BILIARY EXCRETION OF TECHNETIUM-99M-SESTAMIBI IN WILD-TYPE DOGS AND IN DOGS WITH INTRINSIC (ABCB1-1Δ MUTATION) AND EXTRINSIC (KETOCONAZOLE TREATED) P-GLYCOPROTEIN DEFICIENCY

By

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A thesis submitted in partial fulfillment of

the requirements for the Degree

MASTER OF SCIENCE IN VETERINARY SCIENCE

WASHINGTON STATE UNIVERSITY

College of Veterinary Medicine

AUGUST 2008

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of JOANA CHABY LARA SANTOS COELHO find it satisfactory and recommend that it be accepted.

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Chair

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AKNOWLEDGEMENTS

I would like to specially thank Dr. Katrina Mealey for giving me the opportunity to work with her in such an interesting subject and for her unconditional guidance and support throughout all process. I would like to thank Denise Waiting for making this study happen and for her exceptional help. I would also like to thank the members of my committee, Doctors Russell Tucker, John Mattoon and Greg Roberts for their help with this study.

In addition, I would like to acknowledge Professors Rich Alldredge and Nairanjana Dasgupta for their assistance with the statistical analysis of this study.

Funding for this project was provided by the Graduate Research Grant Award 2006 by the department of Veterinary Clinical Studies at Washington State University.

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Abstract

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August 2008

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P-glycoprotein (P-gp), the product of ABCB1 gene, is thought to contribute significantly to biliary excretion of a variety of drugs. Because a number of endogenous (ABCB1 polymorphisms) and exogenous (pharmacological P-gp inhibition) factors can interfere with normal P-gp function, efforts are being made to gain a better understanding of P-gp's role in biliary drug excretion. The objective of this study was to compare biliary excretion of technetium-99m-sestamibi (^{99m}Tc-MIBI), a radio-labeled P-gp substrate, in wild-type dogs (ABCB1 wild/wild), P-gp deficient dogs (ABCB1 mut/mut), and dogs with presumed intermediate phenotype (ABCB1 mut/wild). The effect of pharmacological inhibition of P-gp (ketoconazole) on biliary excretion of ^{99m}Tc-MIBI in ABCB1 wild/wild dogs was also

determined. Results of this study showed that ABCB1 mut/mut dogs have significant decreased biliary excretion of ^{99m}Tc-MIBI compared to ABCB1 wild/wild dogs. Biliary excretion of ^{99m}Tc-MIBI in ABCB1 mut/wild dogs is not significantly different from ABCB1 wild/wild dogs. The P-gp inhibitor ketoconazole significantly decreased biliary excretion of ^{99m}Tc-MIBI. P-gp appears to play a major role in the biliary excretion of ^{99m}Tc-MIBI and likely other P-gp substrate drugs in dogs. This canine model may be useful for further studies delineating the role of P-gp in biliary drug excretion.

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INTRODUCTION

The research reported in this thesis was conducted with and under the guidance of the graduate committee members as part of the Radiology Residency of the Department of Veterinary Clinical Sciences at Washington State University College of Veterinary Medicine. This research has generated one manuscript (Chapter 1) which will be submitted for publication in the journal *Biopharmaceutics & Drug Disposition*. The format of this chapter is as required by that journal.

CHAPTER ONE

Biliary excretion of technetium-99m-sestamibi in wild-type dogs and in dogs with intrinsic (ABCB1-1Δ mutation) and extrinsic (ketoconazole treated) P-glycoprotein deficiency

