

## Molecular Basis for Enhanced Activity of Posaconazole against *Absidia corymbifera* and *Rhizopus oryzae*<sup>∇</sup>

Andrew S. Chau, Guodong Chen, Paul M. McNicholas,\* and Paul A. Mann

Schering-Plough Research Institute, 2015 Galloping Hill Road, 4700, Kenilworth, New Jersey 07033

Received 19 June 2006/Returned for modification 15 July 2006/Accepted 30 August 2006

**Posaconazole and itraconazole were more potent inhibitors of ergosterol synthesis, in both intact cells and cell extracts from *Absidia corymbifera* and *Rhizopus oryzae*, than voriconazole and fluconazole. Similarly, expression of CYP51 from *R. oryzae* in *Saccharomyces cerevisiae* significantly increased resistance to fluconazole and voriconazole but not to posaconazole and itraconazole.**

*Rhizopus*, *Absidia*, *Mucor*, and *Cunninghamella* are the members of the class *Zygomycetes* that most frequently cause invasive fungal infections in humans (12). The incidence of zygomycosis appears to be increasing; an expanded population of immunocompromised hosts coupled with antifungal prophylaxis using agents that have limited activity against these pathogens (such as voriconazole) may be contributory factors (9). Posaconazole, a novel azole antifungal agent, appears to be unique among the azoles in having both in vitro activity (13) and proven clinical efficacy against this class of pathogens (6, 7, 14, 15). In this study we examined the molecular basis for this enhanced activity.

**In vitro susceptibility testing.** Susceptibility testing of *Rhizopus oryzae* ATCC 11886 and *Absidia corymbifera* ND320 was performed as described previously (4). Only posaconazole and itraconazole were active (Table 1). Testing was repeated using the conditions used in the whole-cell labeling assays: malt extract (ME) broth with incubation for 48 h at 30°C (note: as previously reported, hyphal mat formation was reduced in ME broth and consequently labeling of sterols was more efficient [11]). Posaconazole was again active against both strains. Itraconazole was less active in ME against *R. oryzae*; the reason is unknown. Fluconazole and voriconazole again showed no activity against either strain.

**Inhibition of ergosterol synthesis in whole cells.** Sterols in liquid cultures of *R. oryzae* and *A. corymbifera* were labeled with [1-<sup>14</sup>C]acetate and extracted as described previously (11). The principal sterol in both strains was confirmed by gas chromatography-mass spectrometry (GC/MS) to be ergosterol. Exposure of the strains to posaconazole at sub-MIC levels resulted in inhibition of ergosterol synthesis in both strains; GC/MS analysis confirmed that the inhibition products retained a C-14 methyl group, which is consistent with the proposed mechanism of action of posaconazole (data not shown). Three independent measurements of the reduction in the area of the chromatogram peak corresponding to ergosterol were made for each test drug using three drug concentrations (a single concentration of flucon-

TABLE 1. Impact of test media on in vitro activities of antifungal agents against *Rhizopus oryzae* and *Absidia corymbifera*

Organism	MIC (μg/ml)							
	Posaconazole		Itraconazole		Voriconazole		Fluconazole	
	RPMI <sup>a</sup>	ME <sup>b</sup>	RPMI	ME	RPMI	ME	RPMI	ME
<i>R. oryzae</i>	2	2	2	>16	16	>16	>256	>256
<i>A. corymbifera</i>	0.25	1	0.5	0.25	>16	>16	>256	>256

<sup>a</sup> MIC read after 24 h of incubation at 35°C.

<sup>b</sup> MIC read after 48 h of incubation at 30°C; tests were performed three times.

azole was used, since it is not active against molds). Overall, there was a good correlation between the MIC and the amounts of drug resulting in 50% (IC<sub>50</sub>) and 90% (IC<sub>90</sub>) reductions in the ergosterol peak (Table 2). Posaconazole was the most active drug; voriconazole and fluconazole were not active. Consistent with the MIC results in ME broth described above, itraconazole did not inhibit ergosterol synthesis in *R. oryzae*.

