Summary: Toxicological studies on feline cytochrome P450 associated with environmental chemical exposures

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Domestic cats are frequently treated with veterinary drugs and are being increasingly exposed to a variety of environmental chemicals. They are known to be particularly sensitive to some drugs and chemical exposures. Knowledge regarding the biotransformation of xenobiotics in cats is required to better elucidate the species sensitivity to, and toxicity caused by, environmental exposures. Cytochrome P450 (CYP) is one of the most dominant metabolic enzymes in phase I of xenobiotic metabolism and can be induced by numerous substances. Consequently, studies on feline CYP isozyme expression related to chemical exposures are necessary to predict the adverse effects during drug development and veterinary clinical medication. In this study, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and neonicotinoids were chosen as model environmental compounds that cats are exposed to worldwide. This Ph.D. dissertation includes four chapters as follow:

Chapter 1 presents the general metabolic background of cats, the importance of environmental chemicals they are exposed to, and the role of CYPs in cats. The main objective of this study is also included at the end of this chapter.

Pet cats (*Felis catus*) are small carnivorous species belonging to the Felidae family that have become popular companions for humans. Recently, biomonitoring studies on household residues and environmental contaminants in cats are of importance and have been reported increasingly (Dye et al., 2007; Henríquez-Hernández et al., 2017; Serpe et al., 2018). Therefore, it is necessary to have extensive studies regarding the exposure and metabolism of xenobiotics in cats. The knowledge of metabolic pathways related to environmental chemical exposure and other drugs will help to protect cats from the toxicities of xenobiotics and their metabolites.

PCBs and PBDEs are synthetic organic compounds that have been extensively

detected in environments (Kodavanti and Loganathan, 2017); they consist of 209 different compounds that depending on the positions of choline or bromine atoms attached to the biphenyl (Siddiqi et al., 2003). The organohalogens, including PCBs 118, 138, 153, 180, and 187 and PBDEs 47, 99, 153, and 209, are abundant in the sera and hair of domestic cats and dogs (Ali et al., 2013; González-Gómez et al., 2018; Mizukawa et al., 2013; Norrgran et al., 2015; Serpe et al., 2018) as well as in house dust (Braouezec et al., 2016; DellaValle et al., 2013; Guo et al., 2012; Wang et al., 2013; Whitehead et al., 2014). In cats, many findings suggested that the levels of these organohalogen compounds in the blood are linked to feline hyperthyroidism or thyroid hormone disturbance (Mensching et al., 2012; Norrgran et al., 2015; Peterson, 2013).

The hydroxylated PCBs (OH-PCBs), which are formed by oxidation of PCBs with CYP monooxygenase system (Bhalla et al., 2016; Tehrani and Van Aken, 2014), have increased critical environmental concerns because some evidences suggested that they can exert various toxic effects, particularly endocrine disruption, at lower doses than the parent compounds (Kawano et al., 2005; Purkey et al., 2004). Interestingly, although the composition of PCBs is quite similar across all species, the pattern profiles of OH-PCBs in the blood of cats differ (Mizukawa et al., 2013). This report indicated that the differences in PCB metabolism between cats and other species might be associated with CYP expressions and functions. Therefore, determining the interspecies differences of PCB metabolism between cats and other species (e.g., dogs) is greatly required. Furthermore, the feline CYP expression pattern and induction by organohalogen exposure should be clearly understood to determine the risks of PCBs or PBDEs to domestic cats.

In addition to organohalogens, cats can be exposed to veterinary products, including neonicotinoids, which are applied as an effective insecticide to eradicate of ectoparasites (Mehlhorn et al., 2001; Mencke and Jeschke, 2002; Rust, 2005; Vo et al., 2010). Since neonicotinoids act as neurotoxins, they mainly act on the parasympathetic system and the sympathetic system (Selvam and Srinivasan, 2019). Neonicotinoids, especially imidacloprid and clothianidin, can cause many adverse effects at sub-lethal doses in wildlife and freshwater vertebrates, ranging from genotoxic and cytotoxic effects, impaired immune

function, reduced growth and reproductive success (Gibbons et al., 2015). However, the toxicity of neonicotinoid exposure in domestic cats and dogs has not been reported in veterinary medicine. A number of studies indicated that the toxicities in mammals might be related to the metabolic capacity to metabolize neonicotinoids and subsequent accumulation of their metabolites in the brain and other tissues (Casida, 2011; Ford and Casida, 2006; Shi et al., 2009; Thompson et al., 2020). Therefore, investigating interspecies variations in metabolic capacity to neonicotinoid exposure is vital to understand and estimate the toxicity of metabolites in each of the exposed species, including pet cats and dogs. To my knowledge, no information is available on the domestic pet's capability for neonicotinoid metabolism.

