Metabolism of Pyriproxyfen. 3. *In Vitro* Metabolism in Rats and Mice

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In vitro metabolism of pyriproxyfen [4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy)propyl ether, Sumilarv] by several tissues of rats and mice was investigated. Most of the major metabolites of pyriproxyfen observed in studies of *in vivo* metabolism were formed by liver microsomes incubated *in vitro* with β-NADPH for both species. No sex-related differences were observed in mouse microsomes in major *in vitro* metabolic reactions, these being hydroxylation at the 4-position of the terminal phenyl ring, hydroxylation at the 5-position of the pyridyl ring, and cleavage of the propyl pyridyl or propyl phenyl ethers. On the other hand, in rat microsomes there were significant sex-related differences in these major metabolic reactions except for cleavage of the propyl phenyl ether. All of the gender-specific reactions, in which cytochrome P450 enzymes were involved, were inhibited by antisera against male-specific rat P450 CYP2C11 or CYP2C13. Therefore, the results strongly imply that the 2C family of cytochrome P450 is involved in the metabolism of pyriproxyfen in rats.

Keywords: *Metabolism; pyriproxyfen; rat; mouse; in vitro*

INTRODUCTION

Pyriproxyfen [4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy)-propyl ether, Sumilarv] is a new insect growth regulator with insecticidal activities against houseflies, mosquitoes, and cockroaches (Estrada and Mulla, 1986; Hatakoshi *et al*., 1987; Kawada *et al*., 1987, 1988, 1989). This compound is now in the advanced developmental stage and has been undergoing toxicological evaluation, including metabolism studies. The metabolism of this compound in carp, dragonfly, midge, and mosquito larvae was earlier reported by Miyamoto *et al*. (1993).

In vivo metabolism studies of pyriproxyfen in rats and mice have already been conducted, and its metabolic fate has been elucidated as follows: (1) excretion is rapid and complete, (2) tissue residues are small, (3) major metabolic reactions are hydroxylation at the 4- or 2-position of the terminal phenyl ring (4′- or 2′-hydroxylation) and the 5-position of the pyridyl ring (5″-hydroxylation), desphenylation, cleavage of the ether linkages, and conjugation of the resultant phenols with sulfuric or glucuronic acid (Matsunaga *et al*., 1995; Yoshino *et al*., 1995), and (4) slight sex-related differences were observed in extents of 4′- and 5″-hydroxylation and ether cleavage in rats but not in mice.

This report deals with *in vitro* metabolism studies with several tissues of rats and mice, conducted in order to elucidate the sex-related differences in metabolic reactions of pyriproxyfen in more detail.

MATERIALS AND METHODS

Chemicals and Reagents. [*phenoxyphenyl*-^{14}C]Pyriproxyfen with a specific activity of 1.57 GBq/mmol was prepared in the Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd. The radiochemical purity was >99% on thin-layer chromatography (TLC). β-NADPH was purchased from Oriental Yeast Co. (Tokyo, Japan). Piperonyl butoxide was purchased from Aldrich Chemical Co., Inc., Tokyo, Japan. Antibodies to cytochrome P450s CYP2C11 and CYP2C13 were purchased from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan).

Preparation of Subcellular Fractions. SD rats and ICR mice were purchased at 7 weeks of age from Charles River Japan Inc. (Atsugi, Japan). Kidneys, livers, lungs, and small intestines were excised after sacrifice by decapitation. The excised tissues were washed with ice-cold saline and weighed after removal of fat and connective tissues. After homogenation at a concentration of 20% (0.2 g fresh tissue/mL) in ice-cold 0.15 M KCl solution with a Potter-Elvehjem glass−Teflon homogenizer and centrifugation at 10000*g* for 10 min at 4 °C, the 10000*g* supernatants (S10) were adjusted to a concentration of 10% with 50 mM potassium phosphate buffer (pH 7.4). Hepatic microsomal and cytosol fractions were prepared by centrifugation of S10 of liver at 105000*g* for 60 min at 4 °C. The concentration of the supernatant (cytosol) was adjusted to 10% with the buffer. The pellet was resuspended in the same volume of buffer and centrifuged at 105000*g* for 60 min at 4 °C to obtain the microsomal fraction. Protein content was measured by the method of Bradford (1976) using a protein assay kit (Bio-Rad Laboratory, Richmond, CA) and bovine serum albumin as a standard, being 1.0−1.8 mg/mL for all of the 10% hepatic microsomal suspensions in the buffer. The cytochrome P450 content was determined by the method of Omura and Sato (1964).

