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Review Article

## Candida albicans Lanosterol 14 $\alpha$ -demethylase: Structural insights into its molecular mechanisms and mode of inhibition by azole antifungals

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### ABSTRACT

During the past three decades azole compounds have been developed as medical and agricultural agents to combat fungal diseases. During the 1980s they were introduced as orally active compounds in medicine and the number of such azole drugs is likely to expand in the near future. They act by inhibiting cytochrome P450 14 $\alpha$ -demethylase (CYP51), a key enzyme in the fungal ergosterol biosynthesis. Azole antifungals now represent a successful strategy for antifungal development. A universal step in the biosynthesis of membrane sterols and steroid hormones is the oxidative removal of the 14 $\alpha$ -methyl group from sterol precursors by sterol 14 $\alpha$ -demethylase (CYP51). This enzyme is a primary target in treatment of fungal infections in organisms ranging from humans to plants, and development of more potent and selective CYP51 inhibitors (azoles) is an important biological objective. However, their clinical value has been limited by their relatively high risk of toxicity, the emergence of drug resistance, pharmacokinetic deficiencies, and/or insufficiencies in their antifungal activities. Despite recent developments, there is still need for genuinely broad-spectrum and low-toxicity azole antifungal agents. In this review molecular basis of how azoles interact with their target enzyme is discussed. Indeed, the understanding of the molecular basis of such interaction will help in the design of more active CYP51 inhibitors.

**Keywords:** Azole antifungals; Lanosterol 14 $\alpha$ -demethylase; CYP51; Ergosterol biosynthesis.

### INTRODUCTION

During the past two decades the frequencies and types of life-threatening fungal infections have increased dramatically in immunocompromised patients (Anaissie, 1992; Pfaller, 1992; Richardson, 1991). Several factors have contributed to this rise: the expansion of severely ill and/or immunocompromised patient populations with human immunodeficiency virus (HIV) infection, with chemotherapy induced neutropenia, and receiving organ transplant-associated immunosuppressive therapy; more invasive medical procedures, such as extensive surgery and the use of prosthetic devices and vascular catheters; treatment with broad-spectrum antibiotics or glucocorticosteroids; parenteral nutrition; and peritoneal dialysis or hemodialysis (Nafsika H, 1996).

*Candida albicans* and *Aspergillus* spp. account for most of the invasive infections in the neutropenic cancer patient. However, non-*Candida albicans* spp. and previously uncommon opportunistic fungal pathogens

such as *Fusarium* spp., zygomycetes, and dematiaceous molds are observed with increasing frequency in this population.

For many years, the treatment of most invasive fungal infections was essentially limited to amphotericin B (AmB) with or without 5-flucytosine (5-FC). As the use of AmB was associated with infusion-related side effects and dose-limiting nephrotoxicity, the continuous search for newer, less toxic antifungals led to the discovery of the azoles. The therapeutic options did not expand until the late 1980s, when fluconazole and itraconazole were introduced. Triggered by the increasing number of neutropenic patients and patients with AIDS, the past decade, however, has seen a major expansion in antifungal drug research. These efforts have resulted in the discovery or design of several new antifungal compounds that are currently at various stages of clinical investigation (Walsh TJ, 1996).

The azole derivatives, discovered in the late 1960s, are totally synthetic and are the most rapidly expanding group of antifungal compounds (Fromtling R, 1988; Vanden Bossche H, 1985) (Fig. 1a&1b). They are classified as imidazoles or triazoles on the basis of whether they have two or three nitrogens in the five-membered azole ring. The azole antifungals which are currently at various stages of clinical trials are displayed in Fig.3. Depending on the particular compound, azole antifun-

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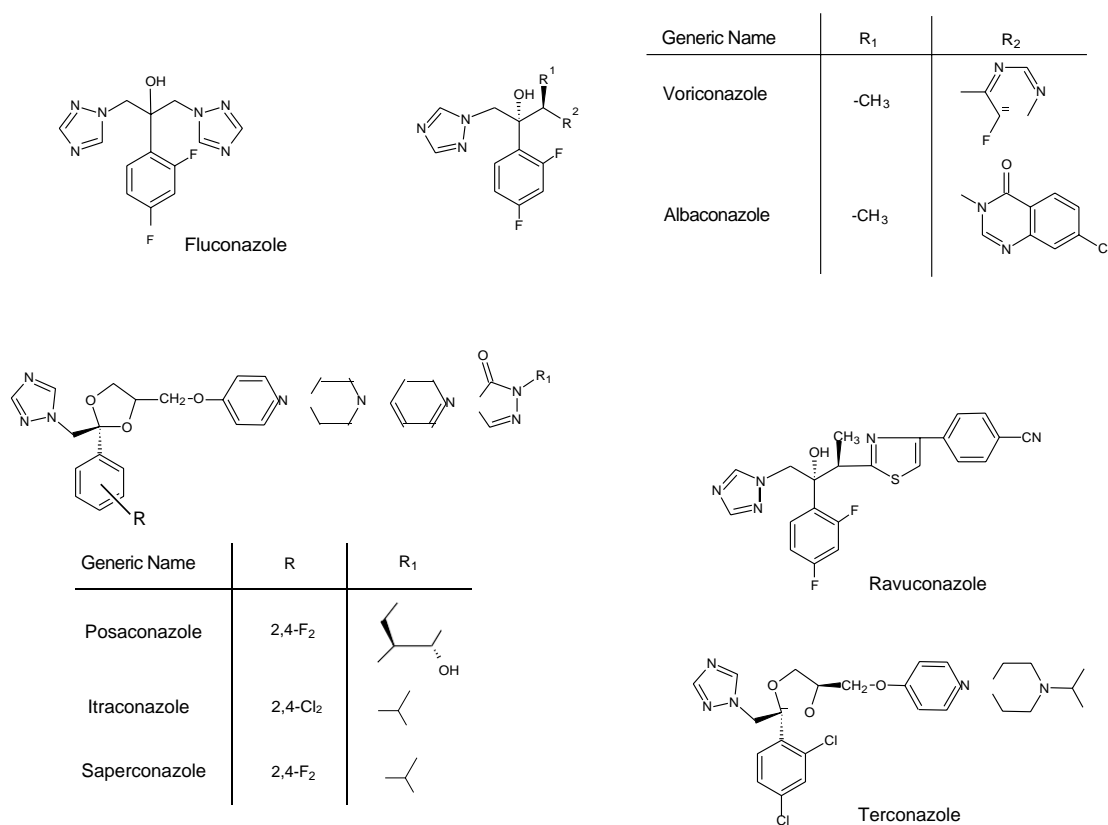


Figure 1: (a) Triazole based antifungals

gal agents have fungistatic, broad-spectrum activity that includes most yeasts and filamentous fungi.

#### AZOLE ANTIFUNGALS IN CLINICAL USE

The azole antifungals are synthetic drugs used in the treatment of various mycoses (see Table.1). The azole antifungal agents in clinical use contain either two or three nitrogens in the azole ring and are thereby classified as imidazoles (e.g., ketoconazole and miconazole, clotrimazole) or triazoles (e.g., itraconazole and fluconazole), respectively. Representative structures of azole derivatives are shown in Fig. 1a&1b, and pharmacokinetic properties are compared in Table 2. With the exception of ketoconazole, use of the imidazoles is limited to the treatment of superficial mycoses, whereas the triazoles have a broad range of applications in the treatment of both superficial and systemic fungal infections. Furthermore the azoles are active against wide range of fungi (see Table 3) (George M Brenner, 2008). Another advantage of the triazoles is their greater affinity for fungal rather than mammalian cytochrome P-450 enzymes, which contributes to an improved safety profile.

