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Ro 09-1470 Is a Selective Inhibitor of P-450 Lanosterol C-14 Demethylase of Fungi

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Ro 09-1470 is a new antifungal agent that belongs to a series of compounds characterized by a tetrahydropyran skeleton with glycine and alkenyl side chains and that inhibits P-450 lanosterol C-14 demethylase ($P\text{-}450_{14DM}$) of fungi (Y. Aoki, T. Yamazaki, M. Kondoh, Y. Sudoh, N. Nakayama, Y. Sekine, H. Shimada, and M. Arisawa, J. Antibiot. 45:160–170, 1992; S. Matsukuma, T. Ohtsuka, H. Kotaki, H. Sawairi, T. Sano, K. Watanabe, N. Nakayama, Y. Itezono, M. Fujiu, N. Shimma, K. Yokose, and T. Okuda, J. Antibiot. 45:151–159, 1992). We have studied the compound's mode of interaction with fungal $P\text{-}450_{14DM}$ and its selectivity for the fungal versus mammalian P-450 enzymes. Ro 09-1470 bound to the *Saccharomyces cerevisiae* $P\text{-}450_{14DM}$ by coordinating to the heme with one-to-one stoichiometry. Unlike the azole compounds, it interacted with both ferric and ferrous heme. It was active also against the $P\text{-}450_{14DM}$ of *Candida albicans*. Ro 09-1470 preferentially inhibited the yeast $P\text{-}450_{14DM}$, showing a 50% inhibitory concentration (IC_{50}) of 0.47 to ~1.1 μM, which is much lower than the IC_{50}s for rat hepatic P-450s catalyzing cholesterol biosynthesis (IC_{50} = 341 μM), *p*-nitroanisol O-demethylation (>1,000 μM), aniline hydroxylation (>1,000 μM), and aminopyrine N-demethylation (920 μM). The degree of selectivity for yeast P-450 was higher than that of ketoconazole.

Sterols are essential cell membrane components of all eukaryotic cells; they function mainly to maintain adequate levels of membrane fluidity and to modulate membrane-bound enzyme activities (13). Ergosterol is a fungus-specific sterol required for the vegetative growth of yeast under all conditions. The key step in ergosterol biosynthesis, removal of the 14α-methyl group from lanosterol, is catalyzed by a cytochrome P-450-containing enzyme named P-450 lanosterol C-14 demethylase ($P\text{-}450_{14DM}$) (2–4, 16).

A number of azole derivatives which inhibit ergosterol biosynthesis by affecting $P\text{-}450_{14DM}$ activity have been developed as potent antifungal agents. Azole-treated fungal cells accumulate C-14 methyl sterols, thereby disrupting membrane structure and function (7, 24, 25). A multicopy plasmid expressing the gene for $P\text{-}450_{14DM}$ has produced resistance to azole antifungal agents in *Saccharomyces cerevisiae* (19) and *Candida albicans* (10) and thus proved that $P\text{-}450_{14DM}$ is the primary target of azole antifungal agents. Yoshida and Aoyama (27) and Rodrigues et al. (20) investigated in detail the direct interaction of azole antifungal agents with purified $P\text{-}450_{14DM}$ and indicated that these compounds formed low-spin stoichiometric complexes with high affinity, indicating that their azole nitrogens interact with the heme iron.

It has been known for some time that azole antifungal agents also potently inhibit many P-450-mediated reactions involved in steroid hormone (5) and cholesterol (23) biosyntheses, as well as in xenobiotic metabolism (8, 12). Actually, endocrinal side effects and hepatotoxicity are serious problems accompanying clinical treatment with azole antifungal agents.

