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 the ABCB1 gene, is a large, glycosylated membrane-spanning protein that belongs to the ATP-binding cassette (ABC) superfamily of membrane transporters (Schinkel & Jonker, 2003). P-gp functions as an ATP-dependent efflux pump, capable of transporting exogenous and endogenous substrates from the inside to the outside of cells (Cornwell, 1991). Originally found in tumour cells, 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, brush border membrane of proximal tubule cells in the kidneys; and capillary endothelial cells of the brain and testis. In the dog, P-gp has been identified in the liver, kidneys, adrenals, colon and capillary endothelial cells of the brain (Ginn, 1996). Along with other factors such as a drug’s lipophilicity, tissue blood flow and metabolism, expression of P-gp is a major determinant of drug disposition and provides a defence mechanism by limiting accumulation of potentially harmful xenobiotics. P-gp has wide substrate specificity, transporting a large number of structurally and pharmacologically unrelated hydrophobic compounds (Schinkel & Jonker, 2003). A number of P-gp substrate drugs are commonly used in veterinary medicine, including loperamide, digoxin, cyclosporin A, ivermectin, milbemycin, doxorubicin, vincristine and others. Adverse drug reactions and drug–drug interactions involving P-gp are especially relevant for drugs with narrow therapeutic indices, where alteration of transporter function can have great impact on drug efficacy and safety. For example,
vincristine-induced bone marrow toxicity is significantly more likely to occur in dogs with the ABCB1-1Δ mutation than in wild-type dogs (Mealey et al., 2008a,b).

The ABCB1-1Δ polymorphism, first described in ivermectin-sensitive collies, consists of a four base-pair deletion mutation (Mealey et al., 2001). The mutation generates a premature stop codon in the ABCB1 gene, resulting in a severely truncated, nonfunctional protein. Thus, dogs with two mutant alleles exhibit a P-gp null phenotype, similar to mdr1a and mdr1b knockout mice. Roughly, 75% of Collies in the United States, France and Australia have at least one mutant allele. Several other breeds have also been found to harbour the ABCB1-1Δ mutation, including the Australian Shepherd, Long-haired Whippet, Silken Windhound, Shetland Sheepdog, Old English Sheepdog, German Shepherd and English Shepherd, among others (Neff et al., 2004).

The clinical effects of the ABCB1-1Δ mutation clearly illustrate the role of P-gp in the blood–brain barrier (Mealey et al., 2001, 2008a,b). However, P-gp’s putative role in biliary excretion of xenobiotics has not been explored. Vincristine, a cytotoxic drug commonly used in chemotherapeutic protocols to treat lymphoma, is primarily excreted through the biliary system (Watanabe et al., 1995; Song et al., 1999). Dogs harbouring the ABCB1-1Δ mutation (i.e. ABCB1 mut/wild and ABCB1 mut/mut) have been shown to be more likely to develop haematologic toxicity, specifically neutropenia and thrombocytopenia after treatment with vincristine than ABCB1 wild/wild dogs (Mealey et al., 2008a,b). As the ABCB1-1Δ mutation produces a nonfunctional protein product, biliary excretion of P-gp substrate drugs such as vincristine would be predicted to be decreased, resulting in increased overall exposure to the drug. Whether or not this is actually the case is not currently known because the impact of P-gp on biliary excretion of substrate drugs has not yet been investigated in dogs.

Technetium-99m-sestamibi (99mTc-MIBI) is a widely used radiopharmaceutical agent for myocardial perfusion imaging studies in humans (Slart et al., 2006). Because it is a P-gp substrate, 99mTc-MIBI is also used to assess P-gp activity in patients with a variety of tumours (Abdel-Dayem, 1997; Fukumoto, 2004). 99mTc-MIBI has been shown to be a sensitive probe of P-gp function in both in vitro and in vivo studies in humans and rodents, and was recently used to compare blood–brain barrier function in ABCB1 wild/wild and ABCB1 mut/wild dogs (Mealey et al., 2008a,b). 99mTc-MIBI undergoes minimal biotransformation and its biliary clearance in humans is markedly reduced in the presence of P-gp inhibitors (Barbarics et al., 1994). 99mTc-MIBI is therefore an excellent probe for evaluating P-gp’s role in biliary drug excretion (Kabasakal et al., 2000).

