Stereoselective interaction of SCH 39304, a triazole, with sterol 14α-demethylase of *Aspergillus fumigatus*

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The inhibitory activity of SCH 39304 and its enantiomers on radial growth and on the target enzyme, sterol 14α-demethylase, in *Aspergillus fumigatus* was studied to assess the role of stereochemistry in the efficacy of the drug. SCH 39304 and the *RR* (+) enantiomer were active in inhibiting the growth while no inhibition in the growth was observed with the *SS* (–) enantiomer. The MIC of SCH 39304 for the growth was about twice that of the *RR* (+) enantiomer. The differences in IC₅₀ s of SCH 39304 and its enantiomers for cell-free ergosterol biosynthesis correlated with their variations in MICs and type II binding spectra indicated the *SS* (–) enantiomer failed to bind to microsomal P450. These results show that the difference between *SS* (–) and *RR* (+) enantiomers in interacting with the target enzyme is the cause for significant difference in the potency between these two forms.

Introduction

A large number ofazole antifungal agents have been developed and used with considerable success in treating mycotic infections in plants as well as in animals.¹,² Studies on mode of action revealed that these drugs act mainly by interfering with the cytochrome P450-dependent enzyme sterol 14α-demethylase, which is responsible for C14 demethylation of sterols in ergosterol biosynthesis. The consequences of this inhibition are depletion of ergosterol and accumulation of 14α-methyl sterols, which cause alterations in membrane fluidity, permeability, activities of membrane-bound enzymes, such as those for nutrient transport and chitin synthesis, and ultimately growth arrest.³ The overall effect ofazole drugs is fungistatic, but at higher concentrations they may be fungicidal in some fungal species.⁴

*A. fumigatus*, which causes invasive aspergillosis, has emerged recently as an important pathogen in immunosuppressed individuals and is a leading cause of mortality in bone-marrow transplant recipients.⁵,⁶ The potencies of differentazole drugs varied between 10- and 1000-fold when tested in in-vitro growth inhibition of *A. fumigatus*. This variation is probably due to differences in either permeability or affinity to the target enzyme (unpublished observation). A zole drug inhibition of sterol 14α-demethylase occurs through the interaction of the nucleophilic N of the ring (N₆ of imidazole or N₄ of triazole) of the drug to the sixth co-ordination position of the ferric haem of cytochrome P450.⁷ However, the selectivity and affinity of an azole drug for fungal cytochrome P450 is determined by the interaction of the N₁ substituent group to the substrate binding region in the apoprotein. This view was mainly based on carbon monoxide displacement studies which showed that exchange of N-heterocycles of ketoconazole or itraconazole did not alter the affinity of the drug to microsomal P450 of *Candida albicans* but deacylation of the N₁ substituent group of ketoconazole did affect the affinity.⁸,⁹ In addition the affinity and selectivity of azoles for P450s is also influenced by stereoisomerism of the N₁ substituents.¹⁰ However, comparatively little is known about stereoselective interaction of azoles with the target enzyme.¹¹ SCH 39304 (racemic mixture) contains *RR* (+) (SCH 42427) and *SS* (–) (SCH 42426) enantiomers (Figure 1) in equal proportion and is a triazole compound that has been developed.

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for oral administration and topical treatment, but was not commercialized. Preliminary studies showed that the RR (+) enantiomer was the active form, not the SS (–) enantiomer and recently studies on binding of the transactive form to heterologous expressed sterol 14α-demethylase of C. albicans have been reported. In the present study we have investigated the stereoselective interaction of SCH 39304 and its enantiomers with sterol 14α-demethylase of A. fumigatus in order to correlate with their antifungal activity.

Materials and methods

Organisms and chemicals

A. fumigatus H06-03 (Pfizer Central Research Culture Collection, Sandwich, UK) was used in this study. Unless otherwise stated all chemicals were purchased from Sigma (Poole, UK). SCH 39304 and its enantiomers were kindly provided by Schering-Plough Research (Bloomfield, NJ, USA).