Ro 09-1470 is one of a new series of compounds having antifungal properties. We investigated the mode of action for Ro 09-1470 and concluded that it inhibits fungal $P\text{-}450_{14DM}$ from several lines of evidence: (i) ergosterol biosynthesis was predominantly inhibited by the compound under conditions in which DNA, RNA, protein, and respiration were not inhibited; (ii) C-14 methyl intermediates of ergosterol biosynthesis were accumulated; (iii) $P\text{-}450_{14DM}$ enzyme activity was inhibited in vitro; and (iv) the compound bound to heme of $P\text{-}450_{14DM}$ (1). The distinguishing feature of Ro 09-1470 is its structure; it does not have an azole moiety as the group coordinating to the heme of $P\text{-}450_{14DM}$. Instead, an amino group of glycine appears to bind to the iron of the heme.

In this paper, we investigated the specificity of Ro 09-1470 for fungal versus mammalian P-450 enzymes. Ro 09-1470 inhibited fungal $P\text{-}450_{14DM}$ more strongly than rat hepatic $P\text{-}450_{14DM}$; its effects on other P-450s were also weak. Ro 09-1470 differed from the azole antifungal agents in its interaction with P-450s. Inhibitory activities against P-450s, affinity for rat microsomes, and stoichiometry in heme binding were compared for Ro 09-1470, ketoconazole, and fluconazole; Ro 09-1470 was more similar to fluconazole than to ketoconazole in those comparative studies.

MATERIALS AND METHODS

Reagents. Pure powdered ketoconazole and miconazole were purchased from Sigma, while fluconazole was purified from tablets of Diflucan (Pfizer). The purity specified by high-performance liquid chromatography was 100%. Itraconazole (9.5% pure) and genaconazole (9.8% pure) were gifts from A. W. Polak (Basel, Switzerland).

Cholesterol biosynthesis assay of rat hepatic cell extract (CE). The assay method of Trzaskos et al. (22) was modified. Male Sprague-Dawley rats obtained from the Charles River Laboratory (10 weeks old) were used in this study. Animals were maintained on a diet containing 3% cholestyramine and on a light cycle of alternating periods of 12-h light and 12-h dark; they were sacrificed at the midpoint of the dark cycle. After 10 days of treatment with cholestyramine, rats were decapitated, and livers were perfused in situ with 100 ml of cold phosphate-buffered saline. Livers were excised and

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homogenized with a loosely fitting Potter homogenizer in 0.1 M potassium phosphate buffer (pH 7.4)–0.5 mM dithiothreitol–10 mM nicotine amide–0.25 M sucrose. CEs were obtained by centrifugation at 600 × *g* for 10 min and at 15,000 × *g* for 30 min. To the CE was added 1/4 volume of glycerol, and the mixture was stored at −80°C. No activity loss was observed for at least several months.

The reaction mixture (1 ml) contained 0.3 M ATP, 0.1 M NADPH, 0.3 M NAD, 0.3 M glucose-6-phosphate, 20 U of glucose dehydrogenase per ml, 0.3 M glutathione, 10 mg of $MnCl_2$ per ml, 10 mg of $MgCl_2$ per ml, 0.25 μCi of [2-^{14}C]mevalonate (Amersham), and 5 mg of CE per ml. The reaction was run at 30°C with continuous shaking for 20 min and stopped by the addition of an equal volume of 15% KOH in 95% ethanol. The saponification, extraction, and development by thin-layer chromatography of sterols were performed by the methods described in a previous paper (1). The radioactivity on the thin-layer chromatography plates was determined with a thin-layer chromatography scanner (Aloka; Radiochromanizer JTC-50). The P-450$_{14DM}$ inhibition activity was calculated from the amounts of 4,4-desmethylsterols relative to those in the control.