**Inhibition of ergosterol synthesis in cell extracts.** Cell extracts were prepared from liquid cultures of *R. oryzae* and *A. corymbifera* as described previously (1). Incorporation of [<sup>14</sup>C]mevalonic acid (note: there was no labeling of sterols when [1-<sup>14</sup>C]acetate was used) into a peak that eluted at the same time as purified ergosterol was time dependent; maximum incorporation was achieved after 4 and 6 h for *R. oryzae* and *A. corymbifera*, respectively (data not shown). Additional peaks that eluted before ergosterol were evident on the radiochromatogram; increasing the incubation period to 24 h did not impact the pattern of peaks (data not

```

                                Y132      F145
Ca.CYP51      HLTIPVFGKGVLYDCPNRSLMEQKKFAKFAITDTSFKRYVVKIREE
Afum.CYP51    PLTTPVFGSDVWYDCPNRSLMEQKKFEKYGLTQSALESHVPLIEKE
Celeg.CYP51    HMTKYVFGPDVWYDAPHAVFMEQKKFEKAGLNSDCFRQHVPMIVQE
Roryz.CYP51A  HMTKYVFGNDIVFDTAHSVFMQKRMKISGLNIEAFRKDVP LI LEE
Roryz.CYP51B  HMTKHVFGPEVWYDAPHSVFMQKRFKAGLNSSEFRQHVPMIVEE
                *   *   *   *   *   *   *   *   *   *   *   *
    
```

FIG. 1. Alignment of CYP51 proteins from *Candida albicans* (Ca), CYP51 (accession no. CAA31658); *Aspergillus fumigatus* (Afum), CYP51A (accession no. AAK73659); *Cunninghamella elegans* (Celeg), CYP51 (accession no. AAF20263); and *Rhizopus oryzae* (Roryz). Residues spanning Y132 and F145 from *C. albicans* are highlighted.

\* Corresponding author. Mailing address: Schering-Plough Research Institute, 2015 Galloping Hill Road, 4700, Kenilworth, NJ 07033. Phone: (908) 740-7644. Fax: (908) 740-3918. E-mail: paul.mcnicholas@spcorp.com.

<sup>∇</sup> Published ahead of print on 11 September 2006.

TABLE 2. Comparison of whole-cell and cell extract IC<sub>50</sub>s and IC<sub>90</sub>s (μg/ml) for ergosterol inhibition in *Zygomycetes*

Ergosterol inhibition	Organism	Mean value (SD) for antifungal agent <sup>a</sup>							
		Posaconazole		Itraconazole		Voriconazole		Fluconazole	
		Whole cell	Cell extract	Whole cell	Cell extract	Whole cell	Cell extract	Whole cell	Cell extract
IC <sub>50</sub> <sup>a</sup>	<i>Rhizopus oryzae</i>	0.5 (0.06)	0.046 (0.005)	>16	0.034 (0.001)	>16	0.3 (0.1)	>256	11 (1.6)
	<i>Absidia corymbifera</i>	0.033 (0.001)	0.004 (0.0007)	0.08 (0.06)	0.007 (0.002)	>16	0.025 (0.0045)	>256	0.14 (0.036)
IC <sub>90</sub> <sup>a</sup>	<i>Rhizopus oryzae</i>	2.2 (0.4)	0.09 (0.003)	>16	0.2 (0.03)	>16	2.5 (0.65)	>256	87 (18.9)
	<i>Absidia corymbifera</i>	0.11 (0.01)	0.01 (0.002)	0.9 (0.44)	0.01 (0.002)	>16	0.16 (0.07)	>256	0.9 (0.36)

<sup>a</sup> IC<sub>50</sub>s and IC<sub>90</sub>s are averages from three independent determinations.

shown). It was not possible to identify the labeled sterols by GC/MS, possibly because they were below the limit of detection. Addition of test drugs to the cell extracts resulted in reductions in the peak corresponding to ergosterol and an increase in the earlier peaks; IC<sub>50</sub>s and IC<sub>90</sub>s were calculated from a minimum of three measurements made across a range of drug concentrations. Although posaconazole and itraconazole were again far more active than voriconazole and fluconazole, in contrast to results with the whole-cell assays, the latter drugs blocked ergosterol synthesis in the cell extract assay (Table 2). These data suggest that the ability to achieve sufficient intracellular levels plays a role in determining the activities of azoles against these two zygomycetes.

