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CYP450-Dependent Biotransformation of the Insecticide Fipronil into Fipronil Sulfone Can Mediate Fipronil-Induced Thyroid Disruption in Rats

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In rats, the widely used insecticide fipronil increases the clearance of thyroxine (T_4). This effect is associated with a high plasma concentration of fipronil sulfone, the fipronil main metabolite in several species including rats and humans. In sheep, following fipronil treatment, fipronil sulfone plasma concentration and thyroid disruption are much lower than in rats. We postulated that fipronil biotransformation into fipronil sulfone by hepatic cytochromes P450 (CYP) could act as a potential thyroid disruptor. The aim of this study was to determine if fipronil sulfone treatment could reproduce the fipronil treatment effects on T_4 clearance and CYP induction in rats. Fipronil and fipronil sulfone treatments (3.4 μmol/kg/day *per os*, 14 days) increased total and free T_4 clearances to the same extent in THX + T_3, euthyroid-like rats. Both treatments induced a 2.5-fold increase in *Ugt1a1* and *Sult1b1* messenger RNA (mRNA) expressions and a twofold increase in UGT1A activity suggesting that T_4 elimination was mediated, at least in part, by hepatic uridine 5′-diphospho-glucuronosyltransferases (UGT) and/or sulfotransferases (SULT) induction. Both treatments induced a 10-fold increase in *Cyp3a1* and *Cyp2b2* mRNA expressions concomitant with a threefold increase in CYP3A immunoreactivity and a 1.7-fold increase in antipyrine clearance, a biomarker of CYP3A activity. All these results showed that fipronil sulfone treatment could reproduce the fipronil treatment effects on T_4 clearance and hepatic enzyme induction in rats. The potential of fipronil sulfone to act as a thyroid disruptor is all the more critical because it persists much longer in the organism than fipronil itself.

Key Words: **fipronil; fipronil sulfone; thyroid disruption; clearance; metabolism; rat.**

Fipronil (5-amino-1-(2,6-dichloro-4-(trifluoromethyl)phenyl)-4-((trifluoromethyl)sulfinyl) pyrazole-3-carbonitrile) is widely used as a phytosanitary product and veterinary insecticide in many countries. It was developed as a substitute for insecticides that are considered more toxic such as lindane and belongs to the second generation of insecticides acting on the γ-aminobutyric acid receptor chloride channels (Cole *et al.*, 1993).

To date, regulatory toxicological evaluations and experimental investigations have shown that fipronil treatment can act as a thyroid disruptor in rats (Agence Française de Sécurité Sanitaire des Aliments [AFSSA], 2005; Leghait *et al.*, 2009). However, these studies did not account for species-specific fipronil hepatic metabolism or address the contribution of fipronil metabolites formed *in vivo* to the overall effect of fipronil treatment on thyroid function. Fipronil is readily metabolized into fipronil sulfone in several mammalian species such as rats, humans, livestock species, and dogs (AFSSA, 2005; Mohamed *et al.*, 2004). This main fipronil metabolic pathway is mediated by hepatic cytochromes P450 (CYP) enzymes, and, fipronil, as many other xenobiotics, can lead to increased CYP expression and activity (Das *et al.*, 2006; Leghait *et al.*, 2009).

In rats, *per os* (po) treatment with fipronil is associated with a high plasma concentration of fipronil sulfone concomitant with a marked increase in the clearance of free and total thyroxine (T_4) (Leghait *et al.*, 2009). This results, at least in part, from an increase in the activity of one type of phase II hepatic enzyme potentially involved in T_4 elimination, the uridine 5′-diphospho (UDP)-glucuronosyltransferases (UGT). The fact that thyroid disruption in the rat is associated with high plasma fipronil sulfone concentrations but with very low plasma fipronil concentrations led us to postulate that the metabolite fipronil sulfone might be responsible for the fipronil-induced thyroid disruption rather than fipronil itself. In agreement with this hypothesis, fipronil-induced thyroid disruption is much more limited in sheep, a species in which fipronil treatment results in a much lower plasma fipronil sulfone concentration concomitant with a much higher plasma concentration of fipronil than in the rat (Leghait *et al.*, 2009, 2010). Thus, the biotransformation of fipronil into fipronil

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sulfone by hepatic CYP enzymes could be a determining factor in fipronil-induced thyroid disruption. Such a hypothesis is supported by *in vitro* data on human hepatocytes indicating that fipronil sulfone is more cytotoxic to hepatocytes and a greater inducer of hepatic enzymes than fipronil itself (Das *et al.*, 2006).

Data in rats and sheep suggest that fipronil sulfone persists much longer in the organism than fipronil itself. For example, in rats, the terminal half-lives were estimated to be 208 and 8.5 h for fipronil sulfone and fipronil, respectively (Mohamed *et al.*, 2004). In sheep, after fipronil treatment, the plasma fipronil sulfone concentration appeared to decrease more slowly than plasma fipronil, so that it remained detectable at high levels long after plasma fipronil became undetectable (Leghait *et al.*, 2010). In humans, data obtained from observations made on acute self-poisoning indicate that fipronil is transformed into fipronil sulfone and disappears rapidly from the blood over the first 15–20 h after ingestion (Mohamed *et al.*, 2004). Because thyroid function is mainly involved in long-term developmental processes, the consequences of an interference with thyroid function on living organisms are more likely to be deleterious if the disruption is sustained for a long period of time. From this standpoint, if the potential of fipronil sulfone as a thyroid disruptor was confirmed, because of its ability to persist much longer in organisms, fipronil sulfone would be of much more concern than fipronil itself.

The goal of this study was to evaluate if treatment with fipronil sulfone, which leads to similar fipronil sulfone internal exposure as with fipronil without a potential effect of the parent fipronil before its biotransformation, has the same effects as fipronil treatment on (1) T_4 clearance and (2) CYP induction. In addition, we sought to gain more insight on which metabolic pathways of thyroid hormones might be triggered by fipronil and/or its metabolite by determining the metabolic profiles of thyroid hormones and quantifying phase I and phase II hepatic enzymes messenger RNA (mRNA) expression and activity.

MATERIALS AND METHODS

Animal Housing, Surgery, Blood, and Tissue Samples

Animal Housing

Experiments were performed on adult female Wistar rats (Wistar HsdHan: WIST Outbred, 225 g body weight) purchased from Harlan (Gannat, France). The rats were housed at two to four per cage at room temperature (21°C ± 1°) with a reversed 12-h light/dark cycle (lights off at 9:00 A.M.). They were acclimatized for at least 2 weeks before surgery. The rats had free access to food (2016 Teklad Diet; Harlan) and tap water and were weighted twice a week. All animal procedures were carried out in accordance with the accepted standards of humane animal care under the agreement number 31-242 for animal experimentation from the French Ministry of Agriculture.

Surgery

Catheters were surgically inserted into one femoral vein under isoflurane anesthesia (AErrane, Baxter SA, Maurepas, France, 2–2.5% isoflurane in O_2) for serial blood sampling at least 2 days before pharmacokinetic investigations. For experiments 2 and 3 (Fig. 1), the animals had been previously thyroidectomized (THX) 1 week before the beginning of fipronil or fipronil sulfone treatments under isoflurane anesthesia. They were treated with daily triiodothyronine (T_3) sc injections at a dose (10 μg/kg/day) calculated to restore T_3 plasma concentrations at steady state close to the physiological ones (Leghait *et al.*, 2009) thus insuring the maintenance of a euthyroid-like physiological status. This model had previously been developed and validated in our laboratory for pharmacokinetic investigations on thyroid hormones in rat (Leghait *et al.*, 2009). Briefly, endogenous T_4 production was suppressed in our model by surgical ablation of the thyroid gland to allow for control of the rate of T_4 entrance into the organism. It is noteworthy that in contrast to immunolabeled thyroxine–based methods (Soldin *et al.*, 2010), our model is the only physiologically relevant one allowing the total T_4 clearance and the free T_4 clearance to be evaluated at the same time *in vivo*. This is important as our previous studies have shown that the clearance of the free T_4 might be an earlier and more sensitive biomarker for metabolism-driven thyroid disruption in different species (Leghait *et al.*, 2009, 2010). Following thyroidectomy, rats were treated with T_3, the bioactive form of thyroid hormones, to ensure a euthyroid-like state compatible with the maintenance of normal hepatic enzyme activities dependent upon the supply of thyroid hormone to the liver (Liddle *et al.*, 1998; Masmoudi *et al.*, 1997).

Blood and Tissue Sampling

After each blood sample (150–200 μl), a volume of physiological saline equivalent to the collected blood volume was administered, followed by 150 μl of heparinized saline (Heparine choay, 50 UI/ml, Sanofi-Aventis, France). Blood samples were centrifuged at 4000 × g for 15 min at + 4°C and the plasma stored at −20°C until assay. For experiments 2 and 3, after the last blood sample, rats were deeply anesthetized with isoflurane, and total blood was collected from the catheter in heparinized tubes for T_3, fipronil, and fipronil sulfone assays. The livers were infused via the catheter or through the posterior vena cava with sterile isotonic NaCl (B Braun Medical, Boulogne, France; 15 ml/min, 20 ml per liver), rapidly frozen in liquid nitrogen, and stored at −80°C until the preparation of hepatic microsomal and cytosolic fractions.