#### AZOLE ANTIFUNGALS IN AGRICULTURE

Every year fungal infections of crops cause hundreds of millions of dollars worth damage to a wide variety of food and other crops. Prior to the development of effective agricultural antifungals crop diseases were the causes of several major famines. The infamous Irish Potato Famine of the 19<sup>th</sup> century was caused by the pathogenic fungus *Phytophthora infestans*.

Tens of thousands of people starved to death and thousands more emigrated to the united states to escape the famine. Today both imidazole and triazole antifungal agents such as imazalil and propiconazole are among the most effective crop protection agents known. There are over 20 azole antifungals used in crop protection and a representative sample is shown (Fig.2). The mechanism of action of agricultural antifungal is identical to that of the agents used for mammalian infections. Infact, they bear a remarkable resemblance to antifungal drugs employed in treating human disease.

#### LANOSTEROL 14 $\alpha$ - DEMETHYLASE: MOLECULAR TARGET FOR AZOLE ANTIFUNGALS

Sterols are essential lipid components of eukaryotes and have been shown to be responsible for a number of important physical characteristics of membranes. Sterol biosynthesis is an essential eukaryotic metabolic pathway in animals (cholesterol biosynthesis), fungi (ergosterol biosynthesis) and plants (biosynthesis of sitosterol and an array of phytosterols) (David Lamb, 1999). A universal step in the biosynthesis of membrane sterols and steroid hormones is the oxidative removal of the 14  $\alpha$ -methyl group from sterol precursors by sterol 14 $\alpha$ -demethylase (CYP51)(Fig.4). This enzyme is a primary target in treatment of fungal infections in organisms ranging from humans to plants, and development of more potent and selective CYP51 inhibitors is an important biological objective (Ali Nasser Eddine, 2008).

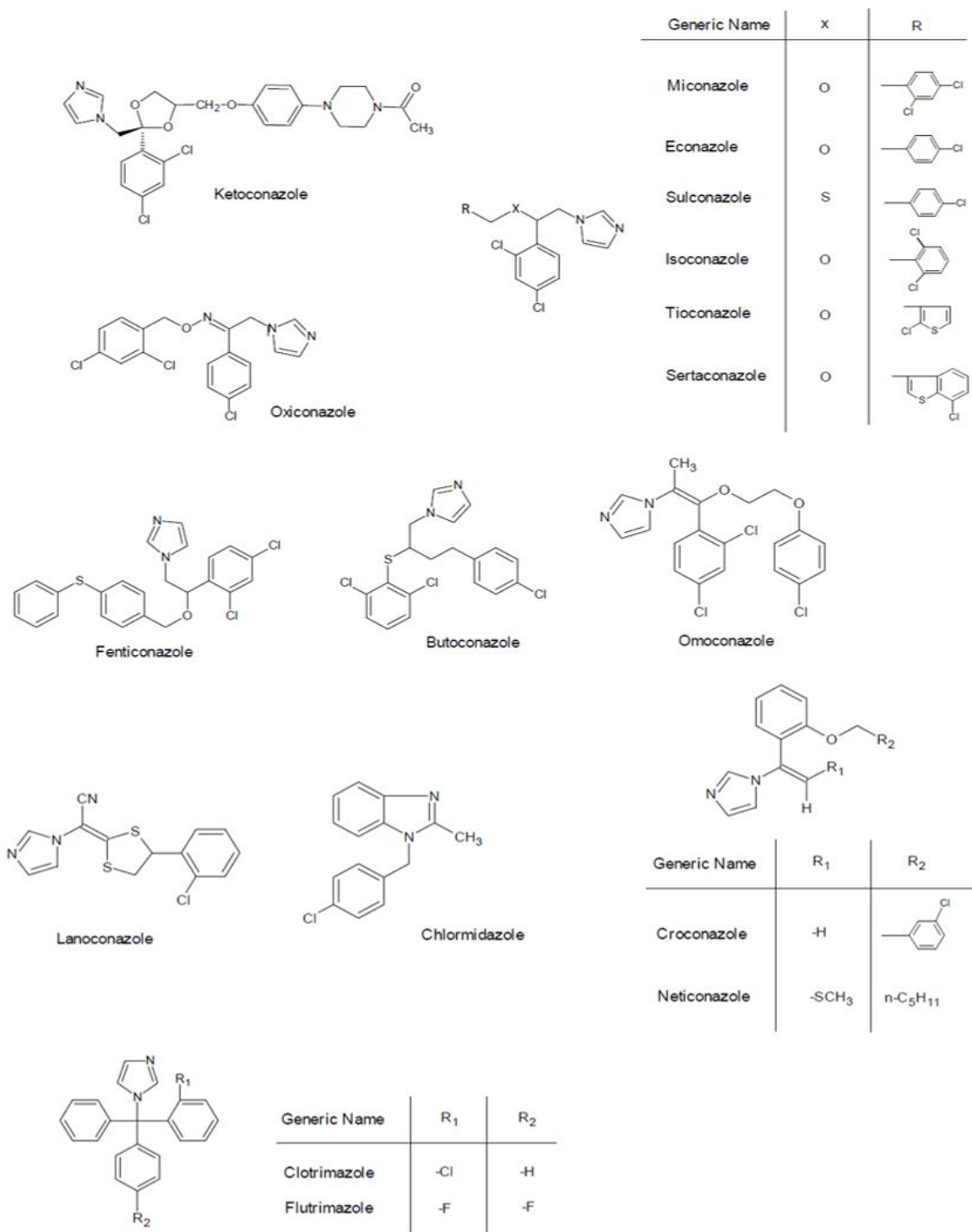


Figure 1: (b) Imidazole based antifungals

Lanosterol 14 $\alpha$ -demethylase (P450<sub>14DM</sub>, CYP51) is a member of the cytochrome P450 (P450, CYP) superfamily, which is a heme thiolate containing enzyme involved in biosynthesis of membrane sterols, including cholesterol in animals, ergosterol in fungi, and a variety of C24-modified sterols in plants and protozoa in most organism in biological kingdoms from bacteria to animals.

