CLOTRIMAZOLE IS A SELECTIVE AND POTENT INHIBITOR OF RAT CYTOCHROME P450 3A SUBFAMILY-RELATED TESTOSTERONE METABOLISM

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ABSTRACT:

In this study, clotrimazole (CTZ) and ketoconazole (KTZ) were evaluated for their inhibition of testosterone metabolism catalyzed by rat hepatic microsomes differentially expressing certain cytochrome P450 enzymes. The objective was to compare the inhibitory potencies using hepatic microsomes from adult female rats treated with dexamethasone (F-DEX) and hepatic microsomes from vehicle-treated adult male rats (M-VEH), which are known to contain high levels of isozymes CYP3A1 (3A23) and 3A2, respectively. The results demonstrate that CTZ is a very potent and selective inhibitor of the 6β-hydroxylation of testosterone, a CYP3A-mediated reaction, in all rat metabolic systems tested. The IC₅₀ value was 9.7 nM in F-DEX, and 6.7 nM in M-VEH for CTZ. The in vitro inhibitory potency for CTZ significantly exceeds the same parameters for KTZ, a well established specific inhibitor of human CYP3A-mediated reactions. It was found that the IC₅₀ values of KTZ in F-DEX and M-VEH were 69 and 780 nM, respectively. These values for KTZ are 10-fold and 100-fold higher, respectively, than for CTZ. CTZ, at the concentration that inhibits 90% and more of CYP3A-mediated reactions (40 nM), has less than a 10% inhibitory effect on the activities of other rat liver enzymes, such as CYP1A1, -1A2, -2A1, -2B1, -2B2, -2C11, and -2E1. In summary, CTZ is a more potent and selective inhibitor of all CYP3A-mediated reactions than KTZ in rat hepatic microsomes.

The cytochrome P450 (P450¹) superfamily of enzymes has important functions in both biosynthesis and oxidative degradation of many physiological and foreign compounds (Conney, 1982). In particular, P450s are responsible for the regio- and stereoselective hydroxylation of sex steroids (Wood et al., 1983; Suchar et al., 1996; Zhu and Conney, 1998). The broad substrate specificity is due in part to the presence of multiple P450s that are the products of distinct genes. Considerable research effort in the P450 field is directed toward answering two important questions. First, how much of a particular P450 is present in a microsomal sample? Second, what is the activity of this P450 in the metabolism of a given substrate? Two tools are frequently used in the study of the above-mentioned problems: specific antibodies and chemical inhibitors. Determination of the P450 content, as a protein, is primarily possible by using specific polyclonal or monoclonal antibodies (Thomas et al., 1983; Cooper et al., 1993). The involvement of a particular P450 in a given catalytic activity can be determined by the use of inhibitory antibodies and/or chemical inhibitors (Clarke, 1998).

One of the most potent classes of P450 inhibitors is imidazole. The N-substituted imidazole antimycotic agents, KTZ and CTZ (Fig. 1), have demonstrated antifungal activity (Henry and Sisler, 1984). Imidazoles impair the integrity of fungal cell membranes by inhibiting the biosynthesis of membrane lipids through inhibition of the P450-dependent 14α-demethylase activity (van den Bossche et al., 1983). Previously, researchers have shown that CTZ and KTZ are potent and selective in vitro inhibitors of human CYP3A activity (Gascon and Dayer, 1991; Baldwin et al., 1995; Bourrie et al., 1996). However, limited data exist concerning any comparison of the potency of CTZ and KTZ in rat hepatic microsomal preparations and the specificity of CTZ for CYP3A enzymes, especially at low concentrations. Enzymes of the CYP3A subfamily are important because they have been implicated in the metabolism of a wide variety of endogenous and exogenous compounds (Sonderfan et al., 1987; Gonzalez et al., 1988; Guengerich, 1995).

