Inhibitory Effects of Ketoconazole and Miconazole on Cytochrome P-450-Mediated Oxidative Metabolism of Testosterone and Xenobiotics in Mouse Hepatic Microsomes — Comparative Study with Cimetidine

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The inhibitory effects of ketoconazole (KCZ) and miconazole (MCZ), imidazole containing antimycotics, on the hydroxylations of testosterone as a model for endogenous steroids, and the N-demethylation of aminopyrine and the hydroxylation of aniline as models for xenobiotics were compared with those of cimetidine in mouse hepatic microsomes. *In vitro*, both KCZ and MCZ inhibited these enzyme activities in a dose-dependent manner. The inhibitory potencies of KCZ and MCZ for testosterone hydroxylations and aminopyrine N-demethylation were much greater, with 50% inhibition concentration (IC_{50}) values 2—3 orders of magnitude lower than those of cimetidine, while the potencies of these antimycotics for aniline hydroxylation were similar to that of cimetidine.

Although KCZ, MCZ and cimetidine produced type II difference spectra, the difference between the antimycotics (405 nm) and cimetidine (392—405 nm) was found in the trough position of the difference spectra. Spectral dissociation constants (K_s) of these antimycotics ($2.2 \times 10^{-7} - 5.4 \times 10^{-6}$ M) were also 1—2 orders of magnitude lower than those of cimetidine ($1.3 \times 10^{-5} - 1.6 \times 10^{-4}$ M), and both KCZ and cimetidine had two kinds of K_s , while MCZ had a single K_s .

Pentobarbital sleeping time was prolonged in a dose-dependent manner by the i.p. administration of 10—50 mg/kg of KCZ, MCZ or cimetidine, and the potencies for the prolongation of sleeping time decreased in the order of MCZ > KCZ > cimetidine.

These results indicated that the inhibitory potencies of KCZ and MCZ for the cytochrome P-450-mediated oxidative metabolism of testosterone and xenobiotics in hepatic microsomes were greater than those of cimetidine, and that the affinities of KCZ and MCZ for some cytochrome P-450 species in hepatic microsomes differed from one another.

Keywords — ketoconazole; miconazole; cimetidine; antimycotics; imidazole derivative; testosterone hydroxylation; aminopyrine N-demethylation; aniline hydroxylation; cytochrome P-450; drug-metabolizing enzyme

Introduction

Ketoconazole (KCZ) and miconazole (MCZ), imidazole containing antimycotics, are used for the therapy of systemic fungal infections, orally and intravenously, respectively (Fig. 1). It has been shown that the pharmacological actions of KCZ and MCZ appear *via* the specific inhibition of 14-demethylation of sterol, which is catalyzed by cytochrome P-450, thereby preventing the conversion of lanosterol to ergosterol in fungi.^{1,2)}

KCZ has been shown to inhibit a number of cytochrome P-450-dependent steroidogenic enzyme activities in gonadal, adrenal and testicular tissues.³⁻⁶⁾ Recently, it has also been shown that KCZ is a potent inhibitor of the oxidative drug-metabolizing enzyme system in

hepatic microsomes⁷⁻¹⁰⁾ and that it affects the clearance of some coadministered drugs in man, *e.g.*, cyclosporine, antipyrine and warfarin.¹¹⁻¹⁵⁾ On the other hand, it is well known that this enzyme system mediates the hydroxylations of various endogenous steroids, such as testosterone and cortisol. Therefore, it is possible that these antimycotics may also have inhibitory effects on cytochrome P-450 species catalyzed oxidations of steroids in hepatic microsomes.

Cimetidine, an imidazole derivative which is widely used for the treatment of peptic ulcer, has been demonstrated to exhibit inhibitory activities for this enzyme system *in vitro* and *in vivo*,¹⁶⁻¹⁹⁾ and affect the oxidative metabolism of a number of clinical drugs.²⁰⁻²³⁾ Previously, we demonstrated that cimetidine inhibits testosterone hydroxylations in mouse

Fig. 1. Chemical Structures of the Drugs Tested

hepatic microsomes²⁴⁾ and cortisol hydroxylation in man.^{25,26)} Therefore, a comparison of cimetidine with certain test drugs seems to be fundamentally significant for elucidating for their inhibitory potencies for the cytochrome P-450-mediated oxidative metabolism of endogenous and exogenous substrates in hepatic microsomes.

