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POSSIBLE PHARMACOKINETIC AND PHARMACODYNAMIC FACTORS AFFECTING PARKINSONISM INDUCEMENT BY CINNARIZINE AND FLUNARIZINE

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Abstract—Potentialities of cinnarizine [1-(diphenylmethyl)-4-(3-phenyl-2-propenyl)piperazine, CZ] and its fluorine derivative flunarizine [1-[bis(4-fluorophenyl)-methyl]-4-(3-phenyl-2-propenyl)piperazine, FZ] to induce parkinsonism as an adverse effect were evaluated pharmacokinetically and pharmacodynamically in rats. In multiple-dose experiments, CZ or FZ was given to rats at a daily dose of 20 μmol/kg for 1, 5, 10, 15, and 30 days, and CZ, FZ, and the ring-hydroxylated metabolites of their cinnamyl moiety [1-(diphenylmethyl)-4-[3-(4′-hydroxyphenyl)-2-propenyl]piperazine, C-2 and 1-[bis(4-fluorophenyl)methyl]-4-[3-(4′-hydroxyphenyl)propenyl]piperazine, F-2] in the plasma and striatum were determined 24 hr after the final dose. Plasma and striatum concentrations of the above compounds except for FZ reached steady state after 10 doses, but their concentrations of FZ continued to increase throughout the experiments. The concentrations obtained after the 30 doses were in the order of FZ > F-2 > CZ > C-2 for the plasma and of F-2 > FZ > CZ > C-2 for the striatum. The ratios of striatum to plasma concentrations of C-2 and F-2 were 2.4 and 3 times higher than those of the parent drugs. Binding affinities of CZ, FZ, and their 10 metabolites for rat striatal dopamine D-2 receptors (D2-R) were assessed by competitive radioligand-binding studies using [^{3}H]-N-[(2RS,3RS)-1-benzyl-2-methyl-3-pyrrolidinyl]-5-chloro-2-methoxy-4-methylamino-benzamide ([^{3}H]-YM-09151-2). The IC_{50}s calculated from their *Ki* values were in the order of F-2 < C-2 < FZ < CZ < C-4 ≪ F-1, indicating that C-2 and F-2 exhibit higher affinities for D2-R than the parent drugs, whereas affinities of other metabolites were 1 to 2 orders of magnitude less than those of C-2 and F-2. These results suggest some important roles of C-2 and F-2 in the development of parkinsonism as active metabolites during chronic medication with CZ and FZ, respectively.

Key words: cinnarizine; flunarizine; parkinsonism; active metabolite; dopamine D-2 receptor; striatum; blood concentration

CZ§ and its fluorine derivative FZ are selective calcium-entry blockers widely used in the treatment of cerebral and peripheral vascular insufficiency [1–3]. Recent clinical studies reported that both CZ- and FZ-induced extrapyramidal side effects, such as parkinsonism, akathisia, and tardive dyskinesia in patients during chronic administration [4, 5]. FZ is also reported to be more potent for the inducement of parkinsonism than CZ [6], but its mechanism remains to be elucidated.

Neuroleptic-induced parkinsonism is known to be due to the blockade of dopamine D2-R in the striatum [7, 8]. CZ and FZ are piperazine derivatives with chemical structures similar to those of the neuroleptics trifluoperazine and fluphenazine, which produce a high incidence of parkinsonism. FZ was reported to competitively displace [^{3}H]spiperone bound to D2-R in rat striatal membranes [9, 10]. We have recently reported that CZ, as well as FZ, had a suppressive effect on the rat stereotypy induced by apomorphine, a dopamine agonist [11]. These results indicate that CZ- and FZ-induced parkinsonism may be due to D2-R blockade in the striatum.

In vivo oxidative metabolism was studied in rats [12, 13] and rabbits [14] for CZ and in rats and dogs for FZ [15, 16]. Lavrijisen *et al.* [17] reported species differences in the oxidative metabolism of FZ using liver subcellular fractions and hepatocytes from rats, dogs, and humans. We have recently reported the oxidative metabolism of CZ and FZ in liver microsomes from rats [18, 19] and humans [20]. The oxidative metabolism of CZ and FZ was shown to consist of 4 and 3 primary metabolic pathways, respectively, in liver microsomes from rats and humans (Fig. 1). Metabolites of CZ and FZ may contribute to adverse effects, as in the case of carbamazepine [21, 22]. However, there has been no report on the effects of CZ and FZ metabolites on D2-R. Investigation of the effects of these metabolites, as well as their parent drugs, on D2-R may help clarify the mechanism causing parkinsonism encountered during chronic administration of CZ and FZ.