Enzymatic Reactions. A 2 μL ethanol solution of [*phenoxyphenyl*-^{14}C]pyriproxyfen, the final concentration of which was adjusted to 0, 0.05, 0.1, 0.5, or 1 mM, was added to 2 mL of 10% S10, 10% hepatic microsome suspension, or 10% cytosol and incubated for 5 min at 37 °C. The reaction was initiated by adding β-NADPH to the preincubated mixture at a concentration of 3 mM, and the mixture was aerobically incubated for another 10 min at 37 °C. The reaction was terminated by addition of 2 mL of ice-cold 1:1 ethanol−acetone to the reaction mixture, which was then centrifuged at 1500*g* for 10 min at 4 °C. The supernatant was concentrated and subjected to TLC with a 3:2 toluene−diethyl ether solvent system. Enzyme activity was calculated from the amounts of identified metabolites. Identification was conducted as reported by Matsunaga *et al*. (1995) and Yoshino *et al*. (1995).

Incubation of Hepatic Microsomes with P450 Antisera. An inhibition study of *in vitro* metabolism with P450 antisera was conducted using the method of Neville *et al*. (1993) with some modifications. A 250 μL microsomal suspension containing 250 pmol of cytochrome P450 was preincubated

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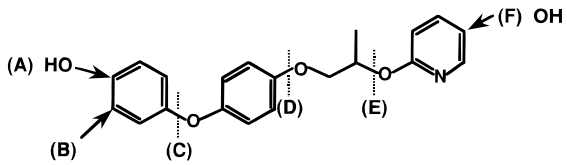

Figure 1. Chemical structure of pyriproxyfen and sites of phase I metabolic reactions in rats and mice: (A) 4′-hydroxylation, (B) 2′-hydroxylation, (C) desphenylation, (D) cleavage of the propyl phenyl ether, (E) cleavage of the propyl pyridyl ether, and (F) 5″-hydroxylation.

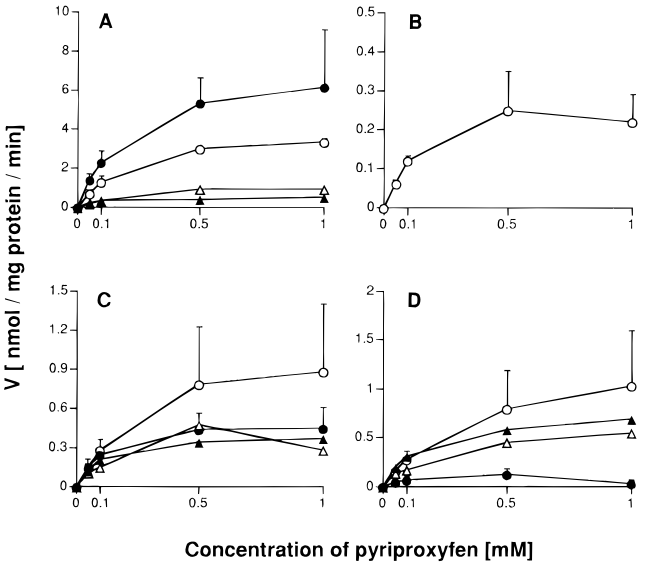

Figure 2. Michaelis−Menten plots of the major metabolic reactions of pyriproxyfen in hepatic microsomes of rats and mice: (A) 4′-hydroxylation, (B) 5″-hydroxylation, (C) cleavage of the propyl phenyl ether, and (D) cleavage of the propyl pyridyl ether. Key: (○) male rats, (●) female rats, (△) male mice, and (▲) female mice. Results are mean ± SE values (n = 3).

for 25 min at 37 °C with 100 μL of cytochrome P450 antisera CYP2C11 or CYP2C13. The reaction was initiated by adding β-NADPH (3 mM), 10 μL of an ethanol solution of [*phenoxyphenyl*-^{14}C]pyriproxyfen (10 μM), and 50 mM potassium phosphate buffer to give a final volume of 1 mL. The reaction mixture was then incubated at 37 °C for 10 min. Reaction mixture without any cytochrome P450 antisera was incubated as a control. The reaction was terminated by adding 2 mL of 1:1 ethanol−acetone. Percent inhibition was calculated by subtracting the percent metabolism by hepatic microsomes preincubated with antisera from control values which were set at 100%.

