Interspecies differences in cytochrome P450-mediated metabolism of neonicotinoids among cats, dogs, rats, and humans

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ABSTRACT

Neonicotinoid insecticides are used for agricultural and non-agricultural purposes worldwide. Pets are directly exposed to neonicotinoids in veterinary products and through environmental contamination. Cytochrome P450 (CYP) is among the most significant xenobiotic metabolizing enzymes that oxidizes several chemicals, including neonicotinoids. However, CYP activities and metabolite compositions of neonicotinoid metabolites are unknown in most domesticated pet species. Our objectives were to reveal the differences in metabolites of neonicotinoids (imidacloprid, clothianidin, and acetamiprid) and CYP activities among common pet species (cats and dogs), humans, and rats. The results indicated that the CYP-mediated neonicotinoid metabolism was different depending on species and each neonicotinoid. Among these four species, the kinetics of imidacloprid metabolism indicated that rats have the highest rate of oxidation of imidacloprid to 4OH-imidacloprid, while the greatest enzyme kinetics of imidacloprid metabolism to 5OH-imidacloprid were found in rats and humans. Clothianidin was rapidly metabolized to 1-methyl-3-nitroguanidine and dm-clothianidin in rats, but cats and humans showed the lowest formation of dm-clothianidin. CYP activities in metabolism of acetamiprid to dm-acetamiprid and N-acetyl-acetamiprid were determined to be significantly higher in humans compared to other species. However, further studies should be targeted at identifying the differences in hepatic metabolism of neonicotinoids in these species using recombinant CYP enzymes.

Keywords: Neonicotinoids, cytochrome P450, species variations, *in vitro* microsomal assay

1. Introduction

Fleas are one of the most common ectoparasites in wildlife and companion animals worldwide, particularly in cats and dogs. They serve as a vector for bacteria (e.g., *Yersinia pestis, Rickettsia felis,* and *Bartonella henselae*) and can be an intermediate host for tapeworms (*Dipylidium caninum*) (Dryden and Rust, 1994). The most common variety of flea found on cats and dogs is the cat flea (*Ctenocephalides felis*) (Dryden and Rust, 1994; Mehlhorn et al., 2001). Clinical symptoms of flea infestation include over-grooming, hair loss, papular dermatitis, seborrhea, and pruritus, as well as secondary bacterial infections in cats and dogs (Koutinas et al., 1995; Mehlhorn et al., 2001). Control measures have been developed to limit flea population and impede their spread on animal skin and the environment by interrupting their life cycle. Interventions focus on the prevention of egg laying and development. Several systemic and topical treatments are available in the form of powder, sprays, and spot-on formulations that can be applied directly to skin of cats and dogs or their collars (Vo et al., 2010). These veterinary products mostly contain various effective insecticide formulations that have toxic potentials in pets, but information on the toxicities and metabolic pathways of these chemicals are highly limited for cats and dogs.

Neonicotinoids, such as imidacloprid, acetamiprid, and clothianidin, belong to a class of neuroactive insecticides linked the activation of post-synaptic nicotinic acetylcholine receptors (nAChR) that are highly selective to some insect species (Casida, 2018; Sheets et al., 2016). The chemical structure of neonicotinoids varies by generation; the first-generation neonicotinoids (imidacloprid, nitenpyram, thiacloprid, and acetamiprid) share a chloropyridine ring, whereas the core structure of second-generation neonicotinoids

(thiamethoxam and clothianidin) has a chlorothiazole group (Thompson et al., 2020). Imidacloprid is one of the most common veterinary insecticide for eliminating fleas in cats and dogs, while acetamiprid and clothianidin are typical neonicotinoids used to control insects and pests in agricultural, commercial, and residential contexts worldwide (Mehlhorn et al., 2001; Simon-Delso et al., 2015; Vo et al., 2010). Non-target vertebrates such as humans, pets, and wildlife can be unintentionally exposed to these neonicotinoid residues in the environments leading to toxicities and adverse effects that have been reported globally (Gibbons et al., 2015; Sheets et al., 2016; Simon-Delso et al., 2015). Neonicotinoids can be absorbed by skin (7.9%-11.4%) and efficiently absorbed through the intestine of humans and animals, causing neurotoxicity, hepatoxicity, and impaired immune function (Aggarwal et al., 2014; Li et al., 2012; Simon-Delso et al., 2015; Thompson et al., 2020). The parent compounds and their metabolites are different in toxicity considerations. In wild birds, the low-dose of imidacloprid could affect their thyroid homeostasis and reproduction (Pandey and Mohanty, 2015). Clothianidin (metabolite of thiamethoxam) and imidacloprid desnitro olefin (metabolite of imidacloprid) played a key role in down regulation of acetylcholine gene expression in Chinese lizards (*Eremias argus*) (Wang et al., 2019). In the 2-year rat study, acetamiprid induced histopathological change in the liver in males and body weight reduction in females, whereas the toxicity of different metabolites of acetamiprid is still unclear (Authority, 2016). The studies indicate that the toxicities of neonicotinoids relate to the metabolism and detoxifying enzymes such as the cytochrome P450 (CYP) inhibitors partially block imidacloprid, thiacloprid and clothianidin metabolism, elevating the levels of the parent compounds in the brain and liver involved in carcinogenicity in mice (Casida,

2011; Ford and Casida, 2006; Shi et al., 2009; Thompson et al., 2020), highlighting the need for a more thorough understanding of interspecies differences in the metabolism of neonicotinoids for phase I and phase II biotransformation.

The metabolism of neonicotinoids has been most commonly studied in mice, rats, and humans (Schulz-Jander and Casida, 2002; Shi et al., 2009; Thompson et al., 2020). *In vivo*, a human pharmacokinetic model for neonicotinoids was established and found that the clothianidin was partly metabolized, whereas acetamiprid and imidacloprid was largely metabolized and excreted in urine (Harada et al., 2016). The liver of mice treated with imidacloprid, thiacloprid, and clothianidin contains metabolites formed by imidazolidine hydroxylation, thiazolidine hydroxylation and desaturation, and N-demethylation (Shi et al., 2009). These studies have found that the formation of many neonicotinoid metabolites is mediated CYP enzymes (Schulz-Jander and Casida, 2002; Thompson et al., 2020). CYP, major metabolic enzyme in phase I reactions, is one of most important enzymes for neonicotinoid metabolism via oxidation reactions. The CYP3 and CYP2 families are responsible for imidacloprid and clothianidin metabolism in humans (Casida, 2011; Schulz-Jander and Casida, 2002). However, the role of CYP in neonicotinoid metabolism in vertebrate species or domestic pets, such as cats and dogs, is not well known. Moreover, studies of neonicotinoid toxicities in vertebrates suggest that there are toxicological risks associated with some neonicotinoid metabolites; understanding the metabolism of neonicotinoids is critical to characterizing potential health risks in each species (Casida, 2011; Thompson et al., 2020). To our knowledge, no information is available on interspecies differences in the role of CYP in neonicotinoid metabolism or the variation in metabolite

composition among species. The objectives of this *in vitro* study were therefore to investigate the interspecies differences among cats, dogs, rats, and humans in neonicotinoid metabolite formation following CYP metabolism, and to elucidate CYP activities in these studied species in the metabolism of first generation (imidacloprid and acetamiprid) and second generation (clothianidin) neonicotinoids, which are the most commonly used insecticides worldwide and their residues are ubiquitous in environments.

