FELINE HEPATIC BIOTRANSFORMATION AND TRANSPORT MECHANISMS

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Feline hepatic biotransformation and transport mechanisms

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FELINE HEPATIC BIOTRANSFORMATION AND TRANSPORT MECHANISMS

Biotransformatie en transportmechanismes in de lever van de kat
(met een samenvatting in het Nederlands)

Proefschrift

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In herinnering aan mijn vader

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SOLA DOSIS FACIT VENENUM

"Only the dose makes the poison", Paracelsus, 1493-1541

CHAPTER 1

GENERAL INTRODUCTION

BACKGROUND

Due to the increasing popularity of cats held as a pet, the cat has become a veterinary relevant target animal species. Where cats used to be kept as animals to hunt rats and mice in the barns to protect the grain stocks, the cat is nowadays being considered as a companion animal and family member. With the increasing emotional value of pets, the owners demand optimal health care for their cats during their entire and increasing life span. In turn, the range of indications for treatment is increasing and requires a closer insight into the options for feline medication. For decades pharmacological research had been devoted predominantly to dogs, since dogs served as a model for human medicine and as a spin-off pharmaceutical products were also developed for dogs as a target veterinary species. In veterinary practice, veterinarians use drugs that are licensed for the use in dogs or humans also in the treatment of cats. Several incidents with adverse drug reactions, however, clearly indicated that cats should not be considered simply as small dogs. Remarkable differences exist between behavior, disease spectrum, therapeutic demands and drug handling between dogs and cats, and feline medicine is now a well-established specialty within companion animal medicine.

The therapeutic outcome of any medication is determined by the pharmacodynamics and pharmacokinetic properties of a drug. In human medicine, response to medications as intended is on average only approximately 50% (Squassina et al., 2010). This low response rate is mainly attributed to inter-individual differences in drug handling, i.e. the variability in pharmacokinetics. Variations in drug pharmacokinetics are likely to be also the major determinant in inter-species differences in drug efficacy and the prevalence of undesirable side effects. Insight into the mechanisms that determine pharmacokinetics is needed to define appropriate dosing regimens that are not only based on interspecies scaling, which neglects drug-specific parameters such as biotransformation. In human medicine, comparison of drug handling in larger groups of patients, denoted as population kinetics, is considered as an inevitable element in pre- and post-marketing surveillance of pharmaceutical products. Such an approach should also become a prominent target in the treatment of individual animals species, particularly dogs and cats as they reach a high age and are treated for a larger variety of indications than any other animal species (Martinez et al., 2013). An evaluation of the available data on population kinetics indicated that drug biotransformation is one of the main drivers of differences in drug efficacy and safety. Drug biotransformation is species-specific, and pre-clinical experiments in rodent species might incompletely predict human drug handling. It can be assumed, that species differences in biotransformation are also the main cause of

variability in drug handling between for example cats and dogs, or cats and humans, if drugs established in human medicine for a certain indication are used in cats.

The aim of this review is therefore to present an overview of the current knowledge about the hepatic biotransformation of cats and the possible clinical implications for feline medicine.

GENERAL PRINCIPLES OF DRUG BIOTRANSFORMATION

Biotransformation includes the processes in the elimination or clearance of endogenous and exogenous compounds, such as drugs and other xenobiotics. Biotransformation involves different phases such as cellular uptake (generally denoted as Phase 0), enzymatic drug biotransformation and conjugation (Phase I and II) and excretion (Phase III). Each phase depends on the physico-chemical properties of the drug such as molecular configuration, lipophilicity and ionization at physiological pH.

Drug biotransformation involves the chemical modification of the parent drug by enzymes and the result is generally a less active metabolite. However, distinct reactions such as the formation of epoxides or imines, increase the reactivity and hence cellular toxicity and are likely to cause direct liver cell injury. The enzymatic modification of drugs by oxidation processes is in general denoted as phase I reactions, and the outcome is a more water-soluble product. If the drug molecule is not having any reactive groups that allows enzymatic oxidation or hydroxylation, the molecule can be conjugated to an endogenous cellular component, such as glutathione, sulfate, methionine and other amino acids. The major conjugation pathway in the liver, however, is the conjugation to glucuronic acid, that is abundantly produced by hepatocytes, but also by enterocytes, lining the intestinal tract. Drug conjugates are generally not biologically active, with some exceptions, such as the hepatic formation of morphine-6-glucuronide that retains biological activity. Again, the outcome of a conjugation reaction, generally noted as phase II reactions, is a more water-soluble product. Considering that both phase I and phase II reactions result in hydrophilic metabolites, it became apparent that cells, such as the hepatocytes, need to express specific transporters to facilitate the excretion of these metabolites that cannot rapidly cross cell membranes. Hepatocytes export drug metabolites via the basolateral and the canalicular membrane and hence possess a broad array of efflux transporters. As the export of metabolites is an inevitable part of drug biotransformation, the active

extrusion of drugs out of a cell is now generally denoted as phase III (for review see Döring & Petzinger, 2014).

The liver is regarded as the most important organ for the biotransformation because of its high content of metabolizing enzymes, and therefore this thesis mainly focuses on this organ. However, other organs, such as the intestines, kidneys and lungs, can play an important role as well. This thesis is particularly focused on the biotransformation and excretion phase (phase I, II and III) in the liver of cats and the following paragraphs present a summary of the current knowledge about the individual phases of drug biotransformation in feline livers. As in feline medicine, little is known about uptake transporters (phase 0) and only an OATP-2 like transporter is suggested in feline liver (Horii et al., 2004), the overview starts considering only phase I and phase II reactions. Figure 1 gives a schematic overview of the different phases of feline biotransformation.

PHASE I BIOTRANSFORMATION REACTIONS AND FELINE DRUG METABOLISM

Phase I biotransformation reactions comprise oxidation, reduction, dealkylation and hydrolysis (Table 1). The transformed products are less biological active with some well-known exceptions such as the formation of epoxides and imines mentioned above. The phase I metabolites can either be directly excreted or undergo a second phase conjugation reaction, described below.

It should be mentioned that these phase I reactions can be used also to convert a pro-drug into a biologically active metabolite. The concept of hepatic activation of products in used in some cases where the biologically active principle show an intestinal instability or a low absorption rate.

Cytochrome P450 (CYP)

The cytochrome P450 enzyme family is the most important and highly preserved groups of enzymes that catalyze phase I reactions of the majority of drugs. Cytochrome P450 enzymes (CYPs) are monooxygenases, found mostly membrane-bound in the endoplasmic reticulum and mitochondria of a mammalian cell. The CYP superfamily is classified into gene families on the basis of their amino acid sequences. Enzymes that are ≥40% identical in their amino acid level belong to a particular family designated by an Arabic numeral, whereas those that are ≥55% identical make up a particular subfamily designated by a letter. At present, 18 CYP families are distinguished in humans (Nebert & Russell, 2002; Zanger & Schwab, 2013). CYPs can be

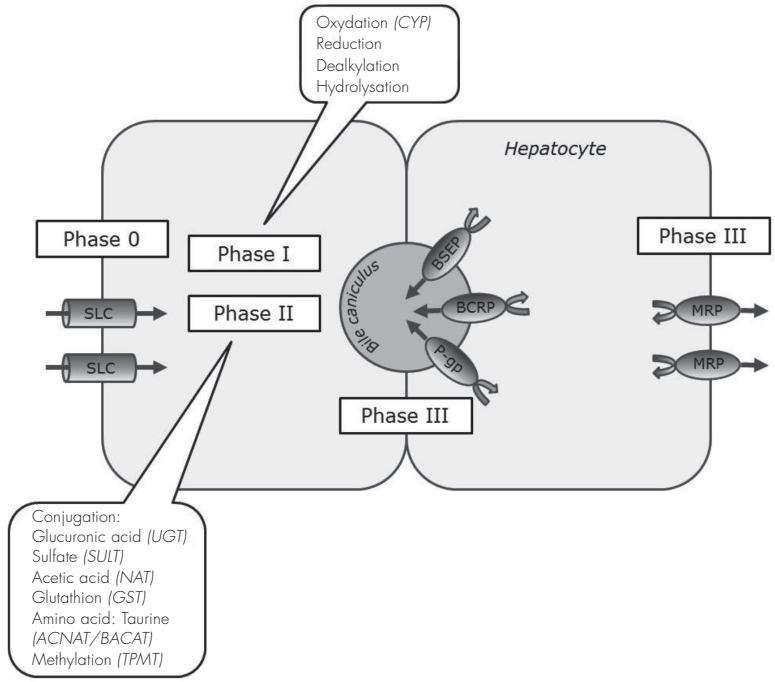
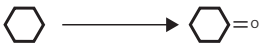
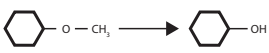
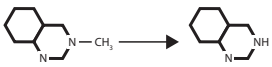
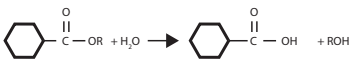


Figure 1. Investigated biotransformation pathways in feline species.
CYP=Cytochrome P450; UGT=UDP-glucuronosyltransferase; SULT=sulfotransferase; NAT=N-acetyltransferase; GST=glutathione S-transferase; ACNAT=acyl-coA: amino acid N-acyltransferase; BACAT=bile acid-coA: amino acid N-acyltransferase; TPMT=Thiopurine S-Methyltransferase; SLC=solute carrier (non-specified); BSEP=Bile Salt Export Pump; BCRP=Breast Cancer Resistance Protein; P-gp=P-glycoprotein; MRP=multidrug resistance-associated protein (non-specified)

Table 1. Most important biotransformation reactions in phase I of the biotransformation.

Reactiontype	**Chemical conversion**
Hydroxylation	
Oxidation	
Dealkylation: *O*-demethylation	
Dealkylation: *N*-demethylation	
Hydrolysis: ester	
Hydrolysis: amide	

compared between species regarding their DNA sequences, and homologous genes are then called orthologs.

Within the numerous CYP450 enzymes that are involved in the synthesis and inactivation of endogenous substrates such as hormones and neurotransmitters, the CYP1, CYP2 and CYP3 families facilitate particularly drug biotransformation (Nebert & Russell, 2002; Zanger & Schwab, 2013). It is estimated, based primarily on *in vitro* studies, that the metabolism of probably 30-50% of drugs used in humans involves CYP3A-mediated oxidation (Thummel & Wilkinson, 1998; Zanger & Schwab, 2013). The most important drug metabolizing CYP subfamilies are considered to be CYP1A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A (Zanger & Schwab, 2013). Each CYP subfamily metabolizes certain substrates, but between these different subfamilies overlap exists in substrate specificity (Brosen & Rasmussen, 1997). Despite this overlapping substrate specificity, many drugs are metabolized by mainly one or a few enzymes, which emphasizes the importance of knowing the function and expression of the responsible isozymes. Specifically for the human liver, the most important isozymes are CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 (Antonovic & Martinez, 2011). In particular the spectrum of drugs known to be metabolized at least partly by human CYP3A4, is extremely broad. Some drugs can decrease or increase the metabolizing capacity of this isozyme by means of enzyme inhibition (ketoconazole, cimetidine and erythromycin) or induction (barbiturates), respectively. Hence, drugs that are administered to a patient simultaneously may result in elevated or diminished plasma levels of one of the interacting drugs.

Methods for measuring the activity or substrate preference of CYPs are based on tissue homogenates (e.g. liver, intestines, kidneys) containing the CYP isozymes. By means of different centrifugation steps and enrichment, the membrane-bound enzymes are obtained. The final sub-mitochondrial fraction contains predominantly microsomes, vesicle-like artifacts re-formed of pieces of endoplasmatic reticulum. Total CYP content and activity in this microsomal fraction was first estimated by Omura and Sato (1964), who discovered that reduced CYPs form complexes with carbon monoxide and thereby produce a unique absorption peak at 450 nm (hence the name CYP450) (Omura & Sato, 1964). Microsomes can be incubated with different (human) marker substrates to measure a specific isozyme activity (Table 2). The products that are formed can be measured by e.g. high pressure liquid chromatography (HPLC), with fluorescence or UV detection. For humans and to a smaller extent for rodents, purified recombinant isozymes are nowadays commercially available, to test which isozyme is mainly responsible for the metabolism of a drug or other xenobiotic.

Table 2. Enzymatic activities associated with human CYPs (Antonovic & Martinez, 2011).

CYP	*Enzyme activity*	*Biochemical process*
CYP1A1	Phenacetin-O-deethylase, ethoxyresorufin O-deethylase	O-demethylation, O-deethylation, N-demethylation, N-hydroxylation
CYP1A2	7-ethoxy-4-trifluoro-methyl-coumarin-O-dealkylase	
CYP2B6		O-depentylation, 16-α-hydroxylation
CYP2C8, 2C9	Tolbutamide hydroxylase	O-demethylation, methyl hydroxylation
CYP2C19	S-mephenytoin-4-hydroxylation	4'-hydroxylation
CYP2D6	Dextromethorphan-O-demethylase, Bufuraolol 1'-hydroxylase	4'-hydroxylation, 1'-hydroxylation, O-demethylation
CYP2E1	Chlorzoxazone-6-hydroxylase, Nitrosodimethylamine N-demethylase	Hydroxylation, 6-hydroxylation, p-hydroxylation, N-demethylation, [ω-1]-hydroxylation
CYP3A4	Testosterone-6-β-hydroxylation, Erythromycin N-demethylase, midazolam 1'-hydroxylase	6-β-hydroxylation, oxidation, N-demethylation, O-debenzylation, hydroxylation

The expression of the individual enzymes is determined by species, age and gender, dietary components and diseases. In addition, and as mentioned above, certain drugs can inhibit or induce individual CYPs, resulting in different pharmacokinetic profiles and in turn in a different clinical outcome. This applies particularly to patients requiring multi-drug treatment. A reduced activity or less expression of certain isozymes results in a longer residence time of a parent drug in the body leading to drug accumulation and toxicity (slow metabolizers). On the other hand, a higher expression or activity of certain isozymes can result in a higher turnover of certain drugs (fast metabolizers), leading to a shorter half-live in the body and possibly to a lower therapeutic effect.

Cytrochrome P450 in cats and dogs

Wide interspecies variations in CYP activity have been reported (Shimada et al., 1997; Nebbia et al., 2003; Baririan et al., 2005; Martignoni et al., 2006). Already in the late 1970s, the total CYP content and activity of feline liver was measured and compared to other species, but no major differences were found between the CYP amounts of cats and dogs (Maugras & Reichart, 1979; Gregus et al., 1983). Chauret *et al.* (1997) investigated for the first time the *in vitro* CYP activity of the cat by means of HPLC. Human marker substrates were used to get a broad idea of the function of the feline isozymes, assuming that these human marker substrates were related to the

feline orthologs. It was recognized that there was no significant difference in CYP1A1/1A2 (phenacetin O-deethylase), CYP2C19 (S-mephenytoin 4'-hydroxylase), CYP2D6 (dextromethorphan O-demethylase) and CYP2E1 (chlorzoxazone 6-hydroxylase) activity between cats, dogs and humans. CYP2C8/2C9 (tolbutamide hydroxylase) activity was lower in cats than in dogs or humans, although CYP3A4 (testosterone 6β-hydroxylase) activity was similar in cats and dogs whereas humans showed a 7-fold higher activity (Chauret et al., 1997). Ten years later, Shah et al. (2007) also found CYP1A (ethoxyresorufin O-deethylation), CYP2C (tolbutamide hydroxylation), CYP2D (bufuralol 1'-hydroxylation) and CYP3A (midazolam 1'- and 4-hydroxylation) activities in feline liver microsomes, although CYP2C activity was negligible (Shah et al., 2007).

Although minor differences exist in CYP activity between cats and dogs, examples of major clinically relevant drug induced adverse reactions due only to these differences in CYP activity are not recognized. Canine CYP3A12 and CYP3A26, canine orthologs for human CYP3A4, are not playing such a prominent role in the hepatic CYP expression like CYP3A in humans (Martinez et al., 2013). For cats, little is known about the importance of CYP3A in the feline liver.

Until now, CYP1A1 and CYP1A2 (Tanaka et al., 2006), CYP2D6 (Komatsu et al., 2010; Schenekar et al., 2011), CYP2E1 and CYP2E2 (Tanaka et al., 2005), CYP3A131 and CYP3A132 (Honda et al., 2011) have been identified in cats. Cats and dogs showed the highest homology in amino acid sequence for CYP2E compared to other species, nevertheless the cat showed significantly lower CYP2E activity than dogs. Cats possess two highly similar CYP2E genes, while most mammals only possess a single gene (Tanaka et al., 2005). Both feline CYP3As showed also the highest homology in their open reading frame (ORF) with that of the dog. In Table 3, the ortholog genes are presented for human, dogs and cats.

In felines, some drugs which are known for their CYP inhibiting properties in man, turned out to be inhibitors of feline CYP3A (midazolam 1'- and 4-hydroxylation) in *in vitro* assays with feline liver microsomes (Shah et al., 2009). However, only ketoconazole did affect the plasma concentration of another CYP3A-metabolizing drug *in vivo*, while cimetidine had no influence (Shah et al., 2009). Ketoconazole in co-administration with cyclosporine A also inhibited the elimination of cyclosporine A in cats (McAnulty & Lensmeyer, 1999). On the other hand, phenobarbital, a well-known human CYP3A inducing drug, did not induce CYP activity or its elimination kinetics in cats after repeated dosing (Truhaut et al., 1978; Cochrane et al., 1990).

Table 3. Cytochrome P450 ortholog genes of human, dog and cat (Antonovic & Martinez, 2011).

Human	*Dog*	*Cat*
CYP1A1	CYP1A	CYP1A1
CYP1A2	CYP1A2	CYP1A2
CYP2B6	CYP2B11	
CYP2C8, 2C9, 2C19	CYP2C21, CYP2C41	
CYP2D6	CYP2D15	CYP2D6
CYP2E1	CYP2E1	CYP2E1, CYP2E2
CYP3A4	CYP3A12, CYP3A26	CYP3A131, CYP3A132

In conclusion, orthologs of isozymes can manipulate substrates similarly compared across species or have a similar reaction to certain compounds, but they can also react quite differently. Therefore, the functionality of each isozyme cannot simply be extrapolated to other species, but instead should be tested separately for each compound or drug within the species of interest.

PHASE II

The acquired functional group from phase I can conjugate with endogenous substances, such as glucuronic acid, acetic acid, glutathion, sulfate, or an amino acid. Compounds can also undergo phase II reactions directly if the drug has a functional group available for conjugation. As a result, these phase II metabolites are larger molecules, highly polar and mostly less pharmacological active than the parent compound, and can be excreted by transporter proteins. These conjugation reactions are catalyzed by various enzyme families. Most of these are embedded in membranes of the smooth endoplasmic reticulum, while some are freely dissolved in the cytosol. More detailed information about these conjugating enzymes in cats, is given below.

UDP-glucuronosyltransferases (UGTs)

The most common conjugating reaction is the glucuronide conjugation accomplished by the enzyme family of the UDP-glucuronosyltransferases (UGTs). UGTs are membrane-bound and conjugate a glycosyl group (e.g. glucuronic acid) to many lipophilic endogenous and exogenous substrates. Until now, four human UGT enzyme families are classified, i.e. UGT1, UGT2, UGT3 and UGT8, based on homology in amino acid sequence, substrate affinity and the difference in the conjugated endogenous molecule. UGT1 and

UGT2 catalyze the addition of UDP-glucuronic acid as a glycosyl group to the compound and are the most abundant enzyme families in the human liver. UGT1 is subdivided into the subfamilies UGT1A and UGT1B, just as UGT2 is subdivided in UGT2A and UGT2B (Mackenzie et al., 2005; Dong et al., 2012).

The activity of UGTs can be measured with the same methodology as for CYPs, as UGTs are also membrane-bound. Again, (human) probe substrates can be used to measure the activity of specific isozymes after microsomal incubation (Table 4), however overlap in substrate affinity between the different UGT isozymes is common (Court, 2005). In human liver, the most important UGT isozymes for hepatic drug and xenobiotic metabolism are: UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 (Ohno & Nakajin, 2009).

Already in the 1970s, it was described that cats have a low glucuronide capacity for phenolic derivatives (Capel et al., 1972a; Capel et al., 1972b; Capel et al., 1974). Decades later, Court and Greenblatt (1997) investigated the reason for this lack of glucuronidation in cats by means of acetaminophen (=paracetamol), also a phenolic derivative. Acetaminophen is notoriously known for its toxicity in cats, as cats can develop methemoglobinemia, cyanosis, respiratory distress, depression and vomiting after administration (Villar et al., 1998; McConkey et al., 2009). Doses of 10 to 50 mg/kg can already trigger these symptoms or can even cause death in cats (Allen, 2003), certainly with repeated dosing, while for humans these doses are generally safe and commonly applied.

Experiments with feline liver microsomes showed only a low formation of acetaminophen-glucuronide compared to dogs and human, suggesting that cats may either completely lack, or express very low levels of a functional species homologue of UGT1A6, the responsible isozyme for acetaminophen glucuronidation (Court & Greenblatt, 1997). Three years later, it was recognized that UGT1A6 is a pseudogene in cats with several deleterious genetic mutations, responsible for the lack of transcription and translation to a functional protein. A very low expression of other hepatic UGT1A isoforms was also suggested in cats, as low levels of UGT1A1 and UGT1A02 were found (Court & Greenblatt, 1997; Court & Greenblatt, 2000).

Since UGT1A6 is a pseudogene and cats lack UGT isoforms, it was suggested that cats can hardly glucuronidate drugs or other chemical substances. However, functional studies discovered some glucuronide formation in cats. Drugs that are well glucuronidated by cats are e.g. phenolphthalein (Pekanmaki & Salmi, 1961; Watkins & Klaassen, 1986), lorazepam (Schillings

Table 4. Potentially useful isoform-selective probe substrates for hepatic human UGTs (Court, 2005).

UGT	*Probe substrate*	*Other hepatic UGTs*
UGT1A1	Bilirubin 17β-Estradiol (3-OH)	None UGT1A3
UGT1A3	R-Lorazepam	None
UGT1A4	Trifluoperazine Imipramine	None None
UGT1A6	1-Naphthol (1-OH) Serotonin	None None
UGT1A9	Propofol	None
UGT2B4	Codeine	UGT2B7
UGT2B7	Morphine (3-OH) Morphine (6-OH) 3′-azidothymidine (AZT)	UGT1A3, 9, 2B4, 15, 17 UGT1A1, 3 UGT2B4, 17
UGT2B15	S-Oxazepam S-Lorazepam	UGT1A1, 6, 2B7 None
UGT2B17	Dihydrotestosterone	UGT2B7, 15

et al., 1975; Elliott, 1976; Ruelius, 1978), pradofloxacin (EMEA/V/C/099), ibuprofen (Magdalou et al., 1990), and telmisartan (Ebner et al., 2013). Telmisartan and ibuprofen are even glucuronidated with a higher rate in feline hepatic microsomes than in other species. Knowledge of the responsible UGT isozymes for the metabolism of all these drugs is not comprehensive, but for the most recently investigated compound telmisartan in cats it is known that in human hepatocytes this drug is metabolized mainly by UGT1A3 (Yamada et al., 2011).

Glucuronides were found for certain drugs, but glucuronidation is also essential for the elimination of endogenous substrates in cats and other animal species. Glucuronides of bilirubin were found in feline bile, mainly as diglucuronide bilirubin but also as monoglucuronide. *In vitro* incubations of cat liver homogenates or microsomal preparations showed also that both bilirubin mono- and diglucuronide could be formed (Fevery et al., 1977). In humans, bilirubin glucuronidation is accomplished by UGT1A1 (Bosma et al., 1994), suggesting a functional UGT1A1 homologue in feline liver (Court & Greenblatt, 1997; Court & Greenblatt, 2000). Moreover, the excretion of thyroxine in cats is mainly in the form of glucuronides (Myant, 1966). In humans, it is known that thyroxine is mainly glucuronidated by UGT1A1 and 1A3 (Kato et al., 2008); again suggestive for a functional UGT1A1 and UGT1A3 homologue in cats. As these glucuronides can be found in cats, the

general opinion that cats are entirely lacking glucuronidation capacity, needs to be revisited. Therefore, the UGT-activity should be investigated in cats more thoroughly for each individual drug. Drugs with a high rate of glucuronidation in human and veterinary species, are listed in Table 5.

Drug glucuronide conjugates require an active transport out of the liver cell. In cats, these transport mechanisms have not been studied as yet.

Sulfotransferases (SULTs)

Sulfation of drugs is performed by sulfotransferases (SULTs), which conjugate 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to an endogenous or exogenous substrate. To date, two classes of human SULTs, cytosolic and membrane-bound, have been discovered and were divided in four families, i.e. SULT1, SULT2, SULT4 and SULT6, which are also divided in different subfamilies. The cytosolic SULTs are responsible for the sulfation of xenobiotics and endogenous compounds such as steroids, bile acids and neurotransmitters. The membrane-bound SULTs are involved in the metabolism of proteins and lipids.

Considering the low glucuronidation capacity in cats, it was suggested that cats have much higher sulfate conjugation capacity than other animals. Indeed, cats show a relative high SULT activity for the substrates taurolithocholate and estrone compared to dogs (Gregus et al., 1983; Watkins & Klaassen, 1986). High amounts of mono- and disulfates were found in the bile of cats after receiving phenolic steroids such as estrone and 17β-estradiol (Quamme et al., 1971). Morphine-3-glucuronide, a marker substrate for human UGT2B activity, was found at very low concentrations in urine and feces after administration of morphine to cats. Morphine-3-ethereal sulfate was found as the major conjugated metabolite (Yeh et al., 1971). Also acetaminophen, which in humans is mainly catalyzed by UGT1A6, is being sulfated to a high extent in cats (Savides et al., 1984). Although data about SULTs of cats are still limited, the current knowledge on the formation of sulfate conjugates in cats supports the notion of an escape mechanism for the low feline glucuronidation capacity compared to other species. However, the sulfation pathway can be easily saturated and cannot fully compensate for the low glucuronidation capacity (Savides et al., 1984).

N-acetyltransferases (NATs)

N-acetyltransferases (NATs) are enzymes responsible for the acetylation of xenobiotics and endogenous compounds. NATs are not well described for cats, but it is known that cats only possess NAT1, although other mammals also have NAT2 (Trepanier et al., 1998). One of the exceptions is the dog, where none of the NATs have been found as they lack both genes (Trepanier et al., 1997). NAT2 deficiency is partly responsible for the sensitivity of paracetamol

Table 5. Drugs that have glucuronidation listed as an important clearance mechanism in human and veterinary species (Miners & Mackenzie, 1991; Williams et al., 2004; Kiang et al., 2005; Kaivosaari et al., 2011). The table contains a selection of drugs which could be prescribed for or (accidentally) taken by companion animals. In bold, drugs with a narrow safety margin in cats.

Drugs with a high percentage of glucuronidation		
Acetaminophen	*Fluoroquinolones*	***Naproxen***
Alprazolam	*(mainly pradofloxacin)*	*Oxazepam*
Benoxaprofen	*Flurbiprofen*	***Phenylbutazone***
Buprenorphine	***Ibuprofen***	*Pirprofen*
Carprofen	*Ketoprofen*	*Propofol*
Codeine	*Levothyroxine*	***Pyrethroids***
Chloramphenicol	***Lindane***	*(e.g.* ***permethrin,***
Cyproheptadine	*Lorazepam*	*except for flumethrin)*
(Dex)medetomidine	*Metoclopramide*	*Salicylate*
Diazepam	*Metronidazole*	*Suprofen*
Diclofenac	*Midazolam*	*Temazepam*
(Ethinyl)estradiol	*Mirtazapine*	*Valdecoxib*
Fenoprofen	*Morphine*	
Firocoxib	*Naloxone*	

in cats. Acetaminophen (APAP) can be metabolized by carboxyesterases forming the deacetylated *para*-aminophenol (PAP) (Song & Chen, 2001), which is normally acetylated back to APAP by NAT2 in the erythrocytes. Because of lack of NAT2 in cats, an excessive amount of PAP is formed and oxidates hemoglobin to methemoglobin (McConkey et al., 2009). Other compounds that are acetylated slowly in feline liver are sulfamethazine, isoniazid and some sulfonamide antimicrobials (Gregus et al., 1983; Noli et al., 1995).

Taurine conjugation

Taurine is the most abundant free amino acid in mammals and is contained in the diet or is synthesized from cysteine or methionine in most species. Taurine, which consists of a sulfonic acid and an amino group, is an essential amino acid in cats as it was found that cats possess very low activities of the enzymes L-cysteine sulfinic acid decarboxylase (CSAD) (de la Rosa & Stipanuk, 1985) and cysteine dioxygenase (CD) (Knopf et al., 1978; Park et al., 1991). The formation of taurine is demonstrated schematically in Figure 2. It was found that cats do not utilize dietary cysteine sulfinic acid as a precursor for taurine. However, cysteic acid may replace partly or entirely the dietary taurine needs of cats, while this is also metabolized by CSAD (Edgar et al., 1994). The

dietary cysteine sulfinic acid appeared to be efficiently transaminated into pyruvate and sulfite, instead of decarboxylated into taurine (Edgar et al., 1998). Moreover, high activities of cysteine desulfhydrases, which produce pyruvate out of cysteine, were found in cats (Park et al., 1991). Therefore, the deficient taurine production in cats is most probably caused by a combination of a low CD activity and a low affinity of CSAD for cysteine sulfinic acid.

Taurine can be conjugated to bile acids, lipids or drugs containing carboxyl groups. The conjugation of taurine is performed by acyltransferases, where acyl-coA: amino acid N-acyltransferase (ACNAT1) serves for the conjugation of taurine to fatty acids, and bile acid-coA: amino acid N-acyltransferase (BAT, BAAT or BACAT) serves for the conjugation of taurine to bile acids, as well as fatty acids. BACAT was found in human liver cytosol (Falany et al., 1994) and in rat hepatic peroxisomes (Rembacz et al., 2010). BACAT has been characterized from several species and shows large species differences in the use of glycine or taurine as an acceptor molecule. Probably, the structural differences of the enzyme account for the differences in substrate specificity (Falany et al., 1994; O'Byrne et al., 2003; Reilly et al., 2007). BACAT is very selective in the amino acids that can be conjugated to bile acids, as simple homologs of taurine and glycine cannot act as a substrate for BACAT (Falany et al., 1994).

The bile acid composition of cat and dog gall-bladder bile revealed that the bile acids in cats and dogs are almost exclusively conjugated with taurine (Washizu et al., 1990). In contrast, in humans the most predominant bile acid is glycine-conjugated (Washizu et al., 1990; Washizu et al., 1991). It is at least remarkable that cats only conjugate their bile acids to taurine, while taurine is an essential amino acid for this species.

Taurine can also be used in the biotransformation of xenobiotics, especially NSAIDs containing carboxyl groups such as the profens. In dogs, diclofenac and fenclofenac are excreted as taurine conjugates (Jordan & Rance, 1974; Stierlin et al., 1979; Tsuchiya et al., 1980). Tremendous taurine and glycine conjugation was observed for 1-naphthylacetic acid in cats (Dixon et al., 1977a). Clofibric acid, a drug used as a hypolipidaemic, is metabolized by glucuronic acid conjugation in rat, pig, rabbit and man, but is extensively metabolized by taurine conjugation in cats, dogs and ferrets. The authors attribute the taurine conjugation to the carnivorous diet of these species (Dixon et al., 1977b; Emudianughe et al., 1983). Although taurine is an essential amino acid for cats, taurine conjugation or conjugation with another amino acid, could be an important mechanism in the biotransformation of drugs in carnivores.