Thin-Layer Chromatography (TLC). TLC analysis was conducted essentially as reported earlier by Saito *et al.* (1991) and Yoshino *et al.* (1993a). Precoated silica gel 60 F_{254} chromatoplates (20 × 20 cm, 0.25 mm layer thickness; Merck, Darmstadt, Germany) were used for TLC analysis. R_f values for pyriproxyfen and its metabolites were reported previously (Matsunaga *et al.*, 1995; Yoshino *et al.*, 1995). Radioactive spots on TLC plates were localized by placing imaging plates (Type BAS-III, Fuji Photo Film Co., Ltd., Japan) on TLC plates at room temperature for 1−2 days followed by visualization with a Bio-Image analyzer (BAS2000, Fuji Photo Film, Co., Ltd.).

Radioanalysis. Radioanalysis was carried out according to the methods of Yoshino *et al.* (1993b). Radioactivity in silica gel regions scraped from TLC plates was quantified by liquid scintillation counting (LSC) with a Tri-Carb 2500TR spectrometer (Packard).

Statistical Analysis. Two-tailed Student's *t*-test was used for statistical data analysis.

RESULTS AND DISCUSSION

Tissue or Subcellular Fractions Involved in Metabolism of Pyriproxyfen. The major phase I metabolic reactions of pyriproxyfen observed *in vivo* in rats and mice are shown in Figure 1. Pyriproxyfen was not metabolized *in vitro* by 10% S10 from kidney, lung, or small intestine for either species, while all of the reactions shown in Figure 1 occurred with liver S10. High metabolic activity was found in hepatic microsomes, whereas that with hepatic cytosol was only slight. On the basis of the above observation, further investigations were conducted using only the hepatic microsome fraction. The activity of the microsomes toward pyriproxyfen was almost completely inhibited by 1 mM piperonyl butoxide, an inhibitor of cytochrome P450 (Anders, 1968); this strongly indicated that pyriproxyfen is metabolized mainly by cytochrome P450 enzymes.

***In Vitro* Metabolism by Hepatic Microsome.** Figure 2 shows Michaelis−Menten plots of the major metabolic reactions of pyriproxyfen in hepatic microsome fractions from rats and mice. These reactions are 4′-hydroxylation, 5″-hydroxylation, and cleavage of the propyl phenyl ether or propyl pyridyl ether. Values for the apparent kinetic constants, V_{max}, K_m, and the first-order rate constant (V_{max}/K_m), are summarized in Table 1. V_{max} and K_m values were calculated from

Table 1. Apparent Kinetic Constants of Metabolic Activities of 4′-Hydroxylation, 5″-Hydroxylation, and Cleavage of the Propyl Phenyl or Propyl Pyridyl Ether of Pyriproxyfen in Hepatic Microsomes of Rats and Mice[a]

metabolic reaction	species	sex	V_{max} [nmol (mg of protein)$^{-1}$ min^{-1}]	K_m (mM)	first-order rate constant (V_{max}/K_m)
4′-hydroxylation	rat	male	5.4 ± 1.7	0.40 ± 0.27	17 ± 6*
	rat	female	7.5 ± 3.3	0.21 ± 0.11	38 ± 8*
	mouse	male	1.3	0.25	5.0
	mouse	female	0.6	0.11	5.4
5″-hydroxylation[b]	rat	male	0.45 ± 0.25	0.34 ± 0.31	1.8 ± 0.6
cleavage of the					
propyl phenyl ether	rat	male	1.3 ± 0.81	0.32 ± 0.15	3.6 ± 1.0
	rat	female	0.51 ± 0.17	0.12 ± 0.07	4.9 ± 1.7
	mouse	male	0.40	0.15	2.6
	mouse	female	0.44	0.12	3.4
propyl pyridyl ether	rat	male	1.8 ± 1.4	0.47 ± 0.35	3.7 ± 0.2**
	rat	female	0.17 ± 0.07	0.18 ± 0.13	1.2 ± 0.5**
	mouse	male	0.54	0.16	3.4
	mouse	female	0.75	0.14	5.4

[a] Data were estimated from Lineweaver−Burk plots. Data for rats show the mean ± SE values (n = 3). For mice, analyses were conducted with pooled microsomes of three mice. [b] 5″-Hydroxylation was not detected in hepatic microsomes of female rats, male mice, and female mice. * These values are significantly different from each other ($P < 0.05$). ** These values are significantly different from each other ($P < 0.005$).