The aim of this study was to evaluate the potentialities of CZ, FZ, and their metabolites to induce parkinsonism in rats. In multiple-dose experiments, rats were given CZ

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§ Abbreviations: CZ, cinnarizine; FZ, flunarizine; D2-R, dopamine D-2 receptor; HPLC, high-performance liquid chromatography; C-1, 1-(Diphenylmethyl)piperazine; C-2, 1-(diphenylmethyl)-4-[3-(4′-hydroxyphenyl)-2-propenyl]piperazine; C-3, benzophenone; C-4, 1-[(4′-hydroxyphenyl)phenylmethyl)]-4-(3-phenyl-2-propenyl)piperazine; CNP, cinnamylpiperazine; F-1, 1-[bis(4-fluorophenyl)methyl]piperazine; F-2, 1-[bis(4-fluorophenyl)methyl]-4-[3-(4′-hydroxyphenyl)propenyl]piperazine; F-3, 4,4′-difluoro-benzophenone; F-5, 4,4′-difluorobenzhydrol; [^{3}H]-YM-09151-2, [^{3}H]-N-[(2RS,3RS)-1-benzyl-2-methyl-3-pyrrolidinyl]-5-chloro-2-methoxy-4-methylamino-benzamide.

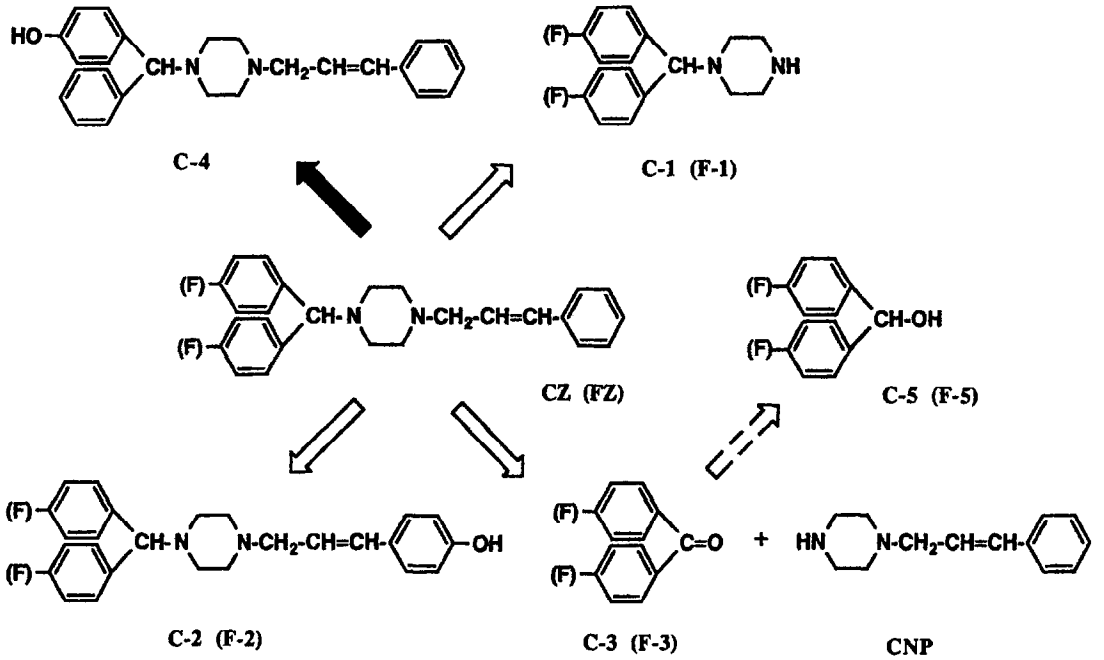

Fig. 1. Metabolic pathways of CZ and FZ in liver microsomes of rat [18, 19] and human [20]. Open arrows are common primary metabolic pathways for CZ and FZ. A closed arrow is a primary metabolic pathway for CZ only. A broken arrow is a secondary metabolic pathway for CZ and FZ.

or FZ at a daily oral dose of 20 μmol/kg for 1 to 30 days, and plasma and striatum concentrations of CZ, FZ, and their metabolites then determined by HPLC. The binding properties of CZ, FZ, and their metabolites for rat striatal D2-R were also examined to evaluate their affinities for the D2-R. On the basis of these pharmacokinetic and pharmacodynamic data, we probed the potentialities of these compounds to induce parkinsonism.

