Effects of Antimycotics on Hepatic Steroid Metabolism

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When male ddY mice were treated with consecutive doses of 10 and 100 mg/kg of miconazole (MCZ) or ketoconazole (KCZ), imidazole-containing antimycotics, once a day for 3 d, a dose-dependent shortening of pentobarbital sleeping time was observed for MCZ, while no change in the sleeping time was observed for KCZ. Even at a low dose (10 mg/kg), MCZ significantly increased cytochrome P-450 content and reduced nicotinamide adenine dinucleotide phosphate cytochrome c reductase activity. Simultaneously, hydroxylase activities of testosterone as a model of endogenous steroids, and aminopyrine N-demethylase and 7-ethoxycoumarin O-deethylase activities were increased, while KCZ lacked inducing properties even at a high dose (100 mg/kg). The change in hepatic oxidative metabolism of cortisol (F) in a patient before, during and after treatment with progressively increasing doses of 2–10 mg/kg/d of MCZ for 14 d was examined by monitoring urinary 6β-hydroxy cortisol (6β-OHF), an oxidative metabolite of F. The ratio of 6β-OHF to F in 24-h urine decreased by 15% from the original level on day 1, and then it began to increase on day 7 to reach 2.4 times the original level on day 14. These results suggest that MCZ, but not KCZ, has inducing activity for hepatic cytochrome P-450-dependent oxidative metabolism of steroids and xenobiotics, in addition to its known inhibitory activity.

Keywords — miconazole; ketoconazole; antimycotic; imidazole derivative; cytochrome P-450; enzyme inhibition; enzyme induction; steroid hydroxylase; testosterone hydroxylation; 6β-hydroxycortisol

Introduction

Miconazole (MCZ) and ketoconazole (KCZ) are imidazole-containing antimycotics used for the therapy of systemic fungal infections, intravenously and orally, respectively. Previously, we reported that MCZ and KCZ are potent inhibitors of the oxidative metabolism of testosterone and xenobiotics in mouse hepatic microsomes, being more potent than cimetidine.1) Therefore, our finding suggests that these antimycotics also have inhibitory activities on the cytochrome P-450-dependent oxidative metabolism of some endogenous steroids such as testosterone, in addition to the known inhibitory action of KCZ on testosterone biosynthesis.2–4)

There have been several investigations regarding the inducing actions of these antimycotics for hepatic microsomal drug-metabolizing enzymes in animals. In the mouse, MCZ (50 mg/kg) had a biphasic effect on pentobarbital sleeping time which was prolonged after a 1-d treatment, but decreased after 3- and 5-d treatments, indicating an inducing effect.5) Furthermore, it has been reported that MCZ behaved as a phenobarbital-type inducer, whereas KCZ did not belong to either category of inducers (phenobarbital- and 3-methylcholanthrene-type inducers) for rat hepatic microsomal oxidative metabolism of xenobiotics.6) Therefore, it is possible that these antimycotics may also have inducing actions on cytochrome P-450-dependent oxidative metabolism of endogenous steroids such as testosterone and cortisol (F) in hepatic microsomes, in addition to their known extraordinary inhibitory actions on the hydroxylases of testosterone1,7) and androstenedione8) in vitro.

The purposes of the present study were to define the inducing effects of MCZ and KCZ on hepatic microsomal oxidation of testosterone as a model of endogenous steroids and xenobiotics in mice, and in part, to examine the change in hepatic oxidative conversion of F to 6β-hydroxycortisol (6β-OHF) in a patient under treatment with MCZ.

Materials and Methods

Materials — The drugs used were kindly
supplied by the following companies: MCZ from Mochida Pharmaceutical Co., Ltd., Tokyo; KCZ from Kyowa Hakko Co., Ltd., Tokyo; 6β-hydroxytestosterone from Teikoku Hormone Mfg. Co., Ltd., Tokyo; 7α-hydroxytestosterone from Shionogi Pharmaceutical Co., Ltd., Osaka. MCZ injection (Florid-F®, 200 mg/ampule) was obtained commercially. Testosterone, aminopyrine, aniline, 7-ethoxycoumarin and pentobarbital sodium were purchased from Nacalai Tesque Inc., Kyoto. 16α-Hydroxytestosterone was purchased from Sigma Chemical Co., Ltd., St. Louis, MO. All other chemicals and solvents used were of analytical grade.

**Treatment of Animals** — Male ddY mice (4–6 weeks old) were used. MCZ and KCZ were dissolved in corn oil and in saline containing 0.1 N HCl (final pH adjusted to 3.0 with 0.1 N NaOH), respectively. Ten or 100 mg/kg of MCZ or KCZ was injected intraperitoneally daily for 3 d. Control animals were treated with the appropriate vehicle alone at the same times. Except for the groups of animals used for the measurement of pentobarbital sleeping time, mice were killed by decapitation 24 h after the last administration, and the livers were perfused with ice-cold 1.15% KCl solution in situ to remove blood.