Test Materials and Chemicals

Fipronil (lot B20050318, purity estimated by the supplier: 95.6%) was purchased from 3B Medical Systems Inc. (Libertyville, IL). Fipronil sulfone for experiment 1 (lot 429-35B, purity estimated by the supplier: 99.3%) was purchased from Crescent Chemical (Islandia, NY). Fipronil sulfone for experiments 2 and 3 was obtained by chemical synthesis from fipronil. Briefly, 3-chloroperbenzoic acid (2.024 g, 11.7 mmol; Sigma-Aldrich, Saint-Quentin Fallavier, France) was added to fipronil (1.684 g, 3.86 mmol) in dichloromethane (40 ml). The white suspension obtained was stirred at room temperature for 3 days. The organic phase was washed successively with 0.2M sodium hydroxide (20 ml, twice), water (20 ml), and sodium chloride saturated aqueous solution (20 ml). After drying with magnesium sulfate, the solvent was evaporated, and dichloromethane was added to the crude product. Fipronil sulfone precipitated as a white solid (1.245 g, 71% yield). The melting point of the powder obtained was 218°C–219°C, corresponding to the known fipronil sulfone melting point (219–221.5°C, Buntain *et al.*, 1988), whereas the fipronil melting point is 195.5–203°C (Connelly, 2001). The purity of fipronil sulfone was evaluated by mass spectrometry (MS). The analysis revealed the parent ion $[M\text{-}H]^-$ at $m/z = 451$ with its typical isotopic abundance at $[M\text{-}H + 2]^-$ and $[M\text{-}H + 4]^-$ linked to the two chlorides. No peak at the m/z transition of 435 could be observed on the spectra confirming that no residual fipronil remained in the preparation.

For iv administrations (experiment 1), the fipronil and fipronil sulfone solutions were obtained by dilution of fipronil or fipronil sulfone in an ethanol/pyrogen-free water solution (vol/vol) to produce a final concentration of 2 mg/ml for fipronil and fipronil sulfone. For administrations po (experiments 1, 2, and 3),

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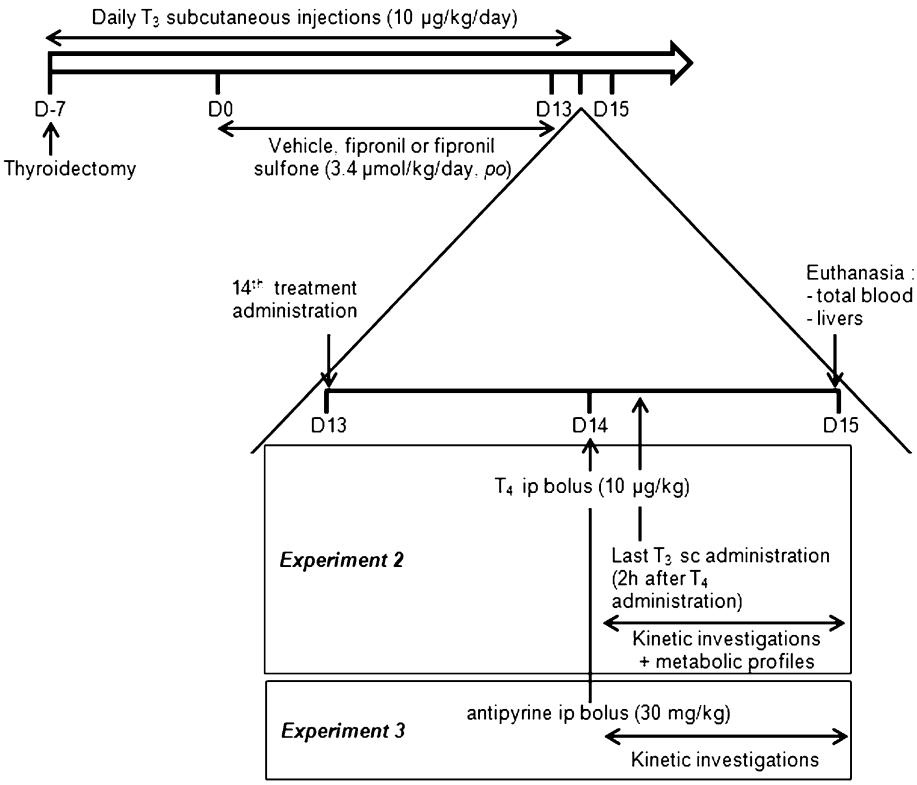

FIG. 1. Experimental designs of experiments 2 (effect of fipronil and fipronil sulfone on T_4 clearance) and 3 (effect of fipronil and fipronil sulfone on CYP induction). Thyroidectomized rats received daily injections of T_3 throughout the experiments. From days 0 to 13, they were treated per os with vehicle, fipronil (3.4 µmol/kg/day), or fipronil sulfone (3.4 µmol/kg/day). At day 14, the rats received an ip bolus of T_4 (10 µg/kg, experiment 2) or antipyrine (30 mg/kg, experiment 3). Total blood and livers were collected after euthanasia to determine T_3 blood concentrations and liver enzyme activity and mRNA expression.

concentrated solutions of fipronil and fipronil sulfone were first prepared in ethanol (0.14 mg/µl). The solutions for administration were obtained by dilution of the concentrated solutions in corn oil to produce final concentrations of 1.50 and 1.55 mg/ml for fipronil and fipronil sulfone, respectively, ensuring that fipronil and fipronil sulfone solutions had the same molar concentration (3.4 µmol/ml). The vehicle in experiments 2 and 3 consisted of a corn oil/ethanol mix. The fipronil and fipronil sulfone solutions were stored protected from light.

For experiment 2, concentrated solutions (1 mg/ml) of T_3 and T_4 (triiodothyronine and L-thyroxine; Sigma-Aldrich) were obtained by dissolving T_3 or T_4 in 0.4M NaOH in 60% ethanol. The final T_3 solution (10 µg/ml) used for sc injections was obtained by a 100-fold dilution of the concentrated solution in saline containing 49.4 mg/ml of sodium bicarbonate. The T_4 solution used for the kinetic experiment (10 µg/ml) was prepared by a 100-fold dilution of the concentrated solution in 10mM PBS pH 7.4 containing 0.1% (wt/vol) bovine serum albumin (BSA). For experiment 3, antipyrine (Sigma-Aldrich) was dissolved in sterile water at a final concentration of 30 mg/ml. The administered volumes were always adjusted to the most recently recorded body weight.

Experimental Schedules

Experiment 1: Determination of the Rates of Absorption of Fipronil and Fipronil Sulfone and of Fipronil Biotransformation into Fipronil Sulfone

To establish the fipronil and fipronil sulfone dosing regimen required to reach similar fipronil sulfone plasma concentrations in experiments 2 and 3 after fipronil or fipronil sulfone treatment po, we determined the fipronil sulfone absorption rate and the rate of fipronil biotransformation into fipronil sulfone. This experiment was carried out on 16 adult female rats (mean body weights ± SD = 218 ± 23 g). The animals were allocated to fipronil- or fipronil sulfone–treated groups ($n = 8$ each). Three rats of each groups received an iv bolus of fipronil (6.9 µmol/kg) or fipronil sulfone (6.6 µmol/kg), and five rats of each group received a po bolus of fipronil (3.4 µmol/kg) or fipronil sulfone (3.4 µmol/kg) through feeding needles. Five rats could not be used until the end of the experiment due to a loss of the catheter function. To monitor the time course of fipronil and fipronil sulfone plasma concentrations, blood (150 µl) was collected in heparinized tubes the day before and 0.08, 0.5, 2, 4, 8, 24, 96, and 144 h after fipronil ($n = 2$) or fipronil sulfone ($n = 2$) iv administrations and the day before and 1, 2, 6, 10, 24, 34, 48, 120, 168, and 216 h after fipronil ($n = 3$) or fipronil sulfone ($n = 4$) po administrations.

Experiment 2: Effect of Fipronil and Fipronil Sulfone on T_4 Clearance

This experiment was carried out on 39 T_3-treated thyroidectomized (THX + T_3) adult female rats. The animals were allocated to vehicle, fipronil-, or fipronil sulfone–treated groups ($n = 13$ each) and received a daily po dose of vehicle, fipronil (3.4 µmol/kg/day), or fipronil sulfone (3.4 µmol/kg/day) through gavage tubes for 14 days. This dose of fipronil was within the range of doses that have been shown to be associated to a thyroid disruption in rats in previous studies (AFSSA, 2005; Leghait *et al.*, 2009). Nine rats could not be used in the study because of complication during the surgical procedure or loss of the catheter function. Twenty-four hours after the last administration, all of the rats received an ip bolus of T_4 (10 µg/kg, $n = 9$, $n = 10$, and $n = 11$ for the vehicle control, fipronil-, and fipronil sulfone–treated groups, respectively) leading to physiologically relevant maximal T_4 plasma concentrations in rats (Leghait *et al.*, 2009).