**Expression of *R. oryzae cyp51* in *Saccharomyces cerevisiae*.** Two *cyp51* orthologues (*cyp51A* and *cyp51B*) were identified from *R. oryzae* genomic sequence ([http://www.broad.mit.edu/annotation/fungi/rhizopus\\_oryzae](http://www.broad.mit.edu/annotation/fungi/rhizopus_oryzae)). Both genes, along with *ERG11* from *S. cerevisiae*, were PCR amplified from cDNA and fused to the *GAL10* promoter on YE<sub>p</sub>51 (2). The resultant plasmids were used to transform the azole-sensitive *Saccharomyces cerevisiae* strain YKKB-13 (2). Expression of *cyp51A* from *R. oryzae* resulted in 2-, 8-, and >32-fold (compared to the same strain expressing the *S. cerevisiae ERG11* gene) decreases in susceptibility to posaconazole, itraconazole, and voriconazole, respectively, but had no impact on susceptibility to the mechanistically unrelated drugs caspofungin and amphotericin B (Table 3). Expression of *cyp51B* did not change susceptibility to any of the drugs. Whether this is because *cyp51B* is poorly expressed in *S. cerevisiae* or is nonfunctional remains to be determined.

The CYP51 sequences from *R. oryzae*, which are 62% identical, were aligned with sequences from *Candida albicans*, *Aspergillus fumigatus* (only CYP51A was used), and *Cunninghamella elegans* (the only available zygomycete se-

quence). By focusing on regions previously associated with azole resistance, we identified two naturally occurring amino acid substitutions, Y132F and F145M (using *C. albicans* numbering), that are unique to CYP51A from *R. oryzae* and that may account for the observed pattern of azole susceptibility (Fig. 1). Previously a *C. albicans* strain harboring a Y132F substitution tested fluconazole resistant and susceptible to itraconazole (5, 8). Similarly, a *Histoplasma capsulatum* isolate with an Y136F substitution (Y136 is analogous to Y132 in *C. albicans*) displayed reduced susceptibility to fluconazole but not posaconazole (16). Finally, a *C. albicans* CYP51 protein harboring Y132H and F145L substitutions was significantly less susceptible to inhibition by fluconazole (10). Together these data suggest that substitutions at Y132 and F145 can strongly impact susceptibility to fluconazole without impacting either posaconazole or itraconazole. Although the effect of substitutions at Y132 and Y145 on voriconazole has not been assessed, prior work has suggested that strains with two substitutions, one at Y132 and a second substitution elsewhere in the protein, display large reductions in susceptibility to voriconazole but remain susceptible to posaconazole (3).

In summary, the data presented here suggest that the superior activities of posaconazole and itraconazole over those of voriconazole and fluconazole against *R. oryzae* and *A. corymbifera* are due to a combination of an increased affinity for the target site coupled with enhanced cellular penetration and/or reduced efflux.

We thank the following SPRI personnel: Birendra Pramanik for advice on GC/MS, Reena Patel and Cara Mendrick for providing assistance with whole-cell IC<sub>50</sub>/IC<sub>90</sub> and MIC determinations, and Li Xiao for performing the sequence alignments.

TABLE 3. Impact of expressing *cyp51* genes from *Rhizopus oryzae* on susceptibility of *Saccharomyces cerevisiae* YKKB-13 to antifungal agents

Expressed gene	MIC (μg/ml) <sup>a</sup>					
	Posaconazole	Itraconazole	Voriconazole	Fluconazole	Caspofungin	Amphotericin B
Vector alone	0.5	0.25	0.063	8	0.125	2
<i>S. cerevisiae ERG11</i>	1	0.5	0.5	64	0.125	2
<i>R. oryzae cyp51A</i>	2	4	>16	>256	0.125	2
<i>R. oryzae cyp51B</i>	0.5	0.25	0.25	16	0.125	2

<sup>a</sup> Triplicate independent MIC determinations were performed with yeast nitrogen base-leucine supplemented with 2% galactose and 1% raffinose and read at 72 h.