The aims of the present study were: to define the inhibitory potencies of KCZ and MCZ on the oxidative metabolism of xenobiotics and testosterone as a model of endogenous steroids in mouse hepatic microsomes; and to ascertain the relative inhibitory potentials between these antimycotics and cimetidine.

Materials and Methods

Materials — The drugs used were kindly supplied by the following companies: KCZ from Kyowa Hakko Co., Ltd., Tokyo, MCZ from

Mochida Pharmaceutical Co., Ltd., Tokyo and cimetidine from Smith Kline and Fujisawa Co., Ltd., Tokyo. Testosterones, aminopyrine and aniline were purchased from Nakarai Chemical Co., Ltd., Kyoto. All other chemicals and solvents were of analytical grade.

Preparation of Mouse Hepatic Microsomes

— Livers from male ddY mice (4–6 weeks old) were homogenized in ice-cold 1.15% KCl (1:4, w/v) and the homogenate was centrifuged at $10000 \times g$ for 15 min. The supernatant fraction was centrifuged at $105000 \times g$ for 60 min to obtain a microsomal pellet. The microsomes were suspended in 0.1 M sodium-potassium phosphate buffer, pH 7.4, to a concentration of 2–5 mg protein/ml. Protein was determined by the method of Lowry *et al.*²⁷⁾

Enzyme Assay — The activities of testosterone 6 β -, 7 α - and 16 α -hydroxylases, aminopyrine N-demethylase and aniline hydroxylase were determined in a cofactor mixture with a final concentration of 5 mM MgCl₂, 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, enzyme preparation (0.5 mg protein/ml), substrate (25 μ M testosterone, 5 mM aminopyrine or 5 mM aniline), 67.5 mM sodium-potassium phosphate buffer, pH 7.4, KCZ or MCZ (0.05 – 100 μ M) or cimetidine (0.01 – 10 mM) to make a final volume of 1 ml, except for the assay of testosterone hydroxylase activities where the final volume was increased to 5 ml. Antimycotics and cimetidine were dissolved in ethanol and the same buffer, respectively, and added to the incubation mixture to make the final concentration described above. The amount of ethanol added to the incubation mixture was 0.1% (v/v). The same volume of solvent was added to the control. The reaction was started by adding the microsomal suspension and was carried out at 37 °C with shaking for 5 min for aminopyrine N-demethylation, 10 min for testosterone hydroxylation and 20 min for aniline hydroxylation. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid, except for the assay of testosterone hydroxylations where 20 ml of dichloromethane was added. Testosterone 6 β -, 7 α -, and 16 α -hydroxylase activities were as-

sayed as described in a previous paper.²⁴⁾ Aminopyrine N-demethylase activity was determined by measuring the rate of formaldehyde formation according to the method of Nash.²⁸⁾ Aniline hydroxylase activity was measured by the method of Imai *et al.*²⁹⁾

Spectrophotometric Determination of the Binding of Antimycotics and Cimetidine to Cytochrome P-450 — Microsomes suspended in 0.1 M sodium-potassium phosphate buffer, pH 7.4, (2 mg protein/ml) were divided into a sample and a reference cuvette. After recording the base line using a double beam spectrophotometer (Shimadzu, Model UV-300), the samples were titrated with an ethanol solution of KCZ or MCZ, or the same buffer solution as that for cimetidine. An equivalent volume of the solvents was added to the reference cuvette. Spectra were recorded between 360 and 500 nm and the absorbance peak minus trough values were used to construct double-reciprocal plots. In the case of non-linear plots, the spectral dissociation constants (K_s) were calculated with an extended least squares non-linear regression program (MULTI)³⁰⁾ run on a microcomputer (NEC, Model PC-9801VM2).

Measurement of Pentobarbital Sleeping Time — KCZ and cimetidine were dissolved in saline containing 0.1 N HCl (final pH adjusted to 3.0 with 0.1 N NaOH) and MCZ was dissolved in corn oil. They were administered as intraperitoneal injections. Pentobarbital sodium dissolved in saline (40 mg/kg) was injected intraperitoneally 30 min after treatment with a single dose of 10, 30 or 50 mg/kg of the drug or vehicles alone.