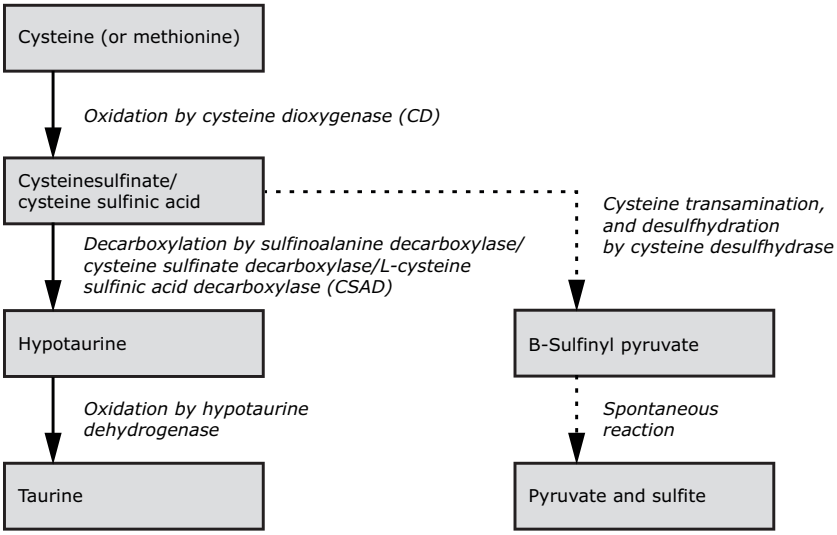


Figure 2. Catabolism of cysteine and metabolism of taurine in mammals. The alternative route of the catabolism of cysteine in cats is indicated by the interrupted line.

Thiopurine S-Methyltransferase (TPMT)

Thiopurine *S*-Methyltransferase (TPMT) is involved in the detoxification of thiopurine drugs such as azathioprine, which is used for anti-cancer therapy and as an immunosuppressant. Cats are very sensitive for azathioprine and can show severe bone marrow suppression (Beale et al., 1992), probably because they possess a low TPMT activity in their erythrocytes compared to other species (Foster et al., 2000; White et al., 2000; Salavaggione et al., 2004). The TPMT activity in feline liver has never been studied but the current knowledge on the low TPMT activity in red blood cells refers to genetic polymorphisms in the feline TPMT gene (Salavaggione et al., 2004).

PHASE III

Efflux transporters in hepatocytes

After passing the first and/or the second phase of the biotransformation, the metabolite is more hydrophilic and needs to be actively excreted by the hepatocyte. Transporter-proteins embedded in the canalicular (apical) or basolateral (sinusoidal) membrane mediate the transport of these drugs or metabolites to the bile or into the sinusoidal blood, respectively. These active transporters were found not only in liver cells but also in various other organs. As they use ATP as energy source for transporting compounds against a concentration gradient, they are collectively called ATP-Binding Cassette (ABC) transporters. Historically, ABC-transporters have been discovered in tumor cells that became resistant to chemotherapy by expressing these efflux transporters. Chemotherapeutic drugs were excreted out of the tumor cells almost as fast as the uptake of the drug, preventing that cytostatics reach their intracellular targets and thus resulting in a reduced efficacy. The best-known mammalian ABC-transporters are P-glycoprotein (P-gp, MDR1, ABCB1), the Breast Cancer Resistance Protein (BCRP/ABCG2), the multidrug resistance-associated protein 2 (MRP2/ABCC2) and 4 (MRP4/ABCC4) and the Bile Salt Export Pump (BSEP/ABCB11).

P-glycoprotein, MDR1 (ABCB1)

The multi-drug resistance protein (MDR1/ABCB1) or permeability glycoprotein (P-gp) is expressed by hepatocytes, intestinal epithelial cells, renal tubular cells and vascular endothelial cells and in various other cell types (Mealey, 2004). The transporter is involved in the efflux of endogenous compounds, such as hormones and neurotransmitters. In 1976, P-gp was described first in Chinese hamster ovary cancer cells which were resistant to colchicines and chemotherapeutic agents (Juliano & Ling, 1976). P-gp is now generally related to multi-drug resistance in anti-cancer therapy (Darby et al., 2011). As a possible solution for the multi-drug resistance in anti-cancer therapy, inhibitors for human P-gp have been developed (Coley, 2010; Choi & Yu, 2014). However, in clinical practice various of these P-gp inhibitors exert undesirable side effects when co-administered with cytostatic agents, and hence are not commonly used (Coley, 2010).

Almost 20 years after the first description of P-gp, Schinkel *et al.* described in 1996 for the first time that not only cytostatics are substrates for P-gp, but also entirely different classes of drugs, such as the antiparasitic drug ivermectin (Schinkel et al., 1996). The observation that P-gp is a functional element of the blood-brain-barrier was a major hallmark in research devoted to the efflux transporters. In veterinary medicine, where ivermectin is widely used, Mealey

et al. identified in 2001 that a polymorphism in the Mdr1 gene accounts for the clinically observed neurotoxic effects, such as vomiting, depression, drooling, ataxia, tremors and coma, observed following ivermectin application in certain dog breeds (Mealey et al., 2001). The dysfunction of Mdr1 in Collie breeds (but also various other sheepdogs) caused by a 4-pair deletion in the Abcb1 gene, is now one of the most prominent clinically relevant polymorphisms of drug transporters in animals. Examples of veterinary important drugs that are P-gp substrates, are steroid hormones (e.g. dexamethasone, methylprednisolone), antimicrobial agents (e.g. erythromycin, fluoroquinolones, doxycycline, itraconazole), opioids (e.g. loperamide, morphine), cardiac drugs (e.g. digoxin, diltiazem, verapamil) and immunosuppressants (e.g. cyclosporine, tacrolimus) (Martinez et al., 2008).

In the liver, P-gp is located on the canalicular membrane of the hepatocytes, where it actively transports neutral and cationic substances into the bile (Fardel et al., 1996). Overlap exists in the substrate affinity between P-gp and CYP3A4 in humans and probably in many animal species, as CYP3A and P-gp share a common transcription factor.

In cats, P-gp was first found *in vitro* in feline lymphoma cells, where it was suggested that the basic structure of this feline ortholog and its role in multi-drug resistance was essentially the same as in other species (Okai et al., 2000). P-gp expression was demonstrated in cats with lymphoma (Brenn et al., 2008), pulmonary carcinoma (Hifumi et al., 2010) and other feline tumours (Van der Heyden et al., 2011), but the rate of P-pg expression was not predictive for remission or survival time. Mdr1 organ distribution has also been investigated in cats. Using a defined antibody (C494) it could be demonstrated that in cats a similar distribution of P-gp was found as in humans and dogs. At the same time, P-gp expression of all well-known biological barriers was confirmed (Van der Heyden et al., 2009).

Functional studies on feline P-gp have not been performed. However, drugs that are inhibiting P-gp function could also cause adverse reactions.

BCRP (ABCG2)

The human Breast Cancer Resistance Protein (BCRP/ABCG2), again first identified in a human breast cancer cell line, is expressed by different organs including the intestines, liver, kidney, brain and retina, and forms a biological barrier for potentially toxic compounds (Mao & Unadkat, 2014). In the liver of mice, Bcrp is responsible for the biliary excretion of hydrophilic sulfate conjugates and glucuronide conjugates. However, the latter are also transported by multidrug resistance-associated protein Mrp2.

In cats, one of the investigated substrates for Bcrp is the antibiotic enrofloxacin, which belongs to the group of fluoroquinolones. Fluoroquinolones are photoreactive compounds and Bcrp protects the retina from phototoxicity caused by these drugs. However, feline retinal Bcrp was found to have only minimal protection against enrofloxacin, causing retinal degeneration and blindness in cats receiving this drug in high doses. The functional defect of feline Bcrp was caused by four feline-specific amino acid changes in highly conserved areas of Abcg2 compared to other species (Ramirez et al., 2011). Orbifloxacin, another fluoroquinolone, also causes retinal damage in cats (Wiebe & Hamilton, 2002).

It is known that in most species Bcrp mediates the biliary excretion of acetaminophen sulfate (Zamek-Gliszczynski et al., 2006), and this effect could occur to a lesser extent in cats when they have dysfunctional hepatic Bcrp. Therefore, after saturation of the glucuronidation and sulfation pathway, the metabolization of acetaminophen turns to the cytochrome P450 pathway, resulting in the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI), causing most adverse side effects in cats. However, specific functional studies on feline hepatic Bcrp or investigations into Bcrp polymorphisms have not been performed as yet.

MRP2 (ABCC2)

The multidrug resistance-associated protein 2 (MRP2/ABCC2), which is expressed on the bile canalicular membrane, actively transports diverse substrates from the hepatocyte into the bile canaliculi (Suzuki & Sugiyama, 2002). MRP2 in humans can also be found on the apical membrane of epithelial cells of enterocytes and on the apical membrane of the renal proximal tubule epithelium, where it actively transports compounds respectively into the intestinal lumen and renal collective ducts. MRP2 can also be found in some tumor tissues, such as ovarian carcinoma, colorectal carcinoma, leukemia, mesothelioma and hepatocarcinoma (Suzuki & Sugiyama, 2002). MRP2 substrates include glucuronides (for example bile salt glucuronides, telmisartan glucuronide, acetaminophen glucuronide and 1-naphtyl-beta-D-glucuronide), glutathione conjugates, sulfated bile salts, but also non-conjugated organic anions such as ampicillin (Suzuki & Sugiyama, 2002). It was found that polymorphisms in the human ABCC2 gene could lead to patients developing a Dubin-Johnson syndrome, which is characterized by conjugated hyperbilirubinemia and chronic jaundice, as bilirubin glucuronides are substrates for MRP2 (Konig et al., 1999).

Mrp2 has been investigated in dogs, where canine Mrp2 was compared to rats. Dogs showed a much lower expression of Mrp2 in the liver than rats, but the canine Mrp2 also showed an additional low-affinity site, which made a major contribution to the transport of glucuronide conjugates (Ninomiya et al., 2005). For cats, little is known about Mrp2 expression, distribution or function. Mrp2 was detected in liver tissue of cats by immunohistochemistry (Ijzer et al., 2009), but functional studies are absent. It could be assumed that cats probably have a limited expression and activity of Mrp2 in the liver, as this species has an impaired production of glucuronides.

OUTLINE OF THE THESIS

As outlined in this chapter (**Chapter 1**), the current knowledge of the different phases involved in the biotransformation of drugs in cats is still very limited. In the subsequent chapters, each phase has been represented by different investigations with the aim to contribute to a better understanding of feline biotransformation. As in daily practice, because of the absence of drugs that are specifically licensed for use in cats, drugs intended for dogs are regularly used, a comparison between cats and dogs is presented to emphasize the differences between these species. Where appropriate, results were compared to results from rodents and human studies, as here the knowledge on drug metabolism is much more advanced.

Chapter 2 focuses on phase I of the biotransformation, with the most important oxidizing enzyme family Cytochrome P450 (CYP). Insight into the species-specific biotransformation by CYPs is of importance in drug development and in the veterinary clinic with the treatment of patients. Drugs can be substrates, as well as inducers or inhibitors of CYP isozymes, which can result in a shortening or a prolongation of the duration of action of a drug, drug-drug interactions and unexpected side effects (Trepanier, 2006; Fink-Gremmels, 2008). It was one of the aims of this study to provide a summation of the activity of the CYP enzymes of cats and dogs, using a rapid screening fluorometric assay, originally developed for human CYP investigation. Our hypothesis was that this assay could be used in a clinical routine, as this assay is fast and does not require extensive analytical skills and technology. In this way, individualized medicine could be implemented into veterinary medicine, which provides optimal drug therapy for each patient.

Chapter 3 aims at the most important chemical reactions of phase II biotransformation, i.e. the conjugation of drugs with glucuronic acid. It is well-known that cats lack glucuronidation capacity due to an inactive gene. In

contrast to this general assumption, for some drugs or endogenous substances, high concentrations of glucuronides can be observed in cats. Therefore, our investigations were intended to re-assess the overall glucuronidation capacity of feline liver, using prototypic substrates, identified to be specific for individual human UGT isozymes, as this approach was expected to contribute to the understanding which specific enzymes are involved in the limited, but clearly observed glucuronidation activity of cats.

Chapter 4 describes the investigation into the feline bile salt export pump (Bsep), which is part of phase III of the biotransformation. Although adverse side effects following medication can be very diverse in appearance in cats, and include anorexia, vomiting and lethargy, jaundice appears to be a typical symptom observed in many feline patients, indicating that cats are sensitive to liver dysfunctions, such as cholestasis, hepatic lipidosis or cholangitis (Center, 1999; Center, 2005; Otte et al., 2011). One of the major efflux transporters facilitating the excretion of bile acids is the Bile Salt Export Pump (BSEP/ ABCB11), which is a member of the ATP-Binding Cassette (ABC) transporters. Inhibition of this transporter has been related to drug-induced liver injury (DILI) in man, as inhibition of BSEP can lead to high intracellular bile acid concentrations and subsequent cytotoxic effects (Kullak-Ublick & Meier, 2000). Therefore, we hypothesized that an impairment of Bsep function may also explain the higher sensitivity of cats to drug-induced impairment of liver functions. However, data on feline Bsep were completely absent. The aim of this research was to investigate the functional characteristics of feline Bsep in comparison with canine and human Bsep/BSEP, with respect to substrate affinities and specific inhibitors of Bsep function. To this end, we conducted a DNA sequence analysis and cloned the obtained feline Bsep (as well as canine and human Bsep for comparison) into cell membrane vesicles for functional analysis.

Chapter 5 is devoted to the biotransformation of diazepam. This drug was selected, as it is well-known that cats are sensitive to diazepam, and readily develop liver injury after repeated dosing. The biotransformation of diazepam is complex and involves phase I oxidation reactions as well as glucuronidation of the parent compound and its metabolites. This implies that also the hepatic efflux transporter Bsep may be involved in the low tolerance of cats to diazepam treatment. Hence we applied the methodological approach used in the previous chapter to investigate the biotransformation of diazepam in more detail in feline *in vitro* models.

Chapter 6 continues with the investigation of diazepam and its metabolites, but is now focused on the export of these compounds out of the cell. Our

hypothesis was that P-glycoprotein (P-gp), another member of the ABC-transporters, could be also responsible for the excretion of diazepam or its metabolites into the bile. Therefore, the excretion of diazepam and its metabolites was tested on feline lymphoma cells overexpressing feline P-gp. Moreover, human and dog marker P-gp inhibitors were also tested with these feline lymphoma cells, to investigate the functional characteristics of feline P-gp.

In Chapter 7 the results of the studies in this thesis are discussed and placed into a broader perspective, with emphasis on the clinical implications in veterinary practice.

Chapter 8 provides a brief summary of the obtained results.

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CHAPTER 2

CYTOCHROME P450-MEDIATED HEPATIC METABOLISM OF NEW FLUORESCENT SUBSTRATES IN CATS AND DOGS

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ABSTRACT

This study aimed to investigate the biotransformation of cat liver microsomes in comparison to dogs and humans using a high throughput method with fluorescent substrates and classical inhibitors specific for certain isozymes of the human cytochrome P450 (CYP) enzyme family. The metabolic activities associated with CYP1A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A were measured. Cat liver microsomes metabolized all substrates selected for the assessment of cytochrome P450 activity. The activities associated with CYP3A and CYP2B were higher than the activities of the other measured CYPs. Substrate selectivity could be demonstrated by inhibition studies with α-naphthoflavone (CYP1A), tranylcypromine/ quercetine (CYP2C), quinidine (CYP2D), diethyldithiocarbamic acid (CYP2E) and ketoconazole (CYP3A) respectively. Other prototypical inhibitors used for characterization of human CYP activities such as furafylline (CYP1A), tranylcypromine (CYP2B) and sulfaphenazole (CYP2C) did not show significant effects in cat and dog liver microsomes. Moreover, IC50 values of cat CYPs differed from dog and human CYPs underlining the interspecies differences. Gender differences were observed in the oxidation of 7-ethoxy-4-trifluoromethylcoumarin (CYP2B) and 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (CYP2D), which were significantly higher in male cats than in females. Conversely, oxidation of the substrates dibenzylfluorescein (CYP2C) and 7-methoxy-4-trifluoromethylcoumarin (CYP2E) showed significant higher activities in females than in male cats. Overall CYP-activities in cat liver microsomes were lower than in those from dogs or humans, except for CYP2B. The presented difference between feline and canine CYP-activities are useful to establish dose corrections for feline patients of intensively metabolized drugs licensed for dogs or humans.

INTRODUCTION

Each year new drugs are developed and licensed for veterinary use. Drugs need to be licensed per animal species, especially drugs for food-producing animals. In companion animals, more substances are available for dogs than for cats, and compounds which are intended for canine or human patients are often also used in feline medicine with only a general adaptation in dosing regimens to (metabolic) body weight (body weight $^{0.75}$). However, extrapolations solely based on metabolic body weight or body surface area may be insufficient for all substances that are extensively metabolized, as significant interspecies variations in cytochrome P450-activity have been reported (Shimada et al., 1997; Nebbia et al., 2003; Baririan et al., 2005).

Cytochrome P450 enzymes (CYPs) are involved in the metabolism of numerous xenobiotics (including drugs) and endogenous substances. The ultimate goal of biotransformation processes is to convert a substance into metabolites, which are less active than the parent compound or even inactive, less lipid-soluble, more polar and more suitable for elimination by renal and/or biliary excretion (for review see: Anzenbacher & Anzenbacherova, 2001; Nebert & Russell, 2002; Zuber et al., 2002; Fink-Gremmels, 2008).

The CYP superfamily consists of a large number of isozymes, which are classified into gene families on the basis of their amino acid sequence. The most important enzyme families for drug biotransformation are CYP1, CYP2 and CYP3, and to a lesser extent CYP4 (Nebert & Russell, 2002). These families are further divided into subfamilies. The five most important drug metabolizing CYP subfamilies in humans are CYP1A, CYP2C, CYP2D, CYP2E and CYP3A. Each CYP subfamily metabolizes a distinct set of substrates but there is a considerable overlap between the different subfamilies in substrate specificity (Brosen & Rasmussen, 1997).

The expression and activity of these isozymes have been investigated primarily in rodents, as surrogates for humans in drug development (Shimada et al., 1997; Zhao & Ishizaki, 1997; Eagling et al., 1998; Bogaards et al., 2000). For example, similar CYP3A activities were found in male rat liver microsomes and human liver microsomes with respect to the 6-hydroxylation of dexamethasone (Tomlinson et al., 1997). Bogaards *et al.* confirmed the similarity in CYP3A activity of male rats, mice and humans by measuring the testosterone 6β-hydroxylase activity. They also found similarities in *7*-ethoxyresorufin *O*-dealkylase activity (CYP1A), *7*-ethoxy-4-trifluoromethyl-coumarin O-dealkylase activity (CYP2B) and diclofenac 4'-hydroxylase (CYP2C9) between these species (Bogaards et al., 2000).

In recent years, the CYP activity has been investigated for many animal species that represent veterinary patients and considerable interspecies variations have been found (Shimada et al., 1997; Nebbia et al., 2003; Baririan et al., 2005). Relatively little is known about the biotransformation of dogs (Chauret et al., 1997; Zhao & Ishizaki, 1997; Roussel et al., 1998; Shou et al., 2003; Lu et al., 2005) and in cats this knowledge is even more limited (Maugras & Reichart, 1979; Chauret et al., 1997; Tanaka et al., 2005; Shah et al., 2007). Insight into the species-specific biotransformation by cytochromes is of importance in drug development and in the veterinary clinic, as drugs can be substrates, as well as inducers or inhibitors of CYP isozymes. This can result in a shortening or a prolongation of the duration of action, drug-drug interactions and unexpected side effects (Trepanier, 2006; Fink-Gremmels, 2008).

For cats, the highest cytochrome activities were found in the phenacetin O-deethylase (CYP1A) and the testosterone 6β-hydroxylase (CYP3A). In dogs, these activities were high as well but comparable to the chlorzoxazone 6-hydroxylase activity (CYP2E) (Chauret et al., 1997) and also the aniline *p*-hydroxylation (CYP2E), benzphetamine *N*-demethylation and nifedipine oxidation (CYP3A) (Shimada et al., 1997).

In consideration of these obvious differences, it was one of the aims of the present study to provide a summation of the activity of the CYP enzymes of cats and dogs. To this end a rapid screening fluorometric assay developed for human CYP investigation was used. The fluorometric assay is based on a cytochrome catalyzed reaction that converts a substrate into a quantifiable fluorescent product (Crespi et al., 1997).

The second aim of this study was the evaluation of the suitability of this assay, which is fast and does not require extensive analytical skills and technology, in a clinical routine.

In a comparative approach the metabolic activity of the isozymes CYP1A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A were measured in cat and dog liver microsomes. The substrates for these isozymes were 3-cyano-7-ethoxycoumarin (CEC), 7-ethoxy-4-trifluoromethylcoumarin (EFC), dibenzylfluorescein (DBF), 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), 7-methoxy-4-trifluoromethylcoumarin (7-MFC) and 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) respectively. Substrate specificity was estimated using human prototypical inhibitors of these isozymes, with furafylline/α-naphthoflavone as inhibitors for CYP1A, tranylcypromine for CYP2B, tranylcypromine/quercetine/sulfaphenazole for CYP2C, quinidine for CYP2D, diethyldithiocarbamic acid (DETC) for CYP2E and ketoconazole for CYP3A.

MATERIALS AND METHODS

Drugs and chemicals

3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), 3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin-HCl (AHMC), Dibenzylfluorescein (DBF) and 7-Hydroxy-4-trifluoromethylcoumarin (7-HFC) were purchased from BD Gentest (Woburn, MA, USA). 7-Benzyloxy-4-(trifluoromethyl)-coumarin (BFC), Diethyldithiocarbamic acid (DETC), 7-Ethoxy-4-trifluoromethylcoumarin (EFC), fluorescein, furafylline, glucose-6-phosphate, ketoconazole, 7-methoxy-4-trifluoromethylcoumarin (7-MFC), NADP, sulfaphenazole, tranylcypromine and quinidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-Cyano-7-ethoxycoumarin (CEC) and 3-Cyano-7-hydroxycoumarin (CHC) were purchased from Ultrafine Chemicals (Manchester, UK).

Quercetine was purchased from Indofine Chemical Company. Glucose-6-phosphate dehydrogenase was obtained from Roche Diagnostics GmbH (Mannheim, Germany), and magnesium chloride hexahydrate was from BDH Chemicals Ltd (Poole, England). All other reagents and solvents used were of analytical grade.

Preparations of submitochondrial fractions

Cats

The isolation of submitochondrial fractions (commonly referred to as microsomes) containing predominantly microsomal proteins followed the procedure as described by Rutten *et al.* with minor modifications (Rutten et al., 1987). In brief, cat liver samples of approximately 10 g were extracted from adult healthy cats (n=10, five males and five females, aged ± 1 year) directly after euthanasia and were quickly frozen by liquid nitrogen and stored at -70°C. The cats had served in an authorized study for the development of FIV vaccines and had been sacrificed as cell donors. The tissue samples were homogenized with 1.15% KCl, containing 0.1 mM EDTA at 4°C. The homogenates were centrifuged at 9000 *g* for 25 min at 4°C, and the supernatant obtained (S9-fraction) was centrifuged at 100,000 *g* for 1 hour and 15 min at 4°C. The microsomal pellet was resuspended in 1.15% KCl 0.05 M phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol, quickly frozen in liquid nitrogen and stored in Eppendorf-cups at -70°C until use.

Dogs

Beagle dog liver microsomes (n=8, four males and four females, pooled, aged ≥ 12 months) were purchased from BD Gentest (Woburn, MA, USA). The microsomes were stored in Eppendorf-cups at -70°C until use.

Humans

Human liver microsomes (n=17, ten males and seven females, pooled) were purchased from BD Gentest (Woburn, MA, USA). The microsomes were stored in Eppendorf-cups at -70°C until use.

The protein concentrations of the microsomal fractions were determined by the method of Lowry using BSA (bovine serum albumin) as a standard (Lowry et al., 1951).

Enzyme assays

The metabolic activity of the isozymes was measured by means of a fluorescence-based method in flat-bottom 96-well plates according to manufacturer's instructions with minor changes to optimize the conditions for the feline and canine microsomes (http://www.bdbiosciences.com). Briefly, assays were performed by incubating liver microsomes (final protein concentration of 0.25 mg/mL) in a 200 µL volume with 0.5 M potassium phosphate buffer (pH 7.4), NADP (1.3 mM), glucose-6-phosphate (3.3 mM), $MgCl_2$ (3.3 mM), glucose-6-phosphate dehydrogenase (0.4 U/mL) and CEC, EFC, DBF, AMMC, 7-MFC and BFC as substrates for CYP1A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A respectively, after a preincubation of 10 min at 37°C (an overview is given in Table 1). After an incubation time of 60 minutes at 37°C for cat and dog liver microsomes, the reactions were stopped by adding 75 µL STOP-solution, BD Gentest (Woburn, MA, USA) (80% acetonitrile/20% 0.5 M Tris base or 2N NaOH). The fluorescence was measured using a Fluostar Optima BMG (B&L Systems, Maarssen, The Netherlands) and the amount of formed product was calculated by means of a standard curve.

To obtain IC50 values, enzyme activity was measured after addition of various concentrations of the inhibitors furafylline/α-naphthoflavone, tranylcypromine, quercetine/tranylcypromine/ sulfaphenazole, quinidine, DETC and ketoconazole for CYP1A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A respectively. IC50 values were calculated using a nonlinear curve fitting program (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA).

Statistical analysis

Data were analysed using a one-way analysis of variance (ANOVA) followed by the Bonferroni post test (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA) with $P < 0.05$ denoting a significant difference.

Table 1. Fluorescent detection parameters of the different hepatic CYP isozymes with their specific substrates, products and inhibitors.

Fluorescent Detection Parameters					
Enzyme	**Substrate**	**Product**	**Inhibitor**	**Excitation** (Bandwidth of filter), nm	**Emission** (Bandwidth of filter), nm
CYP1A1	BzRes	Resorufin	α-Naphthoflavone	409 (20)	460 (40)
CYP1A2	CBC	CHC	Furafylline	409 (20)	460 (40)
CYP2B6	EFC	HFC	Tranylcypromine	409 (20)	530 (25)
CYP2C8	DBF	Fluorescein	Quercetin	485 (20)	538 (25)
CYP2C9	7-MFC	HFC	Sulfaphenazole	409 (20)	530 (25)
	DBF	Fluorescein	Sulfaphenazole	485 (20)	538 (25)
CYP2C19	CEC	CHC	Tranylcypromine	409 (20)	460 (40)
	DBF	Fluorescein	Tranylcypromine	485 (20)	538 (25)
	OMF	Fluorescein	Tranylcypromine	485 (20)	538 (25)
CYP2D6	AMMC	AHMC	Quinidine	390 (20)	460 (40)
	MAMC	HAMC	Quinidine	390 (20)	460 (40)
CYP2E1	7-MFC	HFC	DETC	409 (20)	530 (25)
CYP3A4	7-BQ	Quinolinol	Ketoconazole	409 (20)	530 (25)
	BFC	HFC	Ketoconazole	409 (20)	530 (25)
	BzRes	Resorufin	Ketoconazole	530(25)	590 (35)
	DBF	Fluorescein	Ketoconazole	485 (20)	538 (25)

BzRes = Resorufin benzyl ether; CEC = 3-Cyano-7-ethoxycoumarin; CHC=3-Cyano-7-hydroxycoumarin; EFC = 7-Ethoxy-4-trifluoromethylcoumarin; 7-HFC= 7-Hydroxy-4-trifluoromethylcoumarin; DBF = Dibenzylfluorescein; ; 7-MFC = 7-Methoxy-4-trifluoromethylcoumarin; OMF = 3-O-methylfluorescein; AMMC = 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin; MAMC = 7-Methoxy-4-(aminomethyl)coumarin; AHMC = 3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride; HAMC = 7-Hydroxy-4-(aminomethyl)coumarin; DETC = Diethyldithiocarbamic acid; 7-BQ = Benzyloxyquinolone; BFC = 7-Benzyloxy-4-(trifluoromethyl)-coumarin (BD Biosciences, www.bdbiosciences.com).

RESULTS

Enzyme assays

In a primary validation assay CYP450 enzyme activities measurable in human liver microsomes were compared with published data collected with human liver microsomes (Table 2). The activity measured in the human microsomes is generally lower than in the published data. However, the relative enzyme activities were comparable. The results of the investigated CYP activities of pooled cat, dog and human liver microsomes are summarized in Table 3. The

CYP activities of male and female cats and dogs are shown in Table 4 & 5 respectively.

Cats showed significant lower activities towards the oxidation of the substrates CEC (CYP1A), 7-MFC (CYP2E) and BFC (CYP3A) compared to dogs and humans. The oxidation activities of EFC (CYP2B) and AMMC (CYP2D) were significant lower in dog liver microsomes than in cat, although the activity associated with CYP2D is extremely low in all animal species. The oxidation of DBF (CYP2C) did not differ significantly between cats and dogs, but a 3-fold higher activity was seen in human microsomes. The most striking difference was found in the oxidation of 7-MFC (CYP2E), as cats seem to have a 12-fold lower activity compared to dogs and an 8-fold lower activity compared to humans.

Gender differences in CYP-activity were observed as the oxidation of EFC (CYP2B) and AMMC (CYP2D) showed significant higher activities in male cats than in female cats. Conversely, oxidation of the substrates DBF (CYP2C) and 7-MFC (CYP2E) showed significant higher activities in female than in male cats. For dogs these gender differences were found with the oxidation of CEC (CYP1A), AMMC (CYP2D) and 7-MFC (CYP2E), where female cytochrome activities were significant higher than male activities. Only the oxidation of EFC (CYP2B) was higher in male dogs than in females.

Table 2. Comparison of measured CYP activities in HLM (human liver microsomes) in pmol/(mg protein*min) ± SD and the reference values presented in the literature for HLM.

Isozyme	***Substrate***	***Vmax HLM***	***Vmax as reported by the manufacturer***
CYP1A	CEC	200.5 ± 3.8	542 (Stresser et al., 2002)
CYP2B	EFC	59.0 ± 6.0	45 (Donato et al., 2004)
CYP2C	DBF	95.9 ± 1.4	289 (Stresser et al., 2002)
CYP2D	AMMC	<LOQ	3.12 (Stresser et al., 2002)
CYP2E	7-MFC	326.2 ± 9.9	1744 (Stresser et al., 2002)
CYP3A	BFC	92.2 ± 4.8	205 (Stresser et al., 2002) 40 (Donato et al., 2004)

The SD of the reference values were all within 10% of the mean. Human CYP2D activity was below the limit of quantification.

Table 3. Activity of the different hepatic CYP isozymes in cats (n=10), dogs (n=8) and human (n=17, as in Table 2) in pmol/(mg protein*min) ± SD.

Isozyme	*Activity pooled cats*	*Activity pooled dogs*	*Activity pooled humans*
CYP1A	101.2 (± 5.1)[bc]	204.4 (± 6.3)[a]	200.5 (± 3.8)[a]
CYP2B	131.3 (± 3.1)[bc]	53.5 (± 3.0)[a]	59.0 (± 6.0)[a]
CYP2C	31.2 (± 0.8)[bc]	33.6 (± 0.7)[ac]	95.9 (± 1.4)[ab]
CYP2D	12.6 (± 1.7)[b]	4.9 (± 0.3)[a]	< LOQ
CYP2E	40.5 (± 4.7)[bc]	473.4 (± 16.5)[ac]	326.2 (± 9.9)[ab]
CYP3A	65.0 (± 3.7)[bc]	90.9 (± 4.3)[a]	92.2 (± 4.8)[a]

Values are the mean of triplicate analyses. Statistical significance was determined by a one-way ANOVA with a Bonferroni post test. P<0.05 indicates a significant difference.
a significantly different from cats
b significantly different from dogs
c significantly different from humans

Table 4. Cytochrome P450 activities of male (n=5) and female (n=5) cats in pmol/(mg protein * min) ± SD.

