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Fipronil induces CYP isoforms and cytotoxicity in human hepatocytes

Parikshit C. Das [a], Yan Cao [a], Nathan Cherrington [b], Ernest Hodgson [a,*], Randy L. Rose [a,✠]

[a] *Department of Environmental and Molecular Toxicology, Mail Box 7633, North Carolina State University, Raleigh, NC 27695-7633, USA*
[b] *College of Pharmacy, University of Arizona, Tucson, AZ 85721-0207, USA*

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Abstract

Recent studies have demonstrated the potential of pesticides to either inhibit or induce xenobiotic metabolizing enzymes in humans. Exposure of human hepatocytes to doses of fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile) ranging from 0.1 to 25 μM resulted in a dose dependent increase in CYP1A1 mRNA expression (3.5 to ∼55-fold) as measured by the branched DNA assay. In a similar manner, CYP3A4 mRNA expression was also induced (10–30-fold), although at the higher doses induction returned to near control levels. CYP2B6 and 3A5 were also induced by fipronil, although at lower levels (2–3-fold). Confirmation of bDNA results were sought through western blotting and/or enzyme activity assays. Western blots using CYP3A4 antibody demonstrated a dose responsive increase from 0.5 to 1 μM followed by decreasing responses at higher concentrations. Similar increases and decreases were observed in CYP3A4-specific activity levels as measured using 6β-hydroxytestosterone formation following incubation with testosterone. Likewise, activity levels for a CYP1A1-specific substrate, luciferin CEE, demonstrated that CYP1A1 enzyme activities were maximally induced by 1 μM fipronil followed by dramatically declining activity measurements at 10 and 25 μM. Cytotoxic effects of fipronil and fipronil sulfone were examined using the adenylate kinase and the trypan blue exclusion assays in HepG2 cells and human hepatocytes. The results indicate both that HepG2 cells and primary human hepatocytes are sensitive to the cytotoxic effects of fipronil. The maximum induction of adenylate kinase was ca. 3-fold greater than the respective controls in HepG2 and 6–10-fold in the case of primary hepatocytes. A significant time- and dose-dependent induction of adenylate kinase activity in HepG2 cells was noted from 0.1 to 12.5 μM fipronil followed by decreasing activities at 25 and 50 μM. For fipronil sulfone, cytotoxic effects increased throughout the dose range. The trypan blue assay indicated that cytotoxic effects contributing to an increase of greater than 10% of control values was indicated at doses above 12.5 μM. However, fipronil sulfone induced cytotoxic effects at lower doses. The possibility that cytotoxic effects were due to apoptosis was indicated by significant time- and dose-dependent induction of caspase-3/7 activity in both HepG2 cells and human hepatocytes. Fipronil mediated activation of caspase-3/7 in concurrence with compromised ATP production and viability are attributed to apoptotic cell death.

Keywords: Fipronil; Human hepatic and HepG2 cells; CYP3A4; CYP2B6; CYP1A1; Cytotoxicity; Apoptosis

Abbreviations: AK, adenylate kinase; ATP, nucleotide adenosine triphosphate; Apaf-1, apoptotic protease activating factor-1; E_2, 17β-estradiol; Fas, death receptor; GABA, gamma-amino butyric acid; RXR, retinoid X receptor; XRE, xenobiotic responsive element

* Corresponding author. Tel.: +1 919 515 5295; fax: +1 919 513 1012.
E-mail address: ernest_hodgson@ncsu.edu (E. Hodgson).
✠ Deceased, Dr. Randy L. Rose was killed in a tragic accident on 23 May 2006.

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1. Introduction

Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile) is a relatively new pesticide with widespread utility in control of many agricultural and domestic pests including many lepidopteran species as well as thrips, locusts, ants, cockroaches, fleas, and ticks [1]. Fipronil has also been designated as an alternative pesticide to organophosphates for termite and fire ant control by the U.S. Environmental Protection Agency [1,2]. Because fipronil is used both commercially and in home applications, recent concerns for potential adverse public health effects have been raised [1,3].

Fipronil toxicity is attributed to its ability to act at the GABA receptor as a noncompetitive blocker of the GABA-gated chloride channels of neurons in the central nervous system. The selective toxicity of fipronil to insects relative to vertebrates is primarily the result of differences at the target site [4,38–40]. Fipronil is moderately toxic to rats and mice, highly toxic to aquatic invertebrates, fish and upland game birds, but non-toxic to waterfowl and other bird species [5,6]. Rat organs affected by chronic fipronil exposure include the thyroid, liver and kidney [1]. Multi-generation rat studies showed reproductive toxicity and developmental delays at the higher doses tested, but no evidence of birth defects [6,7].

Relatively little is known of the ability of fipronil to be metabolized in vertebrates. In vivo mammalian studies indicate that the primary metabolic pathway for fipronil involves the oxidative formation of the sulfone metabolite [4]. Recent studies with human liver microsomes and recombinant CYP isoforms demonstrated that the formation of the sulfone is almost exclusively the result of CYP3A4 activity, although limited metabolism by CYP2C19 was also reported [8]. The sulfone metabolite, as well as a photodegradation product, fipronyl desulfinyl, have been reported to be more toxic to insects, mammals, fish and birds than the parent compound itself [5,9].

Fipronil is one of several pesticides known to induce thyroid cancer in rats, likely as a result of its ability to enhance hepatic thyroid hormone metabolism and excretion [10]. An analysis of several rodent cancer studies suggests that liver and thyroid tumors are often correlated, both within and between species [11]. It is possible that induction of microsomal enzymes and the accompanying symptoms associated with induction, including increased liver weights and heptocellular hypertrophy, may play a role in tumor development. The ability of chemicals to induce metabolic enzymes, including cytochrome P450 (CYP), has long been considered one of the most sensitive biochemical cellular responses to toxic insult [12,13], since it often occurs at much lower doses of the chemical than those known to cause lethal or overtly toxic effects.

Recent studies with human and rat hepatocytes have demonstrated that pesticides are capable of inducing many metabolic enzymes in these cells [14,15]. The present study was undertaken to examine the potential of fipronil to induce important xenobiotic metabolizing enzymes in human hepatocytes and to characterize the cytotoxic effects of fipronil that were discovered during the course of the study.

2. Materials and methods

2.1. Chemicals and reagents

Fipronil and fipronil sulfone were purchased from Chem Service (West Chester, PA) and AccuStandard, Inc (New Haven, CT), respectively. Williams E culture medium and medium supplements, dexamethasone and insulin, were obtained from Bio Whittaker (Walkersville, MD). Eagle's medium E without L-glutamine and phenol red, non-essential amino acid solution, L-glutamine solution, and other cell culture related products were purchased from Mediatech, Inc. (Herndon, VA). Certified fetal bovine serum, trypsin–EDTA solution, and HBSS buffers were obtained from GIBCO Invitrogen Corporation (Carlsbad, CA). Tissue culture flasks, 6-, 24- 48-, and 96-well culture plates along with other tissue culture related products, HPLC grade acetonitrile and water were purchased from Fisher Scientific (Pittsburgh, PA). Testosterone and 6β-hydroxytestosterone were purchased from Steraloids (Newport, RI). The ToxiLight™ assay kit was purchased from Cambrex Corporation (East Rutherford, New Jersey). Caspase-Glo™ 3/7 Assay kit was purchased from Promega Corporation (Madison, WI). Actinomycin D and Z-DEVD-FMK are products of Alexis Biochemicals supplied by AXXORA, LLC (San Diego, CA). Rifampicin, phenobarbital, and all other chemicals, unless specified otherwise, were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Polyclonal anti-rat CYP1A1 from goat, monoclonal anti-human CYP3A4 from mice were from BD Biosciences (Bedford, MA) while mouse anti-human Fas was from R&D Systems (Minneapolis, MN). All chemicals and reagents were used and disposed according to the NCSU safety protocols and guidelines.