Assay of other hepatic P-450s. The livers excised from the nontreated rats (male, 10-week-old Sprague-Dawley rats obtained from Charles River Laboratory) were used to prepare the microsome fraction. The CE obtained by the method described above was centrifuged at 105,000 × *g* for 2 h to precipitate the microsome fraction. The fraction was suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM glutathione, 0.1 mM EDTA, and 20% glycerol. The assay methods for *p*-nitroanisol O-demethylation and aminopyrine N-demethylation followed those of Kitagawa (11), whereas that for aniline hydroxylation followed Kato and Jillette (9). Spectrophotometric analysis was done by the method described in the next paragraph. The P-450 concentration in the microsomes used for kinetic analysis was 2.2 nmol/ml. Ro 09-1470, ketoconazole, and fluconazole were dissolved in dimethyl sulfoxide, and the assay was performed using sample concentrations between 10 and 1,000 μM and keeping the dimethyl sulfoxide concentration constant at 2%.

Preparation of *S. cerevisiae* microsomes and spectrophotometric analysis of P-450$_{14DM}$. The microsome fractions of *S. cerevisiae* were prepared by the methods described in a previous paper (1). The P-450 contents of those fractions were determined according to the methods of Omura and Sato (17) by measuring the reduced carbon monoxide (CO) difference spectrum. The extinction coefficient used for estimating P-450 concentrations was 91 cm^{-1} mM^{-1}. Interaction of the compounds with P-450$_{14DM}$ was analyzed spectrophotometrically as described in a previous paper (1). The optical peak height obtained by subtracting the minimum A_{410} value from the maximum A_{428} value in the difference spectrum was used to measure the affinity for P-450$_{14DM}$. The peak height values were 2.08 m^{-1} M^{-1} for ketoconazole, 2.13 m^{-1} M^{-1} for miconazole, 1.42 m^{-1} M^{-1} for fluconazole, 1.38 m^{-1} M^{-1} for itraconazole, and 0.98 m^{-1} M^{-1} for Ro 09-1470. Inhibition of CO binding to ferric P-450$_{14DM}$ was also measured spectrophotometrically. Compounds and/or dimethyl sulfoxide was added to the reference and sample cuvettes containing the same microsome fractions. Two minutes later, the P-450$_{14DM}$ was reduced for 35 s with a few grains of sodium dithionite, and the sample cuvette was bubbled with CO for 30 s and tightly closed. The difference spectrum was recorded after 2 to 5 min with a spectrophotometer (UVICON 860; Kontron). To measure the inhibition of CO binding to ferrous P-450$_{14DM}$, the microsome fractions were reduced by sodium dithionite for 35 s prior to the addition of compounds. The 50% inhibitory concentrations (IC_{50}s) (50% decrease in the change in optical density at 448 to 490 nm) were determined by interpolation.

Preparation of *C. albicans* microsomes and P-450$_{14DM}$ assay. We prepared microsome fractions of *C. albicans* 652 (1) and assayed the P-450$_{14DM}$ by the methods reported for *S. cerevisiae* in a previous paper (1).

RESULTS

Effects on in vitro cholesterol biosynthesis in rat liver. Effects of Ro 09-1470 and ketoconazole on in vitro cholesterol biosynthesis were analyzed with a CE of rat liver in which P-450$_{14DM}$ was induced by cholestyramine treatment. In the control, only 4,4-desmethylsterols were detected, indicating that three methyl groups at C-4α, C-4β, and C-14 were removed. In contrast, 4,4-dimethylsterols were dominant in the samples treated with 600 μM Ro 09-1470 or 15 μM ketoconazole (Fig. 1). This profile was basically identical to that of the fungal cells mentioned in the previous paper (1) and indicated that P-450$_{14DM}$ was the enzyme most sensitive to Ro 09-1470 and the azole antifungal agents in the assay of cholesterol biosynthesis in rat liver.

