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Chemico-Biological Interactions 147 (2004) 319–329
Chemico-Biological Interactions
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In vitro metabolism of fipronil by human and rat cytochrome P450 and its interactions with testosterone and diazepam

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Accepted 5 March 2004

Abstract

Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile) is a highly active, broad spectrum insecticide from the phenyl pyrazole family, which targets the γ-amino butyric acid (GABA) receptor. Although fipronil is presently widely used as an insecticide and acaricide, little information is available with respect to its metabolic fate and disposition in mammals. This study was designed to investigate the in vitro human metabolism of fipronil and to examine possible metabolic interactions that fipronil may have with other substrates. Fipronil was incubated with human liver microsomes (HLM) and several recombinant cytochrome P450 (CYP) isoforms obtained from BD Biosciences. HPLC was used for metabolite identification and quantification. Fipronil sulfone was the predominant metabolite via CYP oxidation. The K_m and V_{max} values for human liver microsomes are 27.2 μM and 0.11 nmol/mg protein min, respectively; for rat liver microsomes (RLM) the K_m and V_{max} are 19.9 μM and 0.39 nmol/mg protein min, respectively. CYP3A4 is the major isoform responsible for fipronil oxidation in humans while CYP2C19 is considerably less active. Other human CYP isoforms have minimal or no activity toward fipronil. Co-expression of cytochrome b_5 (b_5) is essential for CYP3A4 to manifest high activity toward fipronil. Ketoconazole, a specific inhibitor of CYP3A4, inhibits 78% of the HLM activity toward fipronil at a concentration of 2 μM. Oxidative activity toward fipronil in 19 single-donor HLMs correlated well with their ability to oxidize testosterone. The interactions of fipronil and other CYP3A4 substrates, such as testosterone and diazepam, were also investigated. Fipronil metabolism was activated by testosterone in HLM but not in CYP3A4 Supersomes®. Testosterone 6β-hydroxylation in HLM was inhibited by fipronil. Fipronil inhibited diazepam demethylation but had little effect on diazepam hydroxylation. The results suggest that fipronil has the potential to interact with a wide range of xenobiotics or endogenous chemicals that are CYP3A4 substrates and that fipronil may be a useful substrate for the characterization of CYP3A4 in HLM.

Keywords: Fipronil; Metabolism; Human liver microsomes; Cytochrome P450; CYP3A4; Interaction

1. Introduction

Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile) is a highly active, broad spectrum insecticide from the phenyl pyrazole family, which

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doi:10.1016/j.cbi.2004.03.002

targets the γ-amino butyric acid (GABA) receptor [1–5]. Agriculturally, this pesticide has been used on pests of a wide variety of food crops [6–8]. In non-agricultural applications, fipronil is used to control veterinary pests [9] and has also been designated by EPA as one of alternatives to the organophosphates (OP) for termite and fire ant control [10]. Concerns for fipronil effects on public health have been raised because of the wide range of uses of this pesticide [9,11].

Fipronil is moderately toxic to rats and mice with oral LD50's ranging from ca. 40 to 100 mg/kg [12,13], but is much more toxic toward insects than toward mammals [1,12]. Fipronil selectivity is due to its greater potency in blocking insect GABA-gated chloride channels than their mammalian counterparts [12,14]. Limited metabolic studies indicate that the predominant pathway of fipronil metabolism is S-oxidation to form the sulfone (Fig. 1). Fipronil sulfone is the only metabolite reported in mice [12] and in vitro studies suggest that fipronil sulfone is more potent as an antagonist of the GABA receptor than fipronil [14]. In addition to neurotoxicity, fipronil has been reported to have the potential to induce thyroid cancer in rodents by enhancing the hepatic metabolism and excretion of thyroid hormone [15].

Metabolism may be an important determinant of toxicity. Studies of metabolic stability and pathways of pesticide metabolism in humans can provide important information on differences between humans and laboratory animals in metabolism and potential interactions with endogenous chemicals and other xenobiotics. This study was designed to compare the metabolism of fipronil in human and rat liver microsomes (RLM), to identify enzymes responsible for the metabolism of fipronil and to examine potential interactions with chemicals that are substrates for the same enzymes.

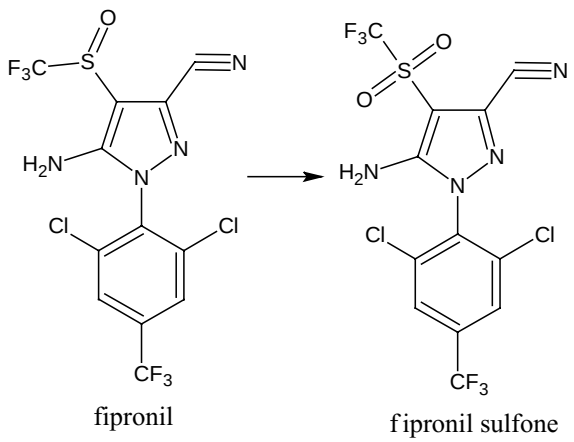


Fig. 1. Cytochrome P450-dependent metabolism of fipronil.