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Introduction

P-glycoprotein (P-gp), the product of ABCB1 gene (formerly known as MDR1) was first discovered in the 1970s, as a prototypic transporter involved in multidrug resistance of neoplastic cells. P-gp is a large (approximately 1280 amino acids, 170 KDa molecular weight) glycosylated membrane-spanning protein that belongs to the ATP-binding cassette (ABC) superfamily of membrane transporters [1,2]. P-gp functions as an ATP-dependent efflux pump, capable of transporting exogenous and endogenous substrates from the inside to the outside of cells [2,3]. Originally found in tumor cells in humans and rodents, P-gp is also expressed physiologically in epithelial cells of organs with excretory or protective function, such as the canalicular membrane of hepatocytes, brush border membrane of enterocytes in the small and large intestines, capillary endothelial cells of the brain and testis and the brush border membrane of proximal tubule cells in the kidneys [2,4,5]. In humans, P-gp has also been identified in hematopoietic cells and in pancreatic, adrenal and placental tissue [6]. In the dog, P-gp has been identified in the liver, capillary endothelial cells of the brain, kidneys, adrenals and colon [7]. The expression of P-gp in these tissues is a major determinant of drug disposition and provides a cellular defense mechanism by limiting intracellular accumulation of potentially harmful xenobiotics [3]. P-gp has wide substrate specificity, transporting a large number of structurally and pharmacologically unrelated hydrophobic compounds. Multiple drugs that are P-gp substrates are commonly used in human and veterinary medicine, including antibiotics, antiemetic drugs, antiparasitics, cardiac glycosides and anticancer agents [1,8-10]. Drug interactions involving P-gp are especially relevant for drugs with narrow therapeutic indices, where induction or inhibition of transporter function can have great impact on drug efficacy and safety. Multiple polymorphisms of ABCB1

gene have been reported in humans. Some of these polymorphisms appear to result in decreased expression of P-gp [2,3,6].

The ABCB1 polymorphism in dogs consists of a four base-pair deletion mutation (ABCB1-1Δ mutation) first described in ivermectin–sensitive Collies [11]. The mutation generates a premature stop codon in the ABCB1 gene, resulting in a severely truncated, nonfunctional protein [11]. Thus dogs with two mutant alleles exhibit a P-gp null phenotype, similar to mdr1a and mdr1b knockout mice [9]. Roughly 75% of Collies in the United States, France and Australia have a least one mutant allele [12-14]. Several other breeds have also been found to harbor the ABCB1-1Δ mutation, including the Australian Shepherd, Long-haired Whippet , Silken Windhound, Shetland Sheepdog, Old English Sheepdog, German Shepherd, English Shepherd, among others [9,11,15,16]. Mutation of the ABCB1 gene in dogs results in a defective blood-brain-barrier that does not promptly excrete P-gp drug substrates, such as ivermectin and loperamide, out of the brain, resulting in neurotoxicity from otherwise standard therapeutic doses [11,17,18].

P-glycoprotein's putative roles also include renal and biliary excretion of xenobiotics [19,20]. Vincristine, a cytotoxic drug commonly used in chemotherapeutic protocols to treat lymphoma, is primarily excreted through the biliary system [21]. Dogs harboring the ABCB1-1Δ mutation (i.e. ABCB1 mut/wild and ABCB1 mut/mut) have been shown to be more likely to develop hematologic toxicity, specifically neutropenia and thrombocytopenia after treatment with vincristine than ABCB1 wild/wild dogs [21]. Since the ABCB1-1Δ mutation results in a non-functional P-gp, it is likely that the dogs harboring the mutant gene have a deficiency of P-gp in the liver, where P-gp plays a role in the excretion of a number of xenobiotics. Thus, increased vincristine-related toxicity in dogs harboring the ABCB1-1Δ mutation may result from

increased exposure to vincristine in these dogs, due to decreased biliary and/or renal excretion of the drug. ABCB1 genotyping can be used to determine if lower doses of these drugs should be administered to canine patients carrying a mutant ABCB1 allele [21]. Currently, these doses would have to be calculated empirically because there are no established doses for dogs harboring the ABCB1-1Δ mutation.

Technetium-99m-sestamibi (^{99m}Tc-MIBI) is a widely used radiopharmaceutical agent for myocardial perfusion imaging studies and to measure P-gp activity in various tumors in humans and in mice [22-25]. ^{99m}Tc-MIBI is a P-gp substrate [24,26] and it undergoes minimal metabolism in the guinea pig [27]. ^{99m}Tc-MIBI has been shown to be a sensitive probe of P-gp function in both *in vitro* and *in vivo* studies [28]. In addition, biliary clearance of ^{99m}Tc-MIBI in humans is markedly reduced in the presence of P-gp inhibitors [29]. Therefore, it is likely that P-gp is the principal mediator of biliary ^{99m}Tc-MIBI excretion in humans [24,26]. By determining the relative biliary excretion of ^{99m}Tc-MIBI in dogs with each ABCB1 genotype, one may then be able to estimate relative dose reductions for other P-gp substrate drugs that undergo primarily biliary excretion (particularly those with a narrow therapeutic index such as vincristine and doxorubicin) for animals harboring the ABCB1-1Δ mutation.