Results

Inhibitory Potencies of KCZ, MCZ and Cimetidine for the Oxidative Metabolism of Testosterone, Aminopyrine and Aniline in Mouse Hepatic Microsomes *in Vitro*

As shown in Fig. 2, KCZ and MCZ markedly inhibited the 6 β -, 7 α - and 16 α -hydroxylations of testosterone in a dose-dependent manner. Comparing the inhibitory potencies of KCZ and MCZ for these enzyme activities at their 50% inhibition concentrations (IC_{50}), the differences in the potencies of KCZ and MCZ for each enzyme were found: that is, the inhibitory potencies of KCZ for the 6 β - and 7 α -hydroxylati

Fig. 2. Dose-Related Inhibition of Testosterone 6 β - (A), 7 α - (B) and 16 α - (C) Hydroxylations in Mouse Hepatic Microsomes by KCZ and MCZ

Each curve is the mean $\pm$ S. D. of experiments with three different microsomal preparations and is presented as a percentage of control activities. The control activities (nmol/mg protein/min; mean $\pm$ S.D.) were: production of 6 β -hydroxytestosterone (3.61 ± 0.16); production of 7 α -hydroxytestosterone (0.77 ± 0.03); production of 16 α -hydroxytestosterone (1.55 ± 0.07). Symbols: $\bullet$, KCZ; $\circ$, MCZ.

Fig. 3. Dose-Related Inhibition of Aminopyrine N-Demethylation (A) and Aniline Hydroxylation (B) in Mouse Hepatic Microsomes by KCZ, MCZ and Cimetidine

Each curve is the mean $\pm$ S.D. of experiments with three different microsomal preparations and is presented as a percentage of control activities. The control activities (nmol/mg protein/min; mean $\pm$ S.D.) were: production of HCHO (9.9 ± 1.8); production of *p*-aminophenol (1.40 ± 0.21). Symbols: $\bullet$, KCZ; $\circ$, MCZ; $\triangle$, cimetidine.

ons were greater than those of MCZ, and the potency of KCZ for the 16α -hydroxylation was much less than that of MCZ. On the other hand, the IC_{50} values of these antimycotics for these enzyme activities were about 3–4 orders of magnitude lower than those of cimetidine (6×10^{-4} M to 10^{-3} M more)¹⁴⁾ which were found

under the same experimental conditions as used in this study. In addition, as shown in Fig. 3, KCZ, MCZ and cimetidine markedly inhibited aminopyrine N-demethylation and aniline hydroxylation in a dose-dependent manner. The data clearly show the extraordinary inhibitory potencies of KCZ and MCZ for aminopyrine N-

TABLE I. Binding and Inhibitory Potencies of KCZ, MCZ and Cimetidine Toward Cytochrome P-450-Mediated Mixed-Function Oxidase System in Mouse Hepatic Microsomes

	50% inhibition concentration (M)					Dissociation constants (M)	
	6 β -Hydroxylation	Testosterone 7 α -Hydroxylation	16 α -Hydroxylation	Aminopyrine N-demethylation	Aniline hydroxylation	K_{s1}	K_{s2}
Cimetidine	6×10^{-4} a)	$>10^{-3}$ a)	$>10^{-3}$ a)	1×10^{-3}	4×10^{-4}	1.3×10^{-5}	1.6×10^{-4}
Ketoconazole	8×10^{-8}	3×10^{-7}	5×10^{-6}	5×10^{-6}	1×10^{-4}	2.2×10^{-7}	5.4×10^{-6}
Miconazole	1.5×10^{-7}	8×10^{-7}	5×10^{-7}	8×10^{-7}	1×10^{-4}	9.5×10^{-7}	ND

a) The values from previous paper.²⁴⁾ ND, Not detected.

Fig. 4. Spectral Changes of Mouse Hepatic Microsomal Cytochrome P-450 Caused by the Addition of KCZ (A), MCZ (B) and Cimetidine (C)

Difference spectra were recorded after the successive additions of $0.05 \mu\text{M}$ (a), $0.2 \mu\text{M}$ (b), $1 \mu\text{M}$ (c), $5 \mu\text{M}$ (d), $10 \mu\text{M}$ (e), $50 \mu\text{M}$ (f), $100 \mu\text{M}$ (g) and $200 \mu\text{M}$ (h) of drugs.

demethylation: that is, their IC_{50} values for this enzyme activity were about 2–3 orders of magnitude lower than that of cimetidine. In addition, the potency of MCZ for this enzyme activity was greater than that of KCZ. On the other hand, the potencies of KCZ and MCZ for aniline hydroxylation were relatively weak and the differences in the IC_{50} values between these antimycotics and cimetidine for this enzyme activity were relatively small. The IC_{50} values obtained are summarized in Table I.

