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FUNCTIONAL EXPRESSION AND COMPARATIVE CHARACTERIZATION OF FOUR FELINE P450 CYTOCHROMES USING FLUORESCENT SUBSTRATES

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ABSTRACT

Cytochrome P450s (CYP) are a major group of metabolizing enzymes for xenobiotics in humans and other mammals. The properties of CYP isoforms in the domestic cat, an obligate carnivore, are largely unknown at present. In this study, we studied relative expression in tissues and enzymatic properties of nine significant feline CYP isoforms. CYP2E2 transcript was most abundant in the feline liver, followed by CYP2A13 and 2E1. Transcripts of CYP3A131, 1A2 and 1A1 were also present in the liver, while CYP2D6 and 3A132 were only slightly expressed. CYP3A131 was a major transcript in the small intestine. Four major CYP isoforms in the feline liver and small intestine (CYP1A2, CYP2A13, CYP2E2 and CYP3A131) were heterologously expressed in *Escherichia coli* to generate functional monooxygenase systems. We carried out screenings of 17 test compounds known to be inhibitors of CYP isoforms in other mammals as well as two anticancer drugs to assess activity modulation of feline CYP isoforms using fluorogenic substrates. These CYP isoforms showed similar selectivity to counterparts in other mammals against inhibitors as a whole but with many exceptions. The present study suggests the usefulness of the feline CYP recombinant system to obtain chemical affinity information and possible drug interactions in CYP metabolism of domestic cats.

Keywords: carnivore; cytochrome P450; domestic cats; drug interaction; felid; xenobiotics

1. Introduction

Drugs approved for use in humans can be prescribed for off-label use in veterinary medicine at the discretion of a veterinarian even in developed countries (Animal Medicinal Drug Use Clarification Act of 1994 in USA). Thus, drugs approved for humans are often also used in companion animals. However, knowledge on the biotransformation of drugs and other xenobiotics in cats is limited. The situation for cats is quite different from that of dogs. Dogs are important experimental animals for the purpose of development of human drugs and much more detailed information on drug metabolism as well as pharmacological and toxicological effects are obtained, especially in drug companies (Bailey et al., 2015). Domestic cats are the most common companion animal and an obligate carnivore, which is dependent on a supply of at least some animal-derived materials in its diet, unlike the dog, which has a relatively developed carbohydrate digestive system (MacDonald et al., 1984). All felids, including domestic cats, must eat meat or tissue of animals. It is well known that cats are very sensitive to some xenobiotics, such as acetaminophen (Court, 2013). This is partly explained by a deficiency of a major part of glucuronidation. For example, it is believed that cats are more susceptible than dogs to acetaminophen toxicity due to lack of UGT1A6 and UGT1A9 (Court and Greenblatt, 2000). Apart from these, significant interspecies variations in drug metabolic activity have been recognized (Nebbia et al., 2003; Baririan et al., 2006). These findings strongly suggest the insufficiency of extrapolations solely based on body weight or body surface from dog and human to cat in determination of drug treatments (Court, 2013).

Cytochrome P450 (CYP) monooxygenase is most important for metabolism of

xenobiotics to polar substances for excretion and to active intermediates, causing toxicological responses in some cases. Among the numerous members of CYP superfamily, CYP1A, 2B, 2C, 2D, 2E, and 3A families are particularly important for xenobiotic metabolism (Lewis and Ito, 2008). However, knowledges on feline CYP are limited. Until now, only CYP1A1/2 (Tanaka et al., 2006), 2A13 (Okamatsu et al., 2015), 2D6 (Komatsu et al., 2010), 2E1/2 (Tanaka et al., 2005), and 3A131/132 (Honda et al., 2011) have been reported to be present in the liver of cats. Characteristically, CYP2B6 transcripts and protein were present in the lung and small intestine, but not in the liver of cats (Okamatsu et al., 2016).

Using liver microsomes, CYP1A-, 2C-, 2D- and 3A-dependent enzymatic activities of CYP families of domestic cats have been previously characterized (Chauret et al., 1997; Pearce et al., 1992; Shah et al. 2007). Enzymatic activities of six CYP families of cats were compared with those of dogs with fluorescent substrates specific for each CYP isoform (van Beusekom et al., 2010). However; in previous studies with cat liver microsomes, the substrate specific to the human orthologues of the enzymes have been used despite the potential for interspecies differences in CYP activities.

The most straightforward method of defining substrate specificity is characterization of CYP isoforms that are prepared by heterologous expression, because CYP isoforms have overlapping metabolic activity for each substrate. *E. coli* is one of the most extensively utilized host in the production of recombinant CYP enzymes. It was reported that the same patterns of apparent allosteric effect are observed with proteins derived from either the liver or from *E. coli* for human CYP3A4 (Ueng et al., 1995). Appropriate N-terminal modifications do not appear to affect enzyme activity, and sometimes introduce desirable characteristics that are likely to facilitate attempts to

crystallize the catalytic domains of microsomal P450s (Yun et al., 2006). Canine CYP1A1, 2B11, 2C21, 2C41, 2D15, 3A12, and 3A26 with canine cytochrome P450 reductase has been heterologously expressed in *E. coli* to study basic metabolic activities (Locuson et al., 2009).

Recently, we prepared recombinant of feline CYP2A13 (Okamatsu et al., 2015) and CYP2B6 (Okamatsu et al., 2016) in *Escherichia coli* (*E. coli*). Although we showed metabolic activity similar to those of human and canine CYP2As for coumarin, 7-ethoxycoumarin, and nicotine by feline CYP2A13, additional properties including interaction with other xenobiotics are largely unknown (Sasaki and Shimoda, 2015). Recombinant CYP isoforms of mice and human heterologously expressed in E. coli has been extensively studied for the metabolic activity and inhibition by xenobiotics using fluorogenic substrates suitable for large-scale screening (McLaughlin et al., 2008; Kajbaf et al., 2011). We previously confirmed metabolic activity of feline CYP2A13 and CYP2B6 using resorufin- and coumarin-based CYP substrates that are the most extensively used substrates (Okamatsu et al., 2015, 2016).

It is important to know that the property of CYP isoforms from domestic cat that shows very different feeding behavior as a strickt carnivore and lacks some major UGT isoform. In this study, we characterized relative expression of nine feline CYPs in the liver and small intestine. Then, we heterologously coexpressed four feline P450s with human P450 oxidoreductase (POR) in *E. coli*, thus generating functional CYP systems *in vitro* for enzyme characterization. For each CYP isoform, we identified an adequate fluorogenic probe substrate with the apparent kinetic parameters. These probe substrates were then used to determine the IC_{50} of 19 test compounds including inhibitors specific for human and canine CYP isoforms and chemotherapeutic agents for each CYP,

allowing us to compare inhibitory potencies as well as affinities to various xenobiotics for each CYP isoform.