2.2. Human hepatocyte primary culture

Primary cultures of mature human hepatocytes were obtained from Vesta Therapeutics (Research Triangle

Park, NC). Hepatocytes were plated (9×10^6 per plate) in six-well culture plates coated with collagen type I and overlayed with Matrigel. Viability of each culture cell at plating was greater than 84% measured by the trypan blue exclusion method. Hepatocytes were cultured in Williams medium E supplemented with 10^{-7} M dexamethasone, 10^{-7} M insulin, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10% FBS and maintained in a humidified incubator at 5% CO_2/95% air at 37 °C for 48 h prior to the initiation of treatment.

2.3. Human hepatoma HepG2 cell line

The human hepatoma HepG2 cell line was a gift from Dr. Andrew Wallace (NCSU, Raleigh, NC). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), and L-glutamine (2 mM). Cells were maintained in a humidified atmosphere containing 5% CO_2/95% air at 37 °C. Cells were sub-cultured every 4–5 days. The initial culture was designated as passage number 1 and all experiments were performed using cells within the first 10 passages.

2.4. Treatment of human hepatocytes and human HepG2 cells

To compensate for differences in sensitivity and limititations and limitations of the assays differences between primary hepatocytes and hepatoma HepG2 cells and in order to maximize reproducibility of the data we have standardized cell densities for different experiments. The culture medium was also used during all cell treatments. Specifics of cell numbers used in different experiments are as follows. Fresh human hepatocytes seeded at 1.5×10^6 cells/well in six-well plate and at 3.75×10^5 cells/well in 24-well plate were exposed to vehicle control (culture medium with solvent DMSO), inducing agents, rifampicin (10 μM) or 3-methyl cholanthrene (10 μM), and increasing concentrations of fipronil (0.1, 0.5, 1, 5, 10 and 25 μM) for 72 h for Branched DNA (bDNA) and Western blot analyses, and CYP1A1 activity and CYP3A4 Metabolism assays, respectively. Likewise, fresh human hepatocytes seeded at 1.875×10^5 cells/well in 48-well plate were used for ToxiLight and Caspase-3/7 assays following exposure to vehicle controls, positive controls like phenobarbital (100 μM), actinomycin D (1 μM), and fipronil (0.1, 0.5, 1, 3.12, 6.25, 12.5, 25 and 50 μM), and fipronil sulfone (0.1, 1, 3.12, 6.25, 12.5, and 25 μM) for 24, 48, and 72 h. Similarly human hepatoma HepG2 cells were plated at 2×10^4 cells/well of 96-well plate and treated with vehicle controls, positive controls such as phenobarbital (100 μM), actinomycin D (1 μM), and fipronil (0.05, 0.1, 0.5, 1, 3.125, 6.25, 12.5, 25 and 50 μM), and fipronil sulfone (0.05, 0.1, 0.5, 1, 3.125, 6.25, 12.5, and 25 μM) for 24, 48, and 72 h for ToxiLight and Caspase-3/7 assays.

2.5. Sample preparation

A 10 μl culture supernatant was collected from either 96-well or 48-well plates for use in the ToxiLight assay, while a mixture of both culture medium and cells were used for the Caspase-3/7 assay. 2–5 μl trypsin–EDTA was used for making a cell suspension in the culture medium in 96-well plates for the trypan blue exclusion assay. For RNA extraction, media was removed from one well of a six-well plate and 200 μl of lysis buffer added before scraping and storage at −80 °C until use. For protein extraction, hepatocytes were harvested from three wells of a 48-well plate or two wells of a six-well plate using a cell scraper and pooled in eppendorf tubes. These were centrifuged at $5000 \times g$ for 3 min and the supernatant discarded. The pellets were suspended in 75 μl chilled cytochrome P450 storage buffer (0.1 M potassium phosphate buffer with 0.1 mM EDTA at pH 7.5) and sonicated twice for 30 s. S9 protein was extracted by centrifuging the sample at $9000 \times g$ for 15 min.

2.6. Branched DNA (bDNA) assay

Recent studies indicated the potential utility of the bDNA assay as a measure of mRNA induction using human hepatocyte tissues [14,15]. Oligonucleotide probe sets specific for human CYP1A1, CYP1A2, CYP3A4, CYP3A5, CYP2B6, CYP2D6, and glyceraldehyde phosphate dehydrogenase were a generous gift from Xenotech LLC (Olathe, Kansas). Reagents required for RNA analysis (i.e., lysis buffer, amplifier/label probe buffer and substrate solution) were supplied in the QuantiGene Discover kit (Genospectra, Fremont, CA). Expression levels were analyzed as described by Hartley and Klaassen [16]. Briefly, specific oligonucleotide probe sets were diluted in lysis buffer. Cell lysate was vortexed and 20 μl was added to each well of a 96-well plate containing capture hybridization buffer (0.05 M HEPES sodium salt, 0.05 M HEPES free acid, 0.037 M lithium lauryl sulfate, 0.5% (v/v) Micr-O-protect, 8 mM EDTA, 0.3% (w/v) nucleic acid blocking agent) and 50 μl diluted probe set (50, 100, and 200 fmol/μl for capture, blocker, and label

probes, respectively). RNA was allowed to hybridize to each probe set containing all probes for a given transcript (blocker probes, capture probes, and label probes) overnight at 53 °C. Subsequent hybridization and post-hybridization wash steps were carried out according to manufacturer's direction, and luminescence was measured with the Quantiplex 320 bDNA Luminometer (Bayer Diagnostics) interfaced with Quantiplex Data Management Software Version 5.02 (Bayer Diagnostics) for analysis of luminescence from 96-well plates. CYP expression data was normalized with respect to glyceraldehyde phosphate dehydrogenase levels and expressed as induction relative to controls.

2.7. Western blotting and quantitation

A 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve S9 proteins as described by Laemmli [17]. Proteins from the gel were electrophoretically transferred to a nitrocellulose membrane at constant voltage (100 V) for 1 h, and stained with Ponceau S for verification of transfer. The blots were blocked in 1% nonfat dry milk for 12 h and incubated in primary antibody (monoclonal anti-human CYP3A4 from mice and mouse anti-human Fas) and secondary antibody IgG-conjugated alkaline phosphatase (anti-goat, or anti-mouse respectively). Specific protein bands were visualized in BCIP/NBT. The intensities of the bands were measured using densitometry and data expressed on a quantitative scale.

2.8. CYP1A1 and 3A4 metabolism assays

Human hepatocytes were treated with varying concentrations of fipronil in 24-well plates at 37 C for 72 h. Fipronil-containing media was refreshed daily. At the conclusion of the exposure period, a 30 min incubation of culture media preceded the addition of media containing the appropriate substrate. For CYP1A1 determinations, Luciferin-CEE, which is a specific substrate for human CYP1A1 and CYP1B1 isoforms, was used according to the manufacturer's recommendations. 50 μM Luciferin-CEE was added to 100 μl culture medium and incubated at 37 °C for 3 h. Following incubation, 45 μl of media was transferred to wells of a 96 well plate to which 45 μl of P450-Glo™ Luciferin detection reagent was added. After incubation at room temperature for 20–30 min, relative luminescence units (RLU) from each well were determined according to the manufacturer protocol (Promega Corporation, Madison, WI).

