Title: Comparison of $^{99m}$Tc-MIBI biliary excretion in dogs with ABCB1 gene mutation versus normal dogs

Research Proposal by:
Joana Chaby Lara Santos Coelho, DVM
Radiology Resident
November 29, 2006
Advisors: Dr. Russell Tucker and Dr. Katrina Mealey

A. Specific Aims

This study represents the first phase (Phase 1) of a larger study that includes a second phase (Phase 2). The aim of Phase 1 is to document the normal hepatobiliary excretion of $^{99m}$Tc-MIBI (technetium-99m labeled sestamibi) by nuclear scintigraphy in dogs homozygous for the normal ABCB1 gene (wild/wild). Once Phase 1 of the project is completed, a grant proposal will be submitted to obtain additional extramural funding to pursue Phase 2 of the study. The aim of Phase 2 is to compare the hepatic excretion of $^{99m}$Tc-MIBI between dogs homozygous for the normal ABCB1 gene (wild/wild), dogs with mutation of one ABCB1 allele (mutant/wild) and dogs with mutation of both ABCB1 alleles (mutant/mutant).

We hypothesize that the hepatobiliary excretion $^{99m}$Tc-MIBI is markedly decreased in dogs with mutation of one or of both ABCB1 alleles (mutant/mutant and wild/mutant) compared to that of dogs homozygous for the normal ABCB1 gene (wild/wild).

B. Background and Significance

P-glycoprotein (P-gp), the product of ABCB1 gene (formerly known as multi-drug resistance gene - MDR1 gene) was first discovered in the 1970s, as a prototypic transporter involved in the multidrug resistance of neoplastic cells (1). P-glycoprotein is a large (approximately 1280 amino acids) glycosylated membrane-spanning protein that functions as a transmembrane efflux pump. Originally found in tumor cells in humans and rodents, P-gp is also expressed in normal tissues that perform excretory or barrier functions, such as the liver, kidneys, intestines, testis and brain endothelial cells (2, 3). In humans, hepatobiliary clearance of $^{99m}$Tc-MIBI is markedly reduced in the presence of P-gp inhibitors (4). Therefore, it is likely that P-gp is the principal mediator of $^{99m}$Tc-MIBI excretion in humans. P-glycoprotein has been identified in various canine organs including liver, kidneys, adrenals, colon and also in the capillary endothelial cells of the brain (5).

In 2001, Mealey et al. demonstrated that a deletion mutation of the ABCB1 gene exists in ivermectin–sensitive collies (6). The mutation generates a premature stop codon in the ABCB1 gene, resulting in a severely truncated, nonfunctional protein. Since then, several other breeds have been found to have mutation of the ABCB1 gene, including Shetland Sheepdog, Australian Shepherd, Old English Sheepdog, English Shepherd, Longhaired Whippet and Silken Windhound among others (6, 7).
Mutation of the ABCB1 gene in dogs results in a defective blood-brain-barrier that does not promptly excrete drugs that are P-gp substrates out of the brain, resulting in neurotoxicity from otherwise standard therapeutic doses (6). P-glycoprotein’s putative roles also include renal and biliary excretion of xenobiotics (8, 9). We hypothesize that dogs with ABCB1 gene mutation may also have decreased hepatobiliary excretion of drugs that are P-gp substrates, which may contribute to the toxicity of therapeutic doses in these dogs. P-glycoprotein has wide substrate specificity and multiple drugs that are P-gp substrates are commonly used in veterinary medicine. These include antibiotics, antiemetic drugs, antiparasitics, cardiac glycosides and anticancer agents. Multiple cases of toxicosis in dogs with the deletion mutation in the ABCB1 gene receiving therapeutic doses of several of these drugs, including ivermectin, doxorubicin, digoxin, mexiletine, loperamide and vincristine, have been reported (6, 10-13). For several of these drugs the liver is the primary organ of excretion. Vincristine and doxorubicin are examples of chemotherapeutic drugs that have been reported to induce gastrointestinal toxicity and myelosuppressive effects at lower doses in dogs with a mutant ABCB1 allele compared to wild type dogs (11).