Table 2. Inhibitory Effect of Antisera against Cytochrome P450 Isozymes CYP2C11 and CYP2C13 on Major Metabolic Reactions of Pyriproxyfen by Hepatic Microsomes of Male Rats[a]

metabolic reaction	inhibition[b] (%) CYP2C11 antiserum	CYP2C13 antiserum
4′-hydroxylation	88	0
5″-hydroxylation	100	64
cleavage of the		
propyl phenyl ether	94	25
propyl pyridyl ether	95	43

[a] Data are the mean values of two trials. [b] Percent inhibition was calculated by subtracting the percent metabolism by hepatic microsomes preincubated with antisera at 37 °C for 25 min from control values by microsomes without antisera which was set at 100%.

Lineweaver−Burk plots. It is well known that the first-order rate constant is proportional to the rate of metabolic reactions at low substrate concentrations (Raxworthy *et al.*, 1986).

As shown in Table 1, no marked sex-related differences were observed for mice. 5″-Hydroxylation was not detected in mice. The first-order rate constants ranged from 3 to 5 for the other three reactions for mice. However, some sex-related differences were found for rats. 5″-Hydroxylation was detected only in male rats. The first-order rate constant of 4′-hydroxylation was significantly larger in female rats (38) than in male rats (17) ($P < 0.05$), and that of cleavage of the propyl pyridyl ether was significantly larger in males (3.7) than in females (1.2) ($P < 0.005$).

Thus, similar tendencies for sex-related differences in metabolism of pyriproxyfen were observed in *in vitro* as well as *in vivo* metabolism studies (Matsunaga *et al.*, 1995; Yoshino *et al.*, 1995), providing significant further evidence of the sex dependence of 4′-hydroxylation, 5″-hydroxylation, and cleavage of the propyl pyridyl ether of pyriproxyfen in rats.

Incubation of Hepatic Microsomes with P450 Antisera. The existence of sex-specific cytochrome P450s in rat liver microsomes is well established (Kato and Yamazoe, 1990; Imaoka *et al.*, 1991). It is currently accepted that sex- and age-related differences in metabolism arise due to changes in the relative composition of the various enzymes comprising the drug-metabolizing cytochrome P450 family (Neville *et al.*, 1993). Accordingly, it is to be expected that sex-related differences in metabolism of pyriproxyfen might have some relation to sex-specific expression of cytochrome P450s. Cytochrome P450 isozymes CYP2C11 and CYP2C13 are male-specific forms, whereas CYP2C12 is female specific (Funae and Imaoka, 1993; Ryan and Levin, 1993). Since antiserum against CYP2C12 was not available, only the antisera against male-specific P450 isozymes were used in this study.

Table 2 shows data for inhibitory effects of antisera on 4′-hydroxylation, 5″-hydroxylation, and cleavage of the propyl phenyl or propyl pyridyl ether of pyriproxyfen by male rat liver microsomes. All of the above four metabolic reactions were inhibited more than 88% by incubation of microsomes with the CYP2C11 antiserum. The CYP2C13 antiserum also inhibited all reactions except 4′-hydroxylation by 25−64%.

These results show that the 2C family of cytochrome P450 is indeed involved in the major metabolic reactions of pyriproxyfen in rats. In conclusion, it can be considered that sex-specific isozymes have essential responsibility for sex-related differences in the metabolism of pyriproxyfen in rats.

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LITERATURE CITED

Anders, M. W. Inhibition of microsomal drug metabolism by methylenedioxybenzenes. *Biochem. Pharmacol.* **1968**, *17*, 2367−2370.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−245.

Estrada, J. G.; Mulla, M. S. Evaluation of two insect growth regulators against mosquitoes in the laboratory. *J. Am. Mosq. Control Assoc.* **1989**, *2*, 57−60.

Funae, Y.; Imaoka, S. Cytochrome P450 in rodents. In *Cytochrome P450*; Schenkman, J. B., Greim, H., Eds.; Springer-Verlag: Berlin, Germany, 1993; Chapter 15.

Hatakoshi, M.; Kawada, H.; Nishida, S.; Kisida, H.; Nakayama, I. Laboratory evaluation of 2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy]pyridine against larvae of mosquitoes and housefly. *Jpn. J. Sanit. Zool.* **1987**, *38* (4), 271−271.

Imaoka, S.; Fujita, S.; Funae, Y. Age-dependent expression of cytochrome P-450 in rat liver. *Biochim. Biophys. Acta* **1991**, *1097*, 187−192.