2. Materials and Methods

2.1. Chemicals

The 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-4-(trifluoromethyl) coumarin (HFC) and 7-ethoxy-4-(trifluoromethyl)coumarin (EFC) were purchased from BD Gentest (Woburn, MA, USA) and 7-ethoxyresorufin, 7-methoxyresorufin, and 7-pentoxyresorufina were obtained from Molecular Probes Europe BV (Leiden, The Netherlands). Coumarin, 7-hydroxycoumarin, resorufin, cyclophosphamide, erythromycin and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO, USA) and 7-benzoxyresorufin was purchased from Roche (Mannheim, Germany). Atipamezole, medetomidine, methoxsalen, miconazole and ticlopidine were obtained from Wako Pure Chemical (Osaka, Japan). 7-ethoxycoumarin and the other reagents use as test compounds were purchased from Tokyo Kasei (Tokyo, Japan). All other reagents used in this study were of analytical grade.

2.2. Quantitative real-time RT-PCR

Tissues of mongrel cats of both sexes (short-haired type; 5 male cats and 6 female cats) collected in our previous study (Okamatsu et al., 2015) were also used in this study. They are all adult (3 years old). Total RNA was extracted from the liver and small intestine of domestic cats using a conventional acid guanidine-phenol-chloroform method (Trizol; Invitrogen, Carlsbad, CA, USA) (Komatsu et al., 2010). We used middle part of small intestine, because RNA degradation of feline small intestine (particularly for upper part) was very rapid in the preliminary experiment. We synthesized cDNA from total RNA using oligo dT primer and reverse transcriptase (QuantiTecht, Qiagen, Hilden, Germany). Quantitative real-time RT-PCR (qRT-PCR) analysis was performed with a real-time PCR detector (Chromo4; Bio-Rad, Hercules, CA, USA) using an SYBR Green-based kit (Thunderbird qPCR Mix, Toyobo, Osaka, Japan). The primer pairs used for CYP1A1, 1A2, 2E1, and 2E2 are listed in Table 1. The sequences of primers for CYP2B6 (Okamatsu et al., 2016), 2A13 (Okamatsu et al., 2015), 2D6 (Komatsu et al., 2010), and 3A131 and 3A132 (Honda et al., 2011) have been previously reported. Standard curves were generated with serial dilutions of PCR products. The concentration of PCR product was quantified with a microchip electrophoresis system (MultiNA, Shimadzu, Kyoto, Japan). The primer sets used for

qRT-PCR were confirmed to produce a single peak in the melting curve as well as a single band by agarose gel electrophoresis. Each CYP expression was normalized per μg total RNA used for reverse transcription.

2.3. Heterologous expression in E. coli

Membrane fractions of feline CYP2A13 with human oxidoreductase (POR) was prepared according to our previous report (Okamatsu et al., 2015). Protein expression in *Escherichia coli* (*E. coli*) using the generated expression plasmids, membrane preparations, and measurement of CYP2A13 content were performed as described previously (Okamatsu et al., 2015).

Heterologous expression of CYP3A131 was carried out according to Okamatsu et al. (2015, 2016). This was based on the method originally developed by Pritchard et al. (2006). Feline full length CYP3A131 was amplified by RT-PCR with primers (3 and 4 in Table 2) and cDNA derived from the liver. To enhance functional protein expression in *E. coli*, bacterial ompA leader sequences were fused to the 5'-end of the complete coding region of feline full length CYP3A131 by PCR with genomic DNA of *E. coli* and the primers 5 and 10 (first-round PCR). After being purified by agarose gel

extraction, resultant PCR product was used as forward primer in the second-round PCR with the CYP3A131 3'-end specific reverse primer 11 and full length feline CYP3A131 as a template. The amplified fragments were subcloned into T-vector (pTAC-2: BioDynamics, Tokyo, Japan) and the plasmid was obtained by transfection to competent cells (DH5α: Frontier Science, Ishikari, Japan) and purified with a plasmid mini kit (FastGene Plasmid Mini Kit, Nippon Genetics). After digestion of the products with Nde I and Xba I of the plasmid were purified by gel elecrophoresis, resultant insert fragment was subcloned, using a DNA Ligation Kit (Mighty Mix, Takara), into pCWOri+ vector (Barnes, 1996), in which the human POR cDNA had already been accommodated (Iwata et al., 1998). The resultant construct was used for transformation with competent cells and CYP3A131 bactosomes were harvested as described previously (Okamatsu et al., 2015). POR activity of bactosomes were measured as reported by Iwata et al. (1998).

Feline CYP1A2 bactosomes were prepared according to Iwata et al. (1998), who replaced the second to seventh N-terminal amino acid regions with bovine CYP17α to facilitate protein expression in *E. coli*. Feline full length cDNA of CYP1A2 was amplified by RT-PCR with liver-derived cDNA and primers (1 and 2 in Table 2). The PCR product was used as a template for the PCR with primers 6 and 7 to add an Nde I

site in N-terminus and Xba I site in C-terminus. The following procedures are the same as those for feline CYP3A131.

Heterologous expression of feline CYP2E2 in *E. coli* was carried out using the expression vector that contained fragment of modified mouse 2C29 N-terminals (Uno et al., 2006), because we failed to obtain functional CYP2E2 by the method described for CYP2A13 and CYP3A131. RT-PCR for the gene encoding mouse CYP2C29 was carried out with cDNA derived from the mouse liver and primer sets (12 and 13 in Table 2). The amplified fragments were added with EcoRI site in N-terminus and Sal I site in C-terminus by the nested PCR with primers (14 and 15) and the PCR product of the previous PCR as a template. The resultant PCR product was further used as a template for the PCR with primers 16 and 15 to extend and add Nde I site in N-terminus. Purified digests with Nde I and Sal I of the plasmid obtained were subcloned into pCWOri+ vector (Modified pCWori+ vector). Otherwise, feline CYP2E2 insert with EcoRI and Sal I sites were obtained with primers (8 and 9) and cat liver cDNA. Purified digests of the two restriction enzymes were subcloned into Modified pCWori+ vector. The following procedures are the same as described above.