Selective inhibition of fungal P-450$_{14DM}$ versus rat liver P-450$_{14DM}$. The inhibitory activities of Ro 09-1470 and the reference azole compounds against rat liver P-450$_{14DM}$ were measured by the method described above (Table 1). The five reference azoles were classified into two groups. One group, comprising ketoconazole, itraconazole, and miconazole, had activity against rat hepatic P-450$_{14DM}$ with an IC_{50} of around 1 μM. The other group, comprising water-soluble fluconazole and genaconazole, showed much weaker activity than the first group. Ro 09-1470 was classified in the latter group with respect to its activity against rat hepatic P-450$_{14DM}$. We also investigated the effect of Ro 09-1470 on P-450$_{14DM}$ activity of *C. albicans*; the results are summarized in Table 1. All the compounds tested showed similar activities with IC_{50}s ranging from 0.038 to 0.47 μM. When the selectivity ratio of Ro 09-1470 for rat liver versus *C. albicans* P-450$_{14DM}$ was compared with those of the azole antifungal agents, the selectivity of Ro 09-1470 was almost the same as that of genaconazole and higher than those of ketoconazole, itraconazole, and miconazole but was lower than that of fluconazole.

Effects on other P-450s of rat liver. To further ascertain the selective inhibition of Ro 09-1470 against fungal P-450$_{14DM}$, we checked its activity against other P-450s. Table 2 represents the IC_{50}s of Ro 09-1470 and the azole antifungal agents against other rat liver P-450s involved in xenobiotic metabolism: *p*-nitroanisol O-demethylation, aniline hydroxylation, and aminopyrine N-demethylation. The inhibitory activities of Ro 09-1470 against all of the three reactions investigated in this study were weaker than those of fluconazole and ketoconazole.

Ro 09-1470 induced a typical type II spectral change in the P-450 of hepatic microsomes prepared from nontreated rats, revealing a difference spectrum with the absorption maximum and minimum at 428 and 392 nm, respectively. Upon spectral titration, linear Hanes-Wilkinson plots (6) were obtained with Ro 09-1470, ketoconazole, and fluconazole (Fig. 2). The K_m value of Ro 09-1470 (39.8 μM) was clearly higher than that of ketoconazole (2.6 μM) and similar to that of fluconazole (39.7 μM). The K_m value of ketoconazole for nontreated microsomes in our experiments was slightly

