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Stereoselective Interaction of the Azole Antifungal Agent SCH39304 with the Cytochrome P-450 Monooxygenase System Isolated from *Cryptococcus neoformans*

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We investigated the stereoselective inhibition of growth and ergosterol biosynthesis by SCH39304 in the pathogenic fungus *Cryptococcus neoformans* obtained from four AIDS patients who failed fluconazole therapy and compared the results to those obtained with a wild-type strain. For all strains, the MICs of the *RR* isomer were approximately half those of the racemate, with the *SS* enantiomer showing no inhibitory activity. The 50% inhibitory concentrations for in vitro ergosterol biosynthesis correlated with the MIC data, indicating stereoselective inhibition of their target P-450 enzyme, sterol 14α-demethylase, as the cause of this difference. The *RR* enantiomer produced classical type II spectra on addition to microsomal extracts of the strains, whereas the *SS* enantiomer showed an absence of binding. Stereo- and regio-specific localization of N-1 substituent groups of SCH39304 within the active site of the enzyme determined the unique discrimination between its two enantiomers, and the inability to bind to sterol 14α-demethylase is also true of other P-450 enzymes contained in the microsomal fraction. As previously observed for other antifungal azoles, isolates obtained following failure of fluconazole therapy showed resistance to SCH39304 and its *RR* enantiomer. This resistance could be associated with an alteration in the sensitivity of ergosterol biosynthesis in vitro. These alterations did not cause any changes allowing the *SS* enantiomer to bind to the P-450 mediating sterol 14α-demethylation.

Cryptococcus neoformans is a pathogenic fungus which profits from impaired immune response systems in humans and hence is prevalent in AIDS patients and organ transplant recipients (4). Current treatment of this fungal infection involves chemotherapy with azole antifungal drugs. Azole antifungal compounds inhibit the cytochrome P-450-dependent 14α-demethylation of lanosterol or 24-methylene-24,25-dihydrolanosterol, resulting in a decreased availability of ergosterol, an essential membrane component, and a corresponding accumulation of 14α-methylated sterols in cell membranes, which eventually leads to cell growth arrest (7). Inhibition occurs through coordination of a nitrogen atom (at N-3 in imidazole and at N-4 in triazoles; Fig. 1) to the heme iron of the enzyme, with the N-1 substituent groups interacting with the apoprotein (13). Additionally, the relative orientations of different N-1 substituent groups can influence antifungal activity, leading to further improvements in efficacy and selectivity (3, 14). Yoshida and Aoyama (15) reported previously on four optical isomers of the agricultural fungicide triadimenol and their inhibition of sterol 14α-demethylase isolated from *Saccharomyces cerevisiae*, with the 1*S*2*R* isomer having the greatest degree of potency. We have also screened a variety of azole antifungal enantiomers for their inhibitory activities against *Candida albicans* sterol 14α-demethylase expressed in *S. cerevisiae* and reported extreme variations in the inhibitory activities of the enantiomers of SCH39304 (6). This report describes the stereospecific interaction of the enantiomers of SCH39304 (Fig. 1) with the cytochrome P-450 monooxygenase system of the human pathogen *C. neoformans* and compares the interaction to that with a series of azole-tolerant *C. neoformans* strains described previously (10).

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MATERIALS AND METHODS

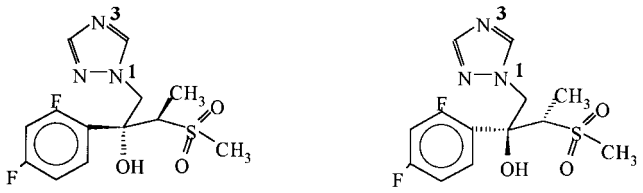
Strains. An azole-sensitive strain, strain B4500 (serotype D) (9), and clinical isolates (serotype A) from four AIDS patients which showed resistance during fluconazole treatment (isolates R715, R716, R717 and R718) were used in the study. The strains were grown on YEPD medium, consisting of 2% (wt/vol) glucose, 2% (wt/vol) Difco peptone, and 1% (wt/vol) Difco yeast extract.

Inhibition of growth. MICs were estimated following inoculation of 5×10^3 cells/ml in YEPD medium containing various doses of racemate or enantiomer. Incubation was at 37°C and 150 rpm in 60-ml containers (Sterilin, Shipley, United Kingdom) with 2 ml of culture. Growth was determined by cell counts and quantitation of the numbers of CFU after plating appropriate dilutions onto solid (2% Difco Bacto Agar) YEPD medium. The azole antifungal racemate and enantiomers were dissolved in dimethyl sulfoxide (DMSO) and were added prior to inoculation.

