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Comparison of the Effects of Feprazone and Phenylbutazone on Testosterone Hydroxylations in Mouse Hepatic Microsomes

KUNIHIKO MORITA, YUKIO HAYASHI, TAKESHI ONO and HARUMI SHIMAKAWA*

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

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The inhibitory and inducing actions of feprazone (FZ), an anti-inflammatory agent, on testosterone hydroxylations in the mouse hepatic microsomal mixed-function oxidase system were compared with those of phenylbutazone (PZ).

As regards inhibitory effects, testosterone hydroxylations were competitively inhibited by addition of FZ and PZ *in vitro*. No significant difference was found between FZ and PZ in inhibitory potency for 6β- and for 16α-hydroxylations, but the inhibitory effect of FZ on 7α-hydroxylation was stronger than that of PZ. However, the three hydroxylations were non-competitively inhibited in microsomes from mice treated with each agent.

As regards the inducing action, only 6β-hydroxylase activity was increased following continuous administration of low doses (75 mg/kg) of FZ and of PZ; in addition, 16α-hydroxylase activity was also increased following continuous administration of high doses (150 mg/kg) of these drugs. 7α-Hydroxylase activity, however, was not significantly affected by either low or high doses. We also found that the patterns and the extents of induction of the three hydroxylase activities by FZ and PZ were different from those by phenobarbital or β-naphthoflavone.

These results indicate that FZ and PZ have essentially identical inducing effects, whereas FZ exhibits a greater inhibitory effect than PZ on the activity of certain P-450 isozymes such as 7α-hydroxylase in the mouse mixed-function oxidase system.

Keywords——testosterone hydroxylation; feprazone; phenylbutazone; mouse hepatic microsome

Phenylbutazone (PZ), a typical pyrazolone derivative which has anti-inflammatory action, is one of a number of drugs with a biphasic action on drug-metabolizing enzymes in the hepatic microsomes.[1] After administration of a single dose, the enzyme activity is first depressed, and then after 12 h, enhanced.[2,3] The inhibitory or inducing effects of PZ on drug-metabolizing enzymes present a problem in clinical use. For example, combined use with PZ causes prolongation of the serum half-life of warfarin or diphenylhydantoin and the enhancement of digitoxin metabolism.[4–6]

On the other hand, feprazone (FZ), a new anti-inflammatory pyrazolone derivative, is reported to be clinically useful, because its adverse effect on gastro-enteric tissue is less than that of PZ, in spite of their similar pharmacological effects.[7–10] However, the effect of FZ on the drug-metabolizing enzyme system remains to be investigated in detail.

It is well known that this enzyme system physiologically mediates hydroxylations of various steroid hormones, such as testosterone. In this study, we compared the inhibitory and inducing activities of FZ on testosterone hydroxylations in mouse hepatic microsomes with that of PZ.

Materials and Methods

Materials——FZ was kindly supplied by Fujisawa Pharmaceutical Co., Ltd., Osaka. PZ and β-naphthoflavone

were purchased from Sigma Chemical Co., Ltd., St. Louis, MO. Phenobarbital sodium salt was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo. Testosterone was purchased from Nakarai Chemical Ltd., Kyoto. All other chemicals and solvents were of analytical grade.

Preparation of Microsomes——Liver tissue from male ddY mice (4—6 weeks old) was homogenized in ice-cold 1.15% KCl (1 : 4, w/v), and the homogenate was centrifuged at 10000 *g* for 15 min. The supernatant fraction was centrifuged at 105000 *g* for 60 min to obtain a microsomal pellet. The microsomes were resuspended in 1.15% KCl and again centrifuged at 105000 *g* for 30 min. This process was repeated twice. The resulting pellet was resuspended in 1.15% KCl to a concentration of about 5 mg protein/ml. Protein was determined by the method of Lowry *et al.*[11]

Treatment Regimens——FZ, PZ, phenobarbital, and β-naphthoflavone were each suspended in corn oil and given as intraperitoneal injections at intervals prior to killing the animals. The regimens used were: FZ (75 mg/kg or 150 mg/kg) or PZ (75 mg/kg or 150 mg/kg), one dose per day on five consecutive days with the final injection 24 h before sacrifice; phenobarbital (80 mg/kg), one dose per day on three consecutive days with the final injection 24 h before sacrifice; β-naphthoflavone (80 mg/kg), one dose 48 h before sacrifice. On the other hand, in the acute studies, animals were given a single administration of 150 mg/kg of FZ or PZ 2 h before sacrifice.