There were two main objectives for this study. The first was to compare biliary excretion of 99mTc-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 99mTc-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 total bilirubin, 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 total bilirubin concentration 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-type ABCB1 allele (ABCB1 wild/wild), two dogs were heterozygous for the ABCB1-1Δ mutation (ABCB1 mut/wild) and three dogs were homozygous for the ABCB1-1Δ mutation (ABCB1 mut/mut). Initially, three ABCB1 mut/wild dogs had been recruited for the study, but one dog in this group was excluded from the study because of abnormalities observed on ultrasonographic evaluation of the liver and kidneys.

ABCB1 genotyping was determined through a commercialized assay (Veterinary Clinical Pharmacology Laboratory, College of Veterinary Medicine, Washington State University, Pullman, Washington, http://www.vetmed.wsu.edu/vcpl/) using previously described methods (Mealey et al., 2003).

There were seven neutered males (six ABCB1 wild/wild and one ABCB1 mut/mut), three spayed females (two ABCB1 mut/wild and one ABCB1 mut/mut) and one 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: six Walker Deer Hounds (ABCB1 wild/wild), two Mongrels (ABCB1 mut/wild) and three Collies (ABCB1 mut/mut).

Nuclear scintigraphy studies

Scintigraphy studies of the hepatobiliary system were performed in all dogs and the same study protocol was used for all scans. 99mTc-MIBI was purchased from a local commercial nuclear medicine supplier (Syncore, Spokane, WA, USA). All procedures involving use of 99mTc-MIBI were approved by the Washington State University Environmental Health & Safety and Radiation Safety Offices. All dogs were fasted for 12 h prior to scans. Animals were anesthetized with desflurane (Suprane®; Baxter International Inc., Deerfield, IL, USA) in oxygen and were positioned in sternal recumbency on the gamma camera (Starcam: General Electric Medical Systems, Milwaukee, WI, USA). The gamma camera was fitted with a low energy, high
resolution, parallel hole collimator. Energy discrimination was accomplished by using a 20% window centred at the 140 keV photopeak of technetium-99m. An intravenous bolus of 272.7–366.3 MBq (7.37–9.9 mCi) 99mTc-MIBI was administered through a saphenous catheter. The precise activity of 99mTc-MIBI injected was calculated by subtracting the residual 99mTc-MIBI activity of the syringe from the activity before injection. A 256 × 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 99mTc-MIBI.

During the second phase of this study only ABCB1 wild/wild dogs were scanned. Ketoconazole (Apotex, Inc. Toronto, ON, Canada) (5 mg/kg PO q12h × 9 doses) was administered to each dog for 4 days prior and in the morning of the day scintigraphy was performed. The protocol used for these scintigraphy scans was the same as described above. The dose of 99mTc-MIBI administered as an intravenous bolus was 210.9–371.11 MBq (5.7–10.03 mCi).

DATA ANALYSIS AND STATISTICS

Change in liver and gallbladder activity throughout time was determined according to standard nuclear scintigraphy protocols. Regions of interest (ROI) around the liver and the gallbladder were drawn during the initial time point and used for each subsequent time point. The automated image analysis software program included in the gamma-camera computer (Starcam; General Electric Medical Systems, Milwaukee, WI, USA) 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. Area under the time–activity curves (AUC), calculated by the trapezoidal method, were created for each study using Excel (Microsoft Office Excel 2007), with time (10–120 min) represented on the x axis and G/L ratios (using mean counts per pixel per ROI) represented on the y axis.