## REFERENCES

1. Ballard, S. A., S. L. Kelly, S. W. Ellis, and P. F. Troke. 1990. Interaction of microsomal cytochrome P-450 isolated from *Aspergillus fumigatus* with fluconazole and itraconazole. *J. Med. Vet. Mycol.* **28**:327–334.
2. Calabrese, D., J. Bille, and D. Sanglard. 2000. A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (FLU1) conferring resistance to fluconazole. *Microbiology* **146**:2743–2754.
3. Chau, A. S., C. A. Mendrick, F. J. Sabatelli, D. Loebenberg, and P. M. McNicholas. 2004. Application of real-time quantitative PCR to the molecular analysis of *Candida albicans* strains exhibiting reduced susceptibility to azoles. *Antimicrob. Agents Chemother.* **48**:2124–2131.
4. Clinical and Laboratory Standards Institute. 2002. Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi. Approved standard M38-A. National Committee for Laboratory Standards, Wayne, Pa.
5. Goldman, G. H., M. E. da Silva Ferreiraqq, Edos Reis Marques, M. Savoldi, D. Perlin, S. Park, P. C. Godoy Martinez, M. H. Goldman, and A. L. Colombo. 2004. Evaluation of fluconazole resistance mechanisms in *Candida albicans* clinical isolates from HIV-infected patients in Brazil. *Diagn. Microbiol. Infect. Dis.* **50**:1:25–32.
6. Greenberg, R. N., K. Mullane, J. A. H. van Burik, I. Raad, M. J. Abzug, G. Anstead, R. Herbrecht, A. Langston, K. A. Marr, G. Schiller, M. Schuster, J. R. Wingard, C. E. Gonzalez, S. G. Revankar, G. Corcoran, R. J. Kryscio, and R. Hare. 2006. Posaconazole as salvage therapy for zygomycosis. *Antimicrob. Agents Chemother.* **50**:126–133.
7. Herbrecht, R. 2004. Posaconazole: a potent extended-spectrum triazole and anti-fungal for the treatment of serious fungal infections. *Int. J. Clin. Pract.* **58**:612–624.
8. Kelly, S. L., D. C. Lamb, and D. E. Kelly. 1999. Y132H substitution in *Candida albicans* sterol 14 $\alpha$ -demethylase confers fluconazole resistance by preventing binding to haem. *FEMS Microbiol. Lett.* **180**:171–175.
9. Kontoyiannis, D. P., M. S. Lionakis, R. E. Lewis, G. Chamilos, M. Healy, M. C. Perego, A. Safdar, H. Kantarjian, R. Champlin, T. J. Walsh, and I. I. Raad. 2005. Zygomycosis in a tertiary-care cancer center in the era of *Aspergillus*-active antifungal therapy: a case-control observational study of 27 recent cases. *J. Infect. Dis.* **191**:1350–1360.
10. Kudo, M., M. Ohi, Y. Aoyama, Y. Nitahara, S. K. Chung, and Y. Yoshida. 2005. Effects of Y132H and F145L substitutions on the activity, azole resistance and spectral properties of *Candida albicans* sterol 14-demethylase P450 (CYP51): a live example showing the selection of altered P450 through interaction with environmental compounds. *J. Biochem.* **137**:625–632.
11. Munayyer, H. K., P. A. Mann, A. S. Chau, T. Yarosh-Tomaine, J. R. Greene, R. S. Hare, L. Heimark, R. E. Palermo, D. Loebenberg, and P. M. McNicholas. 2004. Posaconazole is a potent inhibitor of sterol 14 $\alpha$ -demethylation in yeasts and molds. *Antimicrob. Agents Chemother.* **48**:3690–3696.
12. Roden, M. M., T. E. Zaoutis, W. L. Buchanan, T. A. Knudsen, T. A. Sarkisova, R. L. Schaefele, M. Sein, T. Sein, C. C. Chiou, J. H. Chu, D. P. Kontoyiannis, and T. J. Walsh. 2005. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. *Clin. Infect. Dis.* **4**:634–653.
13. Sabatelli, F., R. Patel, P. A. Mann, C. A. Mendrick, C. C. Norris, R. Hare, D. Loebenberg, T. A. Black, and P. M. McNicholas. 2006. In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. *Antimicrob. Agents Chemother.* **50**:2009–2015.
14. Torres, H., R. Y. Hachem, R. F. Chemaly, D. P. Kontoyiannis, and I. I. Raad. 2005. Posaconazole: a broad-spectrum triazole antifungal. *Lancet Infect. Dis.* **5**:775–785.
15. van Burik, J. H., R. S. Hare, H. F. Solomon, M. L. Corrado, and D. P. Kontoyiannis. 2006. Posaconazole is effective as salvage therapy in zygomycosis: a retrospective summary of 91 cases. *Clin. Infect. Dis.* **42**:61–65.
16. Wheat, L. J., P. Connolly, M. Smedema, M. Durkin, E. Brizendine, P. Mann, R. Patel, P. M. McNicholas, and M. Goldman. 2006. Activity of newer triazoles against *Histoplasma capsulatum* from patients with AIDS who failed fluconazole. *J. Antimicrob. Chemother.* **57**:1235–1239.