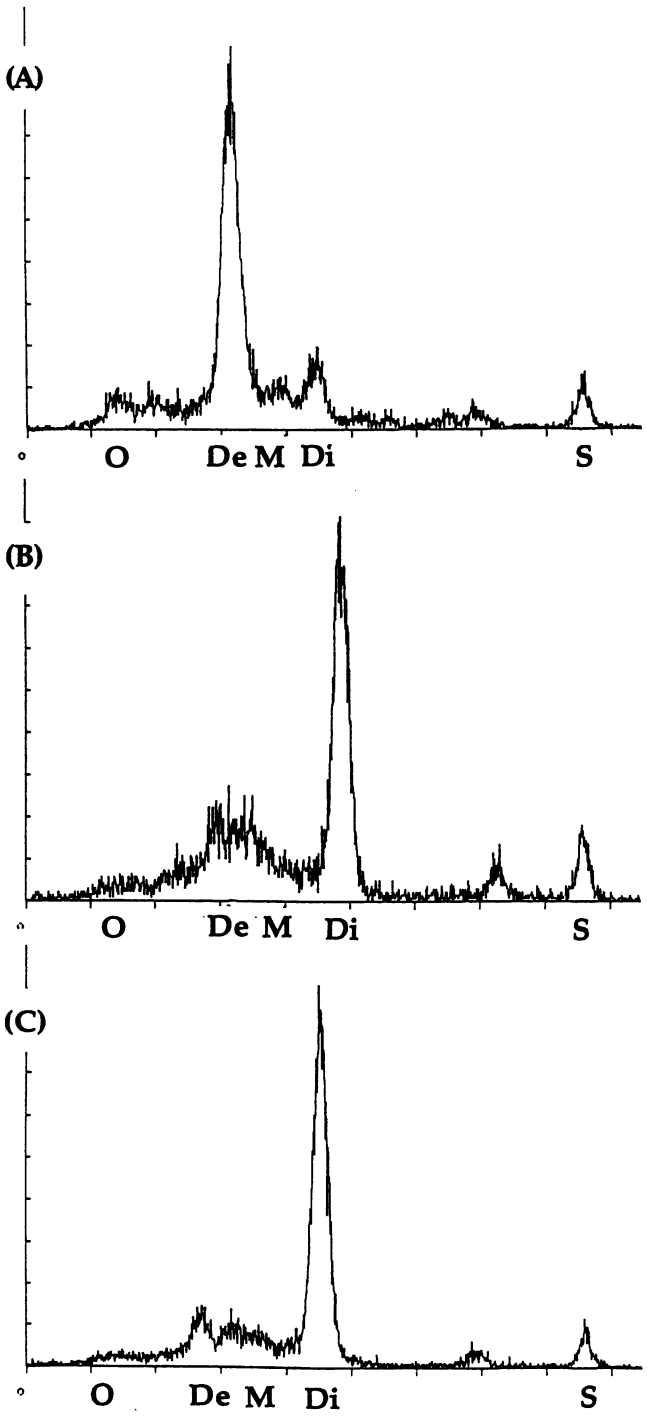

FIG. 1. Effects of Ro 09-1470 and ketoconazole on in vitro cholesterol biosynthesis of rat liver CE: incorporation of [^{14}C]mevalonate into squalene and sterols in the CE of livers from rats treated with cholestyramine. (A) Solvent control; (B) 600 μM Ro 09-1470; (C) 15 μM ketoconazole. Peaks O, De, M, Di, and S represent the origin, 4,4-desmethylsterols, 4α-methylsterols, 4,4-dimethylsterols, and squalene, respectively.

higher than those for the phenobarbital-induced (0.9 μM) and 3-methylcholanthrene-induced (0.8 μM) microsomes reported by Rodrigues et al. (20), while our maximum change in optical density value (0.060/nmol of P-450) was almost the same as theirs (0.050/nmol of P-450). For Ro 09-1470 and fluconazole, the maximum change in optical density values were 0.013 and 0.015/nmol of P-450, respectively.

TABLE 1. Inhibition of fungal and rat hepatic P-450$_{14DM}$ by Ro 09-1470 and azole antifungal agents

Compound	IC_{50} (μM) for P-450$_{14DM}$ from:			Selectivity ratio (rat liver/*C. albicans*)
	Rat liver[a]	*C. albicans*[b]	*S. cerevisiae*[b]	
Ro 09-1470	341	0.47	1.1	726
Fluconazole	1,853	0.065	0.17	28,500
Genaconazole	137	0.12	ND	1,143
Itraconazole	1.98	0.18	ND	11
Ketoconazole	0.46	0.038	0.011	12
Miconazole	0.96	0.32	ND	3

[a] Inhibition of the synthesis of 4,4-desmethylsterol was measured.
[b] Activity of P-450$_{14DM}$ was directly measured. ND, not done.

Interaction with *S. cerevisiae* P-450$_{14DM}$. Ketoconazole was reported to bind to P-450$_{14DM}$ at a ratio of 1:1 (27). We investigated the stoichiometry in the Ro 09-1470 binding to P-450$_{14DM}$. As we have indicated previously (1), Ro 09-1470 induced a marked spectral change in ferric P-450$_{14DM}$ by coordinating to the heme. Figure 3 represents a result of the spectral titration of *S. cerevisiae* P-450$_{14DM}$ with Ro 09-1470 and ketoconazole. The spectral changes were linearly dependent on the amount of Ro 09-1470 and ketoconazole and were saturated when equimolar Ro 09-1470 (0.355 μM) and ketoconazole (0.328 μM) were added to P-450$_{14DM}$ (0.310 μM). This observation clearly indicated that Ro 09-1470 and P-450$_{14DM}$ formed a one-to-one complex, as did ketoconazole and the enzyme.