2. Materials and methods

2.1. Chemicals

Fipronil was purchased from ChemService (West Chester, PA). Testosterone and 6β-hydroxy testosterone were purchased from Steraloids (Newport, RI). Fipronil sulfone was a gift from the Rhône-Poulenc Company (Research Triangle Park, NC). HPLC grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals, unless specified otherwise, were purchased from Sigma (St. Louis, MO).

2.2. Liver microsomes and cytochrome P450 isoforms

Rat liver microsomes were prepared from adult male Long-Evans rats (Charles River Laboratories, Raleigh, NC), according to the method of Cook and Hodgson [16].

Pooled and single-donor human liver microsomes (HLM), human cytochrome P450 (CYP) isoforms and human cytochrome b_5 (b_5) were purchased from BD Biosciences (Woburn, MA). CYP isoforms in Supersomes® obtained were CYP2A6, 2B6, 2C8, 2C9*1, 2C19, 2E1, 3A4, and 3A7, all of which are co-expressed with b_5. Also obtained were CYP1A1, 1A2, 2C9*2, 2C9*3, 2C18, 2D6*1, 2D6*10, 3A4, 3A5, and 4A11 Supersomes®, which are not co-expressed with b_5. All human CYP isoforms in Supersomes® are co-expressed with human CYP reductase.

2.3. In vitro fipronil metabolism

The general method for the in vitro assay was described by Tang et al. [17]. Preliminary experiments determined that incubation times and enzyme amounts were within a linear range. Enzyme kinetics in pooled HLM (1 mg protein), RLM (0.75 mg protein), and

CYP3A4 (20 pmol P450) were assayed by incubation of serial concentrations of fipronil (final concentrations 1.25–80 μM). Activities in single-donor HLM (1 mg protein) or CYP isoforms (18–100 pmol P450) were assayed by incubation of a single concentration of fipronil (final concentration 80 μM). The NADPH-regenerating system used contained NADP (final concentration 0.25 mM), glucose-6-phosphate (final concentration 2.5 mM) and glucose-6-phosphate dehydrogenase (final concentration 2 U/ml).

Reactions were initiated by the addition of the enzyme. Controls were performed in the absence of the NADPH-regenerating system. The reaction was terminated after 30 min of incubation by the addition of ice-cold acetonitrile followed by vortexing. After centrifugation (5 min at 21 000 × *g*), the supernatant was analyzed by HPLC.

To determine the role of CYP3A4 in fipronil metabolism in HLM (0.5 mg protein), a CYP3A4 specific inhibitor, ketoconazole (final concentrations 0–20 μM), was added to the reaction mixture simultaneously with 10 μM fipronil.

To determine the role of b_5 in CYP3A activities, exogenous b_5 was pre-mixed at room temperature with CYP3A4 and 3A5 Supersomes® that were not co-expressed with b_5. The amount of b_5 added to CYP3A4 or 3A5 was the same b_5/CYP3A4 ratio as that in CYP3A4 co-expressed with b_5. Studies involving the pre-mixing of CYP3A4 with b_5 for different times (from 0 to 30 min at room temperature) before the addition of substrate showed that the effect was not time dependent (data not shown). Metabolic activity toward fipronil was compared between CYP3As with and without b_5.

2.4. Interaction with testosterone

The effect of testosterone on fipronil metabolism was assayed in HLM (0.4 mg protein) or CYP3A4 Supersomes® (10 pmol CYP) by co-incubation of fipronil (final concentration 1.25, 5 or 20 μM) with testosterone (final concentrations 0–200 μM) for 15 min. The effect of fipronil on testosterone 6β-hydroxylation was assayed in HLM (0.4 mg protein) by co-incubation of testosterone (final concentration 4, 20 or 100 μM) with fipronil (final concentrations 0–160 μM) for 10 min. Metabolic effects of fipronil and testosterone on each others metabolism were determined by monitoring fipronil sulfone and 6β-hydroxy testosterone (Steraloids, Newport, RI) levels by HPLC, respectively.

2.5. Interaction with diazepam

The effect of diazepam on fipronil metabolism was assayed in HLM (0.4 mg protein) by co-incubation of fipronil (final concentration 5, 20, or 80 μM) with diazepam (final concentrations 0–400 μM) for 15 min. The effect of fipronil on diazepam metabolism was assayed in HLM (0.4 mg protein) by co-incubation of diazepam (final concentration 6.25, 25 or 100 μM) with fipronil (final concentrations 0–160 μM) for 15 min.

