In vitro effect of pimobendan on platelet aggregation in dogs

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Objective—To determine whether pimobendan has in vitro antithrombotic properties through inhibition of platelets in canine blood samples.

Animals—10 healthy adult dogs.

Procedures—Blood samples were collected from each dog into tubes containing hirudin or sodium citrate. Pimobendan was added to blood samples (final concentration, 0.0, 0.01, 0.1, 1.0, or 10.0µM) containing hirudin prior to undergoing collagen- and ADP-induced whole blood impedance aggregometry. Plasma thromboxane concentrations were measured after platelet aggregation. Pimobendan was also added to blood samples (0.0, 0.01, or 10.0µM) containing sodium citrate prior to thromboelastographic evaluation.

Results—Compared with findings for 0.0µM pimobendan, composite platelet aggregation (area under the curve [AUC]) and maximal platelet aggregation (aggregation units [AUs]) at 10.0µM pimobendan were significantly decreased for collagen-induced aggregation (AUC, 349.7 ± 58.4 vs 285.1 ± 72.2; maximal platelet aggregation, 196.2 ± 25.8 AUs vs 161.5 ± 38.0 AUs), and the AUC and velocity of aggregation at 10.0µM pimobendan were significantly decreased for ADP-induced aggregation (AUC, 268.5 ± 35.1 vs 213.4 ± 77.2; velocity of aggregation, 15.7 ± 2.9 AUs/min vs 11.8 ± 3.5 AUs/min). Pimobendan had no significant effect on plasma thromboxane concentration or thromboelastographic variables, regardless of concentration.

Conclusions and Clinical Relevance—In vitro, pimobendan had an antiplatelet effect in canine blood samples at a concentration 1,000-fold higher than that clinically achievable. These antiplatelet properties do not appear to contribute to the positive clinical profile of the drug in dogs. Pimobendan administration would not appear to confer a risk for bleeding and does not have to be avoided in dogs with thrombocytopenia or those concurrently receiving antiplatelet drugs. (Am J Vet Res 2013;74:403–407)

Pimobendan® is a calcium-sensitizing drug with PDE3- and partial PDE5-inhibiting properties that has been shown to improve survival time in dogs with congestive heart failure secondary to dilated cardiomyopathy and chronic valvular heart disease. The beneficial clinical effect of pimobendan has primarily been attributed to its positive inotropic and vasodilatory properties. Additional beneficial effects of pimobendan are not well characterized but may include neurohormonal modulation and platelet inhibition. Platelet inhibition can be achieved by targeting membrane receptors or altering intracellular signaling pathways. Cyclic adenosine 3’5’ monophosphate and cGMP are 2 secondary messengers that exert an inhibitory effect on platelet function. These cyclic nucleotides are regulated by a number of PDEs that hydrolyze the nucleotides into inactive metabolites. Medications that selectively inhibit PDEs may therefore have a beneficial effect by augmenting the platelet inhibitory effects of cAMP and cGMP. Pimobendan and other pyridazinone derivatives inhibit platelet aggregation. Published reports of studies evaluating this effect are limited, but in vitro data for humans have demonstrated a measurable antipla-
thromboxane synthesis. Additionally, a single ex vivo study in adult human males identified a possible anti-thrombotic effect of pimobendan that was not related to platelet inhibition. These limited data have been cited as a possible beneficial clinical effect of pimobendan in humans and dogs.1,8,9 Additionally, concern has been raised about the possibility that pimobendan could cause bleeding complications when administered concurrently with drugs that have antiplatelet properties or when administered to animals that are thrombocytopenic.

To our knowledge, there are no reports of studies evaluating the effect of pimobendan on the coagulation system in dogs. Therefore, the aim of the study reported here was to evaluate the in vitro effect of pimobendan on platelet aggregation and thromboelastographic variables in canine blood samples. We hypothesized that treatment of samples with pimobendan would result in a dose-dependent antiplatelet effect, reflected by reduced platelet aggregation and decreased TXB, release, with no measurable effect on the thromboelastographic profile.

Materials and Methods

Dogs—Ten healthy adult dogs (7 neutered males and 3 spayed females) were recruited for study participation from the staff of the Purdue University Veterinary Teaching Hospital. A physical examination was performed to ensure that the dogs were free of systemic disease; dogs had not been previously diagnosed with systemic disease and were not currently receiving medications other than heartworm prevention. Owners gave informed consent, and the study protocol was approved by the Purdue Animal Care and Use Committee.