Isozyme	*Activity male cats*	*Activity female cats*
CYP1A	102.0 (± 4.7)	99.7 (± 6.0)
CYP2B	158.5 (± 3.6)	142.7 (± 4.9)*
CYP2C	27.9 (± 1.0)	38.2 (± 1.4)*
CYP2D	15.0 (± 1.7)	11.8 (± 1.4)*
CYP2E	35.3 (± 3.2)	46.2 (± 3.3)*
CYP3A	64.9 (± 8.8)	70.2 (± 4.5)

Statistical significance was determined by a T-test, with P < 0.05 as significantly different.
*as significantly different from male cats.

Table 5. Cytochrome P450 activities of male (n=4) and female (n=4) dogs (Beagle) in pmol/(mg protein * min) ± SD.

Isozyme	*Activity male dogs*	*Activity female dogs*
CYP1A	175.4 (± 7.2)	220.6 (± 11.0)*
CYP2B	74.2 (± 0.8)	33.4 (± 1.0)*
CYP2C	34.9 (± 0.5)	35.6 (± 0.5)
CYP2D	2.8 (± 0.2)	5.3 (± 0.5)*
CYP2E	413.2 (±12.3)	624.3 (± 36.4)*
CYP3A	94.5 (± 0.2)	87.8 (± 9.4)

Statistical significance was determined by a T-test, with P < 0.05 as significantly different.
*as significantly different from male dogs.

A general comparison of individual CYP activity revealed that cat liver microsomes metabolized all substrates recommended for the assessment of human CYP activities. The activities associated with CYP1A, CYP2B and CYP3A were the most pronounced enzyme activities. In dogs, all selected substrates were metabolized as well and activities associated with CYP1A, CYP2E and CYP3A were found to represent the most active isozymes.

Inhibition studies

The effect of the prototypical inhibitors on the CYP mediated metabolism in cat and dog liver microsomes is shown in Fig. 1. The initial activities, i.e. without addition of an inhibitor, are set to 100%.

In cats, inhibition of the activity associated with CYP1A could be realized by α-naphthoflavone but not by furafylline. Inhibition of CYP1A activity by furafylline was only seen at concentrations above 10 μM but these concentrations exceeded the maximum solubility of the stock solution in acetonitrile, which was used for all inhibitors. CYP2B activity could be inhibited by addition of high concentrations of tranylcypromine. CYP2C was inhibited by quercetine and high concentrations of tranylcypromine. Sulfaphenazole had no effect on the CYP2C activity in the cat liver microsomes, with DBF as substrate. CYP2D activity declined rapidly after the addition of quinide. CYP2E inhibition could be observed in the presence of DETC and CYP3A was inhibited by ketoconazole.

In dogs, the activity associated with CYP1A was inhibited by high concentrations of furafylline. After addition of the inhibitor tranylcypromine to the dog liver microsomes, the fluorescence indicating for CYP2B activity unexpectedly increased. CYP2C could only marginally be inhibited by tranylcypromine and quercetine. Sulfaphenazole gave no inhibition at all. CYP2D and CYP2E were inhibited by quinidine and DETC respectively. CYP3A was not inhibited by ketoconazole as the fluorescence increased unexpectedly after adding ketoconazole.

Comparing cats and dogs, inhibition of the activities associated with CYP1A by furafylline, CYP2C by quercetine/sulfaphenazole and CYP2E by DETC did not differ. While CYP2B and CYP3A in cat liver microsomes were inhibited by tranylcypromine and ketoconazole respectively, these activities surprisingly increased in dog liver microsomes under the same circumstances. CYP2D activity in cats was more sensitive to quinidine than in dogs.

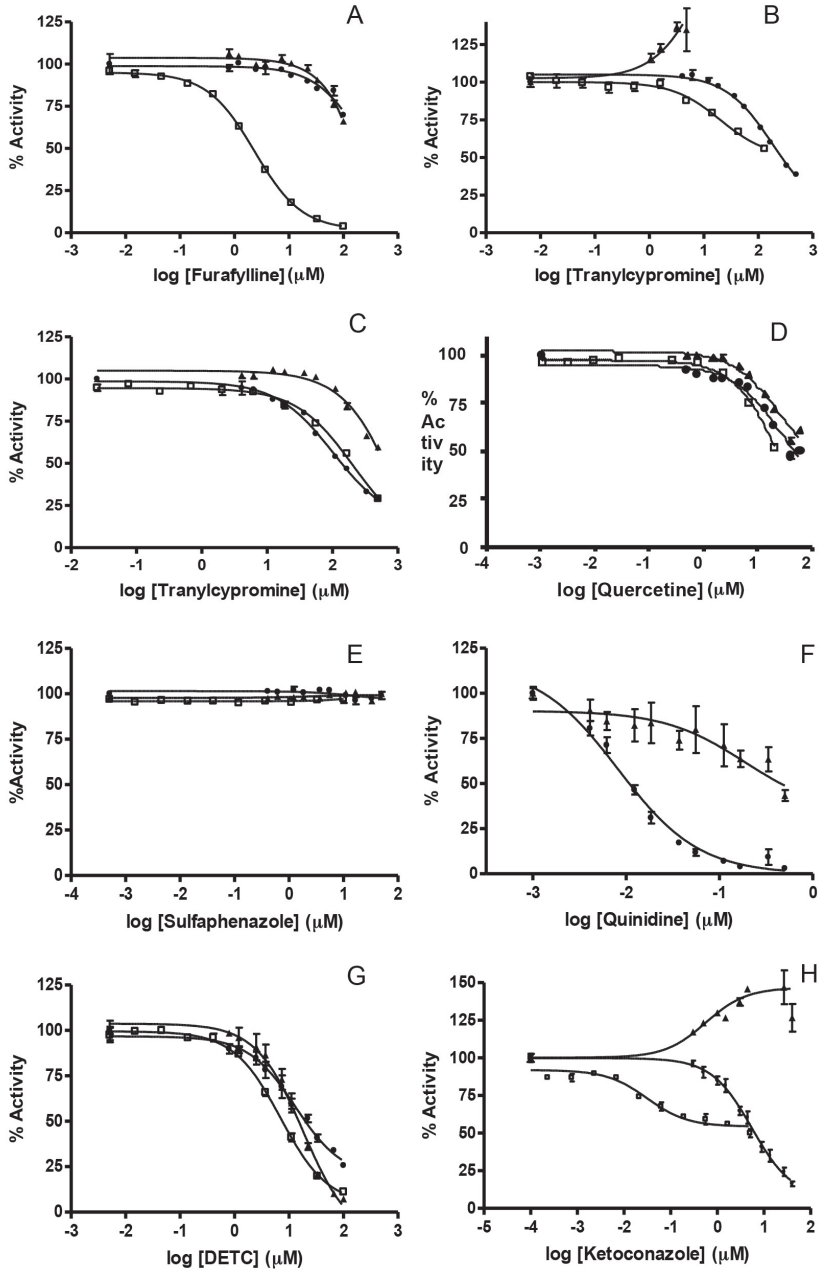

Figure 1. Inhibition profiles of cat (•), dog (▲) and human (□) liver microsomes. Initial activity (i.e. in the absence of inhibitor) was converted to 100%. Inhibition of (A) CYP1A activity by furafylline; (B) CYP2B activity by tranylcypromine; (C) CYP2C19 activity by tranylcypromine; (D) CYP2C8 activity by quercetine; (E) CYP2C9 by sulfaphenazole; (F) CYP2D activity by quinidine; (G) CYP2E activity by DETC; (H) CYP3A activity by ketoconazole.

DISCUSSION

The presented investigations had two objectives: First, to present a comparison of the hepatic CYP isozyme activities in cats and dogs. Secondly, to assess the suitability of the rapid fluorometric assays to measure CYP activities in liver samples obtained from veterinary patients.

Cytochrome P450 activity and inhibition

The presented data of the metabolic CYP activity show that in cats the oxidation of CEC by CYP1A, EFC by CYP2B and BFC by CYP3A represent the highest activities. Similarly, Chauret *et al.* (1997) found the highest activities for phenacetin-*O*-deethylase and testosterone 6β-hydroxylase in cats, associated with CYP1A and CYP3A respectively. Shah *et al.* (2007) found even higher CYP1A activities in cats than in dogs and humans. The oxidation of DBF by CYP2C and AMMC by CYP2D showed the lowest activity. A direct quantitative comparison of the data is not possible, as the substrates differ in the individual assays.

We found gender differences in cytochrome activity, which were not reported by Chauret *et al.* (1997). Other investigations confirm these gender differences in cytochrome activity although only for the isozymes CYP2D and CYP3A (Shah et al., 2007).

Using the same assay with dog liver microsomes, results showed that CYP1A, CYP2E and CYP3A were the most active isozymes. The results are in line with previous investigations (Chauret et al., 1997; Shimada et al., 1997), although Shimada *et al.* (1997) found a lower range of phenacetin-*O*-deethylation activity, representing CYP1A activity.

CYP2D activity was hardly detectable in the liver microsomes of all animal species and these findings are in accordance with previous investigations (Chauret et al., 1997; Shimada et al., 1997). It was found that in dogs, bufuralol-1-hydroxylase activity, associated with CYP2D, was comparable with human CYP2D activity, albeit being lower than in other animals (Sharer et al., 1995; Roussel et al., 1998; Bogaards et al., 2000).

Comparing data of cat and dog liver microsomes, it could be observed that cat liver microsomes show significant lower CYP1A, CYP2E and CYP3A activities than dog liver microsomes. Conversely, dog liver microsomes had significant lower activities of CYP2B and CYP2D than those of cats. In human liver microsomes the CYP2D activity was below the detection level of the fluorometric assay. This might be attributable to the diverse polymorphism

of this isozymes (Heim & Meyer, 1992; Zhou, 2009; Zhou et al., 2009). Both cats and dogs had lower activities of CYP2C than humans, which is in accordance with previous investigations (Chauret et al., 1997). Shah *et al.* (2007) demonstrated that cats have a negligible tolbutamide hydroxylation activity, suggesting unusual low CYP2C activities. It has to be mentioned that the substrate DBF for CYP2C is also metabolized by CYP3A. The activity which is measured is therefore not solely the activity of CYP2C. This could be a reason for the high CYP2C activity in human liver samples, because of the high content of CYP3A in human liver.

In our investigations CYP2E activity of cat liver microsomes was 12-fold lower than in dogs, although data showed that cats and dogs share the highest homology in amino acid sequence for this isoform compared to other animal species (Tanaka et al., 2005). By contrast, Tanaka *et al.* (2005) found a three-fold higher CYP2E activity in cats than in dogs in the 6-hydroxylation of chlorzoxazone. In cats two similar CYP2E genes are present, while in many mammalian species only a single gene exists (Tanaka et al., 2005). CYP2E metabolizes for example acetaminophen (Morgan et al., 1983) and volatile anaesthetics, such as halothane, isoflurane and sevoflurane (Gruenke et al., 1988; Kharasch & Thummel, 1993). Besides the knowledge of the deficient glucuronidation in cats (Court & Greenblatt, 1997; Court & Greenblatt, 2000) this relative low activity of CYP2E may explain the sensitivity of cats for the side effects of previously described drugs and toxins which are substrates for CYP2E.

To demonstrate substrate specificity, defined inhibitors of individual isozymes are commonly applied. To obtain IC50 values, different concentrations of specific inhibitors of the human CYP isozymes were added to the liver microsomes of both species.

CYP1A associated activity was not inhibited by the human prototypical inhibitor furafylline in cat and dog liver microsomes, while in human liver microsomes the activity decreased to zero in the presence of 100 µM furafylline. The absence of inhibition of phenacetin-*O*-deethylase (CYP1A) by furafylline was found in cats, but not in dogs, also by Chauret *et al. (*1997). Our experiments showed that the other well-known inhibitor α-naphthoflavone inhibited CYP1A activity in cats and dogs (data not shown). These findings suggest that either furafylline has a low binding affinity for the isozyme CYP1A, or a second enzyme is involved in the metabolism of the substrate CBC.

CYP2B associated activity in cats was only inhibited by tranylcypromine at high concentrations. In dogs an unexpected rise of the fluorescence of the

CYP2B substrate was found after adding tranylcypromine in increasing concentrations. This phenomenon has been described in rats as well, while EFC, the substrate for CYP2B, was not only metabolized by this isozyme but also for 15% by CYP1A2 and 60% by CYP2E1 (Buters et al., 1993). As two phases could be found in Hanes plots in dog and human microsomes, the involvement of at least two different enzymes for EFC deethylation in these species is suggested (Buters et al., 1993). Hence, we hypothesize that in dogs the fluorescent product is further metabolized by another enzyme resulting in a secondary metabolite which increased fluorescence.

Human CYP2C can be subdivided in CYP2C19, CYP2C8 and CYP2C9. These isozymes can be inhibited by tranylcypromine, quercetine and sulfaphenazole respectively (Crespi et al., 1997; Naritomi et al., 2004). We found that in cat, dog and human liver microsomes CYP2C activity was inhibited by tranylcypromine and quercetine. Sulfaphenazole gave no inhibition of the activity of CYP2C in all three species. This can be caused by the usage of the same substrate DBF for these isozymes. When CYP2C9 only represents a small part of the total CYP2C activity, the other two isozymes are able to convert the substrate and as a consequence no numerical decline in CYP2C activity will be found in the presence of a specific inhibitor. Distinction between these CYP2C isoforms was not possible with the used assay. However, by HPLC a very low activity of tolbutamide hydroxylase, indicating CYP2C9 in humans, was found in cats and dogs (Chauret et al., 1997; Shah et al., 2007). CYP2D associated activity could be inhibited by very low concentrations of quinidine in cats. Dog en human CYP2D activity could hardly be detected and inhibition by quinidine did not give an obvious decline in activity. The low CYP2D activity might be attributable to the high rate of polymorphism of this isozyme, which is known for humans (Fukuda et al., 2000; Ingelman-Sundberg, 2004).

CYP2E associated activity could be reduced by DETC in cat, dog and human in comparable manner and no species differences were found in IC50 values.

The oxidation of BFC, associated with CYP3A, was inhibited by addition of ketoconazole in cat liver microsomes. However, in dogs again an unexpected increase in fluorescence was found. The inhibitor ketoconazole was proven to be specific for human and dog CYP3A (Newton et al., 1995; Kuroha et al., 2002; Lu et al., 2005). Ketoconazole as such did not give any fluorescence. The rise in fluorescence suggests that another isozyme metabolizes the chosen substrate BFC as well.

Assessment of the fluorometric assay

The fluorometric assay was selected in consideration of the obvious advantages of simplicity and the short duration of the assay compared to HPLC analysis or other analytical techniques. It is considered to be a high throughput method for investigation of drug biotransformation and substrate conversion. The most important disadvantage is that this assay is not entirely validated for the use of tissue fractions, such as microsomes. The manufacturer's provision was to use pure isolated isozymes to obtain IC50 values, although Miller *et al.* reported that the CYPs could be introduced in the assay as single, cDNA-expressed enzymes or as enzyme mixtures, such as liver microsomes (Miller et al., 2000). The first evaluation of the assay with mixed human microsomes, did confirm the principle suitability of the assay, but relatively lower enzyme activities were measured in the microsomal fractions. Both assays are normalized for the protein content of the sample, and the CYP-enzyme proteins in the microsomal fractions explain for a large extent the relatively lower values. Moreover, when enzyme mixtures are used as present in the microsomal fraction, the probe substrate may be converted by one or more enzymes, as most CYP450 enzymes have overlapping substrate specificity (Crespi & Stresser, 2000; Miller et al., 2000; Stresser et al., 2002). This overlapping substrate activity is also reflected in the inconsistent results inferred in the inhibition studies. For example BFC, the substrate for CYP3A, should be highly selective for this isozyme and so this substrate could be used with human liver microsomes as a typical substrate (Crespi & Stresser, 2000; Miller et al., 2000; Stresser et al., 2002; Donato et al., 2004). However, when ketoconazole was added in increasing concentrations to human liver microsomes, CYP3A activities did only decline to approximately 50% of the normal activity, indicating that BFC is a substrate for more than one CYP-isozyme.

The final objective was the evaluation of the fluorometric assay for its suitability in a clinical environment where individual patients might need to be investigated for their biotransformation activity of certain drugs. The fluorescent assay requires a smaller amount of microsomal protein as compared to common HPLC-based analyses, but the requested amount of 0.25 mg/mL protein is still high and cannot be obtained from normal thin-needle biopsies.

In conclusion, the presented data provide for the first time a summation of cat CYP450 activities and demonstrate again significant species differences in the activity of individual CYP isozymes between cats and dogs. In clinical practice, the lower CYP activities in cats, combined with the low glucuronidation capacity will result in longer half lives of many drugs that undergo extensive biotransformation reactions. To avoid undesirable side effects and drug toxicity, longer dosage intervals should be considered for

cats when dosage regimes established for dogs or humans are extrapolated to feline patients. The developed assays provide a valuable tool in the preclinical phase of veterinary drug development, as the same protocol can be used for different species, allowing a rapid comparison of results and the identification of species differences.

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CHAPTER 3

COMPARING THE GLUCURONIDATION CAPACITY OF THE FELINE LIVER WITH SUBSTRATE-SPECIFIC GLUCURONIDATION IN DOGS

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ABSTRACT

This study aimed to assess the overall glucuronidation capacity of cats, using prototypic substrates identified for human UDP-glucuronosyltransferases (UGTs). To this end, Michaelis-Menten kinetics were established for the substrates using feline hepatic microsomal fractions and results were compared with similar experiments carried out with dog liver microsomes. Cats are known for their low capacity of glucuronide formation and UGT1A6 was found to be a pseudogene. However, functional studies with typical substrates were not performed and knowledge of the enzymology and genetics of other glucuronidation enzymes in felidae is lacking. The results of this study showed extremely low formation of naphthol-1-glucuronide (1.7 ± 0.4 nmol/mg protein/min), estradiol-17-glucuronide (<0.7 nmol/mg protein/min) and morphine-3-glucuronide (0.2 ± 0.03 nmol/mg protein/min), suggesting a lack of functional UGT1A6 and UGT2B7 homologues in the cat's liver. Dog liver microsomes were producing these glucuronides in much higher amounts. Glucuronide capacity was present for the substrates 17β-estradiol (estradiol-3-glucuronide, 2.9 ± 0.2 nmol/mg protein/min) and 4-methylumbelliferone (31.3 ± 3.3 nmol/mg protein/min), assuming that cats have functional homologue enzymes to at least the human UGT1A1 and probably other UGT1A isozymes. This implies that for new drugs, glucuronidation capacity has to be investigated on a substance-to-substance base. Knowledge of the glucuronidation rate of a drug provides the basis for pharmacokinetic modeling and as a result proper dosing regimens can be established to avoid undesirable drug toxicity in cats.

INTRODUCTION

Cats and other felidae are known for their low capacity for glucuronide conjugation of drugs and toxins. This assumption is based on early data on the biotransformation of phenolic compounds in cats in comparison to other species (Capel et al., 1972a; Capel et al., 1972b; Capel et al., 1974). Subsequently, acetaminophen (paracetamol, a phenolic derivative) is the most prominent example of a drug that is toxic for cats, when common dosing regimens established for dogs or humans are applied. This toxicity has been largely attributed to the fact that cats express an inactive pseudogene UGT1A6, hence being unable to glucuronidate acetaminophen resulting in an accumulation of paracetamol and its phase I metabolites in the liver followed by liver cell damage (Court & Greenblatt, 2000). However, little is known about the enzymology and genetics of other glucuronidation enzymes in felidae.

The formation of glucuronide conjugates is accomplished by a superfamily of enzymes, the UDP-glucuronosyltransferases (UGTs). The UGTs are membrane-bound enzymes that catalyze the conjugation of the glycosyl group of glucuronic acid to many lipophilic endogenous and exogenous substrates (Mackenzie et al., 2005). The resulting water soluble glucuronide conjugates are generally less active than the parent compound and can be excreted into the bile or urine. UGTs have a broad and overlapping substrate specificity albeit with often significant differences in affinity (Radominska-Pandya et al., 1999; Tukey & Strassburg, 2000). The human UGT proteins are classified into two families denoted UGT1 and UGT2, based on evolutionary divergence and homology in amino acid sequence. The UGT1 and UGT2 families are abundant in the liver although UGT activity is also found in extra-hepatic tissue such as the intestines and kidneys. Based on the preference for certain substrates and similarity in amino acid sequences, UGT1 is further subdivided into the subfamilies UGT1A and UGT1B, even as UGT2 is subdivided in UGT2A and UGT2B (Court & Greenblatt, 2000; Mackenzie et al., 2005; Dong et al., 2012). Two other UGT families, denoted UGT3 and UGT8, were also discovered but they conjugate other glycosyl groups to their substrates and are considered unlikely to play a significant role in the detoxification of drugs or other xenobiotics (Mackenzie et al., 2005; Rowland et al., 2013). UGT1A and UGT2B are important isozymes in the human liver responsible for glucuronidation (Miners et al., 2004; Mackenzie et al., 2005; Ohno & Nakajin, 2009; Dong et al., 2012; Harbourt et al., 2012). Substrates for human hepatic UGTs are, amongst others, endogenous steroids, bile acids (Gall et al., 1999), bilirubin (Bosma et al., 1994; Senafi et al., 1994), and many exogenous substances such as drugs, alcohols, phenols, lipid soluble

vitamins, amines and thiols (Ebner & Burchell, 1993; Green & Tephly, 1996; Hashizume et al., 2008). Examples of drugs often used in veterinary medicine, which are substrates for human UGT2B, are various NSAIDs, opioids and benzodiazepines (Miners & Mackenzie, 1991; Jin et al., 1993; Coffman et al., 1997; Stone et al., 2003; Kiang et al., 2005).

A similar classification of UGT enzymes of the cat based on substrate specificities and genetics has not been made. As mentioned above, Court and Greenblatt (2000) initiated the genetic research on UGTs in the cat and showed the inactive pseudogene of UGT1A6. Furthermore, they suggested a limited expression of other hepatic UGT1A isoforms, denoted as UGT1A1 and UGT1A02 (Court & Greenblatt, 1997; Court & Greenblatt, 2000). However, limited data have been published on the activity and substrate preference of these other feline UGTs.

Hence it was the aim of the current study to assess the overall glucuronidation capacity of cats, using prototypic substrates, identified for human UGT isozymes. To this end, Michaelis-Menten kinetics were established for the substrates using feline hepatic microsomal fractions. Results were compared with similar experiments carried out with dog liver microsomes as this could allow a broader interpretation of the clinical relevance of the findings.

MATERIALS & METHODS

Drugs and chemicals

Alamethicin solution, 17β-estradiol water soluble, β-estradiol 3-β-D-glucuronide (E3G), β-estradiol 17-β-D-glucuronide (E17G), 11β-hydroxytestosterone, 1-naphthol, naphthylβ-D-glucuronide (N1G), magnesium chloride hexahydrate, 4-methylumbelliferone (4MU), 4-methylumbelliferyl- β-D-glucuronide hydrate (4MUG), testosterone, and uridine 5'-diphosphoglucuronic acid (UDPGA) were purchased from Sigma Chemical Co. Morphine 3-β-D-glucuronide (M3G) was purchased from Cerilliant Corporation. Morphine hydrochloride was purchased from BUFA B.V.

Tissue samples

Liver tissue was obtained from adult healthy European Shorthair cats (n=8, five males and three females, aged from 11 to 13 months) directly after euthanasia and samples were immediately frozen in liquid nitrogen and stored at -70°C. The cats had served as controls in a study for the development of FIV vaccines and had been sacrificed as cell donors. The same applies to the liver samples of Beagle dogs (n=7, two males and five females, aged from 3.5 to 4.5 years)

that had also served as controls in clinical trials. Animals were sacrificed with permission of the Animal Ethical Committee of the Utrecht University and performed according to the Dutch law on Animal Experiments.

Preparation of submitochondrial fractions

The isolation of submitochondrial fractions (commonly referred to as microsomes) containing predominantly microsomal proteins followed the procedure as described by Rutten *et al.* (1987) with minor modifications. In brief, cat and dog liver samples of approximately 10 g were obtained from adult healthy cats and dogs directly after euthanasia and were quickly frozen in liquid nitrogen and stored at -70°C. The tissue samples were homogenized with 1.15% KCl, containing 0.1 mM EDTA at 4°C. The homogenates were centrifuged at 9000 *g* for 25 min at 4°C, and the supernatant obtained (S9-fraction) was centrifuged at 100 000 *g* for 1 hour and 15 min at 4°C. The microsomal pellet was re-suspended in 1.15% KCl 0.05 M phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol. The microsomes were then quickly frozen in liquid nitrogen and stored in Eppendorf-cups at -70°C until use (Rutten et al., 1987).

The protein concentrations of the microsomal fractions were determined by the method of Bradford using bovine serum albumin (BSA) as a standard (Bradford, 1976) and data were expressed as nmol/mg protein/min. As a quality control for the enzymatic activity of the microsomal preparations, cytochrome P450 activity was measured using 6β-testosterone hydroxylation as described by Chauret *et al.* (Chauret et al., 1997).

Incubation protocols

All glucuronidation assays were performed with pooled microsomes according to standard protocols with only minor modifications (Miners et al., 1988; Furlan et al., 1999; King et al., 2000; Stone et al., 2003; Mano et al., 2004; Court, 2005). Details regarding the specific incubation conditions of each substrate are given in Table 1. Incubation mixtures contained 100 mM phosphate buffer (KH_2PO_4, pH 7.4), 5 mM $MgCl_2$, alamethicin (50 µg/mg protein) and a concentration range of the selected substrates (1-naphthol, 17β-estradiol, morphine, 4MU, respectively) in a total volume of 500 µL. All substrates were dissolved in water, except for 4MU which was dissolved in methanol resulting in a final concentration of 0.5% methanol. Pooled hepatic microsomal protein was added to obtain a protein concentration ranging from 0.1 to 0.5 mg/ml and pre-incubations were performed for 5 min at 37°C. The glucuronidation reactions were initiated by adding UDPGA with a final concentration of 5 mM, and samples remained at 37°C in a heat block for the indicated time and were shaken regularly in order to ensure an equal temperature within

the incubating mixtures. Reactions were terminated by addition of ice-cold acetonitrile, followed by a rapid cooling step. Samples were centrifuged at 13 000 *g* for 5 min and the supernatants were directly injected onto the HPLC column. HPLC conditions are given in Table 2. Assays were tested for linearity in incubation time and protein concentration by duplicate measurements in two independent experiments. All measurements for K_m and V_{max} determination were performed at least in duplicates and the maximum activity measurements were performed in triplicate in three independent experiments. Blanks were obtained from incubations without UDPGA.

Data analysis

Data of the glucuronide formation were fitted by nonlinear regression curve fitting analysis according to the Michaelis-Menten equation by means of GraphPad Prism 6.01 software. Subsequently, K_m and V_{max} were calculated with the same software. Michaelis-Menten enzyme kinetics could not always be applied to the data of the feline liver microsomes due to the low activity of glucuronide formation which was beneath the limit of detection at lower substrate concentrations. Therefore, the maximum activity of glucuronide formation in the feline liver microsomes was measured at substrate concentrations at which the canine microsomal activity was saturated, which is in general assumed to be at a concentration exceeding five times the K_m value. Data of these maximum observed activities were expressed as means ± SD of three independent experiments with samples performed in triplicate. Data of V_{max} or the maximum activity which was measured, were analyzed using an independent two sample Student's t-test with $P < 0.05$ denoting a significant difference.

RESULTS

As a quality control for the enzymatic activity of the microsomal fractions, the cytochrome P450 activity was tested by testosterone metabolism and hydroxylated metabolites were analyzed by HPLC as described by Chauret *et al.* with minor modifications (Chauret et al., 1997). The testosterone hydroxylation (6β-OH TST) activity in the microsomes from cats was approximately one third of the activity in the microsomes from dogs (data not shown).

In initial experiments the linear range for the rate of product formation versus time was established for the incubation period and protein content for each reaction (data not shown). All data were further obtained within the linear phase of product formation.

Table 1. Incubation conditions for the different substrates.

Substrate	Glucuronide formed	Incubation time	Protein concentration	Substrate concentration	Procedure
1-Naphthol	N1G	4 min	0.1 mg/ml	750 µM	Miners et al., 1988 Furlan et al., 1999
17β-Estradiol	E3G E17G	15 min	0.5 mg/ml	3 mM	Court, 2005
Morphine	M3G	90 min	0.25 mg/ml	5 mM	Miners et al., 1988 King et al., 2000 Stone et al., 2003
4-Methyl-umbelliferone	4MUG	4 min	0.1 mg/ml	750 µM	Mano et al., 2004

Incubation mixtures contained 100 mM phosphate buffer (KH_2PO_4, pH 7.4), 5 mM $MgCl_2$, alamethicin (50 µg/mg protein) and different substrate concentrations in a total volume of 500 µL. Pooled hepatic microsomal protein was added and pre-incubations were performed for 5 min at 37°C. Thereafter, UDPGA (final concentration 5 mM) was added and the reaction mixture was incubated at 37 in a heat block for the indicated time.

Table 2. HPLC conditions for the glucuronidation activities of different substrates with their stationary phase, mobile phase and detection conditions.

Glucuronidation activity	Stationary phase	Mobile phase	Detection conditions
1-Naphthol glucuronidation	Phenomenex Synergi, polair (150 x 4.6 mm, 5 µm)[a]	10 mM phosphate buffer, containing 20% acetonitrile (pH 2.7, adjusted with 85% ortho-phosphoracid H_3PO_4)	Fluorometric emission: 330 nm, extinction: 290 nm
β-Estradiol-3-glucuronidation β-Estradiol-17-glucuronidation	Phenomenex Synergi Hydro, polair (250 x 4.6 mm, 4 µm)[b]	A: 20 mM KH_2PO_4 containing 25% acetonitrile B: 100% acetonitrile 10 min A, balance up to 65% B for 4 min, 65% B for 8 min and then to 100% A in 13 min	UV 280 nm
Morphine-3-glucuronidation	Phenomenex Synergi Hydro, polair (250 x 4.6 mm, 4 µm)[b]	10 mM KH_2PO_4, containing 5% acetonitrile (pH 2.1)	UV 220 nm
4-Methylumbelliferyl glucuronidation	Phenomenex Synergi, polair (250 x 4.6 mm, 4 µm)[c]	A: 10 mM ammoniumacetate: acetonitrile (9:1) B: 20 mM ammonium acetate: acetonitrile (6:4) 10 min A, linear from A to B in 10 min, 20 min B and then 20 min A	Fluorometric emission: 365 nm, extinction: 315 nm

a. The HPLC system consisted of two Genkotek High Precision Pumps (model 300), a Gynkotek Autosampler and a Jasco FP920 Fluorescence-detector.

b. The HPLC system consisted of two Genkotek High Precision Pumps (model 480), a Marathon Autosampler and a Shimadzu SPD-10AVP UV-VIS-detector.

c. The HPLC system consisted of two Genkotek High Precision Pumps (model 480), a Marathon Autosampler and a Merck Hitachi F1050 Fluorescence-detector.