MATERIALS AND METHODS

Materials

C-1, C-2, C-4, and CNP were kindly donated by the Eisai Co. (Tokyo, Japan); F-1, F-2, and F-5 were from the Kyowa Hakko Kogyo Co. (Tokyo, Japan). CZ, C-3, benzhydrol (C-5), F-3, haloperidol, chlorpromazine HCl, and poly-L-lysine HBr (molecular mass, 30KD-50KD) were purchased from the Wako Pure Chemical Co. (Osaka, Japan); FZ HCl and thioridazine HCl were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); (+)-butaclamol HCl was from Research Bio-chemicals Inc. (Natick, MA, U.S.A.); a tissue solubilizer Soluene-350 was from Packard Japan (Tokyo, Japan); [^{3}H]-YM-09151-2, 83.9 Ci/mmol, and liquid scintillation cocktail (Econofluor-2) were from New England Nuclear (Boston, MA, U.S.A.). All other chemicals used were of analytical grade.

Multiple-dose experiments

Female Wistar rats (10–14 weeks old, Japan SLC Co., Shizuoka, Japan) were divided into 5 groups (each n = 5). The rats were given CZ or FZ orally at a daily dose of 20 μmol/kg for 1, 5, 10, 15, and 30 days. They were killed by decapitation 24 hr after the final dose, and the blood and striatum collected. The blood sample was centrifuged at 1600 × g for 10 min to separate the plasma fraction. The striatal sample was homogenized in 1.0 mL of distilled water. Plasma (0.2 mL) and striatum concentrations of CZ, FZ, and their metabolites, except for C-1 and F-1 which could not be determined due to some impurities appearing on the chromatograms, were assayed by HPLC as reported previously [18, 19].

Preparation of striatal membranes

Female Wistar rats (10–14 weeks old, Japan SLC Co., Shizuoka, Japan) were killed by decapitation and the striatum quickly removed. Pooled striatal samples from 8–12 animals were homogenized in 20 volumes of 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 12,000 × g for 20 min at 4°C. The pellets were suspended in 30 volumes of the same buffer and recentrifuged. The washed pellets were resuspended in 30 volumes of the same buffer and stored at −80°C until use. Protein concentrations were determined by the method of Lowry *et al.* [23] using bovine serum albumin as standard.

Radioreceptor assay for D2-R

An assay mixture (1.0 mL) consisted of 0.1 mg striatal membrane protein, 0.1 nM [^{3}H]-YM-09151-2, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 50 mM Tris-HCl buffer (pH 7.4). After adding 10 μL of varying concentrations of a displacing drug in methanol to the mixture, the membrane preparation was incubated at 37°C for 20 min. The reaction was terminated by rapid vacuum filtration through a Whatman GF/B glass fiber filter that had been pretreated with 0.1% poly-L-lysine. The filter was then washed 4 times with 4 mL of ice-cold 50 mM Tris-HCl buffer (pH 7.4), and the striatal membranes dissolved in 0.8 mL of Soluene-350 by incubation at room temperature for 30 min. The radioactivity was measured in 10 mL of Econofluor-2 scintil-

lation fluid by an LS-1800 Beckman liquid scintillation counter.

Nonspecific binding was determined in the presence of (+)-butaclamol (10 μM). The specific [^{3}H]-YM-09151-2 binding reached equilibrium within 15 min and remained stable for at least 45 min at 37°C in the presence of radioligand (0.1 nM). Saturation curves were constructed using 7 final concentrations of [^{3}H]-YM-09151-2 ranging from 0.0125 to 0.8 nM, and the maximum binding capacity (B_{max}) and the equilibrium dissociation constant (*Kd*) then determined by the method of Scatchard [24]. The B_{max} and *Kd* values of [^{3}H]-YM-09151-2 for D2-R were 279 ± 23 fmol/mg protein (mean ± SE, n = 3) and 9.6 ± 2.3 pM, respectively. The Hill coefficient of the radioligand was calculated to be 0.98 ± 0.03.