Additions of FZ and PZ *in Vitro*——FZ and PZ were dissolved in 0.1 M phosphate buffer (pH 7.4) and added to the incubation mixture to give final concentrations of 0.05—0.5 mM.

Biochemical Analysis——Testosterone 6β-, 7α- and 16α-hydroxylase activities were assayed as described in a previous paper.[12] The assay mixture contained, in a final volume of 5 ml, a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (0.5 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate, 5 mM $MgCl_2$ and 2 units of glucose-6-phosphate dehydrogenase), phosphate buffer (67.5 mM, pH 7.4), microsomal protein (0.5 mg/ml), testosterone (12.5—100 μM), and FZ or PZ (0.05—0.5 mM) when indicated. The reaction was started by adding 0.5 ml of microsomal suspension and carried out at 37 °C with shaking for 10 min. The reaction was stopped by adding 20 ml of dichloromethane. After addition of 0.25 ml of 0.2 mM digoxin as an internal standard and centrifugation at 900 *g* for 5 min, the organic layer was separated, and the aqueous layer was extracted with a small amount of dichloromethane. The combined organic layer was evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of methanol and subjected to thin-layer chromatography (TLC) procedure.[12]

Cytochrome P-450 was determined in a microsomal suspension containing 1 mg protein/ml 0.1 M phosphate buffer, pH 7.4, by the method of Omura and Sato.[13]

NADPH-cytochrome c reductase activity was measured at 30 °C on a Shimadzu UV-300 dual-beam spectrophotometer by the method of Peters and Fouts.[14]

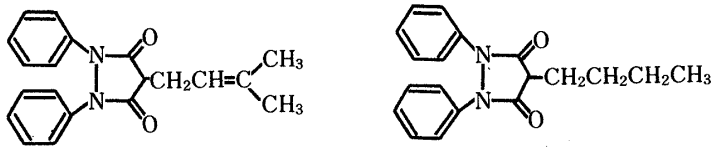
Results

The chemical structures of FZ and PZ are shown in Fig. 1.

Inhibitory Effects of FZ and PZ on Testosterone Hydroxylations *in Vitro* and *in Vivo*

The effects of various concentrations (0.05—0.5 mM) of FZ and PZ on testosterone hydroxylations *in vitro* are shown in Table I. FZ and PZ inhibited each hydroxylation in a dose-dependent manner. The inhibitory effects were investigated in detail by the use of double-reciprocal plots (Fig. 2). The kinetic data indicated that the inhibition of each hydroxylase activity by these compounds was of a competitive type; the apparent K_m was increased without changing the V_{max} of the enzymes. The inhibition constants (K_i) calculated from Fig. 2 are shown in Table II. FZ and PZ showed no significant difference in K_i for 6β- and 16α-hydroxylase activities, but for 7α-hydroxylase activity, FZ had a greater inhibitory effect than PZ.

Figure 3 shows double-reciprocal plots of testosterone hydroxylase activities in micro-



feprazone phenylbutazone

Fig. 1. Structural Formulas of the Drugs Tested

TABLE I. Dose-Related Inhibition of Testosterone Hydroxylations in Mouse Hepatic Microsomes by Feprazone and Phenylbutazone

Drug	mM	Enzyme activity (nmol/mg protein/min)		
		6β-OH	7α-OH	16α-OH
None (control)		3.57±0.15 (100)	0.47±0.03 (100)[a]	1.01±0.07 (100)
Feprazone	0.05	2.66±0.11 (75)	0.39±0.03 (83)	0.94±0.05 (93)
	0.25	1.90±0.16 (53)	0.34±0.01 (73)	0.81±0.06 (80)
	0.50	1.54±0.08 (43)	0.31±0.01 (68)	0.73±0.02 (73)
Phenylbutazone	0.05	3.02±0.20 (85)	0.45±0.02 (96)	0.89±0.05 (88)
	0.25	1.93±0.18 (54)	0.43±0.02 (91)	0.82±0.05 (82)
	0.50	1.51±0.05 (42)	0.40±0.01 (85)	0.75±0.01 (74)

The concentration of testosterone in each experiment was 12.5 μM. Each value is the mean±S.D. of four determinations. *a*) The figure in parenthesis is % of the enzyme activity of the control.