2. Materials and Methods

2.1. Sample and microsome preparation

Table 1 details the samples used in this study. Human microsomes were purchased from Celsis In Vitro Inc. (MD, USA). Sample details of human microsomes are shown in Supplemental Table 1. Cat and dog liver samples were obtained from vehicle control animals in the previous studies that performed at the Faculty of Veterinary Medicine, Hokkaido University, Japan (approval number 14-0054 and 14015) and at the Korea Institute of Toxicology, Korea (approval number 13027), respectively (Khidkhan et al., 2019; Takaguchi et al., 2019). Sprague Dawley (SD) rats (7-week-old) were acquired from Japan SLC, Inc. (Shizuoka, Japan). Microsomal fractions of rats, dogs, and cats were prepared and modified using the method described by Omura and Sato (1964). Livers were homogenized with three volumes of 0.1 M potassium phosphate buffer (KPB, pH 7.4). The homogenates were centrifuged at 11,635 g at 4 ℃ for 20 min. The supernatants were filtered with sterile gauze and centrifuged twice at 92,706 g at 4 °C for 60 min. Microsomal pellets were suspended with 0.1 M KPB and stored at -80 °C until use. The protein concentrations of microsomal fractions were measured using the BCA protein assay kit (Thermo Fisher Scientific, IL, USA),

and CYP concentration was determined using a previously reported method (Omura and Sato, 1964).

2.2. In vitro CYP metabolism of neonicotinoids

Neonicotinoid metabolism assay using liver microsomes was conducted using the methods described in the previous report (Wu et al., 2014). The reaction mixture included 0.1 M KPB (pH 7.4), magnesium chloride ($MgCl_2$, final concentration 3 mM), and glucose 6-phosphate (G6P, final concentration 5 mM) mixed with pooled hepatic microsome (final protein concentration 5 mg/mL) of each species [rats (n=3), dogs (n=3), cats (n=4), and humans (n=10)]. Imidacloprid (Kanto Chemical Co., Inc., Tokyo, Japan), clothianidin (Wako Pure Chemical Co., Osaka, Japan), or acetamiprid (Cosmo Bio Co., Ltd., Tokyo, Japan) in 3% methanol (MeOH) were added (final substrate concentration of 10, 25, 50, 100, 200, and 400 μM) and pre-incubated at 37 °C for 5 min. A mixture of glucose-6-phosphate dehydrogenase (G6PDH, final concentration 2 IU/mL) and β-nicotinamide adenine dinucleotide phosphate (β-NADPH, final concentration 0.5 mM) was added to each sample to start the reaction. After 30 minutes in a shaking water bath, 100% MeOH was added to stop the reaction. Reaction samples were then placed on ice for 15 min before centrifugation at 15,000 g for 10 min, and filtered using the GL Chromato Disk sample filter (pore size 0.2 μM; GL Sciences, Tokyo, Japan). All assays were performed in duplicate for each sample. Appropriate negative controls (the reaction mixture without substrate) were used.

2.3. Chemical analysis

Liquid chromatography mass spectrometer (6495 triple quad LC/MS; Agilent Technologies, Santa Clara, CA, USA) equipped with a 1.7 μm Biphenyl 100 A LC column (2.1*150 mm; Kinetex, Phenomenex Inc, Torrance, CO, USA) was used to quantify the target metabolites of imidacloprid, clothianidin and acetamiprid. The target neonicotinoid metabolites were purchased from Sigma-Aldrich Co. LLC. (Darmstadt, Germany) or synthesized at Toho University (Chiba, Japan) (Supplemental Table 2). Detection methods followed those of previous studies (Ichikawa et al., 2019; Ikenaka et al., 2019). For all analyses, mobile phase A consisted of 0.1% formic acid + 10 mM ammonium acetate in distilled water and mobile phase B consisted of 0.1% formic acid + 10 mM ammonium acetate in 100% MeOH. An injection volume of 5 μL, a flow rate of 0.35 mL/min with gradient elution, and a column temperature of 60 ^{o}C were used for all experiments. The limit of quantification was defined as the concentration of the target compounds sufficient to produce a signal to noise ratio (S/N) higher than 9 under the lowest calibration point (0.5 ppb for imidacloprid and clothianidin; 0.05 ppb for acetamiprid).

2.4. Data analysis

All kinetic parameters (including maximum velocity (V_{max}), Michaelis-Menten constants (K_m), and V_{max}/K_m ratio) were calculated using the Michaelis-Menten equation in GraphPad Prism version 8.0 for Windows (GraphPad Software, CA, USA). Statistical analyses were performed using JMP Pro 13 (SAS Institute, NC, USA). Tukey's HSD test was performed to compare the V_{max}/K_m among species. P value < 0.05 was considered statistically significant in all analyses.

3. Results and Discussion

3.1. Kinetic studies on imidacloprid

In vitro CYP activities for imidacloprid were measured and compared among rats, dogs, cats, and humans in this study. Although many metabolites of imidacloprid (including 4-hydroxy-imidacloprid (4OH-imidacloprid), 5-hydroxy-imidacloprid (5OH-imidacloprid), 5-methoxymethoxy-imidacloprid, 6-CNA, desnitro-dehydro-imidacloprid (dn-dh-imdacloprid), desnitro-imidacloprid (dn-imdacloprid) and imdacloprid olefin) have been reported (Dai et al., 2006; Ikenaka et al., 2018; Simon-Delso et al., 2015; Takahashi et al., 2019), 4OH-imidacloprid, 5OH-imidacloprid, dn-dh-imidacloprid, dn-imdacloprid, and imidacloprid-olefin were detected in all selected species. The kinetic parameters of 4OH-imidacloprid and 5OH-imidacloprid are presented in Table 2; other metabolites were detected in extremely low quantities and were only found in some reactions that involved a high substrate concentration; therefore, these could not be fit to the Michaelis-Menten plot. These results indicated that *in vitro* reactions using liver microsomes in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent system may primarily contribute to hydroxylation of the imidazolidine ring at positions 4 or 5 yielding 4OH-imdacloprid and 5OH-imidacloprid. A recent study indicates 5OH-imidacloprid as the only metabolite of imidacloprid when rat and rainbow trout microsomes are used (Kolanczyk et al., 2020). Fig. 1 demonstrates the Michaelis-Menten plots for CYP activities when imidacloprid was used as a substrate and Table 2 shows the Michaelis-Menten parameters during formation of 4OH-imidacloprid and 5OH-imidacloprid in each species. Significant differences in the V_{max}/K_m values for 4OH-imidacloprid formation were found among species; the rate of oxidation of