Data calculation

The IC_{50} values and Hill coefficients of displacing compounds were obtained from displacing curves by the Hill plot [25]. Inhibition constants (*Ki*) were calculated from the IC_{50} values [26].

RESULTS

Multiple-dose experiments

Figure 2 shows plasma concentrations of CZ, FZ, and their metabolites after repeated oral administration of 20 μmol/kg/day CZ or FZ for different periods of time. CZ and C-2 after CZ dosing and FZ and F-2 after FZ dosing were detected at all the assay time points (1, 5, 10, 15, and 30 days), but F-3 was detected only after 30 doses. The plasma concentrations of CZ, C-2, and F-2 reached steady state after 10 to 15 doses, but those of FZ increased continuously during the treatment. The order of the plasma concentrations on 30 doses was FZ > F-2 > CZ > F-3 > C-2.

The striatum concentrations of the drugs and their metabolites are shown in Fig. 3. The time-courses of their concentrations in the striatum were similar to those in the plasma. The striatum concentrations on 30 doses were in the order of F-2 > FZ > F-3 > CZ > C-2. Ratios of striatum/plasma concentrations (S/P) for the compounds after 30 doses are listed in Table 1. The S/P value

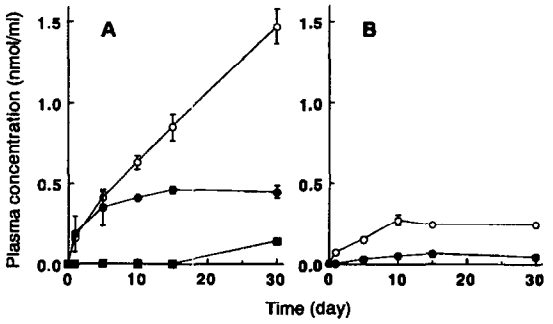

Fig. 2. Time courses of plasma concentrations of CZ, FZ, and their metabolites after repeated oral administration of CZ or FZ in rats. The rats were given CZ or FZ orally at a daily dose of 20 μmol/kg once a day for 1, 5, 10, 15, and 30 days. The concentrations of CZ, C-2, FZ, F-2, and F-3 were determined 24 hr after the final dose. (A) FZ (○), F-2, (●), and F-3 (■); (B) CZ (○), C-2 (●). Each point represents the mean ± SE of 5 animals.

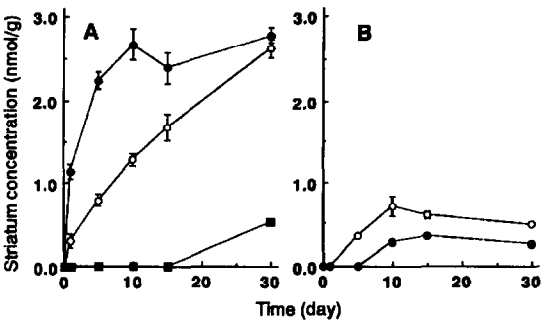

Fig. 3. Time courses of striatum concentrations of CZ, FZ, and their metabolites after repeated oral administration of CZ or FZ in rats. The rats were given CZ or FZ orally at a daily dose of 20 μmol/kg once a day for 1, 5, 10, 15, and 30 days. The concentrations of CZ, C-2, FZ, F-2, and F-3 were determined 24 hr after the final dose. (A) FZ (○), F-2 (●), and F-3 (■); (B) CZ (○), C-2 (●). Each point represents the mean ± SE of 5 animals.

of CZ was 1.2 times higher than that of FZ, and the values of C-2 and F-2 were 2.4 and 3.0 times, respectively, higher than those of the corresponding parent drugs.