^{99m}Tc-MIBI is a cationic lipophilic agent and its uptake into the cells is passive depending on the distribution of regional blood flow and mitochondrial oxidation capacity. ^{99m}Tc-MIBI cationic charge allows its accumulation in mitochondria via interaction with the large negative cytosolic and mitochondrial membrane potentials [24]. Thus, it is retained in organs with high metabolic rates, such as the heart, kidney, lung and liver. The accumulation rates are driven by negative transmembrane potentials but retention of ^{99m}Tc-MIBI is dependent on P-gp activity [24]. In the liver, P-gp is expressed in the canalicular membrane of the

hepatocytes assisting in the active transport of ^{99m}Tc-MIBI from the hepatocyte into the bile [30,31]. ^{99m}Tc-MIBI is therefore an excellent probe for evaluating P-gp's role in biliary drug excretion.

There were two main objectives for this study reported here. The first was to compare the biliary excretion of ^{99m}Tc-MIBI in ABCB1 mut/mut dogs (intrinsic P-gp deficient) and ABCB1 mut/wild dogs (presumed partially P-gp deficient) with ABCB1 wild/wild dogs. The second objective was to compare the biliary excretion of ^{99m}Tc-MIBI in ABCB1 wild/wild dogs before and after administration of ketoconazole (extrinsic P-gp deficiency).

Materials and Methods

Animal procedures

Eleven adult dogs were used in this study. All animal procedures were approved by the Institutional Animal Care and Use Committee of Washington State University. All dogs were healthy based on physical examination, complete blood count, serum biochemistry profile including bilirubin and pre- and postprandial bile acids, urinalysis, radiography of the thorax and abdomen and ultrasound of the abdomen. All dogs had normal hepatobiliary tract morphology based on abdominal ultrasound examination.

The scintigraphy scans of the ABCB1 wild/wild dogs obtained after administration of ketoconazole were performed approximately 6 months after the first studies. Physical examination, complete blood count and serum biochemistry profile including bilirubin were repeated in these dogs prior to the second studies to assure they were still healthy at that time.

The dogs were grouped in three groups according to their ABCB1 genotype. Six dogs were homozygous for the wild ABCB1 allele (ABCB1 wild/wild), 2 dogs heterozygous for the mutant ABCB1 allele (ABCB1 mut/wild) and 3 dogs homozygous for the mutant ABCB1 allele (ABCB1 mut/mut). The ABCB1 genotype was determined through a commercialized assay (Veterinary Clinical Pharmacology Laboratory, College of Veterinary Medicine, Washington State University, Pullman, Washington, www.vetmed.wsu.edu/vcpl/) using previously described methods [32].

There were 7 neutered males (6 ABCB1 wild/wild and 1 ABCB1 mut/mut), 3 spayed females (2 ABCB1 mut/wild and 1 ABCB1 mut/mut) and 1 intact female (ABCB1 mut/mut). The age range was 3-5 years and the weight range 18-30 Kg, with only one dog weighing 30 Kg

and the other dogs weighing between 18 and 23.5 Kg. Dog breeds included: 6 Walker Deer Hounds, 3 Collies and 2 Mongrels.

Nuclear Scintigraphy Studies

Scintigraphy studies of the hepatobiliary system were performed in all dogs and the same study protocol was used for all scans ^{99m}Tc-MIBI was obtained from a local commercial nuclear medicine supplier (Syncore, Spokane, WA). All the procedures involving use of ^{99m}Tc-MIBI were approved by the Washington State University Environmental Health & Safety and Radiation Safety Offices. All dogs were fasted for 12 h prior to the scans. The animals were anesthetized with desflurane (Suprane®, Baxter International Inc., Deerfield, Illinois) in oxygen and were positioned in sternal recumbency on the gamma camera (Starcam, General Electric Medical Systems, Milwaukee, WI).The gamma camera was fitted with a low-energy, high-resolution, parallel hole collimator. Energy discrimination was accomplished by using a 20% window centered at the 140 keV photopeak of technetium-99m. An intravenous bolus of 272.7-366.3 MBq (7.37-9.9 mCi) ^{99m}Tc-MIBI was administered through a saphenous catheter. The precise activity of ^{99m}Tc-MIBI injected was calculated by subtracting the residual ^{99m}Tc-MIBI activity of the syringe from the activity before injection. A 256 x 256 matrix image size was used. Static, 1 min acquisition images of the abdomen, including the whole liver and gallbladder, were obtained at 5-min intervals from 10 min up to 120 min after injection of ^{99m}Tc-MIBI.

