Inhibition of Testosterone Biosynthesis in Testicular Microsomes by Various Imidazole Drugs. Comparative Study with Ketoconazole

Kunihiro MORITA, Takeshi ONO, and Harumi SHIMAKAWA

Hospital Pharmacy, Shiga University of Medical Science, Seta, Ohtsu, 520-21, Japan

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Ketoconazole (KCZ), an imidazole-containing antimycotic, has been demonstrated to inhibit testosterone biosynthesis in man. In this study, the inhibitory activities of various imidazole drugs such as miconazole (MCZ), cimetidine (CIM), ozagrel (OZA) and its metabolites (M-1 and M-2) on the pathway of testosterone biosynthesis in testicular microsomes were compared with that of KCZ in vitro. Additionally, the changes in serum testosterone level in the patients by the treatments with MCZ were followed. KCZ inhibited 17α-hydroxylase and C\textsubscript{17,20}-lyase activities in a dose-dependent manner, while it did not affect 17β-hydroxysteroid dehydrogenase activity. Although the patterns of the inhibitory actions and the interaction of other imidazole drugs with cytochrome P-450 as 17α-hydroxylase and C\textsubscript{17,20}-lyase were similar to those of KCZ, i.e. the inhibitory potencies and affinities for the cytochrome P-450 system decreased in the order of KCZ > MCZ > OZA > M-2 > M-1 > CIM. At the end of the intravenous injection of 200 mg MCZ to the patients, the serum testosterone levels decreased by about 16% of the original level and then returned to the original level 5 h after the end of injection. These results indicate that either imidazole drugs tested could inhibit a cytochrome P-450 enzyme C\textsubscript{17,20}-lyase mainly in testicular microsomes, and suggest that MCZ, a potent inhibitor subsequent to KCZ, induces a slight alteration in the testosterone biosynthesis in its clinical use.

**Keywords** — testosterone biosynthesis; cytochrome P-450; testosterone; imidazole; ketoconazole; miconazole; cimetidine; ozagrel; 17α-hydroxylase; C\textsubscript{17,20}-lyase

Introduction

Ketoconazole (KCZ), an orally administered antifungal imidazole derivative, has been considered a universal inhibitor of cytochrome P-450 enzymes,\(^1\) since it inhibits a number of cytochrome P-450-dependent steroidogeneses in adrenal and testicular tissues.\(^1\) For the typical example, it is well known to lower plasma testosterone level in patients by the inhibitory action of KCZ on the testicular microsomal testosterone biosynthetic pathway.\(^4,6\) Several investigations indicated that its inhibitory action site in this pathway was not 17β-hydroxysteroid dehydrogenase as a non-cytochrome P-450 enzyme but 17α-hydroxylase and/or C\textsubscript{17,20}-lyase as cytochrome P-450 enzymes.\(^7\) However, there is no agreement in the literature as to which cytochrome P-450 enzyme (17α-hydroxylase or C\textsubscript{17,20}-lyase) are inhibited by KCZ. Kan *et al.*\(^7\) and Lambert *et al.*\(^8\) found inhibition of C\textsubscript{17,20}-lyase but not 17α-hydroxylase, in contrast, Sikka *et al.*\(^9\) found similar inhibition of both enzyme activities, whereas Vanden Bossche *et al.*,\(^10\) and Ayub and Levell\(^11\) reported that although KCZ inhibited both enzyme activities, the potency for C\textsubscript{17,20}-lyase was much greater than 17α-hydroxylase.

In addition, a structure-activity study indicated that not only KCZ but other antifungals such as bifonazole, clotrimazole, miconazole (MCZ) *etc.* were the potent inhibitors of this pathway.\(^11\) Although the properties of these drugs as potent inhibitors may be exploited in the treatment of androgen-dependent disorders such as prostatic cancer,\(^11\) the effects of the treatments with these antifungals on testosterone biosynthesis in humans remain to be investigated, except for KCZ.\(^4,6,12\)