In-vitro susceptibility tests

The potencies of SCH 39304 and its enantiomers on A. fumigatus H06-03 mycelial growth were assessed by measuring the radial growth on Sabouraud agar containing various concentrations of drug after transferring a thin mat of mycelium of uniform size (0.4 cm in diameter) on to the agar medium and incubating for 48 h at 37°C.

Sterol isolation and gas chromatography/mass spectrometry analysis

The sterols were extracted as nonsaponifiable lipids from the cultures grown for 24 h at 37°C and analysed by gas chromatography/mass spectrometry with comparison of fragment patterns in the literature.

Inhibition of cell-free ergosterol biosynthesis

Cell extracts were prepared from the 16 h old cultures lysed by mechanical disruption using glass beads in a Braun homogenizer. Cell-free ergosterol biosynthesis was undertaken by following a method described by Ballard et al., i.e. the cell extract was incubated with [2,14C]mevalonate, NADPH and azole drug for 2 h at 37°C, then nonsaponifiable sterols were extracted from the reaction mixture, separated by thin-layer chromatography with a toluene and diethyl ether solvent mixture (9:1, v/v) and the radioactive sterols located by autoradiogram and identified by reference to standards.

Spectrophotometric analysis

Microsomal fraction was isolated with a specific content of 20 pmol of P450 per milligram of protein by a previously published method. P450 content was estimated by using reduced carbon monoxide (CO) difference spectrum. The type II binding spectra were recorded by adding each drug incrementally to 0.1 M microsomal P450. The CO displacement of azole drug was carried out by measuring the reduced CO-difference spectrum for every 5 min after adding 5 μM RR (+) enantiomer and incubating for 2 min on ice.

Protein content estimation

Protein concentrations of cell-free extracts and microsomal fractions were estimated by bicinchoninic acid method using a kit provided by Sigma.

Results

Inhibition of radial growth

The growth of A. fumigatus was inhibited by SCH 39304 in a dose-dependent manner (Figure 2) and complete
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Inhibition was achieved at 10 μM. When individual enantiomers were used for susceptibility studies dramatic differences in their potencies were observed; RR (+) was active in inhibiting the fungal growth while no inhibition in the growth was observed with SS (–) enantiomer. The concentration of RR (+) enantiomer required for complete inhibition of the growth was approximately half that for SCH 39304 inhibitory concentration (5 μM). The concentration of SCH 39304 and its enantiomers required for inhibiting 50% of the growth is shown in the Table. Sterol analysis showed that ergosterol (>80% of the total sterols) is the major sterol in untreated cultures and it was reduced to below 20% of the total sterols after treating with the MIC of the RR (+) enantiomer. A decrease in ergosterol levels and a concomitant increase in C14α- methyl sterols levels (data not shown) in treated cultures were indicative of sterol 14α-demethylase inhibition.

**In-vitro ergosterol biosynthesis inhibition**

Cell-free ergosterol biosynthesis was carried out to study the sterol 14α-demethylase activity and its inhibition by SCH 39304 and its enantiomers. The biosynthesis of ergosterol was estimated by measuring the incorporation of [2-14C]mevalonate into it. The ergosterol biosynthesis was inhibited by SCH 39304 and the RR (+) enantiomer in a dose-dependent manner (Figure 3) but to different degrees and the decrease in ergosterol synthesis correlated with the accumulation of eburicol (data not shown), the sterol 14α-methyl demethylase substrate. However, no inhibition in the biosynthesis was observed with the SS (–) enantiomer. The half inhibitory concentration (IC₅₀) of SCH 39304 was approximately twice that of the RR (+) enantiomer for cell-free ergosterol biosynthesis (Table I). The differences observed in IC₅₀s of SCH 39304 and its enantiomers for sterol 14α-demethylase in cell-free extracts are in agreement with the differences obtained in MICs for the growth.