Inhibition of [^{3}H]-YM-09151-2 binding to D2-R

The *Ki* values and Hill coefficients (n) of CZ, FZ, their metabolites, and 3 neuroleptics, thioridazine, chlorpromazine, and haloperidol for [^{3}H]-YM-09151-2 binding to D2-R in rat striatal membranes are listed in Table 2. The displacing curves were monophasic with Hill coefficients close to unity. The affinity of FZ for D2-R expressed by *Ki* was 3.3 times higher than that of CZ, and the affinities of C-2 and F-2 were 3.4 and 4.4 times, respectively, higher than those of their parent drugs. The *Ki* values of FZ (3.96 nM), F-2 (0.908 nM), and C-2 (3.87 nM) were close to the value of thioridazine (*Ki* = 1.75 nM), one of the typical neuroleptics inducing parkinsonism as an adverse reaction. CZ (*Ki* = 13.2 nM) had 7.5 times lower affinity than thioridazine, and the affinity of C-4 (*Ki* = 46.0 nM) was approximately one third of CZ. C-1 and F-1 showed much lower affinities for D2-R than their parent drugs. At a concentration of 100 μM, CPN produced only 25% inhibition of [^{3}H]-YM-09151-2 binding to D2-R compared with the control, and C-3, F-3, C-5, or F-5 had almost no effect on D2-R under the conditions used. The order of the affinities of the compounds for D2-R was F-2 > C-2 > FZ > CZ > C-4 > F-1 > C-1.

DISCUSSION

The calcium entry-blocking agents CZ and FZ have been reported to cause parkinsonism in patients receiving chronic treatment with these drugs, and the symptoms were reduced after chronic dosing was halted [4, 5]. These findings suggest that FZ or CZ and their metabolites may accumulate in the bodies of patients during chronic treatment, thereby causing the extrapyramidal side effect of parkinsonism. In order to clarify the mechanism causing parkinsonism, we first examined the *in vitro* oxidative metabolism of CZ and FZ using rat and human liver microsomes. We then pointed out the possible involvement of the CYP2D subfamily in the for-

Table 1. Ratios of striatum to plasma concentration (S/P) for CZ, FZ, and their metabolites

Compound	S/P	Compound	S/P
FZ	2.00 ± 0.08 (24)	CZ	2.46 ± 0.12 (20)
F-2	6.09 ± 0.21 (25)	C-2	5.92 ± 0.24 (15)
F-3	4.09 ± 0.33 (5)		

Rats were given CZ or FZ orally at a daily dose of 20 μmol/kg once a day for 1, 5, 10, 15, and 30 days. The concentrations of the compounds were determined 24 hr after the final dose. Values are expressed as mean ± SE. Values in parentheses represent the number of rats.

mation of C-2 from CZ and F-2 from FZ, either of which is one of the major metabolic pathways of the drugs in rats [18, 19] and humans [17, 20].

As a second step further clarifying the mechanism causing parkinsonism, we examined concentrations of CZ, FZ, and their metabolites in the blood and striatum after repeated administration of CZ or FZ in rats to assess the distribution of the drugs and the metabolites in the striatum, which is the primary region to produce neuroleptic-induced parkinsonism *via* dopamine receptors [6, 8]. Female rats were used in this study because a profile of the oxidative metabolism of CZ and FZ in human liver microsomes resembled that in female rat liver microsomes, rather than in males [17–20]. C-2 and F-2 were major metabolites of CZ and FZ, respectively, in the plasma and striatum of female rats given these drugs. The plasma levels of the parent drugs were higher than those of their metabolites from 5 to 30 doses. On the contrary, the striatum concentrations of F-2 were higher than those of FZ, whereas C-2 levels were lower than CZ levels throughout the study. Both the plasma and striatum concentrations of FZ continued to increase during the experiments, whereas concentrations of F-2, CZ, and C-2 reached steady state after the tenth day of repeated administration, suggesting a possible accumulation of FZ in the body. On the other hand, the ratio of striatum to plasma concentrations for C-2 and F-2 were approximately 2 to 3 times higher than the parent drugs after 30 doses indicating higher permeabilities and more effective distribution into the rat brain of the metabolites F-2 and C-2 than the parent drugs.