**Preparation of Mouse Hepatic Microsomes** — Livers from the pretreated mice described above 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 fractions were centrifuged at 105000 × g for 60 min to obtain microsomal pellets. The microsomes were suspended in 0.1 M sodium-potassium phosphate buffer, pH 7.4, to a concentration of 1–5 mg protein/ml. Protein was determined by the method of Lowry et al. 9)

**Biochemical Analysis** — Testosterone hydroxylase activities were assayed as described in a previous paper. 10) Aminopyrine N-demethylase activity was determined by the method of Nash. 11) Aniline hydroxylase activity was determined by the method of Imai et al. 12) 7-Ethoxycoumarin O-deethylase activity was determined by the method of Greenlee and Poland. 13) The assay mixture contained a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (5 mM MgCl₂, 0.5 mM NADP, 5 mM glucose 6-phosphate, 2 units of glucose 6-phosphate dehydrogenase), enzyme preparation (0.5 mg protein/ml), substrate (25 µM testosterone, 5 mM aminopyrine, 5 mM aniline, or 0.5 mM 7-ethoxycoumarin) and 67.5 mM sodium-postassium phosphate buffer, pH 7.4. The final volume was 1 ml, except for the assay of testosterone hydroxylase activities where the final volume was increased to 5 ml.

Cytchrome P-450 was determined in a microsomal suspension containing 1 mg protein/ml 0.1 M of sodium-potassium phosphate buffer, pH 7.4, by the method of Omura and Sato. 14)

NADPH-cytochrome c reductase activity was determined by the method of Peters and Fouts. 15)

**Measurement of the Ratio of 6β-OHF to F in Urine from a Patient** — One male patient (aged 4, body weight 12 kg) hospitalized with pericardosis after radical operation for tetralogy of Fallot was studied. The combination of 200 mg/d of dopamine hydrochloride, 200 mg/d of dobutamine hydrochloride, 50 mg/d of digoxin, 10 mg/d of flavin adenine dinucleotide, 100 mg/d of ascorbic acid and 120 mg/d of furosemide was continuously given to the patient over one month before the test period, during and after the test period. His liver and kidney function was normal as assessed by biological tests. MCZ injection (Florid-F®) was added to the preexisting medications described above. MCZ dosage was adjusted upward over a 14-d period to a maximum daily dosage of 10 mg/kg or 120 mg, given as two divided doses. On day 0 (just before treatment) and days 1, 2, 3, 5, 7, 10, 14 and 17, a 24-h urine sample was taken for the determination of the ratio of 6β-OHF to F. Urine samples were stored frozen at −80°C until assay. Urinary 6β-OHF was determined by a method described previously 16) and urinary F was determined by the fluorescence polarization immunoassay technique (TDX® system, Dainabot kit).

**Results**

Changes in Pentobarbital Sleeping Time Caused by the Consecutive Treatment with
Effects of Consecutive Treatment with MCZ or KCZ on the Monoxygenase System in Hepatic Microsomes

Table I shows the changes in parameters associated with hepatic drug metabolism such as cytochrome P-450 content and NADPH-cytochrome c reductase activity, caused by continuous administrations of low or high doses of MCZ or KCZ. None of the changes in cytochrome P-450 content and NADPH-cytochrome c reductase activity was found after treatment with KCZ even at the high dose, while significant increases in the values of these parameters (26–56% in cytochrome P-450 content and 35–98% in NADPH-cytochrome c reductase activity) were noted after treatment with MCZ. This result paralleled the result obtained in the pentobarbital sleeping time study (Fig. 1).

The effects of these antmycotics on the oxidative metabolism of testosterone and some xenobiotics are shown in Fig. 2. The activities of testosterone 6β-hydroxylase, aminopyrine N-demethylase and 7-ethoxycoumarin O-deethylase were increased up to 1.5- to 1.7-fold over the control activities following treatment with MCZ even at the low dose. Further, at the high dose, these enzyme activities were increased up to 2.1- to 2.8-fold over the control activities. In addition to these enzyme activities, testosterone 7α-hydroxylase was also increased up to 1.8-fold over the control activity following treatment with the high dose of MCZ. However, the inducing potency of MCZ for aniline hydro-