A fast formation of N1G was observed for the dog microsomes with a V_{max} of 61.6 ± 1.3 nmol/mg protein/min (see Figure 1A). The observed data were fitted according to the equation for Michaelis-Menten enzyme kinetics and the calculated K_m and V_{max} are presented in Table 3. The formation of N1G in the microsomes prepared from cat livers was much lower, but above the limit of quantification of the used analytical method. The data could, however, not be fitted according to the Michaelis-Menten equation. The maximum rate of N1G formation that was measured when incubating the feline microsomes with 750 µM 1-naphthol (the substrate concentration at which the canine microsomal activity was approximately saturated) was 1.7 ± 0.4 nmol/mg protein/min.

The formation of E3G from estradiol was observed in both the canine and feline liver microsomes and could be fitted according to the Michaelis-Menten equation. The graphs are shown in Figure 1B and the calculated K_m and V_{MAX} values are given in Table 3. V_{max} for the canine and feline liver microsomes was 10.2 ± 0.4 nmol/mg protein/min and 2.9 ± 0.2 nmol/mg protein/min respectively. The formation of E17G from 17β-estradiol was only observed in the canine microsomes, with a V_{max} of 25.1 ± 1.1 nmol/mg protein/min, as in cats values were below the detection limit.

Morphine was glucuronidated to M3G in dogs with a V_{max} of 34.0 ± 1.5 nmol/mg protein/min. Cats almost completely lacked the capacity to form M3G as they did only produce 0.2 ± 0.03 nmol/mg protein/min after incubation with 5 mM morphine, which was the substrate concentration to obtain approximately the V_{max} in dog liver microsomes. The formation of M3G from morphine could be fitted according to the Michaelis-Menten equation for dogs and is shown in Figure 1C and Table 3. A relative low affinity can be seen for morphine in dogs compared to the other tested substrates. For cats fitting was not possible due to the low M3G activity of the feline microsomes.

4MU is the only substrate which is glucuronidated substantially in cats, as a V_{max} of 31.3 ± 3.3 nmol/mg protein/min was found. Dogs had a V_{max} of 89.6 ± 5.2 nmol/mg protein/min for the same substrate, which is not even three times higher than in cats. The formation of 4MUG from 4MU in both cats and dogs could be fitted according to the Michaelis-Menten kinetics and is shown in Figure 1D and Table 3. It can be observed that the affinity for 4MU is lower in cats than in dogs as the K_m is twice that of dogs.

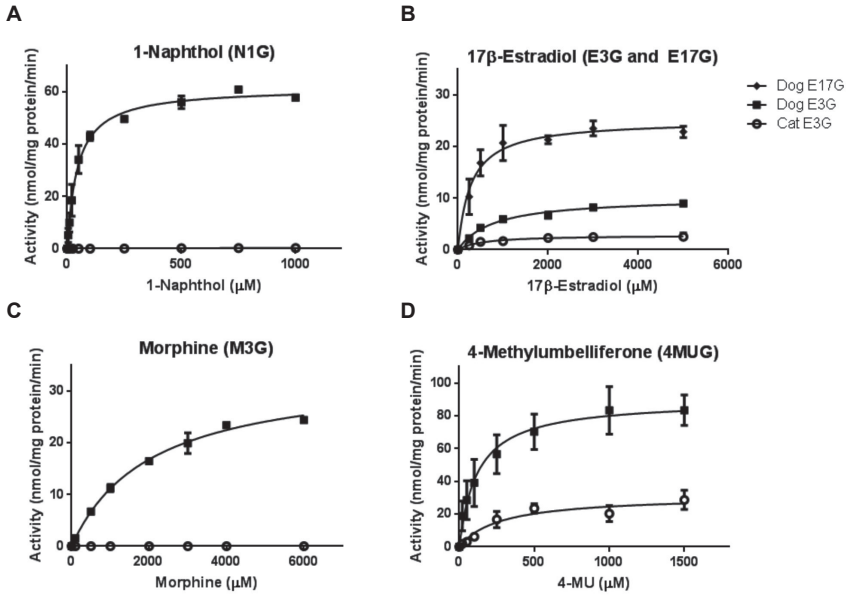

Figure 1. Enzyme kinetics for the glucuronidation of different substrates in pooled cat (○) and dog (■) liver microsomes. A: 1-naphthol; B: 17β-estradiol; C: morphine; D: 4-methylumbelliferone. Data represent the mean ± SD of at least two independent experiments with samples performed in duplicate.

Table 3. Michaelis-Menten kinetics for different formed glucuronides in pooled cat (n=8) and dog (n=7) liver microsomes. Data represent the mean ± SD of at least two independent experiments with samples performed in duplicate.

Glucuronide formed	***Hepatic human UGT isoform***	***Cat***			***Dog***	
		K_m (µM)	*V_{max} (nmol/mg/min)*	*Maximum activity measured (nmol/mg/min)*	*K_m (µM)*	*V_{max} (nmol/mg/min)*
N1G	UGT1A6	-	-	1.7 ± 0.4	45.3 ± 4.4	61.6 ± 1.3*
E3G	UGT1A1	545.2 ± 163.6	2.9 ± 0.2		780.8 ± 108.5	10.2 ± 0.4*
E17G	UGT2B7	-	-	< 0.7	287.9 ± 61.8	25.1 ± 1.1*
M3G	UGT2B7	-	-	0.2 ± 0.03	2045 ± 206.2	34.0 ± 1.5*
4MUG	Non-selective[a]	274.3 ± 83.8	31.3 ± 3.3		120.5 ± 26.8	89.6 ± 5.2*

Due to low glucuronidation activity in the feline liver microsomes, fitting was not possible for N1G, E17G and M3G. Therefore, the maximum glucuronidation activity in cats was measured at the substrate concentration for achieving approximately the V_{max} of dogs. E17G was blow the LOD. These data represent three independent investigations with samples performed in triplicate. P<0.05 denotes a significant difference (*) of V_{max} between cats and dogs.

[a] 4MU is a substrate in the human liver for UGT1A1, 1A3, 1A6, 1A9, 2B7, 2B15, and 2B17.

DISCUSSION

In this study the *in vitro* glucuronidation activity was characterized in cat liver microsomes using typical substrates for human UGT1A and 2B isozymes, as available evidence in human indicates that UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15 are the enzymes of greatest importance in hepatic drug and xenobiotic metabolism (Ohno & Nakajin, 2009; Harbourt et al., 2012). As a control for the assay and for comparison between species, dog liver microsomes were subjected to the same typical substrates as the microsomes from the cats. The results of the current study showed that the overall assumption of cats having a general low glucuronidation capacity needs to be refined.

Probe substrates used for testing the activity of UGT1A enzymes were 1-naphthol, 17β-estradiol and 4-methylumbelliferone (4MU). The feline liver microsomes hardly showed glucuronidation activity for the UGT1A6 probe substrate 1-naphthol, while in the canine liver microsomes a rapid formation of N1G was observed. The low capacity of N1G formation in cats demonstrates that cat liver indeed lacks a functional UGT1A6 homologue. The small amount of product formed in feline liver microsomes likely resulted from the activity of other UGT isoforms that commonly show an overlap in substrate specificity, although with a much lower affinity. The formation of E3G from 17β-estradiol, which in man is predominantly catalyzed by UGT1A1 (Senafi et al., 1994; Soars et al., 2004), was slower in the feline microsomes in comparison to the canine microsomes with the maximum formation rate being approximately 28% of that of the canine microsomes. The substrate 4MU is predominantly glucuronidated by human liver UGT1A6 and 1A9 but is not as specific as the other tested substrates and can therefore be seen as a substrate to test overall glucuronidation capacity (Uchaipichat et al., 2004). The maximum rate of formation of 4MUG in the feline microsomes was approximately 35% from that of the canine liver microsomes.

For the functional characterization of the UGT2B enzyme family in feline liver microsomes, the typical substrates 17β-estradiol and morphine were used. The formation of estradiol glucuronides is not only catalyzed by UGT1A1 but also by UGT2B7 (Gall et al., 1999; Soars et al., 2004) in humans resulting in the E17G product. Feline liver microsomes only formed the glucuronide E3G but E17G could not be detected, as mentioned above. The other probe substrate for UGT2B7 in human is morphine (Coffman et al., 1997), which was used for measuring the formation of M3G. Feline liver microsomes demonstrated a very limited capacity for the formation of M3G compared to the canine liver. This almost lacking capacity of M3G and E17G formation suggests a low expression or absence of an UGT2B7 homologue in the liver of cats.

As yet, the feline homologue to the human UGT1A6 had received most attention. Studies in the 1970s already showed that cats do not form glucuronide conjugates of certain phenolic compounds *in vivo* and genetic analyses by Court and Greenblatt (2000) demonstrated that the feline UGT1A6 is a pseudogene that is not translated into a functional isozyme. However, the liver microsomal activity for glucuronidation of the typical UGT1A6 probe substrate 1-naphthol, had never been studied in cats in detail. The very low capacity of N1G formation that was observed in the current study confirms previous investigations (Watkins & Klaassen, 1986) and further demonstrates that the cat's liver lacks a functional UGT1A6 homologue.

In contrast, formation of glucuronides was found in substantial amounts for the substrates 17β-estradiol and 4MU in the feline liver microsomes, demonstrating that cats have functional homologue enzymes to at least the human UGT1A1 and possibly other isoforms. Previous genetic analyses suggested a low expression of a feline homologue to human UGT1A1, while another feline UGT was named UGT1A02 that was predicted to be homologue to human UGT1A2, 3, 4 and 5 based on phylogenetic analysis (Court & Greenblatt, 2000). In this respect, it is noteworthy to mention that despite a high sequence identity, human UGT1A3 and UGT1A4 are functionally very different as a resultant of only one amino acid difference (Green et al., 1998; Kubota et al., 2007). Moreover, human UGT1A4 has no activity towards 4MU, while human UGT1A3 glucuronidates 4MU but with a very low efficiency (Uchaipichat et al., 2004). Therefore, it can be concluded that a homologue to the human UGT1A3 or another not yet identified UGT, which is specific for the cat, catalyzes the 4MUG formation in the feline liver.

The absent or negligible formation of the glucuronides E17G and M3G found in feline liver microsomes indicates a lack of a functional UGT2B7 homologue in the cat's liver. *In vivo* investigations did also not find M3G in the blood plasma of cats following the application of morphine, nor did they find M6G in all cats in substantial amounts (Taylor et al., 2001). Previously, M3G has been found only at very low concentrations in urine and feces after administration of morphine to cats, and the major conjugated metabolite was found as morphine-3-ethereal sulphate instead (Yeh et al., 1971). An indication that cat livers do not express a functional UGT2B7 homologue is also provided by the low glucuronidation capacity of chloramphenicol, a typical substrate for human UGT2B7, in cats (Watkins & Klaassen, 1986).

In this study a comparison between cat and dog liver microsomes was made using liver specimen of Beagle dogs. This selection was made in consideration of the broad use of Beagles in drug research. However, the limitations of such

an approach are obvious, as variations in glucuronidation capacity can be assumed comparable to those described for differences in cytochrome P450 activity across canine breeds (Martinez et al., 2013).

Our results suggest that the cat does not have functional homologues to the human UGT1A6 and UGT2B7, while glucuronidation capacity in the liver is present that may be catalyzed by UGTs that have functional similarities to the human UGT1A1 and possibly other UGT1A isozymes. Indeed, there are some examples of drugs which are well glucuronidated by cats: phenolphthalein (Pekanmaki & Salmi, 1961; Watkins & Klaassen, 1986), lorazepam (Schillings et al., 1975; Elliott, 1976; Ruelius, 1978), pradofloxacin (EMEA/V/C/099, 2007), ibuprofen (Magdalou et al., 1990), and telmisartan (Ebner et al., 2013) can be found as glucuronides in cats. Telmisartan and ibuprofen are even glucuronidated with a higher rate in feline hepatic microsomes than in other species. Although it is not known which UGT isozymes are involved in the metabolism of all these drugs, for the most recently investigated compound telmisartan in cats it is known that in human hepatocytes this drug is metabolized mainly by UGT1A3 (Yamada et al., 2011).

In conclusion, the present functional data indicate that the cat has a number of functional UGT1A enzymes which are homologues to the human UGT1A isoforms. Other glucuronidation enzymes in the cat remain to be characterized in more detail. This implies that for new drugs, glucuronidation capacity has to be investigated on a substance-to-substance base to provide the basis for PK-modeling to establish proper dosing regimens in cats and to avoid undesirable drug toxicity.

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CHAPTER 4

THE FELINE BILE SALT EXPORT PUMP: A STRUCTURAL AND FUNCTIONAL COMPARISON WITH CANINE AND HUMAN Bsep/BSEP

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ABSTRACT

Background: The bile salt export pump (BSEP/ABCB11) is the primary transporter for the excretion of bile acids from hepatocytes into bile. In human, inhibition of BSEP by drugs has been related to drug-induced cholestasis and subsequent cytotoxic effects. The role of BSEP in canine and feline liver diseases has not been studied in detail, but the same mechanism of inhibition by drugs as in humans could play a role in veterinary medicine. The aim of this study was to investigate the functional characteristics of feline Bsep in comparison with canine and human Bsep/BSEP with respect to substrate affinities and inhibitory potential of model drugs. Orthologs of all three species were cloned and cell membrane vesicles overexpressing feline, canine and human Bsep/BSEP were prepared for functional analyses.

Results: The cDNA sequences of the open reading frames of feline, canine and human Bsep/BSEP showed a high similarity between the species. Functional studies demonstrated for all species a tendency to a higher affinity of BSEP/Bsep for the conjugated bile acid taurocholic acid (TCA) than glycocholic acid (GCA), and a higher affinity for GCA than for the unconjugated cholic acid (CA). The inhibitory potency of the model inhibitors cyclosporine A, troglitazone and ketoconazole was characterized against TCA uptake into BSEP/Bsep containing membrane vesicles. All three substances potently inhibited TCA uptake without significant species differences.

Conclusion: Structure and functional characteristics of cat, dog and human Bsep/BSEP appeared to be very similar, indicating that the properties of this transporter have been highly preserved among the different species. Therefore, inhibition of BSEP by drugs could also be a mechanism in cholestasis and liver disease in veterinary relevant animal species. This model could be used to predict drug-induced liver injury caused by BSEP inhibition at an early stage in veterinary drug development.

INTRODUCTION

An important function of the liver is the formation of bile, which is composed of bile salts, phospholipids and organic anions. Bile salts are amphipathic molecules that have detergent properties and can be highly toxic to hepatocytes if they accumulate intracellularly. Membrane-bound transporter proteins are essential in the transport of bile by the liver. The ATP-binding cassette (ABC) transporter, bile salt export pump (BSEP/ABCB11), is located in the canalicular membrane of the hepatocyte where it actively secretes bile salts into the bile at the expense of ATP hydrolysis.

In human medicine, inhibition of ABC transporters by drugs has been implicated in various adverse drug reactions of which drug-induced liver injury has been related to inhibition of BSEP in the liver. The inhibition of bile acid secretion leads to high intracellular bile acid concentrations and subsequent cytotoxic effects (Kullak-Ublick & Meier, 2000). The clinical relevance of BSEP in bile salt secretion in man has also been demonstrated by several genetic traits, such as progressive familial intrahepatic cholestasis type 2 (PFIC2), benign recurrent intrahepatic cholestasis (BRIC), and intrahepatic cholestasis of pregnancy (ICP) (for review see Nicolaou et al., 2012; Kubitz et al., 2012).

One of the first drugs that turned out to be a BSEP inhibitor was troglitazone and it was withdrawn from the market because of the development of cholestatic liver injury (Funk et al., 2001). To predict drug-induced liver injury caused by BSEP inhibition at an early stage in drug development, *in vitro* assays have been developed using membrane vesicles from genetically engineered cells overexpressing human or rat BSEP/Bsep (Horikawa et al., 2003). Several known cholestatic drugs showed BSEP inhibition in these membrane vesicles, including cyclosporine A, rifampicin and cloxacillin, suggesting that BSEP inhibition is the mechanism behind their hepatotoxic potential (Stieger, 2011).

The causes and prevalence of liver diseases in dogs and cats are mostly unknown (Watson, 2004), but have also been related to drugs (Bunch, 1993; Woodward, 2005). The role of BSEP in canine and feline liver diseases has not been studied in detail, but the same mechanism of inhibition by drugs as in humans could play a role in veterinary medicine. Recently, inhibition of the bile salt export pump and multi-drug resistance-associated protein (mrp) 2 by a novel kinase inhibitor was found to be related to the development of severe hepatotoxicity in dogs (Daniels et al., 2013). Previously, the canine Bsep has been cloned and partly functionally characterized to aid the extrapolation of toxicological data from dogs to humans (Yabuuchi et al., 2008). However, data on feline Bsep is completely absent.

The aim of this study was to investigate the functional characteristics of feline Bsep in comparison with canine and human Bsep/BSEP with respect to substrate affinities and inhibitory potential of model drugs. Knowledge about feline Bsep is lacking and therefore, this is the first study in which the feline Bsep has been cloned and characterized. As a model for *in vitro* cross-species extrapolation of hepatotoxic data, we cloned BSEP/Bsep of all three species and prepared cell membrane vesicles for functional analyses.

MATERIALS AND METHODS

Chemicals and reagents

Tauro[carbonyl-^{3}H]cholic acid (TCA) (5 Ci/mmol) was obtained from Perkin Elmer (Boston, MA). Cholic acid [2,4-^{3}H] (CA) (30 Ci/mmol) and glycocholic acid[glycine-2-^{3}H] (GCA) (40 Ci/mmol) were purchased from Biotrend (Köln, Germany). Adenosine triphosphate (ATP), adenosine monophosphate (AMP), cholic acid, cyclosporine A, glycocholic acid, ketoconazole, taurocholic acid, troglitazone were purchased from Sigma Aldrich (St. Louis, MO, USA). Bac-to-Bac and Gateway systems, Dulbecco's modified Eagle's medium-GlutaMAX-I culture medium and fetal calf serum were obtained from Invitrogen (CA, USA). Triple flasks (500 cm^{2}) were purchased from Sanbio BV Biological Products (Uden, The Netherlands).

RNA isolation and cDNA synthesis

Liver tissue was obtained from adult healthy European Shorthair cats (n=10, five males and five females, aged approximately 1 year) and adult healthy Beagle dogs (n=4, two males and two females, aged from 2 to 3 years) directly after euthanasia and samples were quickly frozen in liquid nitrogen and stored at -70°C. The cats and dogs had served as controls in authorized studies and the animals were sacrificed with permission of the Animal Ethical Committee and according to the Dutch law on Animal Experiments.

RNA was isolated from 30 mg frozen liver tissue by a spin column purification technique (SV Total RNA Isolation System, Promega, Medison, USA). Aliquots of the purified RNA were measured spectrophotometrically and the RNA was stored at -70°C. cDNA was synthesized using the protocol of the SuperScript III Reverse Transcriptase Kit (Invitrogen, California, USA). The reaction mixture, containing 1 µL of 50 µM oligo(dT)-anchor primer or a gene specific primer (Eurogentec S.A., Belgium), 1µg feline RNA or 2 µg canine RNA and 1 µL 10 mM dNTP Mix (Promega, Madison, USA) in a total volume of 13 µL, was incubated for 5 min at 65°C. Hereafter, the mixture was incubated on ice for at least 1 min and 4 µL of 5x First-Strand buffer (250 mM Tris-HCl pH

8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 µL of 0.1 M Dithiothreitol (DTT) and 400 U of SuperScript III Reverse Transcriptase were added to a total of 20 µL reaction volume. This final mixture was incubated for 60 min at 50°C and was inactivated by incubating it for 15 min at 70°C. The cDNA was stored at 4°C until use.

For quantitative Polymerase Chain Reaction (PCR), cDNA was synthesized using the protocol of iScript cDNA Systhesis Kit (Bio-Rad, CA, USA), using 1 µg of feline or canine RNA.

Sequence analysis

The sequence of Bsep cDNA from the canine and feline liver samples was analyzed using canine Bsep (Abcb11) specific primers, which were based on highly conserved regions between human and canine BSEP/Bsep [The National Centre for Biotechnology Information (NCBI) accession numbers for human: NM_003742; for dog: NM_001143932]. The primer sequences are given in Table 1 and were produced by Eurogentec S.A. Belgium. PCR was performed in a reaction mixture containing a final concentration of 1x Phusion Master Mix (2x Phusion Master Mix contained 0.04 U/µL Phusion DNA Polymerase, 2x Phusion HF Buffer with 3.0 mM $MgCl_2$ and 400 µM of each dNTP) (Finnzymes, Espoo, Finland), 1 µM forward primer, 1 µM reverse primer and 1 µL template cDNA in a total of 20 µL. The 3'-end and the 5'-end were obtained by means of a 3'RACE-PCR and 5'-RACE-PCR respectively, using a PCR-anchor primer. After an initial denaturation at 98°C for 30 s, PCR was performed at 98°C for 10 s, at 50-60°C for 30 s, and at 72°C for 30 s for a total of 35 cycles, followed by a final extension of 7 min at 72°C. The PCR-products were stored at 4°C until for further analysis.

The PCR products were separated by gel electrophoresis (1.2% agarose gel stained with ethidium bromide (Bio-Rad, CA, USA)) and the cDNA was extracted and purified from the gel by a spin column technique (Wizard SV Gel and PCR Clean-Up System kit, Promega, Madison, USA). The samples were further processed with the ABI PRISM BigDye Terminatior v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, USA), purified by Sephadex G-50 Superfine (Amersham Biosciences, NJ, USA) and sequenced by an automated DNA sequencer (ABI PRISM 3130xl, Applied Biosystems). The cDNA sequences of the feline and canine Bsep were assembled and the predicted protein sequence was derived from the open reading frame.

Table 1. Designed primers used for PCR and DNA sequencing (Eurogentec S.A., Belgium). The Oligo(dT)-anchor, PCR-anchor and the specific primer BSEP_RACE_A, were used for 3'RACE-PCR. The BSEP_Ar, Oligo(dT)-anchor, PCR-anchor, BSEP_RACE_B, C and D were used for 5'RACE-PCR. Each primer pair is denoted with "f" for the forward primer, and "r" for the reverse primer.

Primer	***Sequence (5'→3')***	***Position (relative to human BSEP [NM_003742])***
Oligo(dT)-anchor	5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT	
PCR-anchor	5'-GACCAGGCGTATCGATGTCGAC	
BSEP_RACE_A	5'-AGTGTTGTTTGCCTGTAGC	3615-3633
BSEP_RACE_B	5'-TGAAGAAAATCGAAACAAT	273-291
BSEP_RACE_C	5'-TGTGCCAAAAATGAGGAG	367-381
BSEP_RACE_D	5'-AGTTCTTGTAATTCAGTGTCA	405-425
BSEP_Af	5'-CTCGACCTGATACGCAAGTTCTGA	3392-3415
BSEP_Ar	5'-AATGGCCCGAGCAATAGCAATAC	3805-3825
BSEP_Bf	5'-CAAGGGAAGGTGATGATAGATGG	3526-3548
BSEP_Br	5'-GATGGGGGCTCCTGTGGTAACT	4065-4086
BSEP_Cf	5'-TGTGCTTCTTCCCCTTCTTGGCT	2840-2862
BSEP_Cr	5'-TGCCCATCTATCATCACCTTCC	3530-3548
BSEP_Df	5'-CAACGCTCCAAGTCTCA	2215-2231
BSEP_Dr	5'-GTGCGGATATTACTGAG	2956-2972
BSEP_Ef	5'-CAAGGCTTGCTACGGATGC	2702-2720
BSEP_Er	5'-TGATTGGGGGTTGTCGATC	3295-3310
BSEP_Ff	5'-GTGGTGGCCAGAAACAAAG	1805-1823
BSEP_Fr	5'-TCTGGCTGAATAAAAAGGCA	2442-2461
BSEP_Gf	5'-TGGATCGAATTAAGGGTGAA	1364-1383
BSEP_Gr	5'-GCGATGAGCAACCGAAATGA	1955-1974
BSEP_Hf	5'-GATGGGATTCTTTACTGGATTC	1089-1110
BSEP_Hr	5'-CCTTCACTGGGGTCATAGAA	1537-1556
BSEP_If	5'-GCAGCTCGTCAGATACAGAA	625-644
BSEP_Ir	5'-CAGAAGGCCAATGCATAACA	1132-1151
BSEP_Jf	5'-TGAAGGCCTATGCCAAAGC	938-956
BSEP_Jr	5'-TTCACCCTTAATTCGATCCA	1364-1383
BSEP_Kf	5'-ATTGTTTCGATTTTCTTCA	273-291
BSEP_Kr	5'-GCTTTGGCATAGGCCTTCA	938-956
BSEP_Lf	5'-AACCCTTGTCCAGATTTTCCTC	1197-1218
BSEP_Lr	5'-ATGATGGGTTTCCGGTCTATTG	1313-1334
FullBsep_felinef	5'-TTGCAATTACCATGTCTGACTCAGTAATTCTTCGC	
FullBsep_feliner	5'-TCAACTGATGGGGGCTCCTGTGATGACTAG	
FullBsep_caninef	5'-TTGCAATTACCATGTCTGATGCAGTAATTCTTCGC	
FullBsep_caniner	5'-TCAACTGATGGGGGCTCCTGTGGTAACTAG	
FullBSEP+AttB1_humanf	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCA CCATGTCTGACTCAGTAATTCTTC	
FullBSEP+AttB1_humanr	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAC TGATGGGGGATCCAGTG	
AttB1_felinef	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCC ACCTTGCAATTACCATGTCTGACTCAGTAATTCTTCGC	
AttB1_feliner	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAC TGATGGGGGCTCCTGTGATGACTAG	

Table 1. Continued

AttB1_caninef	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCC ACCTTGCAATTACCATGTCTGATGCAGTAATTCTTCGC
AttB1_caniner	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAC TGATGGGGGCTCCTGTGGTAACTAG

Hepatic mRNA expression of feline and canine Bsep (Abcb11)

The expression of Bsep cDNA in feline and canine liver samples was evaluated by RT-PCR using SYBR Green Supermix (Bio-Rad, CA, USA) and species-specific primers (Table 1) and gel electrophoresis. After a hot start of 95°C for 3 min, PCR was performed at 95°C for 20 s, at 55-65°C for 30 s, and at 72°C for 30 s for a total of 40 cycles.

Cloning of cDNA encoding Bsep/BSEP (Abcb11/ ABCB11)

To isolate the full-length Bsep encoding sequence, a PCR was performed as described before with primers specific for each animal species. Primers are given in Table 1. For the cat the set of primers consisted of the forward and reverse primers FullBsep_felinef and FullBsep_feliner respectively. For the dog, the forward primer FullBsep_caninef and the reverse primer FullBsep_caniner was used. The product obtained from this first PCR was used for a second PCR, to attach AttB1-primers used for the cloning procedure of Bsep into the membrane vesicles of HEK293 cells (Gateway, Invitrogen, CA, USA). The conditions of the PCR analysis were similar as described above, except for a final concentration of approximately 9% DMSO in the PCR mixture, an annealing temperature of 70°C and a total of 10 cycles. AttB1-primers used for this second PCR are given in Table 1. A PCR reaction to obtain the full-length human BSEP encoding sequence was performed on the ORFEXPRESSTM-ABCB11 vector (LabOmics, GC-H5308) with the forward primer FullBSEP+AttB1_humanf and the reverse primer FullBSEP+AttB1_humanr. After obtaining the BSEP-AttB-product of each species, the product was purified with 30% polyethylene glycol (PEG) 8000/30 mM $MgCl_2$ to remove the surplus of primers.

The pENTR221-BSEP vectors for all species were constructed by performing a BP-reaction. Toxicity problems due to a TATAAT sequence in the human BSEP gene (Noe et al., 2002) were circumvented by introducing a silent mutation at base pair position 81 (AAT→AAC). The sequences of the BSEP/Bsep genes within the pENTR221-BSEP vectors were confirmed, and an LR reaction was

performed with the pENTR221 vectors containing the feline, canine and human Bsep/BSEP and with the destination vector BacMam-VSV-DEST. Enhanced yellow fluorescent protein (eYFP) was used as a negative control, as described previously (Wittgen et al., 2011). Eventually, the expression vectors BacMam-VSV-EX-BSEP were constructed for all species.

Preparation of membrane vesicles of HEK293 cells expressing Bsep/BSEP

After production of recombinant baculo-viruses following the Bac-to-Bac manual, HEK293 cells were transfected and membrane vesicles were isolated as previously described (Wittgen et al., 2011). Briefly, transfected HEK293 cells were lysed with a hypotonic buffer and centrifuged at 100.000*g*. Pellets were resuspended in an isotonic buffer and the membrane vesicles were isolated with spinning steps of 4000*g* and 100.000*g*. After passing the membranes through a 27-gauge needle for 25 times, protein concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad, CA, USA). Presence of BSEP protein in the vesicles was demonstrated by Western blotting using a polyclonal antibody against rat Bsep, which was a generous gift from Dr. B. Stieger (University of Zürich, Switzerland). The antibody was produced in rabbits against the last 13 amino acids of rat Bsep (amino acid sequence: AYYKLVITGAPIS) (Noe et al., 2002) and a secondary goat-anti-rabbit HRP antibody. As a control, Western blotting was first performed on liver tissue of rats, cats and dogs. Protein concentrations were determined according to Bradford (Bradford, 1976).

Functional characterization of Bsep/BSEP-containing membrane vesicles

The membrane vesicles (7.5 µg protein) were incubated in a 30 µL transport mixture with a final concentration of 10 mM Tris Base (pH 7.4), 250 mM sucrose, 10 mM $MgCl_2$, 4 mM adenosine triphosphate (ATP) or adenosine monophosphate (AMP), 1 µM [^{3}H]taurocholic acid (TCA), with or without an inhibitor, in 96-well plates at 37°C. For the concentration-dependent curves 0.15 µCi [^{3}H]TCA, 0.05 µCi [^{3}H]GCA and 0.3µCi [^{3}H]CA was used, supplemented with unlabeled TCA, GCA or CA respectively. The reaction was stopped by placing the 96-well plate on ice-water and by adding 150 µL ice-cold washing buffer (10 mM Tris Base pH 7.4, 250 mM sucrose). The reaction mixture was transferred to a 96-well Multi-Screen HTS filter plate (Millipore, Ireland) and the total mixture was filtered by means of a Multi-Screen HTS vacuum manifold filtration device (Millipore, Etten-Leur, The Netherlands). The filters were washed twice with 200 µL washing buffer, whereafter they were separated from the plate. Two ml scintillation fluid was added and the radioactivity remaining on the filter was measured by a liquid scintillation

analyzer (Tri-carb 2900 TR, Packard). ATP-dependent transport was calculated by subtracting uptake in presence of AMP from that in presence of ATP. The incubation period was checked for linearity in transport rate. Concentration-dependent BSEP transport rates were fitted according to Michaelis-Menten enzyme kinetics and K_m values were calculated by means of GraphPad Prism 6.01 software (San Diego, California, USA).

Statistical analysis

Data were expressed as means ± SD of at least three independent experiments with samples performed in duplicate. Data were analyzed using a one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test (GraphPad Prism 6.01 software, San Diego, California, USA) with $P<0.05$ denoting a significant difference.

RESULTS

cDNA sequence and predicted amino acid sequence of feline and canine Bsep

The cDNA coding sequences of feline and canine Bsep were obtained as described in the Materials and Methods section and demonstrated a high level of homology with an identity of 91.2%. The feline cDNA sequence has been submitted to the NCBI database [NCBI accession number KF_601333]. The open reading frame (ORF) of the obtained canine Bsep sequence was highly identical (99.8%) to the previously reported sequence by Yabuuchi et al. (2008), with only a few non-coding differences in the cDNA sequence resulting in a completely similar amino acid sequence. Feline Bsep cDNA was 89.3% identical to human BSEP sequence [NCBI accession number NM_003742]. In this respect, dogs also share 89.3% identity with human BSEP.