2.6. HPLC methods for analysis of fipronil, testosterone and diazepam

The HPLC method to separate fipronil and its metabolite, fipronil sulfone, was modified from Ngim et al. [18]. Briefly, a Synergi Max C12 column (Phenomenex, Rancho Palos Verdes, CA) was used with an isocratic mobile phase (70% methanol and 30% water containing 0.005 M acetic acid). A Shimadzu (Kyoto, Japan) HPLC system (LC-10AT VP pump, SPD-10A VP UV-VIS detector, SIL-10AD VP auto injector, and SCL-10A VP system controller) was used. The flow rate and detection wavelength were set at 1 ml/min and 275 nm, respectively. Retention times for fipronil and fipronil sulfone were 12 and 18 min, respectively. Peak areas of fipronil and its sulfone metabolite were integrated using Shimadzu Class-VP 7.0 program. The limit of detection was 0.02 pmol for either compound. Concentrations of fipronil sulfone were obtained by extrapolation of peak area from a standard curve ranging from 0.25 to 32 μM. Testosterone and its metabolites eluted several minutes before fipronil and fipronil sulfone.

Testosterone and its metabolites were separated using the method described by Usmani et al. [19]. Fipronil and its metabolite eluted after 6β-hydroxy testosterone and testosterone.

Diazepam and its metabolites were separated by a Synergi Max C12 column using a method modified from Shou et al. [20]. Briefly, a gradient system containing two pumps was used. From 0 to 30 min, pump A (20% acetonitrile, 30% methanol and 50% water) was maintained at 100% to separate diazepam and its

metabolites. After that, the ratio of pump B (70% acetonitrile and 30% methanol) was increased to 100% in 1 min and maintained for 8 min to elute fipronil and its metabolite. The retention times for diazepam, desmethyl diazepam, temazepam and oxazepam were 28, 19, 16, and 11 min, respectively.

2.7. Enzyme kinetic calculations and statistics

All values were expressed as mean ± S.E. (n = 2–3 determination). Enzyme kinetic parameters were determined using SigmaPlot Enzyme Kinetics Module 1.1 (SPSS Inc., Chicago, IL).

3. Results

3.1. Enzyme kinetics of fipronil metabolism in liver microsomes

Fipronil sulfone was the only metabolite detected following incubation of fipronil with either RLM or pooled HLM. While K_m values in human and rat liver microsomes were similar (19.9 and 27.2 μM, respectively), the V_{max} in RLM was 3.8-fold higher than that observed in HLM (0.39 and 0.11 nmol/mg protein min, respectively) (Fig. 2). It should be noted that unless otherwise stated, studies involving human liver microsomes utilized pooled human liver microsomes from BD Biosciences. These preparations have been formulated to be representative of the catalytic activities for an average of many individuals.

3.2. Fipronil metabolism by human CYP isoforms

In a comparison of 15 different CYP isoforms and three polymorphic variants, only CYP2C19 and CYP3A4 showed substantial activity toward fipronil (Fig. 3). The activity of CYP3A4 toward fipronil was five times greater than that of CYP2C19. Kinetic studies of CYP3A4 activity toward fipronil showed that its K_m value was close to that obtained in liver microsomes (Fig. 2).

The high activity of CYP3A4 depended on co-expression of b_5 (Table 1). In the absence of co-expressed b_5 the activity of CYP3A4 toward fipronil is dramatically decreased. The addition of exogenous b_5 to CYP3A4 Supersomes® in which

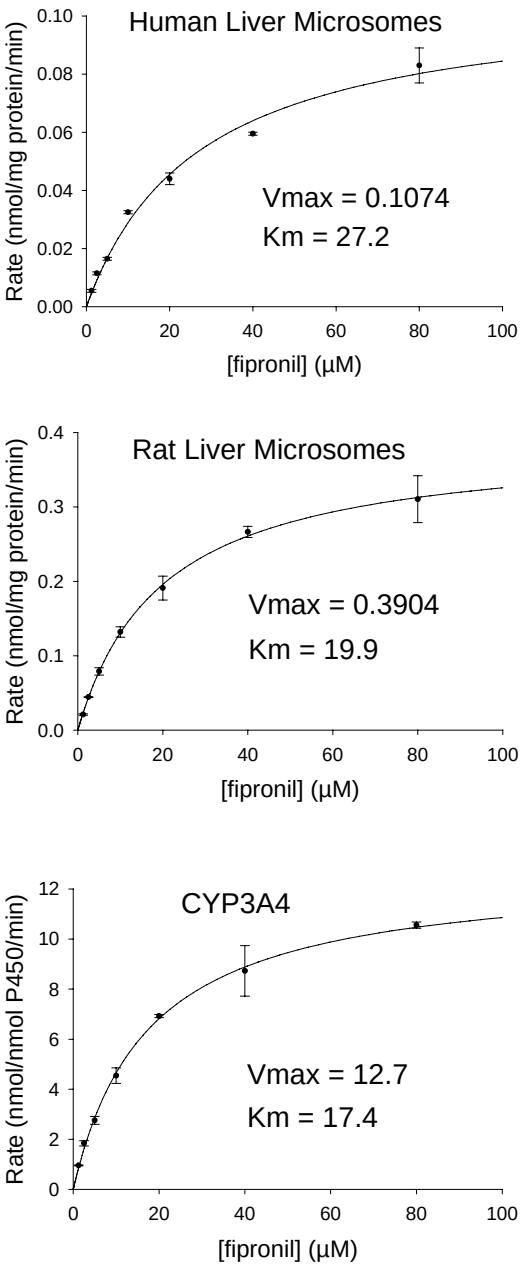


Fig. 2. Kinetic plots of fipronil metabolism in human liver, rat liver and baculovirus-expressed CYP3A4 microsomes. Data points represent the mean of three separate determinations and error bars represent S.E.