Microsomal preparation. Cells from each strain were grown to the late logarithmic phase and were harvested by centrifugation at $1,500 \times g$. Cells were resuspended in 100 mM potassium phosphate buffer containing 1 mM EDTA, 0.5 mM dithiothreitol, and 20% (vol/vol) glycerol. Cells were broken with a Braun homogenizer (Braun GmbH, Mesungen, Germany), and the cell debris was removed by centrifugation at $1,500 \times g$ for 10 min. The resulting supernatant was centrifuged at $10,000 \times g$ to remove mitochondria, and the microsomal pellet was prepared by a final centrifugation step at $100,000 \times g$. Microsomal pellets were resuspended to a final protein concentration of 10 mg/ml, and the mixture was stored at −80°C until use. The protein concentration was determined by the method of Lowry et al. (11).

Cytochrome P-450 content estimation. The cytochrome P-450 concentration was measured as described by Omura and Sato (12). Microsomal suspensions reduced with sodium dithionite were transferred to two quartz cuvettes, and the baseline was recorded with a Philips PU8800 UV/VIS scanning spectrophotometer. The contents in the sample cuvette were bubbled with carbon monoxide for 45 s at a rate of one bubble per second, and the difference spectra were recorded.

Type II binding spectra. Microsomal suspensions (100 pmol/ml) were transferred into each of two quartz cuvettes, and a baseline was recorded from 350 to 500 nm. Difference spectra were then recorded after incremental additions of azole antifungal agents (dissolved in DMSO) to the test cuvette and after the addition of an equal volume of solvent to the reference cuvette by the method of Baldwin and Wiggins (1).



RR-SCH39304 SS-SCH39304

FIG. 1. Chemical structures of the enantiomers of SCH39304.

Sterol biosynthesis assay and inhibition studies. Inhibition of CYP51 by azole antifungal agents was investigated by assessing the cell-free biosynthesis of ergosterol by the methods described previously (2, 10). Cell extracts were prepared as described above for the microsomal preparations. The reaction mixture consisted of a cell extract (924 μl/ml), cofactor solution (50 μl; containing 1 μmol of $NADP^+$, 1 μmol of NADPH, 1 μmol of NAD^+, 3 μmol of glucose-6-phosphate, 5 μmol of ATP, and 3 μmol of reduced glutathione), divalent cation solution (10 μl of 0.5 M $MgCl_2$ and 5 μl of 0.4 M $MnCl_2$), 1 μl of azole solution, and 10 μl of [2-^{14}C]mevalonate (0.25 μCi) (the reaction mixture was adjusted to pH 7 by the addition of 10 M KOH). The mixture was incubated at 37°C for 2 h with shaking (110 rpm), and the reaction was stopped by the addition of 1 ml of saponification reagent (15% [wt/vol] KOH in 90% [vol/vol] ethanol). Sterols were extracted in saponification reagent at 80°C for 45 min. Nonsaponifiable sterols were extracted three times with 5 ml of hexane and were dried under nitrogen. Samples were applied to thin-layer chromatography plates (ART 573; Merck) and were developed in cyclohexane-diethyl ether (5:1 [vol/vol]). Radioactive metabolites were located by autoradiography, and the ergosterol band was excised and radioactivity was assessed by liquid scintillation counting.

Chemicals. All chemicals were obtained from Sigma Chemical Company, Poole, United Kingdom, unless specified otherwise. [2-^{14}C]mevalonate, dibenzethylenediamine salt (specific activity, 53 mCi/mmol), was obtained from Amersham, Amersham, United Kingdom. SCH39304 and pure enantiomers were gifts from Schering-Plough, Bloomfield, Ill. (Fig. 1).

RESULTS

Effects of SCH39304 and enantiomers on growth. Growth rates for the azole-tolerant isolates R715, R716, R717, and R718 in YEPD medium were the same as that for azole-sensitive isolate B4500, with a doubling time of 2 h. By using the racemate of SCH39304 as the inhibitor, a 10-fold difference in susceptibility was observed between the sensitive and resistant isolates (Table 1). Dramatic differences were observed between the individual enantiomers; the *RR* enantiomer was an effective inhibitor of the growth of all strains examined, with the azole-resistant isolates being 2.5- to 10-fold less sensitive than the sensitive strain to the compound. The *SS* enantiomer showed no inhibitory activity on the growth of any of the strains tested. An approximately twofold higher concentration of racemate compared to the concentration of the *RR* enantiomer was required for inhibition, as would be expected if only one enantiomer were active.