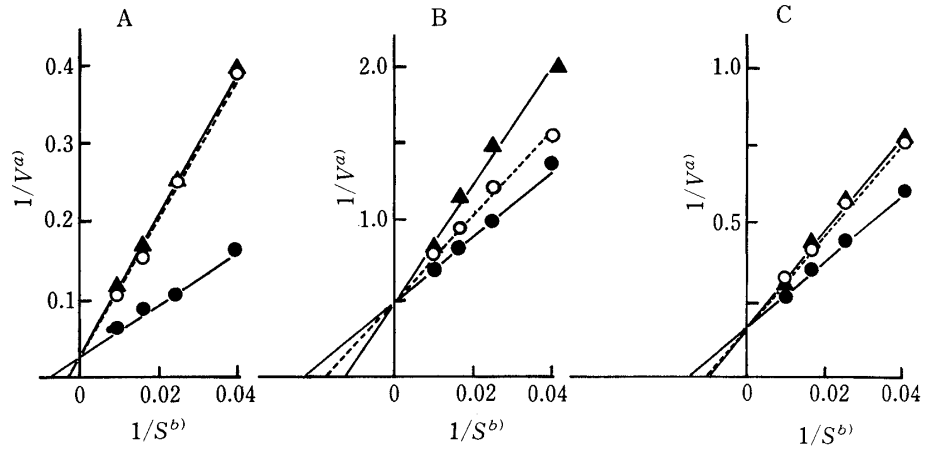


Fig. 2. Double-Reciprocal Plots of Testosterone Hydroxylations Obtained with Mouse Hepatic Microsomes in the Absence and Presence of 0.5 mM Feprazone and Phenylbutazone *in Vitro*

A, 6β-hydroxylation; B, 7α-hydroxylation; C, 16α-hydroxylation.
Symbols: —●—, control; —▲—, feprazone; —○—, phenylbutazone.
a) V=nmol/mg protein/min. *b*) S=concentration of testosterone (μM).

TABLE II. Inhibition Constants (K_i) of Feprazone and Phenylbutazone for Testosterone Hydroxylations in Mouse Hepatic Microsomes

Drug	K_i (mM)[a]		
	6β-OH	7α-OH	16α-OH
Feprazone	0.30±0.03	0.61±0.02[b]	0.99±0.14
Phenylbutazone	0.26±0.02	1.40±0.04	0.99±0.20

Each value is expressed as the mean±S.D. of four determinations. *a*) Calculated from the double-reciprocal plots for testosterone hydroxylations (Fig. 2). *b*) Significantly different from phenylbutazone ($p<0.01$).

somes from mice killed 2 h after administration of 150 mg/kg of FZ and of PZ. In contrast with the results *in vitro*, each hydroxylase activity was non-competitively inhibited; the V_{max} of the enzymes was decreased without changing the apparent K_m.

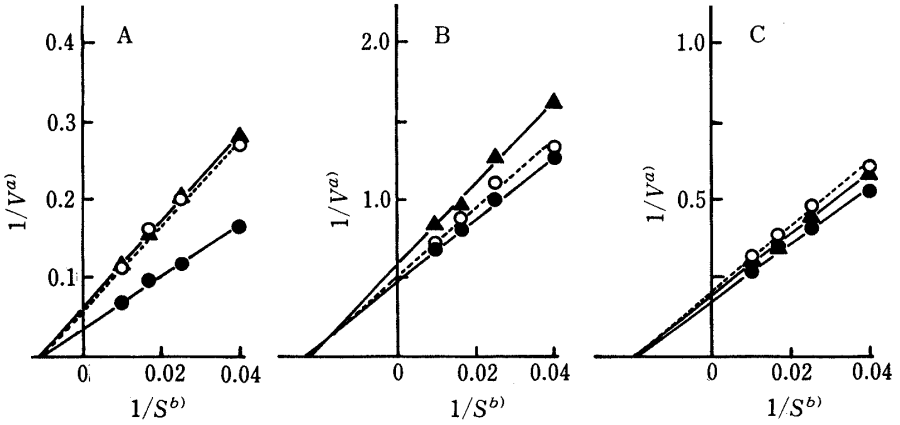


Fig. 3. Double-Reciprocal Plots of the Effects of Feprazone and Phenylbutazone *in Vivo* on the Hydroxylations of Testosterone by Mouse Hepatic Microsomes

Vehicle alone (●), 150 mg/kg of feprazone (▲) and phenylbutazone (○) were given *i.p.* 2 h before killing the mice.
A, 6β-hydroxylation; B, 7α-hydroxylation; C, 16α-hydroxylation.
a) V=nmol/mg protein/min. *b*) S=concentration of testosterone (μM).