CYP3A4 determinations were conducted using the same hepatocyte preparations as used for CYP1A1 assay. Following removal of the Luciferin-CEE containing media, the hepatocytes were incubated in fresh media for 30 min. This media was then replaced with media containing 100 μM concentrations of testosterone which was subsequently incubated at 37 °C for 30 min. Following the incubation period, medium was removed to vials containing equal volumes of methanol, centrifuged at 15,000 rpm and the supernatants analyzed for testosterone metabolites by HPLC. Separation of metabolites utilized a modification of the method described by Usmani et al., [18]. This method utilizes a Prodigy column (3 μl, 150 mm × 4.6 mm, ODS (3), 100 A; Phenomenex, Rancho Palos Verdes, CA), detection at 247 nm with a flow rate of 0.5 ml/min. Mobile phase for pump A was 1% tetrahydrofuran, 99% water; for pump B 100% methanol. The gradient used was: 0–14 min (60–85% B), 14–17 min (85–60% B), 17–20 min (60% B). Concentrations of 6β-hydroxy testosterone were determined from standard curves based on peak area.

2.9. Cell viability and cytotoxicity assay

Cell viability was assessed using cells harvested as a cell suspension in isotonic culture medium. The trypan blue exclusion assay was used semi-quantitatively to determine the viability of cells in all treatment groups and controls using a hemocytometer. Specifically, 100 μl 0.4% trypan blue in PBS (pH 7) was added into 900 μl of cell suspension. 10 μl of this mixture was placed on the hemocytometer for counting under the microscope. Greater than 100 cells per field were examined and the data were expressed as percent viable cells (Hausser Scientific, Horsham, PA).

2.10. ToxiLight assay

ToxiLight™ is a non-destructive luciferase-based bioluminescence cytotoxicity assay used to measure toxicity in mammalian cells and cell lines in culture. The kit quantitatively measures the release of adenylate kinase into the culture medium. The emitted light intensity expressed as RLU value is linearly related to the adenylate kinase activity. The assay was performed according to the manufacturer's protocol (Cambrex Bio Science Rockland, Inc., Rockland, ME). Measurements at different time intervals are all cumulative from 0 time.

2.11. Caspase-3/7 assay

Caspase-Glo™-3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and 7 activities by using a luminometer. Luminescence produced by

luciferase as RLU value is proportional to the amount of caspase-3/7 activity present in the sample. The assay was performed according to the manufacturer's protocol (Tech. Bull. No. 323, Promega Corporation, Madison, WI).

2.12. Statistical analysis

Data are summarized and expressed as mean ± S.E. using Microsoft Excel spread sheet and Sigma Plot graphics programs (Chicago, IL, USA). The significant differences between control and treated data sets were determined by using one-way analysis of variance and Student's *t*-test.

3. Results

3.1. Effect of fipronil on CYP3A4, CYP2B6 and CYP1A1 mRNA transcripts in human hepatocytes

CYP-specific mRNA induction was examined using the bDNA assay. Preliminary experiments in which fresh human hepatocytes were incubated with varying concentrations of a number of pesticides indicated that relatively low concentrations of fipronil induced mRNA expression levels for several CYPs (data not shown). Multiple concentrations of fipronil demonstrated a dose dependent induction of CYP1A1 mRNA levels, with a maximum of 52-fold induction at 25 μM (Fig. 1). Although CYP3A4 mRNA levels were also dramatically induced by fipronil, the dose response was markedly different. In this case, a dose-dependent increase in CYP3A4 mRNA expression was noted with increasing concentrations from 0.1 to 1 μM fipronil, while levels of induction decreased with sequentially higher doses (Fig. 1). Maximal induction of CYP3A4 mRNA expression varied from 10- to 27-fold in hepatocytes from two individuals following 1 μM fipronil exposure (data from second individual not shown). The fipronil mediated induction of CYP3A4 mRNA levels reflected by the bDNA assay was similar to levels observed for the well-known CYP3A4 inducer, rifampicin (20-fold ± 5, $n = 3$). Thus, the overall fipronil induction of each CYP isoform is very much comparable to the induction by the prototypical CYP inducer, rifampicin, where induction was similar to 1A1 (5.0 ± 3.9, $n = 2$), 3A5 (1.6 ± 0, $n = 1$), and 2B6 (2.2 ± 0.24, $n = 2$). The patterns observed using other prototypical inducers such as 3-methylcholanthrene and phenobarbital were as expected. Although the pattern of mRNA induction for CYPs 3A5 and 2B6 were similar to those observed for 3A4, the levels of induction were much less pronounced (Fig. 1). Two primary cultures were used in this study, the representative one shown also expressed CYP3A5 but the second one did not. However, the overall findings were similar regardless of the presence or absence of CYP3A5.

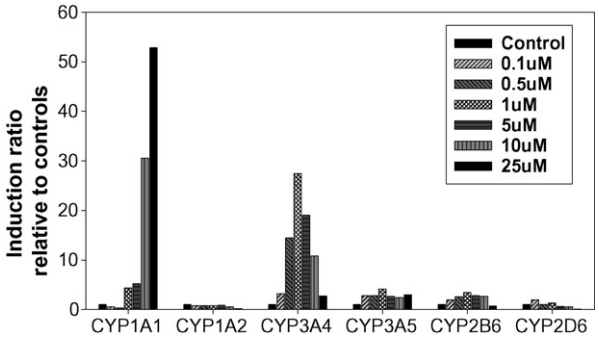


Fig. 1. Dose–response of fipronil effects on mRNA expression of selected CYP isoforms in human hepatocytes. Total RNA was isolated from hepatocytes which had been exposed to varying concentrations of fipronil (0.1–25 μM) or 0.1% DMSO for 72 h. RNA was analyzed by bDNA assay, normalized to glyceraldehyde phosphate dehydrogenase levels and compared to control. Data is one representative sample of two individual preparations.

3.2. Effect of fipronil on CYP3A4 functional protein expression in human hepatocytes

To confirm bDNA results, a dose response from four individual human hepatocyte treatments was examined by Western blotting for CYP3A4. In each case, a dose-dependent increase in CYP3A4 protein expression was noted with increasing concentrations of fipronil from 0.1 to 5 μM; with protein expression becoming progressively lower at higher doses of fipronil (10–25 μM). The highest level of CYP3A4 protein expression occurred at a concentration of 1 μM fipronil (Fig. 2A). Further verification of the induction of functional CYP3A4 protein was obtained using testosterone as a substrate in three hepatocyte preparations. CYP 3A4 activity was normalized to total cell protein. 6β-hydroxytestosterone production essentially mirrored expression levels that were determined by Western blotting. The maximum induction was ∼4-fold at 1 μM fipronil (Fig. 2A). Fresh human hepatocytes from six different individuals showed varying levels of induction ranging from ∼2- to 8-fold in CYP3A4 protein expression (measured by western blotting) following exposure to 1 μM fipronil (Fig. 2B).

3.3. Induction of CYP1A1 activity in fipronil treated human hepatocytes

In contrast with CYP3A4, CYP1A1 mRNA induction was dose-dependent up to 25 μM fipronil (Fig. 1).