During the second phase of this study only the ABCB1 wild/wild dogs were scanned. Ketoconazole (Apotex, Inc. Toronto, Ontario, Canada) (5 mg/Kg PO q12h x 9 doses) was administered to each dog for 4 days prior and in the morning of the day the scintigraphy study was performed. The dose of ketoconazole used in this study is within the range used for

antifungal activity in dogs. The protocol used for these scintigraphy scans was the same as described above. The dose of ^{99m}Tc-MIBI administered as an intravenous bolus was 210.9-371.11 MBq (5.7-10.03 mCi).

Data analysis and Statistics

Change in the liver and gallbladder activity throughout time was determined by manually drawing individual regions of interest (ROI) around the liver and gallbladder for each imaging time point. The automated image analysis software program included in the gamma- camera computer (Starcam, General Electric Medical Systems, Milwaukee, WI) was used to obtain the activity in the liver and gallbladder. A gallbladder to liver activity (G/L) ratio was calculated for each imaging time point using the mean counts per pixel of each ROI. Time-activity curves (TAC) were created for each study using Excel (Microsoft Office Excel 2007), with time (10 - 120 min) represented the on x-axis and G/L ratios (using mean counts per pixel per ROI) represented on the y-axis.

Statistical analysis was performed with SAS (SAS, SAS Institute Inc, Cary, NC) Proc. Mixed one-way ANOVA repeated measures in time using G/L ratios was used to compare ABCB1 mut/mut and ABCB1 mut/wild dogs with ABCB1 wild/wild dogs and to compare ABCB1 wild/wild dogs before and after administration of ketoconazole. A value of $p<0.05$ was considered statistically significant.

Results

Biliary excretion of ^{99m}Tc-MIBI in ABCB1 mut/mut and ABCB1 mut/wild dogs versus ABCB1 wild/wild dogs

This study showed that ABCB1 mut/mut dogs have significantly decreased biliary excretion of ^{99m}Tc-MIBI compared to ABCB1 wild/wild dogs ($p<0.001$) during the 120 min period following intravenous injection of ^{99m}Tc-MIBI. The biliary excretion of ^{99m}Tc-MIBI in ABCB1 mut/wild dogs is not significantly decreased from ABCB1 wild/wild dogs ($p=0.370$), during the same time period.

Ventral images of the abdomen of an ABCB1 wild/wild dog and of an ABCB1 mut/mut dog acquired 120 min after intravenous injection of ^{99m}Tc-MIBI are shown in Figure 1. Intense ^{99m}Tc-MIBI uptake is seen in the region of the gallbladder on the ABCB1 wild/wild dog (Fig.1. a). In contrast, the region of the gallbladder in the ABCB1 mut/mut dog shows a distinct void of activity (Fig.1.b). At the same time, the background activity on the ABCB1 wild/wild dog is subjectively greatly reduced compared to the background activity in the ABCB1 mut/mut dog. These findings are consistent with decreased elimination of ^{99m}Tc-MIBI into the gallbladder in the ABCB1 mut/mut dog with resultant increased circulating ^{99m}Tc-MIBI.

Figure 2 represents the TAC of the mean G/L ratios for each one of the three groups. Progressive increase in G/L ratio throughout the 120 min period is observed in ABCB1 wild/wild dogs and ABCB1 mut/wild dogs. No significant difference was found between the G/L ratios obtained at each imaging time point in the ABCB1 mut/mut dogs ($p=0.999$), which is illustrated in the TAC as an almost flat line.

The least difference between the mean G/L ratios for the three different groups was observed 10 min after ^{99m}Tc-MIBI intravenous injection. The mean G/L ratios values for the different groups at 10 min were: ABCB1 mut/mut dogs (0.81 ±0.06), ABCB1 mut/wild dogs (1.04±0.12) and ABCB1 wild/wild dogs (1.22±0.37). The maximum difference between mean G/L ratios for the three groups was observed at 120 min. The mean G/L ratios for the different groups at 120 min were: ABCB1 mut/mut (0.91±0.36), ABCB1 mut/wild (4.81±1.70) and ABCB1 wild/wild (7.34±2.24).