Inhibition of CO binding to *S. cerevisiae* P-450s. Reduced azole antifungal agent complexes with P-450$_{14DM}$ could be formed by reducing the corresponding ferric complexes with sodium dithionite. The interaction between azole antifungal agents and ferrous P-450$_{14DM}$, however, is very weak when P-450 heme is reduced prior to the addition of the antifungal agents (27). We compared the ability of Ro 09-1470 and azole antifungal agents to inhibit CO binding to ferric and ferrous P-450$_{14DM}$ (Table 3). The CO difference spectrum of the dithionite-reduced P-450 in the microsomal preparation of *S. cerevisiae* showed maximum absorption at 448 nm, as reported by Vanden Bossche et al. (24). Ro 09-1470 showed inhibitory activity against both ferric and ferrous P-450$_{14DM}$s with similar affinities, whereas ketoconazole and fluconazole inhibited ferrous P-450$_{14DM}$ with much lower affinity, as reported by other researchers (27).

TABLE 2. Inhibition of rat hepatic drug-metabolizing P-450s by Ro 09-1470 and azole antifungal agents

Assay system	IC_{50} (μM) of:		
	Ro 09-1470	Ketoconazole	Fluconazole
p-Nitroanisol O-demethylation	>1,000	693	>1,000
Aniline hydroxylation	>1,000	28	278
Aminopyrine N-demethylation	920	77	212

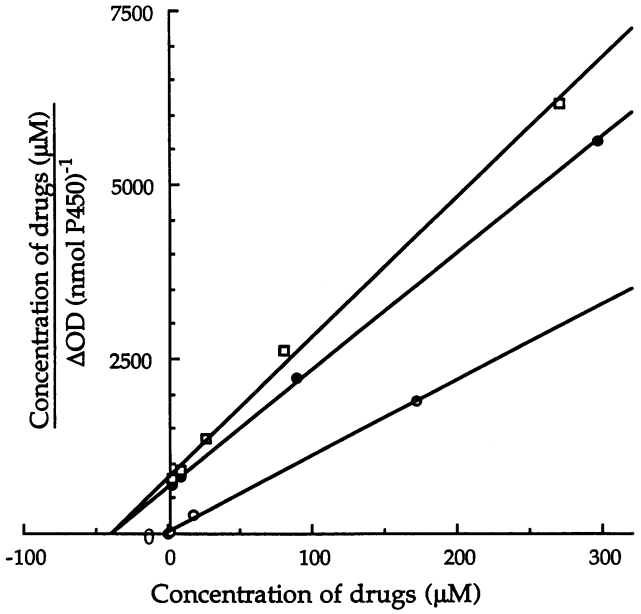

FIG. 2. Hanes-Wilkinson plot of the difference spectrum of rat liver microsome induced by Ro 09-1470 (□), ketoconazole (○), and fluconazole (●).

DISCUSSION

To identify a safe P-450$_{14DM}$ inhibitor as an antifungal agent, it is important to evaluate the selectivity of such an inhibitor for the fungal enzyme.

The P-450$_{14DM}$-inhibiting Ro 09-1470 structurally differs from the azole antifungal agents in that it has glycine as a coordinating group to heme. Its ability to inhibit rat hepatic P-450$_{14DM}$, which has a key role in cholesterol biosynthesis, was stronger than that of fluconazole (triazole), similar to that of genaconazole (triazole), and weaker than those of itraconazole (triazole), ketoconazole (imidazole), and miconazole (imidazole). Therefore, it seems that the inhibitory activity against this enzyme does not depend on the type of heme-coordinating group in the inhibitors.