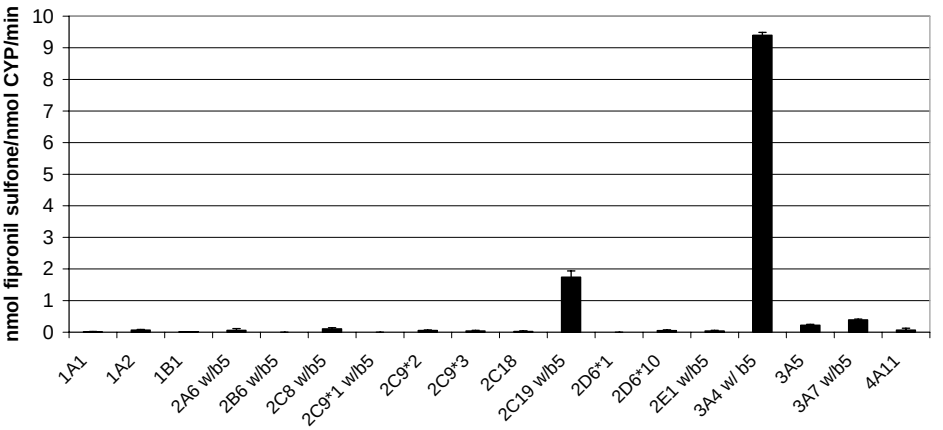


Fig. 3. Metabolic activities (nmol of product/nmol cytochrome P450 min) toward fipronil in human cytochrome P450 Supersomes®. Columns represent the mean of three separate determinations and error bars represent S.E.

Table 1
Effect of cytochrome b_5 (b_5) on CYP3A activity (mean ± S.E., $n = 2$–3 determinations)

	Activity (nmol/nmol CYP min)
CYP3A4 co-expressed with b_5	9.40 ± 0.09
CYP3A4	0.38 ± 0.03
CYP3A4 pre-mixed with b_5	1.27 ± 0.15
CYP3A5	0.22 ± 0.02
CYP3A5 pre-mixed with b_5	0.36 ± 0.01
CYP3A7 co-expressed with b_5	0.39 ± 0.02

b_5 was not co-expressed increased activity of these preparations ca. 3.5-fold, although it must be noted that these activities were still significantly lower than preparations where b_5 was co-expressed. CYP3A5 was less active than CYP3A4 even with the addition of exogenous b_5 (Table 1). CYP3A7 was not very active toward fipronil even though b_5 was co-expressed.

3.3. Fipronil metabolism in single-donor HLM

Fipronil metabolism was analyzed in 19 single-donor HLM. The difference in fipronil metabolism between different individuals is over 40-fold (Fig. 4). Correlation analyses between fipronil sulfoxidase activities and CYP-specific metabolic activities among these individuals (BD Biosciences) demonstrated that the best correlations were for CYP3A4 (r^2 = 0.81) and CYP2B6 (r^2 = 0.66) (Fig. 5). All other

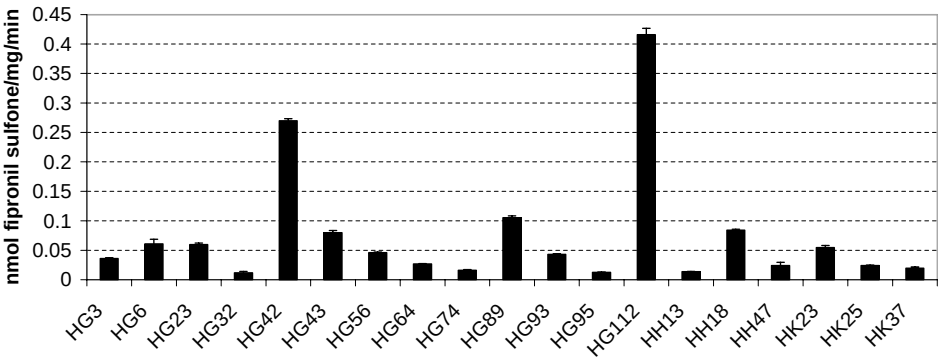
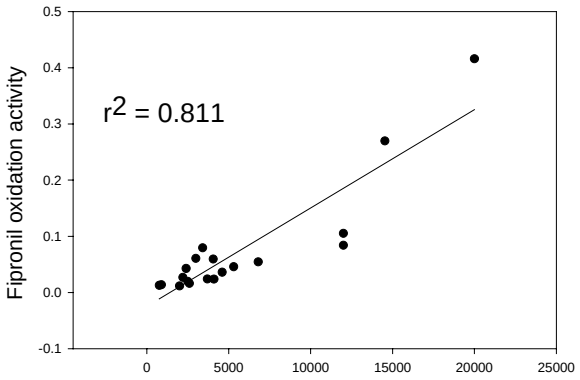
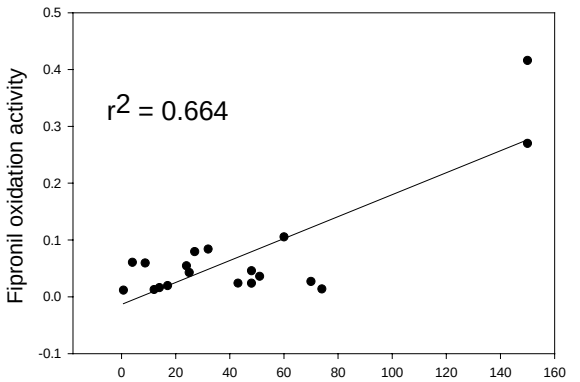