Biliary excretion of ^{99m}Tc-MIBI in ABCB1 wild/wild dogs before and after administration of ketoconazole

Biliary excretion of ^{99m}Tc-MIBI is significantly decreased in ABCB1 wild/wild dogs after administration of ketoconazole (p<0.001). The effect of the ketoconazole administration on the TAC of ABCB1 wild/wild dogs was similar to the effect of natural P-gp deficiency in ABCB1 mut/mut dogs (lower G/B ratios throughout time). However, significant differences were observed between the G/L ratios throughout time in the ABCB1 wild/wild dogs after administration of ketoconazole, contrary to the ABCB1 mut/mut dogs. The mean G/L ratios for the ABCB1 wild/wild dogs progressively increased throughout time, starting at 10 min (0.86±0.57) and reaching a maximum mean G/L ratio at 120 min (2.86±1.86) (Fig. 3).

Discussion

According to our results, P-gp plays a key role in the biliary excretion of ^{99m}Tc-MIBI. The biliary excretion of ^{99m}Tc-MIBI is significantly decreased in ABCB1 mut/mut dogs compared to ABCB1 wild/wild dogs. Decreased biliary excretion of other P-gp substrates in ABCB1 mut/mut dogs, including chemotherapeutic drugs, such as vincristine and doxorubicin is likely and may contribute to the enhanced toxicity of therapeutic doses of these drugs in ABCB1 mut/mut dogs. Indeed, ABCB1 mut/mut dogs with lymphoma are significantly more likely to develop hematologic toxicity after treatment with vincristine, a drug primarily eliminated through the biliary system [21]. Several other P-gp substrate drugs, that rely on biliary excretion, including doxorubicin, ivermectin and loperamide have been associated with toxicosis in ABCB1 mut/mut dogs [11,18,33]. Interestingly, vincristine and doxorubicin have also caused toxicity in ABCB1 mut/wild dogs, but in many cases to a lesser extent than ABCB 1 mut/mut dogs [21]. Thus, it appears that ABCB1 mut/wild dogs may have an intermediate phenotype.

Contrary to our expectations, a significant difference was not identified between the biliary excretion of ^{99m}Tc-MIBI in ABCB1 mut/wild dogs compared with ABCB1 wild/wild dogs. Failure to achieve a significant difference may have been due to low numbers of dogs in this group. Even though, no significant difference was found, the G/L ratios of one of the ABCB1 mut/wild dogs were distinct and non-overlapping with the G/L ratios of the ABCB1 wild/wild dogs starting at 60 min through 120 min after injection of ^{99m}Tc-MIBI. The other ABCB1 mut/wild dog had values that clearly overlapped with those of ABCB1 wild/wild dogs. More dogs in the ABCB1 mut/wild group may have generated data resulting in a significant difference in the biliary excretion of ^{99m}Tc-MIBI in ABCB1 mut/wild dogs compared to ABCB1 wild/wild dogs.

^{99m}Tc-MIBI is a P-gp substrate that also has affinity for the multidrug resistance-associated proteins (MRP) 1 (ABCC1) and 2 (ABCC2), membrane transporters belonging to the same transmembrane transporter superfamily as P-gp [34]. In dogs, as in humans, MRP1 and MRP2 are expressed in the basolateral membrane and canalicular membrane of hepatocytes, respectively [35]. While MRP1-mediated transport of ^{99m}Tc-MIBI has been reported in cell culture systems, it has not been consistently identified *in vivo* [24,36-38]. MRP2-mediate biliary transport of ^{99m}Tc-MIBI has only recently been reported by Hendrikse et al. 2004 [36]. Expression of MRP1 in healthy human liver has been reported to be relatively low [36]. Expression of MRP1 is higher and MRP2 is lower in canine liver as compared to human liver [35]. Results from our study suggest that differences in biliary excretion of ^{99m}Tc-MIBI in ABCB1 mut/mut dogs compared to ABCB1 wild/wild dogs are primarily the result of differences in P-gp activity with minimal contribution of other membrane transporters such as MRP. Furthermore, there is no reason to believe that the P-gp null-phenotype, ABCB1 mut/mut dogs are simultaneously MRP-deficient compared to ABCB1 wild/wild dogs. To evaluate the specific contribution of MRP to the biliary excretion of ^{99m}Tc-MIBI, studies using technetium-99m-disofenin (^{99m}TC-HIDA), a radiopharmaceutical that is a substrate for MRP1 and MRP2, but not P-gp, [36] could be considered in the future.