They catalyze the removal of the 14-methyl group (C-32) of lanosterol via three successive monooxygenation reactions (Fig.4). The first two of these reactions are conventional cytochrome P450 hydroxylations that produce the 14-hydroxymethyl and 14-carboxyaldehyde derivatives of lanosterol. In the final step, the 14-aldehyde group is eliminated as formic acid with concomitant introduction of a  $\Delta^{14,15}$  double bond. P450<sub>14DM</sub> occurs in different kingdoms, such as

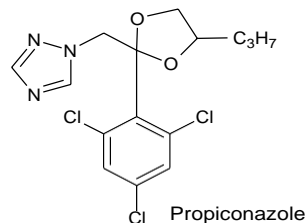
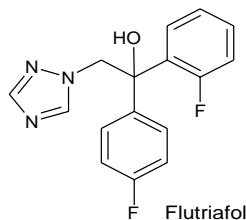
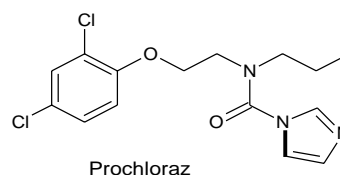
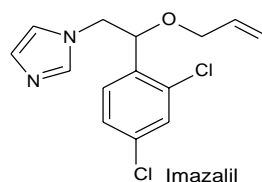


Figure 2: Azole antifungals in agriculture

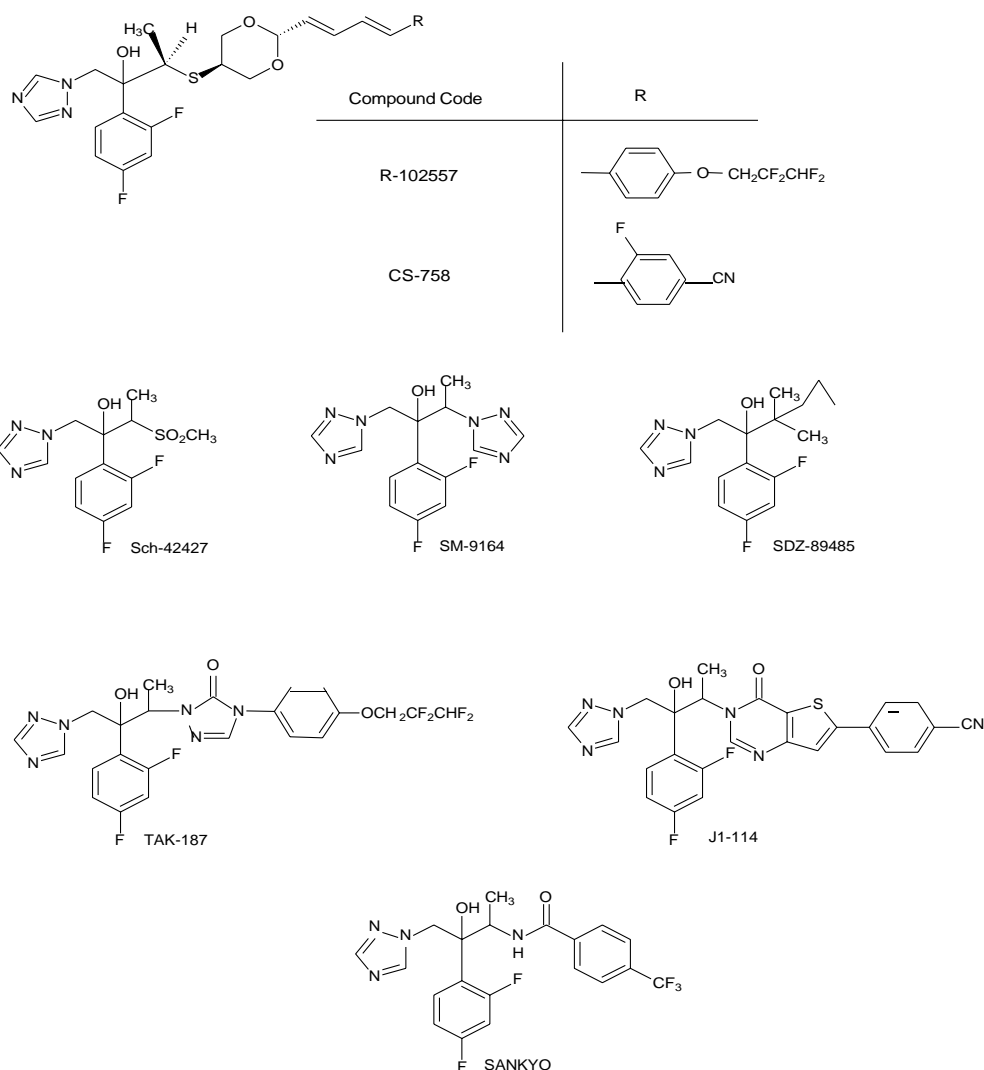


Figure 3: Azole antifungals under clinical investigation

fungi, higher plants, and animals, with the same metabolic role, i.e., removal of the 14-methyl group of sterol precursors such as lanosterol, obtusifoliol, dihydro-lanosterol, and 24(28)-methylene-24,25-dihydrolanosterol, and this is the only known P450 distributed widely in eukaryotes with essentially the same metabolic role (Haitao Ji, 2000).

#### COMPARISON OF LANOSTEROL 14 $\alpha$ -DEMETHYLASE (CYP51) OF FUNGAL, PLANT, AND ANIMAL FOR INHIBITION BY DIFFERENT AZOLES ANTIFUNGALS

Sterol 14-demethylation is included in all sterol biosynthetic pathways of eukaryotes, which had been known as the P450-dependent reactions (Yoshida Y, 1994). Since the first finding of P450 responsible to this func-

**Table 1: Clinical uses of some commonly used azole antifungal agents**

Drug	Systemic and subcutaneous mycoses	Dermatophyte Infections	Superficial candida infection
Clotrimazole	NO	YES	YES
Econazole	NO	YES	YES
Fluconazole	YES	NO	YES
Itraconazole	YES	YES	YES
Ketoconazole	NO	YES	YES
Voriconazole	YES	NO	NO

**Table 2: Pharmacokinetic properties of azole antifungals**

Drug	Route of administration	Oral bioavailability	Elimination half-life	Routes of elimination
Clotrimazole	Topical	NA	NA	NA
Econazole	Topical	NA	NA	NA
Fluconazole	Oral/iv	95%	35 hours	Renal excretion
Itraconazole	Oral	55%	60hours	Biliary, fecal, and renal excretion
Ketoconazole	Oral/topical	Highly Variable	8 hours	Biliary, fecal excretion
Voriconazole	Oral/iv	96%	Dose dependent	Metabolism; renal excretion

tion as Lanosterol 14 $\alpha$ -demethylase (P450<sub>14DM</sub>, later named as CYP51) of yeast by Yoshida and his associates in the late 1970s (Aoyama Y, 1978; Yoshida Y, 1984; Aoyama Y, 1984), numbers of investigations have been done on this P450 species. In 1994, it is been reported that sterol 14-demethylase P450 of rat shows high amino acid sequence identity with yeast P450<sub>14DM</sub> (CYP51) and suggested that yeast and rat P450<sub>14DM</sub> (CYP51) are orthologous enzymes conserved throughout eukaryotic evolution. Furthermore, a gene (AC number; D55681) encoding a protein showing the highest amino acid sequence homology to CYP51 family was found in the genome of *M. tuberculosis*. The product of this gene expressed in *E. coli* showed the characteristics of CYP51 (Aoyama Y, 1998; Bellamine A, 1999). This finding showed the occurrence of CYP51 family in prokaryotic genome, and molecular phylogenetic analysis revealed that CYP51 appeared in the prokaryotic era and then distributed into all biological kingdoms through the divergence of life (Yoshida Y, 1997).