Spectrophotometric Changes in the Binding of KCZ, MCZ and Cimetidine to Cytochrome P-450

Figure 4 shows the difference spectra induced by the addition of antimycotics and cimetidine to mouse hepatic microsomes. All had absorption maxima (peak) at 428 nm and the absorption minima (trough) at 392–405 nm, characteristic of a type II spectral change.³¹⁾ The intensity of the spectral changes caused by KCZ or MCZ were about 3-fold greater than that of

cimetidine. Furthermore, there was a difference between the antimycotics and cimetidine at the trough in the difference spectra. The trough in the cimetidine-induced difference spectrum was displaced from 405 nm to 392 nm, depending upon increase in the concentration, while the troughs in the KCZ- or MCZ-induced spectra became apparent at 405 nm, depending upon increase in the concentration. These spectral changes were converted to double-reciprocal plots in order to obtain the dissociation constants (K_s). As shown in Fig. 5, the plots for KCZ and cimetidine were biphasic and two K_s values (K_{s1} and K_{s2}) were obtained, while the plot for MCZ was a single line. The K_s values obtained are also summarized in Table I. In agreement with their higher inhibitory potencies, the results show that KCZ and MCZ have higher affinities for cytochrome P-450 in hepatic microsomes, because the K_s values of the antimycotics were 1–2 orders of magnitude lower than those of cimetidine.

Fig. 5. Double-Reciprocal Plots of the Changes in Absorbance Produced by the Addition of Various Concentrations of KCZ (A), MCZ (B) and Cimetidine (C)

Bindings of antimycotics and cimetidine to cytochrome P-450 were estimated from the absorbance difference between the peak (428 nm) and the trough (392–405 nm). Each value is the mean of experiments with three different mouse hepatic microsomal preparations.

Fig. 6. Effects of KCZ, MCZ and Cimetidine on Pentobarbital Sleeping Time

Mice were treated with single doses of 10, 30 and 50 mg/kg of KCZ (cross-hatched), MCZ (solid black) or cimetidine (dotted), or vehicles alone (A, saline, pH 3.0; B, corn oil). 40 mg/kg of pentobarbital sodium was injected i.p. 30 min after the i.p. injection of each drug. Value is the mean $\pm$ S.D. from 5–6 animals.

a) Significantly different from the appropriate control ($p < 0.01$).

Changes in Pentobarbital Sleeping Time by the Treatment with Antimycotics and Cimetidine

As shown in Fig. 6, when mice were treated with a single dose of 10–50 mg/kg of KCZ, MCZ or cimetidine, pentobarbital sleeping time was prolonged in a dose-dependent manner. The potencies for the prolongation of sleeping time decreased in the order of MCZ > KCZ > cimetidine.

Discussion

It has been demonstrated that KCZ inhibits testosterone biosynthesis by blocking mainly the testicular microsomal cytochrome P-450-dependent C_{17-20} lyase enzyme complex.⁵⁾ In the present study, we demonstrated that KCZ also inhibited the oxidative metabolism of testosterone in hepatic microsomes *in vitro*, in addition to its known inhibitory action on testosterone biosynthesis (Fig. 2). The inhibitory effects of MCZ on these enzyme activities were generally similar to those of KCZ. The inhibitory potencies of these antimycotics for testosterone hydroxylations are also believed to be much greater than those of cimetidine. Previously, we

found that the inhibitory effects of cimetidine on testosterone hydroxylations in mouse hepatic microsomes are essentially due to its imidazole ring structure²⁴⁾ and the conversion of cortisol to 6 β -hydroxycortisol in human liver is inhibited by the administration of 800 mg/d of cimetidine.^{25,26)} Judging from the similar therapeutic plasma peak levels among KCZ (4–40 μ M),³²⁾ MCZ (2 – 9 μ M)³³⁾ and cimetidine (4 – 8 μ M),^{34,35)} our findings suggest that KCZ and MCZ, 1-imidazole derivatives, may exhibit inhibitory effects on the hepatic oxidative metabolism of various endogenous steroids in man, such as testosterone and cortisol.

