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# Oxidative metabolism of cinnarizine in rat liver microsomes

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**Abstract**—The oxidative metabolism of cinnarizine (CZ) [1-(diphenylmethyl)-4-(3-phenyl-2-propenyl)-piperazine] to 1-(diphenylmethyl)piperazine (M-1), 1-(diphenylmethyl)-4-[3-(4'-hydroxyphenyl)-2-propenyl]piperazine (M-2), benzophenone (M-3) and 1-[(4'-hydroxyphenyl)-phenylmethyl]-4-(3-phenyl-2-propenyl)piperazine (M-4) has been studied in rat liver microsomes. In Wistar rats, kinetic analysis revealed sex differences (male > female) in the $K_m$ values for formation of all the metabolites and the $V_{max}$ values for the formation of M-1, M-3 and M-4. The reactions required NADPH, and were inhibited by carbon monoxide and SKF 525-A. Only M-2 formation was suppressed by sparteine or metoprolol, and was significantly lower in female Dark Agouti rats than in Wistar rats of both sexes. The results suggest that CZ is oxidized by cytochrome P450, and M-2 formation is related to debrisoquine/sparteine-type polymorphic drug oxidation.

Cinnarizine {[1-(diphenylmethyl)-4-(3-phenyl-2-propenyl)-piperazine], CZ*} is a selective calcium entry blocker, and is extensively used in the treatment of cerebral and peripheral insufficiency [1, 2]. However, recent clinical studies demonstrated that CZ and its fluorine derivative flunarizine induce extrapyramidal side-reactions such as Parkinsonism and dystonia when given to patients [3–5]. The mechanism causing these side-effects remains to be elucidated. Drug metabolism should be taken into account for the assessment of toxicities as well as pharmacological effects produced during medication. The *in vivo* metabolic fate of CZ has been studied in rats [6, 7] and rabbits [8]. Soudijn and Wijngaarden [6] reported that radiolabelled CZ was extensively metabolized in the rat after oral administration. They found benzhydrol and its glucuronide to be major metabolites in the urine, and 1-(diphenylmethyl)piperazine (M-1) and benzophenone (M-3) to be major metabolites in the feces of this animal species [6] (Fig. 1). CZ was reported in rabbits also to be metabolized mainly to benzhydrol glucuronide as a urine metabolite [8]. However, no systematic studies on the oxidative metabolism of CZ with liver microsomes of mammals have been reported so far.

We have been studying the roles of cytochrome P450 (P450) isozymes in the oxidative metabolism of various drugs using enzyme kinetic techniques [9–11]. To characterize enzymatic reaction in the oxidative metabolism of CZ, we studied the enzyme kinetics of the formation of its metabolites in liver microsomes of Wistar rats as an initial step to elucidate the mechanisms of side-effects induced during CZ medication. As genetic polymorphism needs to be taken into account in the assessment of the toxicity or pharmacology of drugs [12], we compared CZ metabolism in liver microsomes from female Dark Agouti (DA) rats, an animal model of debrisoquine 4-hydroxylase deficiency [13, 14], with that in those from Wistar rats.

## *Materials and Methods*

*General.* CZ, M-1, 1-(diphenylmethyl)-4-[3-(4'-hydroxyphenyl)-2-propenyl]piperazine hydrochloride (M-2), 1-[(4'-hydroxyphenyl)-phenylmethyl]-4-(3-phenyl-2-propenyl)piperazine (M-4) were donated from the Eisai Co. Ltd (Tokyo, Japan); 4-hydroxy-flunarizine was from the Kyowa Hakko Kogyo Co. (Tokyo, Japan). M-3, benzhydrol, 4,4'-difluorobenzophenone and sparteine sulfate were purchased from the Wako Pure Chemical Co. (Osaka, Japan); metoprolol tartrate was from the Sigma Chemical Co. (St Louis, MO, U.S.A.); and SKF 525-A was from Research Biochemicals Inc. (Natick, MA, U.S.A.). Male and female Wistar and female DA rats (8 weeks old, Shizuoka Laboratory Co., Shizuoka, Japan) were killed by decapitation, and liver microsomes were prepared by the method of Omura and Sato [15]. Protein concentrations were determined by the Lowry method [16].

*Assay methods of enzymatic activities.* Metabolic activities were determined by HPLC. Reaction medium consisted of

* Abbreviations: CZ, cinnarizine, 1-(diphenylmethyl)-4-(3-phenyl-2-propenyl)piperazine; M-1, 1-(diphenylmethyl)piperazine; M-2, 1-(diphenylmethyl)-4-[3-(4'-hydroxyphenyl)-2-propenyl]piperazine; M-3, benzophenone; M-4, 1-[(4'-hydroxyphenyl)-phenylmethyl]-4-(3-phenyl-2-propenyl)piperazine; P450, cytochrome P450; DA, Dark Agouti.