imidacloprid to 4OH-imidacloprid was found to be most rapid when rat liver microsomes were used, followed by that using human, dog, and cat microsomes (Fig. 1A, Table 2). The V_{max}/K_m values for 5OH-imidacloprid formation using rat and human microsomes were significantly higher than those using dog and cat microsomes (Fig. 1B and Table 2). A previous *in vitro* study (Kolanczyk et al., 2020) reports that the clearance of 5OH-imidacloprid in the SD rat (K_m = 158.7 µM, V_{max} = 38.4 pmol/min/mg) is much higher than that in rainbow trout (K_m = 79.2 µM, V_{max} = 0.75 pmol/min/mg). In humans, CYP2D6 and CYP3A4 are selective for the nitro-reduction of imidacloprid and formation of 5OH-imidacloprid, respectively (Casida, 2011; Schulz-Jander and Casida, 2002). Although human CYP3A4 is orthologous to rat CYP3A9 and dog CYP3A12 (Court, 2013a; Martignoni et al., 2006; Xue et al., 2003), our results indicated that CYP3A in rats and humans may have a greater enzymatic affinity to imidacloprid as a substrate and could therefore more efficiently metabolize imidacloprid to 5OH-imidacloprid, as compared to CYP3A in dogs and cats.

3.2. Kinetic studies on clothianidin

The CYP metabolic assay for clothianidin indicated that 1-methyl-3-nitroguanidine and desmethyl-clothianidin (dm-clothianidin) were the major metabolites detected in all analyzed species. In mice, dm-clothianidin is the principal metabolite found in the brain and liver after exposure to clothianidin (Ford and Casida, 2006). As shown in Fig. 2 and Table 2, the clothianidin was rapidly metabolized to 1-methyl-3-nitroguanidine (Fig. 2A) and dm-clothianidin (Fig. 2B) using rat microsomes compared to dog, human, and cat microsomes. A study of human CYP recombinant enzymes in NADPH-dependent reactions reveals that CYP3A4, CYP2C19, and CYP2A6 are responsible for converting clothianidin to dm-

clothianidin (Ford and Casida, 2006; Shi et al., 2009). Among the four chosen species, our findings suggested that CYP3A, CYP2A, and CYP2C in rats may have higher clothianidin substrate-binding capacities and clothianidin demethylation ability than in other species. Interestingly, cats and humans showed no significant difference in clearances (V_{max}/K_m) of these metabolites.

3.3. Kinetic studies on acetamiprid

An *in vitro* acetamiprid metabolism assay using microsomes of the four species revealed the formation of several metabolites including 6-chloronicotinic acid (6-CNA), descyano-acetamiprid (dc-acetamiprid), desmethyl-acetamiprid (dm-acetamiprid), N-acetyl-acetamiprid, and N-acetyl-desmethyl-acetamiprid (N-acetyl-dm-acetamiprid). Among these metabolites (Table 2), the kinetics of N-acetyl-dm-acetamiprid were calculated for the microsomes obtained from humans and cats. 6-CNA, the final metabolites of acetamiprid (Hussain et al., 2016; Marfo et al., 2015; Nomura et al., 2013), was fit to the Michaelis-Menten curve in the reaction using rat microsomes, while it could not be detected in the reactions using dog microsomes. In addition, the levels of 6-CNA were not fit to the Michaelis-Menten equation in the reactions using the microsomes of cats and humans. 6-CNA is indicated as a common metabolite of chloropyridyl neonicotinoids, including imidacloprid and acetamiprid. It is excreted in human urine and has been proposed as an indicator in monitoring exposure to insecticides (Taira et al., 2011; Uroz et al., 2001). However, our findings indicated that 6-CNA may be not a suitable marker in dogs, cats, or humans exposed to acetamiprid and imidacloprid. Dc-acetamiprid was also found in all analyzed species; however, the detected levels were not fit to the Michaelis-Menten plot.

Kolancyzk et al., (2020) report the detection of only a single metabolite, dm-acetamiprid, when acetamiprid is metabolized *in vitro* using rat and rainbow trout microsomes. While the kinetic parameters for microsomal demethylation of acetamiprid could not be quantified in rainbow trout (Kolanczyk et al., 2020). The kinetics of dm-acetamiprid in rats were determined (K_m = 70.9 μM, V_{max} = 10 pmol/min/mg) (Kolanczyk et al., 2020), but these parameters are different from those in our results (K_m = 565 ± 171 μM, V_{max} = 1344 ± 274 pmol/min/mg). Although the microsome of male SD rats was similarly used, the differences in materials and methods, such as microsome and imidacloprid concentrations, buffer, incubation periods, chemical analysis, and detector, are applied and may cause the differences in the kinetics of dm-acetamiprid in rats between our study and previous report (Kolanczyk et al., 2020). In humans, dm-acetamiprid is the dominant metabolite of acetamiprid and is frequently detected in urine samples (Harada et al., 2016). As shown in Figs. 3A and 3B and in Table 2, the V_{max}/K_m values of dm-acetamiprid and N-acetyl-acetamiprid formation were significantly higher in humans than in other examined species. There is currently no published information regarding the specific CYP-mediated acetamiprid demethylation in humans or in other vertebrates. The residues of dm-acetamiprid in urine can be used as a marker to detect environmental exposure to acetamiprid in humans owing to the slow excretion of this metabolite (Harada et al., 2016; Marfo et al., 2015). Therefore, the lower clearance (V_{max}/K_m) of acetamiprid and its metabolism to dm-acetamiprid in rats, dogs, and cats, as compared to humans, suggested that dm-acetamiprid might serve as a marker to indicate environmental exposure to acetamiprid in these species also. However, interspecies differences in excretion of acetamiprid involved in the enzymes

263 in phase II metabolism should be considered as well because it could affect the variation in
264 residue levels of dm-acetamiprid in the blood in each animal species.

265 ***3.4. Interspecies differences of metabolic capacities for neonicotinoid metabolism***

266 In this study, the interspecies differences in neonicotinoid metabolites were analyzed,
267 particularly those in acetamiprid metabolism. Among these chosen species, kinetic
268 parameters of imidacloprid metabolism indicated that the maximum rate of oxidation of
269 imidacloprid to 4OH-imidacloprid was presented in rats, while the highest enzyme kinetics
270 of imidacloprid metabolism to 5OH-imidacloprid were found in rats and humans. The
271 greatest clearances of 1-methyl-3-nitroguanidine and dm-clothianidin was noticed in in rats,
272 but cats and humans showed the lowest formation of dm-clothianidin. CYP activities in
273 metabolism of acetamiprid to dm-acetamiprid and N-acetyl-acetamiprid were determined to
274 be significantly higher in humans in comparison to other species. Phase I metabolism of
275 neonicotinoids is largely dependent on CYP (Simon-Delso et al., 2015; Thompson et al.,
276 2020); however, the CYP isoforms that were involved in the metabolism of neonicotinoids
277 was unclear. Our previous study (Khidkhan et al., 2019) found that the mRNA expressions
278 of CYP1-3 families in the liver of cats are different from those in humans (Rodriguez-Antona
279 et al., 2001), dogs (Martinez et al., 2013) and rats (Jin et al., 2016). In addition, the studies
280 on species differences between mouse, rat, dog, monkey, and human CYP-mediated drug
281 metabolism found that the species-specific isoforms of CYP1A, CYP2C, CYP2D, and
282 CYP3A cause the significant interspecies differences in terms of catalytic activity
283 (Martignoni et al., 2006). Based on our results in present study, it can be concluded that the
284 interspecies differences in the metabolism of neonicotinoids might be due to the differences