2.4. Western blotting

Four feline recombinant CYPs (0.5 pmol except CYP1A2 at 0.26 pmol)and liver microsomes (30 μg) were run on 10% SDS polyacrylamide gels and transferred to cellulose nitrate filters (pore size 0.45μm) (Advantec, Tokyo, Japan) according to our previous study (Okamatsu et al., 2015, 2016). The filters were immunoblotted with primary antibody specific for human CYP protein, followed by incubation with biotinylated rabbit anti-goat IgG antibody or biotinylated goat anti-rabbit IgG antibody (Vectastatin ABC-AP KIT, Vector Labs, Burlingame, CA, USA). The primary antibodies used were rabbit anti-human CYP1A2 antibody (Abgent, San Diego, CA, USA) (1:50), rabbit anti-human CYP2E1 antibody (Abcam, Cambridge, UK) (1:100), and rabbit anti-human CYP3A43 antibody (1:100) (Abgent). After the avidin-biotin reaction, a specific band was visualized using a DCIP/NBT substrate kit (Vector Labs).

2.5. Enzyme activity assay

Metabolic activity of feline recombinant CYPs with coumarin was determined spectrofluorometrically as described previously (Okamatsu et al., 2015). In brief,

recombinant feline CYP proteins (2-8 pmol/100μL) were incubated with coumarin (0-5,000 μM as a final concentration) at 37 °C for 15 min in potassium phosphate buffer (pH 7.4) after 5 min preincubation. The fluorescence of 7-HC (excitation 370 nm and emission 450 nm) was measured with a fluorescence spectrophotometer (650-10S, Hitachi, Tokyo, Japan). The same reaction condition was also used for determining metabolism of BFC (0-1,000 μM) and MFC (0-5,000 μM) . Fluorescence of HFC converted (420 nm for excitation and 535 nm for emission) was determined in a reaction buffer without extraction after the reaction was stopped by addition of 900 μL ice-cold potassium buffer. The detection limit of HFC (3σ) was 0.45 pmol.

Alkoxyresorufin-O-dealkylase activity was determined as previously described (Okamatsu et al., 2016). Alkoxyresorufins (benzyloxyresorufin, ethoxyresorufin, methoxyresorufin, and pentoxyresorufin) (0-75 μM))were incubated with recombinant feline CYP2A (2–8 pmol/100 mL) in potassium phosphate buffer (pH 7.4) at 37 ^{o}C. Resorufin formed by the reaction was measured by an excitation wavelength of 535 nm and emission wavelength of 595 nm. The detection limit of resorufin (3σ) was 0.14 pmol.

2.6. Fluorometric enzyme inhibition assays

Effects of 19 compounds were studied using fluorescent substrates selected from the previous study (McLaughlin et al., 2008). Basically, all incubations were done under the conditions described in the previous section. After test compounds and substrates in 0.1 M potassium phosphate buffer (pH 7.4) were preincubated for 5 min at 37 ^{o}C, reactions were carried out for 20 min after addition of NADPH (final 1 mM). Concentrations of substrates used for enzyme inhibition assays were around the Km values;100µM BFC for CYP1A2, 1.5 µM coumarin for CYP2A13, 300 µM 7-EC for CYP2E2, and 300 µM BFC for CYP3A131. The concentration of test compound as inhibitors were in the range of 0.05 nM – 10 µM (the lower limit) to 500 – 2,000 µM (upper limit), dependent on the effectiveness of compounds in the preliminary experiments. IC_{50} values were calculated in Microsoft Excel as previously described (Okamatsu et al., 2016).

2.7. Statistical methods

Results are presented as means±SE. Significance of differences among groups was determined by one-way ANOVA followed by the Tukey-Kramer test ($p < 0.05$). For linear regression, Eadie-Hofstee plots were generated and data were analyzed in Microsoft Excel using least-squares linear regression to generate the best fit line.

3. Results

3.1. Tissue expression of CYP isoforms

We characterized the relative expression of the CYP isoforms that we identified previously (CYP2A13, 2B6, 2D6, 3A131, 3A132) (Komatsu et al., 2010; Honda et al., 2011; Okamatsu et al., 2015, 2016) and other CYP isoforms (1A1, 1A2, 2E1, 2E2) (Tanaka et al., 2005, 2006) in the liver and small intestine (Fig. 1). Significant sex-dependent difference were not recognized in the amount of transcripts of any isoform in the liver and small intestine. Thus, all data were shown as the mean of all the animals analyzed. In the liver, CYP2E2 transcripts were most abundant, followed by 2A13, 2E1, 3A131 = 1A2 = 1A1, 2D6, and CYP3A132. CYP2B6 was not detected in the liver, which is in agreement with our previous work (Okamatsu et al., 2016). CYP3A131 was a major CYP transcript in the small intestine, while there were few transcripts for other

CYPs except CYP2B6 and 1A1 with slight expressions (Fig. 1B).

3.2. Heterologous coexpression of four major CYP isoforms in E. coli.

We focused on CYP2E2, CYP2A13 and CYP3A131 due to their abundance in the liver and small intestine. Additionally, we prepared CYP1A2 recombinant protein, because CYP1A2 metabolizes a variety of arylamines and heterocyclic arylamines in therapeutic drugs (Zhou et al., 2009).

The reduced-CO difference spectra with these recombinant CYP proteins showed clear absorption peaks at around 450 nm (Fig. 2). We have already reported recombinant protein of CYP2A13 and CYP2B6 (Okamatsu et al., 2015, 2016). Bactosomes of four feline CYP isoforms prepared in this study contained 0.02-0.46 nmoles/mg protein of P450 (CYP1A2 0.03 ± 0.01, 2A13 0.26 ± 0.11, 2E2 0.04 ± 0.02, 3A131 0.03 ± 0.01: n = 3) with 56.6-143.9 nmol/min/mg protein as activities of POR (CYP1A2 82.2 ± 10.8, 2A13 117.1 ± 13.8, 2E2 111.8 ± 11.7, 3A131 83.0 ± 13.3: n = 3).

These recombinant CYP proteins were detected by immunoblotting with each anti-human CYP polyclonal antibodies (human CYP1A2, 2E1 and 3A43) (Fig. 3). These antibodies reacted with feline CYP counterparts but not other feline CYPs including CYP2A13 and CYP2B6 (Fig. 3). Immunoreactivities of CYP1A, CYP2E and CYP3A were detected in the liver microsomes by these antibodies, irrespective of sex (Fig. 3).

3.3. Metabolic activities of recombinant CYPs

We characterized metabolic activities of four recombinant feline CYPs prepared in the previous chapter, along with CYP2A13. For high-throughput screening, fluorogenic substrates were selected by reference to substrates for human CYP isoforms (Donato et al., 2004). None of the recombinant CYP isoforms studied showed O-dealkylase activity of 7-pentoxy resorufin.