TABLE III. Effects of Feprazone, Phenylbutazone, Phenobarbital and β-Naphthoflavone on the Parameters Associated with Hepatic Drug Metabolism in Mice

Treatment	(mg/kg/d)	Cytochrome P-450 nmol/mg microsomal protein	NADPH-cytochrome c reductase nmol/mg microsomal protein/min
None (corn oil)		0.615±0.006	120.7± 5.5
Feprazone	(75)	0.705±0.040	174.3±18.9[a)]
	(150)	0.784±0.013[a)]	212.2±15.5[b)]
Phenylbutazone	(75)	0.778±0.075[a)]	116.5± 3.8
	(150)	0.954±0.045[a)]	176.8± 5.7[b)]
None (corn oil)		0.574±0.007	117.3± 5.8
Phenobarbital	(80)	1.167±0.070[b)]	267.3±17.6[b)]
β-Naphthoflavone	(80)	0.689±0.010[a)]	195.9±13.7[b)]

Treatment regimens and biochemical analysis were described under Materials and Methods. Each value is the mean±S.E. of four determinations. *a*) Significantly different from the appropriate control ($p<0.05$). *b*) Significantly different from the appropriate control ($p<0.01$).

Inducing Effects of FZ and PZ on Testosterone Hydroxylations

In order to investigate the enzyme-inducing characteristics of FZ and PZ, we compared the effects of these drugs on mouse hepatic microsomal systems with those of the widely studied enzyme-inducing agents, phenobarbital and β-naphthoflavone.[15-17)]

Table III 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 administration of FZ, PZ, phenobarbital and β-naphthoflavone. Some increases in the values of these parameters (30—55% in P-450 content and 45—75% in NADPH-cytochrome c reductase activity) were noted after treatments of FZ and PZ, in contrast with the greater increases noted after phenobarbital treatment (100% in P-450 content and 130% in NADPH-cytochrome c reductase activity).

The effects of these drugs on the mixed-function oxygenation of testosterone are shown in Fig. 4. Testosterone hydroxylations were not affected by β-naphthoflavone, but they were

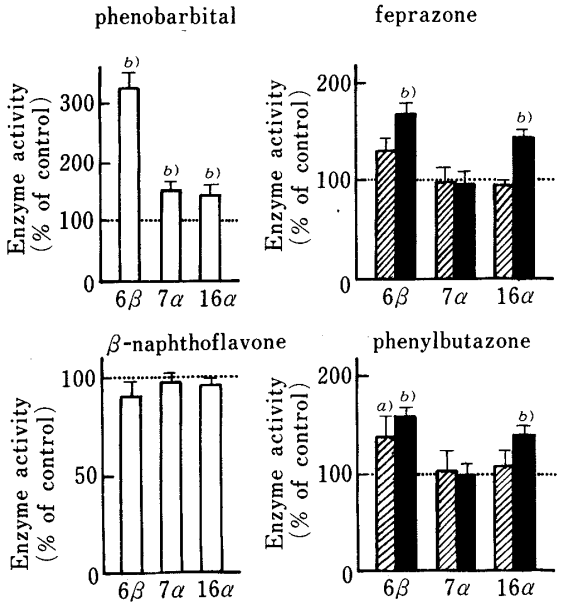


Fig. 4. *In Vivo* Effects of Feprazone, Phenylbutazone, Phenobarbital and β-Naphthoflavone on Testosterone Hydroxylations in Mouse Hepatic Microsomes

Treatment regimens are described under Materials and Methods. Each value is the mean ± S.D. of four separate determinations and is presented as a percentage of the corresponding activity in control animals treated with the vehicle alone. The control activities (nmol/mg protein/min; mean ± S.D.) were: production of 6β-hydroxytestosterone (3.57 ± 0.15); production of 7α-hydroxytestosterone (0.76 ± 0.03); production of 16α-hydroxytestosterone (1.54 ± 0.07). Symbols: ▨, 75 mg/kg; □, 80 mg/kg; ■, 150 mg/kg. *a*) Significantly different from the appropriate control ($p < 0.05$). *b*) Significantly different from the appropriate control ($p < 0.01$).

considerably increased following treatment with phenobarbital. This effect was primarily due to a substantial increase (three-fold) in the activity of testosterone 6β-hydroxylase. On the other hand, in the cases of FZ and PZ, only 6β-hydroxylase activity was increased following treatment with low doses (75 mg/kg), and both 6β- and 16α-hydroxylase activity was increased following treatment with high doses (150 mg/kg) of these drugs, but 7α-hydroxylase activity was not significantly affected in either case. Further, FZ and PZ showed no significant difference in inducing effects on testosterone hydroxylations.