The amino acid sequence deduced from the ORF of ABCB11 of the different species is shown in Figure 1. Differences in amino acids between human, dogs and cats are displayed in small black borders. The amino acid sequences showed a similarity of 92.3% between cats and dogs. Cat Bsep is 88.9% identical to the human protein, and dog Bsep 89.6%. The twelve transmembrane domains, the Walker A and B motifs and the signatures were highly identical between species. The major differences in amino acid pattern were located halfway the ORF sequence (ORF position 661-684), in the "linker domain" of the first nucleotide-binding domain, over a range of 23 amino acids. Moreover, both feline and canine ORF sequences contained four amino acids more than the human BSEP ORF in this region.

Bsep/BSEP gene and protein expression in liver tissue samples

Presence of *Bsep* mRNA in pooled liver samples from cats and dogs was demonstrated by PCR analyses (Figure 2). The bands of *Bsep* mRNA can only be compared qualitatively, but they appeared to be of similar density. Presence of Bsep protein in liver tissue was demonstrated by Western blotting using a rabbit anti-rat Bsep antibody. The expression of Bsep was observed in livers from rat, dog and cat (data not shown).

Bsep/BSEP protein expression in membrane vesicles prepared from native and Bsep/BSEP-overexpressing HEK293 cells

The presence of Bsep/BSEP in membrane vesicles prepared from HEK293 cells overexpressing feline, canine and human Bsep/BSEP was confirmed by Western blotting (Figure 3). No staining was seen in the vesicles serving as negative control that were prepared from mock-transduced HEK293 cells with eYFP, which indicates that both bands in the Western blot (lane C, D, and E) are specific. The double band can be explained by posttranslational modifications (most likely N-glycosylation) of the protein as previously discussed by Gerloff *et al.* (1998) (Gerloff et al., 1998).

Vesicular uptake studies

Vesicular uptake of tritium-labeled TCA, GCA and CA was demonstrated for all samples containing Bsep/BSEP proteins, while uptake into membrane vesicles prepared from mock-transduced HEK293 cells did not exceed background activity (data not shown). The vesicular uptake was ATP-dependent and the rate of uptake was time-dependent (data not shown). To be in the linear range of the uptake rate, samples were incubated for 5 minutes to assess the transport kinetics of TCA and 7.5 minutes to assess the transport kinetics of GCA and CA.

The rate of bile salt uptake into the vesicles was saturable and the data could be described according to Michaelis-Menten kinetics (Figure 4 and 5). K_m values obtained for each bile acid from nonlinear regression analysis were compared between species (Table 2). There were no differences in K_m values of each bile acid between the vesicles prepared from cells overexpressing Bsep/BSEP of the different species. Within species, the K_m value for uptake of CA by the vesicles overexpressing feline or canine Bsep was higher than the corresponding K_m value for TCA uptake. The feline Bsep also had a lower affinity for CA than for GCA.

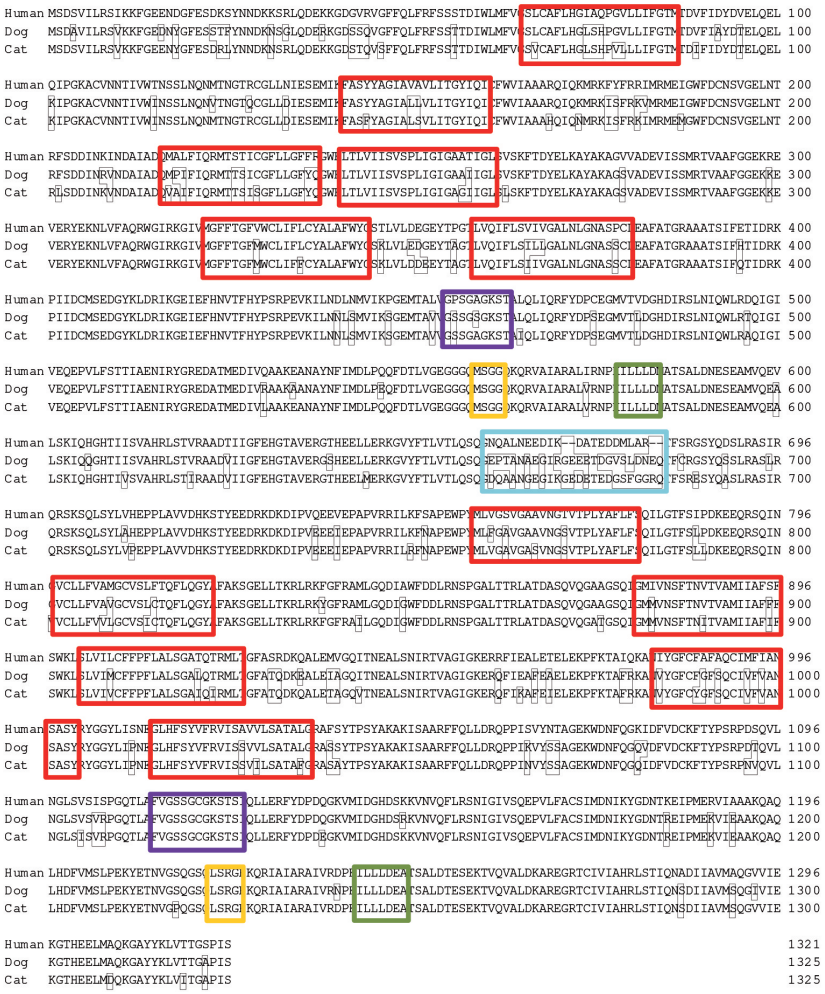

Figure 1. Alignment of human, dog and cat amino acid sequences of BSEP/Bsep ORF. Differences in amino acid pattern relative to the human BSEP sequence are given in black borders. Transmembrane domains were predicted by Yabuuchi et al. (2008) for the dog, and are given in red borders for all species. Walker A is given in purple borders, Walker B in green borders and Signature C in yellow borders. The region of highly different amino acid sequences is given in a blue border.

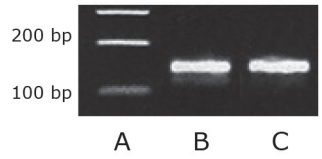

Figure 2. Bsep product of canine and feline liver tissue using primers BSEP_Lf and BSEP_Lr on a 1.8% agarose gel stained with ethidium bromide. A: BenchTop 100 bp DNA Ladder (Promega, USA); B: canine Bsep; C: feline Bsep

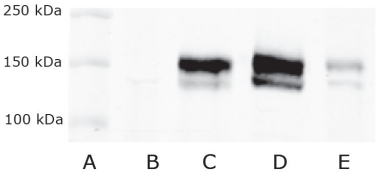

Figure 3. Western Blot of membrane vesicles over-expressing cat, dog and human Bsep/BSEP. A: Precision Plus Protein Dual Color Marker (Bio-rad Laboratories); B: eYFP (control); C: Bsep cat; D: Bsep dog; E: BSEP human. In lanes B, C, D, and E equal amounts of total protein were loaded.

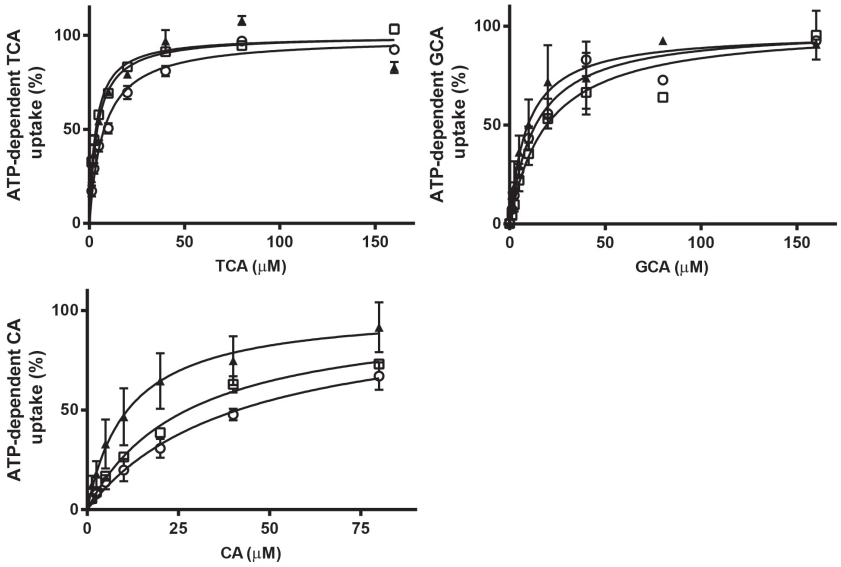

Figure 4. ATP-dependent transport of TCA, CA and GCA in membrane vesicles (7.5 µg protein) expressing BSEP/Bsep of human (▲), dogs (□), and cats (○).Vesicles were incubated in 10 mMTris Base buffer (pH 7.4) containing 250 mM Sucrose, 10 mM $MgCl_2$ and 4 mM ATP or AMP, at 37°C for 5 min (TCA) or 7.5 min (CA and GCA). ATP-dependent transport was calculated by subtracting transport in presence of AMP from that in presence of ATP. Curves were fitted by GraphPad Prism 6.01 software (San Diego, California, USA) and V_{max} was calculated. Measurements were performed in duplicate in at least three independent experiments. The mean of the duplicates of each experiment was transformed as a relative activity to the V_{max} (=100%). Each value in the graph is the mean ± SD of the relative activities of three independent experiments.

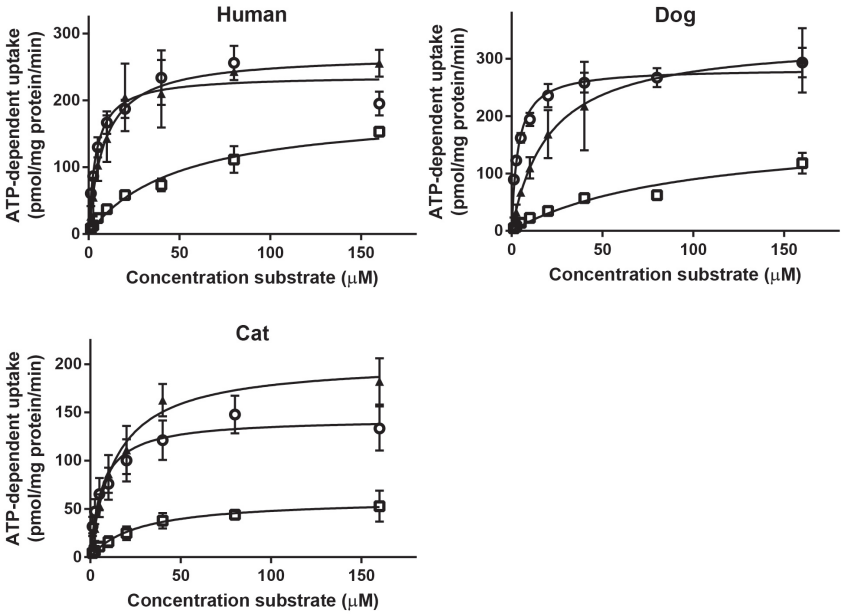

Figure 5. ATP-dependent transport of TCA (○),GCA (▲) and CA (□) in membrane vesicles (7.5 µg protein) expressing human, dog and cat BSEP/Bsep.Vesicles were incubated in 10 mM Tris buffer (pH 7.4) containing 250 mM Sucrose, 10 mM $MgCl_2$ and 4 mM ATP or AMP, at 37°C for 5 min (TCA) or 7.5 min (CA and GCA). ATP-dependent transport was calculated by subtracting transport in presence of AMP from that in presence of ATP. Curves were fitted by using GraphPad Prism 6.01 software (San Diego, California, USA). Measurements were performed in duplicate in three independent experiments and are given in pmol/mg protein/min. Each value in the graph is the mean ± SD of three independent experiments.

Table 2. K_m values for TCA, GCA and CA of human, dog and cat BSEP/Bsep containing membrane vesicles (7.5 µg protein). K_m/V_{max} curves were fitted by GraphPad Prism 6.01 software (San Diego, California, USA) and K_m was calculated. Measurements were performed in duplicate in at least three independent experiments and mean K_m ± SD are given in the table. Statistical significance was determined by a two-way ANOVA followed by the Bonferroni post-hoc test. P<0.05 indicates a significant difference.

	Km (µM)		
	Human	***Dog***	***Cat***
TCA	4.1 ± 0.5	3.4 ± 0.3	7.5 ± 0.7
GCA	9.0 ± 2.0	19.3 ± 3.5	13.4 ± 2.0
CA	11.3 ± 4.3	27.2 ± 3.2[a]	41.2 ± 11.3[b,c]

[a] significantly different from TCA in dogs

[b] significantly different from TCA in cats

[c] significantly different from GCA in cats

Differences in maximum rate of ATP-dependent uptake were seen between uptake of CA versus TCA and GCA. Variations in the maximum rate of ATP-dependent uptake were also seen in the uptake of the bile acids between the species (Figure 5).

The inhibitory effects of three model Bsep inhibitors were subsequently determined on TCA uptake in the membrane vesicles. Cyclosporine A, troglitazone and ketoconazole decreased the uptake of TCA by vesicles prepared from the cells overexpressing Bsep/BSEP of the different species and the data were fitted by nonlinear regression to a one-site competition model (Figure 6). Inhibitory potencies, as given by the IC50 values, were all in the micromolar range and the potency of each compound did not differ significantly between species (Table 3).

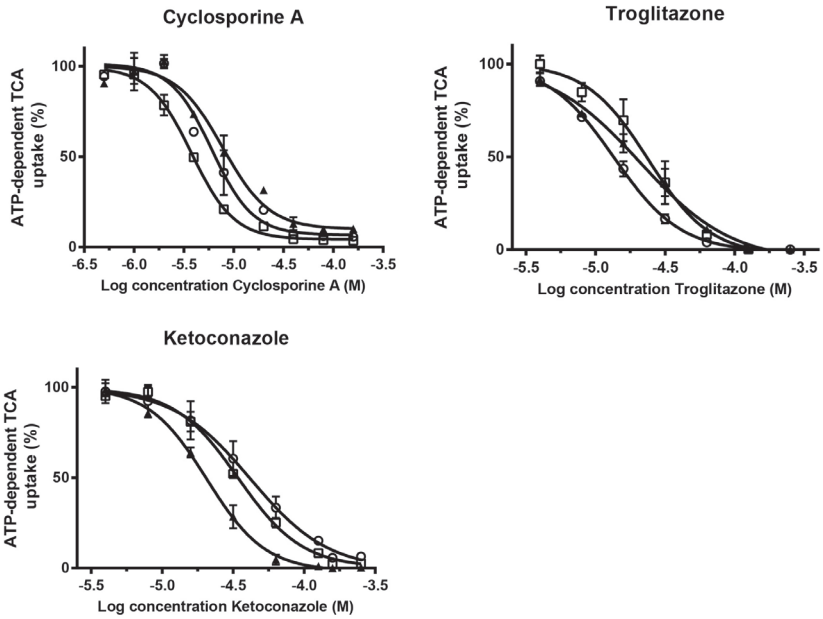

Figure 6. Inhibition of cyclosporine A, troglitazone and ketoconazole on the uptake of 1 μM [^{3}H]TCA in membrane vesicles (7.5 μg protein) expressing human (▲), dog (□) and cat (○) BSEP/Bsep. Vesicles were incubated in 10 mM Tris Base buffer (pH 7.4) containing 250 mM Sucrose, 10 mM $MgCl_2$ and 4 mM ATP or AMP, at 37°C for 5 min. ATP-dependent transport was calculated by subtracting transport in presence of AMP from that in presence of ATP. Inhibition curves were fitted by using GraphPad Prism 6.01 software (San Diego, California, USA) and values are expressed as mean ± SD of percentage uptake of four independent experiments.

Table 3. Inhibitory potency of cyclosporine A, troglitazone and ketoconazole against 1 µM [^{3}H]TCA uptake into membrane vesicles (7.5 µg protein) expressing human, dog and cat BSEP/Bsep.

	IC_{50} (µM)		
	Human	***Dog***	***Cat***
Cyclosporine A	7.8 ± 1.2	3.8 ± 0.2	6.0 ± 0.8
Troglitazone	20.9 ± 2.9	23.9 ± 2.5	13.6 ± 4.2
Ketoconazole	20.6 ± 1.1	34.2 ± 2.0	41.8 ± 6.6

Inhibition curves were fitted by using GraphPad Prism 6.01 software (samples without inhibitor were set on 100%). [^{3}H]TCA uptake in presence of ATP was corrected for uptake in presence of AMP. Measurements were performed in duplicate in four independent experiments. Statistical significance was determined by one-way ANOVA followed by the Bonferroni post-hoc test with $P<0.05$ denoting a significant difference. No significant differences were found between the different species.

DISCUSSION

The aim of this study was to characterize and compare the function of feline bile acid efflux transporter Bsep with canine and human Bsep/BSEP with respect to endogenous bile acids and typical drug inhibitors. To this end, membrane vesicles isolated from genetically engineered cells overexpressing Bsep/BSEP of the different species were made. To our knowledge, this is the first study in which feline Bsep has been cloned and characterized.

The cDNA sequence of the ORFs of feline, canine and human Bsep/BSEP showed a high similarity between the species. Analysis of the translated protein sequences revealed that the twelve transmembrane domains, which are thought to be responsible for substrate specificity, and the Walker A and Walker B motifs, which are needed for binding and hydrolyzation of ATP, appeared to be highly identical. A more divergent part of the protein was observed in the linker region from amino acid 661 to 684, where feline and canine Bsep contains four additional amino acids compared to the human BSEP. The linker region may have a regulatory role in the rate of ATP hydrolysis, as has been described for P-glycoprotein/ABCB1 (Sato et al., 2009), or it could mediate the turnover of the ABC transporter (Kolling & Losko, 1997). Amino acid differences in the linker region could also relate to species differences in post-transcriptional regulation of BSEP/Bsep.

The presence of BSEP/Bsep protein in the membrane vesicles that were prepared from HEK293 cells overexpressing the transporter was confirmed by Western blotting. Although differences in BSEP/Bsep staining intensity were observed between vesicle preparations of the different species, a quantitative estimate of transport protein abundance could not be made. The used antibody was developed against the last 13 amino acids of the ORF of rat Bsep and differences in affinity for the proteins of the different species could be expected. The terminal 13 amino acids of rat Bsep are exactly the same for feline Bsep. Canine Bsep differs one amino acid and human BSEP two amino acids from rat. Variations in transporter abundance in the vesicle preparations may have occurred and a direct comparison of maximum transport activities of BSEP/Bsep between the different species can therefore not be made. However, we observed in different transductions and vesicle preparations consistently lower V_{max} values of cat Bsep for all tested bile acids compared to human and dog BSEP/Bsep. Moreover, in spite of a low staining intensity for human BSEP compared to canine and feline Bsep, we consistently found the highest V_{max} for human BSEP for all substrates compared to the other species.

The functional studies demonstrated that TCA, GCA and CA were substrates of BSEP/Bsep in all three species, showing saturable transport kinetics. K_m values were in the same range as reported by different groups for human BSEP and rat Bsep in different expression systems (Stieger, 2011). In all species there was a tendency to a higher affinity of Bsep for TCA than GCA, and a higher affinity for GCA than CA. The same order of affinity of the bile acids TCA and GCA was previously noted for human BSEP (Byrne et al., 2002; Noe et al., 2002). The lower affinity and maximum transport rate of CA compared to TCA and GCA for feline and canine Bsep, is in accordance with a relatively lower amount of unconjugated bile salts in bile of these species. The conjugated bile acid TCA is a model substrate for BSEP, commonly used in *in vitro* functional studies, although in man bile acids are mainly conjugated to glycine and only to a minor extent to taurine. This is in contrast to carnivore species, like cat and dog, in which bile acids are almost exclusively conjugated to taurine with TCA as the major bile acid in both species (Wildgrube et al., 1986; Washizu et al., 1991; Perwaiz et al., 2001). Since protein sequence and function of Bsep appeared to be highly conserved among human, dog and cat, inter-species variation in the constitution of conjugated bile acids must be more related to the availability of co-substrates or enzymes for conjugation.

The inhibitory potency of the model inhibitors cyclosporine A, troglitazone and ketoconazole was characterized against TCA uptake into BSEP/Bsep containing membrane vesicles. All three substances potently inhibited TCA uptake without significant species differences. A comparable inhibitory

potential of troglitazone was found previously for human and canine BSEP/Bsep (Byrne et al., 2002; Yabuuchi et al., 2008).

CONCLUSIONS

The structure and functional characteristics of cat Bsep appeared to be very similar to dog and human Bsep/BSEP, indicating that the properties of this transporter have been highly preserved among the different species. The methods and results of this study can be used as an *in vitro* model for the assessment of interactions of drugs and other substances with feline and canine Bsep, which is suitable to study the risk of drug-induced cholestasis in these species in more detail.

Inhibition of BSEP by drugs has been related to cholestasis and subsequent drug-induced liver injury in man and could also be a mechanism in cholestasis and liver disease in veterinary relevant animal species.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

CB and JH carried out all the experimental work and CB drafted the manuscript. JK, JS and FR provided valuable information on the subject of ABC-transporters, the design of the study and revised the manuscript. JS and FR coordinated and supervised the study. All authors read and approved the final manuscript.

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CHAPTER 5

FELINE HEPATIC BIOTRANSFORMATION OF DIAZEPAM: DIFFERENCES BETWEEN CATS AND DOGS

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Research in Veterinary Science, manuscript under revision

ABSTRACT

Diazepam induces acute liver failure in cats after repeated dosing. Because hepatotoxic effects of diazepam are rare in humans and dogs, and as diazepam is glucuronidated in these species before excretion, it was hypothesized that cats have a limited hepatic capacity to metabolize diazepam. In addition, since a cholestatic pattern of serum enzyme elevations has been seen in human patients, it was hypothesized that inhibition of the feline bile salt export pump (Bsep) was the underlying mechanism of toxicity.

Marked differences were observed in the biotransformation of diazepam between feline and canine liver microsomes, with a low efficiency of phase I metabolism of diazepam and phase II glucuronidation of diazepam and metabolites in the feline liver microsomes. Diazepam and its phase I metabolites inhibited the feline and canine Bsep.

Diazepam is poorly metabolized in feline liver and inhibits bile salt secretion with the risk of hepatotoxicity.

INTRODUCTION

Diazepam, a benzodiazepine, has been suggested for use in cats as deduced from human medicine for the treatment of a variety of indications, including muscle relaxation and anticonvulsive therapy, treatment of behavioral disorders and anxiety, and appetite stimulation. The dose recommended for treatment of seizures is 0.5-1 mg/kg q8-12h IV or rectally, and for behavioral disorders doses generally comprise 0.2-0.4 mg/kg q12-24h orally, or 1.0-2.5 mg per cat q8-12h orally (Plumb, 2011). However, care should be taken with long-term dosing, because cats have shown to frequently exhibit severe side effects such as depression, anorexia, and acute hepatic failure after repeated dosing (Dez Hughes et al., 1996; Center et al., 1996; Park, 2012).

The underlying mechanism of acute liver injury caused by diazepam in cats is unknown, but could be related to differences in biotransformation of diazepam compared to other species (Park, 2012). Diazepam is converted into three metabolites in most animal species, nordiazepam (=N-desmethyldiazepam), temazepam and ultimately oxazepam, via phase I N-demethylation or hydroxylation. The biotransformation pathways are shown schematically in Figure 1, and all these metabolites are pharmacologically active. Although differences in the relative quantities of these formed metabolites have been observed previously between species, nordiazepam appeared to be the principal metabolite of diazepam in man and dogs (Vree et al., 1979; Chenery et al., 1987; Seddon et al., 1989), and the lipophilic metabolites temazepam and oxazepam are excreted as phase II glucuronides in the urine (Schwartz et al., 1965; Vree et al., 1979).

Cats are particularly known for their sensitivity to phenolic derivatives and it was found that the pseudogene UGT1A6 was responsible for the impaired glucuronidation capacity of cats (Court & Greenblatt, 2000; van Beusekom et al., 2014). Considering that the metabolites of diazepam are conjugated with glucuronic acid in humans and dogs, the impaired feline glucuronidation capacity might result in intrahepatic accumulation of diazepam and phase I metabolites causing liver injury after repeated dosing. In humans, inhibition of the bile salt export pump (BSEP) is one of the causes of drug-induced liver injury (DILI). BSEP, a member of the ATP-binding cassette transporters, is responsible for the active efflux of bile acids from the hepatocytes into the bile canaliculi. Inhibition of this transporter by drugs can cause cholestasis and subsequent cytotoxic effects (Kullak-Ublick & Meier, 2000).

Hence, we hypothesized that species-specific feline hepatic biotransformation and excretion pathways (of bile salts) might explain the sensitivity of cats to

diazepam. Therefore, a series of experiments addressing the biotransformation of diazepam and its metabolites were conducted *in vitro* using cat liver microsomes. Moreover, the inhibition of the bile salt export pumps by diazepam and its phase I metabolites was investigated by membrane vesicles overexpressing feline Bsep and data were compared with those of dogs.

MATERIALS & METHODS

Chemicals and reagents

Adenosine triphosphate (ATP), adenosine monophosphate (AMP), alamethicin solution, cholic acid, cyclosporine A, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glycocholic acid, ketoconazole, magnesium chloride hexahydrate, β-nicotinamide adenine dinucleotide phosphate hydrate (NADP), taurocholic acid, troglitazone and uridine 5′-diphosphoglucuronic acid (UDPGA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diazepam, (*R,S*)-temazepam and (*R,S*)-oxazepam were purchased from BUFA B.V. (IJsselstein, The Netherlands). N-desmethyldiazepam (=nordiazepam) and temazepam glucuronide were purchased from Lipomed (Arlesheim, Switzerland). Oxazepam glucuronide was purchased from Cerilliant Corporation (Round Rock, Texas, USA). Tauro[carbonyl-^{3}H]cholic acid (TCA) (5 Ci/mmol) was obtained from Perkin Elmer (Boston, MA, USA).

Tissue samples

Liver tissue was obtained from adult healthy European Shorthair cats (n=8, five males and three females, aged from 11 to 13 months) directly after euthanasia and samples were immediately frozen in liquid nitrogen and stored at -70°C. The cats had served as controls in a study for the development of FIV vaccines. The same applies to the liver samples of Beagle dogs (n=7, two males and five females, aged from 3.5 to 4.5 years) that had also served as controls in clinical trials. Animals were sacrificed with permission of the Animal Ethical Committee of Utrecht University and euthanasia was performed according to the Dutch law on Animal Experiments.

Preparation of submitochondrial fractions

Submitochondrial fractions (commonly referred to as microsomes) containing predominantly microsomal proteins were isolated according to the procedure by Rutten *et al.* (1987) with minor modifications described in our previous study (van Beusekom et al., 2014). In brief, cat and dog liver samples of approximately 10 g were homogenized with 1.15% KCl, containing 0.1 mM EDTA at 4°C. The homogenates were centrifuged at 9000 *g* for 25 min at

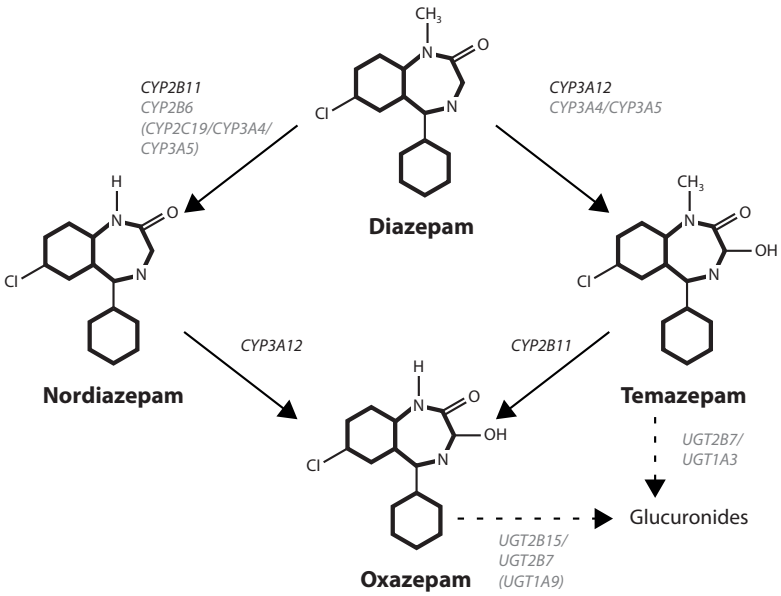

Figure 1. Metabolism pathway of diazepam (Yang et al., 1999; Court et al., 2002; Shou et al., 2003; Acikgöz et al., 2009). The involved isozymes are given in black for dogs, and in grey for humans.

4°C, and the supernatant obtained (S9-fraction) was centrifuged at 100 000 *g* for 1 hour and 15 min at 4°C. The microsomal pellet was re-suspended in 1.15% KCl 0.05 M phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol. The microsomes were then quickly frozen in liquid nitrogen and stored in Eppendorf-cups at -70°C until use (Rutten et al., 1987).

The protein concentrations of the microsomal fractions were determined by the method of Bradford (Bradford, 1976) and data were expressed as nmol/mg protein/min.

Phase I (N-demethylation and hydroxylation) assays of diazepam

For measuring phase I reactions pooled liver microsomes were used. Incubation mixtures contained 100 mM phosphate buffer (KH_2PO_4, pH 7.4), 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, and a concentration range of diazepam, in a total volume of 500 µL and a final concentration of 1% DMSO. Assays were tested for linearity in incubation time and protein concentration. For all substances, an incubation time of 10 min

and a microsomal protein concentration of 0.5 mg/ml was within the linear range. Pre-incubations were performed for 5 min at 37°C, whereafter the reactions were initiated by adding NADP to a final concentration of 1 mM. Samples remained at 37°C in a heat block for the indicated time and were shaken regularly in order to ensure an equal temperature within the incubating mixtures. Reactions were terminated by addition of ice-cold acetonitrile, followed by a rapid cooling step. Samples were centrifuged at 13,000 *g* for 5 min and the supernatants were directly injected onto the HPLC column. All measurements to determine K_m and V_{max} values were performed in duplicates and in at least three independent experiments. Blanks were obtained from incubations without NADP.

Phase II (glucuronidation) assays of diazepam and its metabolites

Glucuronidation assays were performed with pooled liver microsomes. Incubation mixtures contained 100 mM phosphate buffer (KH_2PO_4, pH 7.4), 5 mM $MgCl_2$, alamethicin (50 µg/mg protein) and a concentration range of diazepam, nordiazepam, (*R,S*)-temazepam or (*R,S*)-oxazepam, in a total volume of 500 µL and with a maximum of 5% DMSO or 1% methanol as final concentration. Assays were tested for linearity in incubation time and protein concentration. The chosen incubation times were 30 min for diazepam and (*R,S*)-temazepam, and 60 min for nordiazepam and (*R,S*)-oxazepam, all with a microsomal protein concentration of 0.5 mg/ml. Pre-incubations were performed for 5 min at 37°C, where after the glucuronidation reactions were initiated by adding UDPGA with a final concentration of 5 mM. Samples remained at 37°C in a heat block for the indicated time and were shaken regularly in order to ensure an equal temperature within the incubating mixtures. Reactions were terminated by addition of ice-cold acetonitrile, followed by a rapid cooling step. Samples were centrifuged at 13,000 *g* for 5 min and the supernatants were directly injected onto the HPLC column. All measurements to determine K_m and V_{max} values were performed in duplicates and in at least three independent experiments. Blanks were obtained from incubations without UDPGA.