TABLE 1. MICs and IC_{50}s of the racemate and the *RR* enantiomer of SCH39304 for azole-resistant *C. neoformans* clinical isolates R715, R716, R717, and R718 and azole-sensitive strain B4500[a]

Strain	MIC (μM)		IC_{50} (μM)	
	SCH39304 racemate	SCH39304 *RR* isomer	SCH39304 racemate	SCH39304 *RR* isomer
B4500	5.0	2.0	0.4 ± 0.17	0.18 ± 0.02
R715	20.0	9.0	3.5 ± 0.5	1.5 ± 0.15
R716	10.0	5.0	4.0 ± 0.6	1.8 ± 0.2
R717	20.0	10.0	3.5 ± 0.5	2.0 ± 0.2
R718	50.0	20.0	3.0 ± 0.7	2.0 ± 0.2

[a] No effect was observed in either the MIC or the IC_{50} experiment for the SCH39304 *SS* enantiomer at concentrations up to 10^{-3} M.

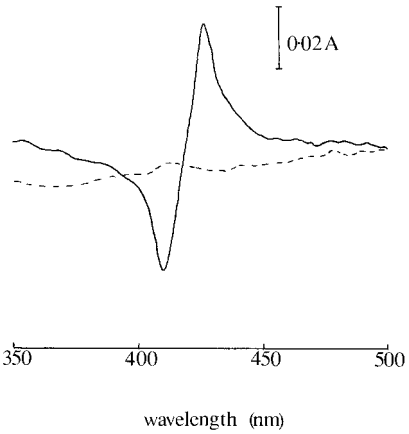


FIG. 2. Type II difference spectrum (——) produced by the interaction of the *RR* enantiomer of SCH39304 with microsomes from the clinical isolate *C. neoformans* R716. – – –, spectral change without the addition of azole.

Inhibition of ergosterol biosynthesis. The inhibitory activities of azole antifungal agents on sterol 14α-demethylation were tested with cell extracts from wild-type strain B4500 and the azole-tolerant isolates by measuring the incorporation of radioactivity from [2-^{14}C]mevalonate into [^{14}C]4-desmethylated sterols. The SCH39304 racemate and the *RR* enantiomer were effective inhibitors of incorporation of [2-^{14}C]mevalonate into desmethylated sterols (Table 1). The *SS* enantiomer showed no inhibitory activity in all tests at concentrations up to 10^{-3} M. The 50% inhibitory concentrations (IC_{50}s) of the racemate and *RR* enantiomer were approximately 10-fold higher for the resistant isolates than for the sensitive isolate.

Stereoselective interaction of SCH39304 and its enantiomers with microsomal extracts. Cytochrome P-450 was detected in microsomal samples isolated from all strains. They displayed reduced carbon monoxide difference spectra, with a Soret maximum of 448 nm. A total of 100 pmol of microsomal cytochrome P-450 from each strain was used in subsequent experiments to investigate azole binding to the P-450(s) that was present. Incremental addition of the racemate or the *RR* enantiomer to microsomal extracts induced type II spectral changes, indicating binding to the heme as a sixth ligand. Induced type II spectra were characterized by a maximum at 428 nm and a minimum at 406 nm for all strains (Fig. 2). The maximum concentration of DMSO used (1% [vol/vol]) caused no change in the spectrum over the region scanned. The *SS* enantiomer failed to induce a type II spectrum with microsomal extracts from all strains, reflecting the incompatibility of the stereochemical arrangement of the N-1 substituent group of this isomer for binding to the cytochrome P-450 apoproteins present in the microsomal fraction.

DISCUSSION

We have previously shown a unique variation in the inhibitory activity of the azole antifungal compound SCH39304 and its enantiomers in their ability to inhibit *C. albicans* sterol 14α-demethylase when it is heterologously expressed in *S. cerevisiae* (6). We investigated here their inhibition of microsomal P-450s present in the pathogenic fungus *C. neoformans*. The results obtained in this work reinforce the stereo- and regioselectivities of the *RR* enantiomer over those of the *SS* isomer in binding and inhibiting sterol 14α-demethylase. Accordingly,