Data is presented as mean (±SD) G/L ratio at individual time points or mean (±SD) AUC of G/L ratio × time (G/L ratio × min) for total biliary 99mTc-MIBI accumulation. Statistical analysis was performed with SAS (SAS Institute Inc, Cary, NC, USA) Proc. Mixed one-way ANOVA repeated measures in time using G/L ratios was used to compare ABCB1 mut/wild and ABCB1 wild/wild dogs before and after administration of ketoconazole. A value of P < 0.05 was considered statistically significant.

RESULTS

Biliary excretion of 99mTc-MIBI in ABCB1 mut/mut vs. ABCB1 mut/wild and ABCB1 wild/wild dogs

Mean biliary accumulation of 99mTc-MIBI in ABCB1 mut/mut dogs (8715 G/L × min) was significantly lower (P < 0.001) than that of ABCB1 wild/wild dogs (469 ± 124 G/L × min) during the 120 min period following intravenous injection of 99mTc-MIBI. Biliary accumulation of 99mTc-MIBI in ABCB1 mut/wild dogs (346; range 281–412 G/L × min) did not appear to be decreased from ABCB1 wild/wild dogs during the 120-min study period.

Ventral images of the abdomen of an ABCB1 wild/wild dog and an ABCB1 mut/mut dog that were acquired 120 min after intravenous injection of 99mTc-MIBI are shown in Fig. 1. Intense 99mTc-MIBI uptake is seen within the gallbladder of the ABCB1 wild/wild dog (Fig. 1a). In contrast, the gallbladder of the ABCB1 mut/mut dog shows a distinct void of activity (Fig. 1b).

Fig. 1. Ventral images of the abdomen acquired at 120 min after intravenous injection of technetium-99m-sestamibi (99mTc-MIBI) (approximately 10 mCi/dog) to an ABCB1 wild/wild dog (a) and to an ABCB1 mut/mut dog (b). Intense gallbladder 99mTc-MIBI uptake (arrow head) is present in (a). A void of activity in the location of the gallbladder (arrow) is present in (b).

Fig. 2. Time–activity curves of mean gallbladder to liver activity ratios [using mean counts per pixel per regions of interest (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).
represents the mean G/L ratios vs. time for each group. Progressive increases in G/L ratio can be observed at each subsequent time point in ABCB1 wild/wild dogs and ABCB1 mut/wild dogs. Conversely, increases in G/L ratios cannot be detected at subsequent time points in ABCB1 mut/mut dogs (i.e., the slope approximates zero).

As would be expected shortly after intravenous injection of $^{99m}$Tc-MIBI, very little difference was observed in mean G/L ratios among the groups at the 10 min imaging time point. The mean G/L ratios values for the three groups at 10 min were: ABCB1 mut/mut dogs (0.81 ± 0.06); ABCB1 mut/wild dogs (1.04; range 0.96–1.12) and ABCB1 wild/wild dogs (1.22 ± 0.37). The mean G/L ratio showed the greatest difference between groups at the 120-min time point. The mean G/L ratio for ABCB1 wild/wild dogs at 120 min was 7.34 ± 2.24. By comparison, the mean G/L ratio for ABCB1 mut/mut dogs (0.91 ± 0.36) was significantly different (P < 0.001), whereas the mean G/L ratio for ABCB1 mut/wild dogs (4.81; range 3.61–6.02) did not differ from ABCB1 wild/wild dogs (P = 0.370).