285 in expression levels and functions of CYP isoforms in rats, dogs, cats, and humans. Previous
286 studies report that glutathione conjugation, glycine conjugation, and glucuronidation in phase
287 II metabolism are important in neonicotinoid clearance (Ford and Casida, 2006; Tomizawa
288 and Casida, 2000). However, cats lack the metabolic capacity for glucuronidation (Court,
289 2013b). Although our results suggested approximately the same V_{max}/K_m values in cats and
290 dogs in the metabolism of neonicotinoids, in reality, cats may display lesser metabolic
291 capacity than dogs. Further studies should be directed at identifying the differences in hepatic
292 metabolism of neonicotinoids in these species using recombinant CYP enzymes. Moreover,
293 to clearly define the species variations in toxicological risks involved in the exposure of
294 neonicotinoids to household pets, the investigations on phase II metabolism, *in vivo*
295 experiments, and biomonitoring exposures are needed.

296 ## 4. Conclusions

297 In summary, this study constituted the first investigation of interspecies variations in
298 CYP-mediated metabolism of neonicotinoids and the specific compositions of neonicotinoid
299 metabolites detected using the hepatic microsomal fractions of rats, dogs, cats, and humans.
300 The rate of formations of most neonicotinoid metabolites (including 4OH-imidacloprid,
301 5OH-imdacloprid, 1-methyl-3-nitroguanidine, and dm-clothianidin) was highest in rats,
302 followed by humans, dogs and cats. The formation of dm-acetamiprid and N-acetyl-
303 acetamiprid was highest in humans, followed by rats, dogs, and cats. These results indicated
304 that the variation of CYP-mediated neonicotinoid metabolism could depend on species and
305 each neonicotinoid, which was confirmed using *in vitro* microsomal assay.

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Conflict of interest

There are no conflicts of interest to declare.

Figure legends

Fig. 1 Michaelis-Menten plots for CYP activity (mean ± SD) of imidacloprid substrates in SD rat (black circle), dog (blue square), cat (green triangle) and human (red triangle) liver microsomes

Fig. 2 Michaelis-Menten plots for CYP activity (mean ± SD) of clothianidin substrates in SD rat (black circle), dog (blue square), cat (green triangle), and human (red triangle) liver microsomes

Fig. 3 Michaelis-Menten plots for CYP activity (mean ± SD) of acetamiprid substrates in SD rat (black circle), dog (blue square), cat (green triangle), and human (red triangle) liver microsomes

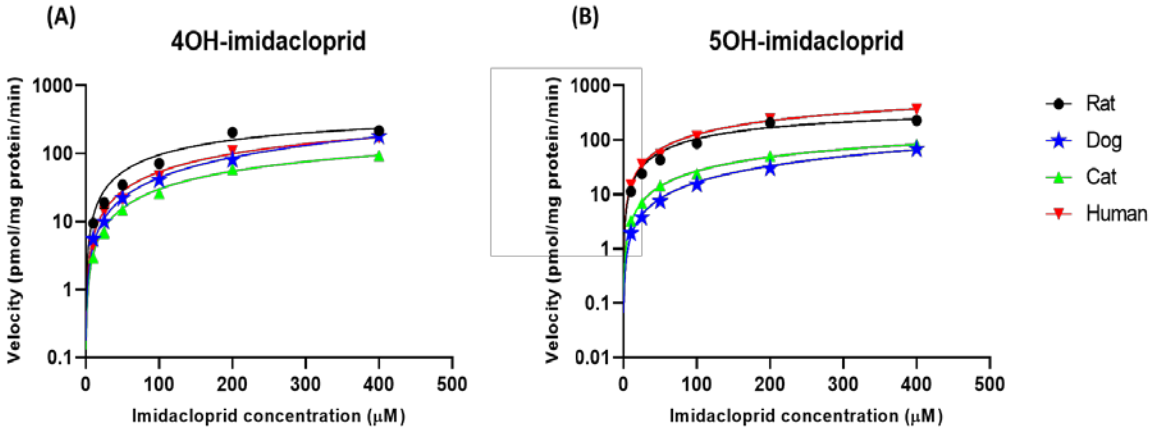
(A)
4OH-imidacloprid
(B)
5OH-imidacloprid
Velocity (pmol/mg protein/min)
Imidacloprid concentration (μM)
Rat
Dog
Cat
Human

Fig. 1

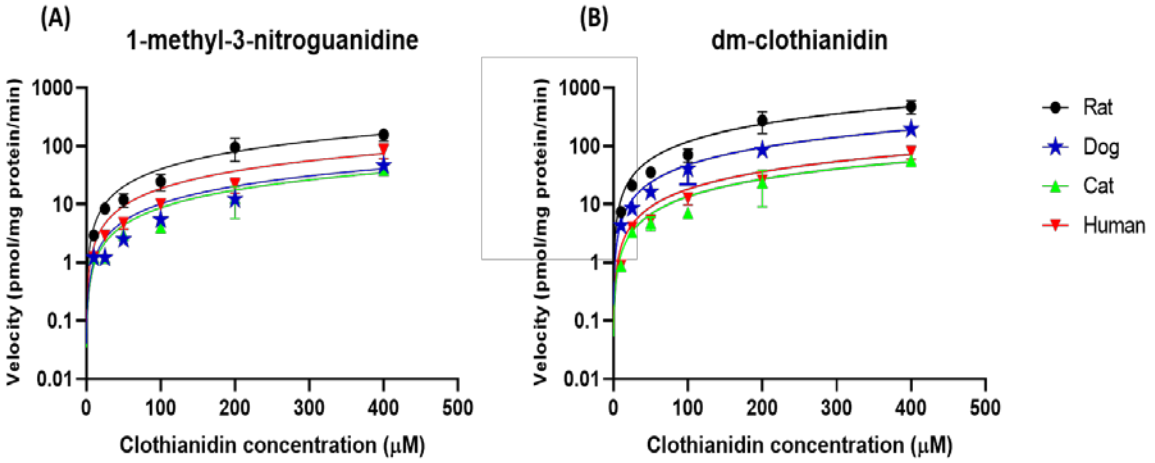
(A)
1-methyl-3-nitroguanidine
(B)
dm-clothianidin
Velocity (pmol/mg protein/min)
Clothianidin concentration (μM)
Rat
Dog
Cat
Human