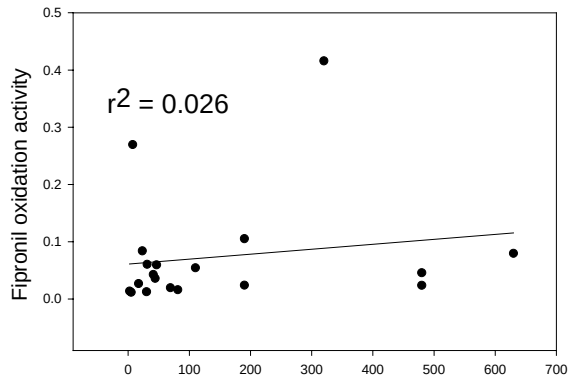
Fig. 4. Metabolic activities (nmol of product/mg protein min) toward fipronil in single-donor HLM. Columns represent the mean of three separate determinations and error bars represent S.E.



(A) Testosterone 6β–hydroxylation activity of CYP3A4



(B) (S)-Mephenytoin N-Demethylation activity of CYP2B6



(C) (S)-Mephenytoin 4'-Hydroxylation Activity of CYP2C19

Fig. 5. Correlation analyses of fipronil sulfoxidation activity with CYP isoforms. Substrate-specific activity levels for each individual were reported by BD Biosciences for (A) CYP3A4 using testosterone 6β-hydroxylation, (B) CYP2B6 using mephenytoin *N*-demethylation, and (C) CYP2C19 using mephenytoin 4′-hydroxylation.

correlations between sulfoxidation activity and specific CYP isoforms were less than 0.17.

3.4. Inhibition of fipronil metabolism in HLM by ketoconazole

The role of CYP3A4 in the metabolism of fipronil was verified by co-incubations of ketoconazole, a specific CYP3A4 inhibitor, with fipronil. In pooled HLM, increasing concentrations of ketoconazole significantly inhibited metabolism of 10 μM fipronil, resulting in 78% inhibition at 2 μM ketoconazole. The IC_{50} at this concentration of fipronil for ketoconazole inhibition was 0.16 μM. In similar experiments involving ketoconazole inhibition of CYP3A4-mediated testosterone 6β-hydroxylation, 50 μM concentrations of testosterone were inhibited by 95% with 2 μM ketoconazole.

3.5. Metabolic interactions of fipronil and testosterone or diazepam in HLM

Co-incubations of varying concentrations of fipronil with increasing concentrations of testosterone in pooled HLM resulted in increased fipronil sulfone production at all three fipronil substrate concentrations tested (Fig. 6). The greatest level of activation was observed at the lowest fipronil concentration (1.25 μM) in combination with a 20 μM concentration of testosterone (ca. 2.3-fold). Incubations of 20 μM fipronil were not as readily activated by increasing testosterone concentrations (control levels for fipronil sulfone production at fipronil concentrations of 1.25, 5 and 20 μM were 0.005 ± 0.000, 0.020 ± 0.002, and 0.042 ± 0.003 nmol/mg protein min, respectively). In contrast with HLM preparations, the activation of fipronil metabolism by testosterone was not observed when using CYP3A4 Supersomes® as the enzyme source (data not shown).