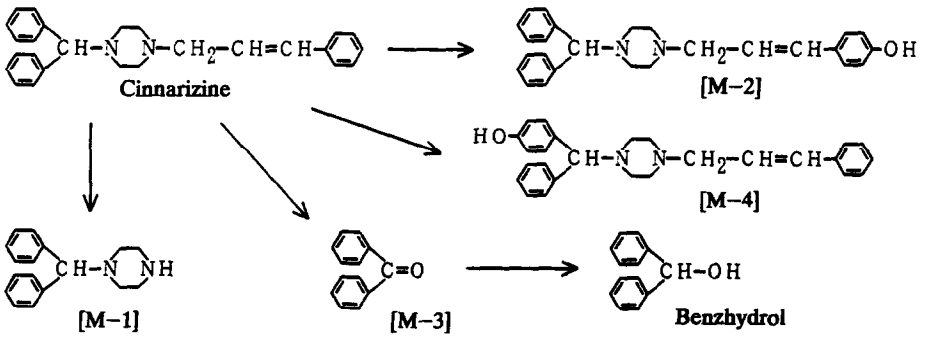


Fig. 1. Proposed oxidative metabolic pathways of CZ in rat liver microsomes.

Table 1. Michaelis–Menten parameters for liver microsomal CZ metabolic activities of male and female Wistar rats

| Metabolite | Male $V_{max}$ (pmol/min/mg) | Male $K_m$ ($\mu$M) | Female $V_{max}$ (pmol/min/mg) | Female $K_m$ ($\mu$M) |
|---|---|---|---|---|
| M-1 | 134.5 ± 23.5 | 42.1 ± 0.6 | 53.8 ± 3.0* | 36.6 ± 0.9† |
| M-2 | 60.4 ± 3.2 | 5.1 ± 0.4 | 60.2 ± 3.1 | 2.4 ± 0.1† |
| M-3 | 1620.0 ± 262.0 | 23.5 ± 3.5 | 120.2 ± 1.9† | 41.8 ± 1.9† |
| M-4 | 197.4 ± 5.4 | 17.1 ± 0.7 | 26.2 ± 1.3† | 1.2 ± 0.1† |

Values are expressed as means ± SE of three to four determinations.
* and † significantly different from mean values of male rats ($P < 0.05$ and $P < 0.01$, respectively).

1 mg of microsomal protein, 0.5 mM NADP, 10 mM glucose 6-phosphate, 2 U of glucose 6-phosphate dehydrogenase, 4 mM $MgCl_2$, 0.5–320 $\mu$M CZ and 100 mM potassium phosphate buffer (pH 7.4) to make a final volume of 1.0 mL. After preincubation at 37° for 5 min, incubation was started by adding NADP and continued for 5 min at 37°. All the assays were performed under conditions that were linear with respect to time of incubation and protein concentration. The reaction was stopped by adding 0.5 mL of 1 M carbonate buffer (pH 10). 4-Hydroxy-flunarizine was added as an internal standard, and metabolites formed were extracted into organic solvent consisting of *n*-hexane/acetone (9:1, v/v). After centrifugation at 1600 *g* for 10 min, the organic layer was evaporated *in vacuo*, and the residue was dissolved in 100 $\mu$L of methanol and subjected to HPLC as described below.

Five metabolites (M-1, M-2, M-3, M-4 and benzhydrol) of CZ were determined under the following conditions: instrument, LC-6A liquid chromatograph equipped with a SPD-6A UV detector and a C-R1B chromatointegrator (Shimadzu, Kyoto, Japan); column, a reversed-phase Capcellpack C18 (4.6 mm i.d. × 25 cm, Shiseido Co., Tokyo, Japan); column temperature, 40°; mobile phase, 50 mM potassium phosphate buffer (pH 3.7)/acetonitrile (55:45, v/v); flow rate, 1.2 mL/min; detector wavelength, 210 nm. The internal standard, mobile phase and flow rate in the inhibition studies using SKF 525-A mentioned below were changed to 4,4′-difluorobenzophenone, 50 mM potassium phosphate buffer (pH 3.7)/acetonitrile (60:40, v/v) and 1.0 mL/min, respectively, because SKF 525-A interfered with the metabolite measurement. The intra-assay coefficients of variation were less than 7.0%. The lowest limits of detection for M-1, M-2, M-3, M-4 and benzhydrol were 0.005, 0.005, 0.02, 0.005 and 0.01 $\mu$M, respectively.

The rate of the N-dealkylation at the 1-piperazine nitrogen forming M-3 was calculated by summing the rates of formation of M-3 and benzhydrol. For inhibition studies, SKF 525-A, sparteine or metoprolol (50 and 200 $\mu$M each) was added to the reaction medium before preincubation. Enzyme kinetic parameters ($K_m$ and $V_{max}$) were analysed by a non-linear least-squares regression analysis program based on a simplex method [17]. Statistical significance was calculated by the Student's *t*-test.