Isobe, N.; Ito, S.; Yoshitake, A.; Matsuo, M.; Miyamoto, J. Metabolism of (*R*)- and (*S*)-isomers of (*E*)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazole-yl-1-penten-3-ol (S-3308L) in rats. *J. Pestic. Sci.* **1987**, *12*, 421−434.

Kato, R.; Yamazoe, Y. Sex-dependent regulation of cytochrome P-450 expression. In *Principle, Mechanisms and Biological Consequences of Induction*; Ruckpaul, K., Rein, H., Eds.; Taylor & Francis: London, 1990; pp 82−112.

Kawada, H.; Dohara, K.; Shinjo, G. Evaluation of larvicidal potency of insect growth regulator, 2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy]pyridine, against the housefly. *Jpn. J. Sanit. Zool.* **1987**, *38* (4), 317−322.

Kawada, H.; Dohara, K.; Shinjo, G. Laboratory and field evaluation of an insect growth regulator, 4-phenoxyphenyl (*RS*)-2-pyridyloxy)propyl ether, as a mosquito larvicide. *Jpn. J. Sanit. Zool.* **1988**, *39*, 339−346.

Kawada, H.; Dohara, K.; Shinjo, G. Laboratory evaluation of a new insect growth regulator pyriproxyfen, as a cockroach control agent. *Jpn. J. Sanit. Zool.* **1989**, *40*, 195−201.

Matsunaga, H.; Yoshino, H.; Isobe, N.; Kaneko, H.; Nakatsuka, I.; Yamada, H. Metabolism of pyriproxyfen in rats. 1. Absorption, disposition, excretion, and biotransformation studies with [*phenoxyphenyl*-^{14}C]pyriproxyfen. *J. Agric. Food Chem.* **1995**, *43* (1), 235−240.

Miyamoto, J.; Hirano, M.; Takimoto, Y.; Hatakashi, M. Insect growth regulators for pest control, with emphasis on juvenile hormone analogs, present status and future prospects. In *Pest Control with Enhanced Environmental Safety*; Duke, S. O., Menn, J. J., Plimmer, J. R., Eds.; ACS Symposium Series 524; American Chemical Society: Washington, DC, 1993; pp 144−168.

Neville, C. F.; Nonomiya, S.; Shimada, N.; Kamataki, T.; Imaoka, S.; Funae, Y. Characterization of specific cytochrome P450 enzymes responsible for the metabolism of diazepam in hepatic microsomes of adult male rats. *Biochem. Pharmacol.* **1993**, *45* (1), 59−65.

Omura, T.; Sato, R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **1964**, *239*, 2370−2378.

Raxworthy, M. J.; Youde, I. R.; Gulliver, P. A. Catechol-*O*-methyltransferase: substrate-specificity and stereoselectivity for β-adrenoceptor agents. *Xenobiotica* **1986**, *16* (1), 47−52.

Ryan, D. E.; Levin, W. Age- and gender-related expression of rat liver cytochrome P450. In *Cytochrome P450*; Schenk-

man, J. B., Greim, H., Eds.; Springer-Verlag: Berlin, Germany, 1993; Chapter 30.

Saito, K.; Kaneko, H.; Sato, K.; Yoshitake, A.; Yamada, H. Age-related changes in metabolism of diethofencarb: relationship between metabolism in rat and hepatic drug-metabolizing enzyme activities. *Xenobiotica* **1991**, *21*, 575−582.

Yoshino, H.; Matsunaga, H.; Kaneko, H.; Yoshitake, A.; Yamada, H. Metabolism in *N*-[chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl-3,4,5,6-tetrahydrophthalimide(S-23121) in the rat: I. Identification of a new, sulphonic acid type of conjugate. *Xenobiotica* **1993a**, *23*, 609−619.

Yoshino, H.; Kaneko, H.; Nakatsuka, I.; Yamada, H. *Metabolism* of *N*-[chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl-3,4,5,6-tetrahydrophthalimide (S-23121) in the rat: II. Absorption, disposition, excretion and biotransformation. *Xenobiotica* **1993b**, *23*, 1075−1084.

Yoshino, H.; Kaneko, H.; Nakatsuka, I.; Yamada, H. Metabolism of pyriproxyfen. 2. Comparison of *in vivo* metabolism between rats and mice. *J. Agric. Food Chem*. **1995**, *43* (10), 2681−2686.

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