A New Combination Therapy for Selective and Prolonged Antiplatelet Effect: Results in the Dog

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SUMMARY This study characterizes the effect of dazmegrel, a thromboxane synthetase inhibitor, on platelet function in the dog and introduces its potential use in combination with aspirin therapy. Ex vivo testing of dazmegrel alone was performed with three dosages and three administration regimens. Platelet aggregation response, malondialdehyde formation and prostaglandin metabolites generation were evaluated. To maintain complete thromboxane A2 inhibition, dazmegrel had to be given 3 times per day at dosages of not less than 6 mg/kg. The same result was achieved with a single daily administration of combined dazmegrel and aspirin in equal dosages of 3 mg/kg. Dazmegrel, both alone and with aspirin, increased and sustained heightened levels of prostacyclin, unlike the simultaneous inhibition of both prostaglandin metabolites seen with aspirin therapy alone. Because the combination of dazmegrel and aspirin effectively blocks thromboxane A2 formation and also enhances prostacyclin formation, the synergistic action of these agents increases their combined antiplatelet effect to a level not attainable by either agent alone. The significance of this combined therapy warrants further experimental study and may soon merit clinical trial for the prevention of stroke and other major thrombotic complications.

THE IMPORTANCE OF PLATELET ACTIVATION in occlusive vascular diseases has led to the development of several antiplatelet therapy protocols. In many clinical trials aspirin (ASA) has decreased the incidence of stroke and myocardial infarction.\textsuperscript{1,2} The non-selective, potent inhibition of high-dose ASA on the prostaglandin metabolites thromboxane A2 (TXA2) and prostacyclin (PGI2) caused investigators to suggest a theoretic advantage to the use of low-dose therapy.\textsuperscript{3,4} Preferred inhibition of pro-aggregatory TXA2 in humans has been limited to single-dose ASA administration\textsuperscript{5} or short-term cumulative effect.\textsuperscript{6}

Selective thromboxane inhibitors were introduced following the development of a better understanding of arachidonic acid metabolism.\textsuperscript{7,8} Thromboxane synthetase inhibitors (TSI) inhibit TXA2 and remarkably enhance the formation of anti-aggregatory PGI2, possibly by redirection of endoperoxide utilization.\textsuperscript{9-11} Therefore, combination therapy of low-dose ASA and TSI could provide an effective antithrombotic treatment which promotes and accentuates the desirable differential inhibition of these prostaglandins. This study evaluates the effect of the imidazole derivative dazmegrel (UK-38,485, Pfizer, Inc.) on platelet behavior and explores its therapeutic potential in combination with low-dose ASA.

Materials and Methods

Subjects and Data Analysis

Blood was drawn from the jugular veins of mongrel dogs which weighed 14 to 30 kg. Samples were anticoagulated with 3.8% sodium citrate.

To allow comparison of each study group, all data were standardized to the relative inhibitory effect, which was represented by the ratio of the difference between pretreatment and post treatment levels divided by the pretreatment value. Data were analyzed by use of a T test or one-way analysis of variance.

Animal care complied with the "Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 80-23, revised 1978).

Platelet Aggregation

Platelet aggregations were performed on a Payton dual channel aggregometer with an Omniscribe B-5000 chart recorder according to the turbidometric method of Born.\textsuperscript{12} Using autologous platelet poor plasma (PPP), the platelet count in platelet rich plasma (PRP) was adjusted to 250,000 ± 25,000 platelets/μl. Following a three-minute incubation period at 37°C, inducing agents were added to 0.45 ml of PRP and the ensuing aggregation was monitored. Inducing agents were adenosine diphosphate (ADP) in final concentrations of 5 and 10 μM and 100 μg collagen. Change in aggregation response was assessed by degree of inhibition with attention paid to the alteration of curve shape for ADP and lag time for collagen.

Malondialdehyde Formation

Using the modified method of Stuart,\textsuperscript{13} production of malondialdehyde (MDA), the stable metabolite of C17 hydroxyacid, was determined from 1 ml of PRP stimulated with 10 μl thrombin (500 NIH/ml). MDA generation was expressed as nmol/10\textsuperscript{9} platelets and measured as the optical density difference at 532 nm between thrombin- and saline-incubated samples (Spectronic 21, Bausch and Lomb). The range for pre-treatment values was 2.5 ± 1.5 nmol/10\textsuperscript{9} platelets.

Prostaglandin Metabolite Determination

Radioimmunoassays of thromboxane B2 (TXB2) and 6-keto-prostaglandin F1α (6-keto-PGF1α), the stable metabolites of TXA2 and PGI2, respectively, were per-
formed using commercially available kits from New England Nuclear. To control any nonspecific effects resulting from other cyclooxygenase split products, calibration standards were prepared with serum from blood samples exposed to indomethacin in concentrations which completely inhibited both metabolites. Under the conditions studied, binding of the radiolabeled tracer was approximately 50% with a 5 pg/0.1 ml limit of the metabolite detection. The cross-reactivity of other prostaglandin-derived products did not exceed 3%. The supernatant from the centrifuged 10 μM ADP platelet aggregation sample was retained for TXB₂ analysis. Serum samples for 6-keto-PGF₁α measurements were obtained from 5 ml of clotted venous whole blood after incubation at 37°C for 30 minutes and centrifugation for 15 minutes at 1,000 g. The range of baseline levels for TXB₂ was 430 ± 180 pg/0.1 ml; for 6-keto-PGF₁α, it was 210 ± 70 pg. All plasma and serum samples were kept frozen until analyzed.