Extensive research has been performed in effort to develop P-gp modulators that increase concentrations of chemotherapeutic drug in tumor cells expressing P-gp [8,39,40]. Understanding how P-gp modulators affect the toxicity and pharmacokinetics of cytotoxic agents is necessary before the initiation of therapeutic trials of P-gp modulators in cancer patients. The second phase of our study demonstrates that ketoconazole, a P-gp modulator, can affect the pharmacokinetics (biliary clearance in particular) of ^{99m}Tc-MIBI and likely of P-gp substrate

chemotherapeutic drugs. The administration of ketoconazole, which has been shown to inhibit P-gp *in vitro* [41] and *in vivo* [10], significantly decreased biliary excretion of ^{99m}Tc-MIBI in ABCB1 wild/wild dogs. Thus, co-administration of ketoconazole with other P-gp substrate drugs that undergo biliary excretion will likely result in increased drug exposure for these animals. Imaging studies of the biliary system using ^{99m}Tc-MIBI may be useful in determining the degree to which biliary excretion is impaired by various P-gp modulators. This type of information could then be used to design safe dosage regimens for P-gp modulators.

Multiple structurally and functionally unrelated drugs routinely used in veterinary and human medicine are substrates for P-gp [8,9]. ^{99m}Tc-MIBI imaging appears to be a powerful tool for *in vivo* monitoring of P-gp activity in the canine liver. Imaging studies such as those reported here may help in developing mathematical models to determine appropriate dosages of P-gp substrates for ABCB1 mut/mut and ABCB1 mut/wild dogs. These studies may also be used to determine doses of P-gp substrate drugs which may be safely co-administered with a P-gp modulator such as ketoconazole. Imaging studies such as hepatobiliary scintigraphy also have the advantages of being *in vivo* studies, not requiring multiple venous samplings and involving relatively low doses of radiation. However, it should be taken into account that drug elimination is a complex process and any single isolated study is unlikely to be completely predictive of P-gp function and its role in drug clearance.

The ABCB1-1Δ canine model appears to represent a good model for studying the role of P-gp in the biliary excretion of substrate drugs. Humans and dogs have a single ABCB1 gene whereas rodents have two genes that encode P-gp (abcb1a and abcb1b) [24,38] which potentially makes the dog a better model than rodents for the study of P-gp activity. Also image analysis is easier to perform in dogs compared to rodents given their greater size.

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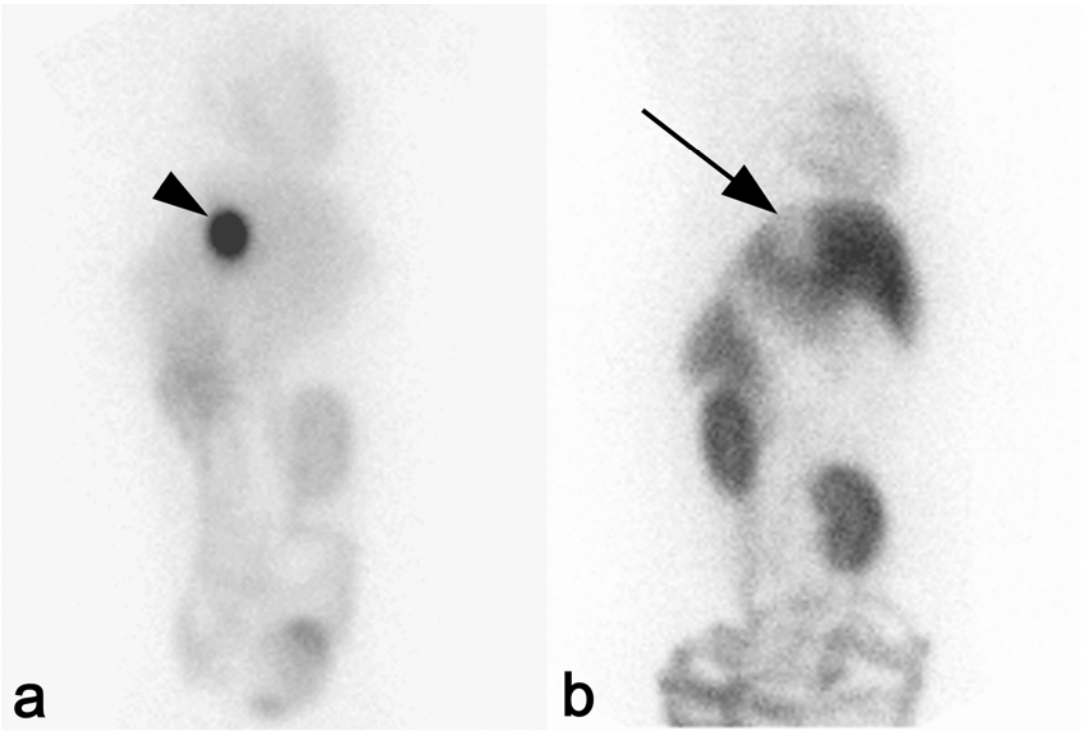