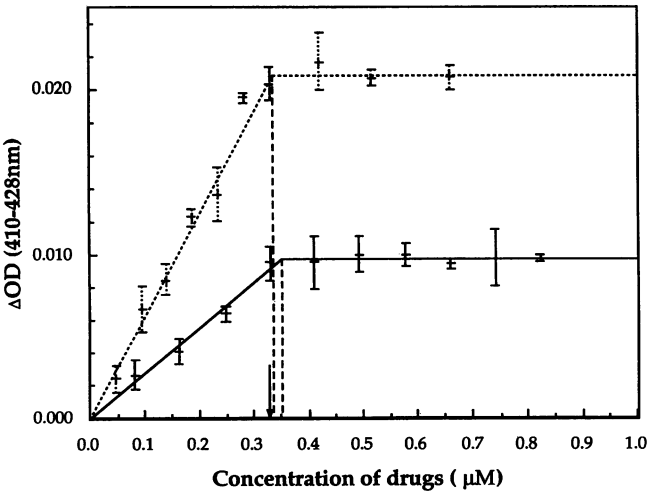

FIG. 3. Titration of the difference spectrum of *S. cerevisiae* microsome induced by Ro 09-1470 (solid line) and ketoconazole (dashed line). The arrow indicates the concentration of P-450 used.

TABLE 3. Inhibition of CO-binding to P-450 in *S. cerevisiae* microsome fractions by Ro 09-1470 and azole antifungal agents

Compound	IC_{50} (μM) for:		Ratio (ferrous/ ferric)
	Ferric P-450	Ferrous P-450	
Ro 09-1470	0.47	1.9	4
Ketoconazole	0.062	111	1,790
Fluconazole	0.19	23	121

The rank order of selectivity for fungal P-450$_{14DM}$ and mammalian P-450$_{14DM}$ was essentially the same as that for anti-rat hepatic P-450$_{14DM}$ activity. Itraconazole, ketoconazole, and miconazole are known to be lipophilic compounds, whereas fluconazole, genaconazole, and Ro 09-1470 are hydrophilic (data not shown). The lipophilic azoles exhibited lower enzyme selectivity than the hydrophilic ones. That difference, however, would not be explained by a higher concentration of the lipophilic compounds in membranes that might occur because of high partition coefficients, because microsomes were used in both fungal and rat liver assays and the membrane distribution efficiency of each compound was compensated for by calculating the selectivity ratios.

It has been reported that ketoconazole and miconazole have similar inhibitory activities against rat and human hepatic P-450$_{14DM}$s, with IC_{50}s ranging from 0.5 to 1 μM (18), but fluconazole does not (23). Furthermore, the kinetic parameters of rat and human hepatic P-450$_{14DM}$s were very similar (18). Under our assay conditions in which ketoconazole and miconazole showed strong inhibitory activities against rat hepatic P-450$_{14DM}$ and fluconazole did not, Ro 09-1470 exhibited a moderate inhibition. Ro 09-1470 is thus expected to have lower inhibitory activity against the human enzyme than do the azole antifungal agents, except for fluconazole.

The inhibitory potency of azole antifungal agents against mammalian P-450s is by no means restricted to P-450$_{14DM}$, since inhibition also occurs against a number of cytochrome P-450 activities, including the hepatic microsomal metabolism of a variety of xenobiotic compounds. Ketoconazole, miconazole, and clotrimazole, all nonplanar molecules, were especially more potent inhibitors of the phenobarbital-inducible cytochromes P-450 (in the rat, cytochromes P-450$_{2B1}$ and P-450$_{2B2}$), which interact with globular molecules, than of the polyaromatic hydrocarbon-inducible cytochromes P-448 (in the rat, P-450$_{1A1}$ and P-450$_{1A2}$), which interact essentially with planar molecules but not with bulky members among the two main P-450 groups of gene families (14). We investigated the effects of Ro 09-1470 and azole antifungal agents on xenobiotic metabolism using *p*-nitroanisol, aniline, and aminopyrine as substrates. Ketoconazole showed more potent inhibition against aminopyrine N-demethylation catalyzed mainly by P-450$_{2B1}$ than against *p*-nitroanisol O-demethylation catalyzed by P-450$_{1A1}$ (21), which agreed with the phenomena described above. Under these conditions, the tendency of the inhibition profile of Ro 09-1470, a nonplanar molecule, was essentially similar to that of ketoconazole but the inhibitory activity of Ro 09-1470 was clearly lower than those of both fluconazole and ketoconazole.