Fig. 2

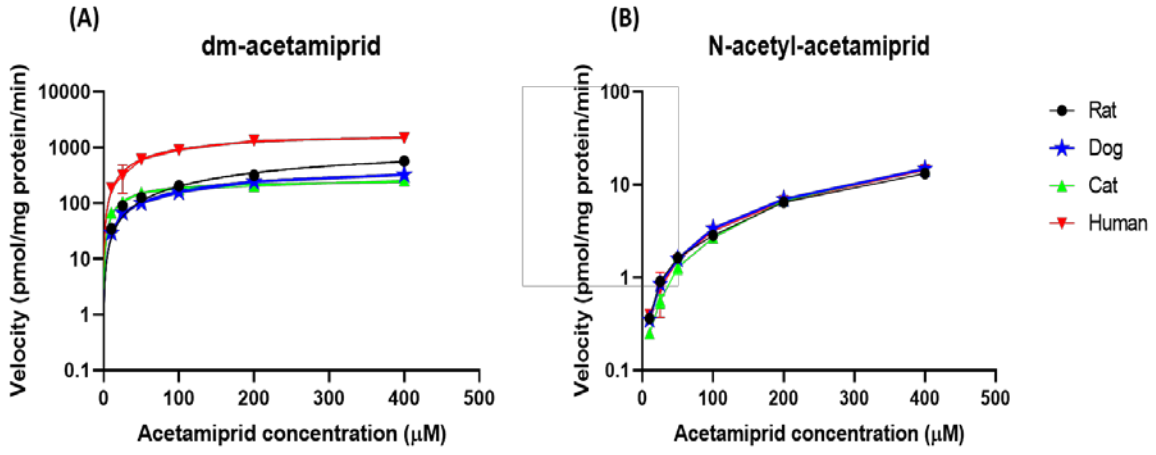

Fig. 3

Table 1 Details of animals used to obtain liver microsomes

Species	SD Rat	Dog (Beagle)	Cat	Human
Age	7 weeks	5-7 months	24-28 months	19-77 years
Sex	Male	Male	Male	Male and Female
Number of samples	4	3	4	10
Sample procurement	Purchased	Previous study (Takaguchi et al., 2019)	Previous study (Khidkhan et al., 2019)	Purchased

372 **Table 2** Michaelis-Menten kinetic parameters (V_{max} (pmol/min/mg), K_m (μM), V_{max}/K_m, (μL/min/mg), mean ± SD) for CYP
373 metabolism of neonicotinoids

Substrate	Metabolite	Parameter	Rat	Dog	Cat	Human
Imidacloprid	4OH-imidacloprid	V_{max}	454 ± 2	465 ± 606	336 ± 15	668 ± 68
		K_m	374 ± 2	1166 ± 1557	1025 ± 42	1142 ± 43
		V_{max}/K_m	1.2 ± 0.1 [A]	0.5 ± 0.1 [B]	0.3 ± 0.0 [B]	0.6 ± 0.0 [B]
	5OH-imidacloprid	V_{max}	440 ± 45	78 ± 53	283 ± 73	1042 ± 56
		K_m	320 ± 61	468 ± 358	958 ± 308	721 ± 17
		V_{max}/K_m	1.4 ± 0.1 [A]	0.2 ± 0.0 [B]	0.3 ± 0.0 [B]	1.5 ± 0.0 [A]
	5-methoxymethoxy-imidacloprid	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND
	6-CNA	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND
	dn-dh-imidacloprid	V_{max} K_m V_{max}/K_m	NF	NF	NF	NF
	dn-imidacloprid	V_{max} K_m V_{max}/K_m	NF	NF	NF	NF
	Imidacloprid-olefin	V_{max} K_m V_{max}/K_m	NF	NF	NF	NF
Clothianidin	1-methyl-3-nitroguanidine	V_{max}	26 ± 13	8 ± 6	8 ± 2	19 ± 15
		K_m	56 ± 31	113 ± 101	91 ± 28	141 ± 122
		V_{max}/K_m	0.5 ± 0.0 [A]	0.1 ± 0.0 [B]	0.1 ± 0.0 [B]	0.2 ± 0.0 [B]
	dm-clothianidin	V_{max}	105 ± 37	78 ± 1	23 ± 11	58. ± 13
		K_m	100 ± 30	189 ± 9	188 ± 107	391 ± 39
		V_{max}/K_m	1.1 ± 0.1 [A]	0.4 ± 0.0 [B]	0.1 ± 0.0 [C]	0.2 ± 0.0 [C]
	Clothianidin-urea	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND
	dm-clothianidin-urea	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND
	dm-dn-clothianidin	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND

374 **Table 2** Michaelis-Menten kinetic parameters (V_{max} (pmol/min/mg), K_m (μM), V_{max}/K_m, (μL/min/mg), mean ± SD) for CYP

375 metabolism of neonicotinoids (continued)

Substrate	Metabolite	Parameter	Rat	Dog	Cat	Human
Acetamiprid	6-CNA	V_{max}	56 ± 12	ND	NF	NF
		K_m	310 ± 107			
		V_{max}/K_m	0.2 ± 0.0			
	dc-acetamiprid	V_{max}	NF	NF	NF	NF
		K_m				
		V_{max}/K_m				
	dm-acetamiprid	V_{max}	1344 ± 274	472 ± 20	260 ± 5	1940 ± 204
		K_m	565 ± 171	183 ± 5	36 ± 5	110 ± 38
		V_{max}/K_m	2.4 ± 0.2 ^{B}	2.5 ± 0.0 ^{B}	7.2 ± 1.2 ^{B}	17.7 ± 4.2 ^{A}
	dm-dc-acetamiprid	V_{max}	ND	ND	ND	ND
		K_m				
		V_{max}/K_m				
	N-acetyl-acetamiprid	V_{max}	11 ± 2	19 ± 16	5 ± 4	5 ± 0
		K_m	271 ± 5	527 ± 459	173 ± 158	96 ± 11
		V_{max}/K_m	0.039 ± 0.000 ^{AB}	0.037 ± 0.002 ^{B}	0.027 ± 0.001 ^{C}	0.047 ± 0.004 ^{A}
	N-acetyl-dm-acetamiprid	V_{max}	NF	NF	0.4 ± 0.3	0.5 ± 0.5
		K_m			244 ± 210	61 ± 152
		V_{max}/K_m			0.002 ± 0.001	0.008 ± 0.007

376 Different characters (A, B, and C) indicate statistically significant differences of V_{max}/K_m (Tukey's HSD test, $P < 0.05$), NF;

377 Not fit to Michaelis-Menten plot, ND; Not detected

Supplemental Table 1 Tested details of human liver microsomes (10- donor mixed gender pool)

Test	Result
Protein concentration	21.6 mg/mL
Total CYP concentration	0.303 nmol/mg
CYP1A2 activity: rate of formation of acetaminophen	358 pmol/min/mg
CYP2A6: total rate of formation of 7-hydroxycoumarin and metabolites	295 pmol/min/mg
CYP2B6: rate of formation of hydroxybuproprion	189 pmol/min/mg
CYP2C8: rate of formation of desethylamodiaquine	100 pmol/min/mg
CYP2C9: rate of formation of 4'-methylhydroxytolbutamide	36.6 pmol/min/mg
CYP2C19: rate of formation of 4'-hydroxymephenytoin	47.0 pmol/min/mg
CYP2D6: rate of formation of dextrorphan	32.7 pmol/min/mg
CYP2E1: rate of formation of 6-hydroxychlorzoxazone	231 pmol/min/mg
CYP3A4: rate of formation of 6β-hydroxytestosterone	1304 pmol/min/mg
CYP3A4: rate of formation of 1-hydroxymidazolam	396 pmol/min/mg
UGT: rate of formation of 7-hydroxycoumarin glucuronide	1685 pmol/min/mg

391 **Supplemental Table 2** The substrate and target metabolites of imidacloprid, clothianidin and acetamiprid used in this study.