Amino acid sequence alignment of all known CYP51s indicates that the overall amino acid sequence identity between CYP51s of evolutionary distant species is about 30% (see table. 4) (Yuri Aoyama, 2005). The structural examinations with X-ray crystallographic analysis of CYP2C5 (Williams P A, 2005) and CYP2C9 (Williams P A, 2003) and site directed mutagenesis revealed that the putative SRSs (substrate recognition sites) are actually located at the active-site responsible for the interaction with substrates.

It can thus be concluded that CYP2 family has been diversified through alterations of the SRSs for adapting to metabolize wide variety of organic compounds. In contrast, CYP51 might have suppressed structural alte-

rations of SRSs to conserve its essential role in sterol biogenesis by eukaryotes. However, it is noteworthy that substrates undergoing 14- $\alpha$  demethylation in fungal, plant, and animal sterol biosynthetic pathways are different (Fig. 5). This fact suggests a possibility that a slight difference may exist among substrate specificities of fungal, plant, and animal CYP51s, although amino acid sequences of their SRSs are highly conserved (Yuri Aoyama, 2005).

In animals and humans, a downstream product of Lanosterol 14 $\alpha$ -demethylation is cholesterol, which is necessary for the synthesis of bile acids, mineralocorticoids, glucocorticoids and sex steroids. While cholesterol can, in principle, be supplemented with food intake, inhibition of CYP51 results in a lack of FF-MAS and its following metabolite testis-meiosis activating steroid (T-MAS). These direct products of the CYP51 reaction act as meiosis-activating steroids on ovaries and testes (Byskov A G, 2002) so that inhibition of CYP51 in human may affect the endocrine system (Zarn J A, 2003). Eva R. Trosken et al., (Eva R Trosken, 2006) established an assay allowed for an optimal comparison of inhibitory potencies of azole compounds towards human and fungal CYP51. For a number of compounds. e.g. epoxiconazole, tebuconazole, bifonazole, clotrimazole, ketoconazole and miconazole, the difference between the inhibition of human CYP51 and fungal CYP51 is smaller than by a factor of 10. For imazalil, fluconazole and itraconazole, on the other hand, the difference between the inhibition of fungal and human enzyme is by more than a factor of 400, indicating high specificity for the fungal enzyme compared to the human analogue. This is a desirable feature for all antifungal azole compounds since production of FF-MAS and T-MAS in humans should not be inhibited.

**Table 3: Causes and management of selected systemic and subcutaneous mycoses**

Mycosis	Pathogens	Treatment
Aspergillosis	<i>Aspergillus fumigatus</i>	Voriconazole, caspofungin, or both; Amphotericin B
Blastomycosis	<i>Blastomyces Dermatitidis</i>	Itraconazole or Amphotericin B
Candidiasis	<i>Candida albicans</i> , <i>C. glabrata</i> , <i>C. parapsilosis</i> , <i>C. tropicalis</i> , <i>C. Krusei</i>	Fluconazole, voriconazole, Amphotericin B ± flucytosine, or caspofungin
Coccidioidomycosis	<i>Coccidioides immitis</i>	Itraconazole or fluconazole (mild to moderate disease); Amphotericin B (severe disease); fluconazole for meningitis
Cryptococcosis	<i>Cryptococcus neoformans</i>	Amphotericin B + flucytosine followed by fluconazole for meningitis; fluconazole or amphotericin B for nonmeningeal infections
Fusariosis	<i>Fusaria species</i>	Voriconazole
Histoplasmosis	<i>Histoplasma Capsulatum</i>	Itraconazole for moderate disease; amphotericin B for severe disease and meningitis
Mucormycosis	<i>Absidia</i> , <i>rhizopus</i> , and <i>Rhizomucor species</i>	Amphotericin B
Pseudallescheriasis	<i>Pseudallescheria boydii</i>	Voriconazole, itraconazole, or miconazole, with surgery
Sporotrichosis	<i>Sporothrix schenckii</i>	Itraconazole, fluconazole, or saturated potassium iodide solution

Examination was done on substrate specificities of rat and yeast CYP51s resulting in data that showed that 24,25-dihydrolanosterol and 24-methylene dihydrolanosterol were the specific substrates for rat and yeast CYP51s, respectively, although lanosterol was a good substrate for both of them (Aoyama Y, 1991). These substrate specificities of yeast and rat CYP51s correlate well with their intrinsic substrates in the sterol biosynthetic pathways (Fig.5). The sterol biosynthetic pathway of plants, whose natural substrate of CYP51 is obtusifoliol (Fig.5), is different from those of fungi and animals. CYP51s of maize, Sorghum bicolor, and wheat favorably metabolized obtusifoliol but showed no activity for lanosterol and 24,25- dihydrolanosterol (Taton M, 1991; Kahn R A, 1996; Cabello Hurtado, 1999). These facts clearly indicate that CYP51s of fungi, plants, and animals have different substrate specificities relating to the sterol precursors undergoing 14- demethylation in their sterol biosynthetic pathways.

#### MECHANISM OF ACTION OF AZOLE ANTIFUNGALS

Azole antifungal agents prevent the synthesis of ergosterol, a major component of fungal plasma membranes, by competitively inhibiting the cytochrome P450-dependent enzyme lanosterol 14 $\alpha$ -demethylase (P450<sub>14DM</sub>, CYP51), (Georgopapadakou N H, 1996; Ghannoum M A, 1997; Koltin Y, 1997; White T C, 1998)(Fig.6). This enzyme also plays an important role in cholesterol synthesis in mammals. When azoles are present in therapeutic concentrations, their antifungal efficacy is attributed to their greater affinity for fungal P450<sub>14DM</sub> than for the mammalian enzyme. Exposure of fungi to an azole causes depletion of ergosterol and accumulation of Lanosterol and other 14 $\alpha$ -methylated sterols. This interferes with the “bulk” functions of ergosterol in fungal membranes and disrupts both the