Recently, certain imidazole derivatives have been developed to expect their inhibitory activities for cytochrome P-450 in mammalian cells. Ozagrel (OZA), a thromboxane synthase inhibitor, is the typical example. Its anti-thrombotic action appears via the specific inhibition of thromboxane A\(_2\) synthesis\(^13\) which is catalyzed by cytochrome P-450 isozyme.\(^14\) On the other hand, cimetidine (CIM), an H\(_2\)-receptor antagonist, is well known to inhibit hepatic microsomal cytochrome P-450-dependent monooxygenase
activities.\textsuperscript{15-20} If these drugs also act as the potent universal inhibitors of various cytochrome P-450 isozymes which are widespread in various mammalian cells, the attention for the changes in some cytochrome P-450-dependent physiological reactions such as steroidogenesis in its clinical use should be given.

In this paper, we focus on the objects as follows: to reconfirm the site(s) and potency of the inhibitory action of KCZ on the testicular microsomal testosterone biosynthetic pathway; to examine the inhibitory effects of MCZ, CIM, OZA and its metabolites (M-1 and M-2)\textsuperscript{21} on this pathway in comparison with KCZ (Fig. 1), and to determine whether or not the treatment with MCZ affects the serum testosterone level in patients.

\textbf{Materials and Methods}

\textbf{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, OZA (hydrochloride monohydrate form), M-1 and M-2 from Kissei Pharmaceutical Co., Ltd., Matsumoto and Ono Pharmaceutical Co., Ltd., Osaka. CIM was purchased from Sigma Chemical Co., Ltd., St. Louis, MO. Progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone were purchased from Nacalai Tesque, Inc., Kyoto. All other chemicals and solvents were of analytical grade.

\textbf{Preparation of Rat Testicular Microsomes} — Testis from male SD rats (200 – 250 g) were decapsulated and homogenized in ice-cold 1.15% KCl (1:4 w/v). The homogenate was centrifuged at 10000 \( \times g \) for 15 min and 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 1 – 5 mg protein/ml. Protein concentration was determined by the method of Lowry et al.\textsuperscript{22}

\textbf{Enzyme Assay} — The steroidogenic enzyme reactions were performed in a mixture of 92.5 mM sodium-potassium phosphate buffer (pH 7.4), 0.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), microsomal preparation (0.25 mg protein/ml), each substrate (0.5 \( \mu \text{M} \)) and drug (KCZ and MCZ were 0.1 – 30 \( \mu \text{M} \), and others were 1 – 1000 \( \mu \text{M} \)) in 5 ml volumes at 37°C for 30 min under an atmosphere of 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \) (v/v). Progesterone, 17α-hydroxyprogesterone or androstenedione were used as the substrates for assay of 17α-hydroxylase, \( C_{17,20} \)-lyase or 1β-hydroxysteroid dehydrogenase activities, respectively. The activities of these enzymes were found to be linear with respect to protein concentration. Antimycotics and other drugs were dissolved in
ethanol and the same buffer, respectively, and added to the incubation mixture to make the final concentrations described above. The same volume of solvents was added to the control. The amounts of ethanol added to the incubation mixture was 0.1% (v/v). The reaction was started by adding the microsomal suspension and stopped by adding 20 ml of methylene chloride. Steroids were completely extracted with two additional methylene chloride washes and the organic layer was evaporated to dryness under reduced pressure. The residue was dissolved in 0.2 ml volume of methylene chloride and injected into a high performance liquid chromatography (HPLC) column as described below.

**Chromatographic Conditions** — Steroid analyses were performed on HPLC (Shimadzu, model LC-3A) equipped with ultraviolet (UV) detector. The absorption peak areas at 240 nm and retention times were determined with an integrator (Shimadzu, Chromatopac C-R2AX). A Cosmosil 10SL column, 25 cm × 4.6 mm, (Nacalai Tesque, Inc., Kyoto) operated at ambient temperature was used to separate steroids, which were eluted with methylene chloride–acetonitrile–isopropanol (88 : 10 : 2, v/v/v) at a flow rate of 1 ml/min according to the method of Nozu et al. Progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone separated were identified by comparison with authentic standards (retention times in HPLC). It was confirmed that the drugs tested did not interfere in the steroid analysis with HPLC.