HPLC conditions

The HPLC system was a Prominence HPLC and consisted of an auto injector, two pumps, a column oven, an UV-detector (set at a wavelength of 230 nm), all from Shimadzu ('s-Hertogenbosch, The Netherlands), and a C18 column (250 x 4.6 mm, 5 µm, RP, Gemini, Phenomenex). The mobile phase consisted of two solutions: (A) 20 mM phosphate buffer (KH_2PO_4, pH 4.5); (B) acetonitrile. The gradient for the phase I incubations started with 75% A and 25% B for 4 min, whereafter it was balanced up to 40% B in 5 min. The concentration

Table 1. Incubation conditions for the different substrates and HPLC retention times for the substrates and formed products.

Substrate	***Product formed***	***Incubation time***	***Protein concen-tration***	***Retention time (phase I protocol)***	***Retention time (phase II protocol)***
(*R*,*S*)-Oxazepam		60 min	0.5 mg/ml		21.4 min
	R-oxazepam glucuronide				7.4 min
	S-oxazepam glucuronide				8.3 min
(*R*,*S*)-Temazepam		30 min	0.5 mg/ml		22.6 min
	R-temazepam glucuronide				12.9 min
	S-temazepam glucuronide				13.3 min
Diazepam		10 min (phase I) 30 min (phase II)	0.5 mg/ml	24.6 min	24.1 min
	Oxazepam			14.2 min	21.4 min
	Temazepam			17.7 min	22.6 min
	Nordiazepam			18.7 min	22.7 min

Mobile phase: (A) 20 mM phosphate buffer (KH_2PO_4, pH 4.5); (B) acetonitrile.
Protocol phase I: start with 25% B for 4 min, balance up to 40% B in 5 min, stay at 40% B for 6 min. Gradually decrease back to 25% B in 1 min and stay at 25% B for 4 min. Balance up to 60% B again in 1 min, stay at 60% B for 9 min, and decrease back to 25% in 2 min.
Protocol phase II: start with 25% B for 15 min, balance up to 60% B in 5 min, stay at 60% B for 7 min, then gradually decrease back to 25% B in 1 min.

of 40% B remained for 6 min and gradually decreased back to 25% B in 1 min. The purpose of using 40% B was the separation of temazepam and nordiazepam, because with higher acetonitrile concentrations these peaks merged. The concentration of 25% B remained for 4 min and was increased to 60% B in 1 min and remained for 9 min, to accelerate the retention time of diazepam. Thereafter the concentration of B was decreased to 25% in 2 min. The retention times of all substrates and metabolites are shown in Table 1. The gradient for the phase II incubations started with 75% A and 25% B for 15 min, whereafter it was balanced up to 60% B in 5 min. The concentration of 60% B remained for 7 min and gradually decreased back to 25% B in 1 min.

Inhibition of Bsep transport activity by diazepam and its metabolites

Membrane vesicles were produced by transfected HEK293 cells overexpressing feline Bsep as described in our previous study (van Beusekom et al., 2013).

Bsep transport activity was determined by measuring ATP-dependent taurocholic acid (TCA) uptake into the membrane vesicles. The membrane vesicles (7.5 µg protein) were incubated in a 30 µL transport mixture with a final concentration of 10 mM Tris Base (pH 7.4), 250 mM sucrose, 10 mM $MgCl_2$, 4 mM adenosine triphosphate (ATP) or adenosine monophosphate (AMP), 1 µM [^{3}H]taurocholic acid, and a concentration range of diazepam or one of its metabolites, in 96-well plates at 37°C for 5 min. The reaction was stopped by placing the 96-well plate on ice-water and by adding 150 µL ice-cold washing buffer (10 mM Tris Base pH 7.4, 250 mM sucrose). The reaction mixture was transferred to a 96-well Multi-Screen HTS filter plate (Millipore, Ireland) and the total mixture was filtered by means of a Multi-Screen HTS vacuum manifold filtration device (Millipore, Etten-Leur, The Netherlands). Filters were washed twice with 200 µL washing buffer, where after they were separated from the plate. Scintillation fluid was added and the radioactivity that remained on the filter was measured by a liquid scintillation analyzer (Tri-carb 2900 TR, Packard). ATP-dependent transport was calculated by subtracting uptake in presence of AMP from that in presence of ATP, and the Bsep transport activity of TCA without diazepam or one of its metabolites was set on 100%.

Statistical analysis

Data were expressed as means ± SD of at least three independent experiments with samples performed in duplicate. Data of the phase I and phase II experiments were analyzed by nonlinear regression analysis according to the Michaelis-Menten equation using GraphPad Prism 6.04 software (San Diego, California, USA). Data of Bsep inhibition were fitted according to a one-site binding competition model with the same software. Subsequently, K_m, V_{max} and IC_{50} values were calculated and statistically evaluated using an independent two-sample Student's t-test with $P < 0.05$ denoting a significant difference.

RESULTS

Phase I (N-demethylation and hydroxylation) assays of diazepam

Incubations of the microsomes with a concentration range of diazepam resulted in the formation of the metabolites nordiazepam and temazepam. Cat liver microsomes produced mainly temazepam, while in dog microsomes relatively more nordiazepam was found. Data were fitted according to Michaelis-Menten enzyme kinetics, and the calculated K_m and V_{max} are presented in Figure 2 and Table 2. The velocity of temazepam production was significantly different between cat and dog microsomes, with V_{max} values of 1.1 ± 0.3 and 2.5 ± 0.5 nmol/mg protein/min ($p<0.05$), respectively. The level of

nordiazepam formed was under the limit of quantification (LOQ, which was 1 µM for nordiazepam) in cats, while in dogs a high velocity of 3.1 ± 0.1 nmol/mg protein/min was achieved with a low K_m value of 11.6 ± 2.7 µM. In dogs, after more than 10 min of incubation with concentrations of diazepam ranging from 25 to 500 µM, the formation of oxazepam was observed (data not shown).

For the phase I incubations a final concentration of 1% DMSO was used to dissolve diazepam, similar to the method used for the phase II incubations with temazepam and oxazepam. As Nishiya *et al.* (2010) demonstrated that organic solvents influence the inhibition of recombinant CYP3A4 activity, which is necessary for the metabolism of diazepam, we evaluated this potential effect and found up to a concentration of 1% DMSO had no significant effect on the rate of biotransformation (Nishiya et al., 2010).

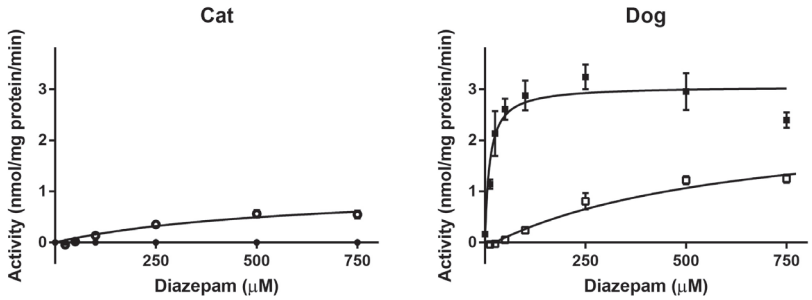

Figure 2. Enzyme kinetics for demethylation of diazepam to nordiazepam (●,■), and hydroxylation of diazepam to (*R,S*)-temazepam (○,□) in pooled cat (circles) and dog (squares) liver microsomes. Nordiazepam production in cats was below the LOQ.

Table 2. Michaelis-Menten kinetics for nordiazepam and temazepam after incubation with the substrate diazepam in pooled cat (n=8) and dog (n=7) liver microsomes.

Substrate diazepam, formed metabolite	***Cat***		***Dog***	
	K_m (µM)	V_{max} (nmol/mg/min)	K_m (µM)	V_{max} (nmol/mg/min)
Nordiazepam	< LOQ	< LOQ	11.6 ± 2.7*	3.1 ± 0.1*
Temazepam	590 ± 260	1.1 ± 0.3	630 ± 230	2.5 ± 0.5*

Data represent the mean ± SD of at least three independent experiments with samples performed in duplicate. LOQ= Limit of quantification. * denotes a significant difference ($P<0.05$) between cat and dog values.

Phase II (glucuronidation) assays of diazepam and its metabolites

After an incubation period of 30 min with concentrations of diazepam ranging from 250 µM up to 1 mM, no glucuronide conjugates were formed in the feline liver microsomes. Incubations with nordiazepam for 60 min resulted also in the lack of any measurable production of glucuronides. Similar results were obtained with the canine liver microsomes. Incubations with a concentration range of (*R,S*)-temazepam resulted in the formation of *R*-temazepam and *S*-temazepam glucuronides, and incubations with a concentration range of (*R,S*)-oxazepam resulted in *R*-oxazepam and *S*-oxazepam glucuronides. All data were obtained within the linear phase of product formation for incubation time and protein content. The data were fitted according to Michaelis-Menten enzyme kinetics, and the calculated K_m and V_{max} values are presented in Table 3.

A fast formation of *S*-temazepam glucuronide was observed for the dog liver microsomes after incubation with racemic (*R,S*)-temazepam, with a V_{max} of 3.0 nmol/mg protein/min (Figure 3). Cat liver microsomes hardly glucuronidated racemic (*R,S*)-temazepam, resulting in a 100-fold lower V_{max} for *S*-temazepam glucuronide compared to dogs. In contrast, albeit limited, the formation of *R*-temazepam glucuronide was significantly higher in cats than in dogs, with a V_{max} of 0.51 ± 0.04 and 0.25 ± 0.02 nmol/mg protein/min ($p<0.05$), respectively. However, the canine K_m value for *R*-temazepam glucuronide was lower than the feline K_m.

Cats showed a very low formation of both *R*- and *S*-oxazepam glucuronides after incubation with racemic (*R,S*)-oxazepam, where *S*-oxazepam glucuronide was even below the LOQ (for *S*-oxazepam the LOQ was 0.1 µM). Dogs showed a much higher formation of both glucuronides, with a V_{max} of 3.2 ± 0.4 and 4.0 ± 0.6 nmol/mg protein/min for *R*- and *S*-oxazepam glucuronide respectively.

Inhibition of Bsep transport activity by diazepam and its metabolites

The inhibitory effects of diazepam, nordiazepam, temazepam and oxazepam were determined on [^{3}H]TCA uptake in membrane vesicles overexpressing feline or canine Bsep. Diazepam and its metabolites all decreased the uptake of TCA and data were fitted according to a one-site competition model (Figure 4). IC_{50} values were calculated and are given in Table 4. Calculation of an IC_{50} value for the highly lipophilic metabolite oxazepam was not possible, due to solubility problems at higher concentrations. Inhibitory potencies were significantly different between cats and dogs, where dogs had slightly lower IC_{50} values for all substrates than cats.

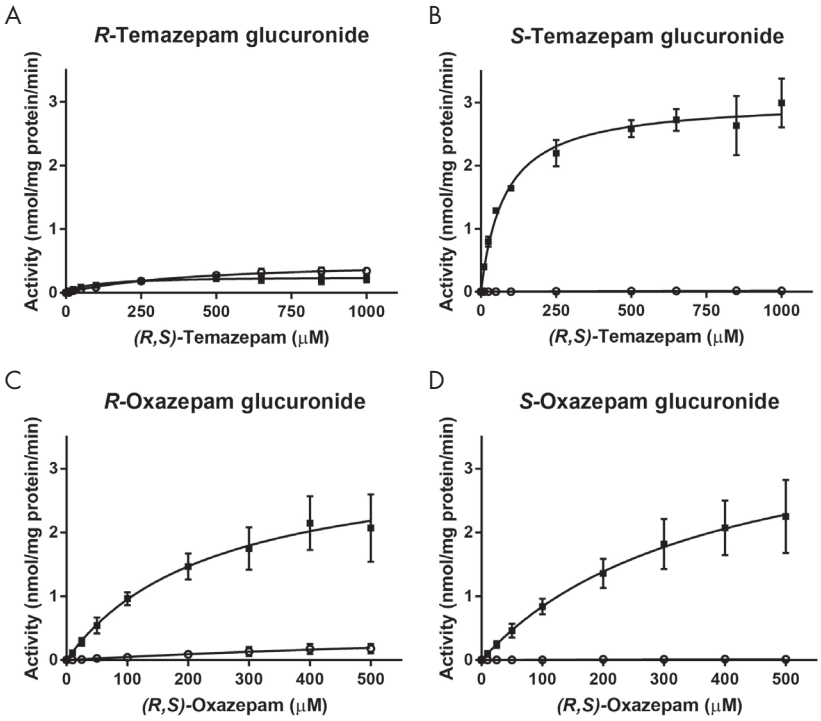

Figure 3. Enzyme kinetics for the glucuronidation of (*R,S*)-temazepam and (*R,S*)-oxazepam in pooled cat (○) and dog (■) liver microsomes. (A) formation of *R*-temazepam glucuronide; (B) formation of *S*-temazepam glucuronide; (C) formation of *R*-oxazepam glucuronide; (D) formation of *S*-oxazepam glucuronide. Data represent the mean ± SD of at least three independent experiments with samples performed in duplicate.

Table 3. Michaelis-Menten kinetics for different formed glucuronides in pooled cat (n=8) and dog (n=7) liver microsomes.

Substrate and formed glucuronide	***Cat***		***Dog***	
	K_m (μM)	V_{max} (nmol/mg/min)	K_m (μM)	V_{max} (nmol/mg/min)
Temazepam				
R glucuronide	441 ± 84	0.51 ± 0.04	94 ± 29*	0.25 ± 0.02*
S glucuronide	920 ± 490	0.030 ± 0.009	79 ± 11*	3.04 ± 0.09*
Oxazepam				
R glucuronide	>1000	ND	240 ± 57	3.2 ± 0.4*
S glucuronide	< LOQ	< LOQ	380 ± 110*	4.0 ± 0.6*

Data represent the mean ± SD of at least three independent experiments with samples performed in duplicate. ND= could not be determined. LOQ= Limit of quantification. * denotes a significant difference (P<0.05) between cat and dog values.

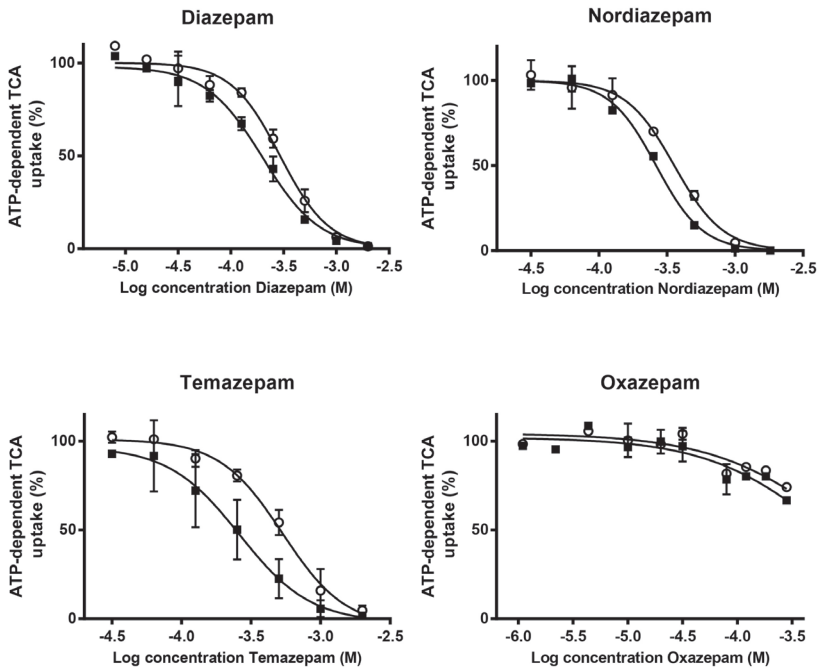

Figure 4. Diazepam and its metabolites inhibit the uptake of 1 μM [^{3}H]TCA in membrane vesicles (7.5 μg protein) expressing cat (○) and dog (■) Bsep. Vesicles were incubated in 10 mM Tris Base buffer (pH 7.4) containing 250 mM Sucrose, 10 mM $MgCl_2$ and 4 mM ATP or AMP, at 37°C for 5 min. ATP-dependent transport was calculated by subtracting transport in presence of AMP from that in presence of ATP. Values are expressed as mean ± SD of percentage uptake of at least three independent experiments with samples performed in duplicate.

Table 4. Inhibitory potency of diazepam and its metabolites.

Inhibitor	***IC_{50} (μM)***	
	Cat	**Dog**
Diazepam	293 ± 23	200 ± 19*
Nordiazepam	375 ± 22	268 ± 18*
Temazepam	533 ± 66	262 ± 60*
Oxazepam	-	-

Measured by the uptake of 1 μM [^{3}H]TCA into membrane vesicles (7.5 μg protein) expressing cat and dog Bsep. Data represent the mean ± SD of four independent experiments.
* denotes a significant difference ($P<0.05$) between cat and dog values.

DISCUSSION

Based on experience in human medicine, diazepam has been used effectively for the treatment of a variety of indications in cats. However, severe side effects have been observed in this species after repeated dosing, which have not been described for man or dogs. The livers of these cats showed centrilobular hepatic necrosis, profound biliary ductile proliferation and hyperplasia, and suppurative intraductal inflammation (Dez Hughes et al., 1996; Center et al., 1996).

Diazepam is a highly lipophilic compound and plasma clearance is dependent on the hepatic formation of phase I and II metabolites to facilitate its excretion into urine and bile. The cat is well-known for its limited hepatic capacity to form glucuronide conjugates in comparison to dogs and other species (van Beusekom et al., 2014). Our current data demonstrate that cats also have a low capacity to form glucuronide conjugates of diazepam and its phase I metabolites. Interestingly, besides the differences found in the phase II biotransformation assays, striking differences were also observed in the phase I biotransformation reactions between cats and dogs.

Diazepam was converted into temazepam in the liver microsomes from both cats and dogs with a comparable affinity (K_m) and capacity (V_{max}). However, temazepam was the principle phase I metabolite formed in the feline liver microsomes, while formation of nordiazepam was observed in the canine liver microsomes with a higher efficiency (V_{max}/K_m) than for the temazepam formation. Moreover, dog liver microsomes produced oxazepam that could not be detected in the feline samples. Temazepam and nordiazepam formation from diazepam is catalyzed in dogs by mainly CYP3A12 and CYP2B11, respectively (Shou et al., 2003; Lu et al., 2005). Further biotransformation of temazepam into oxazepam in dogs is catalyzed by CYP2B11. The lack of quantifiable formation of nordiazepam and the lack of observable formation of oxazepam in the feline liver microsomes suggests a feline CYP2B11 orthologue gene with different characteristics in comparison to dogs and human.

CYP2B6 has a major role in the formation of nordiazepam from diazepam in humans (Yang et al., 1998; Yang et al., 1999; Acikgöz et al., 2009). CYP2B6 is primarily expressed in the liver and genetic variations have been observed resulting in inter-individual variations in enzyme activity and even complete loss of expression (Wang & Tompkins, 2008; Mo et al., 2009). A search through nucleotide databases using the BLASTN program (NCBI) did not retrieve results to obtain a full-length feline cDNA orthologue to human CYP2B6 cDNA (data not shown). Interestingly, another drug that is metabolized by CYP2B6

is ketamine, which is poorly metabolized in cats (Hanna et al., 1988). It thus seems that cats might have a CYP2B6 protein with characteristics different from dogs (CYP2B11) and human. However, further studies are needed to identify the feline CYP enzymes determining the metabolic profile of drugs in cats.

Next, glucuronide conjugation of diazepam and metabolites was assessed in the liver microsomes after addition of diazepam, nordiazepam, temazepam or oxazepam as racemic mixtures. Diazepam and nordiazepam were neither glucuronidated in cat nor in dog liver microsomes. Glucuronide conjugates of temazepam and oxazepam were formed in both canine and feline liver microsomes, but with major differences between species. *S*-temazepam, *R*-oxazepam and *S*-oxazepam glucuronidation were formed with a much lower affinity and capacity in feline than in the canine liver microsomes. *R*-temazepam was conjugated with glucuronic acid in cats at a rate comparable to the dog liver microsomes, but with a lower affinity, while formation of the minor metabolite *R*-temazepam glucuronide occurred at the lowest maximum rate (V_{max}) measured for all conjugates in the canine liver microsomes. Thus, feline liver microsomes have a very limited capacity to form phase II glucuronide conjugates of diazepam hydroxyl metabolites in comparison to dogs.

As yet, the UGT isoforms that catalyze the conjugation of diazepam phase I metabolites in dogs or cats are not known. In humans, UGT2B isoforms, UGT2B15 and UGT2B7, and to a lesser extent UGT1A9 catalyze the conjugation of *S*- and *R*-oxazepam, respectively (Court et al., 2002; He et al., 2009). We have previously suggested that cats have a very limited UGT2B7-like activity that is substantiated here and the current data indicate that UGT2B15 homologue activity is also low in the cat. Temazepam was suggested to be also a substrate for UGT1A3 (Oechsler & Skopp, 2010). The formation of *R*-temazepam glucuronide in feline liver microsomes may therefore be dependent on an active UGT1A3 orthologous gene in cats, that might be the feline UGT1A02 found in earlier investigations (Court & Greenblatt, 2000). This is also indicated by the previous finding of the high formation of glucuronide conjugates of the human UGT1A3 substrate telmisartan in feline liver microsomes (Yamada et al., 2011; Ebner et al., 2013).

Previous *in vivo* studies showed the appearance of temazepam, oxazepam and nordiazepam in the blood plasma of cats and dogs after IV administration of diazepam (Cotler 1984; Driessen 1987; Vree 1979). Although the formation of oxazepam is much slower in the cat than in the dog, it can ultimately be formed. However, no glucuronide metabolites of oxazepam were found in the blood plasma and only traces were detected in the urine, in contrast to dogs

where high levels of oxazepam glucuronides were found in both blood plasma and urine. The latter also accounts for temazepam. The highest concentrations of the metabolites reached in the blood plasma appeared to be nordiazepam in cats, which is similar to dogs. Nordiazepam was very slowly eliminated, being the reason for the accumulation in the blood and resulting in a very long elimination half-life of 21 hours (Cotler et al., 1984). Nordiazepam can be formed in the cat's liver although we detected very low amounts of nordiazepam in our samples. However, formation of nordiazepam and all other metabolites could in principle also be formed in extra-hepatic tissues. The appearance of the active metabolite nordiazepam in the blood contributes to the duration of action of diazepam that has little relationship with the elimination half-life of diazepam itself. Moreover, the elimination half-lives of diazepam and its metabolites in blood plasma are generally longer in cats than in dogs (Table 5) and therefore, from a pharmacokinetic point of view, the dosage interval could be longer in cats than in dogs to achieve the same effect. The low extent of hepatic biotransformation suggests a risk for intrahepatic accumulation and subsequent adverse effects as has been clinically observed.

Our data demonstrate that the feline liver has a low capacity to metabolize diazepam, either by phase I or II metabolism. We hypothesized that diazepam hepatotoxicity was related to inhibition of bile acid secretion. Clinically apparent liver injury is rare in humans, but the onset of injury also occurs after long term dosing, and patterns of serum enzyme elevations have typically been cholestatic or mixed (Cook & Sherlock, 1965; Fors & Nilsson, 1968;

Table 5. Elimination half-lives of diazepam and its metabolites in cats and dogs.

	Cats		***Dogs***	
Diazepam	3.5 h	(Driessen et al., 1987)	1.33 h	(Vree et al., 1979)
	5.5 h	(Cotler et al., 1984)	1.0 h	(KuKanich & Nauss, 2012)
			2.07 h	(Platt et al., 2000)
			3.2 h	(Loscher & Frey, 1981)
			7.6 h	(Klotz et al., 1976)
Nordiazepam	21 h	(Cotler et al., 1984)	2.4 h	(KuKanich & Nauss, 2012)
			3.6 h	(Loscher & Frey, 1981)
			10 h	(Vree et al., 1979)
Temazepam	4.5 h	(Driessen et al., 1987)	3 h	(Vree et al., 1979)
Oxazepam	-		3 h	(Vree et al., 1979)
			5.7 h	(Loscher & Frey, 1981)
			6.2 h	(KuKanich & Nauss, 2012)

Franks & Jacobs, 1975). Therefore, we studied the inhibitory potential of diazepam and metabolites against Bsep-mediated taurocholate transport. To our knowledge, this is the first study which describes inhibition of both feline and canine Bsep transport activity by diazepam and its metabolites. The inhibitory potency of diazepam and its metabolites on Bsep transport is not as strong as common marker inhibitors such as cyclosporine A or troglitazone (van Beusekom et al., 2013), and was rather comparable between the feline and canine Bsep. However, in combination with the impaired glucuronidation, high concentrations of non-glucuronide metabolites could accumulate intracellularly, and hence inhibition of Bsep is likely to occur in feline liver. On the other hand, diazepam appeared to be more potent in its toxic effects than e.g. paracetamol on human hepatocytes *in vitro* by means of a relative straight forward cytotoxicity assay (Jover et al., 1992) and further studies are needed to unravel the mechanism of toxicity that would assist in a therapy plan for cats showing signs of diazepam induced liver injury.

In conclusion, cats seem to have a different preference for phase I reactions than dogs and a limited capacity to glucuronidate diazepam hydroxyl metabolites. Therefore, high concentrations of non-glucuronidated metabolites might accumulate in the hepatocyte with probably a long residence time. These non-glucuronidated metabolites can inhibit Bsep-mediated efflux of bile acids. In turn, the intracellular accumulation of bile acids will lead eventually to liver injury. However, other mechanisms could also be involved in liver injury induced by repeated administration of diazepam in cats. To prevent cats from having drug-induced liver injury, caution should be taken with the use of diazepam. In a clinical setting, diazepam or its metabolites should only be used when there are no alternatives for this drug. When using diazepam, we recommend to administer the drug with the lowest effective dose, a proper dosing interval, and for a short period of time.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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CHAPTER 6

A FUNCTIONAL MODEL FOR FELINE P-GLYCOPROTEIN

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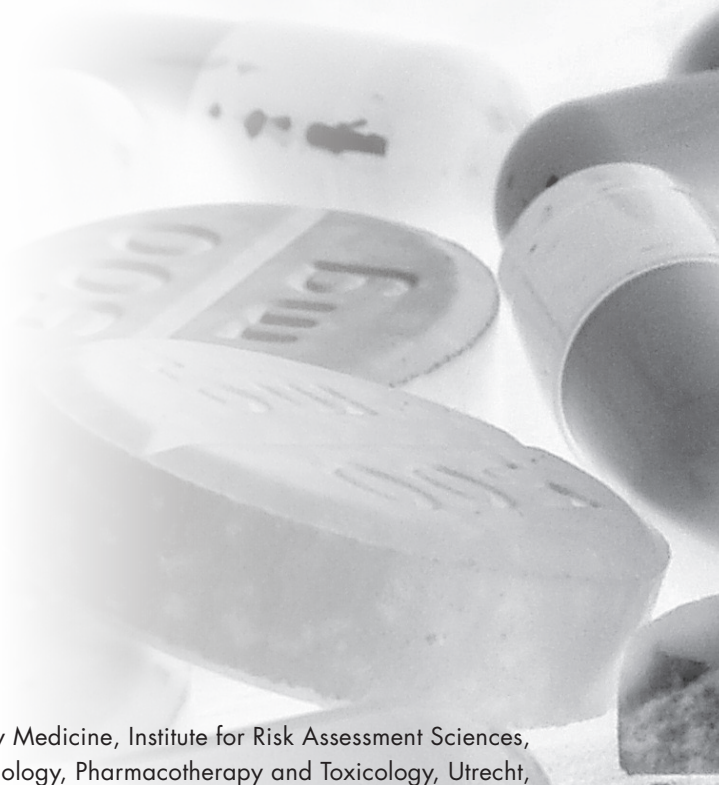

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ABSTRACT

P-gp (ABCB1) belongs to the group of export transporters that is expressed in various species at biological barriers. Inhibition of P-gp can lead to changes in pharmacokinetics of drugs (drug-drug interactions), which can lead to toxicity and adverse side effects. This study aimed to investigate the inhibitory potential of a selection of veterinary drugs on feline P-gp by means of fluorescence-associated flow cytometry of feline lymphoma cells. In this model, PSC833 and ivermectin potently inhibited P-gp function, cyclosporine and verapamil moderately inhibited P-gp function, whereas ketoconazole, itraconazole, diazepam, nordiazepam, temazepam and oxazepam had no effect on P-gp function. This model can be used for testing the inhibitory potency of (new) drugs on feline P-gp.

The multi-drug resistance protein (MDR1/ABCB1) or permeability glycoprotein (P-gp) is a member of the ATP-binding cassette (ABC) transporters, and can be found in e.g. liver, intestines, kidneys, and the brain, in many animal species (Mealey, 2004). Drugs that are substrates for P-gp are prevented from entering or excreted to the luminal side of these organs and therefore P-gp serves as a part of the biological barrier. Its role in pharmacokinetics is exemplified by a well-known veterinary clinical example of a dysfunction of P-gp in ivermectin sensitive Collie breeds (Mealey et al., 2001), and by multi-drug resistance in anti-cancer therapy (Darby et al., 2011). Polymorphisms as well as drug-drug interactions at the level of P-gp are of clinical concern in both human and veterinary medicine (Schrickx & Fink-Gremmels, 2008; Zakeri-Milani & Valizadeh, 2014).

In cats, P-gp was first studied *in vitro* in feline lymphoma cells, where it was suggested that the basic structure of the feline ortholog and its role in multi-drug resistance was essentially the same as in other species (Okai et al., 2000). Organ distribution (Van der Heyden et al., 2009) and P-gp expression in various tumors was later demonstrated in cats (Brenn et al., 2008; Hifumi et al., 2010; Van der Heyden et al., 2011).

This study aimed to investigate the inhibitory potential of a selection of veterinary drugs on feline P-gp, by means of fluorescence-associated flow cytometry of feline lymphoma cells. The feline T-cell lymphoma cell lines were a generous gift from Y. Goto-Koshino (University of Tokyo, Japan). The FT-1 cell line (without expression of P-gp) was derived from an FeLV-positive thymic lymphoma of a cat, and the ADM-resistant subline FT-1/ADM (with major P-gp expression) was selected from the FT-1 cells after several passages in a medium containing adriamycin (ADM) (Okai et al., 2000).

Initial experiments showed low uptake of rhodamine 123 (Rh123) in the FT-1/ADM cell line, due to the high efflux of the fluorescent dye. Therefore, instead of an efflux assay, an uptake assay was performed, as previously described for canine cells (Schrickx, 2014). In 96-well plates, a total number of 0,5 x 10^6 viable cells/well were incubated with 8 µM Rh123 and a concentration range of the selected drug simultaneously, for 30 min at 37°C in an atmosphere of 5% CO_2. After washing with PBS, cell-associated fluorescence was measured with a flow cytometer (Accuri C6, BD Biosciences, USA). Differences of Rh123 fluorescence intensity between control cell samples (FL_c) and cell samples with the test compound (FL_s) were calculated according to equation 1. The background fluorescence intensity (FL_B) of cell samples that were not loaded with Rh123 was subtracted from each value.

Relative uptake = (FLS – FLB) / (FLC - FLB) eq. 1

Concentration dependent inhibitory effects were presented by sigmoidal curves, calculated in similar ways as Schrickx (2014). Incubation of the FT-1/ADM cells with 10 µM of the marker P-gp inhibitor PSC833 (Novartis Pharma) (Boesch et al., 1991) was regarded as the maximum Rh123 efflux inhibition (I_{max} or 100% inhibition). The mean uptake of Rh123 by cells incubated in control medium (without PSC833 or any other of the tested compounds) was the minimum inhibition of Rh123 efflux (I_{min} or 0% inhibition). Inhibition curves with a variable slope were fitted for the concentration ranges and IC_{50} values were calculated by using GraphPad Prism 6.01 software (San Diego, California, USA) and values are expressed as mean ± SD of percentage uptake of at least three independent experiments.