ABCB1 genotyping can be used to determine if lower doses of these drugs should be administered to canine patients carrying a mutant ABCB1 allele (6). Currently, these doses would have to be calculated empirically because there are no established normal doses for dogs harboring a mutant ABCB1 gene. By determining the relative hepatobiliary excretion of $^{99m}$Tc-MIBI in dogs with each ABCB1 genotype, one can then estimate relative dose reductions for other P-gp substrate drugs (particularly those with a narrow therapeutic index such as vincristine and doxorubicin) for animals harboring the ABCB1 mutation.

Technetium-99m is the most frequently used radionuclide for in vivo imaging studies in nuclear medicine. $^{99m}$Tc-MIBI is a lipophilic radioactive compound (radiopharmaceutical) that is a substrate for P-gp. $^{99m}$Tc-MIBI has been widely used for myocardial perfusion imaging in humans and in research to measure P-gp activity in humans and in mice (4). The uptake of $^{99m}$Tc-MIBI depends on the distribution of regional blood flow and on mitochondrial oxidation capacity because its cationic charge allows accumulation in mitochondria via interaction with the large negative cytosolic and mitochondrial membrane potentials. 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.

C. Current Research/Preliminary Data

A preliminary $^{99m}$Tc-MIBI brain scintigraphy study was performed at the WSU Veterinary Teaching Hospital in six adult collies (3 mutant/mutant dogs and 3 wild type (wild/wild) dogs for the ABCB1 gene). All dogs had unremarkable blood work and no history or clinical signs indicative of hepatobiliary disease. This study showed significant (p-value < 0.05) decreased blood-brain-barrier excretion of $^{99m}$Tc-MIBI in mutant/mutant compared to the wild type dogs. In two of these dogs the liver was also imaged and showed dramatic decrease in biliary excretion of $^{99m}$Tc-MIBI in the mutant/mutant dogs compared to the wild type dogs (see Fig. 1 and 2).
D. Research Design and Methods

The total population of the study (including Phases 1 and 2) will include 12 adult dogs: 6 dogs homozygous for the wild ABCB1 allele (wild/wild), 3 dogs heterozygous for the mutant ABCB1 allele (wild/mutant) and 3 dogs homozygous for the mutant ABCB1 allele (mutant/mutant). Phase 1 will consist of hepatobiliary studies in the 6 dogs homozygous for the wild ABCB1 allele (wild/wild) to establish normal values for hepatobiliary excretion of $^{99m}$Tc-MIBI.

In a preliminary study we found a statistically significant (p-value < 0.05) difference between the retention of $^{99m}$Tc-MIBI activity in the brain of dogs with and without mutation of ABCB1 gene. In a study by Joseph et al. hepatobiliary excretion in normal rodents (mice and rat) was compared to that of animals with mutation of mdr1a (single knockout) or both mdr1a and mdr1b (14). These genes are analogs of the ABCB1 gene in humans and dogs, codifying P-gp. Intrasplicic injection was performed and $^{99m}$Tc-MIBI was rapidly incorporated into the liver. Statistically significant differences (p-value < 0.05) were found between all groups and there was no overlap in any measured values of liver activity between wild type animals and mdr1 knockout animals at any point in time. In this study, 13 animals without mdr1 gene mutation, 12 animals with a single mutation of either mdr1a or mdr1b and 6 animals with mutation of both mdr1a and mdr1b genes were used.

If our hypothesis is correct, we believe that distinct, non-overlapping values of liver and gall bladder activity will be obtained between wild type, mutant/wild and mutant/mutant dogs. If this is the case, the number of dogs proposed will be sufficient to show statistical significance.
The procedures that follow will be applied to all wild type dogs and, if extramural funding is obtained to dogs with ABCB1 gene mutation.

Animal procedures. Each dog will be housed in a run measuring 4 x 8 feet with chain link fencing, a concrete floor, and a raised bed. Facilities exceed the minimal requirements specified by USDA guidelines.