CYPs are the biggest superfamily enzymes that are involved in metabolism, such as xenobiotic oxidation and clearance of several compounds in phase I (Otyepka et al., 2011; Zuber et al., 2002). The purpose of the CYP biotransformation process is to convert a substance into inactive metabolites, which are less lipid-soluble, and highly water-soluble so that they are suitable for renal and/or biliary excretion (van Beusekom et al., 2010; Zuber et al., 2002). Among the various CYP families, the CYP1, CYP2, and CYP3 families play an essential role in detoxifying drugs and exogenous chemicals (Tomaszewski et al., 2008; Zanger and Schwab, 2013). The expression and activity of these CYP families for drug uses have been primarily elucidated in rodents, such as mice and rats, as a surrogate for humans in new drug development (Bogaards et al., 2000; Eagling et al., 1998). However, CYP expression and activity differ among age, gender, genetic polymorphism, and animal species (Graham and Lake, 2008; Martignoni et al., 2006; Sadler et al., 2016; Tomaszewski et al., 2008; Zuber et al., 2002). Consequently, investigations on CYP1–CYP3 activities and expression profiles will provide valuable information for predicting environmental chemicals and the effects of pharmaceutical exposure for each species.

The objectives in this study were (1) to elucidate the mRNA expression of the CYP1-3 families in cat tissues that are useful in defining the specific metabolic capacity in each tissue; (2) to investigate the CYP mRNA expression related to in vivo organohalogen exposures, including chronic BDE-209 exposure and acute PCB exposure, that can support my knowledge on the toxicity and clinical signs of cats exposed to these chemicals; (3) to

estimate the pathways of feline CYP-mediated PCB metabolism to clarify the specific CYP isoforms for PCB in cats compared to dogs; and (4) to study the interspecies differences in CYP metabolic abilities for neonicotinoids between cats and other species that will provide significant evidence to evaluate the capacity of CYP activity and clearance of these chemicals in cats. These objectives will allow us to better understand the chemically induced CYP, and in the future, progress to toxicity prediction and the ability of CYP metabolism after exposure to several compounds in cats since they are frequently exposed to drugs and environmental pollutants.

Chapter 2 discusses studies regarding tissue distribution and mRNA expression of feline CYP isoforms associated with *in vivo* organohalogen exposures.

In this part, I aimed to elucidate the existing isoforms of the CYP1–CYP3 families in various cat tissues including the liver, kidney, heart, lung, small intestine (duodenum, jejunum, and ileum), and brain (cerebrum, cerebellum, hypothalamus, midbrain, pons, and medulla). I also investigated the CYP mRNA expression related to acute PCB exposure in cats using the cDNA cloning and quantitative real-time RT-PCR (qRT-PCR) techniques. To estimate the possible PCB congener-induced CYPs, the correlation between the CYP mRNA expression and DL-PCBs toxic equivalent (TEQ) in the liver was analyzed. Furthermore, I examined the hepatic CYP mRNA expression in cats treated BDE-209 for long-term exposure. In cats, the greatest abundance of CYP1–CYP3 was expressed in the liver, but some extrahepatic isozymes were found in the kidney (CYP1A1), heart (CYP1B1), lung (CYP2B11 and CYP2S1) and small intestine (CYP3A131). In cats, CYP1A1, CYP1A2 and CYP1B1 were significantly upregulated in the liver as well as in several tissues exposed to PCBs, indicating that these CYPs were distinctly induced by PCBs. The strong correlations between 3,3',4,4'-tetrachlorobiphenyl (CB77) and CYP1A1 and CYP1B1 mRNA expressions were noted, demonstrating that CB77 could be a potent CYP1 inducer. All selected CYP isoforms showed no significant difference in mRNA expressions between control and BDE-209 exposure groups. However, CYP3A12 and CYP3A131 revealed a trend that was two times higher in the BDE-209 exposure group compared to control group. The present results indicate that the acute exposure of PCBs could clearly upregulate CYP1

family expression, while chronic exposure of BDE-209 could not alter CYP expression in the liver of cats.