Neonicotinoids	Target metabolite	Retention time (min)	MRM (*m/z*)	CE	Source
Imidacloprid	Imidacloprid	4.89	256.06>175.10	24	Kanto Chemical
	4OH-imidacloprid	4.41	272.01>191.20	24	Toho University
	5OH-imidacloprid	4.29	272.01>191.20	16	Toho University
	5-Methoxymethoxy-imidacloprid	5.21	316.71>317.30	0	Toho University
	6-CNA	3.77	158.00>122.20	20	Sigma-Aldrich
	Desnitro-dehydro-imidacloprid	3.54	209.10>126.00	20	Toho University
	Desnitro-imidacloprid	3.70	236.00>132.00	24	Sigma-Aldrich
	Imidacloprid-olefin	4.42	254.00>236.10	8	Sigma-Aldrich
Clothianidin	Clothianidin	4.26	250.02>169.00	12	Wako Pure Chemical
	1-Methyl-3-nitroguanidine	1.20	119.10>73.20	4	Sigma-Aldrich
	Clothianidin-urea	3.84	206.01>132.10	16	Toho University
	Desmethyl-clothianidin	3.93	236.00>132.00	16	Toho University
	Desmethyl-clothianidin-urea	3.30	192.01>132.10	16	Toho University
	Desmethyl-desnitro-clothianidin	2.05	191.01>132.00	16	Toho University
	Desnitro-clothianidin	2.65	205.01>132.1	20	Toho University
Acetamiprid	Acetamiprid	5.50	223.08>126.00	24	Cosmo Bio
	6-CNA	3.71	158.00>122.20	20	Sigma-Aldrich
	Descyano-acetamiprid	3.43	198.01>126.10	28	Toho University
	Desmethyl-acetamiprid	4.91	209.06>125.8	20	Sigma-Aldrich
	Desmethyl-descyano-acetamiprid	2.35	184.01>126.10	20	Toho University
	N-acetyl-acetamiprid	5.13	199.01>126.10	20	Toho University
	N-acetyl-desmethyl-acetamiprid	4.20	185.01>126.00	20	Toho University

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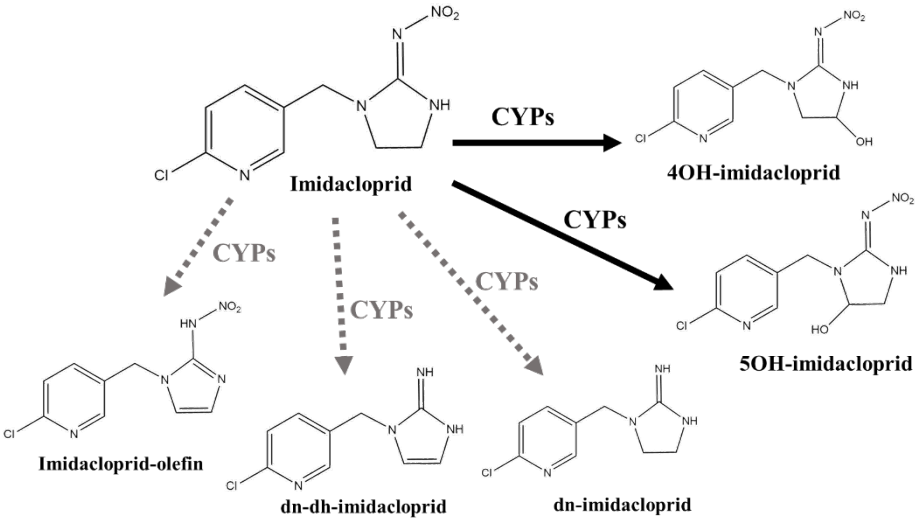
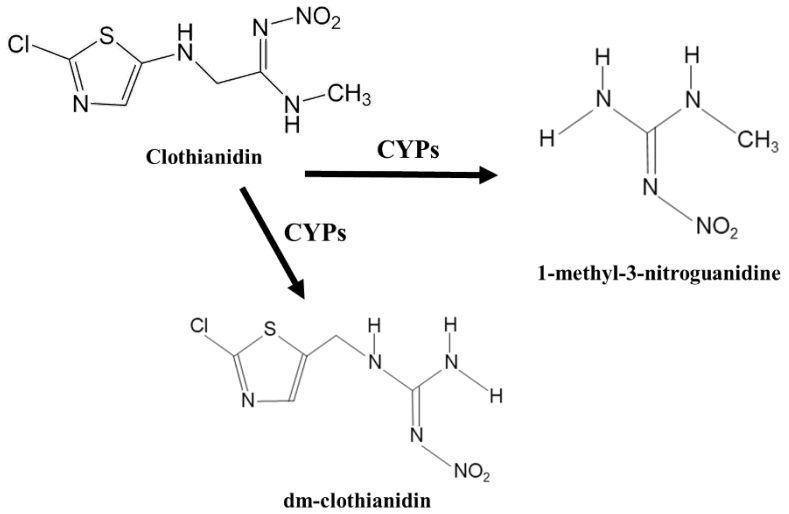
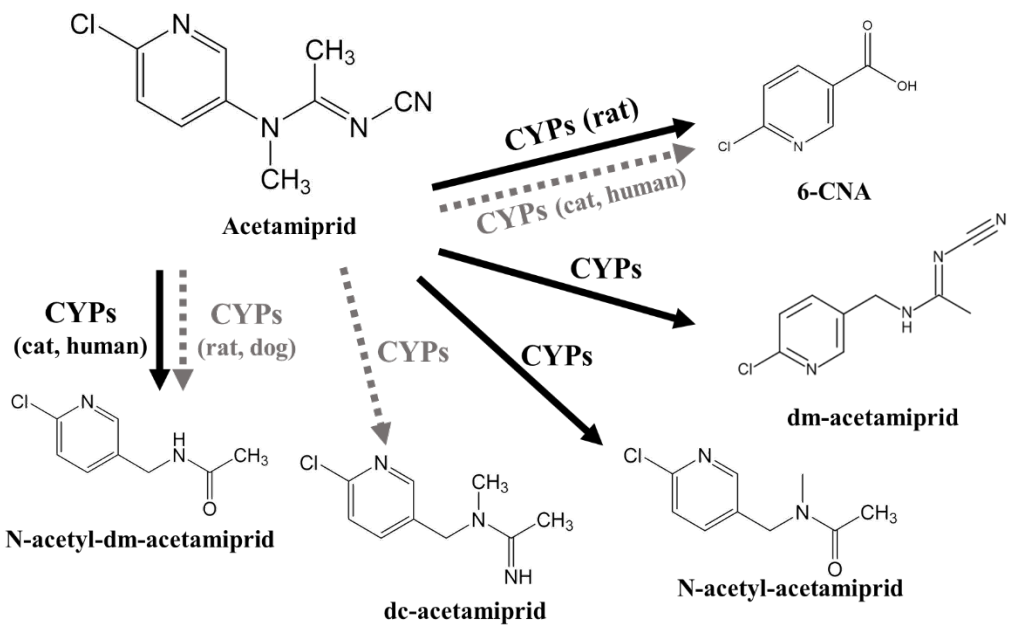