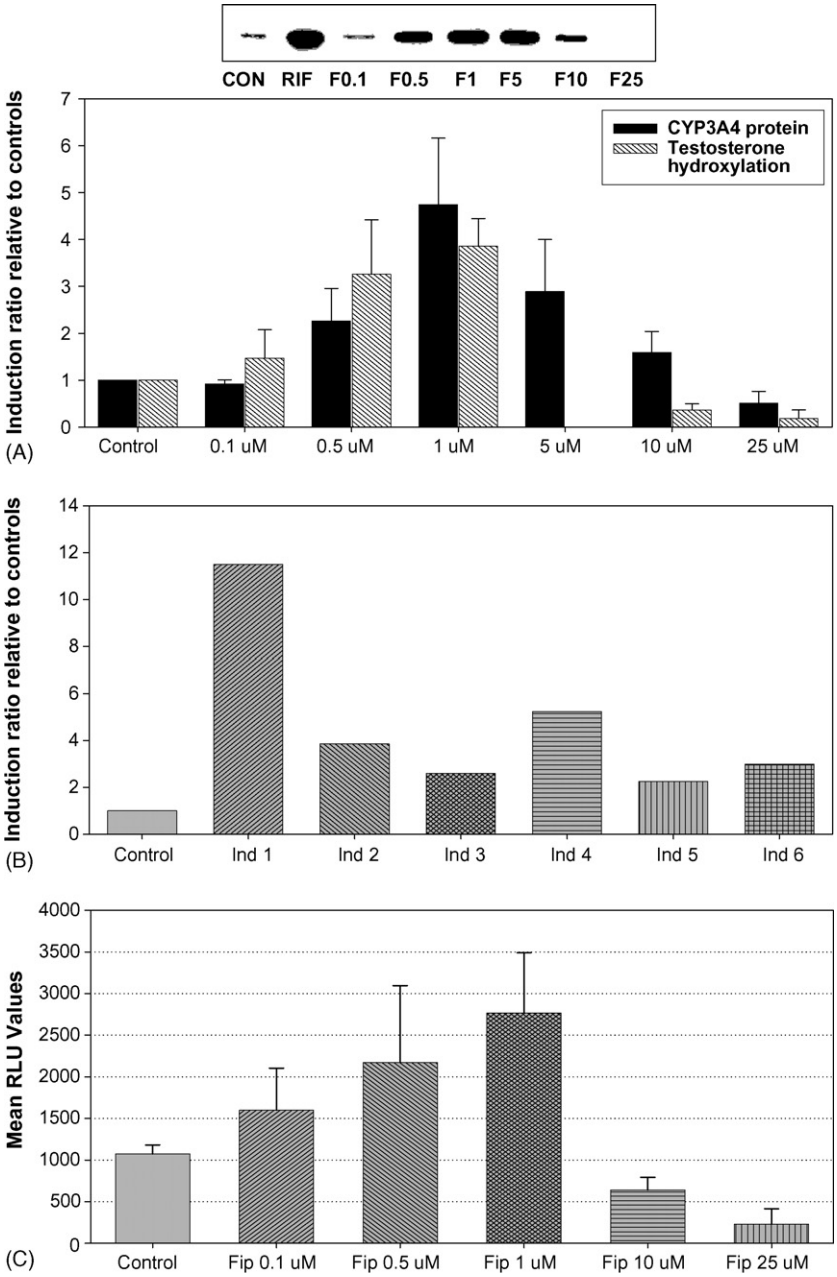


Fig. 2. (A) Dose–response of fipronil effects on CYP3A4 protein and activity levels in human hepatocytes. Human hepatocytes were exposed to varying concentrations of fipronil (0.1–25 μM) or 0.1% DMSO for 72 h and CYP3A4 protein levels and testosterone hydroxylation activities were determined by western blot and HPLC analysis, respectively. Protein levels are represented as induction relative to controls following densitometric analysis of western blots from four individuals. CYP3A4 activity levels are represented by 6β-hydroxytestosterone formation following incubation of hepatocytes with testosterone from three individuals. Error bars are representative of the means and S.E.s of the mean. Representative Western blot. Lanes, from left to right—con: control; RIF: rifampicin: F0.1–F25: fipronil dose in μM. (B) Fipronil-mediated induction of CYP3A4 protein in human hepatocytes from six individuals. CYP3A4 protein levels were determined following exposure of hepatocytes to fipronil (1 μM) for 72 h by western blot analysis. Each bar represents the induction relative to controls following densitometric analysis and is representative of duplicate determinations. (C) Dose–response effect of fipronil on CYP1A1 enzyme activity levels in human hepatocytes. Human hepatocytes were exposed to varying concentrations of fipronil (0.1–25 μM) or 0.1% DMSO for 72 h and CYP1A1 activity measured using the Luciferin-CEE assay. The data are presented as mean RLU ± S.E. from two individual preparations, with triplicate determinations from each individual.