To determine which dogs have ABCB1 gene mutation, DNA samples will be extracted from buccal swabs and analyzed with a polymerase chain reaction (PCR) using previously described methods (15). To be eligible for this study, the dogs must be physiologically normal and have no previous history of renal or liver disease. This will be determined by obtaining a clinical history and performing physical examination, complete blood count (CBC), serum chemistry profile, pre- and postprandial bile acids, urinalysis, abdominal radiographs, and abdominal ultrasound exam. Dogs with any blood work abnormalities or abnormalities on physical examination, abdominal radiography or ultrasonography that may be correlated with liver and/or renal disease will be excluded from this study and replacement dog will be recruited.

All animals will be fasted for 12 hours before the nuclear scintigraphy examination. Each dog will be sedated immediately prior to the procedure with medetomidine hydrochloride (Domitor®, Pfizer, Animal Health, Exton, PA) (2 µg/kg i.v.) administered through an intravenous 22 gauge, catheter placed in the cephalic vein. Medetomidine will be used as a sedative because it is a non P-gp substrate drug, so it will not confound the results. At the end of the examination, sedation will be reversed with atipamazole hydrochloride (Antisedan®, Pfizer, Animal Health, Exton, PA) (2 µg/kg i.v.).

If heavy sedation is found to be insufficient for achieving immobility during the nuclear scintigraphy study, general anesthesia will be performed with isoflurane (IsoFlo®, Abbott Laboratories, North Chicago, IL) by mask induction and maintained after tracheal intubation. In one study in dogs, the use of general anesthesia did not affect the hepatic extraction efficiency compared to heavy sedation (16). We consider that the degree of hypotension caused by general anesthesia should not significantly affect the distribution of the radiopharmaceutical, especially because it will be administered by intrasplenic injection rather than by intravenous injection.

The dogs will be positioned in sternal recumbency over a large field of view gamma camera fitted with a high resolution, low-energy, all-purpose parallel hole collimator (Starcam, General Electrical Medical Systems, Milwaukee, WI). A radioactive marker (Co-57) will be placed at the level of the xiphoid to aid identification of the liver. Once adequate sedation is achieved, ultrasound (ATL HDI 3000, Philips Corporation, Grand Rapids, MI) guided intrasplenic injection of a bolus of 10 mCi of $^{99m}$Tc-MIBI, as previously described will be performed (17). The injection site will be examined ultrasonographically to monitor for hemorrhage. The precise activity of the $^{99m}$Tc-MIBI bolus administered will be calculated by subtracting the residual $^{99m}$Tc-MIBI activity in the syringe post-injection from the activity before injection (17).

A dynamic frame-mode acquisition will be initiated after successful needle placement within the spleen (based on ultrasonographic observation of the needle within the splenic parenchyma) and approximately 1-2 s prior to injection of the radionuclide. Dynamic images will be acquired at one frame/second rate for the first minute and then static images will be
acquired every 10 minutes (1 minute acquisition per image), starting at 10 minutes post-administration of $^{99m}$Tc-MIBI, up to 90 minutes.

As part of the radiation safety protocol, the dogs will stay in isolated housing after the nuclear scintigraphy study until their body radioactivity, measured with a Geiger-Muller survey meter, is less or equal to 0.2 mR /hour at 1 meter. We anticipate that the dogs will be released from isolation the day following the procedure. In isolation, the dogs will have the same level of treatment care (as when not in isolation), although physical contact between the animals and the care taker will be minimized and hazardous radioactive materials will be properly disposed (e.g. urine and feces).

All procedures involving use of $^{99m}$Tc-MIBI will be approved by the Washington State University Environmental Health and Safety and Radiation Safety Offices.