Sample collection—The dogs were restrained manually in a gentle fashion to facilitate atrumatic blood sample collection. From each dog, blood was collected from an external jugular vein with a winged collection set10 fitted with a needle at the distal end. Once blood flow was evident, the first 2 mL of blood was discarded and then the distal needle was inserted into 2 tubes containing hirudin to a final concentration of 25 µg/mL and 1 tube containing 3.2% sodium citrate to a final concentration of 9 parts blood to 1 part citrate (vol/vol). The total volume of blood collected was approximately 11 mL. The samples were gently rocked and then stored at 22°C for 30 minutes prior to analysis. All measurements were performed within 2 hours after sample collection.

In vitro preparation—A stock solution of pimobendan was created by dissolving a 1.25-mg tablet in 50% methanol with a validated method.10 Serial dilutions of the stock solution with 50% methanol were used so that similar volumes of the pimobendan solution were added to each aliquot of blood used for analysis. For each dog, pimobendan was added to achieve final concentrations of 0.0 (ie, methanol only), 0.01, 0.1, 1.0, or 10.0µM in the aliquots of the blood sample containing hirudin and incubated for 5 minutes prior to whole blood aggregation. Pimobendan was also added to achieve final concentrations of 0.0 (ie, methanol only), 0.01, and 10.0µM in aliquots of the blood sample containing sodium citrate and incubated for 5 minutes prior to thromboelastographic evaluation.

Whole blood platelet aggregation—Platelet aggregation was performed with a multichannel whole blood impedance aggregometer3 according to the manufacturer’s recommendations. Irrigation saline (0.9% NaCl) solution11 was preheated to 37°C, and 300 µL was pipetted into single-use test cells containing duplicate impedance sensors and a polytetrafluoroethylene-coated magnetic stirrer. Aliquots (300 µL each) of pimobendan-spiked hirudin-containing blood were pipetted into the test cells, and aggregation was induced with 20 µL of collagen (final concentration, 3.2 µg/mL) or 20 µL of ADP (final concentration, 6.5µM). The impedance change was measured for 15 minutes. Measurements were performed in duplicate, and the computer software automatically reported the mean of each variable, including a composite of amplitude and time of platelet aggregation (determined as AUC, maximum amplitude of aggregation (determined as AUs), and velocity of aggregation (determined as AUs/min).

Thromboelastography—Thromboelastography was used to evaluate the effect of pimobendan on the global coagulation cascade in an attempt to determine whether pimobendan had an antithrombotic effect that was not caused by platelet inhibition. Calcium concentrations in the aliquots of pimobendan-spiked citrate-containing blood were returned to physiologic concentrations via the addition of 0.2M calcium chloride, and thromboelastography12 tracings were obtained without the use of any coagulation activator. Four variables were recorded for analysis: R-time (R), K-time (K), α-angle, and maximum amplitude. Native citrate-containing blood samples (ie, no methanol or pimobendan) and blood samples containing pimobendan at concentrations of 0.0 (ie, methanol only), 0.01, and 10.0µM were evaluated. Aliquots were evaluated within 2 hours after collection.

Assessment of plasma TXB2 concentration—Whole blood was recovered from the test cells after collagen- or ADP-induced platelet aggregation, and plasma was obtained via centrifugation (2,000 × g for 10 minutes at 22°C) to measure TXB2, concentration. All plasma samples were stored at –80°C for 1 month prior to analysis. Each sample was diluted (1:50) with ultrapure water, and TXB2 concentration was measured with a commercial ELISA kit according to the manufacturer’s recommendations. All plasma samples were analyzed in duplicate.

Statistical analysis—All statistical analyses were performed with commercial statistical software.1 Data in subgroups (classified on the basis of pimobendan concentration) for each platelet aggregation variable and plasma TXB2 concentration were evaluated for normality via the Shapiro-Wilk test. Assumptions of normality were not violated for AUC, maximum platelet aggregation, and TXB2, concentration. Data in each subgroup had normal distributions and differences among the groups were evaluated via repeated-measures ANOVA. Thromboelastography variables were compared via the Friedman test. Significance for all analyses was set at
Table 1—Mean ± SD values for platelet aggregation variables determined via collagen- and ADP-induced impedance aggregometry in blood samples collected from 10 healthy adult dogs and subsequently exposed to various concentrations of pimobendan.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pimobendan concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Collagen-induced platelet aggregation</td>
<td>AUC</td>
</tr>
<tr>
<td>Maximal platelet aggregation (AUs)</td>
<td>196.2±25.8</td>
</tr>
<tr>
<td>Velocity of aggregation (AUs/min)</td>
<td>17.2±4.9</td>
</tr>
<tr>
<td>ADP-induced platelet aggregation</td>
<td>AUC</td>
</tr>
<tr>
<td>Maximal platelet aggregation (AUs)</td>
<td>159.2±18.2</td>
</tr>
<tr>
<td>Velocity of aggregation (AUs/min)</td>
<td>15.7±2.9</td>
</tr>
</tbody>
</table>