*Results and Discussion*

If a drug has several metabolic pathways in microsomal oxidation, enzyme kinetics is a good method for estimating the degree of contribution of each pathway to its metabolism. We have examined the enzyme kinetics of the oxidative metabolism of CZ at the substrate concentration range of 0.5–320 $\mu$M in liver microsomes of Wistar rats of both sexes. The rates of formation of M-1, M-2, M-3 and M-4 were represented by a single Michaelis–Menten equation. Table 1 lists the kinetic parameters calculated. In the oxidative metabolism of CZ in rat liver microsomes, a significant sex difference was observed. That is, $K_m$ values of all the metabolites examined were significantly greater in male than in female rats. $V_{max}$ values in male rats were also significantly greater than those in female rats, except

Table 2. Effects of inhibitors on CZ metabolic activities in liver microsomes of male Wistar rats

| Inhibitor | Activity (%) M-1 | M-2 | M-3 | M-4 |
|---|---|---|---|---|
| Complete | 100 | 100 | 100 | 100 |
| Without NADPH | 0 (0.0)† | 0 (0.0)† | 0 (0.0)† | 0 (0.0)† |
| CO/O (4:1) | 31 (4.4)† | 43 (3.1)† | 16 (1.6)† | 29 (2.5)† |
| SKF 525-A 50 $\mu$M | 40 (2.0)† | 25 (1.4)† | 54 (0.5)† | 30 (1.2)† |
| 200 $\mu$M | 18 (0.5)† | 6 (2.9)† | 17 (0.4)† | 9 (0.4)† |
| Sparteine 50 $\mu$M | 91 (4.4) | 53 (3.5)† | 90 (2.0) | 90 (1.9) |
| 200 $\mu$M | 92 (1.5) | 45 (2.7)† | 90 (4.0) | 86 (1.9) |
| Metoprolol 50 $\mu$M | 92 (1.1) | 79 (1.2)* | 104 (4.3) | 93 (0.8) |
| 200 $\mu$M | 91 (1.2) | 64 (2.3)* | 107 (1.6) | 88 (1.2) |

In the "Complete" system, CZ (50 $\mu$M) was incubated with liver microsomes in the presence of an NADPH-generating system.
Each value represents an activity (%, mean of three determinations) relative to the activity in the "Complete" system. Each value in parentheses represents SE.
* and † significantly different from "Complete" systems ($P < 0.05$ and $P < 0.01$, respectively).

for M-2, the $V_{max}$ of which was almost the same in both sexes. The most striking difference was seen in the $V_{max}$ values for the formation of M-3: the $V_{max}$ in male rats was 13.5-fold of that in female rats. The oxidative metabolic pathways of CZ as determined in the present study are summarized in Fig. 1.

Most drug oxidations are catalysed by the microsomal cytochrome P450 (P450) system [18–20]. The oxidation of CZ is thought to be catalysed by this system, and thus the effects of various inhibitors of the oxidation reaction mediated by P450 on the formation of CZ metabolites were investigated in rat liver microsomes. As summarized in Table 2, all of the oxidations required NADPH as a cofactor, and were inhibited significantly with carbon monoxide and SKF 525-A, typical inhibitors of P450. These results indicate that P450 catalyses the N-dealkylations and the ring hydroxylations of CZ in rat liver microsomes. Addition to the incubation medium of sparteine and metoprolol, known substrates for P450 isozymes belonging to the CYP2D subfamily [21], suppressed only the formation of M-2 from CZ significantly. The inhibition of the ring hydroxylation (M-2 formation) by sparteine and metoprolol suggests that debrisoquine 4-hydroxylase may be involved in the formation of M-2 from CZ. However, the incomplete suppression with these inhibitors also indicates the possibility of involvement of some other P450 isozyme(s) in CZ oxidation.