The Effect of Dazmegrel on Platelet Function
In Vivo Single Administration Group
To study the inhibitory action of dazmegrel on platelet function in relation to dose requirements and duration of effect, three groups of five animals were tested. Single oral doses of 3, 6 and 12 mg/kg were administered. Blood samples for all subjects were obtained before drug administration, 2-3 hours afterward, and daily thereafter until the platelet response returned to baseline level. In the 6 and 12 mg/kg groups more frequent sampling was done on the first day of testing to establish when the peak inhibitory action of dazmegrel occurred.

In Vivo Daily Administration Group
To establish an adequate daily regimen that would maintain maximum inhibition of TXA₂ formation, two groups of six animals each were studied. The first three were given 6 mg/kg of dazmegrel every eight hours and the second three were given 12 mg/kg once per day. Animals in each group received dazmegrel for one week with monitoring before treatment, three times during treatment, and on the third day after the drug was discontinued.

The Effect of Combined Dazmegrel and ASA on Platelet Function
We tested a combination of 3 mg/kg ASA and 3 mg/kg dazmegrel; this investigation was prompted by our observation that the lowest tested dosage of dazmegrel selectively inhibited TXA₂ and enhanced PGI₂ formation, and we assumed that complete inhibition of TXA₂ would be accomplished by the established dose of ASA. Three mg/kg was found to be the minimum dose of ASA in this model for complete inhibition of TXA₂ production.¹⁸

In Vivo Single Administration Group
Two groups of six animals were studied. Each subject received 3 mg/kg of each drug. In the first group the ASA was given two hours after dazmegrel and in the second group drug administration was simultaneous. These regimens were chosen in order to assess any differences between the combination therapies with regard to enhancement of PGI₂ generation. For all subjects blood sampling was done prior to any drug ingestion, two hours after ASA administration, daily for the first four days following treatment, and again on the seventh day.

In Vivo Daily Administration Group
To investigate the potential of this combination therapy for maintaining a favorable in vivo TXA₂ and PGI₂ balance, a group of six animals was given 3 mg/kg of each drug for eight days. Blood samples were collected prior to drug administration, periodically during treatment, and on the third day after the therapy was discontinued.

Results
Evaluation of Dazmegrel's Effect on Platelet Function
In Vivo Single Administration Group
Data for animals included in this part of the study are represented in figures 1, 2a, and 2b. Inhibition of MDA and TXB₂ formation was significantly induced by 3 mg/kg and maximized by 6 mg/kg, with baseline recovery three days and one day later respectively. Although dazmegrel was found to be an effective inhibitor of TXB₂ formation, its blocking action lasted only about eight hours. However, 6-keto-PGF₁α production was dramatically enhanced for more than three days following treatment in each regimen tested. For all dosages tested, there was no significant difference in terms of the percent aggregation response for either ADP or collagen-induced reactions. However, the lag time of the collagen response was prolonged in the 6 and 12 mg/kg groups for up to six hours after treatment.

In Vivo Daily Administration Group
The results from the 6 mg/kg every eight hours and 12 mg/kg once per day study groups are presented in...
treatment level

**Figure 2.** The effects of single administration of dazmegrel on MDA formation and prostaglandin metabolites synthesis: a) 6 mg/kg; b) 12 mg/kg (mean ± SE); *p < 0.01 for the differences between post treatment and pretreatment levels.

As in the single administration study, platelet aggregability in both groups was unaffected following treatment. MDA formation was completely eliminated. To maintain maximum inhibition of TXB₂ and enhancement of 6-keto-PGF₁α, 6 mg/kg were required three times per day. For dogs given 12 mg/kg/day, dazmegrel was effective in enhancing 6-keto-PGF₁α formation, while only maintaining about 70% inhibition of TXB₂ production. In both study groups, baseline recovery for TXB₂ and MDA formation occurred about three days after cessation of treatment. However, 6-keto-PGF₁α enhancement was still present at this time.

**Evaluation of the Effect of the Dazmegrel and ASA Combination on Platelet Function**

**In Vivo Single Administration Group**

Data for the two groups given 3 mg/kg ASA and 3 mg/kg dazmegrel simultaneously and two hours apart are shown in figures 4a and 4b. Similar results were found for both groups with no apparent differences detected for the two methods of drug administration. ADP-induced platelet aggregations were inhibited ~40% (p < 0.01) with complete elimination of the secondary wave in subjects experiencing biphasic reaction patterns. The collagen response was affected only in terms of a prolonged lag phase. MDA generation was completely eliminated one day following treatment. TXB₂ formation was totally inhibited two hours after ASA administration with about 90% inhibition remaining the next day. The most important aspect of this combination therapy was the marked enhancement of 6-keto-PGF₁α formation accompanying TXB₂ inhibition following treatment.