### Table 1. Effects of Consecutive Doses of MCZ and KCZ on the Parameters Associated with Hepatic Microsomal Monoxygenase Activity in Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytochrome P-450 (nmol/mg protein)</th>
<th>NADPH-cytochrome c reductase (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (corn oil)</td>
<td>0.73 ± 0.09</td>
<td>112 ± 16</td>
</tr>
<tr>
<td>Miconazole 10 mg/kg</td>
<td>0.92 ± 0.10 (\alpha)^a)</td>
<td>151 ± 21 (\alpha)^a)</td>
</tr>
<tr>
<td>Miconazole 100 mg/kg</td>
<td>1.14 ± 0.10 (\beta)^a)</td>
<td>221 ± 36 (\beta)^a)</td>
</tr>
<tr>
<td>Control (saline, pH 3.0)</td>
<td>0.74 ± 0.13</td>
<td>117 ± 16</td>
</tr>
<tr>
<td>Ketoconazole 10 mg/kg</td>
<td>0.75 ± 0.08</td>
<td>113 ± 12</td>
</tr>
<tr>
<td>Ketoconazole 100 mg/kg</td>
<td>0.69 ± 0.04</td>
<td>128 ± 26</td>
</tr>
</tbody>
</table>

Mice were treated with 10 or 100 mg/kg of MCZ or KCZ once a day for 3 d. \(\alpha\) Significantly different from the appropriate control \((p < 0.05)\). \(\beta\) Significantly different from the appropriate control \((p < 0.01)\).
Enzyme Induction by Miconazole

Fig. 2. Effects of Treatment with Antimycotics on Monoxygenase Activities in Hepatic Microsomes

Mice were treated with 10 mg/kg (L) or 100 mg/kg (■) of KCZ (left) or MCZ (right) once a day for 3 d. Each enzymatic reaction was started by adding the microsomal suspension from pretreated mice and was carried out at 37°C with shaking for 5 min for aminopyrine N-demethylation and 7-ethoxycoumarin O-deethylation, 10 min for testosterone hydroxylations and 20 min for aniline hydroxylation. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid, except for the assay of 7-ethoxycoumarin O-deethylation and testosterone hydroxylations where 0.125 ml of 15% trichloroacetic acid and 20 ml of dichloromethane were added, respectively. Each value is the mean ± S.D. of four determinations and is presented as a percentage of the corresponding activity in control animals treated with vehicle alone. The control activities in the microsomes from the corn oil- or saline-treated mice (nmol/mg protein/min; mean ± S.D.) were: 9.9 ± 1.8 or 10.5 ± 1.9 for the production of HCHO; 1.39 ± 0.21 or 1.42 ± 0.23 for the production of p-aminophenol; 2.06 ± 0.25 or 2.16 ± 0.35 for the production of 7-hydroxycoumarin; 3.60 ± 0.26 or 3.61 ± 0.18 for the production of 6β-hydroxytestosterone; 0.76 ± 0.07 or 0.68 ± 0.13 for the production of 7α-hydroxytestosterone; 1.55 ± 0.17 or 1.61 ± 0.11 for the production of 16α-hydroxytestosterone, respectively. a) Significantly different from the appropriate control (p < 0.05). b) Significantly different from the appropriate control (p < 0.01).

xylase activity was relatively weak, and testosterone 16α-hydroxylase activity was little affected by the treatment with MCZ even at the high dose. In contrast to MCZ, KCZ did not affect any enzyme activity even at the high dose, except for testosterone 7α-hydroxylase activity, which was decreased to about 50% of the control activity.

Effect of MCZ on Oxidative Metabolism of F in a Patient

Measurement of the ratio of 6β-OHF to F or 17-hydroxycorticosteroids (17-OHCS) in urine is useful as a noninvasive method for evaluating changes in human hepatic monoxygenase activity such as enzyme inhibition\(^{17-21}\) and induction\(^{22-27}\) because 6β-OHF is a polar metabolite formed by the oxidative metabolism of F by hepatic microsomes. By the use of this method, we examined whether MCZ affects hepatic oxidative metabolism of F in a patient.

The change in the ratio of 6β-OHF to F in urine from a patient, before, during and after 14 d of MCZ treatment is shown in Fig. 3. The ratio was at first decreased to 85% of the original level (day 0) by the treatment with the dose of 2 mg/kg/d of MCZ on day 1, and then it returned to around the original level with the increasing dose (4—6 mg/kg/d) during day 3 to 5. Furthermore, the ratio began to increase on day 7 to reach 240% of the original level at the doses of
7—10 mg/kg/d during days 10—14. The ratio decreased to 180% of the original level 3 d after the cessation of treatment with MCZ. On the other hand, there was no marked change in the amount of urinary F throughout the entire period of the experiment.