Feline CYP1A2 heterologously expressed in *E. coli* caused concentration-dependent hydroxylation of 7-benzoxyresorufin (BR), 7-ethoxyresorufin (ER), and 7-methoxyresorufin (MR). Coumarin and 7-ethoxycoumarin were not studied. Values of Km and Vmax obtained from the dose-response curves of hydroxylation of some substrates by CYP1A2 together with other CYPs are given in Table 3. CYP1A2 showed a high-affinity hydroxylation with BR and ER compared to that with MR with lower maximal activity (Table 3). CYP1A2 also hydroxylated 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) with lower affinity but higher Vmax.

Feline CYP2A13 showed significant hydroxylation activity with ER, coumarin, and 7-ethoxycoumarin (7-EC) (Table 3). Among these substrates, coumarin showed the highest affinity, while 7-EC showed the highest activity but lowest affinity.

Feline CYP2E2 showed significant metabolic activity with MR, coumarin, and 7-EC (Table 3). However, hydroxylation of coumarin by CYP2E2 needed a very high concentration of substrates and a Vmax value was not obtained even at 5 mM. Only very low activity was recognized with MR.

Feline CYP3A131 hydroxylated BFC into hydroxycoumarin, while it showed significant but lower affinity in metabolic activity with coumarin and EFC (Table 3).

Thus, we selected BFC for CYP1A2 and CYP3A131, coumarin for CYP2A13 and 7-EC for CYP2E2 as fluorogenic substrates to test the effects of xenobiotics in the following study.

3.4. Determination of ligand IC_{50}

We selected 17 compounds that are reported to inhibit CYP activity in mice and humans, including selective inhibitors for CYP isoforms. We also selected two anticancer drugs as a pilot study. We used 5–7 concentrations of the substrates for calculation of Km and Vmax values in triplicate measurements, after we tested 7–9 concentrations of substrates to determine an appropriate range in preliminary experiments. The IC_{50} data obtained for each CYP isoform are indicated in Table 4.

Overall, there was no compound that showed activation of CYP isoforms studied (Table 4). IC_{50} values of most chemicals, with exceptions, exceeded 100 µM on CYP2E2 in cat. Other CYP isoforms, CYP1A2, 2A13, and 3A131 showed their characteristic sensitivity to these 19 chemicals.

Three azole antifungals, clotrimazole, ketoconazole and miconazole showed very strong inhibitory activity on feline CYP3A131 at around the submicromolar range. These antifungals moderately inhibited CYP1A2, following CYP3A131. CYP2A13 was relatively insensitive to these antifungals. The antifungals hardly affected CYP2E2 activity except miconazole that showed weak inhibition.

Fluvoxamine, a selective serotonin reuptake inhibitor (SSRI) inhibited activity of feline CYP1A2 in the micromolar range. By contrast, the other SSRI, sertraline, showed relatively strong inhibition on feline CYP3A131 much more than CYP1A2.

Two selective inhibitors for adenosine receptor (P2Y) that are used as antiplatelet agents, clopidogrel and ticlopidine, were generally ineffective on these feline CYP isoforms. Quinidine, an antiarrhythmic agent, showed some modulation to CYP3A131 only.

Methoxsalen that is used in treatment of various skin diseases inhibited feline CYP2A13 activity at the submicromolar range. Methoxsalen also strongly inhibited CYP1A2 and 3A131.

Two antiulcer agents, cimetidine, a H_2 histamine receptor blocker and omeprazole, a proton pump inhibitor, hardly affected feline CYP isoforms, although omeprazole showed weak inhibition on CYP3A131. Erythromycin, a macrolide antibiotic agent weakly inhibited feline CYP3A131.

Tryptamine, a monoamine alkaloid, showed weaker inhibition on feline CYP2A13 but relatively strong effect on feline CYP1A2. Quercetin, a flavonol found in many plants, inhibited feline CYP1A2 and CYP3A131.

Medetomidine and atipamezole are frequently used for sedative hypnotics and the antidote, respectively, in veterinary medicine. Both imidazole derivatives strongly inhibited most feline CYP isoforms except CYP2E2. CYP2A13 was particularly sensitive to these agents with an IC_{50} of 0.2 μM. Effects of dexamethasone were not observed practically.

Finally, the effects of two anti-cancer agents, cyclophosphamide and tamoxifen, were studied. The result was that both drugs showed no or only weak inhibition on CYPs examined (Table 4).

4. Discussion

We determined relative expression of nine CYPs in the liver and small intestine of cats. The results showed that CYP2E2, CYP2A13, and CYP2E1 are three major transcripts in the feline liver. These three transcripts accounted for about 60% of nine CYPs transcripts addressed. In our previous study, CYP2B subtype was not present in the feline liver but present in the lung and small intestine as both transcript and immunoreactivity of CYP2B6. The expression pattern of CYP isoforms in the feline liver was very different to that of canine liver containing CYP2E1 (26%), CYP2A25, CYP2D15 (25% for both CYP isoforms), CYP2C21 (14%), CYP3A12 (5%) and CYP2B11 (2%) (Martinez et al., 2014). The expression pattern of CYP isoforms in the feline liver was also different to that of the human liver containing CYP2E1 (c.a. 55%), CYP3A4 (11%), 2C8 (9), 2C9 (8), 1A2 (6), 2A6 (3) (Bièche et al., 2007). Although CYP2E1 was the most major transcript of CYP isoforms that were involved in xenobiotics metabolism (c.a. 57%) and contribution of CYP3A4/5 was only about 12% (Bièche et al., 2007), estimated CYP3A protein level (c.a. 29%) exceeded that of CYP2E1 (c.a. 7%) in the human liver, as revealed by immunoblotting (Shimada et al., 1994). This discrepancy was partially explained by negative regulation of CYP2E1 protein level by miR-378, a microRNA (Mohri et al., 2010). Thus, protein levels of CYPs in the feline liver should be studied in the following study. Overall, however, it is suggested that a few CYP isoforms are majorly expressed in the liver of cat compared to those of dog and human at least in mRNA level.

CYP3A131 was the overwhelmingly predominant transcript of all CYP isoforms studied in the feline small intestine in our data. In dog, CYP3A12 is the most abundant

CYP isoform in the intestine, using multiple reaction monitoring mass spectrometry (Heikkinen et al., 2012). Of two CYP3A isoforms in dog, CYP3A26 is a major transcript of the CYP3A isoform compared to CYP3A12 in the liver, but the reverse is the case in the small intestine (Mealey et al., 2008). In cat, transcripts of CYP3A131 are a hundred times as high as those of CYP3A132 in both the liver and small intestine (Honda et al., 2011). Expression of CYP3A131 in the small intestine was comparable with that in the liver of cat, suggesting the importance of CYP3A131 in oral drug administration in cat (Honda et al., 2011). As the highest concentration of CYPs in the small intestines of other mammals is found in the duodenum (Heikkinen et al., 2012), more CYP3A131 transcripts could be detected in the upper part of the small intestine, because w used middle part of the small intestine. Abundance and activity of both CYP isoforms were higher in the liver than in the small intestine of dog (Mealey et al., 2008). In human, the intrinsic activities of hepatic and intestinal CYP3A4 were comparable for the 16 drug metabolism (Galetin and Houston, 2006), although abundance of CYP3A4, a major isoform also in the small intestine, is lower than that in the liver based on immunoblotting studies (Paine et al., 2006).