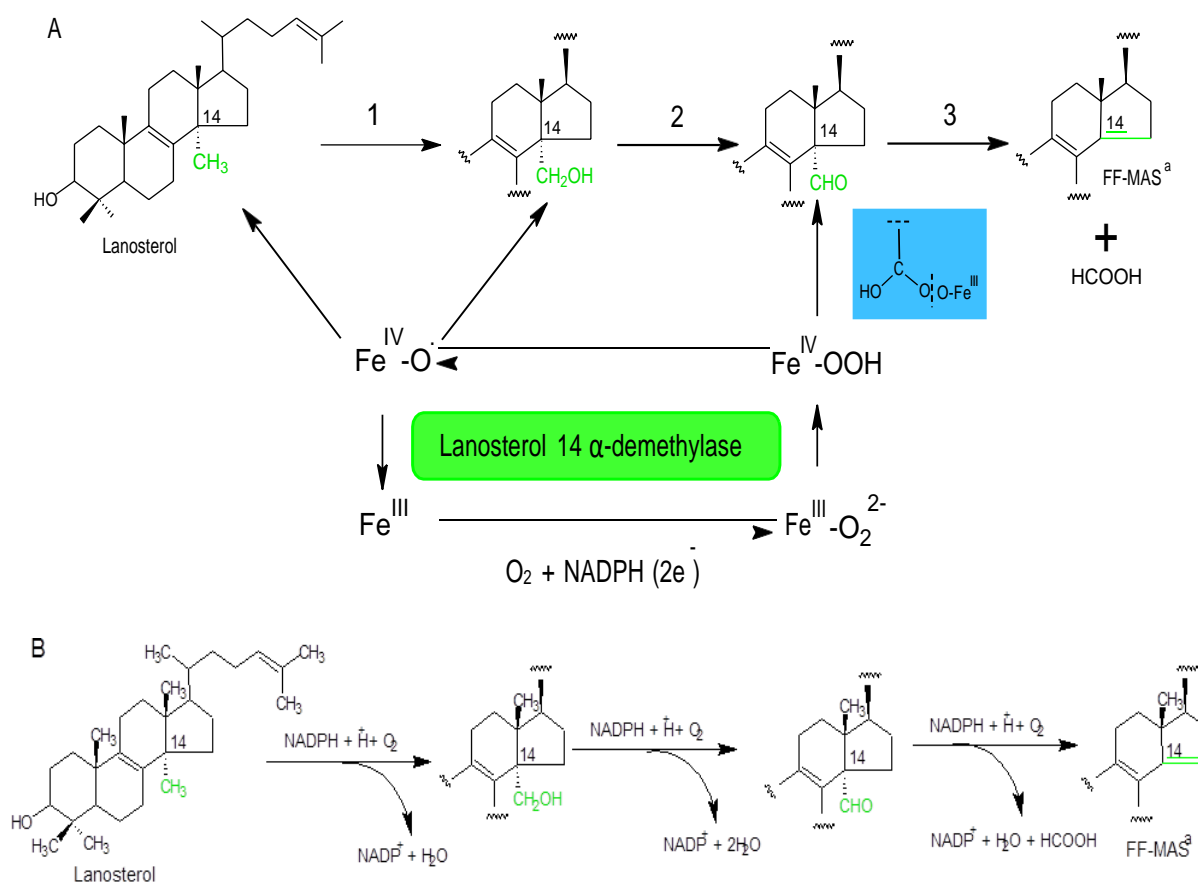
structure of the membrane and several of its functions such as nutrient transport and chitin synthesis. The net result is the growth inhibition of fungal cells. Ergosterol also has a hormone like (“sparking”) function in fungal cells, which stimulates growth and proliferation (Chunquan Sheng, 2006; Daniel J Sheehan, 1999). This function may be disrupted when ergosterol depletion is virtually complete (>99%).

The basic Nitrogen atom of the imidazoles (N<sub>3</sub>) and triazoles (N<sub>4</sub>) forms a bond with the heme iron of the P450<sub>14DM</sub> prosthetic group in the position normally occupied by the activated oxygen, (Fig.7). The remainder of the azole antifungal forms bonding interactions with the apoprotein in a manner which determines the relative selectivity of the drug for the fungal demethylase versus other CYP450 enzymes (David A Williams, 2007).

As explained earlier, since biosynthesis of the mammalian membrane sterol cholesterol also employs a CYP450 14  $\alpha$ -demethylase, why don't 14 $\alpha$ -methyl sterols accumulate in human cell membranes? The reason is the relative strength of inhibition of the same enzyme from different species. The IC<sub>50</sub> value for ketoconazole against the enzyme from *Candida albicans* is approximately 10<sup>-9</sup>M versus approximately 10<sup>-6</sup>M for the human enzyme. This three orders of magnitude difference in strength of inhibition provides the therapeutic index with respect to this particular enzyme (David A Williams, 2007).

#### Structure-Activity Relationship Of Azole Antifungals

Antifungal activity of azoles are mainly attributed to the coordination abilities of the nitrogen atom (N<sub>3</sub> of



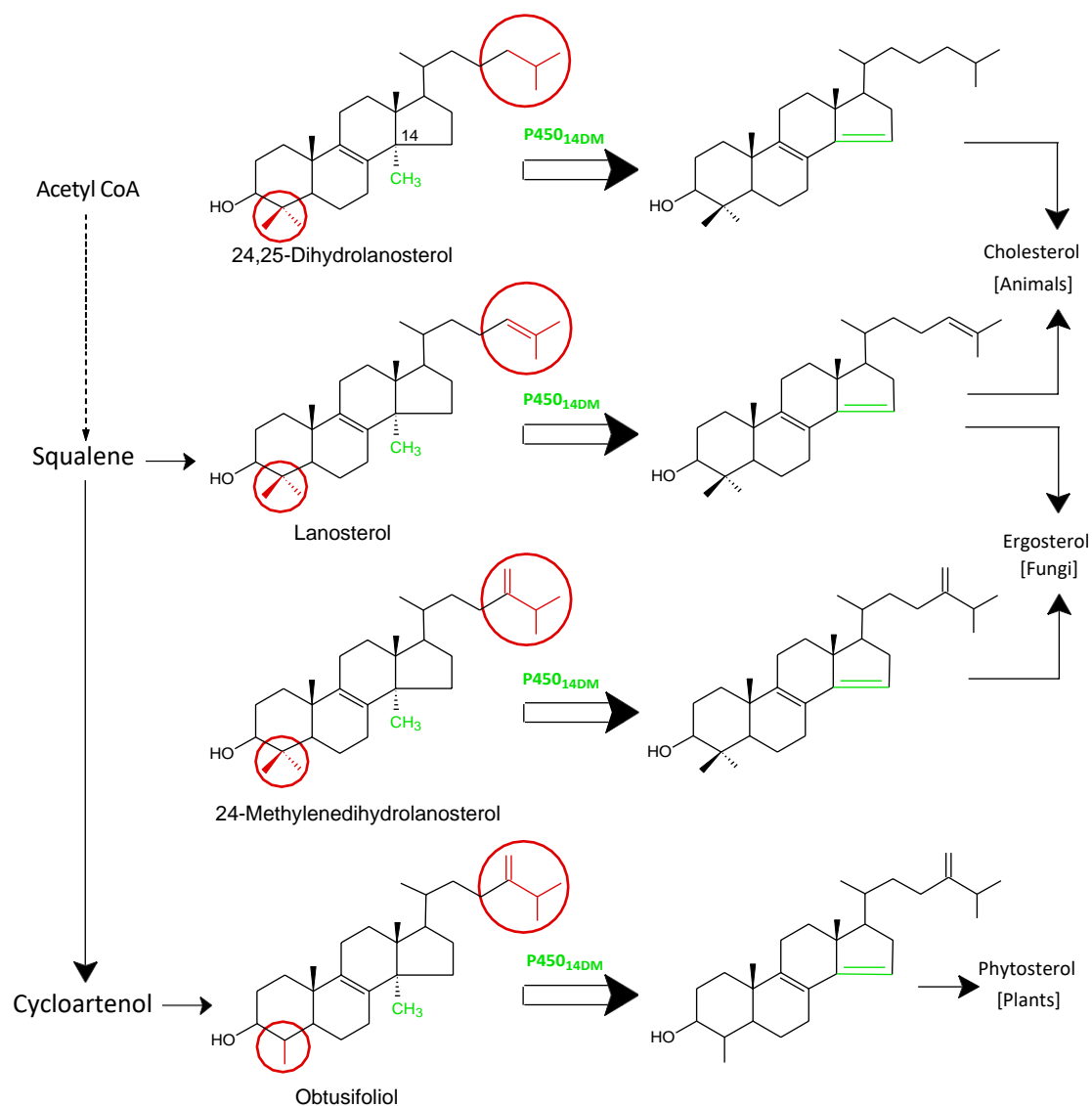
<sup>a</sup>Follicular fluid meiosis-activating sterol