In contrast to the fipronil results, testosterone metabolism was inhibited by increasing concentrations of fipronil at all three dose levels examined (Fig. 7). At the two lowest concentrations of testosterone (4 and 20 μM) fipronil concentrations of 100 μM resulted in approximately 50% inhibition of the 6β hydroxy-testosterone product (control values for 6β-hydroxy testosterone metabolite production at testosterone substrate concentrations were $0.050 \pm$

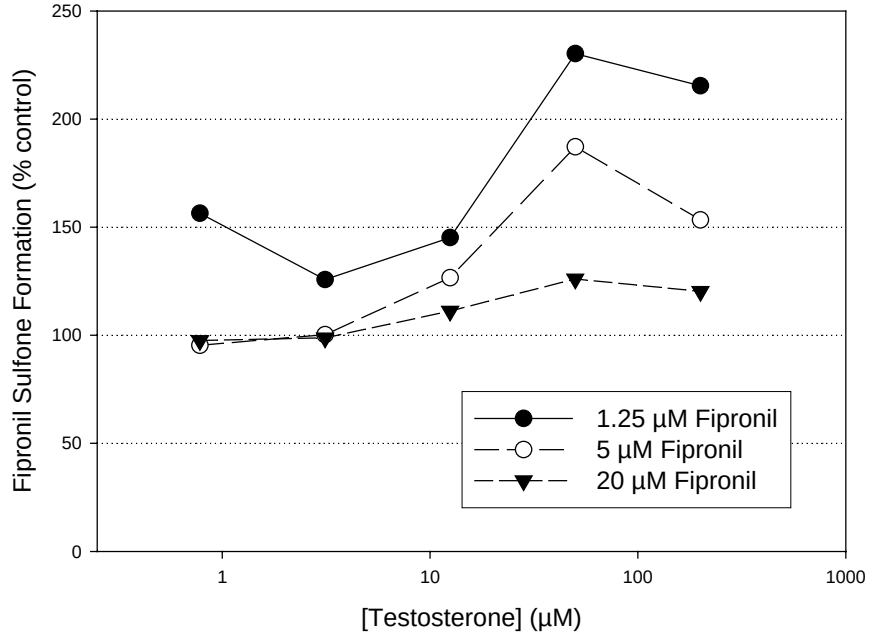


Fig. 6. The effects of testosterone on fipronil sulfone formation in HLM. Data presented is based on the percent of fipronil sulfone formation when compared to control (0 μM testosterone) with each data point representing the mean of two separate determinations.

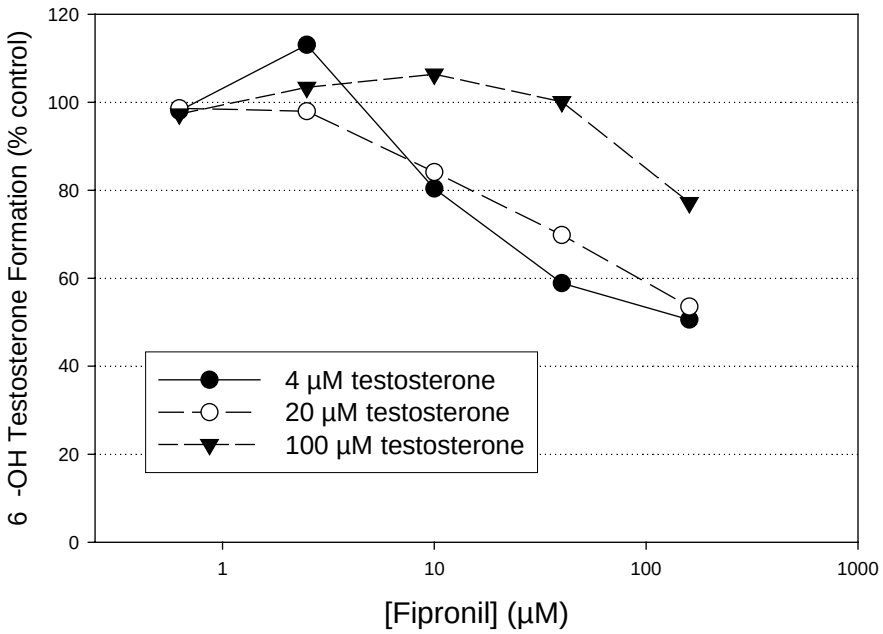
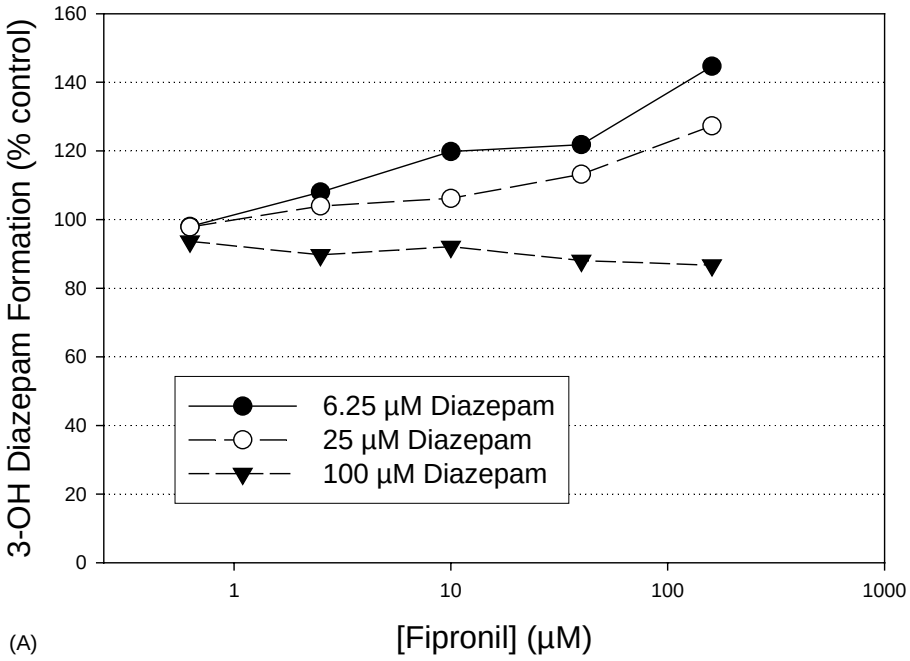