Blood samples were collected from each dog into tubes containing hirudin; pimobendan (final concentration, 0.0, 0.01, 0.1, 1.0, or 10.0 µM) was added before sample aliquots (300 µL) underwent collagen- and ADP-induced impedance aggregometry (aggregation was induced with 20 µL of collagen [final concentration, 3.2 µg/mL] or 20 µL of ADP [final concentration, 6.5µM]). *Value is an outlier value (black circles), and whiskers define the range of values except when there were no data for given variables, mean values are significantly (P < 0.001, P = 0.001, P = 0.01, and P = 0.02) different, compared with the corresponding mean value for 0.0 µM pimobendan.

Results

Platelet aggregation—The mean ± SD values for collagen- and ADP-induced platelet aggregation were summarized (Table 1). Collagen-induced AUC (Figure 1) and maximal platelet aggregation were significantly reduced for 10.0 µM pimobendan, compared with findings for 0.0 µM pimobendan (P < 0.001 and P = 0.001, respectively). No significant changes in velocity of aggregation induced by collagen were detected among the 5 evaluated concentrations of pimobendan. The ADP-induced AUC and velocity of aggregation were significantly reduced for 10.0 µM pimobendan, compared with findings for 0.0 µM pimobendan (P = 0.01, respectively). No significant changes in maximal platelet aggregation induced by ADP were detected among the 5 evaluated concentrations of pimobendan.

Thromboelastography—The median and range for thromboelastography variables were summarized (Table 2). There were no significant differences in R, K, α-angle, or maximum amplitude among the 5 evaluated concentrations of pimobendan (0.0, 0.01, or 10.0 µM pimobendan). However, compared with findings for native blood (no methanol), there was a significant (P < 0.05) increase in R for 0.01µM pimobendan and a significant (P < 0.05) decrease in α-angle for both 0.0 and 0.01µM pimobendan.

Plasma TXB₂ concentration—The plasma TXB₂ concentrations in blood samples that had undergone collagen- or ADP-induced platelet aggregation were summarized (Table 3). No significant difference in plasma TXB₂ concentration was detected among the 5 evaluated concentrations of pimobendan.

Discussion

In the present in vitro study, the effect of pimobendan (at 5 concentrations) on platelet aggregation and thromboelastographic variables in canine blood samples was investigated. Results indicated that a significant reduction in platelet aggregation, compared with findings for 0.0µM pimobendan, occurred at the highest concentration of pimobendan (10.0µM) only. In addition, there was no evidence that pimobendan...
inhibited thromboxane release from activated platelets. It is important to note that 10.0µM pimobendan is approximately 1,000 times the mean peak plasma concentration of pimobendan achieved in dogs via clinical dosing protocols (0.01µM; 3.09 ng/mL).6,13 This finding is similar to that reported for human platelets, in that pimobendan appears to have a mechanistic effect on platelets but only at concentrations considerably higher than those clinically achievable.8,9,b

Thromboelastography revealed that there was a significant increase in R for blood samples containing 0.01µM pimobendan and a significant decrease in α-angle for blood samples containing 0.0 or 0.01µM pimobendan, compared with findings for native blood (no methanol), suggesting an antithrombotic effect. However, there was no significant difference in any thromboelastography variable among the 3 evaluated concentrations of pimobendan (0.0, 0.01, or 10µM pimobendan). Additionally, albeit not significant, the median R was almost doubled in all blood samples containing methanol, compared with the value for native blood samples, and all tracings were noticeably different from tracings obtained from native blood samples. This suggested that methanol, not pimobendan, was most likely responsible for the thromboelastographic changes.