**Figure 4: The sequence of reactions catalyzed by lanosterol 14 $\alpha$ -demethylase**

\*\*A+ Demethylation of the 14  $\alpha$ - methyl group from Lanosterol carried out by the cytochrome P450 enzyme sterol 14 $\alpha$ -demethylase (CYP51). The mechanism involves three successive heme-catalyzed insertions of activated oxygen into the three carbon-hydrogen bonds of the 14 $\alpha$ -methyl group. The first and the second steps are ordinary monooxygenation driven by single oxygen activated at the heme iron. However, the last step is driven by the attack of peroxide on the heme iron. C-C bond fission is achieved by cleavage of peroxide linkage of the intermediate as shown in the sky blue box. The group is finally eliminated as formic acid to create a double bond between carbons 14 and 15. [B] The role of NADPH in the three steps is been shown in molecular level. FF-MAS is then converted into T-MAS and T-MAS undergoes various enzymatic conversion and finally gives ergosterol.

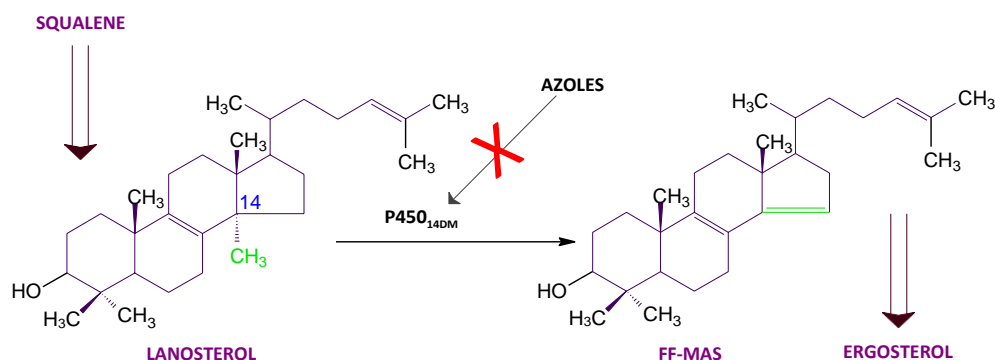
**Table 4: Amino acid sequence identity between given pair of CYP51s from various species**

Species	Human	Rat	<i>C. Albicans</i>	<i>S. Cerevisiae</i>	<i>U. Maydis</i>	<i>S. Pombe</i>	<i>D. Discoideum</i>	<i>S. Bicolor</i>	<i>A. Thaliana</i>	<i>M. Tuberculosis</i>
Human	100	93.4	38.0	36.6	38.4	39.7	34.5	35.4	37.7	33.3
Rat		100	38.4	35.7	39.3	39.9	34.3	36.0	38.3	32.0
<i>C. Albicans</i>			100	64.8	45.6	46.8	27.2	27.8	26.3	37.8
<i>S. Cerevisiae</i>				100	47.5	49.3	30.7	29.7	30.7	28.1
<i>U. Maydis</i>					100	32.3	29.2	28.5	34.4	26.9
<i>S. Pombe</i>						100	32.3	30.4	30.9	30.4
<i>D. Discoideum</i>							100	38.6	39.5	30.5
<i>S. Bicolor</i>								100	76.1	33.8
<i>A. Thaliana</i>									100	37.9
<i>M. Tuberculosis</i>										100



**Figure 5: Sterol biosynthetic pathways of eukaryotes**

\*The group shown in green color indicates the 14-methyl group removed by CYP51. The local structures characteristic for the natural substrates of animal, plant, and fungal 14-demethylation steps are indicated by red circle.

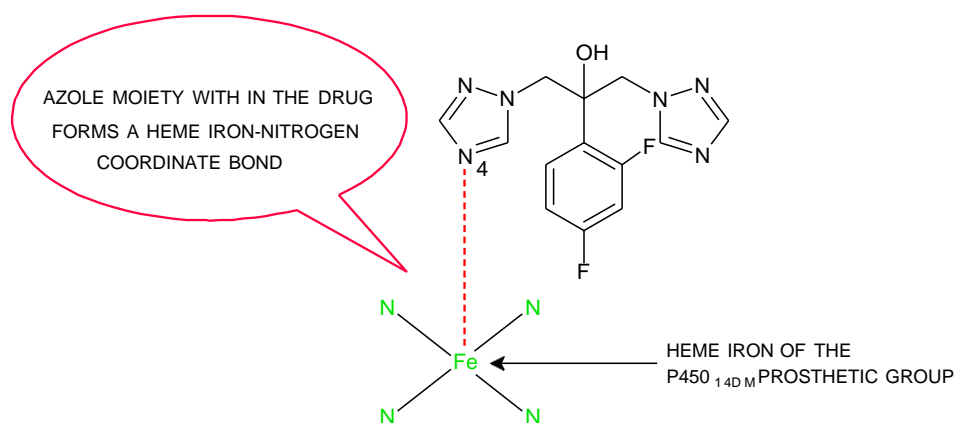


**Figure 6: Inhibition of fungal lanosterol 14 $\alpha$ -demethylase by azoles**

imidazole and N<sub>4</sub> of triazoles) of the azole ring to the iron atom of heme. The halogenated phenyl group of azole inhibitor is deep in the hydrophobic binding cleft within the heme environment of the enzyme. Additionally, the chirality at C2 and C3 atoms are important for the antifungal activity (35). The hydroxyl group at-