Eagling et al. (1998) demonstrated that KTZ is not a specific inhibitor of CYP3A subfamily isozymes in hepatic microsomes of male rats. It was concluded that P450 inhibitors do not exhibit the same selectivity in rat as is demonstrated in human hepatic microsomes. KTZ was a very potent and selective inhibitor of CYP3A4 activity in human hepatic microsomes (IC₅₀ = 40 nM), as evaluated by the 6β-hydroxylation of testosterone. In contrast, the CYP3A0090-9556/01/2906-837–842$3.00

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Eagling et al. (1998) demonstrated that KTZ is not a specific inhibitor of CYP3A subfamily isozymes in hepatic microsomes of male rats. It was concluded that P450 inhibitors do not exhibit the same selectivity in rat as is demonstrated in human hepatic microsomes. KTZ was a very potent and selective inhibitor of CYP3A4 activity in human hepatic microsomes (IC₅₀ = 40 nM), as evaluated by the 6β-hydroxylation of testosterone. In contrast, the CYP3A
activity in rat hepatic microsomes was inhibited with an IC$_{50}$ of 290 nM, and other P450 reactions were inhibited at concentrations greater than 1 µM (Eagling et al., 1998).

Many studies exist comparing the effects of various imidazole drugs on multiple microsomal preparations using various substrates and marker reactions. CTZ appears to be equally or more potent than KTZ in the systems tested. One study compared the inhibition of multiple P450s by known specific inhibitors in hepatic microsomes from possum, rat, rabbit, sheep, and chicken. CTZ was found to be more potent than KTZ in all cases (Olkowski et al., 1998). Sheets et al. (1986) tested the abilities of KTZ and CTZ to inhibit the oxidative metabolism of androst-4-ene-3,17-dione by hepatic microsomes of PB-treated male rats. Both KTZ and CTZ are potent inhibitors of 6β-, 16β-, and 16α-hydroxylation activities, with 50% inhibition between 0.1 and 10 µM (Sheets et al., 1986). KTZ, CTZ, and other N-substituted imidazole agents were compared in their abilities to inhibit 7-ethoxycoumarin O-deethylase, aminopyrine N-demethylase, and aniline 4-hydroxylase activities. Hepatic microsomes from male rats were used at inhibitor concentrations of 1, 10, and 100 µM, and CTZ was found to be only slightly more potent than KTZ (Murray and Zaluzy, 1988).

In this study, KTZ and CTZ were tested to determine their selective inhibition of CYP3A-mediated reactions, i.e., 6β-hydroxylation of testosterone, in rat hepatic microsomes compared with other P450s. The concentrations of CTZ used here are less than researchers have previously described, making them achievable in vivo and pharmacologically relevant (Pappas and Franklin, 1993). The particular objective was to determine the inhibitory potency and selectivity toward metabolism supported by liver microsomal CYP3A isozymes compared with that supported by other P450s (e.g., CYP1A1, CYP1A2, CYP2A1, CYP2B1, CYP2B2, CYP2C11, and CYP2E1).

Materials and Methods

Chemicals. Testosterone was purchased from Fisher Scientific (Pittsburgh, PA). CTZ and β-NADPH were purchased from Sigma (St. Louis, MO). KTZ was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Mono-hydroxylated metabolites of testosterone and 11α-hydroxyprogesterone were purchased from Steraloids (Wilton, NH). Acetonirole, methanol, and methylene chloride, all HPLC grade, were purchased from Fisher Scientific (Fairlawn, NJ). The 4-MA was a gift from Dr. G. H. Rasmussen of Merck, Sharp, and Dohme (Rahway, NJ).

Micosomes and cDNA. Hepatic microsomes were prepared from adult Sprague-Dawley rats treated with DEX, pregnenolone-16α-carbonitrile, PB, 3-MC, ISN, or VEH (corn oil) as previously described (Thomas et al., 1983; Cooper et al., 1993). Microsomal preparations of insect cells infected with recombinant baculovirus expressing rat CYP3A1,2 -3A2, and -2B1 cDNA were obtained from GENTEST (Woburn, MA).