In this study, the properties of metabolism of nine fluorescent substrates by four major feline CYP isoforms were characterized. Fluorescent substrates are useful because of the easy and simple procedures adequate for high-throughput screening (Crespi et al., 1997: van Beusekom et al., 2010). Although substrates strictly specific to each CYP isoform should be explored, there is no information on gold standard substrate in feline CYP isoform. Fluorescent substrates are useful because of the easy and simple procedures adequate for high-throughput screening (Crespi et al., 1997: van Beusekom et al., 2010). In this study, functional CYP1A2 could be prepared based on

the method by Iwata et al. (1998). Although feline functional CYP3A131 also could be prepared with the same method (Iwata et al., 1998), we selected the method used by Pritchard et al. (2006), because usage of OmpA leader sequences improved the amount of recombinant CYP3A131 protein. Functional feline CYP2E2 was not obtained by the same method for CYP1A2 and 2A13, but by the method according to Uno et al. (2006).

Overall, Km values of the four CYP isoforms to chemicals addressed were similar to those of human and dog using recombinant systems; however, some differences were also confirmed. Km values of metabolic activities of resorufin derivatives, BR and MR by feline CYP1A2 were similar to those of human CYP1A2, while Km in metabolism of BFC, a coumarin derivative of feline CYP1A2, is seven times as high as that by human CYP1A2 (Stresser et al., 2002). Feline CYP3A131 showed ten times lower affinity to BFC than that of human CYP3A4 (Stresser et al., 2002). Feline CYP2A13 catalyzed coumarin with similar affinity to human CYP2A6 and canine CYP2A13, while canine CYP2A25 did not show significant metabolic activity of coumarin (Stresser et al., 2002; Zhou et al, 2010). Information on fluorescent substrates metabolism by CYP2E is very limited. Although human CYP2E1 catalyzed MFC in both bactosome of *E. coli* and microsomes of immortalized human liver epithelial cells individually expressing human CYP2E1 (Stresser et al, 2002; Donato et al., 2004), feline CYP2E2 did not show any activity in this study. None of the four CYP isoforms studied catalyzed MFC. This is in contrast to the study with feline liver microsomes, showing significant metabolic activity of MFC (van Beusekom et al., 2010). As MFC is known as a good substrate for CYP2C9 and CYP2E1 in human (Donato et al., 2004), we should consider possible difference of substrate specificity of feline CYP isoform to MFC as well as possible involvement of CYP isoform other than four CYP isoforms we

tested. Feline CPY2C has not been reported and recombinant feline CYP2E1 is not obtained yet. Of these nine fluorescent substrates we studied, BFC for CYP1A2 and CYP3A131, coumarin for CYP2A13, and 7-EC for CYP2E2, which can be used as substrates for drug-drug interaction studies.

In this study, we developed fluorescence-based drug-drug interaction systems with four major feline CYP isoforms and their fluorescent substrates. We confirmed possible interactions of four major CYP orthologues with 19 chemicals, most of which were reported to inhibit enzymatic activities of CYP isoforms in other mammals. Two thirds of them are medical drugs used for feline patients (Papich, 2010; Plumb et al., 2011). *In vitro* screening studies for inhibition on CYP isoforms heterologously expressed in *E. coli* with fluorescent substrates were extensively carried out in human (Kajbaf et al., 2011) and mice (McLaughlin et al., 2008). We selected twelve test chemicals from the drugs previously examined in these two studies. IC_{50} values of these overlapping chemicals in our feline experiments were compared with those of human and mouse in Table 4. Kajbaf et al. (2011) tested the effects of a large number of drugs on metabolism of some substrates by bactosome expressing human CYP1A2, 2C9, 2C19, 3A4 and human lymphoblastoid cells expressing human CYP3A4. Kajbaf et al. (2011) confirmed fairly good correlation was confirmed between substrates with some exceptions. They also showed a reasonable agreement between IC_{50} values obtained with bactosomes and microsomes of human lymphoblastoid cells. Additionally, Donato et al. (2004) also reported the same conclusion in primary human hepatocytes or in human liver microsomes after comparison and the results obtained with fluorescent probes and other specific probes. Furthermore, they also concluded that correlation between bactosome and human *in vivo* data is even better than that between

lymphoblastoid preparation and *in vivo* data. It was reported that inhibitory effect of a drug on enzymatic activity was dependent on the substrate employed in human CYP3A4 (Kenworthy et al., 1999; Yuan et al., 2002). Additionally, we used human POR not feline one, although the species of POR used in the single CYP systems may be of some importance in regards to enzyme kinetics (Locuson et al., 2009). There is a possibility that the system could be a good *in vivo* representation based on findings from similar studies in humans but would require evaluation of feline microsomes and *in vivo* data. Thus, we should be careful about the data obtained with limited substrates; however, the data could be worth evaluating in the screening system.

In this study, it was CYP3A131 that was highly sensitive to the largest number of drugs out of four feline CYP isoforms assayed. Human CYP3As are involved in metabolism of a variety of drugs and are regarded as one of the most sensitive CYP isoforms to chemicals, and thus the site of many drug-drug interaction. Human CYP3A isoforms have the largest active site and is associated with the greatest magnitude of diversity in substrates (Lewis et al., 1998). By contrast, CYP2E2 which was the most abundant transcript of CYP isoforms studied in the feline liver, was insensitive to the xenobiotics examined in this study. Feline CYP2A13 was very sensitive to a limited number of xenobiotics with submicromolar IC_{50} values.

It is well known that azole fungicides exert potent inhibition on most CYP isoforms in the human. Particularly, ketoconazole preferentially inhibits human CYP3A4/5, while miconazole shows relative selectivity to human CYP2 C19 and CYP3A4 (Zhang et al., 2002). Bactosome preparations of murine and human CYP3As were similarly modulated by ketokonazole (IC_{50}: CYP3A11 < CYP2A4 < CYP1A2 << CYP2E1 no inhibition) (McLaughline et al., 2008). Similar to the case in mouse, low

concentrations of ketoconazole also inhibited feline CYP3A131 preferentially. The inhibitory effect of ketokonazole was moderate to feline CYP1A2 and weak for both feline CYP2A13 and CYP2E2 in this experiment. We also confirmed similar selective inhibition by miconazole and clotrimazole to feline CYP3A131 and CYP1A2 with somewhat different potency. Serum concentration of ketoconazole could reach 10 μM in adult human (Huang et al., 1986). Information on pharmacokinetic ketoconazole is not available in cat. Quite recently, however, it was reported that the Cmax for posaconazole in healthy cats that were orally administered (15 mg/kg) is approximately 1.7 μM (Mawby et al., 2016). These suggest that 3A131 inhibition is indeed a concern by azole antifungals in cat.