Blood (200 µl) was collected in heparinized tubes the day before and 0.25, 1, 2, 4, 8, 12, and 24 h after T_4 administration to monitor the time course of

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plasma T_4 concentrations. Livers were collected 48 h after the last vehicle, fipronil, or fipronil sulfone administration to determine the effect of fipronil and fipronil sulfone on UGT1A and phenol SULT activities and on *Ugt1a1* and *Sult1b1* mRNA expressions. Plasma fipronil and fipronil sulfone concentrations were determined 22.5 ± 0.9 h after the last fipronil or fipronil sulfone administration. Total plasma T_3 concentrations were determined at euthanasia, 23.6 ± 0.5 h after the last T_3 sc injection and 25.7 ± 0.1 h after the T_4 ip administration. The experimental design of experiment 2 is shown on Figure 1.

Experiment 3: Effect of Fipronil and Fipronil Sulfone on CYP Induction

This experiment was carried out on 21 THX + T_3 adult female rats. The animals were allocated to vehicle control, fipronil-, or fipronil sulfone–treated groups ($n = 7$ each) and treated as described in experiment 2. Six rats could not be used for the study because of complication during the surgical procedure or loss of the catheter function. Twenty-four hours after the last administration, all of the rats received an ip bolus of antipyrine (30 mg/kg, $n = 5$ for vehicle, $n = 4$ for fipronil-treated, and $n = 6$ for fipronil sulfone–treated groups), an acknowledged surrogate to test the oxidative capacity of the liver *in vivo* (Tanaka *et al.*, 1985) and more specifically the activity of CYP enzymes (Matthew and Houston, 1990).

Blood (200 µl) was collected in heparinized tubes the day before and 0.5, 1, 2, 3, 5, 7, and 9 h after antipyrine administration to monitor the time course of plasma antipyrine concentrations. Livers were collected 48 h after the last vehicle, fipronil, or fipronil sulfone administration to study the effect of fipronil and fipronil sulfone on CYP3A protein expression and on *Cyp1a1*, *Cyp1a2*, *Cyp2b2*, *Cyp3a1*, and *Cyp3a2* mRNA expressions. Plasma fipronil and fipronil sulfone concentrations were determined 33.4 ± 2.0 h after the last fipronil or fipronil sulfone administration. The experimental design of experiment 3 is shown in Figure 1.

Assays

Fipronil and Fipronil Sulfone Assay

For the iv administration in experiment 1 and the po administrations in experiments 2 and 3, plasma fipronil and fipronil sulfone concentrations were determined by high-performance liquid chromatography (HPLC) coupled with an ultraviolet (UV)/MS detection method as previously described (Lacroix *et al.*, 2010). For the po administration in experiment 1, the extraction method was the same as that above but fipronil and fipronil sulfone plasma concentrations were assayed by liquid chromatography (LC)/MS with an Acquity ultra performance liquid chromatography (UPLC) coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA). The mean within and between day precision was below 12.0% for both fipronil (three different Quality Control [QC] pools) and fipronil sulfone (five different QC pools) for both methods. The limit of quantification (LOQ) was 2.5 ng/ml for both fipronil and fipronil sulfone. The between-method variability, estimated through the determination of the mean between-method precision of the same QC pools assayed with both methods, was 16.0%.

Subcellular Fractionation and Protein Assays

The preparation of hepatic microsomal fractions was carried out as previously described (Zalko *et al.*, 2006). Supernatants obtained after centrifugation of the S9 fractions were kept as cytosolic fractions. The protein content of the microsomal and the cytosolic fractions was determined in experiment 2 and 3 by the method of Bradford (1976) with BSA as a standard.

Experiment 2: effect of fipronil and fipronil sulfone on T_4 clearance.

Thyroid Hormone Assays. Total and free plasma T_4 (TT_4 and FT_4) and total plasma T_3 (TT_3) concentrations were determined using radioimmunoassay kits from Siemens (Coat-a-count kits, Siemens Healthcare Diagnostics, Los Angeles, CA). All assays were performed according to the manufacturer's instructions. The mean intra-assay coefficient of variation for three QC pools was 8.1 and 11.6% for TT_4 and FT_4, respectively, and the mean inter-assay coefficient of variation for these three QC pools was 9.4 and 11.7% for TT_4 and FT_4, respectively. All the T_3 samples were quantified in the same run and the mean intra-assay coefficient of variation for three QC pools was 10.4%. The limits of quantification for the total T_4 and total T_3 assays were validated at 5 and 0.1 ng/ml, respectively. The limit of detection of the free T_4 assay was set at the lowest value of the standard curve, i.e., 1 pg/ml.

Thyroid Hormone Metabolic Profiles. In an attempt to determine if metabolic pathways other than conjugation might have been involved in the increased T_4 clearance, we characterized the profile of thyroid hormone metabolites in plasma during the T_4 kinetic experiment. The remaining plasma samples from experiment 2 were pooled by treatment to obtain two different pools for each time point. T_4, T_3, T_2 (diiodothyronine), and rT_3 (reverse triiodothyronine) were assayed by LC/MS with an Acquity UPLC coupled to a Xevo triple quadrupole mass spectrometer (Waters). Thyroid hormones samples spiked with T_4-d_5 as an internal standard were purified by solid-phase extraction with Strata X-C cartridges (Phenomenex, LePecq, France). The extracted samples were separated on an Acquity BEH RPC18 column (2.1 × 100 mm, 1.7 µm; Waters) with an acidified (0.05% formic acid) H_2O/AcN gradient elution. Samples were ionized in positive electrospray ionization mode (ESI^+) and detected with the following multiple reaction monitoring transitions : T_4 m/z 778 < 605; T_3 and rT_3 m/z 652 < 606; T_2 m/z 526 < 480; and T_4-d_5 m/z 783 < 201. The calibration curves ranged from 5 to 100 ng/ml for T_4, from 0.2 to 100 ng/ml for 3′,5′-T_2 and T_3 and from 1 to 100 ng/ml for rT_3. The selectivity of the method for the T_2 isomers 3,3′-diiodothyronine and 3′,5′-diiodothyronine was not evaluated.

Determination of *Ugt1a1* and *Sult1b1* mRNA Expression by RT-qPCR. In the liver of female rats, UDP-glucuronosyltransferase 1A1 (UGT1A1) and sulfotransferase 1B1 (SULT1B1) are key isoenzymes for the conjugation of T_4 (Kester *et al.*, 2003; Wu *et al.*, 2005). We, thus, focused the investigation of mRNA expression primarily on these two enzymes. Total hepatic RNAs were extracted with TRIzol reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's protocol, and 2 µg were reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). RT-qPCR was performed using the SYBR Green TaqMan Universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France). Fluorescence was monitored and analyzed on a 7300 Real-Time PCR detection system instrument (Applied Biosystems). RT-qPCR data were normalized by TATA box–binding protein (*Tbp*) mRNA levels. Results are expressed as $\frac{2^{(Ct_{Tbp} - Ct_{gene})}{}_{\text{sample}}}{\text{mean}[2^{(Ct_{Tbp} - Ct_{gene})}]_{\text{control group}}}$, where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold. All the primers used (Table 1) were validated for RT-qPCR efficiency.

UGT1A Activity Toward 4-Nitrophenol. To verify that the increase in *Ugt* mRNA expression correlated with an increase in UGT enzymes activity, we determined UGT1A activity toward 4-nitrophenol. This enzyme activity can be considered as a relevant index for the capacity of the liver to glucuronoconjugate thyroid hormones. Indeed, UGT isoforms involved in T_4 glucuronidation are the bilirubin and phenol UGT1A (Visser *et al.*, 1993), which catalyze the conjugation of simple phenols like 4-nitrophenol (Peters *et al.*, 2003). UGT activity toward 4-nitrophenol was assayed by an optimization of the method of Burchell and Weatherill (1981) on microsomal fractions as previously described (Leghait *et al.*, 2009) with a substrate (4-nitrophenol) concentration of 4mM.