**Spectrophotometric Determination of the Binding of Imidazole Drugs to Cytochrome P-450** — Microsomes suspended in 0.1 M sodium-potassium phosphate buffer, pH 7.4 (1 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 the ethanol solutions of KCZ or MCZ, or the same buffer solutions of CIM, OZA, M-1 or M-2. 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.

**Human Study** — Two male patients (A and

![Fig. 2. Effects of Various Imidazole Drugs on Testosterone Biosynthesis in Testicular Microsomes](image-url)

In retrocaudal order the figures represent the amounts of progesterone converted (A) and the productions of 17α-hydroxyprogesterone (B), androstenedione (C) and testosterone (D) when the microsomes were incubated with 0.5 µm progesterone as substrate with NADPH in the presence of various concentrations of imidazoles. Each value is the mean of experiments with three different rat testicular microsomal preparations. Standard deviations for different data points were between 3 and 10%. a) Values were estimated as the amounts of progesterone added minus the remaining progesterone after the incubation for 30 min. Symbols: O, KCZ; ●, MCZ; △, OZA; ▲, M-2; □, M-1; ■, CIM.
B, aged 46 and 53, respectively) were studied. Patients A and B were infected with *Candida albicans* within the periods of hospitalization with leiomyosarcoma and multiple liver tumor, respectively. The combination of 1 g/d of cefitiozoxime and 20 mg/d of morphine hydrochloride was given continuously to the patients over one month before the test periods, during and after the test periods. Twenty ml of MCZ solution (Florid-F, 200 mg/ampule) was mixed with 250 ml of 5% glucose and then was injected intravenously by gravity drip for 1 h. Blood samples were collected, before, and 1 and 6 h after, the initiation of the injection. The blood samples were immediately centrifuged and the serum was stored at -20 °C until assay. Serum total testosterone was determined by specific radioimmunoassay technique (Commissariat A L Energie Atomique, Testosterone Direct Radioimmunoassay-Kit).

**Results**

**Effects of Various Imidazoles on Testicular Microsomal Steroidogenic Enzyme Activities**

First, the effects of KCZ, MCZ, CIM, OZA, M-1 and M-2 on the pathway of testosterone biosynthesis in testicular microsomes were examined by using progesterone as initial substrate. As shown in Fig. 2, the productions of androstenedione (C) and testosterone (D) decreased in the drugs dose-dependent manner. The productions of 17α-hydroxyprogesterone (B) were biphasic in behavior in response to the concentrations of KCZ, MCZ, OZA and M-2: that is, the productions increased at their relatively lower concentration regions and decreased at their relatively higher concentration regions. The progressive decreases in the amounts of progesterone converted (A) occurred at the relatively higher concentrations of KCZ, MCZ, OZA and M-2. To identify the site and the potency of inhibitory actions of KCZ, MCZ, CIM, OZA, M-1 and M-2 on this pathway, the effects of these drugs on the enzyme activities at the individual steps were examined. Each drug inhibited both of \( \text{C}_{17,20}^{\alpha} \)-lyase and 17α-hydroxylase activity in a dose-dependent manner, while no drug had affect on 17β-hydroxysteroid dehydrogenase activity (data not shown). The 50% inhibitory concentrations (IC\(_{50}\)) of these drugs for the activities of 17α-hydroxylase and \( \text{C}_{17,20}^{\alpha} \)-lyase and the production of testosterone are summarized in Table I. Although the differences in the IC\(_{50}\) values among the drugs extended 1 — 4 orders of magnitude, the values of each drug for 17α-hydroxylase and \( \text{C}_{17,20}^{\alpha} \)-lyase activities pararelled their values for the testosterone production; the IC\(_{50}\) values of KCZ were the lowest of all and the values increased in the order of MCZ, OZA, M-2, M-1 and CIM. The IC\(_{50}\) values of the drugs on 17α-hydroxylase activity were about 20 to 30-fold greater than those on \( \text{C}_{17,20}^{\alpha} \)-lyase activity.