It was reported that medetomidine, a substrate for canine CYP2B11 showed strong inhibition on canine CYP2B11 that was heterologously expressed in *E. coli* (IC_{50}: 0.0048 μM for racemic form) as well as other CYP isoforms (IC_{50}: 1.1 μM for CYP1A1 and CYP2D15) (Baratta et al., 2010). In this and previous studies (Table 4, Okamatsu et al., 2016), medetomidine effectively inhibited activities of feline CYP1A2, CYP2B6 (IC_{50}: 0.024 μM), CYP2A13, and CYP3A131, although CYP2B6 was not expressed in the feline liver (Okamatsu et al., 2015). Additionally, atipamezole, an antidote for medetomidie also showed strong inhibition on activities of CYP1A2, CYP2A13, CYP2B6 (IC_{50}: 0.11 μM) (Okamatsu et al., 2016) and CYP3A131 but not CYP2E2 (Table 4). According to company's instructions, atipamezole is used in a five-fold concentration of medetomidine to feline patients, sounding the alarm on the possible drug-drug interactions that should be considered for many drugs in veterinary medicine, although we do not have any information on serum and the exact-free hepatic concentrations of medetomidine and atipamezole concentrations in cat.

Fluvoxamine, a specific inhibitor for human CYP1A2 and CYP2C19 (Madhusoodanan et al., 2014), showed reasonably comparable affinity to CYP1A2 and CYP3A131 in cat, while it required about 300-fold concentrations to obtain IC_{50} on CYP3A4 compared to CYP1A2 in human (Kajbaf et al., 2011). Methoxsalen, an inhibitor specific to human CYP2A6 (Zhang et al., 2001) showed strong inhibition on feline CYP2A13 and also on feline CYP1A2 and 3A131, while tryptamine, another inhibitor for CYP2A6/13 (Stephens et al., 2012) showed weaker inhibition on feline CYP2A13 but not CYP3A131. Tryptamine also inhibited feline CYP1A2 activity. We already reported that clopidogrel and ticlopidine, inhibitors specific to human CYP2B6, also inhibit feline CYP2B6 enzymatic activity in lower concentrations (IC_{50}: 0.33 μM for clopidogrel, 5.82 μM for ticlopidine) (Okamatsu et al., 2016). In this study, these xenobiotics hardly affected activities of all feline CYP isoforms addressed, suggesting the selective inhibition on feline CYP2B6. Sertraline, a SSRI, inhibits human CYP2B6 (IC_{50}: 3.2 μM) (Walsky et al., 2006). Feline CYP2B6 also was very sensitive to this drug (IC_{50}: 0.49 μM) (Okamatsu et al., 2016). Sertraline exerted relatively strong inhibition on feline CYP3A131 in this study. Recently, sertraline was reported to show mechanism-based inhibition on human CYP3A4 (Masubuchi and Kawaguchi, 2013). The proton pump inhibitor, omeprazole, is a metabolism-dependent reversible inhibitor of human CYP2C19 and CYP3A4 (Ogilvie et al., 2011). Feline CYP3A131 showed similar or moderate sensitivity to omeprazole compared to corresponding isoforms in mouse and human in general, although different IC_{50} values were reported for human CYP3A4 by substrates used (Table 4). Another group of antiulcer drug, cimetidine, is well known as a weak inhibitor for various human CYP isoforms including CYP1A2, 2D6, and CYP3A4, at least with human liver microsomes (Martínez et al., 1999). On

the contrary, human CYP isoform-expressing bactosomes or lymphoblastoid cells are only inhibited by cimetidine at the concentration of 100 μM or higher (Kajbaf et al., 2011). In this study, cimetidine did not affect activity of feline CYP1A2 and showed only weak inhibition on feline CYP3A131 with an IC_{50} of about 188 μM. Erythromycin, a classical antibiotic, inhibits human CYP3A4 using bactosomes of *E. coli* and microsomes of human β-lymphoblastoid cell lines (Table 4). We confirmed weaker inhibitory effects of erythromycin in feline CYP3A131 in this study (Table 4).

CYP2C isoforms have not been reported yet in cat. We were not able to generate a functional feline CYP2D6 recombinant preparation using the same strategies used for the other five feline CYP isoforms in *E. coli*. Nevertheless, we also compared the specificity of inhibitors for these CYP isoform with feline CYP orthologs we had. Quercetin, a flavonoid, is known as an inhibitor specific to human CYP2C8 (Hosaka et al., 2015). Quercetin showed strong inhibition on other CYP isoforms in mouse (IC_{50}: CYP1A2 < CYP3A11 < CYP2A4 = CYP2E1) (Table 4). The present results in feline CYP isoforms showed a similar rank order of potencies as that in mouse, although lower affinities. Quinidine, a well-known inhibitor specific to human CYP2D6, showed only weak inhibition on feline CYP isoforms except CYP3A131. IC_{50} values for quinidine are relatively variable with recombinant human CYP3A4 bactosomes of *E. coli* ranging from 5.8 - 60 μM, dependent on substrates used (Table 4).

Dexamethasone is widely known as an inducer of human CYP3As, but this has not been observed in cultured canine hepatocytes (Lu and Li, 2001). It was reported that clinical oral doses of dexamethasone decreases intrinsic clearance of quinidine, a canine CYP3A substrate possibly through reduction of α1-acid glycoprotein (AGP) and inhibition of CYP3A activity (Zhang et al., 2006). Significant changes in the

pharmacokinetic parameters of oleandomycin were observed after intramuscular injection of dexamethasone to cats but not after oral administration (Milanova and Lashev, 2002). In this study, dexamethasone showed only weak inhibition on feline CYP3A131 (IC_{50}: 137.1 μM), similar to human CYP3A4 (Table 4).