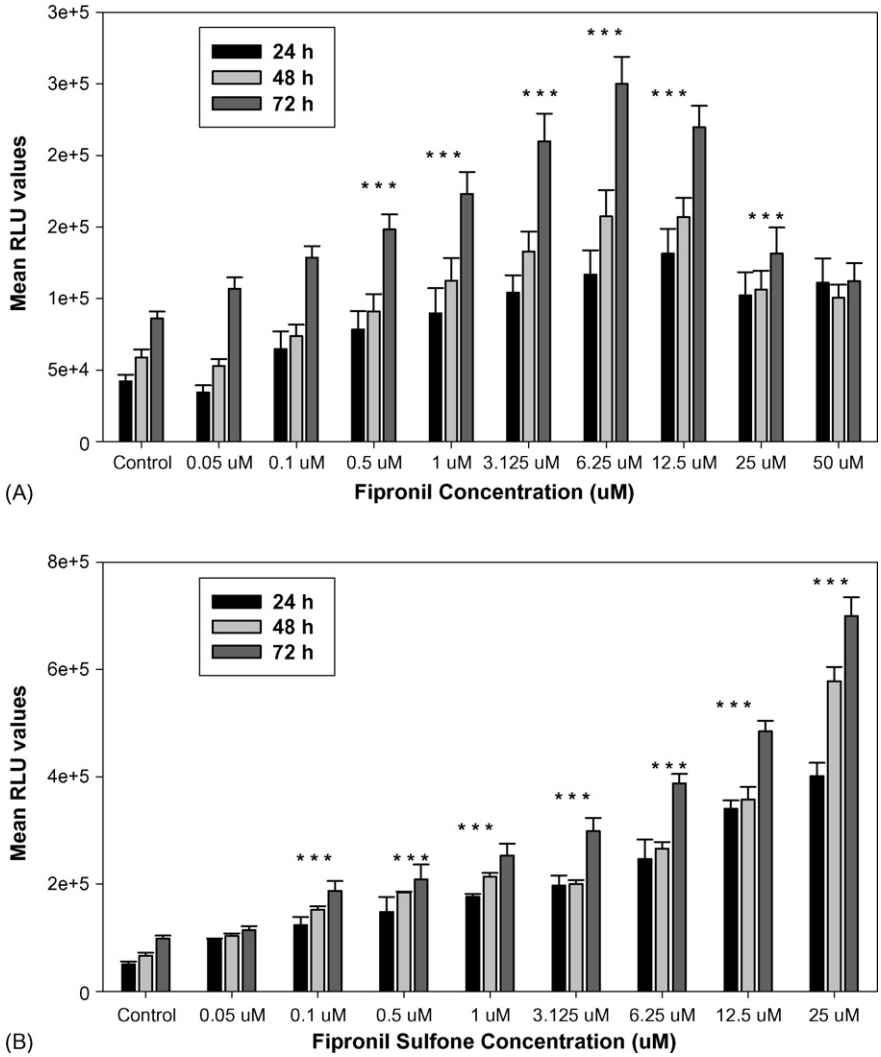


Fig. 3. (A) Dose- and time-dependent effects of fipronil on adenylate kinase activity in HepG2 cells. Cultured HepG2 cells were exposed to increasing concentrations of fipronil (0.05–50 μM) or 0.1% DMSO for 72 h and adenylate kinase activity was measured by ToxiLight™ assay kit at 24 h intervals. Data is expressed as mean RLU ± S.E. from three separate determinations, conducted in triplicate, where asterisk (*) indicates level of significance at $p \leq 0.05$. (B) Dose- and time-dependent effects of fipronil sulfone on adenylate kinase activity in HepG2 cells. Cultured HepG2 cells were exposed to increasing concentrations of fipronil sulfone (0.05–25 μM) for 72 h and assayed for adenylate kinase activity using the ToxiLight™ assay kit at 24 h intervals. Data is expressed as RLU from one determination conducted in triplicate.

To determine if this induction resulted in functional protein, CYP1A1 activity was measured in fipronil treated human hepatocytes and normalized to total cell protein. Results of these assays using a CYP1A1 specific substrate indicated that CYP1A1 activity exhibited a similar response as observed for CYP3A4, in that maximum induction of ∼3-fold was observed at 1 μM which dramatically tapered off at higher concentrations (Fig. 2C).

3.4. Effect of fipronil and fipronil sulfone on adenylate kinase activity and cell viability in HepG2 cells and human hepatocytes

Adenylate kinase activity was monitored following treatment with fipronil or fipronil sulfone in cultured HepG2 cells using the ToxiLight assay. Increasing doses of fipronil caused increased release of adenylate kinase from cellular membranes into the media, peaking at

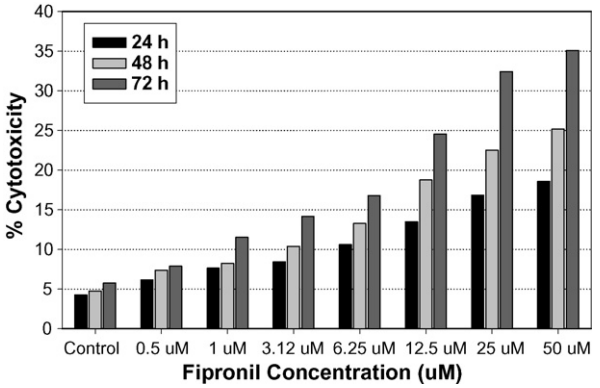


Fig. 4. Dose- and time-dependent effects of fipronil on the HepG2 cell viability. Cultured HepG2 cells were exposed to increasing concentrations of fipronil (0.5–50 μM) for 72 h and cell viability assessed by the trypan blue exclusion assay at 24 h intervals. Data is expressed as percent cytotoxicity where each bar represents the value derived from counting 150–200 cells per sample.

the fipronil concentration of 6.25 μM with a level of activity of approximately 2.5-fold above control levels (Fig. 3A). Concentrations of fipronil above 6.25 μM had diminishing responses of adenylate kinase activity with increasing dose. The degree of induction was time and dose dependent except at 25 and 50 μM fipronil (Fig. 3A). Adenylate kinase activity was also significantly increased by increasing doses of fipronil sulfone, achieving approximately a seven-fold increase at the highest concentration (25 μM) (Fig. 3B).

To more fully evaluate the cytotoxic effects of fipronil, the trypan blue exclusion assay was used. At the concentration causing the greatest adenylate cyclase activity (6.25 μM, 72 h) cytotoxicity was 10% higher than in controls (Fig. 4). At higher concentrations, where adenylate cyclase activity dramatically dropped, cytotoxicity was 20% greater than in the respective controls.

Parallel experiments were conducted using human hepatocytes from two different individuals. In both cases, adenylate kinase activity remained relatively constant up to 12.5 μM, but markedly increased at 25 and 50 μM following 48 and 72 h exposures (Fig. 5A, B). In comparison, significant increases in adenylate kinase activity were observed with fipronil sulfone at the lower dose of 12.5 μM (Fig. 5C).

3.5. Effect of fipronil and fipronil sulfone on caspase-3/7 activity in HepG2 cells and human hepatocytes

Caspase-3/7 activity is an important marker of the cellular apoptotic process. In order to characterize whether fipronil mediated cell death was triggered through the known apoptotic pathway, cultured HepG2 cells were exposed to increasing concentrations of fipronil and fipronil sulfone for varying time periods prior to caspase-3/7 activity measurements and the activity was normalized to cell number. Time- and dose-dependent induction of caspase-3/7 activity was noted from 0.1 to 6.25 μM fipronil, while slowly decreasing from 12.5 to 50 μM fipronil. The maximum induction ranged from two- to three-fold at 48 and 72 h, respectively and was significantly different from the respective control at all doses except at 25 and 50 μM fipronil exposure (Fig. 6A). In comparison with fipronil, fipronil sulfone caused immediate induction of caspase-3/7 activity not only at lower concentrations but also at earlier time points (Fig. 6B). The most striking difference between fipronil and fipronil sulfone was the immediate response observed within 24 h of exposure to the sulfone, an observation not apparent with fipronil.

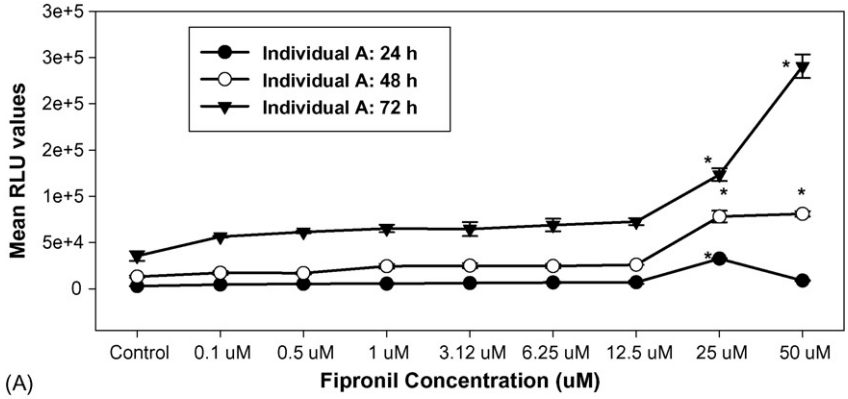
As a comparison with HepG2 cells, fresh human hepatocytes were exposed similarly to increasing concentrations of fipronil or fipronil sulfone. In the two individual hepatocyte preparations examined, caspase 3/7 activity was not significantly affected until doses exceeded 12.5 μM (Fig. 7A and B), suggesting that hepatocytes are less sensitive than HepG2 cells. At the 25 μM concentration, both individuals had significant increases in caspase 3/7 activity; which declined substantially with increasing dose. In a similar manner, as observed in HepG2 cells, fipronil sulfone significantly induced caspase-3/7 activity at lower concentrations than were observed for fipronil (Fig. 7C). Actinomycin D, a known caspase 3/7 inducer, induced caspase-3/7 levels from 8- to 25-fold above controls (data not shown). In contrast, a specific caspase-3/7 inhibitor Z-DEVD-FMK completely abrogated the fipronil-induced caspase-3/7 response (data not shown).