It is well established that debrisoquine 4-hydroxylase oxidizes sparteine and metoprolol, and that the metabolism of sparteine and metoprolol is impaired in debrisoquine-poor metabolizers [22, 23]. As described above, debrisoquine 4-hydroxylase may also be involved in the formation of M-2 from CZ. Therefore, the oxidative metabolism of CZ in liver microsomes from female DA rats, which are known as an animal model for debrisoquine 4-hydroxylase deficiency [13, 14], was examined at substrate concentrations of 1 and 16 $\mu$M in linear and saturated regions, respectively, to compare the Michaelis–Menten kinetics of the formation of M-2 with those in liver microsomes from Wistar rats. Figure 2 shows the formation rates of M-1, M-2, M-3 and M-4 in liver microsomes from female DA rats at the two substrate concentrations together with the results obtained in Wistar rats of both sexes for reference. At the higher substrate concentration (Fig. 2A), the formation rate of M-3 was highest in female DA rats, followed by the formation rates of M-1, M-2 and M-4. At the lower substrate concentration (Fig. 2B), only the formation of M-1 and M-2 was detected in female DA rats, and the formation rates of M-3 and M-4 were negligible. The N-dealkylation forming M-3 was a main metabolic pathway of CZ in male Wistar, whereas the aromatic ring hydroxylation forming M-2 was predominant in female Wistar rats at both the substrate concentrations. The rates of formation of M-2 and M-4 in female DA rats were significantly ($P < 0.01$) lower than those in female Wistar rats at the two substrate concentrations. Total activities of the formation of metabolites in female DA rats were 74.4 and 6.5% of those in female Wistar rats at the substrate concentrations of 16 and 1 $\mu$M, respectively.

Our *in vitro* results showed a strain difference (Wistar > DA) in the aromatic ring hydroxylation activities forming M-2 and M-4. Taking into account the inhibitory effects of sparteine and metoprolol for the formation of M-2 only, it is indicative that this oxidation forming M-2 from CZ is related to debrisoquine/sparteine-type polymorphic drug oxidation involving P450 isozymes belonging to the CYP2D subfamily. On the other hand, the formation of M-4 was not inhibited by sparteine or metoprolol, although a significant strain difference in this enzyme activity was observed (Wistar > DA). This result suggests that female DA rats, known as an animal model for debrisoquine-poor metabolizers, may lack other P450 isozyme(s) responsible for the formation of M-4 as well as that belonging to the CYP2D subfamily.

We found in the present study that CZ was extensively metabolized in rat liver by the microsomal monooxygenase system, including P450. It is noteworthy that aromatic hydroxylations of CZ forming M-2 and M-4 are impaired in the female DA rat, an animal model of CYP2D subfamily deficiency [13, 14], as compared to the female Wistar rat. Koymans *et al.* [24] recently proposed a predictive model for substrates of human P450 2D6 which has one or two basic nitrogen atoms and exhibits a coplanar conformation near the oxidation site. In this model, oxidation sites are 5 to 7 Å apart from the basic nitrogen atom [24]. Considering the structure of CZ in this hypothesis, it is possible that p-hydroxylations of the phenyl rings of both the cinnamyl (M-2 formation) and diphenylmethyl (M-4 formation) moieties of CZ are mediated by CYP2D6 in the human. This raises the possibility that aromatic

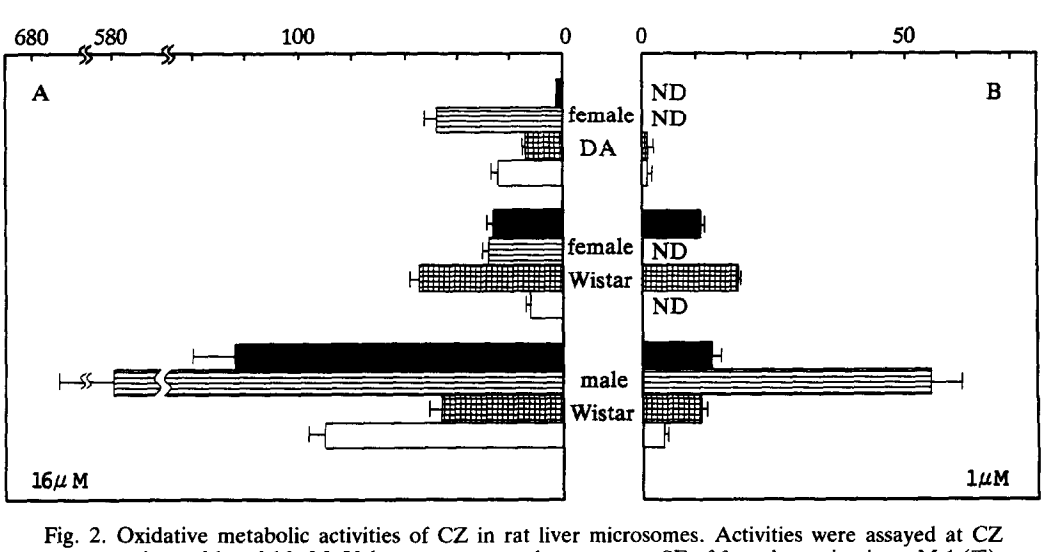


Fig. 2. Oxidative metabolic activities of CZ in rat liver microsomes. Activities were assayed at CZ concentrations of 1 and 16 $\mu$M. Values are expressed as means ± SE of four determinations. M-1 (□), M-2 (▤), M-3 (☰), M-4 (■). ND, not detectable.

hydroxylations are defective in human debrisoquine-poor metabolizers. We are now pursuing this possibility.

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