Testosterone Metabolism and Other Assays. Assay conditions and HPLC methods were adapted from previously described methods (Wood et al., 1983; Sonderfan et al., 1987). Rat hepatic microsomes (100–250 μg of protein) were placed into aqueous solution with MgCl$_2$ (3 mM), KPO$_4$ buffer, pH 7.4 (50 mM), and testosterone (250 nmol in 20 µl of methanol) in a final volume of 1 ml. The linearity of the observed activity of the microsomes was tested and confirmed over the range of time and protein used. The incubation mixture with microsomes from female samples also contained 10 µM 4-MAN to inhibit steroid 5α-reductase activity (Sonderfan and Parkinson, 1988). Inhibition studies were conducted adding CTZ or KTZ, dissolved in 10 µl of acetonitrile, to the microsomal solution. The samples were preincubated at 37°C for 3 min. The reaction was initiated by addition of 100 µl of 10 mM NADPH and incubated for 10 to 20 min at 37°C, and then terminated by the addition of 6 ml of methylene chloride. To correct for incomplete recovery, each sample was spiked with 2 nmol of 11α-hydroxyprogesterone (50 µl in 30% acetonitrile), mixed vigorously for 1 min, and centrifuged. The aqueous phase was discarded, and the organic phase (5 ml) was evaporated under nitrogen; the residue was dissolved in 200 µl of 30% acetonitrile, and 50 µl was injected into the HPLC.

Testosterone metabolites were resolved on a reverse phase C$_{18}$ column (5 µ, 25 cm x 4.6-mm i.d., Jones Chromatography, Lakewood, CO, maintained at 51°C) with a LC-6A binary gradient HPLC system using high-pressure mixing and equipped with a SIL-6B autosampler and a SCL-6B system controller (Shimadzu Scientific Instruments, Columbia, MD). The column was eluted at a constant flow rate of 1.0 ml/min with water/methanol/acetonitrile under the following conditions: 0.1-min isocratic at 58:41:1, a 37-min concave gradient (SCL-6B time/program menu no. 5) to 18:80:2, maintained to 39 min and then a 2-min linear gradient to 58:41:1, which was maintained until the next injection (12 min). The column eluate was monitored at 254 nm with an UV detector (Shimadzu SPD-4A), and the metabolites were quantified by comparing their peak areas (integrated by a Shimadzu C-R4A recording data processor) with those of authentic standards. IC$_{50}$ data calculated using GrafFit software (Eriphus Software, London, UK).

The assay conditions for p-nitrophenol metabolism were as previously described (Duescher and Elfarra, 1993). The assay conditions for ethoxyresorufin metabolism were as previously described (Burke et al., 1994), with 5 µM ethoxyresorufin.

Results

The specificity of the inhibitory effects of low concentrations of CTZ has been evaluated for different rat liver P450s. In this respect, testosterone metabolism represents a very useful method of analysis because it has been shown that the formation of regio- and stereospecific hydroxylated testosterone metabolites is directly related to the catalytic activity of specific rat liver P450s (Wood et al., 1983; Sonderfan et al., 1987).

In Table 1, the rates of testosterone hydroxylation for different P450 enzymes are presented for the rat hepatic microsomes that were evaluated in this study. In accordance with published data (Sonderfan et al., 1987; Yamazoe et al., 1988; Hulla and Juchau, 1989; Niwa et al., 1995; Ghosal et al., 1996), F-VEH catalyzes very low 6β-hydroxylation of testosterone, which is consistent with very low or undetectable expression levels of both CYP3A1 and -3A2 isozymes (Cooper et al., 1993; Ghosal et al., 1996). DEX treatment of female rats induces the expression of CYP3A1, and thus a considerable increase in the 6β-hydroxylation of testosterone, while the CYP3A2 content in F-DEX is still very low. Substantial 6β-hydroxylation of testosterone is observed in addition to the increase in CYP3A2 content. Moreover, there is significant induction of hepatic CYP3A1 in male rats treated with DEX, such that the same protein encoded by P450(12β)B. It is with this background that we use the original designation “CYP3A1” in this manuscript.
The IC50 for recombinant expressed CYP3A1 and -3A2 were tested, and the results were similar to reactions, as catalyzed by F-VEH. Additionally, recombinant cDNA- the IC50 value was 9.7 nM for the CYP3A1-dependent reactions catalyzed by M-VEH. The IC50 was 0.95 nM for CYP3A9/18-dependent analyzed by F-DEX, and it was 6.7 nM for the CYP3A2-dependent reactions catalyzed by F-VEH. The protein (Cooper et al., 1993) but has been shown to express CYP3A9 and -3A18 based on mRNA (Mahnke et al., 1997; Cheesman and Reilly, 1998).