Phenol SULT Activity Toward 2-Naphthol. To verify whether the increase in *Sult* mRNA expression correlated with an increase in the activity of SULT enzymes, we determined phenol SULT activity toward 2-naphthol. This enzyme activity can be considered as a relevant index for the capacity of the liver to sulfoconjugate thyroid hormones as both the sulfoconjugation of thyroid hormones and 2-naphthol are carried out by phenol sulfotransferases (Gamage *et al.*, 2006). SULT activity toward 2-naphthol was assayed by optimizing the method of Sekura *et al.* (1981) as modified by Sheng *et al.* (2001). Briefly, the 2-naphthol substrate (0.25mM) and the 3′-phosphoadenosine 5′-phosphosulfate cofactor (0.2mM) were incubated at 37°C with 220 µg of cytosolic proteins in a sodium acetate buffer (0.5M, pH 5.5). The reaction was stopped after 10 min with sulfuric acid containing methylene blue (final concentrations in the mix of

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TABLE 1
Oligonucleotide Sequences for RT-qPCR

Gene	NCBI RefSeq	Forward primer (5′–3′)	Reverse primer (5′–3′)
Tbp	NM_001004198	CCACACCAGCCTCTGAGAGC	AGCCAAGATTCACGGTGGAT
Ugt1a1	NM_012683	CTTCAGAAAAAAGCCCTATCCCA	CCAAAGAGAAAACCACGATGC
Sult1b1	NM_022513	GAACAAAATGTTCCTGGAGCAAG	TGATGGAGTTTTCTTCAAGAGTTCAA
Cyp1a1	NM_012540	CAGTCCTTCCTCACAGCCAAA	AGGCTGGGAATCCATACACAGA
Cyp1a2	NM_012541	TGAGGACTCAGTCCTTCCCTCA	GGACAACTTGTAAGGATCAGCTCTCT
Cyp2b2	XM_001062335	CTCCGCTATGGTTTCCTGCT	AGGGATGGTGGCCTGTGA
Cyp3a1	NM_013105	TTCTGCAAAACTTCTCCTTCCAG	CTTTAGAATAATGGGTTTTGTTGGTTG
Cyp3a2	NM_153312	TTCTCCTTCCAGCCTTGCA	CAATAAAATTCCTTGAGGGAAAGTCAG

0.1mM and 0.15nM, respectively). Methylene blue forms paired ions with the 2-naphtylsulfate formed by the sulfoconjugation reaction. The resulting sulfuric acid ester was extracted with chloroform and was quantified by reading the absorbance at 651 nm. The SULT activity was calculated as the nanomoles of 2-naphtylsulfate formed per time unit and milligram of hepatic cytosolic proteins.

Experiment 3: Effect of fipronil and fipronil sulfone on CYP induction. Antipyrine Assay. Plasma antipyrine concentrations were determined by HPLC coupled with a UV detection method as previously described (Leghait *et al.*, 2009). The mean within-day precision for three QC pools was 4.3%, and the mean between-day precision for these three QC pools was 6.4%. The LOQ was validated at 0.3 μg/ml.

Determination of *Cyp1a1*, *Cyp1a2*, *Cyp2b2*, *Cyp3a1*, and *Cyp3a2* mRNA Expression by RT-qPCR. Human CYP1A1, CYP1A2, CYP2B6, and CYP3A4 are responsible for fipronil metabolism (Das *et al.*, 2006; Tang *et al.*, 2004) and/or involved in antipyrine metabolism (Engel *et al.*, 1996). Thus, we determined the mRNA expression of the corresponding rat orthologs *Cyp1a1*, *Cyp1a2*, *Cyp2b2*, *Cyp3a1*, and *Cyp3a2* by RT-qPCR in vehicle, fipronil-, and fipronil sulfone–treated groups. RT-qPCR were performed on those mRNA as described above. All the primers used (Table 1) were validated for RT-qPCR efficiency.

CYP3A Protein Expression by Western Blot. As CYP3A4 is the predominant isoform responsible for the *S*-oxidation of fipronil in humans (Tang *et al.*, 2004), we determined if the increase in mRNA expression as a result of fipronil and/or fipronil sulfone treatment was associated with increased protein expression of the equivalent orthologs in rats. Western blot analyses were done with 10 μg of heat-denaturated microsomal proteins. The electrophoresis was run on precast gels (80V, 30 min; 150V, 90 min), and the proteins were transferred onto polyvinylidene fluoride membranes (120V, 60 min). After blocking for nonspecific binding (120 min), the membranes were incubated for 60 min with a mouse monoclonal anti-human CYP3A primary antibody (Santa Cruz Biotechnology Inc, Heidelberg, Germany). After washing, the membranes were incubated for 40 min with a goat anti-mouse secondary antibody coupled to an infrared fluorophore (goat anti-mouse 770 CW IgG, Biotium, Interchim, Montluçon, France). After washing, the staining was quantified with an infrared imaging system (Odyssey infrared imaging system, Licor Biosciences, Les Ulis, France).

A molecular weight marker and three reference pools loaded at 2, 6, or 12 μg of proteins were repeated on all the gels. Each gel included 10 μg of microsomal proteins from two different animals of each treatment group. A calibration curve of the staining intensity versus quantity of protein was obtained through quantification of the reference pool signals and used to calculate the theoretical signal for 10 μg of protein. This value was then used to normalize the signal intensity between membranes. The relative intensity of the signal for each sample was defined as the ratio of the observed signal intensity to the calculated 10 μg theoretical signal and was expressed per gram of fresh tissue.

The specificity of the primary antibody directed to human CYP3A for rat CYP3A was checked by preincubations of the antibody mix with rat CYP3A1 and rat CYP3A2 Supersomes at different concentrations (2–80 μg; BD Biosciences, Le Pont-De-Claix, France). Preincubation with either supersomes for 5 h resulted in a marked supersome concentration–dependent decrease of immunoreactivity in microsomal fractions thus attesting that the signal obtained in our microsomal pool was specific for the rat CYP3A family. The specificity of the secondary antibody was checked by omitting the primary antibody and verifying that no signal above the background could be detected.

Pharmacokinetic Analysis

The pharmacokinetic analyses for experiment 1 were performed by noncompartmental analysis. AUCs (Areas under the plasma concentration time curves) for fipronil and fipronil sulfone after fipronil or fipronil sulfone iv or po administrations were determined using the log-linear trapezoidal rule. The fraction f_{po} of fipronil that was not transformed by first-pass metabolism into fipronil sulfone was calculated according to $f_{po} = \frac{AUC^{po}_{fipronil} / AUC^{iv}_{fipronil}}{AUC^{po}_{fipronil\ sulfone} / AUC^{iv}_{fipronil\ sulfone}}$, where AUCs for fipronil and fipronil sulfone were determined after fipronil iv or po administrations (Weiss, 1988). $(1-f_{po})$ characterized the degree of first-pass formation of the metabolite as the liver is the main organ involved in fipronil elimination (AFSSA, 2005), assuming that $f_{po} = F_H$ (fraction of fipronil that escapes a single passage through the liver) and $(1-f_{po}) = E_H$ (hepatic extraction ratio). E_H is a good estimator of fipronil biotransformation into fipronil sulfone. The fractions of fipronil or fipronil sulfone absorbed, $f_{fip.abs}$ and $f_{sulf.abs}$ were defined as $f_{abs} = \frac{AUC^{po}_{fipronil\ sulfone}}{AUC^{iv}_{fipronil\ sulfone}} \times \frac{Dose_{iv}}{Dose_{po}}$, where AUCs for fipronil sulfone were determined after fipronil iv or po administrations to estimate $f_{fip.abs}$ and after fipronil sulfone iv or po administrations to estimate $f_{sulf.abs}$ (Weiss, 1990).

The pharmacokinetic analyses for experiment 2 and 3 were performed by least-squares regression analysis using WinNonlin software as previously described (Leghait *et al.*, 2009). The time course of plasma antipyrine and total and free T_4 concentrations were fitted using a biexponential equation corresponding to a one-compartment model with a first-order absorption phase. The data were weighted by the inverse of the fitted values for free T_4, but no weighting was applied to the total T_4 and antipyrine data. The terminal half-life ($t_{1/2}$), the time (T_{max}) at the maximal concentration (C_{max}), and the apparent clearance were determined.

Statistical Analysis

The effect of the treatments on the mean plasma T_3 concentrations, TT_4, FT_4 and antipyrine PK parameters, UGT and SULT activities, CYP3A protein expression, *Ugt*, *Sult*, and *Cyp* mRNA expressions, and animal and liver

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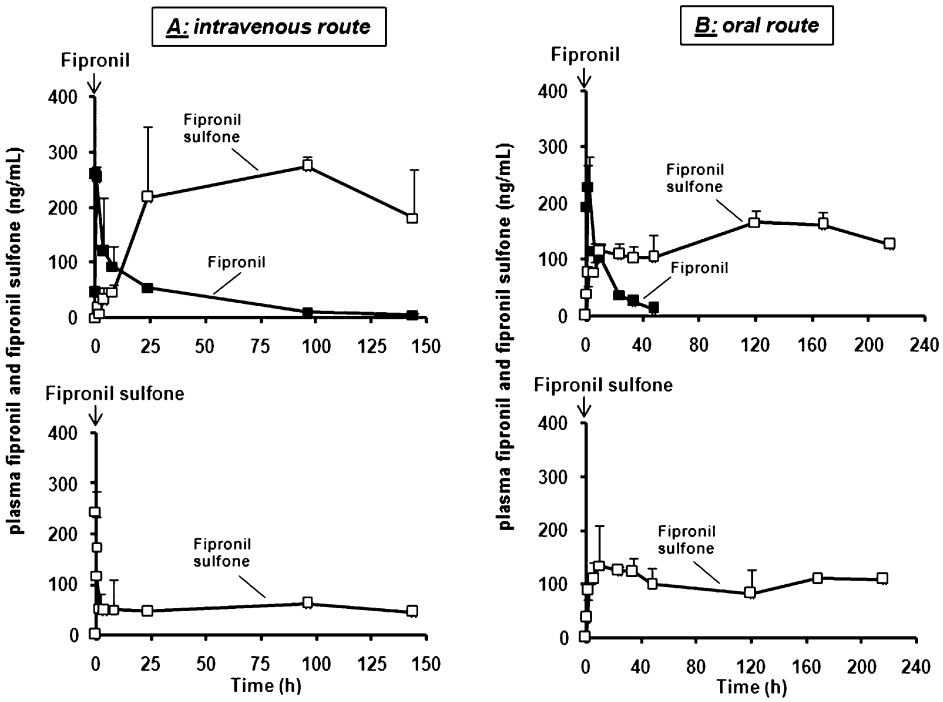

FIG. 2. Time course of mean (±SD) plasma fipronil (■) and fipronil sulfone (□) concentrations after an iv (A) or a po (B) bolus of fipronil (top graphs) or fipronil sulfone (bottom graphs). Fipronil and fipronil sulfone plasma concentrations were measured in blood samples collected the day before and 0.08, 0.25, 0.5, 2, 4, 8, 24, 96, and 144 h after fipronil or fipronil sulfone iv administrations and the day before and 1, 2, 6, 10, 24, 34, 48, 120, 168, and 216 h after fipronil or fipronil sulfone po administrations.

weights was analyzed using a one-way ANOVA with treatment (vehicle control, fipronil, fipronil sulfone) as fixed effect factor, followed by a Bonferroni post hoc comparison. These analyses were computed using R software (R 2.6.2, R Development Core Team, Vienna, Austria).