Via thromboelastography, there appeared to be an effect of methanol on coagulation, but it could not be definitively determined whether methanol had an impact on platelet function because platelet aggregometry was not performed on canine blood samples that did not contain methanol. On the basis of reports of prior studies8,9 involving human platelets, pimobendan must be solubilized in methanol; thus, the presence of methanol to determine the effect of pimobendan on platelet function is required. In our opinion, methanol in the blood samples did not affect platelet function for multiple reasons. First, in the previous in vitro studies8,9 evaluating the effect of pimobendan on human platelets, methanol was present in the blood samples and the reports do not suggest that methanol had an effect on platelet function. Second, although methanol did have an effect on the thromboelastography tracings in the present study, it was limited to the enzymatic activity of the coagulation factors, and the maximal amplitude (a variable that is primarily associated with platelet function) was not altered. Third, the platelet aggregation values obtained for the blood samples containing 0.0µM pimobendan (methanol only) were comparable to the values observed in our laboratory for blood samples from other healthy dogs.

Phosphodiesterases are a family of isoenzymes that hydrolyze cyclic nucleotides, such as cAMP and cGMP, into inactive metabolites. Eleven PDEs have been identified, all with variable expression and distribution in mammalian cells.6,12,13 Treatments that target specific PDE isoenzymes can be used for the treatment of various diseases, including congestive heart failure, inflammatory airway disease, and pulmonary hypertension.1,12–14 Ancillary properties of PDE inhibitors are also being evaluated as potential therapeutic targets. For example, milrinone, a PDE3 inhibitor used for congestive heart failure in humans, has been shown to alter function of both human and equine platelets in vitro.8,15–17 Pimobendan is a PDE3 and partial PDE5 inhibitor that is commonly used for management of congestive heart failure in dogs because of its positive inotropic and vasodilatory properties.8,12 Previous in vitro studies10 involving human platelets have revealed a measurable pimobendan-associated antiplatelet effect, which has been cited as a possible beneficial clinical effect of this drug. Concentrations of pimobendan used

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### Table 2—Median (range) values for thromboelastography variables determined in blood samples collected from 10 healthy adult dogs and subsequently analyzed as native (untreated) blood samples or blood samples exposed to various concentrations of pimobendan.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Native blood (no methanol)</th>
<th>0.0µM pimobendan</th>
<th>0.01µM pimobendan</th>
<th>0.1µM pimobendan</th>
<th>1.0µM pimobendan</th>
<th>10.0µM pimobendan</th>
</tr>
</thead>
<tbody>
<tr>
<td>R (min)</td>
<td>2.2 (1.2–4.0)</td>
<td>4.0 (2.0–5.9)</td>
<td>6.2 (2.8–8.9)</td>
<td>4.9 (2.1–6.3)</td>
<td>6.2 (2.9–8.6)</td>
<td>6.2 (2.9–8.9)</td>
</tr>
<tr>
<td>K (min)</td>
<td>1.5 (1.3–2.0)</td>
<td>2.2 (1.8–3.0)</td>
<td>2.7 (2.3–3.5)</td>
<td>3.7 (2.8–4.5)</td>
<td>4.9 (3.2–5.8)</td>
<td>4.9 (3.2–5.8)</td>
</tr>
<tr>
<td>α-angle (°)</td>
<td>68.9 (54.8–74.4)</td>
<td>57.6 (36.5–63.6)</td>
<td>52.4 (32.9–71.9)</td>
<td>58.8 (54.3–65.2)</td>
<td>58.8 (54.3–65.2)</td>
<td>58.8 (54.3–65.2)</td>
</tr>
<tr>
<td>Maximum amplitude (mm)</td>
<td>65.4 (42.4–80.6)</td>
<td>60.9 (50.4–66.5)</td>
<td>51.3 (28.1–81.4)</td>
<td>59.4 (42.3–78)</td>
<td>59.4 (42.3–78)</td>
<td>59.4 (42.3–78)</td>
</tr>
</tbody>
</table>

Blood samples were collected from each dog into tubes containing sodium citrate; samples were treated with pimobendan (0.0, 0.01, or 10.0µM) and methanol (required for solubilization of pimobendan) or remained untreated. The presence of pimobendan (0.0, 0.01, or 10.0µM) and methanol did not affect platelet function for multiple reasons. First, in the previous in vitro studies8,9 evaluating the effect of pimobendan on human platelets, methanol was present in the blood samples and the reports do not suggest that methanol had an effect on platelet function. Second, although methanol did have an effect on the thromboelastography tracings in the present study, it was limited to the enzymatic activity of the coagulation factors, and the maximal amplitude (a variable that is primarily associated with platelet function) was not altered. Third, the platelet aggregation values obtained for the blood samples containing 0.0µM pimobendan (methanol only) were comparable to the values observed in our laboratory for blood samples from other healthy dogs.