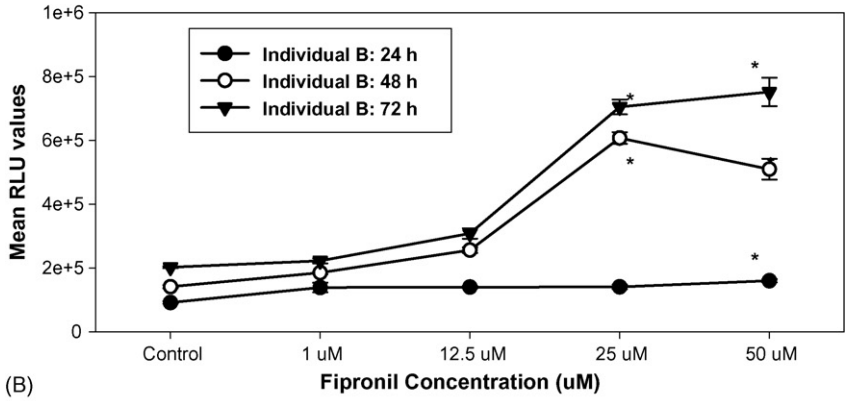
3.6. Effect of fipronil on Fas protein expression in HepG2 cells

Cultured HepG2 cells were treated with various doses of fipronil (0.1–50 μM) for 72 h to determine if fipronil mediated apoptotic cell death signals through the major membrane associated signaling protein Fas. Western blot analysis indicated that fipronil at 12.5 μM and lower doses slightly induced Fas protein expression, while reducing expression at the higher doses of 25 and 50 μM (data not shown).

4. Discussion

Analysis of fipronil metabolism in humans demonstrated only a single metabolite, fipronil sulfone, and





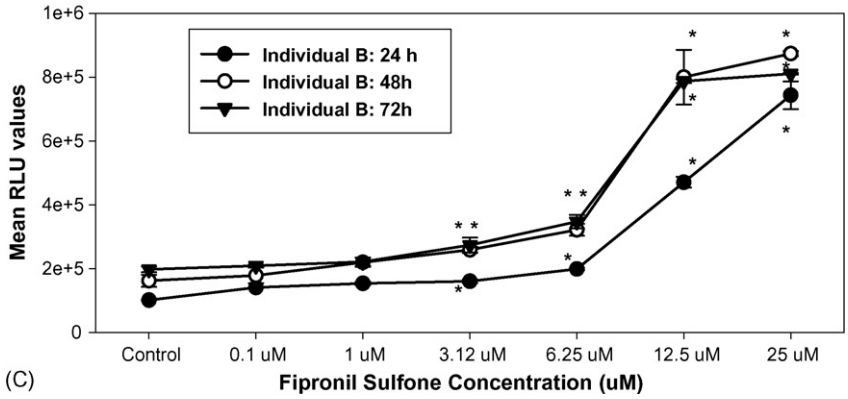


Fig. 5. (A) Dose- and time-dependent effects of fipronil on adenylate kinase activity in human hepatocytes. Human hepatocytes from individual A were exposed to varying concentrations of fipronil (0.1–25 μM) or 0.1% DMSO for 72 h and assayed for adenylate kinase activity using the ToxiLight^{TM} assay kit at 24 h intervals. Data is expressed as mean RLU ± S.E. from a minimum of two separate determinations. The * indicates the level of significance at $p \leq 0.05$. (B) Dose- and time-dependent effects of fipronil on adenylate kinase activity in human hepatocytes. Human hepatocytes from individual B were exposed to varying concentrations of fipronil (0.1–25 μM) or 0.1% DMSO for 72 h and assayed for adenylate kinase activity using the ToxiLight^{TM} assay kit at 24 h intervals. Data is expressed as mean RLU ± S.E. from a minimum of three separate determinations. The asterisk (*) indicates the level of significance at $p \leq 0.05$. (C) Dose- and time-dependent effects of fipronil sulfone on adenylate kinase activity in human hepatocytes. Human hepatocytes from individual B were exposed to varying concentrations of fipronil sulfone (0.1–25 μM) or 0.1% DMSO for 72 h and assayed for adenylate kinase activity using the ToxiLight^{TM} assay kit at 24 h intervals. Data is expressed as mean RLU ± S.E. from a minimum of three separate determinations. The * indicates the level of significance at $p \leq 0.05$.