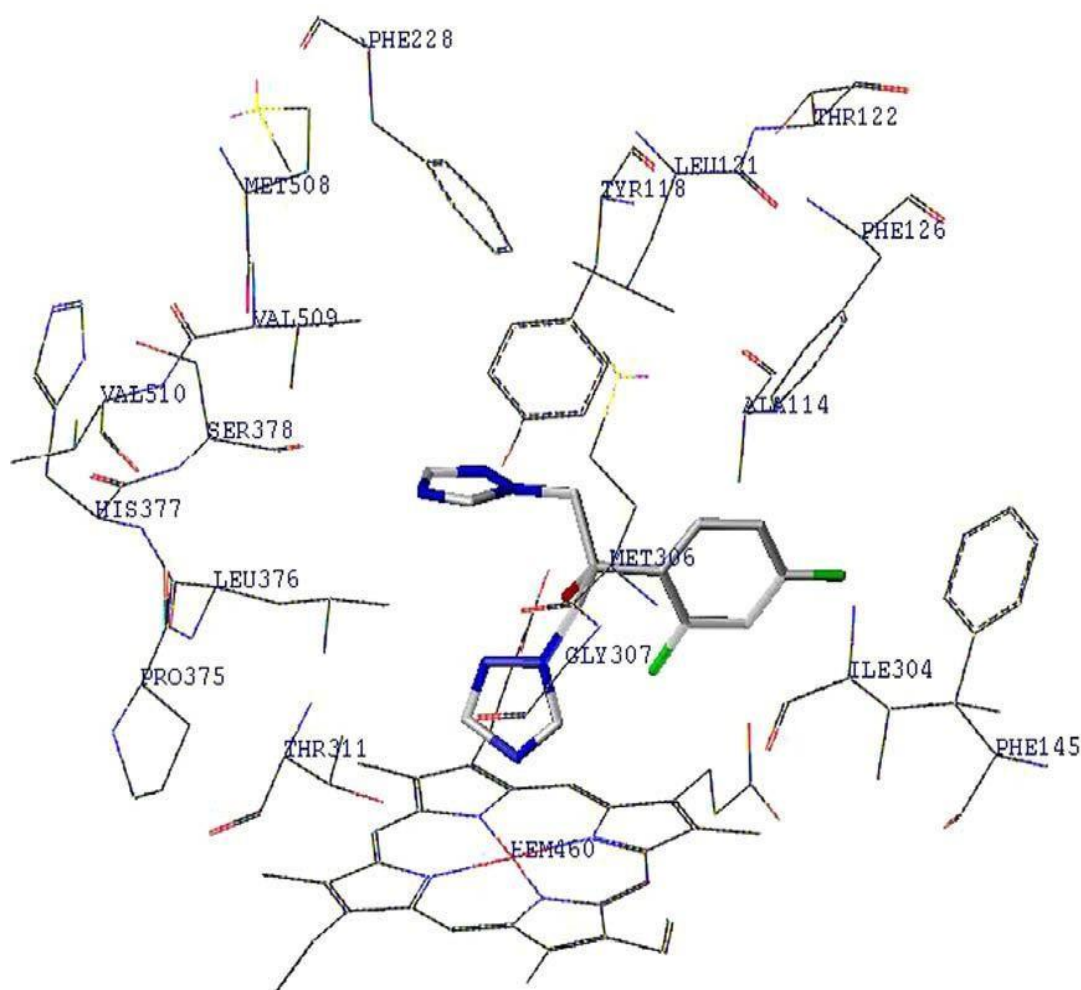
tached to C2 atom and methyl group attached to the C3 position have been favorable for antifungal activity. However, until now any interaction involving the oxygen atom of hydroxyl group attached to C2 was not found. It is because the active site is so large that there





**Figure 7: Mechanism of azole-P450<sub>14DM</sub> binding**

\*The basic 'N' of azole antifungal agent (Fluconazole) form a coordinate bond to the heme iron of CYP450 enzyme, preventing the enzyme from oxidizing its substrate.

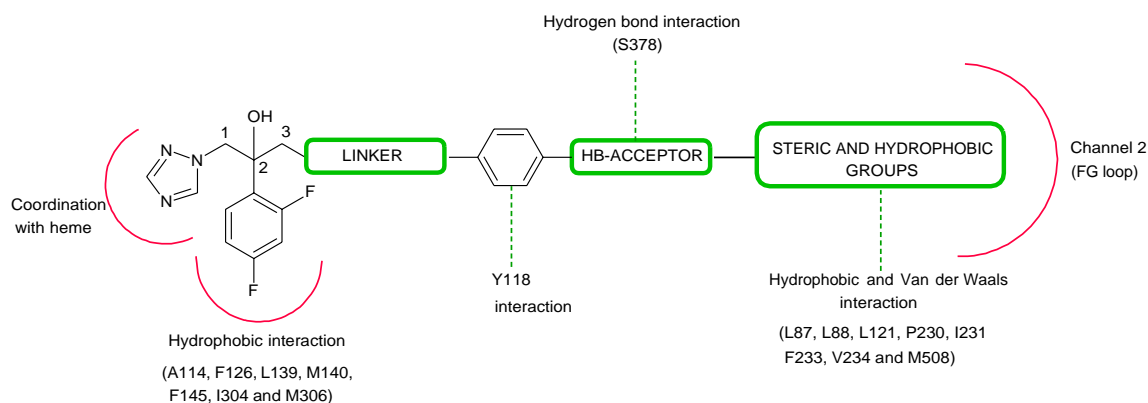


**Figure 8: Computed binding geometry of the clinically used inhibitor fluconazole in the active site of Lanosterol 14 $\alpha$ -demethylase**

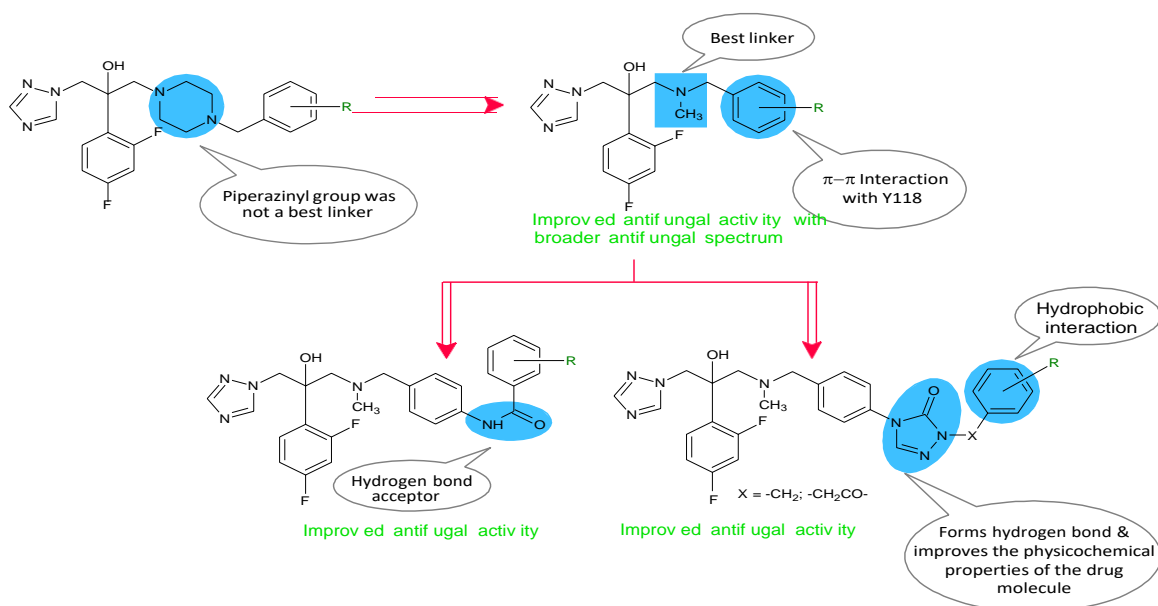
is a significant distance between the oxygen atom and the groups around it (Haitao Ji, 2000).

All the fungal CYP51 proteins that had been characterized were membrane-bound and it is difficult to solve their crystal structures. Up to date the only available solved X-ray crystal structure of CYP51 was obtained from *Mycobacterium tuberculosis*, but the explicit information about the enzyme binding site of pathogenic

fungi is not available yet (Alicja Nowaczyk, 2008). According to this the study of the interaction between azoles and fungal CYP51 can only be done by molecular modeling (e.g, Xiaoyun Chai et al.,) (Fig.8) (Xiaoyun Chai, 2009). Several three-dimensional (3D) models of CYP51 and their interaction with azole antifungals has been reported (Fausto Schiaffella, 2005; Xiaoying Che, 2009; Rajeshri G Karki, 2003; Xiaoyun Chai, 2009; Ree-



**Figure 9: Interaction model of azole antifungals with amino acid residues of Lanosterol 14 $\alpha$ -demethylase**



**Figure 10: Azole antifungals showing variable activities on substitutions**

na Gollapudy, 2004; Antonio Macchiarulo, 2002; Renata Fringuelli, 2009; Xiaoyun Chai, 2009; Francis Giraud, 2008; Francis Giraud, 2009; Ju Zhu, 2006; Zhi Gang Dan, 2009, Qiu Qin He, 2007).

