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Fipronil induces CYP isoforms in rats

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ABSTRACT

The goal of the present study was to evaluate fipronil effects on the activities of drug metabolizing enzymes in rat liver microsomes. Rats were orally treated with fipronil at doses of 1, 5, 10 and 15 mg/kg bw/day for 6 days. Determinations of cytochrome P450 (CYP) enzyme activities were carried out in hepatic microsomes isolated from treated rats. The activities of some members of CYP2E, CYP1A, CYP2A, CYP2B and CYP3A subfamilies significantly increased after fipronil treatment in a dose-dependent manner as compared to control. The major effects were observed in the *O*-deethylation of ethoxyresorufin and *O*-demethylation of methoxyresorufin (reflecting CYP1A1/2 activities), in the *O*-depenthylation of pentoxyresorufin and 16β-hydroxylation of testosterone (reflecting CYP2B1/2 activities), and in the *N*-demethylation of erythromycin and 6β-hydroxylation of testosterone (reflecting CYP3A1/2 activities). Immunoblot studies revealed that fipronil increased the apoprotein levels of CYP1A1. Our results suggest that fipronil is an inducer of hepatic phase I CYP enzymes, causing an increased potential to interact with a wide range of xenobiotics or endogenous chemicals that are substrates of the CYP1A, CYP2B and CYP3A subfamilies. Further investigations are required to *in vivo* evaluate the potential of the metabolite fipronil sulfone as an inducer of phase I CYP enzymes.

1. Introduction

Fipronil is a highly active, broad spectrum insecticide from the phenylpyrazole family. In agricultural applications, this pesticide has been used on pests of a wide variety of food crops (Scharf and Siegfried, 1999; Ngim and Crosby, 2001; Wilde et al., 2001). In non-agricultural applications, it is used to control many domestic and veterinary pests including several lepidopteran species as well as thrips, locusts, ants, cockroaches, fleas and ticks (Tingle et al., 2003; Beugnet, 2004). Concerns for fipronil effects on public health have been raised because of the wide range of uses of this pesticide (Tingle et al., 2003). Fipronil toxicity is attributed to its ability to act at the γ-aminobutyric acid (GABA) receptor as a noncompetitive blocker of the GABA-gated chloride channels of neurons in the central nervous system (Cole et al., 1993; Ratra et al., 2001, 2002). The selective toxicity of fipronil to insects relative to vertebrates is primarily the result of differences at the target site (Hainzl et al., 1998; Grant et al., 1998). The parent compound might to have higher potency at the insect *versus* the mammalian GABA receptor, but also might be dependent on the relative rates conversion to the more persistant and selective sulfone metabolite.

Relatively little is known of the ability of fipronil to be metabolized in vertebrates. Under normal use conditions for fipronil, there are three toxicants to consider: the parent compound, its major sulfone metabolite and the desulfinyl photoproduct (Hainzl and Casida., 1996). Studies indicate that the pathways of fipronil metabolism are reduction to sulfoxide, S-oxidation to form the sulfone (predominant pathway) and photolysis to desulfinyl product (Bobe et al., 1998a, b; Ramesh and Balsubramanian, 1999; Tomlin et al., 2000). Desulfinyl fipronil, although is not a metabolite, is the principal photoproduct on plants and soils and it is as potent as or more potent than fipronil in toxicity to mice and houseflies (Hainzl and Casida., 1996). Fipronil sulfone is the major metabolite in insects and vertebrates and the main metabolite formed *in vivo* in humans and can persist much longer in the organism that fipronil itself. It is more toxic than the parent compound as an antagonist of the GABA receptor (Hainzl et al., 1998). This main fipronil metabolic pathway is mediated by hepatic cytochrome P450 (CYP) enzymes and fipronil as many other xenobiotics can lead to increased CYP enzyme expression and activity. *In vitro* studies with human liver microsomes demonstrated that fipronil sulfone was the predominant metabolite via CYP oxidation

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and CYP3A4 was the major isoform responsible for fipronil oxidation (Tang et al., 2004). It is not clear if metabolic oxidation to the sulfone is a required bioactivation step in vertebrates or just yields and additional toxicant.

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 (Hurley et al., 1998). Fipronil treatment has been associated with a significant increase in the incidence of thyroid gland tumors concomitant with increased plasma concentrations of thyroid-stimulating hormone (TSH) and decreased thyroxine (T4) concentrations (Hurley et al., 1998). It has been proposed that fipronil-induced thyroid disruption results from an increased rate of T4 elimination likely mediated by increased hepatic enzyme activities. Recently, Roques et al. (2013) indicated that constitute androstane receptor (CAR) and pregnane X receptor (PXR) are key modulators of the hepatic gene expression profile following fipronil treatment which, in rats, may contribute to increase thyroid hormone clearance. *In vivo* data in rats assessing the antipyrine clearance, which is considered a relevant biomarker, but non specific, for phase I CYP enzymes involved in hepatic xenobiotic metabolism in different species (St Peter et al., 1991; Engel et al., 1996; Chan and Yeung, 2006; Anadón et al., 2013), demonstrated in thyroid-intact rats that fipronil treatment increased antipyrine clearance (Leghait et al., 2009; Roques et al., 2012). Fipronil treatment was also associated with an increase in hepatic microsomal 4-nitrophenol UDP-glucuronosyltransferase activity, hepatic phase II enzyme involved in T4 glucuronidation, as well as with plasma concentrations of fipronil sulfone at least 20-fold higher than those of fipronil (Leghait et al., 2009). The fact that thyroid disruption in rats is associated with high plasma fipronil sulfone concentrations but with very low plasma fipronil concentrations has led to the suggestion that fipronil biotransformation into fipronil sulfone by hepatic CYP enzymes could be a determining factor in fipronil-induced thyroid disruption. Such hypothesis is supported by *in vitro* data on human hepatocytes, both compounds resulted in a dose-dependent increase in CYP1A1 and CYP3A4 mRNA expressions, but fipronil sulfone was more cytotoxic to hepatocytes than fipronil itself (Das et al., 2006). It is noteworthy, however, that in recent *in vivo* study, both fipronil and fipronil sulfone treatments (3.4 μmol/kg/day *per os* for 14 days) in rats induced a significant increase in CYP3A1 mRNA expression, but not in CYP1A1 and CYP1A2 mRNA expressions (Roques et al., 2012).