Inhibition of Rh123 efflux was observed in the FT-1/ADM cell line, and not in the FT-1 cell line, which suggests that all inhibitory effects are related to P-gp. The inhibitory potencies of the different drugs on Rh123 efflux in the FT-1/ADM cells are shown in Figure 1. PSC833 and ivermectin were the most potent inhibitors, while cyclosporine inhibited Rh123 efflux to a lesser extent. Verapamil inhibited Rh123 efflux only for a small percentage at the highest soluble concentration (≤0.4% DMSO), whereas ketoconazole, itraconazole and the benzodiazepines diazepam, nordiazepam, temazepam and oxazepam (not in graph) had no influence on Rh123 efflux in the FT-1/ADM cells at the given concentrations.

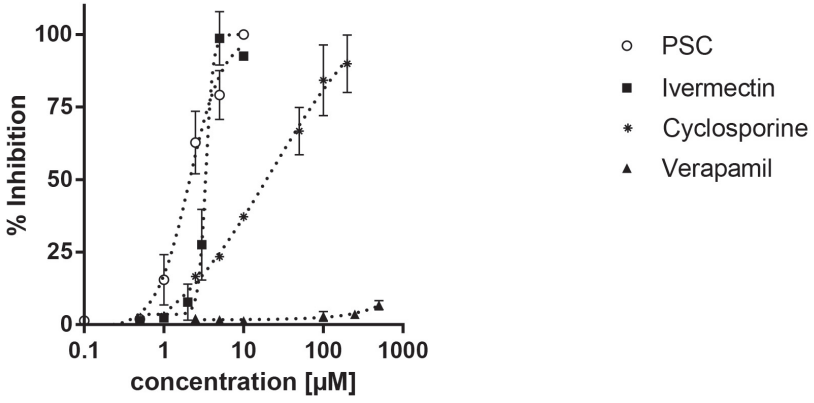

Figure 1. Inhibitory effect of the marker inhibitor PSC833 and tested drugs (ivermectin, cyclosporine and verapamil) on feline P-gp. Data represent the mean ± SD of at least three independent experiments with samples performed in duplicate.

The expression of P-gp cDNA was also investigated by PCR for the different cell-lines using SYBR Green Supermix (Bio-Rad, CA, USA), forward primer 5'-GAA-GTT-AAG-ATC-TTG-AAG-GGC-C and reverse primer 5'-CAT-TGG-CTT-CCT-TGA-CAG-C (Okai et al., 2000). After a hot start of 95°C for 3 min, PCR was performed at 95°C for 15 s, at 55-60°C for 30 s, and 72°C for 30 s for a total of 40 cycles. PCR products showed indeed a very high expression of P-gp in the FT-1/ADM cell-line, and a low expression in the FT-1 cell-line (Figure 2).

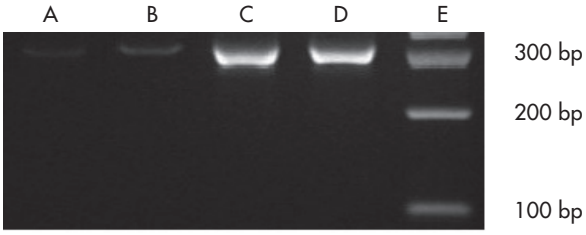

Figure 2. P-gp product of FT-1 (A and B) and FT-1/ADM (C and D) cells on a 2% agarose gel stained with ethidium bromide. A: FT-1 (PCR annealing temperature 55°C); B: FT-1 (PCR annealing temperature 60°C); C: FT-1/ADM (PCR annealing temperature 55°C); D: FT-1/ADM (PCR annealing temperature 60°C); E: BenchTop 100 bp DNA Ladder (Promega, USA).

Compared to the canine lymphoid cell-line (Schrickx, 2014), P-gp in the feline lymphoma cell-line (FT-1/ADM) was much more resistant to drug inhibition by these known human/canine P-gp inhibitors. IC_{50} values for PSC and ivermectin in the feline cells were 2.09 ± 0.18 µM and 3.37 ± 0.16 µM, respectively. The IC_{50} value for cyclosporine could not be calculated accurately, as 100% inhibition could not be established by the tested concentration range. In general, IC_{50} values are much higher for the feline cells, but the ranking order of inhibitory potency of the different compounds is similar to dogs. These findings suggest similar P-gp characteristics for the selection of inhibitors, albeit with an apparently lower affinity for the feline P-gp. Whether these higher IC_{50} values result from a real difference in affinity, or the seemingly high expression of P-gp in feline lymphoma cells, or to confounding differences in cell permeability for the selected P-gp inhibitors compared to the canine cells, needs further investigation.

In conclusion, the lymphoma cell assay used in this study can serve as an easy accessible *in vitro* model for testing the inhibitory potency of (new) drugs on feline P-gp.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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CHAPTER 7

GENERAL DISCUSSION

CONTEXT OF THE THESIS

The aim of this thesis was to contribute to the knowledge about hepatic biotransformation and transport of drugs in cats. The increasing role of cats in daily veterinary practice and the need for advanced therapeutic intervention for various disease conditions, demands an understanding of general mechanisms involved in drug handling in cats. Not for all indications in feline species specific drugs are licensed, and this shortage of registered products enforces veterinarians to use drugs which are available and licensed for other species, like dogs or humans. While there are prominent examples of drug intolerance and toxic effects in cats occurring already at common dosing regimens, also mild side effects and lack of efficacy are certainly an undesirable outcome of therapeutic interventions and veterinarians often observe these unexplainable effects when treating feline patients. Adverse side effects of drugs can be very diverse in appearance, but cats have a tendency to show signs related to liver dysfunction. Cholestasis, hepatic lipidosis or cholangitis is often observed in cats, correlating with symptoms such as anorexia, vomiting, lethargy, and jaundice.

Unpredicted side effects in drug therapy are often based on differences in pharmacokinetics, particularly in veterinary medicine, where species and even breed differences account for a high variability in biotransformation and rate of elimination. Therefore, cat-specific knowledge on factors contributing to such variations in pharmacokinetics needs to be obtained to support the prescribing veterinarian in defining the best dosing regimen, avoiding undesirable side effects. Moreover, considering the above mentioned shortage of licensed drugs for cats, extrapolation of the dose from other species needs to take into account feline-specific pharmacokinetic parameters. In contrast to dogs, for which the knowledge on e.g. CYP activities is becoming comprehensive, comparable insight in cats is lacking as yet.

The aim of this thesis was therefore to provide a close insight into feline hepatic biotransformation and drug transport mechanisms. To this end, a variety of techniques and assays were used, comprising enzyme substrate specificity and enzymes kinetics (phase I and phase II enzymes) as well functional studies measuring transport activity by means of cell-membrane vesicles or feline lymphocytes with radio-active or fluorescent substrates. We chose for an *in vitro* approach as a start, as it is less invasive and requires less laboratory animals than *in vivo* studies, and still provides us with fundamental knowledge about mechanisms on enzymatic or transporter function.

FELINE HEPATIC BIOTRANSFORMATION

Chapter 1 provided an overview of the current knowledge about feline hepatic biotransformation. Reviewing the available literature, it became evident that the overall knowledge regarding these processes in cats is limited and insufficient to provide a sound basis for safe and effective dosing regimens. Based on these data, we designed a series of experimental approaches that address the main phases of drug metabolism.

Comparison of CYP450 biotransformation capacity in cats and dogs

Chapter 2 was devoted to phase I metabolism, focusing on different cytochrome P450 (CYP450 or CYP) enzyme activities. Drug metabolism involves mainly the CYP450 enzyme families CYP1 to CYP4, and significant interspecies differences are observed (Nebbia et al., 2003; Fink-Gremmels, 2008). Moreover, the individual activity of CYP450 enzymes is modulated by genetics (breed and gender) and within a species by age, nutrition and exposure to environmental contaminants. We therefore designed a study that addressed two different aspects, the comparison of the overall feline-specific biotransformation capacity and the application of a rapid assay procedure that might be applicable in population studies and in the clinical routine by veterinarians. Therefore we selected a series of fluorescent substrates, specific for individual CYP450 enzymes in other species, as test compounds. The fluorometric assay was selected for its simplicity and short duration compared to the common analysis of reaction products by HPLC analysis. Using a standard protocol for *in vitro* assays with hepatic microsomes, we determined the rate of conversion of these proto-typic substrates under the conditions of Michaelis-Menten kinetics, and compared the results with comparable assays using canine microsomes, which were tested under the same conditions. This in depth analysis of CYP activities in feline and canine liver microsomes showed that CYP1A, CYP2B and CYP3A represent the highest CYP enzyme activities in feline liver. Cats seem to have more CYP2B and CYP2D than dogs, whereas they had a lower activity of CYP1A, CYP2E and CYP3A than dogs. Besides the species differences, we also observed gender differences in CYP activity for cats as well as for dogs. Different (human) marker inhibitors were used to determine the substrate specificity of the feline and canine isozymes. Again, differences could be observed between species in the inhibitory potency of individual inhibitors. These results are in line with findings in other investigations devoted to species- and gender differences in CYP activity (Shimada et al., 1997; Nebbia et al., 2003; Baririan et al., 2005; Martignoni et al., 2006).

Next to the description of the feline-specific activity of individual CYP450 enzymes, our intention with this study was that veterinarians could use this rapid fluorometric assay for the determination of the drug metabolizing enzyme activity in individual critical patients, for example patients with pre-existing hepatic disease conditions. In such patients, liver biopsies are often needed to establish a diagnosis, and hence we postulated that biopsy material could be also used to characterize the biotransformation capacity of an individual patient. However, when we varied the assay using different concentrations of microsomal proteins, it became apparent that although the required amount of microsomal proteins was smaller as compared to common HPLC-based analysis, it is still too high to allow the use of fine needle biopsies available in patients in daily practice. Despite this limitation, the results obtained remain of interest for the prediction of drug metabolism in cats, particularly in comparison with dogs and humans, as we identified distinct differences that may be of clinical relevance.

Another example of differences between cats and dogs in the CYP450-dependent biotransformation is provided in **Chapter 5**, where a comparison between feline and canine hepatic biotransformation of diazepam is presented. Diazepam was selected as it is known to cause adverse hepatotoxic effects in cats, generally attributed to a low glucuronidation capacity of cats as discussed below. Using again the standard microsome incubation assay we observed that diazepam was mainly converted into temazepam in cats, while in dogs nordiazepam is the principle metabolite. In addition, in dog liver microsomes oxazepam appeared, which could not be detected in feline liver microsomes. In dogs, temazepam and nordiazepam formation from diazepam is catalyzed by CYP3A12 and CYP2B11, respectively (Shou et al., 2003; Lu et al., 2005). Further biotransformation of temazepam into oxazepam in dogs is catalyzed by CYP2B11. The lack of quantifiable formation of nordiazepam and oxazepam in feline liver microsomes therefore suggests that the feline CYP2B11 ortholog has a different substrate spectrum. This is supported by the investigations in **Chapter 2**, which describe a higher CYP2B (EFC = 7-Ethoxy-4-trifluoromethylcoumarin) activity in feline liver microsomes as compared to canine microsomes, but a different response to the inhibition by tranylcypromine. High concentrations of tranylcypromine inhibit the conversion of EFC to HFC (7-Hydroxy-4-trifluoromethylcoumarin) in cat liver microsomes, but in dog liver microsomes an unexpected rise of the EFC-like fluorescence was found.

Comparison of the glucuronidation capacity in cats and dogs

Chapter 3 was devoted to phase II and particularly to the glucuronidation capacity in cats. Previous investigations had already reported that cats (feline species) all carry a mutation in the UGT1A6 gene, resulting in a glucuronidation capacity that is extremely low and alters significantly the elimination of all drugs and dietary (and/or toxic) substances that require glucuronidation for a rapid excretion. Our investigations showed also the lack of functional UGT1A6 isozymes, as the probe substrate 1-naphthol was not glucuronidated in feline liver microsomes. Moreover, we confirmed some glucuronidation capacity in cats, albeit this activity reached only 25-30% of the capacity of dogs. We also investigated UGT2B-like activity in the cat's liver, and suggest a lack of functional UGT2B7 homologs, as only a very limited capacity for morphine-3-glucuronide and no estradiol-17-glucuronide formation was found. We even tried to measure the expression of UGT2B homologs in feline liver by PCR, but we could hardly detect any UGT2B-like expression (data not shown).

On the other hand, there is clear evidence that cats possess some glucuronidation capacity as endogenous compounds, such as bilirubin and thyroxin (Myant, 1966; Fevery et al., 1977) are found as glucuronides in cats. This applies also to the model substrate phenolphthalein (Pekanmaki & Salmi, 1961; Watkins & Klaassen, 1986), and certain drugs such as lorazepam (Schillings et al., 1975; Elliott, 1976; Ruelius, 1978), pradofloxacin (EMEA/V/C/099), ibuprofen (Magdalou et al., 1990), and telmisartan (Ebner et al., 2013) which are all glucuronidated in cats.

In **Chapter 5** we describe a comparison in the biotransformation of diazepam between cats and dogs. Besides differences in phase I metabolism, as described above, we found a remarkable high glucuronidation rate for *R*-temazepam, which was statistically significant higher in cats than in dogs (V_{max} of 0.5 ± 0.04 nmol/mg protein/min in cats and 0.3 ± 0.02 nmol/mg protein/min in dogs). The three other diazepam metabolites, including *S*-temazepam and *R*- and *S*-oxazepam had an extremely low glucuronidation rate in cats. In humans, the conjugation of oxazepam is catalyzed by UGT2B isoforms (Court et al., 2002; He et al., 2009), while temazepam is also suggested to be a substrate for UGT1A3 (Oechsler & Skopp, 2010). The formation of *R*-temazepam glucuronide in feline liver microsomes in our studies is therefore apparently attributable to an active UGT1A3 orthologous gene in cats.

In contrast to dogs, cats have shown to exhibit severe adverse side effects after repeated dosing of diazepam, including centrilobular hepatic necrosis,

profound biliary ductile proliferation and hyperplasia, and suppurative intraductal inflammation (Dez Hughes et al., 1996; Center et al., 1996). The results presented here indicate that a combination of the different preference for phase I metabolites as well as the low phase II capacity, resulting in an intracellular accumulation of lipophilic metabolites, may contribute to the observed hepatotoxicity. This accumulation of toxic metabolites is supported by their inhibitory effects of the bile salt efflux transporters (BSEP) as discussed below.

FELINE HEPATIC TRANSPORT MECHANISMS

BSEP (Bile salt export pump), the ABCB11 transporter

Phase I and phase II metabolism generally result in the formation of more polar, water-soluble substances, which cannot easily cross the cell membrane of hepatocytes, neither the canalicular membrane nor the basolateral membrane. As the significance of these transport mechanisms have for a long time been underestimated, data on the transport capacity of drugs into and out of the hepatocyte are very scarce. The only drug transporter investigated in the feline liver is P-gp (Van der Heyden et al., 2009), although no functional analyses were performed on this transporter. In humans, polymorphisms or genetic defects resulting in a lack of function of efflux transporters can result in severe clinical symptoms caused by the intracellular accumulation of endogenous and exogenous substances. A prominent example is the hyperbilirubinemia in people with Dubin-Johnson syndrome. In these patients the excretion of bilirubine glucuronides into the biliary ducts is hampered, as a mutation in the MRP2 gene results in the expression of a non-functional MRP2 protein (Konig et al., 1999). Another well-known example is drug-induced cholestasis, caused by an inhibition of the efflux of bile acids out of the hepatocyte. Intracellular accumulation of bile acids results in hepatotoxicity (Kullak-Ublick & Meier, 2000).

A major efflux transporter, facilitating the efflux of bile acids from hepatocytes, is the so-called Bile Salt Export Pump (BSEP), a protein that is expressed in the canalicular membrane of the hepatocyte. It acts independently from other transporter proteins that are needed for the canalicular secretion of other bile components. BSEP belongs to the family of ATP-binding cassette efflux (ABC) transporters, that can transport substrates against a concentration gradient by hydrolyzing ATP as an energy source, and is also denoted as ABCB11.

A functional characterization of canine Bsep was conducted by Yabuuchi *et al.* with the aim to aid the extrapolation of toxicological data from dogs to humans (Yabuuchi et al., 2008). However, data on feline Bsep are entirely

lacking. Therefore, **Chapter 4** aimed to present a structural and functional comparison of feline Bsep with canine and human Bsep/BSEP. As a first step, the cDNA coding sequences of the canine and feline Bsep were established and a high level of homology with an identity of 91.2% was observed. The feline Bsep had also an identity of 88.9% with the human BSEP sequence, similar to the degree of homology between dogs and humans.

For the functional characterization, reverse membrane vesicles prepared from HEK293 were transfected with the feline, canine and human BSEP and the ATP-dependent uptake of [3H$^+$]Taurocholic acid (TCA), [3H$^+$]Cholic acid (CA) and [3H$^+$]Glycocholic acid (GCA) was measured to characterize the transport capacity. Subsequently, the inhibition of TCA transport by the prototypic inhibitors cyclosporine A, troglitazone and ketoconazole was measured. No remarkable differences in this comparison of the three types of vesicles were observed. This might be attributable to the common transfection model applied to all three tested species, quenching subtle differences in transport activity.

It is worthwhile to mention that the conjugated bile acid TCA is the prototypic model substrate for BSEP commonly used in *in vitro* functional studies, although in humans bile acids are mainly conjugated to glycine and only to a minor extent to taurine. In carnivorous species such as dogs and cats, however, bile acids are almost exclusively conjugated to taurine with TCA as the major bile acid in both species (Wildgrube et al., 1986; Washizu et al., 1991; Perwaiz et al., 2001). Such a high extent of taurine conjugation in cats is remarkable as taurine is an essential amino acid for this species (Knopf et al., 1978; de la Rosa & Stipanuk, 1985; Park et al., 1991).

Transfection models missing regulatory mechanisms cannot reliably predict species differences in efflux transport capacity, but can be a valuable tool to predict an inhibitory effect of a (new) drug and/or its metabolites on BSEP activity and hence the risk of liver cell injury. To verify our assumption that the BSEP model can be used for drug testing, we studied the metabolism and excretion of the drug diazepam. As mentioned before, diazepam is one of those examples of a drug, whereby cats have shown to develop severe side effects after repeated dosing, while clinically apparent liver injury is hardly recognized in humans and dogs. **Chapter 5** described not only the comparison between feline and canine hepatic biotransformation of diazepam, but also its effect on BSEP activity. It could be shown that in both species, diazepam and its metabolites inhibited BSEP function. Comparable results have been obtained for human BSEP (data not shown). It can be assumed that this inhibition significantly contributes to the risk of liver cell injury, as observed in cats, that exhibited only a minor glucuronidation capacity to the biologically active and hepatotoxic diazepam metabolites.

In humans, drug-induced liver injury (DILI) has been related to inhibition of BSEP. As cats easily show signs of hepatotoxicity following drug application, it is prudent to hypothesize that these adverse side effects could be related to the same mechanism that occurs in humans (Center, 1999; Center, 2005; Otte et al., 2011).

P-gp (P-glycoprotein), the MDR1 ABC-transporter

The canalicular transport of endogenous and exogenous compounds involves not only BSEP, but also the transporters BCRP (ABCG2), MRP2 (ABCC2), MATE-1 (multi-drug extrusion protein 1, previously denoted SLC47A1) and P-gp (MDR1, ABCB1). In veterinary medicine, P-gp is of particular interest, as in certain dog breeds (from the Collie shepherd lineage) a clinically relevant polymorphism resulting in a non-functional P-gp is prevalent. The main clinical effect associated with this polymorphism is not associated with hepatic transport mechanisms, but with the unique function of P-gp as functional element of the blood-brain barrier. If P-gp is not functioning, drugs and toxic substances that otherwise cannot cross the blood-brain barrier, can reach in these cases the central nervous system, resulting in unexpected and even fatal neurotoxic effects. Clinical relevant examples are the endectoparasitic drug ivermectin and the opioid loperamide that is used in the treatment of hypermotility of the intestines (Mealey et al., 2001). These P-gp mutations have been observed as yet only in dogs, but comprehensive investigations on P-gp substrate specifity in cats are missing.

In **Chapter 6** we present a feline P-gp model, comparable to the canine P-gp model that was developed for the prediction of clinically relevant drug-drug-interactions (Schrickx, 2014). Our study aimed to investigate the inhibitory potential of a selection of veterinary drugs on feline P-gp by means of fluorescence-assisted flow cytometry of feline lymphoma cells. The known substrates PSC833 and ivermectin were the most potent inhibitors, while cyclosporine and particularly verapamil inhibited Rh123 efflux to a lesser extent. In this study we also included for comparison diazepam and its metabolites, nordiazepam, temazepam and oxazepam, but none of these substances had an influence on Rh123 efflux in the FT-1/ADM cells at the given concentrations. Taken together, these data show that compared to the canine lymphoid cell-line, the feline lymphoma cell-line (FT-1/ADM) was much more resistant to drug inhibition by known human/canine P-gp inhibitors.

Besides the experiments on feline transporters described above, we made an attempt to measure the expression of different ABC-transporters in feline liver by PCR analysis. We found evidence for expression of the most common hepatic ABC-transporters P-gp, Bsep, and Mrp2 in feline liver samples (data

not shown), which indicates that cats, as expected, possess these transporters. Mrp2 was also detected in liver tissue of cats by immunohistochemistry (Ijzer et al., 2009). Recently, inhibition of Bsep and Mrp2 by a novel kinase inhibitor was found to be related to the development of severe hepatotoxicity in dogs (Daniels et al., 2013). This emphasizes that functional studies of hepatic ABC-transporters are still needed to confirm their presence and activity in the liver and to understand their role in drug transport in cats.

UNRESOLVED CLINICAL QUESTIONS IN FELINE PRACTICE

Although various mechanisms are elucidated behind drug-induced adverse reactions, a few cases are still unsolved. Such idiosyncratic reactions, in which the mechanism does not involve the known pharmacological properties of the drug, are suggested to be explained by reactive metabolites binding to proteins in the body and resulting in "foreign" material that is attacked by the immune system. In man, drug-induced liver injury (DILI) can also be related to idiosyncratic drug reactions, while DILI can be caused by multiple mechanisms and has many risk factors (Hussaini & Farrington, 2007). In general, these idiosyncratic reactions do not follow a classic dose-response relationship (Antonovic & Martinez, 2011; Trepanier, 2013). However, the term idiosyncratic can also be used for rare adverse reactions with unknown origin of a drug, or adverse reactions that occur only in a very small percentage of cats treated with that drug. These reactions seem to be hypersensitivity reactions, with or without an adaptive immune response (Trepanier, 2013).

One of the known examples of drugs with idiosyncratic reactions in cats is permethrin, used to treat ectoparasites (Linnett, 2008). Permethrin, like other pyrethroids, is metabolized by CYPs and carboxylesterases, and the resultant metabolites undergo different conjugation reactions with e.g. glucuronic acid, sulfate, taurine and glycine. The percentage of glucuronide formation relative to the dose differs for each pyrethroid. Until now, it was assumed that the low feline glucuronidation is the cause for the sensitivity of cats for pyrethroids. However, mainly the parent compound is significantly toxic and hydrolysis of the ester bond makes the metabolites less toxic (Kaneko, 2011). Therefore, the low glucuronidation capacity in cats seems not to be the only reason for their specific sensitivity. It is more likely that cats also have a reduced esterase activity preventing the rapid hydrolysis of pyrethroid-esters or that they produce toxic metabolites (Richardson, 2000). This hypothesis is supported by the clinical experiences with flumethrin, a related pyrethroid. This drug is well accepted in cats and does not exhibit adverse symptoms except for some local

reactions on the spot of administration (Stanneck et al., 2012). Flumethrin is believed to be rapidly hydrolysed and the main metabolite flumethrin acid is less toxic (Stanneck et al., 2012).

Another example for a drug that can induce idiosyncratic liver toxicity in cats is methimazole, a prodrug of carbimazole, used for the treatment of hyperthyroidism (Trepanier, 2013). Hepatotoxicity due to antithyroid drugs was also observed in humans (Otsuka et al., 2012), mice and rats, in which glutathione depletion turned out to be a major risk factor, and the use of N-acetylcysteine or taurine reduced the hepatotoxicity (Mizutani et al., 2000; Heidari et al., 2012). Methimazole is metabolized by CYPs and flavin-containing monooxygenases which probably results in a metabolite that needs glutathione conjugation for detoxification (Mizutani et al., 1999; Heidari et al., 2013). In cats, no correlation was found between glutathione levels and methimazole toxicity, although glutathione concentrations were not measured in liver tissue (Branter et al., 2012). Hematologic abnormalities e.g. thrombocytopenia and neutropenia, can also occur in cats treated with methimazole or carbimazole, probably due to immune mediated mechanisms (Peterson et al., 1988).

5-Fluorouracil (5-FU) is being used in anti-cancer therapy in humans, but turned out to be neurotoxic for cats. One of the reasons for this sensitivity of cats, is the deficient dihydropyrimidine dehydrogenase (DHD) activity in some cats. DHD is responsible for the metabolism of 5-FU and is in high concentrations abundantly expressed in the liver. However, only 23% of the tested cats had elevated 5-FU values related to a low DHD activity, thus the authors suggested that DHD deficiency was not the sole explanation for the lack of tolerability of this drug in cats (Saba et al., 2013). The other reasons for the sensitivity to 5-FU could not be explained yet.

CONCLUDING REMARKS AND CLINICAL RELEVANCE OF THE INSIGHT INTO FELINE DRUG BIOTRANSFORMATION

Cats need to be considered as an important animal species in small animal practice, as the population of pet cats is increasing rapidly in The Netherlands. Owners bring their cats to a veterinary clinic for a variety of indications and hence the spectrum of drugs that are used in feline practice is rapidly increasing. As not for all indications, veterinary medicinal products are especially licensed for cats, treatment involves drugs that are used in canine or human medicine. It is therefore essential to identify feline-specific features

of drug biotransformation and excretion that may require an adjustment of the dosing regime in cats. As yet, the low glucuronidation capacity of cats is the only parameter known to cause undesirable side effects, generally associated with hepatic toxicity in cats. In the present investigations, we also identified significant differences in CYP450 metabolism between cats, dogs and humans. Moreover, we not only confirmed the low overall glucuronidation capacity of cats, but also identified exceptional cases, where cats exert a high glucuronidation capacity for individual substances or drug metabolites, which are substrates for the glucuronosyltransferase ortholog of the human UGT1A3. This finding is of special interest in the testing and development of new drugs for cats, as such a sufficient glucuronidation capacity would contribute to a higher safety profile of these substances.

Previously often neglected was the possible influence of hepatic transporters on drug safety. Investigating BSEP as one of the most prominent bile acid transporters, facilitating the efflux of various conjugates from the cytoplasm of hepatocytes through the canalicular membrane into the bile, we describe that for example diazepam and its metabolites inhibit this transporter. It can be assumed that this efflux transporter inhibition, which result in an accumulation of intracellular bile acids, significantly contributes to the liver cell injury in cats observed following repeated administration of diazepam. Furthermore, we established next to the standardized microsomal assays used in the first line for drug biotransformation studies, an expression system to measure the transport capacity of BSEP and P-gp. Such *in vitro* models allow the screening of a large number of drugs and toxins, and might also serve the development of new drugs and can be used to predict drug-drug interactions at the level of hepatocellular transport.

Considering the importance of biotransformation and drug excretion in the safety profile of a given drug, it needs to be mentioned that enzyme activities and (ATP-dependent) efflux transporters are influenced in their activity by genetic factors such as breed, age and gender, but even more importantly by diseases that result in a generalized inflammatory response. Since pet animals seem to get older and older nowadays, the medical care of this geriatric population of cats is a special challenge. In these elderly cats, co-administration of drugs is generally performed in clinical practice, again pointing to the need of a close understanding of drug metabolism and potential drug-drug interactions.

There is certainly a need for more medicinal products registered for cats and many examples can be given for drugs that are still not licensed for cats, such as drugs which decrease blood pressure or stimulate appetite, several opioids for pain control, diuretics, antibiotics, muscle relaxants and chemotherapeutics.

Drug biotransformation studies, as a basis to understand kinetics and hence to be able to establish appropriate dosing regimens, are needed and may use one or more of the *in vitro* systems presented in this thesis.

Finally, it should be mentioned that cats apparently share with humans a higher sensitivity for adverse drug reactions, particularly non-specific and idiosyncratic liver cell injury. In human, this drug-induced liver injury (DILI) is often an acquired phenomenon, occurring with a higher prevalence in females in the post-menopausal age, but also in males and females at younger age (Hussaini & Farrington, 2007). The underlying mechanisms are still not entirely elucidated, but among the most prominent risk factors are polymorphisms in biotransformation enzymes, together with a reduced capacity of the bile acid transporter BSEP (Stephens et al., 2014). In this study we also demonstrate the prominent role of BSEP in feline hepatic drug metabolism, and it can be suggested that cats may be an interesting model species to explore the etiology of DILI.

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CHAPTER 8

SUMMARY

SUMMARY

The aim of this thesis was to contribute to the insight in hepatic biotransformation and transport of drugs in cats. Cats need to be considered as an important animal species in veterinary practice, as the population of pet cats is increasing rapidly. Veterinary medicinal products are not licensed for their use in cats for all indications yet, and veterinarians are enforced to use drugs which are available and licensed for dogs or for humans. However, drug intolerance, toxic effects or a lack of efficacy has been observed when treating feline patients with drugs intended for other species. Species differences in pharmacokinetics are supposed to be the main cause of the variability in drug handling by individual patients, and therefore insight into cat-specific mechanisms that determine pharmacokinetics is essential to define appropriate and safe dosing regimens.

Drug biotransformation, the process to facilitate the elimination of drugs from the body, is generally divided in different phases. Cellular uptake of drugs is generally considered as phase 0, phase I and II comprise enzymatic drug conversions and conjugation reactions, whereas phase III describes the cellular excretion of the drug or its metabolites into the bloodstream, bile duct, intestinal lumen or urine. In this thesis, we designed a series of experimental *in vitro* approaches that address the major phases of drug metabolism. A variety of techniques and assays were used, comprising enzyme studies using microsomal fractions (phase I and phase II enzymes), as well as transport studies using cell-membrane vesicles overexpressing the feline bile salt export pump (Bsep) or feline lymphocytes overexpressing P-glycoprotein (phase III).

The main findings of this thesis can be summarized as follows:

Phase I

- Cytochrome P450 (CYP) activities and substrate specificities differ between feline and canine liver microsomes. Also gender differences are observed.
- Diazepam, a drug that is regularly associated with undesirable hepatic side effects in cats, is converted in feline hepatic microsomal fractions mainly into temazepam, while in dog microsomes nordiazepam appears to be the principle metabolite. Other major canine metabolites such as oxazepam, could not be detected in incubations with feline liver microsomes. The lack of quantifiable formation of nordiazepam and oxazepam in feline liver microsomes suggests a feline CYP2B11 ortholog that significantly differs from the corresponding enzyme in dogs.

- The fluorometric assay intended for rapid analysis of CYP activity of patient-derived liver biopsies was found to be non-realistic for a clinical routine as the amount of liver tissue needed is still too high to allow the application in clinical diagnostics.