Both KCZ and MCZ have also been found to exhibit potent inhibitory effects on aminopyrine N-demethylation and aniline hydroxylation in hepatic microsomes *in vitro*, however, the differences in the inhibitory potencies between antimycotics and cimetidine for aniline hydroxylation were less than those for aminopyrine N-demethylation (Fig. 3 and Table I). The weaker inhibitory potencies of KCZ and MCZ for aniline hydroxylation obtained in this study agree with the *in vitro* and *in vivo* findings in a study which investigated the effects of KCZ on this enzyme reaction in rat.⁹⁾ Therefore, it is suggested that both KCZ and MCZ also markedly inhibit the many cytochrome P-450-mediated oxidative drug-metabolizing enzyme activities, moreover, they might have especially higher affinities for some cytochrome P-450 species in hepatic microsomes, in contrast to cimetidine which has relatively nonspecific affinities and weaker inhibitory potencies. Actually, although KCZ, MCZ and cimetidine produced essentially type II spectral changes which are strongly suggestive of the formation of a nitrogenous ligand to the cytochrome P-450,³¹⁾ *i.e.*, most likely the interaction of the imidazole groups in these drugs with the heme moiety, the differences between the antimycotics and cimetidine were found in the intensities and trough positions in their difference spectra (Fig. 4). It has been demonstrated that 1-alkylimidazole-induced type II difference spectra result from a combination of type I and type II changes, and when the type II compounds bind to a type I or type II site in cytochrome P-450, the troughs in the difference

spectra are observed around 410 nm or 390 nm, respectively.³⁶⁾ Accordingly, this suggests that at lower concentrations, cimetidine first binds to a type I site. Later, it binds to a type II site according to the increase in its concentration, whereas the binding of KCZ and MCZ to a type I site is enhanced according to the increases in their concentrations. This finding might be one of the reasons for the extraordinary inhibitory potencies of these antimycotics for the oxidative metabolism of type I compounds such as testosterone and aminopyrine.³⁷⁾

On the other hand, certain differences between the inhibitory potencies of KCZ and MCZ for testosterone hydroxylations and aminopyrine N-demethylation were found (Figs. 2 and 3, Table I). It was reported that separate enzyme systems catalyze the hydroxylations of testosterone in microsomes,³⁸⁾ and that different cytochrome P-450 isozymes hydroxylate testosterone at the 6 β -, 7 α - and 16 α -positions in mouse hepatic microsomes.³⁹⁾ The IC₅₀ values of KCZ for these four enzyme activities had a more broader range (9×10^{-8} – 5×10^{-6} M) than those of MCZ (1.5×10^{-7} – 8×10^{-7} M). These results parallel the results obtained in the spectrophotometric binding study of these antimycotics for cytochrome P-450: differences in the kind of binding site in cytochrome P-450 among them were found, and the affinities were found to decrease in the order of KCZ (K_{s1}) > MCZ (K_{s1}) > KCZ (K_{s2}) (Fig. 5 and Table I). Consequently, these findings indicate that KCZ and MCZ differ from one another in their affinities for some cytochrome P-450 species. Thus, this remains to be investigated in detail through kinetic analysis of enzyme inhibitions by these antimycotics.

The prolongation of pentobarbital sleeping time after i.p. administrations of antimycotics or cimetidine were found (Fig. 6). Although it is not clear whether the interaction between these drugs tested and pentobarbital on the absorption site occurs, the potent inhibitory activities of KCZ and MCZ obtained *in vitro* study may be reflected in the changes in pentobarbital sleeping time *in vivo*: that is, the results suggest that the inhibitory potencies of KCZ and MCZ for hepatic oxidative drug metabolism *in vivo* are also

greater than that of cimetidine. However, because it has been reported that KCZ has no effect on theophylline clearance in man, and is not, in general, an inhibitor of hepatic oxidative drug metabolism as cimetidine appears to be,⁴⁰⁾ further detailed investigations involving the differences among mouse and humans are required.

In conclusion, the present study suggests that the affinities of KCZ and MCZ for some cytochrome P-450 species differ from one another and that these antimycotics are more potent inhibitors of the cytochrome P-450-mediated oxidative metabolism of endogenous steroids and xenobiotics in hepatic microsomes than is cimetidine. Attention concerning the interaction between these antimycotics and endogenous or exogenous substrates for hepatic microsomal cytochrome P-450 may be necessary at the onset of therapy.

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