Supplemental Fig. 1 The estimated pathways of total imidacloprid metabolite formation in rats, dogs, cats, and humans (black arrow: kinetics calculated, gray dash arrow: not fit to Michaelis-Menten plot)



Supplemental Fig. 2 The predicted pathways of total clothianidin metabolite formation in rats, dogs, cats, and humans (black arrow: kinetics calculated)



Supplemental Fig. 3 The predicted pathways of total acetamiprid metabolite formation in rats, dogs, cats, and humans (black arrow: kinetics calculated, gray dash arrow: not fit to Michaelis-Menten plot)

References

Aggarwal, M., Battalora, M., Fisher, P., Hüser, A., Parr-Dobrzanski, R., Soufi, M., Mostert, V., Strupp, C., Whalley, P., Wiemann, C., Billington, R., 2014. Assessment of in vitro human dermal absorption studies on pesticides to determine default values, opportunities for read-across and influence of dilution on absorption. Regulatory toxicology and pharmacology : RTP 68, 412-423.

Authority, E.F.S., 2016. Peer review of the pesticide risk assessment of the active substance acetamiprid. EFSA Journal 14, e04610.

Casida, J.E., 2011. Neonicotinoid metabolism: compounds, substituents, pathways, enzymes, organisms, and relevance. J Agric Food Chem 59, 2923-2931.

Casida, J.E., 2018. Neonicotinoids and Other Insect Nicotinic Receptor Competitive Modulators: Progress and Prospects. Annual review of entomology 63, 125-144.

Court, M.H., 2013a. Canine cytochrome P450 (CYP) pharmacogenetics. The Veterinary clinics of North America. Small animal practice 43, 1027-1038.

Court, M.H., 2013b. Feline drug metabolism and disposition: pharmacokinetic evidence for species differences and molecular mechanisms. The Veterinary clinics of North America. Small animal practice 43, 1039-1054.

Dai, Y.J., Yuan, S., Ge, F., Chen, T., Xu, S.C., Ni, J.P., 2006. Microbial hydroxylation of imidacloprid for the synthesis of highly insecticidal olefin imidacloprid. Applied microbiology and biotechnology 71, 927-934.

Dryden, M.W., Rust, M.K., 1994. The cat flea: biology, ecology and control. Veterinary Parasitology 52, 1-19.

Ford, K.A., Casida, J.E., 2006. Unique and common metabolites of thiamethoxam, clothianidin, and dinotefuran in mice. Chemical research in toxicology 19, 1549-1556.

Gibbons, D., Morrissey, C., Mineau, P., 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environmental science and pollution research international 22, 103-118.

Harada, K.H., Tanaka, K., Sakamoto, H., Imanaka, M., Niisoe, T., Hitomi, T., Kobayashi, H., Okuda, H., Inoue, S., Kusakawa, K., Oshima, M., Watanabe, K., Yasojima, M., Takasuga, T., Koizumi, A., 2016. Biological Monitoring of Human Exposure to Neonicotinoids Using Urine Samples, and Neonicotinoid Excretion Kinetics. PloS one 11, e0146335-e0146335.

Hussain, S., Hartley, C.J., Shettigar, M., Pandey, G., 2016. Bacterial biodegradation of neonicotinoid pesticides in soil and water systems. FEMS microbiology letters 363.

Ichikawa, G., Kuribayashi, R., Ikenaka, Y., Ichise, T., Nakayama, S.M.M., Ishizuka, M., Taira, K., Fujioka, K., Sairenchi, T., Kobashi, G., Bonmatin, J.M., Yoshihara, S., 2019. LC-ESI/MS/MS analysis of neonicotinoids in urine of very low birth weight infants at birth. PLoS One 14, e0219208.

Ikenaka, Y., Fujioka, K., Kawakami, T., Ichise, T., Bortey-Sam, N., Nakayama, S.M.M., Mizukawa, H., Taira, K., Takahashi, K., Kato, K., Arizono, K., Ishizuka, M., 2018. Contamination by neonicotinoid insecticides and their metabolites in Sri Lankan black tea leaves and Japanese green tea leaves. Toxicology Reports 5, 744-749.

Ikenaka, Y., Miyabara, Y., Ichise, T., Nakayama, S., Nimako, C., Ishizuka, M., Tohyama, C., 2019. Exposures of children to neonicotinoids in pine wilt disease control areas. Environmental toxicology and chemistry 38, 71-79.

Jin, S.E., Ha, H., Seo, C.-S., Shin, H.-K., Jeong, S.-J., 2016. Expression of Cytochrome P450s in the Liver of Rats Administered with Socheongryong-tang, a Traditional Herbal Formula. Pharmacogn Mag 12, 211-218.

Khidkhan, K., Mizukawa, H., Ikenaka, Y., Nakayama, S.M.M., Nomiyama, K., Yokoyama, N., Ichii, O., Darwish, W.S., Takiguchi, M., Tanabe, S., Ishizuka, M., 2019. Tissue distribution and characterization of feline cytochrome P450 genes related to polychlorinated biphenyl exposure. Comparative biochemistry and physiology. Toxicology & pharmacology : CBP 226, 108613.

Kolanczyk, R.C., Tapper, M.A., Sheedy, B.R., Serrano, J.A., 2020. In vitro metabolism of imidacloprid and acetamiprid in rainbow trout and rat. Xenobiotica; the fate of foreign compounds in biological systems 50, 805-814.

Koutinas, A.F., Papazahariadou, M.G., Rallis, T.S., Tzivara, N.H., Himonas, C.A., 1995. Flea species from dogs and cats in northern Greece: environmental and clinical implications. Veterinary Parasitology 58, 109-115.

Li, C.X., Li, M., Feng, X.L., Cao, P., Wang, X.D., Liu, S., Xu, H.B., 2012. [Study on dermal absorption of Imidacloprid in vitro]. Zhonghua lao dong wei sheng zhi ye bing za zhi = Zhonghua laodong weisheng zhiyebing zazhi = Chinese journal of industrial hygiene and occupational diseases 30, 604-607.