Fig. 7. The effects of fipronil on 6β-OH testosterone formation in HLM. Data presented is based on the percent of 6β-OH testosterone formation when compared to control (0 μM fipronil) with each data point representing the mean of two separate determinations.



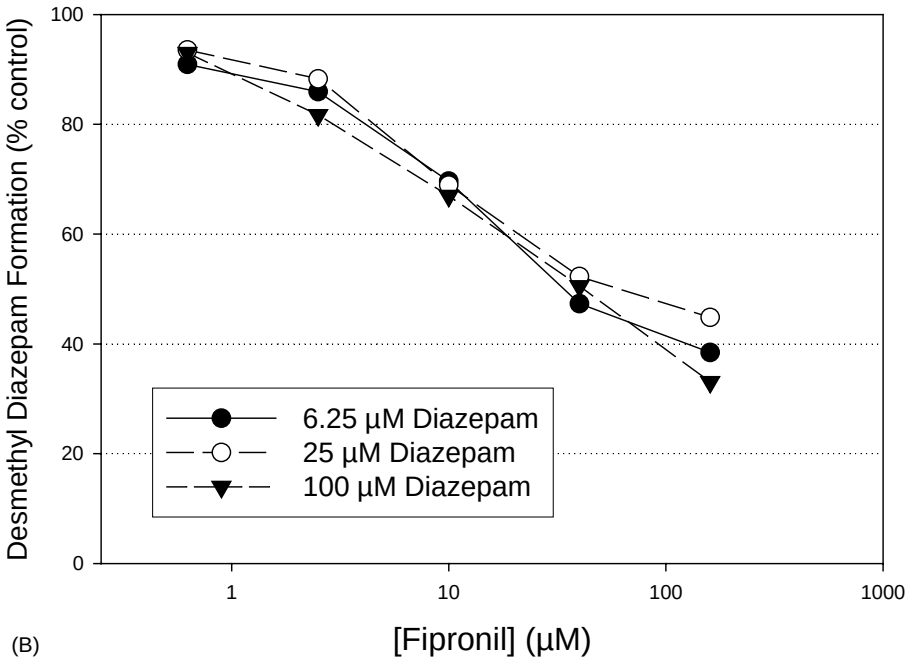


Fig. 8. The effects of fipronil on diazepam metabolism in HLM. (A) The percent of 3-hydroxy diazepam formation when compared to control (0 μM fipronil) with increasing fipronil concentrations; data points represent the mean of two separate determinations. (B) The percent of desmethyl diazepam formation when compared to control (0 μM fipronil) with increasing fipronil concentrations; data points represent the mean of two separate determinations.

0.002, 0.980 ± 0.018, and 2.680 ± 0.169 nmol/mg protein min, respectively).

Co-incubations of HLM with varying concentrations of diazepam, a known CYP3A4 substrate, in the presence of three doses of fipronil only slightly increased fipronil sulfone levels at the higher dose levels examined, but had small effects on lower concentrations of fipronil (data not shown). The opposite experiment, in which increasing concentrations of fipronil were incubated with three dose levels of diazepam produced different results depending on the diazepam metabolite examined (Fig. 8). At the two lower concentrations of diazepam, increasing concentrations of fipronil slightly increased levels of the 3-OH metabolite, while at the highest diazepam concentration there was slight inhibition (Fig. 8A). For the desmethylated product, increasing concentrations of fipronil consistently inhibited diazepam metabolism, with approximately 60% inhibition reported for all three diazepam concentrations examined. Control values for 3-hydroxy diazepam formation at diazepam concentrations of 6.25, 25, or 100 μM in the absence of fipronil were 0.008 ± 0.001, 0.043 ± 0.004, and 0.298 ± 0.057 nmol/mg protein min, respectively. Desmethyl diazepam formation in the absence of fipronil from these same concentrations of diazepam were 0.027 ± 0.002, 0.100 ± 0.004, and 0.340 ± 0.028 nmol/mg protein min, respectively.