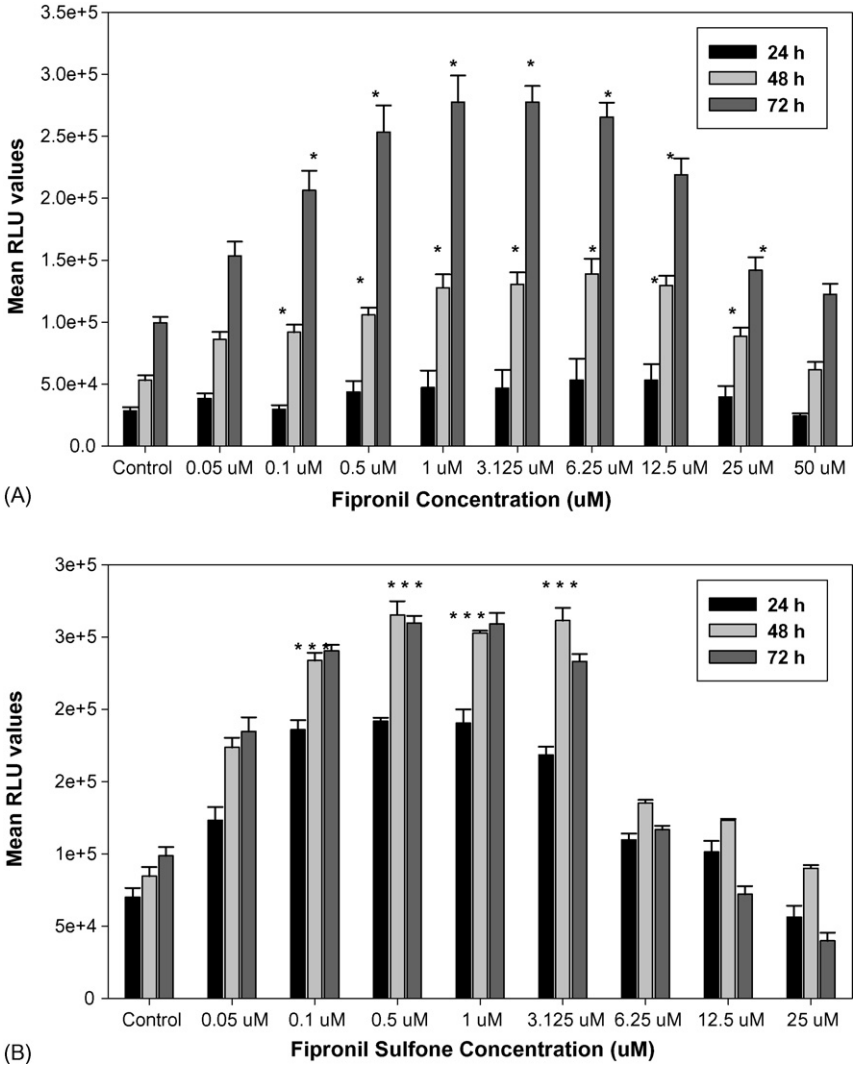


Fig. 6. (A) Dose- and time-dependent effects of fipronil on caspase-3/7 activity in HepG2 cells. Cultured HepG2 cells were exposed to increasing concentrations of fipronil (0.5–50 μM) and caspase-3/7 activity was determined using the Caspase Glo®-3/7 assay kit at 24 h intervals. Data is expressed as mean RLU ± S.E. from triplicate determinations from three independent experiments. The asterisk (*) indicates the level of significance at $p \leq 0.05$. (B) Dose- and time-dependent effects of fipronil sulfone on caspase-3/7 activity in HepG2 cells. Cultured HepG2 cells were exposed to increasing concentrations of fipronil sulfone (0.5–25 μM) and caspase-3/7 activity was determined using the Caspase Glo®-3/7 assay kit at 24 h intervals. Data is expressed as mean RLU ± S.E. from triplicate determinations from one experiment. The asterisk (*) indicates the level of significance at $p \leq 0.05$.

that CYP3A4 is primarily responsible for its production, although CYP2C19 also plays a minor role [8]. Earlier studies had suggested that fipronil sulfone production is also the principal pathway in mice [4] although, in neither species, has metabolism of fipronil to fipronil sulfone been well characterized with reference to cytotoxicity. Variations in fipronil metabolism observed among several individuals were as much as 40-fold, corresponding with the known variability of CYP3A4 expression [8]. It seems appropriate to state, at the outset, that given the differences between cell types, time courses and threshold doses, the assumption that CYP induction and cytotoxicity are mechanistically related is premature and one that will need further investigation.

Fipronil mediated induction of CYP3A5 and CYP2B6 was small relative to CYP1A1 and CYP3A4, and the response patterns were more or less similar to that of CYP3A4. Pesticides that interact with the elements involved in the regulatory process of enzyme induction such as aryl hydrocarbon receptor (AhR) in CYP1A

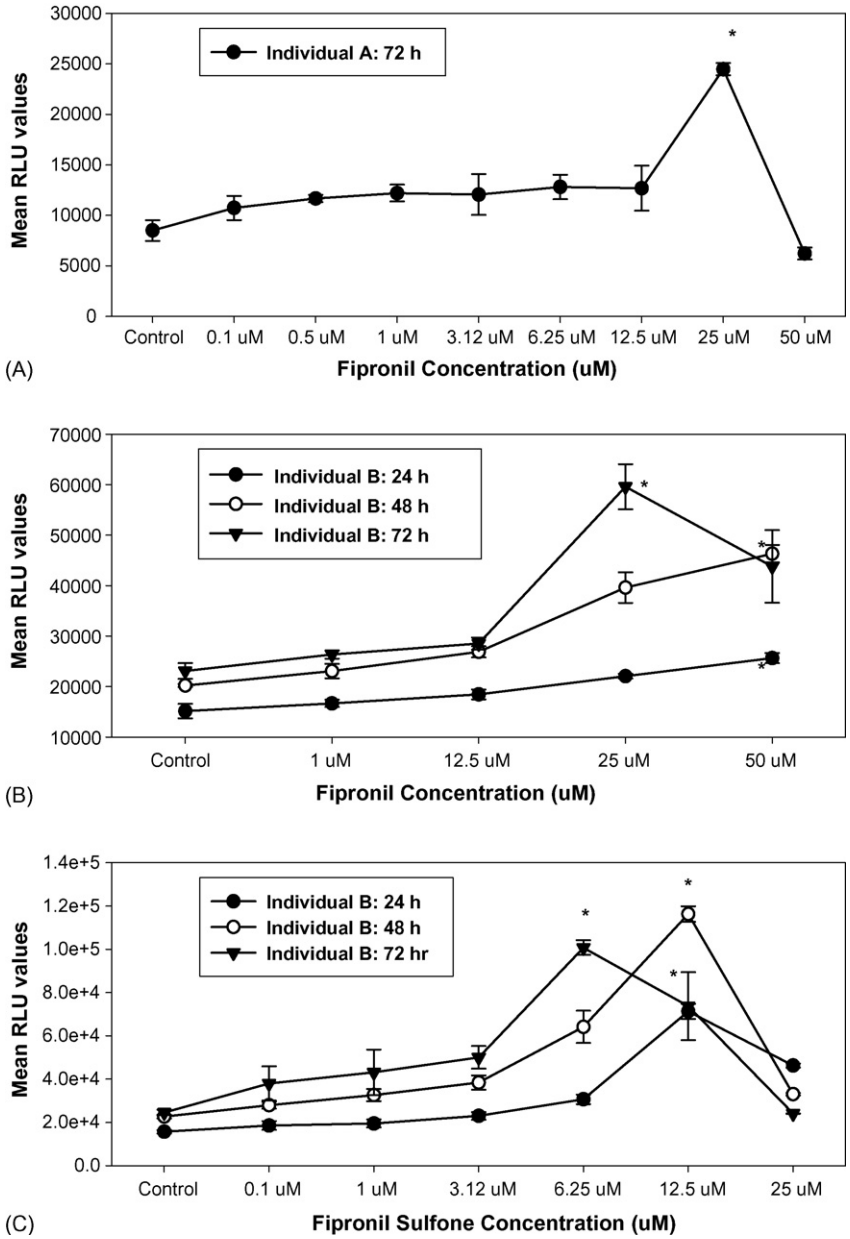


Fig. 7. (A) Dose- and time-dependent effects of fipronil on caspase 3/7 activity in human hepatocytes. Human hepatocytes from individual A were exposed to varying concentrations of fipronil (0.1–50 μM) or 0.1% DMSO for 72 h and assayed using the Caspase Glo-3/7 assay kit. Data is expressed as mean RLU ± S.E. from a minimum of two determinations. The asterisk (*) indicates the level of significance at $p \leq 0.05$. (B) Dose- and time-dependent effects of fipronil on caspase 3/7 activity in human hepatocytes. Human hepatocytes from individual B were exposed to varying concentrations of fipronil (0.1–50 μM) or 0.1% DMSO for 72 h and assayed at 24 h intervals using the Caspase Glo-3/7 assay kit. Data is expressed as mean RLU ± S.E. from a minimum of two determinations. The asterisk (*) indicates the level of significance at $p \leq 0.05$. (C) Dose- and time-dependent effects of fipronil sulfone on caspase 3/7 activity in human hepatocytes. Human hepatocytes from individual B were exposed to varying concentrations of fipronil sulfone (0.1–25 μM) or 0.1% DMSO for 72 h and assayed at 24 h intervals using the Caspase Glo-3/7 assay kit. Data is expressed as mean RLU ± S.E. from a minimum of two determinations. The asterisk (*) indicates the level of significance at $p \leq 0.05$.

induction [36] and pregnane X receptor (PXR) variation in CYP3A induction, as well as variations in their respective regulatory proteins [27], and variation in CAR mediated induction of CYP2B and CYP3A genes [25,26] can potentially have an impact on the degree of induction. Rifampicin, a model inducer of CYP isoforms induced expression of CYP3A4 ∼20-fold probably via modulation of regulatory receptor elements CAR and PXR.