Ji *et al.* (Haitao Ji, 2000) built a homologous 3D model of CYP51 from *C. albicans* based on the crystal coordinates of all four known prokaryotic P450s. With this model they found that the halogenated phenyl group of azole inhibitors is deep in the hydrophobic binding cleft and the long side chains of some inhibitors such as itraconazole and ketoconazole surpass the active site and interact with the residues in the substrate access channel. Another 3D molecular model constructed by Lewis *et al.* (Lewis D F V, 1999b) also showed that typical azole inhibitors were able to fit the putative active site of CYP51 by a combination of heme coordination, hydrogen bonding,  $\pi$ - $\pi$  stacking and hydrophobic interactions within the heme environment of the enzymes. Recently, modelling data of Li *et al.* (Li Xiao, 2004) suggest that the long chain of posaconazole and itraconazole occupies a specific channel within CYP51 and that this additional interaction serves to stabilize the binding of these azoles to the mutated CYP51 proteins. Models generated by Fukuoka *et al.* (Takashi Fukuoka,

2003) predicted that voriconazole was a more potent inhibitor than fluconazole because the additional methyl group of voriconazole resulted in stronger hydrophobic interaction with the aromatic amino acids in the substrate binding site and filled the site more extensively.

Shuang Hong Chen (Shuang Hong Chen, 2007) studied the 3D model of CYP51 from *Candida albicans* (CACYP51) was constructed. In addition, the binding mode of the substrate with fungal CYP51 was also investigated. This model predicted a hypothesis that the phenyl group of the C-3 side chain of azole antifungal compounds interacts with the phenol group of Tyr118, a highly conserved residue in CYP51 family, through the formation of  $\pi$ - $\pi$  face-to-edge interaction (Fig.9). To demonstrate this hypothesis and verify the reliability of the derived pharmacophore model, a series of novel azole compounds on the basis of this model were synthesized and tested for their *in vitro* antifungal activities in their laboratory. One of the major objectives of this study is to reveal that these azole compounds will have excellent *in vitro* activities and the structure-activity relationship correlated with the model. As a result, the synthesized compounds may not only be

used to perform further biological evaluations to develop new candidates of antifungal drugs, but also can be used as “probes” to investigate the structure-function relationship of CYP51 family.

According to the model proposed by Chunquan Sheng Et al., (Chunquan Sheng, 2006), first a linker is necessary to restrict the side chain in proper conformation and adjust the physicochemical properties of whole molecule. Second, a phenyl group is a suitable attachment to the linker because it can form a  $\pi$ - $\pi$  interaction with Y-118. Third, a hydrogen bond acceptor is favored at the para-position of the phenyl, which could form hydrogen bonding interaction with S-378. Last, steric and hydrophobic group is necessary because the corresponding residues are in the ligand access channel 2 (FG Loop) (Fig.9).

First they attempted to change the linker between the C3 and the phenyl group. Docking results revealed that the piperazinyl group was not the best choice and a smaller linker would be more favorable for the  $\pi$ - $\pi$  interaction of the phenyl group with Y118 (Fig.10). After testing various linkers based on 3D-QSAR predictive values and the interaction energy with CACYP51, they chose the  $-N-CH_3$  group for synthesis because the improved flexibility of the molecule allows the phenyl group to more easily lock its proper position, and the methyl group could form additional van der waals interactions with the surrounding A117, L376, and I379 (Fig.10). A total of 13 compounds with benzylamine side chain were synthesized. The *in vitro* antifungal activity assay indicated that their antifungal activities were higher than those of corresponding compounds with piperazinyl groups. Furthermore these compounds showed a broader antifungal spectrum with good activity against both systemic pathogenic fungi (*C.albicans*, *Cryptococcus neoformans*, *A.fumigatus*) and dermatophytes, (e.g. *Trichophyton rubrum* and *Trichophyton mentagrophytes*).

Second they extended the side chain of the benzylamine analogues on the basis of the pharmacophore model. Amide was selected as the hydrogen bond acceptor to be attached to the phenyl group, which was designed to form a hydrogen bond with S378 (Fig.10). Then they chose substituted phenyl groups as steric and hydrophobic groups to attach the amide group in order to form additional hydrophobic and van der Waals interaction with the residues in the ligand access channel 2 (FG Loop). A total of 10 amide-benzylamine compounds were synthesized and tested for *in vitro* antifungal activity. Most of them showed improved antifungal activity in comparison with the benzylamine compounds, which is attributed to the additional hydrogen bonding and hydrophobic interaction with the target enzyme. Most of the compounds showed better activity against *C.albicans* and *Crypt. neoformans* than fluconazole, itraconazole, and ketoconazole, with their MIC<sub>80</sub> values in the range of 0.125  $\mu$ g/mL to 0.008  $\mu$ g/mL.

Third they chose the triazolone group to replace the amide group as a hydrogen bond acceptor because triazolone group can not only form a hydrogen bond with S378 but also can adjust the physicochemical properties of the molecules and improve the water solubility (Fig.10). Various substituted benzyl groups and substituted 2-oxophenethyl groups were attached to the triazolone group to form hydrophobic interaction with the residues in the ligand access channel 2 (FG Loop). They synthesized 34 novel azoles with triazolone-benzylamine side chains. *In vitro* antifungal activities were improved again, and most of them showed the best antifungal activities among the synthesized azoles with their MIC<sub>80</sub> values against *C.albicans* in the range of 0.004  $\mu$ g/mL to 0.001  $\mu$ g/mL.

## CONCLUSION

Azoles (imidazoles and triazoles) are the most broadly known CYP51 inhibitors. They act by inhibiting cytochrome P450 14 $\alpha$ -demethylase (CYP51), a key enzyme in the fungal ergosterol biosynthesis. Azoles play a pivotal role in the treatment of systemic and dermal mycoses. Azoles are less toxic than other antifungals and inexpensive and broadly available, yet have several disadvantages. Their long-term usage can inhibit other P450 enzymes and leads to resistance allowing the drugs tolerance in the pathogen. We have presented here a summary of the molecular aspects of azoles and its target, CYP51. We expect that this information will continue to expand in the coming years, particularly more detailed analysis of CYP51 structure/function relationships. Advances in cell and molecular biology, microbiology, biochemistry and recombinant DNA technology have made it possible for drug research to characterize molecular targets. New computational and experimental methods for the determination of the spatial structure of molecular targets have brought structure-based drug design within reach. This has immensely contributed to a better understanding of target-substrate/inhibitor interactions and has provided essential information for structure-activity relationships and drug design. Structure-based and computer-aided drug design has now become important, essential and powerful constituents of modern drug research.

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