Chapter 3 describes the interspecies differences in specific CYP isoforms responsible for PCB metabolism and investigations on CYP activity for neonicotinoid metabolism in cats compared to other species.

To be able to understand the interspecies differences among CYPs involved in PCB and neonicotinoid metabolism, the objectives of this section were to compare the production of OH-PCBs by CYP-mediated metabolism, to examine whether feline CYPs can metabolize high Cl-PCBs (7–8 Cl), and to reveal the differences in neonicotinoid metabolites and CYP activities among common pet species. The metabolic assay for 12 PCB (3–8 Cl) and highly Cl-PCBs (7–8 Cl) mixtures were conducted *in vitro* using hepatic microsomes of control cats, PCBs-exposed cats, control dogs, and PCBs-exposed dogs. The OH-PCB profiles between cats and dogs were similar for low Cl-OH-PCBs (3–5 Cl), especially 4′OH-CB18 as the major metabolite. These results, combined with *in silico* docking simulation, indicated that cat CYP3A and dog CYP3A/1A1 mainly contribute to the metabolism of PCBs, particularly PCB18. However, CYP1A1 in cats and CYP1A2/2B in dogs may be minor players for the metabolism of some PCB congeners that led to their metabolites being formed in small amounts. Variations in neonicotinoid metabolism were found among species; enzyme kinetics indicated noticeably high Vmax/Km values in rats and humans in neonicotinoid metabolism, while the CYP activity in neonicotinoid metabolism was low in cats and dogs. The feline glucuronidation deficiency, together with these findings, suggested that neonicotinoids and PCBs were metabolized less in cats compared to other species. Therefore, the PCB contaminations in households and using drugs containing neonicotinoids in pets, especially cats, should be considered carefully in toxicology and veterinary medicine.

Chapter 4 concludes all outcomes in this study and provides future perspectives based on the results presented in this thesis.

Overall, CYP3A was the dominant subfamily in the liver. Some CYP isoforms (CYP1A1 and CYP2B11) were highly expressed in the extrahepatic tissues, whereas CYP2B,

one of the most important isoforms for the metabolism of several substances, was not detected in the liver of cats. Chronic oral exposure (one year) to BDE-209 could not induce CYP1–CYP3 mRNA expression in the liver of cats, whereas the results after a single exposure to twelve PCB mixtures revealed that CYP1A1, CYP1A2 and CYP1B1 mRNA expression could be clearly induced by PCBs and may be strongly induced by CB77 in several tissues. However, the findings of *in vitro* PCB metabolism combined with in silico docking simulation indicated that feline CYP3A and canine CYP3A/1A1 mainly contribute to PCB metabolism, particularly PCB18 to 4′OH-CB18, which is the predominant metabolite in both cats and dogs. In addition, CYP1A1 in cats and CYP1A2/2B in dogs could be minor players for the metabolism of some PCB congeners (PCB28, PCB70, PCB77, PCB101, and PCB187) that led to their metabolites being formed at very small amounts. The significantly lower concentrations of OH-PCBs formation using the cat microsome suggested that cats have a lower capacity for PCB metabolism compared to dogs. The *in vitro* study on the interspecies differences in CYP activities for neonicotinoid metabolism indicated that cats and dogs have a low capacity for CYP-mediated neonicotinoid metabolism compared to rats and humans. In addition to CYP metabolism, phase II conjugation also plays an essential role in detoxification and excretion of chemicals. I suggested that the biotransformation of these studied compounds may occur less in cats compared to dogs or other species because cats not only have glucuronidation deficiency but also presented missing CYP2B in the liver as well as low CYP activity for these substances.

Recently, cats are popular pets that can be exposed to several environmental chemicals like humans, but the information on CYP isoforms involved in exposure and metabolism of numerous chemicals in cats is limited. Findings regarding detailed mechanisms of feline CYPs induced by environmental contaminants and further studies to define specific CYP isoforms for the metabolism of chemicals using recombinant feline CYP protein expression are particularly important. Moreover, the in-silico analyses, including docking simulation and molecular dynamics, will provide useful data for estimation of pathways and possible toxicities of CYP-mediated chemical metabolism in cats.

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