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

Ketoconazole treatment significantly (P < 0.01) decreased biliary accumulation of $^{99m}$Tc-MIBI ABCB1 in wild/wild dogs (260.4 ± 103.2 G/L · min) compared with pretreatment values (469 ± 124 G/L · min). Ketoconazole appears to partially disrupt P-gp function in ABCB1 wild/wild dogs creating a phenotype somewhat similar to the effect of natural P-gp deficiency in ABCB1 mut/mut dogs (lower G/L ratios over time). Unlike the G/L ratio vs. time curve generated in ABCB1 mut/mut dogs, however, significant differences were observed between the G/L ratios at subsequent time points in ABCB1 wild/wild dogs after administration of ketoconazole (i.e., the slope is positive). Mean G/L ratios for ketoconazole-treated ABCB1 wild/wild dogs progressively increased over 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 in dogs. The biliary excretion of $^{99m}$Tc-MIBI is significantly decreased in ABCB1 mut/mut dogs compared with 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 in humans and rodents (presumably dogs). 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 (Mealey et al., 2001, 2008a,b; Sartor et al., 2004). Whether or not decreased biliary excretion of these drugs is the primary mechanism responsible for their enhanced toxicity in ABCB1 mut/mut dogs is not known. It is possible that deficient P-gp function in additional locations also contributes to the enhanced toxicity observed in ABCB1 mut/mut dogs.

Interestingly, vincristine and doxorubicin have also caused toxicity in ABCB1 mut/wild dogs, but in many cases to a lesser extent than ABCB1 mut/mut dogs (Mealey et al., 2003). Thus, it appears that ABCB1 mut/wild dogs may have an intermediate phenotype. Because clinical toxicity has been observed in ABCB1 mut/wild dogs with P-gp substrate drugs, we expected to find a significant difference in biliary excretion of $^{99m}$Tc-MIBI in ABCB1 mut/wild dogs compared with ABCB1 wild/wild dogs. Contrary to our expectations, a significant difference was not identified. Failure to achieve a significant difference may have been due to low numbers of dogs in this group. Perhaps 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 with ABCB1 wild/wild dogs. It is also possible that gender differences among groups contributed to intra-group variability.

The second phase of this study was intended to determine whether or not drug–drug interactions might affect P-gp-mediated biliary drug excretion. In this part of the study ketoconazole, an antifungal drug that also inhibits P-gp function, significantly decreased biliary accumulation of $^{99m}$Tc-MIBI. By extension it is reasonable to assume that P-gp substrate drugs with a comparable biliary excretion profile would be affected similarly. Thus, co-administration of ketoconazole with P-gp substrate drugs that undergo biliary excretion will likely result in increased drug exposure for these animals. A number of
drugs used in veterinary medicine for treating a variety of diseases are known to inhibit P-gp, including ketoconazole, cyclosporin A, tamoxifen and others (Nobili et al., 2006; McDevitt & Callaghan, 2007). Concurrent use of P-gp inhibitors with P-gp substrates, particularly those with a narrow therapeutic index such as vincristine or doxorubicin, may delay biliary excretion of the P-gp substrate. This could result in clinically significant adverse drug–drug interactions. Because $^{99m}$Tc-MIBI undergoes minimal biotransformation, 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 inhibitors.

One potential confounding factor that should be mentioned is the breed distribution of the three ABCB1 genotypes. All ABCB1 mut/mut dogs were Collies while all ABCB1 wild/wild dogs were Walker Deer Hounds. While it is possible that differences in biliary excretion of $^{99m}$Tc-MIBI observed between these two groups resulted from an undetermined, breed-related phenomenon besides P-gp, this possibility seems unlikely. This possibility was partially addressed in the second part of the study, which involved only Walker Deer Hounds. Ketoconazole, a P-gp inhibitor, was used to create a P-gp null phenotype. Ketoconazole also inhibits cytochrome P450 3A, but as $^{99m}$Tc-MIBI undergoes minimal metabolism, any differences observed in biliary accumulation of $^{99m}$Tc-MIBI in the pre- and posttreatment groups can be attributed to the effects of ketoconazole on P-gp.

Multiple structurally and functionally unrelated drugs routinely used in veterinary and human medicine are substrates for P-gp. $^{99m}$Tc-MIBI imaging appears to be a powerful, noninvasive 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 dogs with intrinsic or extrinsic dysfunction of P-gp.

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**REFERENCES**


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