Pharmacokinetic modeling of $^{99m}$Tc-MIBI hepatic elimination. We will use a compartmental model to represent the region from which the radioactivity is measured. In brief, the liver vasculature is assumed to be a constant fraction of the body vasculature space. The image, therefore, will represent the summation of the liver parenchyma and vascular spaces. Biliary radioactivity will be calculated separately from liver radioactivity. Because uptake of $^{99m}$Tc-MIBI from the spleen into the liver is extremely rapid and it is concentrated there, the efflux from the liver into the vasculature will be assumed to be negligible. If required, either 2-compartmental or 3-compartmental models will be used, depending on which model is most appropriate for the data based on Akaike’s criteria.

Data analysis and statistics. Change in hepatic radioactivity over time will be determined for each animal by outlining two regions of interest (ROI): one will be surrounding the liver and another will be surrounding the gallbladder. Each ROI will be expressed in counts per minute per pixel. The total radioactivity of the area will be determined by the scintigraphy computer software (Camstar, General Electrical Medical Systems, Milwaukee, WI) and will be adjusted for the natural physical decay of the radioisotope. The results will be displayed as time versus activity curves of $^{99m}$Tc-MIBI hepatic excretion.

In Phase 2 of the study, the data will be collected as described above for the other two groups of dogs, wild/mutant and mutant/mutant and areas under the curve (AUC) will be calculated by the trapezoidal method for each one of the three groups. The differences between the three groups (wild/wild, mutant/wild and mutant/mutant) will be tested for significance using a one-way ANOVA, a p-value of < 0.05 being considered statistically significant.

This study will give us valuable information about the role of P-gp in the hepatobiliary excretion of multiple drugs commonly used in canine patients. It will also represent a baseline of hepatobiliary excretion of $^{99m}$Tc-MIBI for future studies which could include dogs with inflammatory hepatic disease (failure) and hepatic tumors. In clinical practice, it is often assumed that dogs with liver disease have increased biologic half-life of drugs that are excreted and/or metabolized by the liver and for this reason their doses are often empirically decreased to avoid secondary toxic effects. Contrary to this assumption, in one study, mice with severe liver disease did not have altered $^{99m}$Tc-MIBI hepatobiliary excretion compared to normal mice (14). Future $^{99m}$Tc-MIBI hepatobiliary excretion studies could evaluate the hepatic excretion of drugs substrates of P-gp in dogs with liver disease and to determine if lower doses are required in order to prevent toxicity. Also, dogs with liver tumors may have increased
excretion of drugs due to the reported overexpression of P-gp in tumor cells. If this is the case, administration of higher doses of chemotherapeutic drugs substrate of P-gp should be considered in order to obtain the best therapeutic results.

This project makes part of the primary investigator’s Masters Project. We intend to present it at the 2008 American College of Veterinary Radiology (ACVR) Conference, in Chicago and to publish at least one article in the journal of Veterinary Radiology & Ultrasound and/or in the American Journal of Veterinary Research. The anticipated time table for Phase 1 of the study is three weeks with two dogs being studied per week.

E. Vertebrate Animals

IACUC approval will be obtained for this project. The dogs used in this study will be purchased as part of another research study conducted by Dr. Mealey.
F. Literature Cited

G. Budget

**Nuclear Scintigraphy**
The $^{99m}$Tc-MIBI will be ordered from the pharmaceutical company Cardial Health, Spokane, WA.

$^{99m}$Tc-MIBI: $109.00/ dose x 6 = $654.00
Nuclear Scintigraphy exam: $209 (1-30 mCi) x 6 = $1254
Total: $1908.00

**Radiography Abdomen** (2 view exam)
$82.50 x 6 = $495.00

**Abdominal Ultrasonography**
$143.00 x 6 = $858.00

**Blood Work:**
CBC $14.75 x 6 = $88.50
Total bilirubin $12.25 x 6 = $73.50
Small animal profile $37.50 x 6 = $225.00
Pre-prandial bile acids $24.25 x 6 = $145.50
Post-prandial bile acids $24.25 x 6 = $145.50
Urinalysis $ 12.25 x 6 = $73.50
Total: $751.50

**Anesthesia** (includes induction and 2 hours of anesthesia time)
Total = 137.50 x 6 = $825.00 (this value may be overestimated since not all dogs may require general anesthesia)

**Total:** $4837.50