Phase II

- UDP-glucuronosyltransferase (UGT) 1A6 activity, quantified by the prototypic substrate 1-naphthol, was almost negligible in feline liver microsomes, confirming a deficient functional UGT1A6 homologue activity. A very limited capacity for morphine-3-glucuronide and the lack of estradiol-17-glucuronide formation suggest also a lack of functional UGT2B7 homologs in the cat livers.
- The formation of estradiol-3-glucuronide and 4-methylumbelliferone (4MU)-glucuronide, suggests a functional UGT1A1 and probably other UGT1A homologs in cats, albeit with a lower capacity than dogs.
- Studies with diazepam and its metabolites suggested that cats lack a functional UGT2B homologous gene, but express an active UGT1A3 ortholog.
- The overall low glucuronidation capacity in cats still remains of clinical importance in pharmacotherapy and toxicology.

Phase III

- Structure and functional characteristics of the feline bile salt export pump (BSEP) was described for the first time and appeared to be very similar between cats, dogs and humans.
- BSEP is essential for the transport of bile acids out of the hepatocyte, and inhibition of BSEP, as demonstrated for example for diazepam and its metabolites, seems to contribute to the observed hepatotoxicity after repeated dosing. Inhibition of BSEP transport capacity can be measured conveniently in everted membrane vesicles transfected with feline Bsep.
- Feline lymphoma cells express at a high rate the efflux transporter P-glycoprotein (P-gp) and hence serve as a valid model to study P-gp inhibition and drug-drug interactions at the level of P-gp transport.

In conclusion, the cat remains a unique animal species because of its specific and low glucuronidation capacity, that is of pivotal relevance for the selection and establishment of dosing regimens of drugs for feline patients. However, the specific sensitivity for hepatic side effects on drugs in feline species, might be attributable also to other mechanisms, such as expression, function and inhibition of drug efflux transporters in the liver, an area that has been neglected in the past.

SAMENVATTING VOOR NIET-VAKGENOTEN

ACHTERGROND

Het doel van het onderzoek beschreven in dit proefschrift, was een bijdrage te leveren aan de kennis op het gebied van de omzetting en de eliminatie van medicatie (biotransformatie) in de lever van de kat. Door de almaar groeiende populatie katten in Nederland en het stijgende aanbod van katten in de veterinaire praktijk, vervult de kat een steeds grotere rol binnen de diergeneeskunde. De kat wordt, net als de hond, steeds meer als gezinslid beschouwd, waardoor de emotionele waarde van het dier groter is en dus ook de wil van de eigenaar om de beste medische zorg te leveren wanneer het dier dat nodig heeft. Door deze stijgende populariteit van de kat, ontstaat er een behoefte of zelfs een noodzaak om de medische kennis over katten uit te breiden.

Van oudsher werd de hond altijd als belangrijkste diersoort gezien op het gebied van onderzoek. Ten eerste omdat de hond diende als proefdier voor onderzoeken m.b.t. de mens, maar later ook als doeldier voor onderzoek naar bijvoorbeeld nieuwe diergeneesmiddelen. De kat bleef echter achter in deze ontwikkeling. De kosten voor het ontwikkelen en registreren van nieuwe diergeneesmiddelen zijn erg hoog, en daarom worden diergeneesmiddelen in eerste instantie vaak ontwikkeld en geregistreerd voor één specifieke diersoort. Momenteel zijn er veel minder diergeneesmiddelen geregistreerd voor katten dan voor honden of mensen. In de veterinaire praktijk wordt daarom door dierenartsen vaak gebruik gemaakt van geneesmiddelen die geregistreerd zijn voor bijvoorbeeld honden of mensen, om zieke katten te behandelen. Voor deze katten is er dan geen geneesmiddel voor handen wat voor deze indicatie bij katten geregistreerd is. Dierenartsen dienen deze geneesmiddelen dan toe met aanpassing van de dosering naar het lichaamsgewicht van de kat, maar inmiddels weten we dat de kat niet gezien kan worden als “kleine hond”. Van verscheidene medicatie is gebleken dat deze erg giftig kan zijn in katten wanneer de voorgeschreven dosis voor honden of mensen wordt toegepast. Het meest bekende voorbeeld hiervan is paracetamol.

Verschillen in farmacokinetiek worden verondersteld de onderliggende mechanismes te zijn voor zulke vaak onvoorspelbare effecten. Daarom is het zo belangrijk om specifieke kennis te hebben over de kat en over de factoren die bij kunnen dragen aan het verschil in farmacokinetiek tussen de verschillende diersoorten. Dit alles om de praktiserende dierenarts te ondersteunen in het kiezen van de beste medicatie en het vaststellen van het beste doseringsschema voor de kat, en daarnaast om de farmaceutische industrie te stimuleren in het ontwikkelen van medicatie specifiek voor de kat.

OPZET VAN HET PROEFSCHRIFT

Bij het starten van het onderzoek wat geleid heeft tot dit proefschrift, zijn we begonnen met een overzicht te maken van de huidige kennis op het gebied van biotransformatie in de lever van de kat. Het werd al snel duidelijk dat de algemene kennis over deze processen in katten erg gelimiteerd is en dat er vele gaten in de literatuur gevuld moeten worden om een goede basis te creëren voor veilige en effectieve medicamenteuze behandelingen. Daarom hebben we, gebaseerd op de beschikbare data, een serie van experimenten ontwikkeld die een bijdrage leveren aan de kennis over de verschillende fases van de biotransformatie van de kattenlever.

Biotransformatie is dus het proces om het uitscheiden van geneesmiddelen of lichaamseigen stoffen (bijv. hormonen) uit het lichaam makkelijker te maken. Het doel hierbij is om stoffen minder actief te maken en veelal meer wateroplosbaar, zodat uitscheiding via bijv. urine of gal kan plaatsvinden. Biotransformatie gebeurt in verschillende fasen. Tot op heden werden voornamelijk de enzymatische omzettingen in fase I en II onderzocht. Inmiddels is gebleken dat ook de opname in een cel (fase 0) en het transport uit de cel (fase III) van groot belang zijn voor het kinetische gedrag en de veiligheid van een medicijn voor het dier.

In dit proefschrift ligt de focus op de lever, aangezien dit het belangrijkste orgaan is waar biotransformatie plaatsvindt. We hebben een verscheidenheid van technieken en analyses gebruikt, waaronder enzymstudies (fase I en II enzymen), en transportstudies in celmembraanblaasjes of kattenlymfocyten met radioactieve of fluorescerende substraten (fase III). We hebben gekozen voor een *in vitro* benadering, aangezien dit minder invasief is en minder proefdieren vereist vergeleken met *in vivo* studies. Desondanks voorziet deze *in vitro* benadering ons wel van fundamentele kennis over mechanismes in enzym- en transportfunctie.

BELANGRIJKSTE BEVINDINGEN

Fase I

- Cytochroom P450 (CYP) activiteit en specificiteit voor enzymsubstraten verschillen tussen katten- en honden-levermicrosomen. Hierbij worden ook verschillen in geslacht waargenomen.
- Diazepam, een medicijn dat vaak wordt geassocieerd met ongewenste bijwerkingen aan de lever van katten, wordt voornamelijk omgezet tot temazepam in katten-levermicrosomen. Daarentegen wordt nordiazepam bij

honden-levermicrosomen als voornaamste metaboliet waargenomen. Andere metabolieten, zoals oxazepam, werden niet waargenomen in incubaties met levermicrosomen van de kat. Het gebrek aan kwantificeerbare hoeveelheden van gevormd nordiazepam en oxazepam in de levermicrosomen van de kat, suggereert een katten CYP2B11 ortholoog dat significant verschilt van het corresponderende enzym in honden.
- De fluorometrische assay die bedoeld was als snelle analyse van CYP-activiteit van leverbiopten van patiënten, is niet geschikt bevonden voor het gebruik als klinische routine in de veterinaire praktijk. De hoeveelheid leverweefsel dat nodig is om de assay te kunnen gebruiken, is namelijk te hoog voor toepassing in klinische diagnostiek.

Fase II

- UDP-glucuronosyltransferase (UGT) 1A6 activiteit, gekwantificeerd door het prototypische substraat 1-naphthol, was bijna verwaarloosbaar in levermicrosomen van de kat, waardoor de deficiëntie van een functioneel UGT1A6 homoloog wordt bevestigd. Een erg gelimiteerde capaciteit voor morfine-3-glucuronide en een gebrek aan estradiol-17-glucuronide suggereert bovendien ook een gebrek aan functionele UGT2B7 homologen in de kattenlevers.
- De vorming van estradiol-3-glucuronide en 4-methylumbelliferone (4MU)-glucuronide, suggereert een functioneel UGT1A1 homoloog en mogelijk andere UGT1A homologen in katten, hoewel met een lagere capaciteit dan in honden.
- Studies met diazepam en haar metabolieten suggereren dat katten een functioneel UGT2B homoloog gen missen, maar dat zij een actief UGT1A3 homoloog tot expressie brengen.
- De algehele lage glucuronideringscapaciteit in katten blijft nog steeds van klinisch belang in farmacotherapie en toxicologie.

Fase III

- Structuur en functionele eigenschappen van de galzuurtransporter BSEP (bile salt export pump) zijn voor het eerst beschreven bij de kat en deze transporter heeft grote overeenkomsten tussen katten, honden en mensen.
- BSEP is essentieel voor het transport van galzuren uit de levercel, en remming van BSEP, zoals beschreven is voor diazepam en haar metabolieten, lijkt bij te dragen aan de hepatotoxiciteit die wordt waargenomen na herhaalde toediening van diazepam aan katten. Inhibitie van BSEP-transportcapaciteit kan worden gemeten in celmembraanblaasjes, getransfecteerd met katten-BSEP.
- Lymfoomcellen van katten bevatten grote hoeveelheden P-glycoproteïne (P-gp) efflux transporters en dienen daarom als een gevalideerd model

voor P-gp-inhibitie en medicijn-medicijn-interacties op het niveau van P-gp-transport.

Samengevat blijft de kat een unieke diersoort vanwege haar specifieke en lage glucuronideringscapaciteit, wat van cruciaal belang is voor de selectie en de dosering van medicatie voor kattenpatiënten. Echter, de specifieke gevoeligheid van de kattenlever voor bijwerkingen van medicatie kan ook worden toegeschreven aan andere mechanismes, zoals expressie, functie en inhibitie van medicijn-efflux-transporters in de lever, een gebied dat in het verleden onderbelicht is gebleven.

SAMENVATTING VOOR PRAKTISERENDE DIERENARTSEN

Biotransformatie en transportmechanismes van geneesmiddelen in de lever van de kat

Klinische implicaties van farmacokinetiek binnen de kattengeneeskunde

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ACHTERGROND

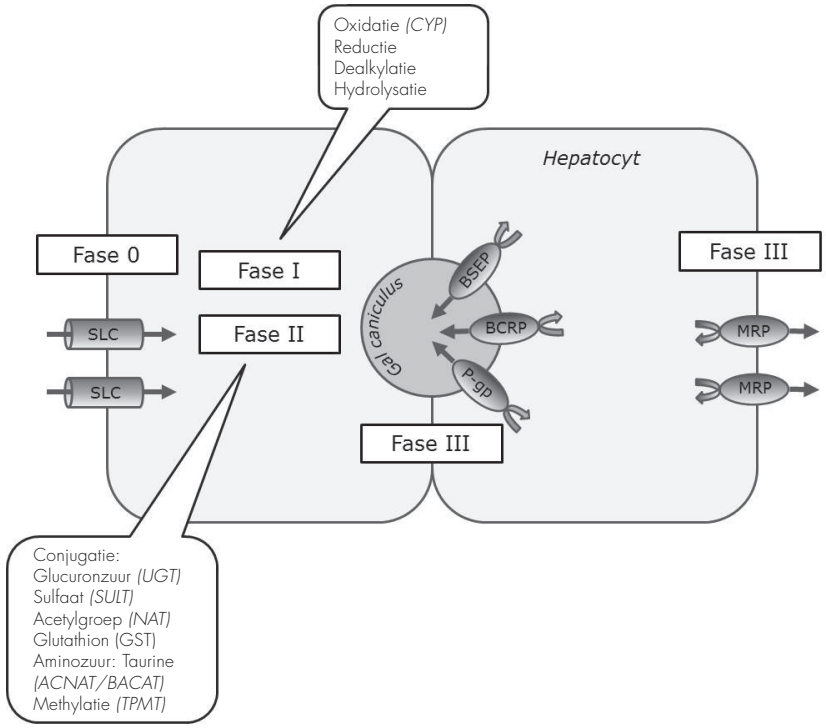
Door de almaar groeiende populariteit van de kat en dus het stijgende aanbod van katten in de veterinaire praktijk, vervult de kat een steeds grotere rol binnen de diergeneeskunde. De stijgende emotionele waarde van katten zorgt er dan ook voor dat eigenaren bereid zijn om de beste medische zorg te leveren wanneer hun kat dat nodig heeft. Helaas is er niet voor iedere indicatie een diergeneesmiddel voor handen dat geregistreerd is voor de kat. Dierenartsen zullen daardoor soms gedwongen zijn uit te wijken naar medicatie die geregistreerd is voor honden of voor mensen. Een aanpassing van de dosering naar het lichaamsgewicht van de kat wordt dan gemaakt, maar inmiddels weten we dat de kat niet gezien kan worden als "kleine hond". Van verscheidene medicatie is gebleken dat deze erg toxisch kan zijn in katten wanneer de voorgeschreven dosis voor honden of mensen wordt toegepast.

Verschillen in farmacokinetiek worden verondersteld de onderliggende mechanismes te zijn voor zulke vaak onvoorspelbare effecten. Daarom is het zo belangrijk om specifieke kennis te hebben over de kat en over de factoren die bij kunnen dragen aan het verschil in farmacokinetiek tussen de verschillende diersoorten. Cyrina van Beusekom is daarom haar promotieonderzoek destijds gestart om een begin te maken deze kennis over de kat uit te breiden. Haar openbare verdediging is gepland op 29 januari 2015. Dit alles om de praktiserende dierenarts te ondersteunen in het kiezen van de beste medicatie en het vaststellen van het beste doseringsschema voor de kat, en daarnaast om de farmaceutische industrie te stimuleren in het ontwikkelen van medicatie specifiek voor de kat.

BIOTRANSFORMATIE IN DE LEVER VAN DE KAT

Biotransformatie omvat de processen welke endogene (bijv. hormonen) en exogene (bijv. geneesmiddelen) stoffen elimineren. Het doel hierbij is om stoffen minder actief te maken en meer hydrofiel, zodat uitscheiding via bijv. urine of gal kan plaatsvinden. Biotransformatie bestaat uit verschillende fasen. Tot op heden werden voornamelijk de enzymatische omzettingen in fase I en fase II onderzocht. Inmiddels is gebleken dat ook de opname in een cel (fase 0) en de excretie vanuit bijv. de levercel naar de galgangen of de (hepatische) circulatie (fase III) van groot belang zijn voor het kinetische gedrag en de veiligheid van een medicijn voor het dier. Elke fase is afhankelijk van de fysisch-chemische eigenschappen van de stof, zoals moleculaire configuratie, lipofiliteit en ionisatie bij fysiologische pH.

In Figuur 1 wordt een overzicht gegeven van de verschillende fasen van de biotransformatie beschreven in de kattenlever, met de bijbehorende enzymen en transporter-eiwitten. Fase 0 is bij de kat nauwelijks beschreven (opname-transporters), maar de overige fasen worden in het kort hieronder beschreven, met de huidige kennis hierover bij de kat.



Figuur 1. CYP=Cytochroom P450; UGT=UDP-glucuronosyltransferase; SULT=sulfotransferase; NAT=N-acetyltransferase; GST=glutathion S-transferase; ACNAT=acyl-coA: amino acid N-acyltransferase; BACAT=bile acid-coA: amino acid N-acyltransferase; TPMT=Thiopurine S-Methyltransferase; SLC=solute carrier (niet gespecificeerd); BSEP=Bile Salt Export Pump; BCRP=Breast Cancer Resistance Protein; P-gp=P-glycoprotein; MRP=multidrug resistance-associated protein (niet gespecificeerd).

Fase I: oxidatie door Cytochroom P450

Fase I wordt veelal gekenmerkt door oxidatieprocessen uitgevoerd door de enzymfamilie Cytochroom P450 (CYP450 of CYP). Metabolieten van fase I zijn vaak beter wateroplosbaar en mogelijk al minder biologisch actief dan de moederstof, maar daarentegen kan een stof juist ook geactiveerd worden. Deze activatie kan gunstig zijn voor de therapeutische werking (denk aan prodrugs zoals prednison), maar kan ook ongunstig zijn zoals bij de vorming van epoxides of imines (carcinogeen of cytotoxisch).

De CYP familie wordt geclassificeerd op basis van gelijkenis in aminozuur sequentie. Enzymen geclassificeerd in een familie worden aangegeven met een cijfer, en enzymen gegroepeerd tot een subfamilie, worden aangegeven met een letter. Inmiddels zijn er voor mensen al 18 CYP families onderscheiden en is gebleken dat vooral CYP1, CYP2 en CYP3 betrokken zijn bij het omzetten van geneesmiddelen. De subfamilies CYP1A, CYP2B, CYP2C, CYP2D, CYP2E en CYP3A blijken hierbij het belangrijkst (Nebert & Russell, 2002; Zanger & Schwab, 2013). Iedere subfamilie metaboliseert bepaalde substraten, maar er bestaat overlap in substraatspecificiteit. Er is gebleken dat tussen verschillende diersoorten grote verschillen bestaan in CYP-activiteit, maar ook binnen een diersoort kunnen verschillen bestaan door genetische factoren, leeftijd, geslacht, ziekte en omgevingsfactoren. Ook kunnen sommige medicijnen de activiteit van CYPs remmen (bijv. ketoconazol of cimetidine) of juist induceren (bijv. fenobarbital), wat in geval van co-medicatie kan leiden tot respectievelijk verhoogde of verlaagde plasmaconcentraties van het geneesmiddel. Het beoogde effect van het geneesmiddel kan dan dus compleet anders uitvallen.

Al in de jaren '70 is de totale CYP-activiteit van katten vergeleken met die van honden of andere species en er werden geen grote verschillen gevonden tussen kat en hond (Maugras & Reichart, 1979; Gregus et al., 1983). Specifiekere studies naar de verschillende CYP subfamilies lieten zien dat er kleine verschillen bestaan in de activiteit en functie van de CYPs tussen katten, honden en mensen (Chauret et al., 1997; Shah et al., 2007). Ook onze onderzoeken bevestigen dit (van Beusekom et al., 2010). Echter ondanks deze verschillen, zijn duidelijke klinisch relevante bijwerkingen van medicatie niet geheel toe te schrijven aan enkel een verschil in CYP-activiteit.

Fase II: conjugatie-reacties

De verkregen functionele groep vanuit fase I kan conjugeren met een endogeen substraat, zoals glucuronzuur, een acetylgroep, glutathion, sulfaat of een aminozuur. Stoffen kunnen echter ook direct fase II ondergaan als het molecuul al een functionele groep bevat. De belangrijkste conjugatiereactie is het koppelen van een glucuronzuur en dit zogenaamde glucuronideren wordt

gekatalyseerd door de enzymfamilie UDP-glucuronosyltransferases (UGTs). De gevormde glucuronides zijn nog meer wateroplosbaar en over het algemeen niet biologisch actief.

UGTs worden net als CYPs onderverdeeld in families en subfamilies, waarbij UGT1A en UGT2B de belangrijkste subfamilies zijn. Voor het omzetten van medicatie in de humane lever zijn de enzymen UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 en 2B15 het belangrijkst (Ohno & Nakajin, 2009).

Het is inmiddels algemeen bekend bij dierenartsen dat katten slecht kunnen glucuronideren, waarvan intoxicatie met paracetamol het beste klinische voorbeeld is. Er is aangetoond dat UGT1A6 activiteit nodig is voor het glucuronideren van paracetamol, maar bij de kat blijkt dit gen een zogenaamd pseudo-gen te zijn, wat dus niet leidt tot transcriptie en translatie van een functioneel UGT1A6 enzym (Court & Greenblatt, 1997; Court & Greenblatt, 2000). Echter, aangezien UGTs uit meerdere (sub)families bestaan, betekent afwezigheid van een functioneel UGT1A6 enzym nog niet dat katten helemaal niet kunnen glucuronideren, terwijl dit wel vaak wordt verondersteld.

Inmiddels is gebleken dat katten wel degelijk glucuronides aanmaken, maar wel slechts voor een zeer beperkt aantal stoffen. Een voorbeeld is telmisartan, een angiotensine II receptor antagonist (Semintra®) voor het behandelen van proteïnurie door bijv. chronisch nierfalen (Ebner et al., 2013). Ook lichaamseigen stoffen zoals bilirubine of thyroxine kunnen door de kat geglucuronideerd worden (Myant, 1966; Fevery et al., 1977). In onze eigen onderzoeken zien wij glucuronideringsactiviteit in de kattenlever, hoewel het wel een stuk beperkter is dan bij honden. Er blijkt activiteit te zijn voor UGT1A1, en mogelijk een andere 1A isovorm, waarbij het waarschijnlijk om een homoloog van UGT1A3 van de mens gaat. UGT2B, welke belangrijk is voor het glucuronideren van bijv. opiaten of NSAIDs, lijkt bij de kat ook maar weinig activiteit te vertonen (van Beusekom et al., 2014).

Kortom, de algemene opinie over het ontbreken van glucuronideringsactiviteit bij de kat heeft wat nuancering nodig, maar het feit dat de kat een lagere glucuronideringscapaciteit heeft dan de hond, blijft bestaan. Het is dus belangrijk te weten welke medicijnen voornamelijk na glucuronidering worden uitgescheiden (zie Tabel 1), want voor deze stoffen bestaat het risico van ongewenste bijwerkingen bij de kat, m.n. bij herhaalde toediening.

Tabel 1. Medicatie waarvoor beschreven is dat glucuronidatie een belangrijk mechanisme is voor de uitscheiding van deze stoffen bij mens en dier (Miners & Mackenzie, 1991; Williams et al., 2004; Kiang et al., 2005; Kaivosaari et al., 2011). De tabel bevat een selectie van geneesmiddelen welke kunnen worden voorgeschreven of (per ongeluk) worden toegediend aan gezelschapsdieren. Vetgedrukt, de stoffen met een nauwe therapeutische breedte.

Medicatie met een hoog percentage glucuronidatie		
Acetaminophen	*Firocoxib*	*Naloxon*
(=paracetamol)	*Fluoroquinolonen*	***Naproxen***
Alprazolam	*(vnl pradofloxacine)*	*Oxazepam*
Benoxaprofen	*Flurbiprofen*	***Phenylbutazone***
Buprenorphine	***Ibuprofen***	*Pirprofen*
Carprofen	*Ketoprofen*	*Propofol*
Codeine	*Levothyroxine*	***Pyrethroiden***
Chlooramfenicol	***Lindaan***	*(bijv.* ***permethrine****,*
Cyproheptadine	*Lorazepam*	*maar niet flumethrine)*
(Dex)medetomidine	*Metoclopramide*	*Salicylaat*
Diazepam	*Metronidazol*	*Suprofen*
Diclofenac	*Midazolam*	*Temazepam*
(Ethinyl)estradiol	*Mirtazapine*	*Valdecoxib*
Fenoprofen	*Morphine*	

Fase III: efflux uit de hepatocyt

Doordat zowel fase I als fase II leidt tot hydrofiele metabolieten, werd duidelijk dat cellen zoals hepatocyten specifieke transporters moeten hebben om de excretie van deze metabolieten te bewerkstelligen, aangezien deze metabolieten niet gemakkelijk de celmembraan kunnen passeren. Hepatocyten exporteren metabolieten via de basolaterale en canaliculaire membraan richting respectievelijk bloedbaan (voor excretie via de nieren) en galgangen (voor excretie via de gal). Deze transporters gebruiken ATP als energiebron om actief te kunnen pompen tegen een concentratiegradiënt in, en worden ATP-Binding Cassette (ABC) transporters genoemd.

ABC-transporters zijn ontdekt in tumorcellen die resistent werden voor chemotherapeutica doordat zij deze efflux transporters tot expressie brachten. Later bleek echter dat deze transporters ook een fysiologische rol vervulden als barrière in vele organen, waaronder de lever, nieren en bloed-hersen-barrière, om weefsels te beschermen tegen invloeden van (toxische) stoffen. Inmiddels zijn er veel ABC-transporters ontdekt, waarbij de benaming vaak de eerste

onderzoekslijn weerspiegelt en soms misleidend lijkt te zijn. Voorbeelden zijn P-glycoprotein (P-gp, MDR1, ABCB1), de Breast Cancer Resistance Protein (BCRP/ABCG2), de multidrug resistance-associated protein 2 (MRP2/ABCC2) en 4 (MRP4/ABCC4), en de Bile Salt Export Pump (BSEP/ABCB11).

P-gp kennen we in de diergeneeskunde het beste van het polymorfisme in het Mdr1 gen bij o.a. de Collie-achtigen. Deze honden hebben geen functionele P-gp-transporters in de bloed-hersen-barrière die het hersenweefsel beschermen tegen de toxische invloed van bijv. ivermectine (Mealey et al., 2001). Bij katten is maar weinig bekend over P-gp. Wij hebben een model ontwikkeld waarbij de functie van feline P-gp getest kan worden in katten-lymfoomcellen, zodat er beter kan worden beoordeeld of medicatie een remmende werking kan hebben op P-gp. Inhibitie van P-gp door co-medicatie kan namelijk leiden tot verhoogde concentraties van medicatie in de cel en mogelijk tot cytotoxiciteit.

BSEP is de transporter in de lever die verantwoordelijk is voor het transporteren van galzuren vanuit de hepatocyt naar de galgangen. Inhibitie van BSEP door medicatie kan leiden tot een verhoogde intracellulaire concentratie van galzuren, welke erg toxisch zijn voor de hepatocyt (Kullak-Ublick & Meier, 2000). Aangezien er over BSEP bij katten nog niets bekend was, hebben wij de functionele eigenschappen van de feline BSEP geanalyseerd en zagen grote overeenkomsten in structuur en functionele eigenschappen met BSEP van honden en mensen (van Beusekom et al., 2013).

De functie van BCRP bij de kat is alleen beschreven voor het oog, waarbij katten BCRP maar minimale bescherming geeft aan de retina tegen bijv. het foto-reactieve antibioticum enrofloxacine. Dit verklaart dat katten na herhaalde toediening van hoge doses enrofloxacine blind kunnen worden (Ramirez et al., 2011). De functie van BCRP in de lever is bij katten niet beschreven, hoewel bij mensen deze transporter voornamelijk betrokken is bij de efflux van sulfaat- en glucuronideconjugaten.

Onopgeloste klinische vraagstukken in de kattengeneeskunde

Hoewel steeds meer mechanismes ontdekt worden achter de bijwerkingen van medicijnen, zijn er nog steeds onopgeloste vraagstukken. We noemen dit idiosyncratische reacties, waarbij het mechanisme niet past bij de bekende farmacologische eigenschappen van het medicijn, en waarbij verondersteld wordt dat er reactieve metabolieten binden aan eiwitten in het lichaam, waarbij dit complex als vreemd lichaam wordt gezien en aangevallen wordt door het immuunsysteem. Over het algemeen volgen idiosyncratische reacties geen klassieke dosis-respons relatie (Antonovic & Martinez, 2011; Trepanier,

2013). De term idiosyncratisch wordt echter ook gebruikt voor zeldzame bijwerkingen met onbekende oorzaak, of bijwerkingen die maar voorkomen bij een erg klein percentage katten dat behandeld wordt met dat geneesmiddel.

Een voorbeeld van een medicijn dat een idiosyncratische reactie bij de kat geeft is permethrine, wat gebruikt wordt tegen ectoparasieten. Permethrine wordt gemetaboliseerd door CYPs en carboxylesterases, en de metabolieten hiervan ondergaan verschillende conjugatiereacties. Er werd gedacht dat de deficiënte glucuronideringscapaciteit van de kat de oorzaak was voor de bijwerkingen van permethrine. Echter, het is vooral de hydrolyse van de ester-verbinding van de moederstof welke de stof minder toxisch maakt (Kaneko, 2011), dus mogelijk hebben katten een verminderde esterase-activiteit of vormen ze toxische metabolieten (Richardson, 2000).

Een ander voorbeeld van idiosyncratische reacties bij de kat is de acute leverschade na herhaalde toediening van diazepam. Weer werd gedacht aan de deficiënte glucuronideringscapaciteit van katten, aangezien de metabolieten van diazepam bij andere diersoorten worden geglucuronideerd. Wij hebben ontdekt dat de metabolieten temazepam en oxazepam inderdaad nauwelijks worden geglucuronideerd in de kattenlever, maar ook dat diazepam en haar metabolieten de galzuurtransporter BSEP van katten remt. Onze hypothese is dus dat de verhoogde concentraties van diazepam en haar metabolieten zorgen voor een inhibitie van BSEP, waardoor het transport van galzuren belemmerd wordt en er een intrahepatische cholestase ontstaat en aldus leverschade.

CONCLUSIE

- De kat vervult een steeds belangrijkere rol binnen de diergeneeskunde, maar de ontwikkeling van medicatie specifiek voor de kat loopt nog achter op deze ontwikkeling.
- Het toedienen van geneesmiddelen die geregistreerd zijn voor honden of mensen aan katten, omvat meer dan alleen een wijziging van de dosis op basis van het lichaamsgewicht.
- Veel factoren zijn van invloed op de farmacokinetiek (absorptie, distributie, metabolisme, eliminatie (ADME)), en het onderzoek naar membraantransporters (opname en excretie) zal nog verder onderzocht moeten worden in de kat.
- De glucuronideringsdeficiëntie blijft van cruciaal belang voor de selectie en de dosering van medicatie in kattenpatiënten.

DANKWOORD

Met dank aan promotor prof.dr. Johanna Fink-Gremmels, copromotor dr. Jan Schrickx, de technische hulp van de divisie Veterinaire Farmacologie, Farmacotherapie en Toxicologie (VFFT) van het Institute for Risk Assessment Sciences (IRAS) van de Universiteit Utrecht, en het departement Farmacologie en Toxicologie van de Radboud Universiteit Nijmegen.

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DANKWOORD

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Cyrina

ABOUT THE AUTHOR

CURRICULUM VITAE

Cyrina van Beusekom was born in Culemborg, the Netherlands on July 24, 1982 and finished secondary school (Gymnasium at Koningin Wilhelmina College, Culemborg) in 2000. She started studying veterinary medicine at the Rijksuniversitair Centrum Antwerpen, Belgium and completed the first year of her studies with honors. After completing her first year in Belgium, she continued her studies at the Faculty of Veterinary Medicine of Utrecht University. She passed the first four years of this study with merit ("met genoegen") and was offered an honors program, consisting of performing one year of research in veterinary science. In 2005 she started her research in feline biotransformation at the division of Veterinary Pharmacology, Pharmacotherapy and Toxicology. She presented the results of this research with an oral presentation during the 10th European Association of Veterinary Pharmacology and Toxicology (EAVPT) Conference, held in Turin, Italy in 2006. After completing the clinical internship for finalizing the study in 2008, she graduated as a veterinarian with a clinical differentiation into companion animals. She obtained a position as a veterinary practitioner in a clinic for companion animals in Veenendaal, whilst continuing research as a PhD candidate. Since 2009, she has been combining her PhD work that has led to this thesis, with being a veterinary practitioner in a clinic for companion animals in Vianen (including working in night- and weekendshifts). In 2014, she presented parts of the results of this thesis at the European Veterinary Conference Voorjaarsdagen in Amsterdam, where she won the first prize of the Boehringer Ingelheim Research Award. Aside from being a researcher, she also worked as a lecturer in veterinary pharmacology, pharmacotherapy and toxicology at the Faculty of Veterinary Medicine, Utrecht University.

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AWARD

Boehringer Ingelheim Research Award, 1st prize
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QUOD MEDICINA ALIIS, ALIIS EST ACRE VENENUM

"What is medicine to some, is bitter poison to others"