4. Discussion

The predominant metabolic pathway for fipronil in both HLM and RLM is the oxidation of the thioether group to generate fipronil sulfone, as was previously reported in mice [12]. The enzyme kinetic studies indicated that K_m values for fipronil metabolism are similar in HLM and RLM, but RLM has higher V_{max} values than HLM, suggesting a higher intrinsic clearance of fipronil in rats than in humans. Fipronil sulfone is more toxic than fipronil to birds but not to mice although in both cases the sulfone is more persistent than the parent compound in the GABA receptor [14]. It is not known whether sulfone production enhances toxicity of fipronil in humans.

These studies provide strong evidence that CYP3A4 is the predominant isoform responsible for S-oxidation of fipronil. Of the 15 isoforms screened for activity, only CYP3A4 and 2C19 significantly metabolized fipronil. Correlation analysis conducted on 19 single-donor HLM samples revealed significant correlations for CYP3A4 and CYP2B6. Examination of isoform levels as reported by BD Biosciences for this group of individuals indicated that those with high levels of CYP2B6 were often the same individuals with high CYP3A4 activity and vice versa. Further verification of the importance of CYP3A4 in the metabolism of fipronil was obtained by demonstrating 78% inhibition of fipronil sulfone production in HLM by 2 μM ketoconazole, a specific CYP3A4 inhibitor. In similar experiments with testosterone, a known CYP3A4 substrate, the same concentration of ketoconazole inhibited up to 95% of testosterone 6β-hydroxylation. The remaining fipronil sulfone activity following ketoconazole inhibition may be attributed to either incomplete inhibition or to CYP2C19 activity, the only other CYP isoform with significant fipronil metabolizing activity.

As demonstrated previously with some, but not all substrates, CYP3A4 activity based on fipronil sulfone production requires the presence of b_5 [21–23]. Co-expression of b_5 has much greater impact than the addition of equivalent amounts of exogenous b_5 on CYP3A4 activity toward fipronil. The length of exogenous b_5 pre-incubation time with CYP3A4 has little effect on CYP3A4 activity. Co-expressed b_5 is likely better integrated into the microsomal membrane with CYP3A4 than exogenous b_5 to facilitate the function of CYP3A4. Although CYP3A7 also was co-expressed with b_5 it apparently is not effective in the oxidation of fipronil. It is unclear whether co-expression of b_5 would have such dramatic effects on fipronil metabolism by CYP3A5 as it has on CYP3A4, however, if not fipronil may be a great substrate to differentiate between CYP3A4 and CYP3A5 activities.

CYP3A4 is usually the most abundant isoform in human liver and has broad substrate specificity. Because fipronil is predominantly metabolized by CYP3A4, it could potentially interact metabolically with many other CYP3A4 substrates, both endogenous and exogenous, such as testosterone and diazepam. The usual interaction between two different substrates for the same enzyme is competitive inhibition. However, interactions between CYP3A4

substrates are complicated because of the allosteric characteristics of CYP3A4 [20,24,25].

Kenworthy et al. [26] proposed multisite kinetic models for the interaction of testosterone and diazepam in CYP3A4. The interaction between fipronil and testosterone in HLM that we observed displays a similar pattern as observed for the interaction between diazepam and testosterone [26], i.e., testosterone activated fipronil metabolism while increasing doses of fipronil inhibited testosterone metabolism. Although in our study, 2.3-fold levels of activation of fipronil metabolism by testosterone in HLM were similar in nature to activation levels reported for diazepam and testosterone in lymphoblast-expressed CYP3A4 microsomes [26] the same phenomenon was not observed when using CYP3A4 Supersomes® as the enzyme source. This difference between HLM and CYP3A4 Supersomes® needs further scrutiny. It is postulated that the human lymphoblast-expressed CYP3A4 has a greater similarity to CYP3A4 in HLM than the insect baculovirus-expressed CYP3A4 Supersomes® used in this study.

Because fipronil and diazepam display the same pattern in their interactions with testosterone, we also examined the interactions between these two compounds. When examining fipronil sulfone production at low fipronil substrate concentrations co-incubation of diazepam had little effect, but at high fipronil concentrations there was a slight activation of metabolism. This is different from the interactions with testosterone, where low substrate concentrations are more sensitive to the modifier. Also, the two pathways of diazepam metabolism [20] respond differently to the presence of fipronil. In the case of the 3-OH diazepam metabolite, increasing concentrations of fipronil resulted in small increases in metabolism, while for desmethyl diazepam high concentrations of fipronil caused up to 60% inhibition. Differences in the response of these two diazepam metabolites are likely the result of conformational changes in the active site brought about by increasing concentrations of fipronil.

In conclusion, CYP3A4 is the major enzyme responsible for fipronil metabolism. Co-expression of b_5 is essential for CYP3A4 to have high activity toward fipronil. Interactions between fipronil and other CYP3A4 substrates display activation, inhibition and regio-selectivity effects, in agreement with previously reported multisite kinetic analysis of atypical interactions of CYP3A4 [26,27].

Acknowledgements

This project was supported by NIOSH Grant OH07551-ECU.

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