RESULTS

Experiment 1: Determination of Rates of Fipronil and Fipronil Sulfone Absorption and of Fipronil Biotransformation into Fipronil Sulfone

Figure 2 shows the time course of the mean (±SD) fipronil and fipronil sulfone plasma concentrations after fipronil or fipronil sulfone iv administration (Fig. 2A) and fipronil or fipronil sulfone po administration (Fig. 2B). The fraction of fipronil that escapes a liver first-pass effect f_{po} was 0.25 and the hepatic extraction ratio E_H was estimated at 0.75 indicating that 75% of fipronil was biotransformed into fipronil sulfone. The absorption coefficients were similar for fipronil and fipronil sulfone (f_{abs} = 0.98 and 0.96 for fipronil and fipronil sulfone, respectively), and both molecules were well absorbed after a po administration. From those results, it was clear that fipronil was highly converted into fipronil sulfone after a fipronil po administration and that the absorption rate after the po route was similar for both molecules. These results clearly indicate that equimolar treatments with either fipronil or fipronil sulfone are likely to lead to similar fipronil sulfone concentrations at steady state.

Experiments 2 and 3: Plasma Fipronil and Fipronil Sulfone Concentrations at the Time of the T_4 or Antipyrine Kinetic Investigations

The plasma fipronil and fipronil sulfone concentrations observed at the end of the two experiments are listed in Table 2. The fipronil levels were below the LOQ of the assay in the fipronil-treated groups. The ratio of fipronil sulfone to fipronil plasma concentrations could be roughly estimated by the ratio of fipronil sulfone plasma concentration to the fipronil LOQ of the assay indicating that it was at least 400 for experiments 2 and 3. As expected from the results of experiment 1, plasma fipronil sulfone concentrations were similar in the fipronil- and in the fipronil sulfone–treated groups for both experiments.

Experiment 2: Effect of Fipronil and Fipronil Sulfone on T_4 Clearance

Characterization of the euthyroid-like state. The mean (±SD) total plasma T_3 concentrations were 0.51 ± 0.16, 0.30 ± 0.07, and 0.30 ± 0.13 ng/ml for the vehicle, fipronil-, and fipronil sulfone–treated groups, respectively, 23.6 ± 0.5 h after the last T_3 sc injection and 25.7 ± 0.1 h after the T_4 ip administration. All these concentrations were within the physiological range of total T_3 concentrations in rats (Döhler *et al.*, 1979). However, a significant treatment effect ($p < 0.05$)

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TABLE 2
Mean (±SD) Plasma Fipronil and Fipronil Sulfone Concentrations in Fipronil-Treated (3.4 μmol/kg/day *per os* for 14 days) or Fipronil Sulfone–Treated (3.4 μmol/kg/day *per os* for 14 days) THX + T_3, Euthyroid-Like Rats

	Experiment 2, 22.5 ± 0.9 h after the last fipronil or fipronil sulfone administration, $n = 21$		Experiment 3, 33.4 ± 0.2 h after the last fipronil or fipronil sulfone administration, $n = 10$	
	Fipronil group, $n = 10$	Sulfone group, $n = 11$	Fipronil group, $n = 4$	Sulfone group, $n = 6$
Plasma fipronil (ng/ml)	< LOQ	< LOQ	< LOQ	< LOQ
Plasma fipronil sulfone (ng/ml)	1502 ± 394	1680 ± 557	1214 ± 547	1285 ± 244

Note. LOQ (2.5 ng/ml for fipronil).

was observed. Fipronil and fipronil sulfone treatments produced a significant 1.7-fold decrease in total plasma T_3 concentrations ($p < 0.05$), and there was no significant difference in total plasma T_3 concentrations between the two treated groups ($p > 0.05$).

Effect of treatments on body and liver weights. The mean body weights (±SD) were similar at the beginning (236 ± 13, 237 ± 11, and 240 ± 12 g) and at the end (239 ± 20, 240 ± 9, and 246 ± 10 g) of the experiment for the vehicle, fipronil-, and fipronil sulfone–treated groups, respectively. The mean (±SD) liver weights expressed as a percentage of body weight were 3.18 ± 0.29%, 4.18 ± 0.54%, and 3.83 ± 0.55% at the end of the experiment for the vehicle, fipronil-, and fipronil sulfone–treated groups, respectively, indicating a significant 31.5% and 20.4% increase in liver weight in the fipronil- and fipronil sulfone–treated groups ($p < 0.01$).

Effect of treatments on total and free T_4 pharmacokinetic parameters. T_4 was undetectable in blood samples collected before T_4 administration, confirming the T_4-free status of the THX + T_3 rats. Figure 3 shows the time course of the mean (±SD) total (Fig. 3A, left) and free (Fig. 3B, left) plasma T_4 concentrations and the resulting apparent clearances for total (Fig. 3A, right) and free (Fig. 3B, right) T_4. The mean total and free plasma T_4 concentrations were higher in the vehicle control group than in the treated groups at all time points. The maximum total T_4 concentration for the control group was within the physiological range for rats (Topo *et al.*, 2010). The apparent clearances of total and free T_4 were about 2.5- and 2.8-fold higher in the two treated groups than in the vehicle group. The other total and free T_4 pharmacokinetic parameters are presented in Table 3. There was no significant difference in TT_4 and FT_4 pharmacokinetic parameters between the fipronil- and fipronil sulfone–treated groups ($p > 0.05$). For total T_4, the

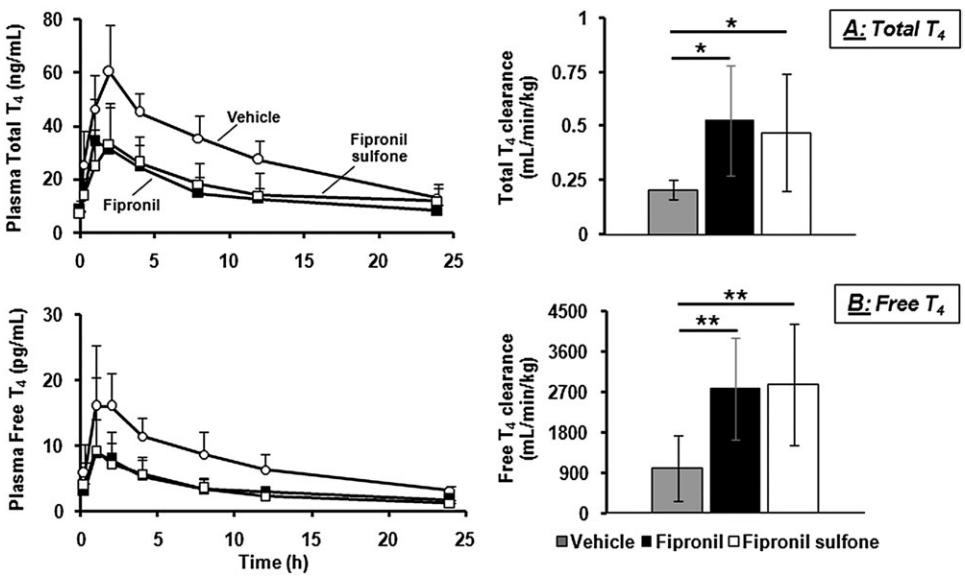

FIG. 3. Time course of mean (±SD) plasma total T_4 (A, left) and free T_4 (B, left) concentrations following an ip T_4 bolus (10 μg/kg) to vehicle ($n = 9$, ○), fipronil-treated (3.4 μmol/kg/day *per os* for 14 days, $n = 10$, ■), and fipronil sulfone–treated (3.4 μmol/kg/day *per os* for 14 days, $n = 11$, □) THX + T_3, euthyroid-like rats. Total and free T_4 concentrations were measured in blood samples collected before and at 0.25, 1, 2, 4, 8, 12, and 24 h after the T_4 bolus. The bar graphs represent mean (±SD) total T_4 (A, right) and free T_4 (B, right) clearances in vehicle control (gray bars), fipronil-treated (black bars), and fipronil sulfone–treated (white bars) groups. Significantly different from control: $*p < 0.05$, $**p < 0.01$.