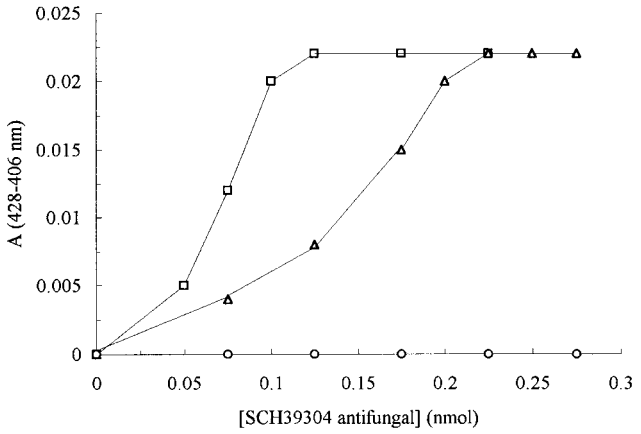


FIG. 3. Spectral titration of microsomal *C. neoformans* P-450 with SCH39304 *RR* enantiomer (□), SCH39304 racemate (△), and SCH39304 *SS* enantiomer (○). P-450 (0.1 nmol/ml) was titrated with increasing amounts of azole, and the magnitude of the resulting difference spectrum was recorded.

the *R* configuration of the substituents at the azole chiral center prove decisive in interacting with the target P-450 enzyme. However, it is likely that a significant quantity of sterol 22-desaturase (P45061) is also present in fungal microsomal fractions (8), so the results presented here have further implications than those presented previously (6) and were also extended to consideration of binding to the more resistant enzyme detected in isolates R715, R716, R717, and R718.

Azole inhibition of sterol biosynthesis by using cell extracts reflected the pattern of activity obtained by treatment of cells. Disruption of cell membranes to produce the extract removes the possibility that influx and efflux mechanisms that select for the *RR* enantiomer and that remove the *SS* enantiomer, respectively, are explanations for the stereoselective inhibition. The *RR* enantiomer was shown to inhibit ergosterol biosynthesis in both the wild-type and resistant isolates, while the racemate was less effective in inhibiting ergosterol biosynthesis and the *SS* enantiomer was inactive. Again, this was explained by the fact that the *RR* form is the active inhibitor molecule of the racemate toward sterol 14α-demethylase.

The *RR* enantiomer was shown to produce type II spectra on binding to microsomal P-450 isolated from each strain. However, the *SS* enantiomer failed to produce a binding spectrum, indicating no binding to P-450. The binding of the racemate to microsomal P-450 was observed to reach saturation at twice the molar concentration at which the *RR* enantiomer exhibited saturation (Fig. 3). This is explained by the fact that the racemate consists of a 50:50 mixture of the *RR* and *SS* enantiomers, with only the *RR* enantiomer being an active inhibitor.

We have already shown that the azole tolerance of resistant isolates R715, R716, R717, and R718 toward ketoconazole, itraconazole, and fluconazole is most likely due to alterations at the active site of the target enzyme, sterol 14α-demethylase, or to changes in the level of the enzyme(s) (10). The results presented here augment this observation. It can also be concluded that if an alteration(s) in the apoprotein is the cause of resistance, the alteration is subtle, with the overall active-site conformation remaining conserved and the *SS* enantiomer continuing to be incapable of binding.

The enantiomers may interact with sterol 14α-demethylase apoprotein through the chlorophenyl ring and the sulfonyl group. Molecular modeling of these enantiomers to the *C. albicans* enzyme suggests that the *RR* enantiomer is favored over the *SS* enantiomer by the sulfonyl moiety, which is positioned in the more hydrophilic channel leading towards the enzyme active site for the *RR* enantiomer (6). This deduction seems more plausible when comparing binding and inhibitory data for another azole antifungal compound, cyproconazole (5). This molecule differs from SCH39304 by the replacement of the sulfonyl group for a cyclopropyl ring. Both the *RR* and the *SS* enantiomers of cyproconazole bind sterol 14α-demethylase and have antifungal activities (5). Consequently, it can be reasoned that in the *SS* enantiomer of SCH39304, the sulfonyl group is positioned into a hydrophobic region within the active site, which cannot be tolerated due to its polarity, and thus, the molecule fails to bind or show inhibitory activity against the treated cells.

It is concluded that stereo- and regiochemical differences within isomeric azole antifungal molecules have a profound effect on efficacy and selectivity. These differences may be exploited for selective inhibition of the fungal enzyme. The absence of binding of the *SS* enantiomer to sterol 14α-demethylase of *C. neoformans*, as well as similar observations for sterol 14α-demethylases isolated from a variety of different species, suggests a conservation of the three-dimensional structure around the active site of this enzyme throughout evolution.

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