by 16–64-fold. On the other hand, compounds **8c** and **8i** cannot form hydrogen-bonding interaction with Arg381, but they have the same activity as compound **8n**. From the docking results, compounds **8c** and **8i** have similar interaction energies with CACYP51 (Table 1) as compound **8n,** because the halogen substitutions (Cl or Br) at position 4 can form additional hydrophobic interaction with surrounding Val404, Met508 and Leu403. If this position is substituted by a less hydrophobic fluorine (compound **8g**), the antifungal activity is decreased to some extent.

In summary, computer modeling was successfully used to the rational design of novel antifungal azoles. In vitro antifungal activity assay indicates that the new azoles show excellent activity against both systemic pathogenic fungi and dermatophytes. Most of the compounds show higher activity than fluconazole and itraconaozle with MIC_{80} values in the range of 0.0156–0.001 μg/mL. In particular, the most active compounds **8c**, **8i** and **8n** show broad antifungal spectrum and are more potent than fluconazole, itraconazole and voriconazole, which are promising leads for the development of novel antifungal agents. Moreover, the therapeutic side effects of azole antifungal agents are partly due to the interactions of azoles with human CYP51. Ser378 is conserved across the fungal CYP51 enzymes and the corresponding residue in human is Ile379. Because the synthesized compounds can form hydrogen-bonding interaction with Ser378, they may show better selectivity toward fungal CYP51 than marketed azole antifungal agents. Further pharmacological and toxicological evaluation of these highly potent compounds is in progress.

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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.2009.07.144.

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