**Discussion**

In the present study, first, we demonstrated that MCZ, but not KCZ, exhibits inducing action for monooxygenase activities in mouse hepatic microsomes. Dose-dependent reduction in pentobarbital sleeping time and increases in cytochrome P-450 content and NADPH-cytochrome c reductase activity were found during continuous administrations of MCZ (Fig. 1 and Table 1). We have already reported that when mice were treated with a single dose of 10—50 mg/kg of MCZ, KCZ, or cimetidine, the pentobarbital sleeping time was prolonged in a dose-dependent manner and the potencies for the prolongation of the sleeping time decreased in the order of MCZ > KCZ > cimetidine.\(^1\) These results clearly indicate that MCZ exhibits both inhibitory and inducing actions on the hepatic microsomal monooxygenase system, even at its antifungally active dose (10 mg/kg) in the mouse.\(^28\)

Furthermore, it was found that the inducing effect of MCZ on this enzyme system is not only for the metabolism of xenobiotics, but also for that of endogenous steroids such as testosterone (Fig. 2). In particular, testosterone 6β-hydroxylase activity was markedly increased by an antifungally active dose of MCZ. Previously, we demonstrated that MCZ and KCZ markedly inhibit the testosterone 6β-, 7α- and 16α-hydroxylase activities in mouse hepatic microsomes *in vitro*.\(^1\) Therefore, it is suggested that MCZ, but not KCZ, also exhibits biphasic action on some hydroxylase activities towards endogenous steroids in hepatic microsomes. In the case of KCZ, a considerable reduction of testosterone 7α-hydroxylase activity in the microsomes prepared 24 h after consecutive treatment with the high dose for 3 d (Fig. 2) might be due to irreversible binding of KCZ or its metabolites to the microsomes. Although the reason why KCZ has no inducing activity despite being an
imidazole derivative similar to MCZ is not clear, the difference in inducing capacity between them appears to be due to the difference in their side chain structures, particularly the number of dichlorobenzene group (MCZ > KCZ), because it was reported that chlorinated benzenes have inducing activity on hepatic microsomal drug-metabolizing enzymes.29)

On the other hand, in contrast to the significant increases of aminopyrine N-demethylase, 7-ethoxycoumarin O-deethylase and testosterone 6β- and 7α-hydroxylase activities, the increases of aniline hydroxylase and testosterone 16α-hydroxylase activities caused by the treatment with MCZ were considerably weak (Fig. 2). It was reported that different cytochrome P-450 isozymes hydroxylate testosterone at the 6β-, 7α- and 16α-positions in mouse hepatic microsomes.30) Therefore, these findings indicate that the inducing effect of MCZ does not appear in the whole cytochrome P-450 population in hepatic microsomes. When the inducing pattern of MCZ for testosterone hydroxylations in mouse is compared with those of so-called enzyme-inducing agents such as phenobarbital, phenylbutazone and β-naphthoflavone,31) it seems that the inducing character of MCZ is similar to that of phenobarbital. Actually, there was no hypochromic shift in the CO-difference spectrum of reduced MCZ-microsomes (data not shown). Similarly, in the rat, it has been reported that MCZ behaves as a phenobarbital-type inducer for hepatic microsomal cytochrome P-450 isozymes.61)

Several clinical cases of drug interaction with MCZ which appeared to be due to its inhibitory action on the hepatic microsomal monoxygenase system were reported.32) On the other hand, it has been reported that urinary D-glucaric acid, a cytochrome P-450-independent substance and an indicator of enzyme induction,33–35) is increased up to 2.3-fold over the original level by treatment with 1000–1500 mg/d of MCZ for 1 week in patients.36) However, it had remained to be examined whether MCZ actually affects hepatic microsomal cytochrome P-450-dependent system in humans. Although it is still a preliminary result, we found that the urinary excretion of 6β-OHF, in a patient cytochrome P-450-dependent metabolite of F, decreased by 15% from the original level on day 1 and then increased up to 2.4-fold over the original level on day 14 during treatment with therapeutic dosage (2–10 mg/kg/d) of MCZ for 2 weeks (Fig. 3). Judging from the facts that a considerable change in the urinary excretion of F itself during the treatment with MCZ was not found (Fig. 3) and that MCZ exhibits biphasic effects on the mouse hepatic microsomal cytochrome P-450 system, the result obtained in the patient suggests that, at least in the initial phase of treatment, MCZ has an inhibitory activity and after continuous treatment it has an inducing activity for this enzyme system in humans. However, further detailed study is, of course, necessary to confirm the biphasic effects of MCZ on the hepatic microsomal oxidative metabolism of various endogenous steroids and xenobiotics in humans.

Although it is still difficult to estimate the alteration of the metabolic balance of steroids in vivo accompanying the treatment with MCZ from the findings at the hepatic microsomal level obtained in this study, in conclusion, it is clearly demonstrated that MCZ, but not KCZ, exhibits inducing action on the hepatic microsomal cytochrome P-450-dependent metabolism of testosterone and xenobiotics in mice, in addition to its inhibitory action, and it is suggested that these actions of MCZ on this enzyme system may also appear in humans.

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References
3) R. J. Santen, H. Van den Bossche, J. Symoens, J. Brug-