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TABLE 3
Mean (±SD) Total and Free T_4 Pharmacokinetic Parameters in Vehicle Control (n = 9), Fipronil-Treated (3.4 μmol/kg/day *per os* for 14 days, n = 10), or Fipronil Sulfone–Treated (3.4 μmol/kg/day *per os* for 14 days, n = 11) THX + T_3, Euthyroid-Like Rats

	Vehicle, (n = 9)	Fipronil, (n = 10)	Fipronil sulfone, (n = 11)
Total T_4			
$t_{1/2}$ (h)	9.5 ± 4.1	8.0 ± 5.4	8.6 ± 4.5
T_{max} (h)	1.8 ± 0.5	1.6 ± 0.4	2.0 ± 1.0
C_{max} (ng/ml)	56.5 ± 12.6	32.1 ± 13.8 *	32.3 ± 13.4 *
Free T_4			
$t_{1/2}$ (h)	8.1 ± 3.5	4.7 ± 2.0 *	4.5 ± 1.9 *
T_{max} (h)	2.0 ± 0.5	1.8 ± 0.4	2.0 ± 0.9
C_{max} (pg/ml)	15.5 ± 5.3	7.9 ± 2.3 *	8.7 ± 5.6 *

Notes. Twenty-four hours after the last vehicle, fipronil, or fipronil sulfone administration, total and free T_4 were assayed in blood samples collected before and at 0.25, 1, 2, 4, 8, 12, and 24 h after a T_4 ip administration (10 μg/kg). Total and free T_4 parameters were calculated using a one-compartment model for extravascular administration with first-order absorption and elimination phases. Significantly different from control: *$p < 0.05$.

C_{max} was significantly lower in the fipronil- and fipronil sulfone–treated groups than in the vehicle group, while the half-life and T_{max} were unaffected by the treatment. For free T_4, the C_{max} and T_4 half-life were significantly lower in fipronil- and fipronil sulfone–treated groups than in the vehicle control group, while the T_{max} was unaffected by the treatment.

Effect of treatments on T_4 metabolic profiles. Figure 4 shows the time course of the mean (±SD) T_4 (A), T_3 (B), and T_2 (C) plasma concentrations in plasma pooled by treatment (two different pools for each time point) following T_4 administration. rT_3 plasma concentrations were below the LOQ of the assay. Overall, those results on total T_4 concentrations were consistent with results obtained by assaying T_4 in individual rats by radioimmunoassay. T_4 plasma concentrations for the vehicle pool were higher than for fipronil- and fipronil sulfone–treated pools at all time points. T_3 plasma concentrations rose well above the physiological range just after the T_3 sc injection but decreased rapidly to physiological levels for all pools. However, plasma T_3 concentrations appeared to be higher for the vehicle pool for each time point. The plasma concentrations of T_2 did not appear to vary over time even after the T_4 or T_3 administration. Interestingly, plasma T_2 concentrations were higher for the vehicle pool than for the fipronil- and fipronil sulfone–treated ones at all time points.

Effect of treatments on Ugt1a1 and Sult1b1 mRNA expressions and on UGT1A and phenol SULT activities. The bar graphs on the left of Figure 5 represent the mRNA expressions for *Ugt1a1* (Fig. 5A, left) and *Sult1b1* (Fig. 5B, left). Significant 3.1- and 4-fold increases were recorded in *Ugt1a1* mRNA expression in the fipronil- and fipronil sulfone–treated groups, respectively ($p < 0.01$). Similarly, *Sult1b1* mRNA expression showed significant 2.5- and 2.9-fold increases in the fipronil- and fipronil sulfone–treated groups, respectively ($p < 0.01$).

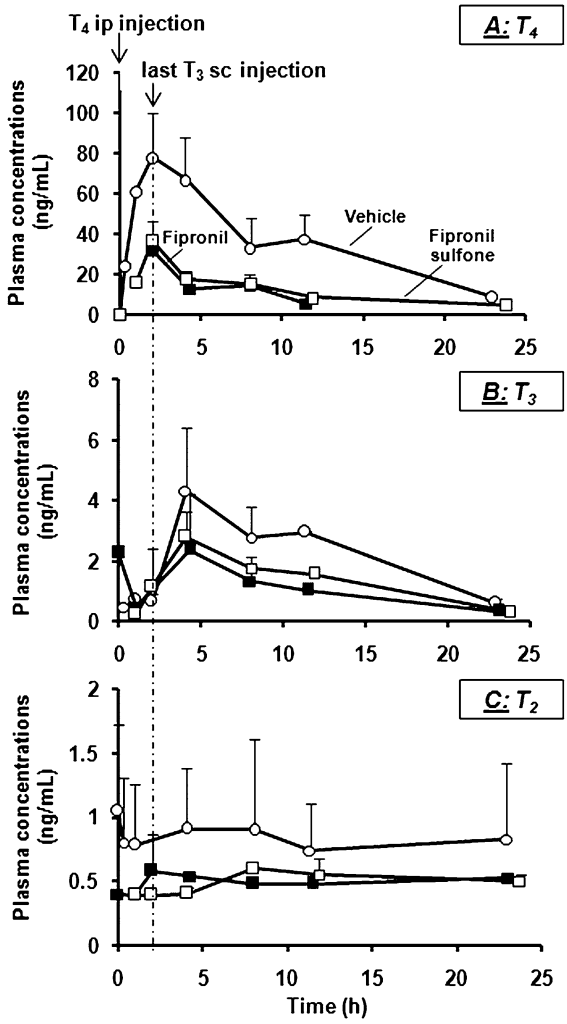

FIG. 4. Time course of mean (±SD) plasma T_4 (A), T_3 (B), and T_2 (C) concentrations following an ip T_4 bolus (10 μg/kg) to vehicle control (○), fipronil-treated (3.4 μmol/kg/day *per os* for 14 days, ■), and fipronil sulfone–treated (3.4 μmol/kg/day *per os* for 14 days, □) THX + T_3, euthyroid-like rats. Blood samples collected for T_4 kinetic investigations were pooled by treatment to obtain two pools for each treatment (n = 4 to 6 animals by pool) and time point (before and at 0.25, 1, 2, 4, 8, 12 and 24 h after the T_4 bolus). Values are mean (±SD) of plasma concentrations of the two pools for each point.

The bar graphs on the right of Figure 5 show the mean (±SD) UGT1A activity toward 4-nitrophenol (Fig. 5A, right) and the mean (±SD) phenol SULT activity toward 2-naphthol (Fig. 5B, right). UGT1A activity was significantly higher in the fipronil- and fipronil sulfone–treated groups ($p < 0.01$) with a 1.9- and 2.1-fold increases as compared with the vehicle group. Surprisingly, none of the treatments had an effect on SULT activity toward 2-naphthol ($p > 0.05$).

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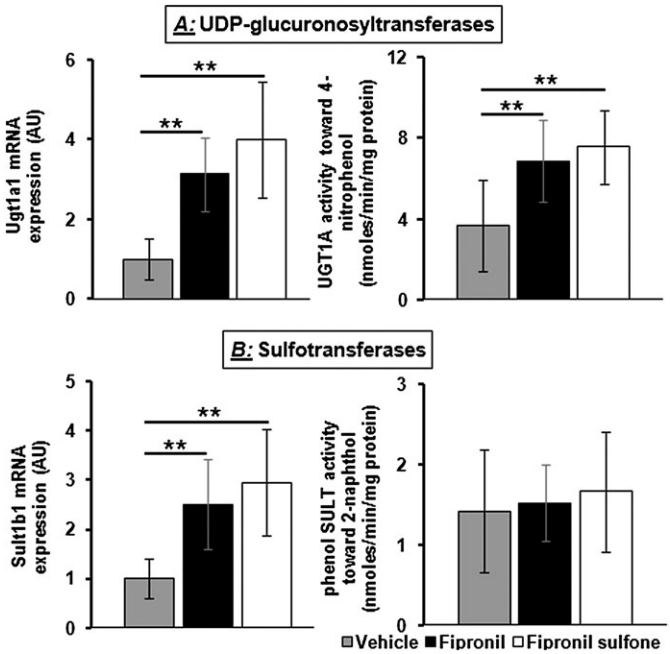

FIG. 5. Mean (±SD) mRNA expression (left) of *Ugt1a1* (A) and *Sult1b1* (B) and hepatic activities (right) of microsomal UGT1A (A) and cytosolic phenol SULT (B) in vehicle ($n = 9$, gray bars), fipronil-treated (3.4 μmol/kg/day *per os* for 14 days, $n = 10$, black bars), or fipronil sulfone–treated (3.4 μmol/kg/day *per os* for 14 days, $n = 11$, white bars) THX + T_3, euthyroid-like rats. Significantly different from control: $^{**}p < 0.01$.