**In Vivo Daily Administration Group**

The results for daily simultaneous administration of 3 mg/kg each of dazmegrel and ASA are shown in figure 5. Complete inhibition of both MDA and TXB₂ formation was observed during treatment. The inhibitory influence over platelet aggregability was similar...
to that shown in the single administration group and this initial effect lasted for the duration of therapy. Full baseline recovery for these platelet parameters was achieved three days following cessation of treatment. Again, the enhancement of 6-keto-PGF$_{1\alpha}$ generation was observed and remained present during therapy and for at least three days after it was discontinued.

**Discussion**

Both in vitro and in vivo experiments have shown that imidazole derivatives are highly selective inhibitors of thromboxane synthesis. A comparison of the relative inhibitory strength of seventeen derivatives on thromboxane synthetase from human platelets was done by Tai and Yuan. In vitro testing with thromboxane synthetase inhibitor UK-34787 showed inhibition of TXB$_2$ and MDA generation in human platelets with no apparent effect on platelet aggregation and release reaction. The first work that described administration of an imidazole derivative (UK-37,248-01) to man was performed by Tyler, et al who established that production of TXB$_2$ in serum was reduced in a dose-related manner. Our study also indicated that in vivo inhibition of TXB$_2$ in canine plasma was dose dependent and occurred soon after oral administration of dazmegrel.

It has been suggested that the pro-aggregatory and anti-aggregatory actions of TXA$_2$ and PGI$_2$ respectively play an important role in regulating thrombotic events. Because these products are derived from common intermediates, the possibility exists that one may be synthesized at the expense of the other. Dreyfus, et al reported that dazoxiben (UK-37,248) reduces TXA$_2$ production with simultaneous enhancement of PGI$_2$ generation in human whole blood after it was exposed to collagen. The same phenomenon was observed in leucocytes from angina patients and healthy volunteers. This redirection of prostaglandin metabolite synthesis was also demonstrated in vivo in humans and in several experimental animal models. Comparable results were also reported in different species after oral administration of the agent used in this study.

The endothelial lining of the vascular system and blood components, mainly leucocytes, may contribute to this PGI$_2$ increase. In a separate study we obtained tubular segments of aorta and jugular veins from controls and from dogs pretreated with ASA or dazmegrel; the harvested vessels were perfused with Gibco medium 199 in a closed circuit pump mechanism and 6-keto-PGF$_{1\alpha}$ levels in the medium were measured at five-minute intervals for 30 minutes. We found that inhibition of vascular PGI$_2$ from ASA-pretreated animals was dose dependent: 3 mg/kg induced complete inhibition. In the cases of dazmegrel pretreatment, PGI$_2$ generation was increased in comparison to the controls, implicating vascular endothelium as a source of this enhancement (unpublished data). It has also been demonstrated that monocytes possess a considerable capacity to synthesize PGI$_2$ and that during selective inhibition of thromboxane synthesis they have the ability to utilize platelet-derived endoperoxides.

Numerous publications have confirmed that ASA inhibits cyclooxygenase and prevents the formation of both TXA$_2$ and PGI$_2$. The inhibition of the latter is theoretically undesirable. Thromboxane synthetase inhibitors block TXA$_2$ formation and redirect the metabolism in favor of PGI$_2$ production. Evidence from in vitro studies has indicated that elimination of TXA$_2$ synthesis by these inhibitors does not modify platelet aggregation behavior but does increase the platelets' susceptibility to very low concentrations of ASA. The comparative evaluation of dazoxiben and ASA's effect on platelet function and prostaglandin metabolism in humans reported by Dale, et al corresponds with our results in dogs for separate administrations of dazmegrel and ASA. In earlier work with ASA, using ex vivo monitoring techniques similar to those described in this study, we established that ASA's inhibitory effect is dose dependent with parallel reduction of both prostaglandin metabolites. In the canine model the minimum dose for complete inhibition was 3 mg/kg. Our subsequent experience with combination dazmegrel and ASA in dogs for separate administrations of dazmegrel and ASA. In earlier work with ASA, using ex vivo monitoring techniques similar to those described in this study, we established that ASA's inhibitory effect is dose dependent with parallel reduction of both prostaglandin metabolites. In the canine model the minimum dose for complete inhibition was 3 mg/kg. Our subsequent experience with combination dazmegrel and ASA suggests that the maximum benefit of both drugs can be achieved by inhibiting the platelet aggregation response and TXA$_2$ synthesis while simultaneously enhancing PGI$_2$ formation. The prolonged TXA$_2$ suppression observed with the combination therapy can be attributed to the action of ASA. This also holds true for the observed reduction in platelet aggregability which cannot be achieved with dazmegrel alone. It has been shown that platelet aggregability assessed in vitro can serve as an index for ongoing thrombotic events. It is likely that the suggested antplatelet combination therapy possesses an antithrombotic potential superior to that of either drug alone. Despite the recognized species differences in platelet reactivity, these experimental studies appear to have clinical implications. However, testing to determine the long-term safety of dazmegrel needs to be addressed prior to clinical trials.

**References**


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