**Spectrophotometric interaction studies**

The investigation was extended further by studying the interaction of SCH 39304 and its isomers with microsomal cytochrome P450 of A. fumigatus. SCH 39304 and RR (+) enantiomer produced a typical type II spectrum indicative of a low spin state haem iron in the P450 resulting from the binding of azole to haem as a sixth ligand. This spectrum was characterized by an absorbance maximum at

<table>
<thead>
<tr>
<th>Compound</th>
<th>radial growth</th>
<th>IC₅₀ (μM) sterol synthesis</th>
<th>type II binding spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH 39304 (+)</td>
<td>1.00</td>
<td>0.24</td>
<td>0.21</td>
</tr>
<tr>
<td>SS (–)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RR (+)</td>
<td>0.48</td>
<td>0.11</td>
<td>0.09</td>
</tr>
</tbody>
</table>

ND = Not detected.
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![Graph](Image)

The relation between the magnitude of type I binding spectra of A. fumigatus microsomal P450 and concentration of SCH 39304 (■), RR (+) (○) and SS (−) (▲). ΔA is the absorbance difference between 426–430 nm and 406–410 nm.

426–430 nm and minimum at 406–410 nm upon binding ofazole with the microsomal P450. The SS (−) enantiomer, however, failed to produce a type II binding spectrum, reflecting an inability to interact with the P450. The spectral response steadily increased with increasing concentration of the drug and reached saturation at 1 μM and 2 μM with the RR (+) enantiomer and SCH 39304, respectively (Figure 4). The half saturating concentrations (IC₅₀) of SCH 39304 and its enantiomers for type II binding spectra are shown in the Table. The RR (+) enantiomer’s inhibitory effect on CO–P450 complex formation was studied to determine the relative affinity of microsomal P450 for the drug. The drug displacement by CO increased gradually with time and reached maximum at 30 min when only 20% of the P450 was azole-bound.

Discussion

Studies on the inhibition of cell growth, in-vivo and in-vitro ergosterol biosynthesis by SCH 39304 and its enantiomers, and their interaction with microsomal P450 show an extreme variation in the activity between RR (+) and SS (−) enantiomers. The potency of SCH 39304 is approximately half that of the RR (+) enantiomer and the results explain this, indicating that the SS (−) enantiomer is inactive due to its inability to interact with the target enzyme. However, its presence in the racemic mixture (SCH 39304) has not altered the activity of RR (+) enantiomer. In type II binding studies the spectral response with the active form was saturated at a 1:10 molar ratio of the microsomal P450 and the compound, respectively. This result confirms the observation of a previous study examining the interactions of A. fumigatus microsomal P450 with itraconazole and fluconazole. It is known thatazole drugs bind with sterol 14α-demethylase of C. albicans and Saccharomyces cerevisiae in 1:1 stoichiometry. However, in this and another study, stoichiometric interaction was not observed. This might be explained by the presence of a variety of P450 isozymes in the microsomal fraction of A. fumigatus which could differ in the affinity for azoles. This view is supported by others who demonstrated that A. fumigatus microsomal P450 expressed constitutively was able to hydroxylate benzo(a)pyrene at the 3 position and progesterone at the 11α position.

Recent reports on stereoselective interaction of azole drugs with S. cerevisiae sterol 14α-demethylase suggested that the relative orientation of the hydrophilic group in the N1-substituent to the nucleophilic nitrogen of the ring is important to fit theazole drug in the active site of the target enzyme. In addition many studies carried out to evaluate the antifungal activity of enantiomers of severalazole drugs indicated that the R-enantiomeric forms were more active than S-enantiomeric forms. The present study also revealed extreme variation between RR (+) and SS (−) enantiomers in their potencies. Molecular modelling predicted that the inability of SS (−) enantiomer to interact with C. albicans sterol 14α-demethylase is due to interference by the hydrophilic sulphonyl group of the N1-substituent with the interaction of theazole and the hydrophobic substrate binding pocket of the P450. The data presented here could be useful in further understanding the active site of A. fumigatus, but the absence of binding by the SS (−) enantiomer appears to extend to all the P450 enzymes in the microsomal fraction of A. fumigatus which include a sterol Δ22-desaturase, a benzo(a)pyrene 3-hydroxylase and a progesterone 11α-hydroxylase.

Acknowledgement

K.V. is a recipient of Commonwealth Postgraduate Scholarship jointly administered by the Commonwealth Scholarship Commission and the British Council.

References


Received 29 May 1996; returned 5 August 1996; revised 24 September 1996; accepted 14 November 1996

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