Discussion

The present study shows that both FZ and PZ are capable of producing inhibitory and inducing effects on testosterone hydroxylase activities in mouse hepatic microsomes.

FZ and PZ can inhibit the hydroxylations of testosterone by hepatic microsomes in two ways. These drugs competitively inhibited 6β-, 7α- and 16α-hydroxylase activities when incubated with microsomes *in vitro*, and non-competitively inhibited these reactions when injected into animals. Cho *et al.*[18] reported that the ethylmorphine-demethylating enzyme activity in microsomes from PZ-treated rats was non-competitively inhibited, while addition of PZ to the microsomal incubation mixtures caused competitive inhibition. They demonstrated that the non-competitive inhibition was associated with irreversible binding of PZ, or its metabolites, to microsomes.

It was reported that the half-life of PZ in mouse was 1.5—3 h.[19] In our study, non-competitive inhibitions of testosterone hydroxylations were found in the microsomes prepared 2 h after administration of FZ and PZ. Cho *et al.* reported that a washing procedure with Tris–KCl buffer removed 95% of ^{14}C-labeled PZ, but the ethylmorphine demethylase activity was not restored.[18] Thus, our results obtained after washing the microsomes with KCl solution may be due to irreversible binding of these drugs or their metabolites to the microsomes. Further studies will be necessary to establish the mechanism of the inhibitory effects.

On the other hand, it was reported that separate enzyme systems catalyze the hydroxylation of testosterone in microsomes;[20] and that different cytochrome P-450 isozymes hydroxylate testosterone at the 6β-, 7α- and 16α-positions in mouse liver microsomes.[21] Accordingly, it is suggested that FZ does not have the same affinity as PZ for certain P-450

isozymes, because FZ had a greater inhibitory effect than PZ on 7α-hydroxylase activity *in vitro* (Fig. 2, Table II). However, it is not clear whether the inhibitory potencies of the two drugs on these hydroxylations *in vivo* were similar or not, because the actual amounts of FZ and PZ in the microsomes were not determined.

We also compared the effects of FZ and PZ on the mouse hepatic microsomal mixed-function oxidase system with those of phenobarbital and β-naphthoflavone as typical P-450-type and P-448-type inducing agents. The three hydroxylation reactions were not affected by β-naphthoflavone; in contrast, these hydroxylations were markedly enhanced by phenobarbital treatment, particularly the 6β-hydroxylase activity (Fig. 4). These findings are similar to the results obtained in male C57B16 mice by Tredger *et al.*[17] Therefore, among mouse hepatic microsomal cytochrome P-450 isozymes, P-448-type isozymes induced by β-naphthoflavone are not considered to be associated with the testosterone hydroxylations. On the other hand, both FZ and PZ induced only 6β-hydroxylase activity following treatment with low doses (75 mg/kg) of the anti-inflammatory agents and also induced 16α-hydroxylase activity following treatment with high doses (150 mg/kg) (Fig. 4). 7α-Hydroxylase activity, however, was not affected following treatments with either dose. These results indicate that the patterns and extents of induction of the three hydroxylase activities by FZ and by PZ are quantitatively similar, but are markedly different from those by phenobarbital. It has been reported that treatment of immature rats and dogs with PZ increases several-fold the activities of testosterone hydroxylases in liver microsomes.[22] The ability of foreign compounds to stimulate steroid hydroxylases in liver microsomes of experimental animals is paralleled by enhanced steroid hydroxylations in man.[20] Kuntzman *et al.*[23] reported that PZ increased the formation of 6β-hydroxycortisol from cortisol by induction of oxidative metabolism in man. Consequently, our observations seem to suggest that FZ induces steroid hydroxylation activities in the same way as PZ in man.

In conclusion, these results indicate that FZ is essentially identical to PZ in terms of inducing effects, but FZ exhibits a greater inhibitory effect than PZ on the activity of certain P-450 isozymes such as testosterone 7α-hydroxylase in the mouse hepatic mixed-function oxidase system. It is, of course, difficult to extrapolate these findings directly to man, but the possibility that a similar interaction could occur in patients seems to require investigation.

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