<table>
<thead>
<tr>
<th>IC(_{50}) (μM)</th>
<th>Testosterone production</th>
<th>( K_s ) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-Hydroxylase(^a)</td>
<td>( \text{C}_{17,20}^{\alpha} )-Lyase(^b)</td>
<td></td>
</tr>
<tr>
<td>KCZ</td>
<td>7.0</td>
<td>0.2</td>
</tr>
<tr>
<td>MCZ</td>
<td>22</td>
<td>1.1</td>
</tr>
<tr>
<td>OZA</td>
<td>700</td>
<td>25</td>
</tr>
<tr>
<td>M-2</td>
<td>&gt;1000</td>
<td>100</td>
</tr>
<tr>
<td>M-1</td>
<td>ND</td>
<td>550</td>
</tr>
<tr>
<td>CIM</td>
<td>ND</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Values are expressed as the mean of three determinations.

\( a\) 17α-Hydroxylase activity was determined as the total amounts of 17α-hydroxyprogesterone, androstenedione and testosterone as the products from progesterone as a substrate. The control activity, mean ± S.D., was 61.6 ± 5.9 pmol/mg protein/min.

\( b\) \( \text{C}_{17,20}^{\alpha} \)-Lyase activity was determined as the total amounts of androstenedione and testosterone as the products from 17α-hydroxyprogesterone as a substrate. The control activity, mean ± S.D., was 49.1 ± 5.1 pmol/mg protein/min.

\( c\) Values were estimated from the data shown in Fig. 2D.
Fig. 3. Spectral Changes of Rat Testicular Microsomal Cytochrome P-450 Caused by the Additions of KCZ, MCZ or OZA (Left), and M-1, M-2 or CIM (Right)

Difference spectra were recorded after the successive additions of 0.01—200 μM of drugs. The each curve represents the typical spectra obtained for the additions of 0.5 μM of KCZ (a), MCZ (b) or OZA (c), or 20 μM of M-2 (d), M-1 (e) or CIM (f).

**Spectrophotometric Changes in the Binding of the Drugs to Testicular Microsomal Cytochrome P-450**

To examine the interactions of the drugs with cytochrome P-450 in testicular microsomes, the difference spectra were recorded. Figure 3 shows the difference spectra induced by the additions of the drugs to rat testicular microsomes. Although the intensities of spectral changes among the drugs tested varied extensively, all had absorption maxima (peak) at 430 nm and the absorption minima (trough) at 410 nm, characteristic of a type II spectral change. These spectral changes were converted to double-reciprocal plots in order to obtain the dissociation constants ($K_s$). The $K_s$ values obtained are also summarized in Table I. The $K_s$ values of these drugs for cytochrome P-450 paralleled the IC$_{50}$ values in their inhibitory actions for 17α-hydroxylase and C$_{17,20}$-lyase activities.

**Changes in Serum Testosterone Levels in Humans by the Treatment with MCZ**

The effect of the administration of 200 mg of MCZ serum testosterone levels in humans is shown in Table II. At the end of injection of MCZ for 1 h, the serum testosterone levels in patients A and B decreased to 82.4 and 85.3% of the original levels, respectively, and then returned to the original levels 5 h after the end of injection at latest.

**Discussion**

In this study, we reconfirmed that KCZ exhibited the inhibitory effects on both C$_{17,20}$-lyase and 17α-hydroxylase activities, but not on 17β-hydroxysteroid dehydrogenase activity. It has been demonstrated that one cytochrome P-450 on testicular microsomes possesses both C$_{17,20}$-lyase and 17α-hydroxylase activities in its one active site. Actually, KCZ produced type II spectral change, which strongly indicates the direct interaction of the imidazole group with heme moiety in cytochrome P-450 (Fig. 3). On the other hand, we also confirmed that the inhibitory potency of KCZ for C$_{17,20}$-lyase was much greater than that for 17α-hydroxylase.