Many neuroleptics induce extrapyramidal side effects, such as parkinsonism, akathisia and tardive diskinesia, and neuroleptic-induced parkinsonism has been shown to be due to the dopamine receptor blockade in the striatum [7, 8]. The clinical features of CZ- and FZ-induced extrapyramidal side effects [4–6] are similar to those induced by neuroleptics, suggesting that CZ and FZ may also cause parkinsonism through a mechanism similar to that for neuroleptics. As the third step to elucidating the mechanism whereby parkinsonism was induced, we examined the affinities of CZ, FZ, and their metabolites for D2-R in striatal fractions from female rats using [^{3}H]-YM-09151-2 as a ligand for D2-R. Among the 10 metabolites of CZ and FZ examined, F-2 and C-2 were found to exhibit higher affinities for D2-R than the corresponding parent drugs. In particular, the *Ki* values of F-2 and C-2 for D2-R were found to be comparable to those of chlorpromazine and thioridazine, typical neuroleptics that induce parkinsonism as a side effect after chronic medication. In contrast, affinity for the receptors of C-4, a metabolite produced by the *p*-hydroxylation of the diphenylmethyl moiety, was lower than that of CZ. The affinities are in the order of F-2 > C-2 > FZ > CZ > C-4 > F-1 > C-1. Other metabolites produced by N-dealkylations (C-3, F-3, C-5, F-5, and CNP) scarcely affected the 2 receptor.

Recently, the existence of 5 subtypes of dopamine receptors (D1, D2, D3, D4, and D5) has been demonstrated through molecular biology techniques [27–31]. [^{3}H]-YM-09151-2, used in the present study, is known to have high affinities for both D2 and D4 receptors [29, 32]. Among the 5 receptors, D2-R is reported to be a predominant dopaminergic receptor type in the striatum closely linked to neuroleptic-induced parkinsonism [8, 32]. Furthermore, striatal muscarinic cholinergic receptors are also known to be involved in neuroleptic-induced parkinsonism [7]. Complementary DNAs of multiple subtypes have recently been cloned for this type of receptors as well [33, 34]. Potentialities of drugs causing parkinsonism as a side effect should be assessed from

Table 2. Competition by CZ, FZ, their metabolites, and typical neuroleptics for [^{3}H]-YM-09151-2 binding in rat striatal membranes

Compound	*Ki* (nM)	nH
CZ	13.2 ± 0.4	1.16 ± 0.03
C-1	5621 ± 151	1.19 ± 0.06
C-2	3.87 ± 0.03	1.02 ± 0.01
C-3	>8760	N.D.
C-4	46.0 ± 3.3	1.18 ± 0.09
C-5	>8760	N.D.
FZ	3.96 ± 0.08	1.01 ± 0.02
F-1	517 ± 22	1.36 ± 0.05
F-2	0.908 ± 0.06	1.00 ± 0.02
F-3	>8760	N.D.
F-5	>8760	N.D.
CNP	>8760	N.D.
Thioridazine	1.75 ± 0.13	1.08 ± 0.02
Chlorpromazine	0.675 ± 0.088	0.96 ± 0.04
Haloperidol	0.125 ± 0.029	0.98 ± 0.02

The membranes (0.1 mg/mL) were incubated with 0.1 nM [^{3}H]-YM-09151-2 and varying concentrations of the compounds for 20 min at 37°C. Each value represents the mean ± SE of 3 independent experiments conducted in duplicate. Hill coefficients were obtained from the competition curves by Hill plots [25]. The inhibition constants (*Ki*) were calculated from IC_{50} values by the method of Cheng and Prusoff [26]. N.D., not determined.

the total balance between their affinities for dopaminergic and muscarinic cholinergic receptors [7]. Therefore, development of specific ligands for each subtype of dopamine receptors and muscarinic cholinergic receptor is necessary to fully understand which subtype(s) of the receptors in striatum is responsible for parkinsonism induced by FZ, CZ, and their metabolites.

In conclusion, the multiple-dose study demonstrated that after repeated administration of FZ or CZ at a daily dose of 20 μmol/kg for 1 to 30 days, the striatum concentrations of F-2 were higher than those of the parent drug, whereas C-2 concentrations were lower than CZ concentrations in the same brain region. In addition, the ratios of striatum to plasma concentrations were higher for F-2 and C-2 than for their parent drugs. The radioreceptor binding assay for D2-R revealed that affinities of C-2 and F-2 were higher than those of the respective parent drugs in rat brain striatum, and the affinities of the metabolites were comparable to those of chlorpromazine and thioridazine. These results indicate that on the basis of the equimolar dosing performed here, FZ is a more potent inducer of parkinsonism than CZ, and that together with the parent drugs C-2 and F-2, metabolites with the cinnamyl moiety whose phenyl ring is *p*-hydroxylated contribute to cause parkinsonism as active metabolites.

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