Marfo, J.T., Fujioka, K., Ikenaka, Y., Nakayama, S.M.M., Mizukawa, H., Aoyama, Y., Ishizuka, M., Taira, K., 2015. Relationship between Urinary N-Desmethyl-Acetamiprid and Typical Symptoms including Neurological Findings: A Prevalence Case-Control Study. PLOS ONE 10, e0142172.

Martignoni, M., Groothuis, G.M., de Kanter, R., 2006. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert opinion on drug metabolism & toxicology 2, 875-894.

Martinez, M.N., Antonovic, L., Court, M., Dacasto, M., Fink-Gremmels, J., Kukanich, B., Locuson, C., Mealey, K., Myers, M.J., Trepanier, L., 2013. Challenges in exploring the cytochrome P450 system as a source of variation in canine drug pharmacokinetics. Drug metabolism reviews 45, 218-230.

506 Mehlhorn, H., Hansen, O., Mencke, N., 2001. Comparative study on the effects of three insecticides
507 (fipronil, imidacloprid, selamectin) on developmental stages of the cat flea (Ctenocephalides felis
508 Bouché 1835): a light and electron microscopic analysis of in vivo and in vitro experiments.
509 Parasitology research 87, 198-207.
510 Nomura, H., Ueyama, J., Kondo, T., Saito, I., Murata, K., Iwata, T., Wakusawa, S., Kamijima, M.,
511 2013. Quantitation of neonicotinoid metabolites in human urine using GC-MS. Journal of
512 Chromatography B 941, 109-115.
513 Omura, T., Sato, R., 1964. THE CARBON MONOXIDE-BINDING PIGMENT OF LIVER
514 MICROSOMES. I. EVIDENCE FOR ITS HEMOPROTEIN NATURE. The Journal of
515 biological chemistry 239, 2370-2378.
516 Pandey, S.P., Mohanty, B., 2015. The neonicotinoid pesticide imidacloprid and the dithiocarbamate
517 fungicide mancozeb disrupt the pituitary–thyroid axis of a wildlife bird. Chemosphere 122, 227-
518 234.
519 Rodriguez-Antona, C., Donato, M.T., Pareja, E., Gomez-Lechon, M.J., Castell, J.V., 2001.
520 Cytochrome P-450 mRNA expression in human liver and its relationship with enzyme activity.
521 Archives of biochemistry and biophysics 393, 308-315.
522 Schulz-Jander, D.A., Casida, J.E., 2002. Imidacloprid insecticide metabolism: human cytochrome
523 P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction.
524 Toxicology Letters 132, 65-70.
525 Sheets, L.P., Li, A.A., Minnema, D.J., Collier, R.H., Creek, M.R., Peffer, R.C., 2016. A critical
526 review of neonicotinoid insecticides for developmental neurotoxicity. Critical reviews in
527 toxicology 46, 153-190.
528 Shi, X., Dick, R.A., Ford, K.A., Casida, J.E., 2009. Enzymes and inhibitors in neonicotinoid
529 insecticide metabolism. J Agric Food Chem 57, 4861-4866.

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C.H., Liess, M., Long, E., McField, M., Mineau, P., Mitchell, E.A.D., Morrissey, C.A., Noome, D.A., Pisa, L., Settele, J., Stark, J.D., Tapparo, A., Van Dyck, H., Van Praagh, J., Van der Sluijs, J.P., Whitehorn, P.R., Wiemers, M., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environmental science and pollution research international 22, 5-34.

Taira, K., Aoyama, Y., Kawakami, T., Kamata, M., Aoi, T., 2011. [Detection of chloropyridinyl neonicotinoid insecticide metabolite 6-chloronicotinic acid in the urine: six cases with subacute nicotinic symptoms]. Chudoku kenkyu : Chudoku Kenkyukai jun kikanshi = The Japanese journal of toxicology 24, 222-230.

Takaguchi, K., Nishikawa, H., Mizukawa, H., Tanoue, R., Yokoyama, N., Ichii, O., Takiguchi, M., Nakayama, S.M.M., Ikenaka, Y., Kunisue, T., Ishizuka, M., Tanabe, S., Iwata, H., Nomiyama, K., 2019. Effects of PCB exposure on serum thyroid hormone levels in dogs and cats. Science of The Total Environment 688, 1172-1183.

Takahashi, K., Tsurumi, T., Inami, M., Li, Z., Kusakabe, T., Kikkawa, S., Azumaya, I., Tominaga, N., Ikenaka, Y., Arizono, K., Kato, K., 2019. Syntheses of 4-OH and 5-OH Imidacloprids. ChemistrySelect 4, 7343-7345.

Thompson, D.A., Lehmler, H.-J., Kolpin, D.W., Hladik, M.L., Vargo, J.D., Schilling, K.E., LeFevre, G.H., Peeples, T.L., Poch, M.C., LaDuca, L.E., Cwiertny, D.M., Field, R.W., 2020. A critical review on the potential impacts of neonicotinoid insecticide use: current knowledge of environmental fate, toxicity, and implications for human health. Environmental Science: Processes & Impacts.

553 Tomizawa, M., Casida, J.E., 2000. Imidacloprid, thiacloprid, and their imine derivatives up-regulate
554 the alpha 4 beta 2 nicotinic acetylcholine receptor in M10 cells. Toxicol Appl Pharmacol 169,
555 114-120.
556 Uroz, F.J., Arrebola, F.J., Egea-González, F.J., Martínez-Vidal, J.L., 2001. Monitoring of 6-
557 chloronicotinic acid in human urine by gas chromatography-tandem mass spectrometry as
558 indicator of exposure to the pesticide imidacloprid. The Analyst 126, 1355-1358.
559 Vo, D.T., Hsu, W.H., Abu-Basha, E.A., Martin, R.J., 2010. Insect nicotinic acetylcholine receptor
560 agonists as flea adulticides in small animals. Journal of veterinary pharmacology and therapeutics
561 33, 315-322.
562 Wang, Y., Zhang, Y., Li, W., Han, Y., Guo, B., 2019. Study on neurotoxicity of dinotefuran,
563 thiamethoxam and imidacloprid against Chinese lizards (Eremias argus). Chemosphere 217, 150-
564 157.
565 Wu, X., Kammerer, A., Lehmler, H.J., 2014. Microsomal oxidation of 2,2',3,3',6,6'-
566 hexachlorobiphenyl (PCB 136) results in species-dependent chiral signatures of the hydroxylated
567 metabolites. Environ Sci Technol 48, 2436-2444.
568 Xue, L., Zgoda, V.G., Arison, B., Almira Correia, M., 2003. Structure–function relationships of rat
569 liver CYP3A9 to its human liver orthologs: site-directed active site mutagenesis to a progesterone
570 dihydroxylase. Archives of biochemistry and biophysics 409, 113-126.

571