Regardless of the degree of induction, fipronil being a primary substrate for the CYP3A4 isoform requires co-expression of b_5 [8] for metabolic activity. Fipronil showed 27-fold induction of CYP3A4 in an individual where CYP3A5 co-expressed and 10-fold induction where CYP3A5 co-expression is absent, although the general pattern of induction was similar in both cases. Unlike CYP3A4 fipronil dose-dependent induction of CYP1A1 expression reaching its highest level at 25 μM. CYP1A1 induction occurs following interaction with the Ah-receptor and subsequent activation of regulatory mechanisms, which suggests that fipronil may interact directly with the AhR to augment CYP1A1 transcription

Fipronil induction of CYP3A4 mRNA levels was supported by protein expression studies in several individuals (Fig. 2A and B). CYP3A4 not only metabolizes numerous xenobiotics, but also is important in the metabolism of endogenous substrates including testosterone, progesterone and estradiol [18,22–24]. This study demonstrated that hepatocyte exposure to fipronil doses as low as 1 μM were sufficient to cause five-fold increases in protein levels as measured by western blotting and a corresponding four-fold increased capacity for testosterone hydroxylation (Fig. 2A). Previous studies in which several pyrethroid, organochlorine and organophosphorus pesticides were screened at 10 μM concentrations demonstrated average induction of CYP3A4 of approximately two-fold [19,20]. The induction response observed for fipronil occurred at one-tenth the dose and was two-fold greater than that for any of the pesticides examined previously. The implications of low level chronic exposure to fipronil and consequent induction of metabolic enzymes including CYP3A4 and 1A1 are unknown.

Like CYP3A4, fipronil also induced CYP2B6 mRNA transcripts in human hepatocytes, with the highest induction up to 3.5-fold with some inter-individual variation (Fig. 1). Since CYP3A4 and CYP2B6 are often co-regulated as a result of activation of the human pregnane X receptor (hPXR) [25,26] our results suggest that fipronil activates this receptor. Induction of hPXR by organochlorine pesticides including chlordane, dieldrin and endosulfan has previously been demonstrated [28]. A recent study reported that fipronil was one of several pesticides capable of inducing hPXR [29]. Since CYP3A4 and 2B6 are involved extensively in drug metabolism, fipronil mediated induction of these isoforms has the potential to impact drug metabolism in exposed individuals. Recent studies have demonstrated that pesticides have the potential to inhibit drug metabolism [8,30] and hormone metabolism [18,31,43–46]. However, few, if any pesticide studies have explored potential drug/pesticide interactions which may result from induction of pesticide metabolizing enzymes.

Among the CYP isoforms, CYP1A1 is a ubiquitous polycyclic aromatic hydrocarbon (PAH)-responsive monooxygenase enzyme [32,33] that is involved in the activation of pro-carcinogens and pro-mutagens into reactive genotoxic metabolites [34]. Its induction is governed by genetic polymorphisms [35]. Because CYP1A1 is readily activated and is not constitutively expressed in the liver, its induction is often used as an indicator of harmful effects of environmental chemicals [36]. A screen of several pesticides in rat hepatocytes demonstrated significant induction of CYP1A1 levels by tetrachlorvinfos, diflubenzuron, cypermethrin and carbaryl [37]. The present study demonstrated a dose-dependent induction of CYP1A1 mRNA levels by fipronil, with highest expression occurring at 10 and 25 μM (Fig. 1). However, an examination of metabolic activity levels using the CYP1A1-specific substrate, Luciferin-CEE, demonstrated that active protein levels were considerably reduced at fipronil concentrations above 1 μM (Fig. 2C). The over-expression of mRNA at concentrations higher than 1 μM is likely due in part to compensatory mechanisms of the hepatocytes to overcome the cytotoxic effects of higher doses. Furthermore, the highest expression of CYP1A1 transcripts was probably due to early and rapid transcriptional activation where translation was not affected. A similar phenomenon was not apparent for transcripts of the other CYP isoforms probably due to basic differences in the mechanisms of activation, a subject that needs further investigation.

Observations of the cytotoxicity of fipronil at doses lower than those observed for many pesticides prompted further investigations. Doses of fipronil which maximally induced CYP3A4 mRNA expression in hepatocytes also elicited significant cytotoxicity in HepG2 cells, as measured by adenylate kinase activity, though not in human hepatocytes where changes in adenylate kinase activity were not observed until doses of 25 μM or above. However, observations relating to enzyme activity levels for both CYP1A1 and 3A4 indicated possible cytotoxic effects at doses above 1 μM. The data suggest that treatment of primary hepatocytes with higher than 1 μM fipronil shows compromised metabolic activity (Fig. 2A), however limited data generated for adenylate kinase and capase-3/7 activities show peak induction at either 12.5 or 25 and tapering off at the highest dose (Figs. 5 and 7). Thus the overall data suggest that primary hepatocytes lost the ability to induce metabolic

enzyme activity, i.e., CYP3A4, before they were compromised for the production and release of adenylate kinase and caspase-3/7 activity at the highest doses of fipronil.

The cytotoxic responses observed for fipronil relative to fipronil sulfone for both adenylate kinase and caspase-3/7 activities are not extensive enough to decide whether or not sulfoxidation may be considered an activation reaction for cytotoxicity.

The possibility that the cytotoxic response of HepG2 cells and hepatocytes to fipronil was due to apoptosis was also investigated. The two main pathways leading to apoptosis include the receptor-mediated pathway (Fas ligand or TNFα-mediated) and the mitochondria-initiated pathway that involves a release of mitochondrial proteins which commits hepatocytes to apoptosis [47]. The caspase-3/7 assay does not differentiate between these pathways, but potentially is activated by both. Fipronil at lower doses was demonstrated to induce caspase-3/7 activity, reaching a plateau near 3 μM in HepG2 cells. In comparison, hepatocytes were less susceptible, exhibiting maximum caspase 3/7 activity at 25 μM. In both cases, caspase 3/7 activity was enhanced at lower doses of fipronil sulfone than with fipronil. Dose-dependent fipronil mediated activation followed by down regulation of caspase 3/7 in concurrence with compromised ATP induction is attributed to apoptotic cell death. The general lack of response observed in limited trials with the anti-Fas antibody suggested that fipronil does not interact through the Fas ligand mediated pathway.

Human hepatocytes in primary culture are considered to be one of the most suitable models for CYP induction studies [41,42]. The ability to predict in vivo disposition of interactions resulting from induction is hindered not only by the substantial inter-individual differences between individuals but also by the pharmacokinetic characteristics of the pesticides examined [21]. This study also demonstrated that although human hepatoma HepG2 cells exhibited similar toxicity profiles as human hepatocytes, there were differences with respect to the sensitivity of the two cell types to the pesticides examined. For these reasons it would be unwise to assume that the inductive effects of fipronil and fipronil sulfone are necessarily equivalent to in vivo experimental conditions in humans.

Studies in our laboratory have demonstrated that fipronil and fipronil sulfone are significantly more toxic to hepatocytes than other pesticide classes we have examined (unpublished data). Fipronil is a substrate of CYP3A4 [8] and has the potential to significantly induce both CYP1A1 and CYP3A4 resulting in an increased potential to interact with a wide range of xenobiotics and endogenous hormones. In the absence of human in vivo pharmacokinetic data on low-level long-term exposure to populations involved in manufacturing and farming, and predictive data from PBPK models on fipronil toxicity, it is difficult to come to conclusions about potential risks of fipronil exposure to the human population.

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