The results in Table 2 show that CTZ is a very potent inhibitor of the 6β-hydroxylation of testosterone in all metabolic systems tested. Indeed, the IC50 value was 9.7 nM for the CYP3A1-dependent reactions catalyzed by F-DEX, and it was 6.7 nM for the CYP3A2-dependent reactions catalyzed by M-VEH. The IC50 was 0.95 nM for CYP3A9/18-dependent reactions, as catalyzed by F-VEH. Additionally, recombinant cDNA-expressed CYP3A1 and -3A2 were tested, and the results were similar to those demonstrated in the microsomes. The IC50 for recombinant CYP3A1 is 6.5 nM and for CYP3A2 is 7.9 nM.

Such an in vitro inhibitory potency of CTZ was found to significantly exceed the same parameters for KTZ, a well established specific inhibitor of human CYP3A-related activities in vitro and in vivo (Gascon and Dayer, 1991; Baldwin et al., 1995; Bourrie et al., 1996; Eagling et al., 1998). Data in Table 2 and Fig. 2A show that the IC50 for KTZ in F-DEX is 70 nM, which is 7-fold higher than that for CTZ.

The IC50 for KTZ in M-VEH is 780 nM, which is more than 100-fold higher than that for CTZ (Fig. 2B). Figure 3, A and B, allows for comparison of the specificity of the inhibitors upon hepatic microsomal CYP3A1 and -3A2, respectively. F-VEH has little or no CYP3A1 or -3A2 protein (Cooper et al., 1993) but has been used to evaluate the effect of inhibitors upon specific inhibitor of human CYP3A-related activities in vitro and in vivo. CTZ is a more effective inhibitor of all known rat CYP3A subfamily isoforms than KTZ. It appears that the slope of the inhibitor curve for F-VEH is less steep than either F-DEX or M-VEH for either of the inhibitors. Since the IC50 of 63 nM for KTZ with F-VEH was found to be similar to the reaction by CYP3A1, KTZ appears to be more selective for CYP3A1 and -3A9/18 than for -3A2.

Table 2 shows the effects of CTZ and KTZ on other P450s. Specific hydroxylated testosterone metabolites that represent the activity of CYP2A1 and -2C11 have IC50 values for both CTZ and KTZ that are greater than that for CYP3A. Microsomes from PB-treated rats were used to evaluate the activity of CYP2B1/2 since this activity in microsomes from DEX- and VEH-treated rats is low. Importantly, we observed modest inhibition of CYP2B1/2 activity by 1% acetonitrile used as a solvent for CTZ and KTZ. The level of activity in M-PB and F-PB was decreased 44% by acetonitrile, and rat cDNA-expressed CYP2B1 activity was decreased 32% (data not shown). As a result, samples with 1% acetonitrile but no inhibitors were used as the control values. Correcting for this, CYP2B1/2 were the second most sensitive enzymes to inhibition by both CTZ and KTZ. Our findings concerning the 1% acetonitrile effect resemble previous data wherein the effects of different solvents on human liver microsomes (Hickman et al., 1992).

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et al., 1998) and human cDNA-expressed P450s were shown (Busby et al., 1999).