As assumed from the spectrophotometric estimation of the affinity to P-450s in rat liver microsomes, Ro 09-1470 inter-

acted with lower affinity than did ketoconazole but with almost the same activity as did fluconazole.

We have demonstrated in this paper that, on the whole, Ro 09-1470 is a potent inhibitor of fungal P-450_{14DM} but interacts with only low affinity against rat hepatic cytochrome P-450s involved in cholesterol biosynthesis and xenobiotic metabolism. This suggests that Ro 09-1470 might have a larger therapeutic window, if pharmacokinetics are not otherwise different.

In this report we have also demonstrated that Ro 09-1470 combined with ferric P-450_{14DM} heme with one-to-one stoichiometry, as did ketoconazole (27). The difference spectrum of Ro 09-1470 was also superimposable on that of ketoconazole (1), meaning that the spectral change induced by Ro 09-1470 was the same as that induced by ketoconazole. Ketoconazole was reported to induce a marked type II spectral change of ferric P-450_{14DM}, which was characterized by redshifts and hypochromicity of the Soret and α bands and marked enhancement of the δ band (27). This type of spectral change is characteristic for the binding of a basic amino nitrogen to the sixth coordination position of the heme iron (26, 28). It can thus be concluded that the amino nitrogen in the glycine moiety of Ro 09-1470 bound to the sixth coordination position of the heme iron of P-450_{14DM}.

The magnitudes of the difference spectra in the presence of saturated concentrations of compounds were quite different from one another in our experiments (see Materials and Methods). Two imidazoles (ketoconazole and miconazole) induced the largest difference in the heme spectrum, followed by the triazoles (fluconazole and itraconazole) and the glycine-containing compound (Ro 09-1470). It was reported that the triazole compound-induced difference spectrum was smaller than that induced by the imidazole compound (27). Our experiments further showed that these differences must depend on the physicochemical properties, such as the basicity, of the coordinating groups of the compounds.

A Ro 09-1470 complex of ferrous P-450_{14DM} interfered with CO binding (Table 3). The rank order of IC_{50}s of Ro 09-1470, ketoconazole, and fluconazole was essentially the same as that of P-450_{14DM} inhibition (Table 1). It was reported that the inhibitory effects on demethylation activity by azole antifungal agents could be presumed by their ability to interfere with the binding of CO to ferrous P-450_{14DM} (24). This also seems to be the case for other coordinating groups.

Ro 09-1470 could directly interact with both ferrous and ferric forms of P-450_{14DM} with a similar affinity, whereas ketoconazole and fluconazole showed a difference (Table 3). Yoshida and Aoyama (27) discussed in their report that larger-molecular azole antifungal agents such as ketoconazole and itraconazole could not interact directly with ferrous P-450_{14DM} and suggested that the crevice of the ferrous enzyme is narrower than that of the ferric enzyme. The fact that the small compound fluconazole also show low affinity for ferrous P-450_{14DM}, however, suggested that (i) the low affinity for the ferrous enzyme of larger-molecule azoles cannot be simply ascribed to the bulkiness of such compounds in the crevice or (ii) the Ro 09-1470 molecule is so small or flexible that it can slip into the active site through the narrow crevice, which is too narrow even for fluconazole.

Although the coordination with heme is similar for both Ro 09-1470 and the azole antifungal agents, this fact alone cannot lead to the conclusion that the modes of interaction are also similar. Further investigation is certainly needed to understand the precise binding topography and the action mode.

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