Finally, we also tested two representative anticancer drugs in veterinary medicine as these drugs are used on geriatric cats, generally possessing, lower metabolic ability, over long time periods. In the results, cyclophosphamide showed slight inhibition on CYP2A13 only, while tamoxifen slightly inhibited most CYP isoforms except CYP2A13. Although cyclophosphamide exerts moderate inhibition on both mouse CYP2A4 (IC_{50} 87.1 μM) and CYP2C9 (IC_{50} 60.7 μM) using bactosome systems (Table 4; McLaughlin et al., 2008), a low concentration of tamoxifen inhibits human CYP3A4 (IC_{50} 1.6 μM) in contrast to feline CYP3A131 (Table 4). Tamoxifen strongly inhibits mouse CYP1A1 (IC_{50} 1.8 μM) and CYP2B20 (IC_{50} 0.86 μM) and moderately inhibits some CYP isoforms at 100 μM, while it showed similar affinity to mouse CYP3A11 (IC_{50} 28 μM) (McLaughlin et al., 2008; Kajbaf et al., 2011). Further experiments with other anticancer drugs should be undertaken to determine modulation of feline CYP isoforms.

In conclusion, we established an *in vitro* screening system for possible drug-drug interactions on four major feline CYP isoforms that were heterologously expressed in *E. coli*. Four feline CYP isoforms showed similar selectivity to homologues in other mammals against fluorescent substrates and inhibitors as a whole but with many exceptions. A different expression pattern of CYP isoforms was confirmed in the liver and small intestine in cat, compared to those in human and dog. Many drugs used in veterinary practice are off-label human drugs and pharmacokinetic data in cats are

extremely limited at the present time. Therefore, prediction of drug-drug interactions from *in vitro* data is of great value. Although we are not able to know how these recombinant feline CYP isoforms reflect endogenous ones, the present study is a first step to clarify this important problem.

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Figure legends

Fig. 1. Expression of nine CYP isoforms in the feline liver (A) and small intestine (B). Results are expressed as mean ± SEM (copies/pg total RNA used for reverse transcription as template). Eleven cats (male 5 and female 6) were used for each tissue except small intestine (n = 10: male 4, female 6).

Fig. 2. CO difference spectra for recombinant feline CYP1A2 (A), 2E2 (B) and 3A131 (C). Wavelength of the spectrophotometer was shown in abscissa. Vertical lines at the upper right of each panel indicate absorbance sizes (0.0025 for A, 0.005 for B and 0.01 for C). Contents of CYP isoforms in bactosomal protein shown are 0.02 (A), 0.05 (B) and 0.03 (C) (nmol/mg protein).

Fig. 3. Immunoblot analysis of recombinant proteins of three CYP isoforms and microsomes from the feline livers. Recombinant CYP proteins (Recombinant CYPs: 0.25 pmols CYP1A2, 0.5 pmols CYP2A13, 0.5 pmols CYP2E2, 0.5 pmols CYP3A131)

and liver microsomes for both sexes (♂ 1-3, ♀1-3: 30 μg) were loaded to each lane. Size in Kda was indicated in the left of band images for each antibody.

Fig. 1

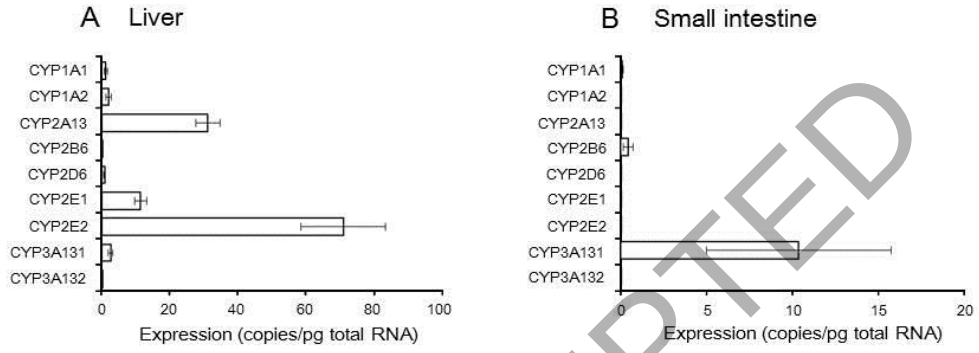

Fig. 2

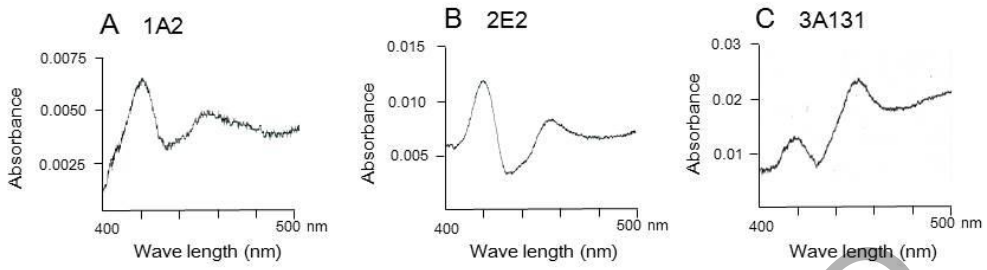

Fig. 3

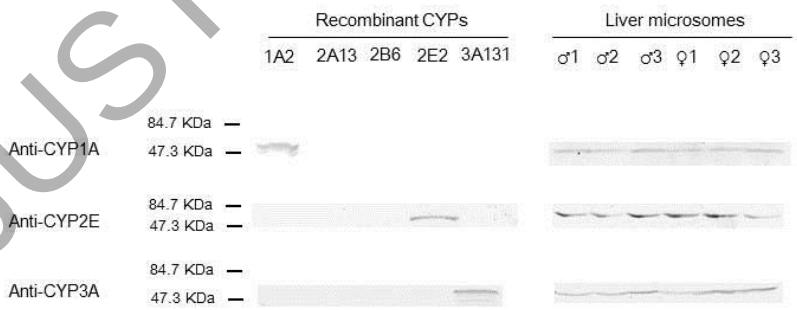

Table 1. Primers used for qRT-PCR

Primer name	
CYP1A1_F	GCGCTATGACCATGATGACC
CYP1A1_R	TATCCCGAATGTGACCCTTC
CYP1A2_F	AGGAACAGATGGCAGAGGTC
CYP1A2_R	TGTCCTGGATGTTGTTCTCG
CYP2E1_F	ACTATGGCATGGGGAAGaGC
CYP2E1_R	AGTTGGTGCTGAGCAGGTGG
CYP2E2_F	GAAGCTTGCCAATGAGGAGAG
CYP2E2_R	AGAGGATGTCCGAGATGACG