The 7-ethoxyresorufin O-deethylase and methoxyresorufin O-demethylase reactions of microsomes from male 3-MC-treated rats are catalyzed selectively by CYP1A1/2. p-Nitrophenol hydroxylation, a reaction specific for CYP2E1, was tested with M-ISN. The IC50 values for CYP1A1/2 and -2E1 were also much greater than those demonstrated for CYP3A.

Figure 4A shows the inhibitory effect of CTZs on the hydroxylated testosterone metabolite production by hepatic microsomes from rats treated with various compounds known to selectively increase certain P450s. It can be concluded from this data that at 40 nM CTZ, all known rat CYP3A activities can be inhibited 90% without inhibiting any other enzyme greater than 10%. KTZ, on the other hand, is not as specific for rat CYP3A, such that there is no concentration where all CYP3A activities can be inhibited 90% without inhibiting any other enzyme greater than 10% (Fig. 4B).

**Discussion**

Our data clearly indicate that CTZ is a specific and effective inhibitor of the 6β-hydroxylation of testosterone at nanomolar concentrations. It is important to note that the 6β-hydroxylation of testosterone catalyzed by F-DEX and M-VEH are supported by the distinct CYP3A isozymes, -3A1 and -3A2, respectively (Cooper et al., 1993). The CTZ IC50 as catalyzed by F-DEX is 9.7 nM, and for M-VEH is 6.7 nM. This confirms that CTZ is equally potent at inhibiting both CYP3A1 and -3A2. We confirmed the data obtained by Eagling et al. (1998) and found that the IC50 for KTZ in M-VEH is 784 nM, whereas the IC50 of KTZ in F-DEX is significantly less (69 nM). This demonstrates that CYP3A1 is more sensitive to the inhibitory effects of KTZ than CYP3A2, despite their high sequence homology. The IC50 for KTZ is 10-fold higher than that for CTZ in the experiments with CYP3A1-containing F-DEX.

The comparative studies of the specificity of these inhibitors were performed with hepatic microsomes from rats treated with different inducers so as to be enriched with certain P450s. In Table 2, the CTZ IC50 values are 10 nM or less for the formation of hydroxylated metabolites of testosterone formed by CYP3A enzymes (6β-, 2β-, and 15β-hydroxylated testosterone); but 100 to 3000 nM for metabolites 16β-, 2α-, 16α-, and 7α-hydroxylated testosterone formed by CYP2B1/2, -2C11, -2C11, and -2A1, respectively. CYP2B enzymes

**Fig. 2.** Comparative effect of CTZ and KTZ on rat hepatic microsomal testosterone 6β-hydroxylation activity expressed as a percentage of control activity where control contains no inhibitor in F-DEX (A) and M-VEH (B).

**Fig. 3.** Effect of CTZ (A) or KTZ (B) on testosterone metabolism to 6β-hydroxylated testosterone by liver microsomes from rats differentially expressing CYP3A subfamily isozymes, expressed as a percentage of control activity where control contains no inhibitor.
are second to CYP3A in their sensitivity to the inhibitory effects of CTZ.

Pappas and Franklin (1993) found that CTZ treatment of male rats prolonged the hexobarbital sleep time, which is accomplished by inhibition of liver microsomal hexobarbital metabolism. The peak concentration of CTZ in the liver (53 μg/g of wet tissue; estimated as 153 μM assuming uniform distribution) occurred at 2.5 h following dosing (75 mg/kg, intragastrically), t_{1/2} = 11 h. We have found that CTZ at 40 nM inhibits 90% or more of the testosterone 6β-hydroxylase activity in rat liver microsomes, while less than 10% of any other reaction of testosterone supported by CYP2A1, -2C11, or -2B1/2 is inhibited (Fig. 4, A and B). Also, judging from the high IC_{50} for CTZ with other P450s in Table 2, CTZ at 40 nM should have little or no effect on catalysis by these P450s (Shou et al., 1994; Ueng et al., 1997). In many cases, the CYP3A activity with several substrates does not follow the classical Michaelis-Menten kinetics. In that case, the use of inhibitory constant, K_i, is not always suitable and the IC_{50} parameter better describes the potency of the inhibitor. As also noted by Wang et al. (2000), CYP3A4 was different from other P450s, in that drug-drug interactions by this enzyme was more dependent on the substrate than with other P450 enzymes.