Figure 1. Ventral images of the abdomen acquired at 120 min after intravenous injection of ^{99m}Tc-MIBI to an ABCB1 wild/wild dog (a) and to an ABCB1 mut/mut dog (b). Intense gallbladder ^{99m}Tc-MIBI uptake (arrow head) and relatively low background activity is present in (a). A void of activity in the location of the gallbladder (arrow) and relatively high background activity is present in (b)

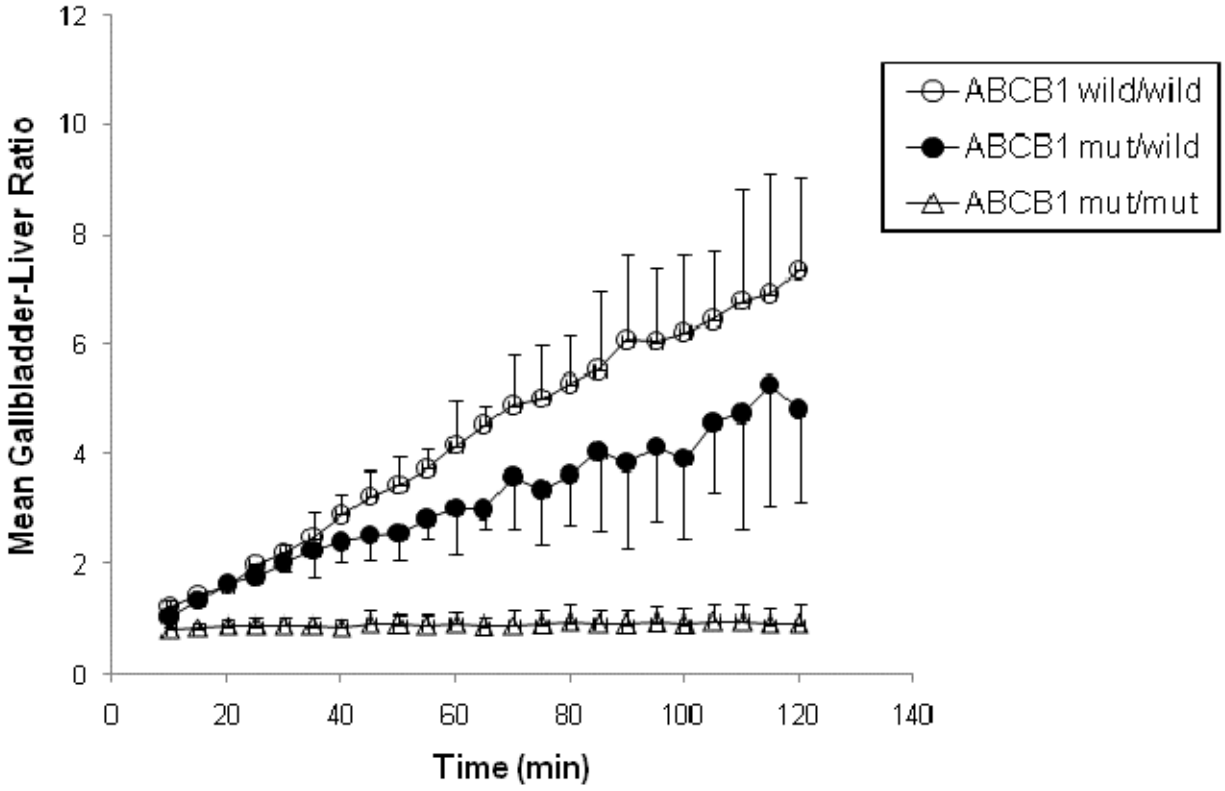