### Table 3—Mean ± SD plasma TXB2 concentration (ng/mL) in blood samples collected from 10 healthy adult dogs and subsequently analyzed as native (untreated) blood samples or blood samples exposed to various concentrations of pimobendan and had undergone collagen- or ADP-induced impedance aggregometry.

<table>
<thead>
<tr>
<th>Agonist added to blood sample for aggregometry</th>
<th>Pimobendan concentration (µM)</th>
<th>0.0</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>111.1 ± 56.1</td>
<td>111.3 ± 56.9</td>
<td>103.1 ± 68.5</td>
<td>98.9 ± 43.4</td>
<td>84.4 ± 42.3</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>10.5 ± 4.9</td>
<td>10.2 ± 5.1</td>
<td>8.9 ± 4.6</td>
<td>8.5 ± 4.5</td>
<td>8.5 ± 5.2</td>
<td></td>
</tr>
</tbody>
</table>

Blood samples were collected from each dog into tubes containing hirudin; pimobendan (final concentration, 0.0, 0.01, 0.1, 1.0, or 10.0µM) was added before sample aliquots (100µL) underwent collagen- and ADP-induced impedance aggregometry (aggregation was induced with 20µL of collagen [final concentration, 3.2µg/mL] or 20 mL of ADP [final concentration, 6.5µM]).
in those studies\(^8,9\) ranged from 0.05 to 150µM, with an antiplatelet threshold concentration of approximately 1.0µM, which is approximately 100 times the mean peak plasma concentration of pimobendan achieved in dogs via clinical dosing protocols.

One limitation of the present study was that it evaluated the in vitro effect of pimobendan, similar to previous studies in humans. In dogs, pimobendan is metabolized in the liver to UDCCG-212, which is a more potent inhibitor of PDE3 than is the parent compound.\(^1\) One of the commonly cited in vitro studies\(^2\) involving human platelets evaluated both the parent compound and UDCCG-212 and found a dose-dependent decrease in platelet aggregation for concentrations of the metabolite from 0.05 to 10µM. However, those concentrations are also considerably higher than the mean peak plasma concentration of UDCCG-212 that is achieved in dogs (0.01µM; 3.66 ng/mL).\(^7\) In the human in vitro study,\(^9\) changes in single platelet counts were assessed, whereas in the present study, maximum platelet aggregation was compared. Therefore, the inhibitory threshold in the 2 studies may have been different as a result of different experimental methods or species variability. An ex vivo study of pimobendan in dogs may be more appropriate to determine whether UDCCG-212 has a clinical antiplatelet effect. Another limitation of the present study was that pimobendan was incubated with the platelets for a limited period of time (5 minutes). The exposure of pimobendan to platelets would be much longer in vivo, which could result in greater drug-platelet interaction. However, the 5-minute exposure was similar to that used in human in vitro studies\(^8,9\) that have been used to support the suggestion that pimobendan exerts an antiplatelet effect. Platelet counts were not evaluated prior to assessment of platelet aggregation. Therefore, all of the pimobendan concentrations were evaluated in aliquots of each collected blood sample. Therefore, any alteration in the platelet count should have affected each concentration in a similar manner and should not have affected comparisons of effects among concentrations. Lastly, blood samples were collected for evaluation from only 10 dogs, which could have resulted in an underpowered study. However, when the means of collagen- and ADP-induced AUC were compared for 0.0µM pimobendan and for 1.0µM pimobendan (the latter being the highest concentration of drug with no significant effect on platelet function) with an assumption of 80% power, sample sizes of > 150 and 131,000 would be required, respectively, to identify a significant effect on platelet function. Therefore, we believe that the data and conclusions regarding the effect of pimobendan on canine platelets derived from the present study are valid, despite the limited number of dogs in the study.

Results of the present study indicated that pimobendan exerts a mild in vitro inhibitory effect on aggregation of canine platelets at a concentration approximately 1,000 times the mean peak plasma concentration reportedly achievable in dogs. Therefore, it would appear that the beneficial clinical profile of pimobendan in dogs with congestive heart failure is not related to antiplatelet effects. Moreover, there was no evidence that bleeding complications or interactions with other antiplatelet drugs should be of concern when pimobendan is administered to dogs in a clinical setting.

References