Experiment 3: Effect of Fipronil and Fipronil Sulfone on CYP Induction

Effect of treatments on body and liver weights. The mean body weights (±SD) of the rats were similar at the beginning (237 ± 6, 241 ± 12, and 243 ± 17 g) and at the end (243 ± 9, 244 ± 9, and 244 ± 18 g) of the experiment for the vehicle, fipronil-, and fipronil sulfone–treated groups, respectively. The mean (±SD) liver weights expressed as a percentage of body weight were 3.41 ± 0.35%, 3.89 ± 0.40%, and 3.67 ± 0.22% at the end of the experiment for the vehicle, fipronil-, and fipronil sulfone–treated groups, respectively, and did not differ significantly between groups ($p = 0.11$).

Effect of treatments on antipyrine pharmacokinetic parameters. Figure 6 (left) shows the time course of the mean (±SD) plasma antipyrine concentrations in vehicle, fipronil-, and fipronil sulfone–treated groups after antipyrine ip administration. The mean plasma antipyrine concentrations in the vehicle group were higher than in the treated groups at all time points. The treatment significantly affected apparent antipyrine clearance (Fig. 6, right), which was 1.7-fold higher in the two treated groups ($p < 0.01$). The other antipyrine pharmacokinetic parameters are given in Table 4. A significant 1.7-fold decrease in antipyrine half-life was observed in the fipronil- and fipronil sulfone–treated groups ($p < 0.01$). Whatever the parameter, there was no difference between the two treated groups ($p > 0.05$).

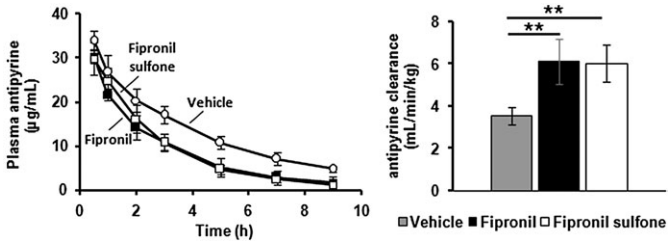

FIG. 6. Time course of mean (±SD) plasma antipyrine (left) concentrations following an ip antipyrine bolus (30 mg/kg) to vehicle ($n = 5$, ○), fipronil-treated (3.4 μmol/kg/day *per os* for 14 days, $n = 4$, ■), and fipronil sulfone–treated (3.4 μmol/kg/day *per os* for 14 days, $n = 6$, □) THX + T_3, euthyroid-like rats. Antipyrine was measured in blood samples collected at 0.5, 1, 2, 3, 5, 7, and 9h after the antipyrine bolus. The bar graphs represent mean (±SD) antipyrine clearance (right) in vehicle control (gray bars), fipronil-treated (black bars), and fipronil sulfone–treated (white bars) groups. Significantly different from control: $^{**}p < 0.01$.

Effect of treatments on Cyp1a1, Cyp1a2, Cyp2b2, Cyp3a1, and Cyp3a2 mRNA expressions and on CYP3A protein expression. *Cyp1a1* (A), *Cyp1a2* (B), *Cyp2b2* (C), *Cyp3a1* (D), and *Cyp3a2* (E) mRNA expressions are shown in Figure 7. The fipronil and fipronil sulfone treatments induced a significant increase in *Cyp3a1* and *Cyp2b2* mRNA expression ($p < 0.01$) as compared with the vehicle group. *Cyp3a1* mRNA expression was 22.1- and 18.7-fold higher and *Cyb2b2* mRNA expression was 10.1- and 7.0-fold higher in the fipronil- and fipronil sulfone–treated groups, respectively. The treatments had no significant effect on the expression of *Cyp1a1*, *Cyp1a2*, and *Cyp3a2* mRNA ($p > 0.05$).

Figure 8A shows a representative example of a Western blot with labeling of CYP3A immunoreactivity for one representative rat of each group from experiment 3. One immunoreactive band was identified at about 52 kDa as a CYP3A immunoreactive band. The bar graphs of the Figure 8B show the mean (±SD) relative intensity of the CYP immunoreactive

TABLE 4

Mean (±SD) Antipyrine Pharmacokinetic Parameters in Vehicle Control ($n = 5$), Fipronil-Treated (3.4 μmol/kg/day *per os* for 14 days, $n = 4$), or Fipronil Sulfone–Treated (3.4 μmol/kg/day *per os* for 14 days, $n = 6$) THX + T_3, Euthyroid-Like Rats

	Vehicle, ($n = 5$)	Fipronil, ($n = 4$)	Fipronil sulfone, ($n = 6$)
$t_{1/2}$ (h)	2.8 ± 0.4	1.7 ± 0.4 **	1.6 ± 0.2 **
T_{max} (h)	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.2
C_{max} (μg/ml)	32.9 ± 3.9	31.4 ± 2.7	31.0 ± 3.7

Notes. Twenty-four hours after the last vehicle, fipronil, or fipronil sulfone administration, antipyrine was assayed in blood samples collected at 0.5, 1, 2, 3, 5, 7, and 9 h after an antipyrine ip administration (30 mg/kg). Antipyrine parameters were estimated using a one-compartment model for extravascular administration with first-order absorption and elimination phases. Significantly different from control: $^{**}p < 0.01$.

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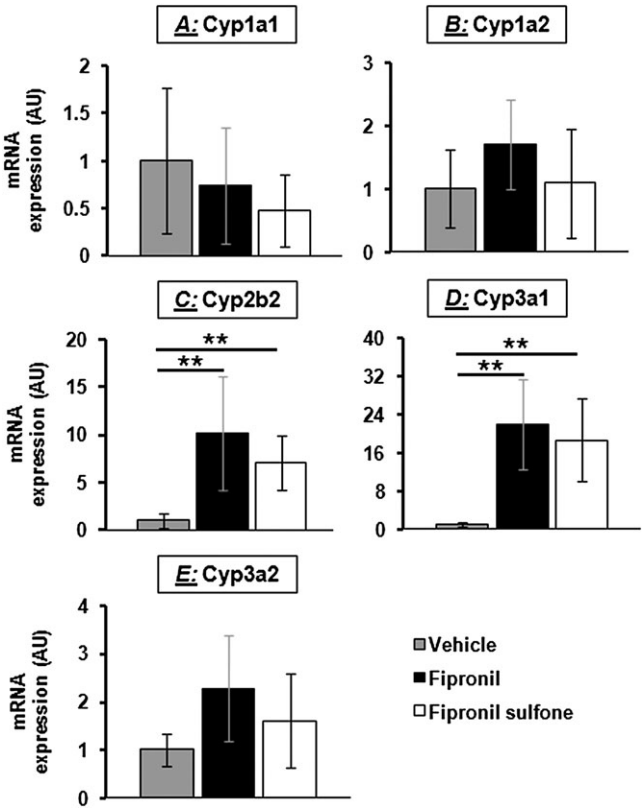

FIG. 7. Mean (±SD) mRNA expressions of *Cyp1a1* (A), *Cyp1a2* (B), *Cyp2b2* (C), *Cyp3a1* (D), and *Cyp3a2* (E) in vehicle control ($n = 5$, gray bars), fipronil-treated (3.4 µmol/kg/day *per os* for 14 days, $n = 4$, black bars), or fipronil sulfone–treated (3.4 µmol/kg/day *per os* for 14 days, $n = 6$, white bars) THX + T_3, euthyroid-like rats. Significantly different from control: $^{**}p < 0.01$.

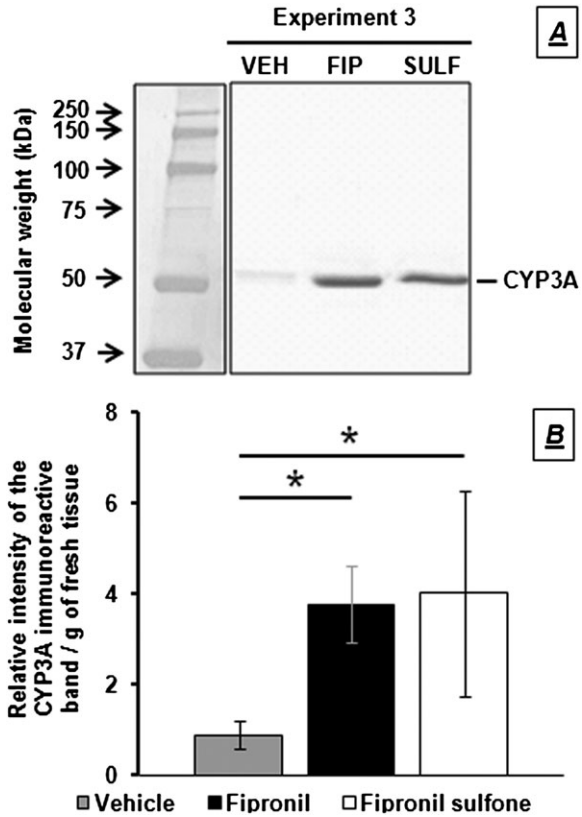

FIG. 8. Western blot (A) with labeling for the quantification of CYP3A protein expression for one representative THX + T_3, euthyroid-like rat of vehicle (VEH), fipronil-treated (FIP), or fipronil sulfone–treated (SULF) groups from experiment 3. Immunoreactivity was quantified with an infrared imaging system. The bar graphs represent mean (±SD) CYP3A immunoreactivity (B) in the vehicle control (gray bars, $n = 5$), fipronil-treated (black bars, $n = 4$), and fipronil sulfone–treated (white bars, $n = 6$) groups. CYP3A immunoreactivity was normalized according to a theoretical value for a reference pool. Significantly different from control: $^{*}p < 0.05$.

band in the three groups. A significant effect of treatment ($p < 0.05$) was observed, resulting in a significant 4.2- and 4.5-fold increase in CYP3A expression in the fipronil- and fipronil sulfone–treated groups, respectively ($p < 0.05$).