In our study, the primary goal was to find an effective and specific chemical inhibitor for CYP3A subfamily enzymes in rat hepatic microsomes, and we did not investigate the mechanism of the inhibitory action. Recently, Gibbs et al. (1999) demonstrated that the inhibitory potential for CTZ and KTZ in human hepatic microsomes greatly depends on the binding of the inhibitor to the microsomal proteins with possible depletion of unbound inhibitor available for the enzyme. If the same phenomena as found by Gibbs et al. (1999) for humans exists in rat hepatic microsomes, it is reasonable to conclude that our results could underestimate the inhibitory potential of CTZ in comparison with KTZ. In other words, the actual IC_{50} for CTZ could be lower than the apparent IC_{50} values in Table 1. Because of the very low apparent IC_{50} values for CTZ with rat CYP3A enzymes, it would be technically quite challenging to obtain the actual IC_{50} values for CTZ under conditions where I >> E and still have data points above and below the IC_{50} values. Nevertheless, for the microsomal protein content that we used in this study, CTZ is clearly a more potent and selective inhibitor for rat CYP3A enzymes than KTZ.

The inhibitory effect of CTZ and KTZ on the 6β-hydroxylation of testosterone in F-VEH needs special comment. First, in accordance with published data, the rate of the 6β-hydroxylation of testosterone in these preparations is very low. The current explanation of this phenomenon is the low, or practically undetectable, levels of either CYP3A1 or -3A2 expression in hepatic microsomes of mature female rats (Cooper et al., 1993; Ghosal et al., 1996). However, the indirect evidence that exists suggests that another CYP3A subfamily enzyme, other than -3A1 or -3A2, might be functionally active in the hepatic microsomes of untreated female rats (Strotkamp et al., 1993). Mahnke et al. (1997) demonstrated expression of specific mRNAs, designated as CYP3A9 and -3A18, in the livers of untreated female rats. These data were confirmed in independent experiments (Cheseman and Reilly, 1998). In a study conducted in our lab on the covalent binding...
of cyclosporin A to microsomal proteins, it was found that the microsomal preparations from untreated adult female rats actively catalyze this reaction, and CYP3A inhibitors (KTZ and troglodycin) inhibit the binding (Sadrieh and Thomas, 1994). We suggested that a female-specific enzyme with high sensitivity to CYP3A inhibitors might exist in hepatic microsomes of untreated female rats (Sadrieh and Thomas, 1994). In general, the data presented here pertaining to the exceptional sensitivity of the 6β-hydroxylation of testosterone to CTZ (IC₅₀ less than 1.0 nM) support the existence of an uncharacterized female-specific enzyme that is likely related with known CYP3A subfamily members, possibly CYP3A9 and/or 3A18. The work to obtain cDNA-expressed catalytically competent female-specific CYP3A isozyme is now in progress in our laboratory.

In conclusion, our findings can be summarized as follows: 1) CTZ is a highly effective, specific, and similarly potent inhibitor of all known rat CYP3A-dependent reactions, including CYP3A1, -3A2, and female-predominant -3A9/18 isozymes; 2) the IC₅₀ of CTZ to inhibit CYP3A1- and -3A9/18-related activities is nearly 1 order less, and CYP3A2-related activity is 2 orders less than the IC₅₀ of KTZ; 3) CTZ, at the concentration that inhibits at least 90% of the CYP3A-related activities (40 nM), has less than 10% inhibitory effect on the activities of other major rat hepatic P450s, i.e., CYP1A1, -1A2, -2A1, -2B1, -2B2, -2C11, and -2E1.

References