The ability of chemicals to induce metabolic enzymes has long been considered one of the most sensitive biochemical cellular responses to toxic insult, since it often occurs at much lower dose of the chemical than those known to cause lethal or overtly toxic effects. To our knowledge, the *in vivo* and *in vitro* data concerning the ability of fipronil to induce hepatic phase I enzymes are conflicting respect to CYP1A1/2 induction. *In vitro*, fipronil increases CYP1A1 and 3A4 activities in human hepatocytes (Das et al., 2006) whereas no clear effect of fipronil was evidenced on the activities of microsomes obtained from fipronil-treated rats, rabbits or mice (AFSSA, 2005; Roques et al., 2012). Accordingly, in an attempt to contribute to the literature with new data of the role of fipronil to induce metabolic phase I CYP enzymes, the present study examined the effect of oral doses of fipronil on CYP1A1/2, CYP2B1/2, CYP2E1, CYP2C11, CYP2A1, CYP3A1/2 and CYP4A1/2 enzyme activities in liver microsomes from treated rats.

2. Materials and methods

2.1. Chemicals

Fipronil, (5-amino-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[1(*R*,*S*)-(trifluoro-methyl) sulfinyl]-1H-pyrazole-3-carbonitrile, purity estimated by the supplier 97.9%, testosterone, 2α-hydroxytestosterone, 6β-hydroxytestosterone, aniline, aminopyrine, erythromycin, lauric acid, ethoxy- and pentoxyresorufins, resorufin, NADPH, NAD^+, cytochrome *c*, bovine serum albumin, and all cofactors were purchased from Sigma Chemical Company (St. Louis, MO). [1−^{14}C]Lauric acid was supplied by the Radiochemical Centre (Amersham, Buckinghamshire, UK). Methoxyresorufin was obtained from Molecular Probes, Inc. (Eugene, OR). The testosterone metabolites 7α-hydroxytestosterone and 16β-hydroxytestosterone were purchased from Steraloids, Inc (Wilton, NH).

Polyclonal anti-CYP1A1 was generous gift from Dr. C. Ioannides, University of Surrey. The antibodies to CYP1A1 detect both the CYP1A1 and the CYP1A2 proteins (Nebert and Jones, 1989). Prestained molecular weight marker (SDS-PAGE Standards #161-0317) was supplied by Bio-Rad (Madrid, Spain).

All other chemicals were obtained from usual commercial sources and were of the highest grade available.

2.2. Animals and experimental design

All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Complutense University of Madrid.

Male Wistar rats (Charles River Inc. Margate, Kent, UK), weighing initially 190–200 g of 8 weeks of age were used. The animals were individually housed in polycarbonate cages with sawdust bedding and were maintained in environmentally controlled rooms (22 ± 2 °C and 50 ± 10% relative humidity) with a 12 h light/dark cycle (light from 08:00 to 20:00 h). Food (A04 rodent diet, Panlab SL, Barcelona, Spain) and water were available *ad libitum*. The animals were assigned randomly to 9 groups of 6 animals each, one control group, four fipronil treated groups, and four pair-fed groups. Experimental animals were treated with single daily oral administrations of fipronil at dose levels of 1, 5, 10 and 15 mg/kg bw [equivalent to 1/100, 1/20, 1/10 and 1/6.5 of the LD_{50} (mean oral LD_{50} of 96.5 mg/kg bw, in corn oil, was previously calculated)] for 6 days. Dose of 3 mg fipronil/kg/day *per os* for 14 days has been shown to be associated to a thyroid disruption in rats in previous study (Leghait et al., 2009). The lower doses were chosen to reflect a possible no-observed-adverse-effect level. Fipronil was dissolved in 0.4 mL of corn oil and was daily administered orally by gavage (gastric intubation using a thin plastic tube attached to a syringe) (n = 6 rats per dose). The fipronil solutions were protected from light and were daily prepared before each administration. Pair-fed and *ad libitum*-fed control animals (n = 6 animals in each group) received 0.4 mL of corn oil orally by gastric intubation once daily for 6 consecutive days. Following treatment, the animals were individually housed in metabolism cages where body weight and food (rat chow) and water consumption were daily recorded. Animals received food and water *ad libitum*, with the following exception: pair-fed animals received the same amount of food that their dosed partners ate during the previous 24 h.

Fipronil-treated and *ad libitum*-fed control animals were sacrificed 24 h after the last administration, while pair-fed animals were sacrificed the following day. The animals were sacrificed by cervical dislocation and the livers were surgically exposed and removed. The liver was blotted dry and weighed, and perfused with 0.9% (w/v) sodium chloride to remove hemoglobin prior to homogenization in 0.25 M sucrose using a Potter-Elvjhem type homogenizer. Microsomal pellets were prepared and resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.25), containing 20% (v/v) glycerol, as previously described (Tamburini et al., 1984) and stored at −80 °C in 1-mL aliquots. All handing was performed at 0–4 °C.