Figure 2. Time-activity curves of mean gallbladder to liver activity ratios (using mean counts per pixel per ROI) for ABCB1 mut/mut dogs (Δ: mean G/L ratio + SD, n=3), ABCB1 mut/wild dogs (●: mean G/L ratio – SD, n=2) and ABCB1 wild/wild dogs (○: mean G/L ratio + SD, n=6)

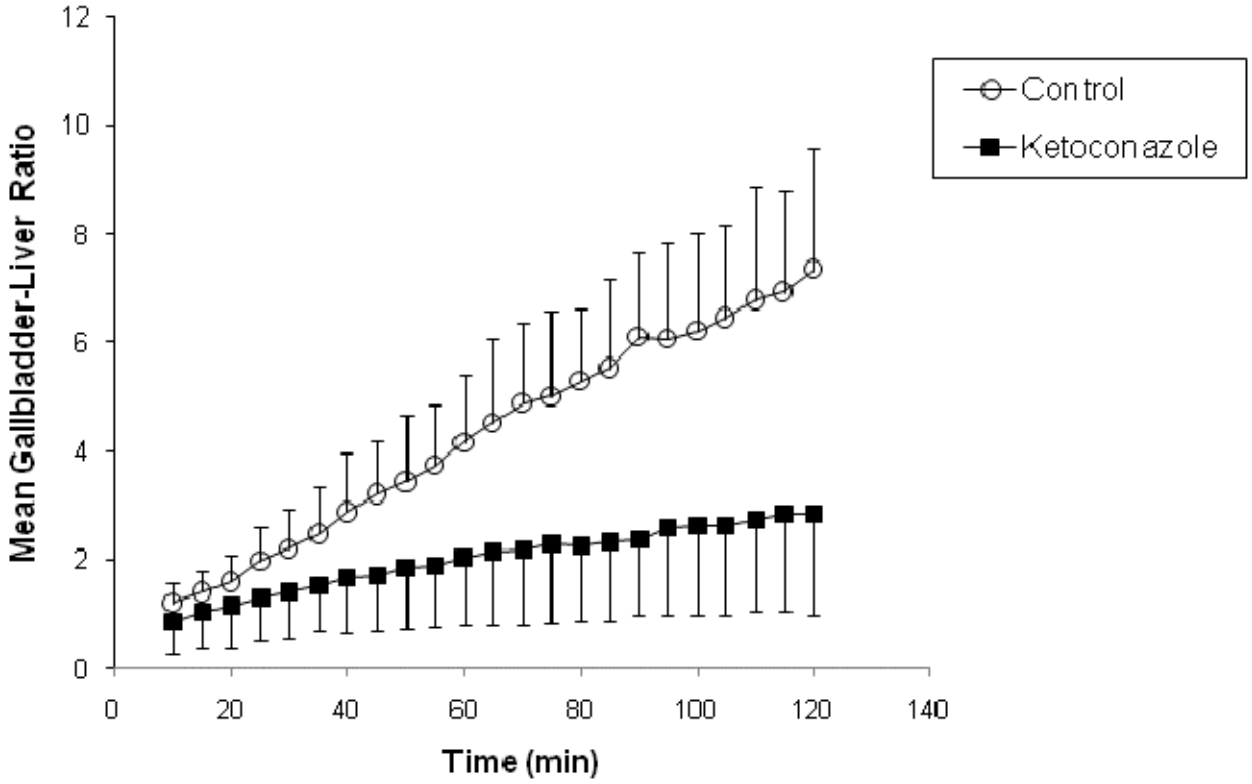

Figure 3. Time-activity curves of gallbladder to liver activity ratios (using mean counts per pixel per ROI) for ABCB1 wild/wild dogs before (○: mean G/L ratio + SD, n=6) and after (■: mean G/L ratio – SD, n=6) administration of ketoconazole