**Table II. Changes in Serum Testosterone Levels in the Patients by the Treatment with MCZ**

<table>
<thead>
<tr>
<th>Time after the initiation of the injection (h)</th>
<th>Serum testosterone concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient A</td>
</tr>
<tr>
<td>0 (just before injection)</td>
<td>6.8 (100.0)</td>
</tr>
<tr>
<td>1 (end of injection)</td>
<td>5.6 (82.4)</td>
</tr>
<tr>
<td>6</td>
<td>6.7 (98.5)</td>
</tr>
</tbody>
</table>

The figure in parenthesis is the percentage of the original (just before treatment) levels in each subject.
Effects of Imidazoles on Steroidogenesis

(Table I). It has also been reported that the affinity of progesterone for the cytochrome P-450 is greater than that of 17α-hydroxyprogesterone. This may cause the difference in the inhibitory potencies of KCZ for these two enzyme activities. These findings indicate that the alterations of the productions of each steroid at a lower concentration region of KCZ (Fig. 2B—D) were due to its inhibitory action on C₁₇,₂₀-lyase activity, whereas those at higher concentration region of KCZ (Fig. 2A and B) were due to its inhibitory action on 17α-hydroxylase activity. These results are in agreement with the patterns of the changes in plasma progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone in humans treated with low (200 mg) or high (400 mg) dose of KCZ.

Secondarily, we also found that all of MCZ, CIM, OZA and its metabolites inhibited the testicular microsomal steroidogenesis with the same mechanism that KCZ does; that is, the patterns of the inhibitory actions and the spectral binding properties of these drugs on this enzyme system were similar to that of KCZ (Figs. 2 and 3). On the other hand, by comparing the IC₅₀ and Kₘ values among these drugs tested, it was clearly shown that the inhibitory potencies and the affinities for cytochrome P-450 as 17α-hydroxylase and C₁₇,₂₀-lyase decrease in the order of KCZ > MCZ > OZA > M-2 > M-1 > CIM (Table I). It has been reported that the drugs having one or more aromatic rings on the imidazole side chain are the potent inhibitors of these enzyme activities, but the drugs having an aliphatic side chain on the imidazole ring are not. A similar result was also obtained in our study; that is, KCZ and MCZ having aromatic ring(s) were the potent inhibitors while CIM having an aliphatic side chain was not so. OZA or its metabolites having both aromatic ring and aliphatic side chain appear the intermediate types in the inhibitory potencies. Interestingly, the order of the inhibitory potencies and affinities among OZA, M-1, M-2 and CIM on the testicular microsomal monoxygenase system was different from that on the system in hepatic microsomes, where the order was M-2 > OZA ≈ CIM > M-1. The findings suggest a qualitative difference between cytochrome P-450 isozymes in testicular microsomes and those in hepatic microsomes.

It has been reported that the serum levels of MCZ reached to the peak at the end of the injection, and then decreased rapidly. The degrees of the alteration in serum testosterone levels at the end of the injection (decrement by 14.2—17.6% of original levels) (Table II) were only about 1/3 to 1/4 as low as that in the treatment with a same dosage of KCZ (decrement by 50% of the original level). This seems to agree with the results obtained in in vitro study, that is, the IC₅₀ values of MCZ for C₁₇,₂₀-lyase activity and testosterone production were 3 to 5-fold greater than those of KCZ (Table I). Furthermore, it has been shown that the inhibitory potency of MCZ for testicular mitochondrial cytochrome P-450scC which is another enzyme involved in testosterone biosynthesis is smaller than that for microsomal cytochrome P-450 (17α-hydroxylase and C₁₇,₂₀-lyase). Although it is still preliminary data, the result obtained in vivo study suggests that MCZ exhibits a slight inhibitory effect on testicular microsomal cytochrome P-450-dependent testosterone biosynthesis in humans, but this action is transitory and this minimizes the significance of the hormonal effects during normal antifungal therapy.

In conclusion, the present study indicates that although several imidazole drugs belonging to various therapeutic categories could also possess the inhibitory actions for testicular microsomal cytochrome P-450-dependent enzyme activities in a manner similar to KCZ, their inhibitory potencies are generally smaller than that of KCZ. Of the drugs tested, MCZ, a potent inhibitor subsequent to KCZ, is capable of a slight inhibition of testosterone biosynthesis in its clinical use.

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References