DISCUSSION

In agreement with our previous results (Leghait *et al.*, 2009), the current study demonstrates that fipronil treatment leads to an increase in free and total T_4 clearances in THX + T_3, euthyroid-like rats. In Leghait's (2009) experiment, the thyroid disruption in fipronil-treated rats could have resulted from either a direct action of fipronil before its hepatic biotransformation into fipronil sulfone or from an action of fipronil sulfone, which persists much longer in the organism than fipronil itself. To determine if fipronil sulfone could be responsible for thyroid disruption, we chose in the current study to compare the thyroid-disrupting effects of fipronil and fipronil sulfone treatments that gave similar plasma fipronil sulfone concentrations. In order to establish the dosing regimen, we first carried out pharmacokinetic investigations of both molecules given by the iv and po routes. For the first time, a precise determination of the rate of fipronil biotransformation into fipronil sulfone and of the absorption of fipronil and fipronil sulfone were established. From our results, it was clear that (1) hepatic first-pass metabolism of fipronil is very important after the po route and (2) fipronil is largely transformed into fipronil sulfone for both routes of administration. This indicated that equimolar fipronil and fipronil sulfone dosing regimen should result in similar fipronil sulfone concentrations, which led us to use this equimolar dosing regimen in our main experiments.

As results from our laboratory and from non-peer–reviewed toxicological evaluations (AFSSA, 2005; Leghait *et al.*, 2009) suggested that conjugation mechanisms associated with biliary clearance were primarily involved in fipronil-induced thyroid disruption in rats, we first studied T_4 conjugation pathways. We demonstrated that the mRNA expression of *Ugt1a1* was increased by both fipronil and fipronil sulfone treatments suggesting that UGT1A1 might be involved in increasing T_4

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elimination. This hypothesis was strengthened by the fact that both treatments increased not only *Ugt1a1* gene expression but also UGT1A activity.

Sult1b1 mRNA expression was increased by fipronil and fipronil sulfone treatments but none of the two treatments had a significant effect on phenol SULT activity toward 2-naphthol. This apparent discrepancy could be explained by the fact that measurement of phenol SULT activity might result from the activity of several other SULT1 isoforms. Indeed, SULT1A1, SULT1A3 and SULT1C2 that are less potent in mediating T_4 sulfoconjugation in the liver of female rats than SULT1B1 (Kester *et al.*, 2003) also sulfoconjugate 2-naphthol (Falany *et al.*, 2005). Thus, if only SULT1B1 activity was upregulated by fipronil and/or fipronil sulfone treatments as suggested by increased mRNA *Sult1b1* expression, this effect might have been diluted by the activities of the other SULT isoforms measured by the assay.

Our results on *Ugt1a1* and *Sult1b1* mRNA expressions and on UGT1A activity suggest that fipronil and fipronil sulfone treatments similarly increase T_4 conjugation pathways. However, the involvement of other T_4 metabolic pathways, including deiodination cannot be excluded. In an attempt to gain more insights on deiodination pathways potentially involved in mediating fipronil- and/or fipronil sulfone–induced thyroid disruption, we focused on the effects of the treatments on plasma T_3 and T_2 concentrations in our THX + T_3, euthyroid-like rats. In the sample collected approximately 24 h after T_4 and 22 h after T_3 administrations, T_3 concentrations were significantly lower in treated animals in experiment 2. The T_3 source in this sample could not be purely exogenous as T_3 could result from the deiodination of T_4 administered for the kinetic investigation. However, at the time at which T_3 was measured, almost no T_4 remained in the blood, suggesting that the T_3 assayed was most likely from exogenous origin. Thus, it can be reasonably assumed that the difference in plasma T_3 concentrations observed at this time reflected an increase in exogenous T_3 elimination rather than a decreased production rate through T_4 deiodination. Accordingly, plasma T_3 and T_2 concentrations in pooled samples were always lower in the treated groups than in the vehicle control group. This strengthens the hypothesis that T_3 and T_2 clearances might have also been increased by fipronil and fipronil sulfone treatments. Alternatively, it cannot be excluded that those treatments might have been associated to a decrease in T_2 and/or T_3 production rate. Such an inhibition of the thyroid-regulating deiodinases has been described after treatment of human liver with halogenated phenolic contaminants, which, as fipronil, disrupt circulating levels of thyroid hormones (Butt *et al.*, 2011). Finally, it is clear that even though an induction of deiodination pathways might have occurred, it was not high enough to balance a potential increase in T_2 and/or T_3 elimination rates. In conclusion, we were not able to provide evidence from this study that metabolic pathways other than conjugation might also be involved in fipronil- or fipronil sulfone–induced thyroid disruption in rats. However, for all the parameters that we evaluated (metabolic profiles, enzyme activity, mRNA or protein expression), exclusive fipronil sulfone treatment led to the same effects as fipronil treatment.

Both fipronil and fipronil sulfone treatments increased antipyrine clearance, which is considered as a relevant biomarker for CYP activity *in vivo* in different species (Chan and Yeung, 2006; St Peter *et al.*, 1991). In agreement with data on antipyrine hepatic metabolism (Engel *et al.*, 1996), this increase in CYP activity was associated with increased rat *Cyp3a1* and *Cyp2b2* mRNA expressions. This was consistent with data obtained on human hepatocytes (Das *et al.*, 2006) in which fipronil induced the human orthologs *Cyp3a4* and *Cyp2b6*. The increased *Cyp3a1* mRNA expression in response to fipronil and fipronil sulfone treatments was associated with an increased protein expression of CYP3A. Thus, it is very likely that fipronil sulfone induces its own formation and that during a fipronil treatment, a dramatic increase in fipronil biotransformation into fipronil sulfone might increase the risk of fipronil sulfone exposure. Moreover, although *Cyp1a1* mRNA expression was increased by fipronil treatment in human hepatocytes (Das *et al.*, 2006), our experiment did not reveal an effect of fipronil and/or fipronil sulfone on the rat *Cyp1a1* ortholog. It is noteworthy, however, that induction of *Cyp1a1* in human hepatocytes occurred at fipronil concentrations in the culture medium mostly much higher than the plasma fipronil or fipronil sulfone concentrations present in our study *in vivo*. Overall, our results clearly showed that fipronil sulfone is a CYP inducer at least as efficient as fipronil itself and can target the same isoenzymes.

An increase in liver weight was observed with fipronil and fipronil sulfone treatments in experiment 2. The hepatomegaly is consistent with results of regulatory toxicological evaluations that showed an increase in rat liver weight after a fipronil treatment (AFSSA, 2005). It is noteworthy that both hepatomegaly and increased activity of hepatic enzymes have been shown to result from the activation of different types of xenosensor nuclear receptors. For example, the activation of the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) have been shown to be associated with hepatomegaly (Huang *et al.*, 2005: Ross *et al.*, 2010) and to regulate hepatic phase I enzymes, especially CYP2B and CYP3A, and phase II enzymes involved in hepatic xenobiotic and thyroid hormone metabolism (Konno *et al.*, 2008). Interestingly, fipronil has been shown to be a ligand of the human PXR *in vitro* (Lemaire *et al.*, 2006). It can thus be postulated that PXR and/or CAR can mediate fipronil- or fipronil sulfone–induced thyroid disruption.

In conclusion, fipronil sulfone treatment in rats can reproduce the effect of fipronil treatment on hepatic T_4 catabolism and on all CYP and conjugation enzyme expressions and activities, suggesting that the metabolite is at least as efficient as the parent molecule. It is noteworthy that, in the current experiment, rats were treated with relatively high doses

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of fipronil or fipronil sulfone. Our conclusions might thus be limited to the maximal effect of fipronil and fipronil sulfone. A full dose-response experiment would be required to determine the relative potencies (concentration leading to half the maximal effect) of the two molecules and definitely conclude on their respective potentials as thyroid disruptors. Furthermore, the dose-response experiment would also be required to determine the full range of fipronil or fipronil sulfone concentrations that can induce an increased elimination of thyroid hormones. This could determine if the thyroid disrupting potential of fipronil or fipronil sulfone should be expressed at lower plasma concentrations, i.e., in the ng/ml range, such as those likely to occur in humans from the acknowledged domestic or professional uses of fipronil (AFSSA, 2005; Herin *et al.*, 2011) and/or accidental intoxication (Mohamed *et al.*, 2004). Further evaluations of the role of fipronil metabolism as a determining factor for the potential of fipronil as a thyroid disruptor appears to be a critical issue. Indeed, in regulatory toxicological evaluations, the potential contribution of fipronil sulfone to fipronil-induced thyroid disruption has not been addressed despite the fact that fipronil sulfone is the main metabolite formed *in vivo* in humans and can persist much longer in the organism than fipronil itself.

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