Table 2. Primers used for making expression vector

No.	Primer name	Sequence（5'to3'）	Usage
1	CYP1A2_F	TCTCAAGCACCTGCCTCTAC	Cloning of CDS of CYP1A2 (NM_001048013)
2	CYP1A2_R	TGCCTTGGCATGCTGGTGTC	do.
3	CYP3A131_F	GTAGAGGAGATTCACAGAGGG	Cloning of CDS of CYP3A4 (NM_001246278)
4	CYP3A131_R	GAAGTCTCTGGTGTTCTGGG	do.
5	ompA F	GGAATTCCATATGAAAAAGACAGCTATCGCG	Preparation of expression vector
6	CYP1A2_express_F	CATATGGCTCTGTTATTAGCAGTTTTTCTGTTCTGCCTGGTGTTC	Preparation of expression vector
7	CYP1A2_express_R	TCTAGATCACTTGATGGAGAAACGTGGC	do.
8	CYP2E2_express_F	GAATTCTACCCCCTGGCCCTTTC	do.
9	CYP2E2_express_R	GTCGACTCACACTCCCGAACG	do.
10	ompA-CYP3A131 Linker	AAAGCTTGGGATCAGGTCCATCGGGACGGCCTGCGCTACGGTAGCGA	do.
11	CYP3A131 express R	TCTAGATCAGGCTCTACTCACACTC	do.
12	Express construct_F1	CAGAGCTCTGGGAGAGGGAAGCTCC	Preparation of exression vector for CYP2E2
13	Express construct_R1	TTAGAGAGGAATGAAGCAGAGCTG	do.
14	Express construct_F2	AATCTTCTGGACGAGGAATTCTCCCTCCTG	do.
15	Express construct_R2	GGGTCGACTTAGAGAGGAATGAAGCAGAGCTG	do.
16	Express construct_F3	CCATATGGCAACGCAATCTTCTGCTCGAGG	do.

Table 3. Apparent kinetic parameters of four feline CYP isoforms coexpressed with human NADPH-CYP reductase for a series of fluorescent probe substrates

	Benzyloxyresorufin		Ethoxyresorufin		Methoxyresorufin		Coumarin		7-Ethoxycoumarin		BFC	
	Km	Vmax	Km	Vmax	Km	Vmax	Km	Vmax	Km	Vmax	Km	Vmax
CYP1A2	2.1±0.08	0.4±0.03	1.8±0.3	0.5±0.07	11.1±0.5	0.1±0.01	ND	ND	ND	ND	128.6±17.6	7.7±0.6
CYP2A13	-	-	1.9±0.05	0.3±0.03	-	-	1.6±0.4	19.8±2.1	7.1±0.9	218.1±36.6	-	-
CYP2E2	-	-	-	-	21.4±1.6	0.08±0.01	>1000	>14	517.5±46.7	19.2±1.8	-	-
CYP3A131	-	-	-	-	-	-	>1000	>10	>500	>60	413.4±35.4	8.7±1.0

Values are indicated in µM (Km) and pmol/min/pmol P450 (Vmax) with ± SE values. N=3 (three different bactosome preparations). When CYP isoforms did not show significant activiry with10 µM resorufrin derivatives (benzyloxyresorufin, ethoxyresorufin, methoxyresorufin), mark - was used. When CYP species did not show significant activity with100 µM coumarin and their derivatives (7-ethoxycoumarin, methoxyresorufin), mark - was used. ND means that corresponding experiment was not carried out. None of these CYPs did not metabolize 7-methoxy-4-(trifluoromethyl)coumarin and 7-ethoxy-4-(trifluoromethyl)coumarin (100 µM for both).

Table 4. IC_{50} data for 19 test compounds with four feline CYP isoforms in comparison with four mouse and two human CYP isoforms

	Cat				Mouse				Human		
	CYP1A2	CYP2A13	CYP2E2	CYP3A131	CYP 1A2	CYP 2A4	CYP 2E1	CYP 3A11	CYP 1A2	CYP 3A4	CYP3A4 [a]
Atipamezole	1.2±0.06	0.2±0.02	59.7±28.2	3.2±0.8	-	-	-	-	-	-	-
Cimetidine	NI	747.7±51.9	256.9±11.9	188.3±39.5	-	-	-	-	>100	-	>100, >100
Clopidogrel	529.2±37.2	NI	NI	98.6±4.6	-	-	-	-	-	-	-
Clotrimazole*	9.3±0.8	73.6±20.3	372.1±49.8	1.1±0.2	-	-	-	-	-	-	-
Cyclophoshamide	NI	165.0±6.6	NI	NI	210	87.1	NI	NI	-	>1000	-
Dexamethasone	NI	NI	258.3±36.8	137.1±32.4	-	-	-	-	ACT	-	>100, >100
Erythromycin	NI	NI	180.0±70.5	40.0±14.8	NI	NI	ACT	NI	>100	4.7	1, 3
Fluvoxamine	3.8±0.8	88.9±5.2	93.8±24.6	18.2±0.3	-	-	-	-	0.1		27, 30
Ketoconazole	14.2±1.8	202.3±5.2	151.7±42.0	0.3±0.3	4.82	1.1	NI	0.02	11	0.005	0.1, 0.2
Medetomidine	5.6±3.5	0.2±0.02	350.6±107.0	0.6±0.1	-	-	-	-	-	-	-
Methoxsalen*	1.3±0.3	0.3±0.02	NI	2.8±0.3	-	-	-	-	-	-	-
Miconazole*	3.5±1.8	21.5±2.4	68.2±52.5	0.2±0.1	-	-	-	-	1		0.1, 07
Omeprazole	89.0±7.0	NI	292.0±54.8	20.6±8.0	190	NI	NI	61.7	>100	4.5	>100, 60
Quercetin*	5.3±1.8	147.7±19.5	73.4±30.1	10.3±0.06	0.02	14	10.4	2.7	-	2.7	-
Quinidine	924.0±56.2	350.5±55.0	245.9±36.4	21.6±0.7	NI	NI	NI	480	>100	5.8	18, 60
Sertraline	180.3±10.2	308.1±83.4	274.8±99.6	8.2±4.7	-	-	-	-	-	-	-
Tamoxifen	202.0±28.1	NI	127.4±12.7	51.6±13.0	20.9	43.6	58.5	28	-	1.6	-
Ticlopidine*	312.6±21.2	NI	NI	262.3±36.9	-	-	-	-	7	-	40, 53
Tryptamine*	14.1±2.6	44.8±1.0	155.2±14.9	313.1±43.4	-	-	-	-	-	-	-

IC_{50} values for 19 compounds with feilne CYP isoforms are indicated in μM with ± SEM. N=3 (three different bactosome preparations). Murine and human data were quoted from McLaughlin et al. (2008) (all murine CYP isoforms and human CYP3A4) and Kajbaf et al. (2011) (human CYP1A2 and CYP3A4 [a]). Two IC_{50} values are shown in CYP3A4[a] by two substrates, diethoxyfluorescein and 7-benzyloxyquinoline. NI, not inhibitory; ACT, compound caused activation of probe substrate; (-), not tested. Compounds with asterisk are not used for feline patient.