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^{-8} 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 steroidaligenic 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
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Fig. 1. Chemical Structures of the Drugs Tested

hepatic microsomes and cortisol hydroxylation in man. 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. Testosterons, 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 × g for 15 min. The supernatant fraction was centrifuged at 105000 × 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.

Enzyme Assay — The activities of testosterone 6β-, 7α- and 16α-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 μM testosterone, 5 mM aminopyrine or 5 mM aniline), 67.5 mM sodium-potassium phosphate buffer, pH 7.4, KCZ or MCZ (0.05 – 100 μM) or cimetidine (0.01 – 10 mM) to make a final volume of 1 ml, except for the assay of testosterone hydroxylase activities were 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 6β-, 7α-, and 16α-hydroxylase activities were as-
sayed as described in a previous paper. Ami-
noptyrin N-demethylase activity was de-
temined by measuring the rate of formalde-
yde formation according to the method of Nash. Ani-
line hydroxylase activity was measured by the
method of Imai et al.

Spectrophotometric Determination of the
Binding of Antimycotics and Cimetidine to Cyto-
chrome 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 spectropho-
tometer (Shimadzu, Model UV-300), the sam-
ples 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 disso-
ciation constants ($K_d$) were calculated with an
extended least squares non-linear regression
program (MULTI) 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 intra-
peritoneal injections. Pentobarbital sodium dis-
solved in saline (40 mg/kg) was injected intra-
peritoneally 30 min after treatment with a single
dose of 10, 30 or 50 mg/kg of the drug or vehi-
cles alone.

Results

Inhibitory Potencies of KCZ, MCZ and Cime-
tidine for the Oxidative Metabolism of Testos-
erone, Aminopyrine and Aniline in Mouse
Hepatic Microsomes in Vitro

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

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

Each curve is the mean ± S. D. of experiments with three different microsomal preparations and is presented as a per-
centage of control activities. The control activities (nmol/mg protein/min; mean ± S.D.) were: production of 6β-
hydroxytestosterone (3.61 ± 0.16); production of 7α-hydroxytestosterone (0.77 ± 0.03); production of 16α-hydroxytes-
tosterone (1.55 ± 0.07). Symbols: ○, KCZ; , MCZ.
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 ($6 \times 10^{-4}$ M to $10^{-3}$ M more) 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

<table>
<thead>
<tr>
<th></th>
<th>50% inhibition concentration (M)</th>
<th>Dissociation constants (M)</th>
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<tr>
<td></td>
<td>6β-Hydroxylation</td>
<td>7α-Hydroxylation</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>$6 \times 10^{-4}$ a)</td>
<td>$&gt;10^{-3}$ a)</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>$8 \times 10^{-8}$</td>
<td>$3 \times 10^{-7}$</td>
</tr>
<tr>
<td>Miconazole</td>
<td>$1.5 \times 10^{-7}$</td>
<td>$8 \times 10^{-7}$</td>
</tr>
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a) The values from previous paper.24) ND, Not detected.
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 antymycotics 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 an Cimetidine to Cytochrome P-450**

Figure 4 shows the difference spectra induced by the addition of antymycotics 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 antymycotics 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 antymycotics were 1—2 orders of magnitude lower than those of cimetidine.
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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.

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. 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 structure and the conversion of cortisol to 6β-hydroxycortisol in human liver is inhibited by the administration of 800 mg/d of cimetidine. Judging from the similar therapeutic plasma peak levels among KCZ (4–40 μM) and MCZ (2–9 μM) and cimetidine (4–8 μM), 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 antymycotics and cimetidine for aniline hydroxylation were less than those for aminopyrine N-demethylation (Fig. 3 and Table 1). 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. 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 noncovalent ligand to the cytochrome P-450, i.e., most likely the interaction of the imidazole groups in these drugs with the heme moiety, the differences between the antymycotics 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. Accordingly, this suggests that at lower concentrations, cimetidin 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 antymycotics for the oxidative metabolism of type I compounds such as testosterone and aminopyrine.

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 1). It was reported that separate enzyme systems catalyze the hydroxylations of testosterone in microsomes, and that different cytochrome P-450 isoforms hydroxylate testosterone at the 6β-, 7α- and 16α-positions in mouse hepatic microsomes. 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 antymycotics 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 (Ks1) > MCZ (Ks2) > KCZ (Ks2) (Fig. 5 and Table 1). 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 antymycotics.

The prolongation of pentobarbital sleeping time after i.p. administrations of antymycotics 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, 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 antymycotics 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 antymycotics and endogenous or exogenous substrates for hepatic microsomal cytochrome P-450 may be necessary at the onset of therapy.

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References


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