Biomarker Optimization to Track the Antithrombotic and Hemostatic Effects of Clopidogrel in Rats

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ABSTRACT

We determined the dose response of the ADP antagonist clopidogrel (0.3–50 mg/kg p.o.) in rat models of thrombosis and provoked bleeding and correlated these activities to ex vivo platelet activation. Carotid artery thrombosis was induced by FeCl₂. Bleeding time was measured by mesenteric vessel puncture and renal cortex or cuticle incision. Platelet biomarkers included standard ADP-induced aggregation, P2Y₁₂ receptor occupancy, and phosphorylation of vasodilator-stimulated phosphoprotein. Clopidogrel decreased thrombus weight up to 78%, caused maximal prolongation of cuticle and mesenteric bleeding, but had little effect on renal bleeds. Due to the steep dose-response curve, the half-maximal inhibitory dose (ED₅₀) for bleeding was 10.5 ± 3.4 mg/kg. Increased bleeding was intermediate (3-fold) at 10 mg/kg and maximal (6-fold) at 30 mg/kg. All biomarkers were affected, but with differing sensitivity. ED₅₀ for peak platelet aggregation to 10 μM ADP (11.9 ± 0.4 mg/kg) and the vasodilator-stimulated phosphoprotein index (16.4 ± 1.3 mg/kg) approximated the higher ED₅₀ for bleeding. ED₅₀ for ligand binding (3.0 ± 0.3 mg/kg) and late aggregation (5.1 ± 0.4 mg/kg) better matched the lower ED₅₀ for antithrombotic activity. Aspirin exerted lesser effects on bleeding (42–70% increase in all models) and thrombosis (24% inhibition). In summary, antithrombotic doses of clopidogrel have limited effects on bleeding and standard measures of platelet aggregation. Other biomarkers may be more sensitive for tracking antithrombotic efficacy.

The thienopyridine clopidogrel is an ADP receptor antagonist that, upon conversion to an active metabolite, irreversibly blocks the platelet P2Y₁₂ receptor (Savi and Herbert, 2005). The structure of the active metabolite and the identity of the platelet receptor for clopidogrel were not known before phase III clinical trials had been completed (CAPRIE Steering Committee, 1996). In fact, the P2Y₁₂ receptor was referred to as the thienopyridine receptor in the original publication that detailed its molecular identification (Hollopeter et al., 2001). The primary activity biomarker for tracking the effects of clopidogrel has been ex vivo inhibition of ADP-induced platelet aggregation (IPA) (Nguyen et al., 2005). It is well recognized that the standard 75-mg maintenance dose of clopidogrel reduces clinical cardiovascular events exerts a submaximal effect on ex vivo IPA (Thebault et al., 1999). The antiplatelet effect of clopidogrel in man requires oral bioavailability of parent drug, its bioactivation to the active metabolite, and productive blockade by that metabolite of platelet P2Y₁₂ receptors. Its effects will not prevent ADP-mediated platelet stimulation through P2Y₁ receptors. Our goal was to understand whether IPA is in fact a good predictor of clopidogrel’s efficacy and bleeding liability in an experimental animal setting. Aspirin was also studied as a comparator to better validate the experimental procedures employed.

The hypothesis that IPA is a robust biomarker of clopidogrel’s antithrombotic activity was addressed in dose-response studies conducted in rats that were orally dosed with clopidogrel for 3 days. These rats were anesthetized and subjected to either an acute thrombosis or provoked bleeding procedure or used to measure ex vivo platelet function. Occlusive thrombosis was induced in the carotid artery by topical application of iron chloride, an oxidative injury widely used in experimental rodent models (Leadley et al., 2000). Provoked bleeding times were measured in response to mes-
enteric vessel puncture, incision of the renal cortex, or cuticle incision. These bleeding models were selected because they had been found to be sensitive to aspirin and represent bleeding responses in a variety of vascular tissues. In addition to IPA, P2Y12 receptor occupancy (Baurand et al., 2000) and phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Geiger et al., 1999) were studied in the hope of calibrating these sensitive biochemical indicators of clopidogrel activity.

Materials and Methods

Chemicals. Clopidogrel (Plavix; Savi and Herbert, 2005) was synthesized by Sanofi-Aventis Company (Paris, France). Aspirin and MRS-2179 were obtained from Sigma (St. Louis, MO) and ADP from Chrono-Log Corp. (Havertown, PA). The vehicle for clopidogrel and aspirin was water (1 ml/kg). BMS-189664 (Das et al., 2002) was synthesized at Bristol-Myers Squibb (Pennington, NJ), and its vehicle was saline.

General Treatment and Surgical Procedure. Each procedure was approved by the Bristol-Myers Squibb Institutional Animal Care and Use Committee and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health. To study clopidogrel, male Sprague-Dawley rats (310–450 g) were dosed once daily for 3 days with a 1 ml/kg oral gavage of either vehicle or clopidogrel (0.3–50 mg/kg). These animals were not fasted. One hour after the final dose, they were anesthetized with Na-pentobarbital (50 mg/kg i.p.), and the trachea was intubated with polyethylene-205 tubing to ensure airway patency. Temperature was maintained with a warming table and/or heat lamp. A polyethylene-50 catheter was inserted into the left carotid artery in those animals selected for biomarker measurement. Other animals were subjected to the arterial thrombosis, venous thrombosis, cuticle bleeding time, mesenteric bleeding time, or renal cortex bleeding time procedure. Only one procedure was conducted on an animal, and none exceeded 90 min in total duration.

To study aspirin, the same surgical preparation was used except that aspirin or vehicle was administered as a 10 mg/kg infusion over 5 min into a polyethylene-50 jugular vein catheter at 10 to 15 min before inducing thrombosis or measuring bleeding time. The 10 mg/kg dose of aspirin was previously shown to cause maximal inhibition of platelet cyclooxygenase, as revealed by a >99% inhibition of thromboxane B2 generation during the clotting of rat blood (Schumacher et al., 1993).

Arterial Thrombosis. The right carotid artery was exposed and fitted with an electromagnetic flow probe attached to a model MDL 1401 flowmeter (Skalar, Delft, Netherlands) or a transit time Doppler probe attached to a T206 flowmeter (Transonic Systems Inc., Ithaca, NY). A piece of parafilm “M” (American National Can, Greenwich, CT) was inserted under the vessel and, following baseline flow measurements, a 2-× 5-mm strip of filter paper saturated with a 50% solution of FeCl2 was placed on top of the artery for 10 min. The carotid artery was dissected free 60 min after filter paper application and opened lengthwise to expose the thrombus, which was removed, blotted dry, and weighed on an AE50 balance (Mettler, Toledo, IN). Total blood flow was measured as an area under the curve (AUC) and normalized as percentage of baseline (0 min) flow over 60 min to provide a measure of integrated blood flow during thrombus formation.

Treatment groups consisted of vehicle (n = 14) and clopidogrel doses of 0.3 (n = 9), 1 (n = 8), 3 (n = 8), 10 (n = 8), 20 (n = 8) or 30 (n = 5) mg/kg. In separate experiments, aspirin (10 mg/kg, n = 6) or vehicle (n = 5) were tested.

Mesenteric Bleeding Time. The abdomen was opened via a midline incision, and the small intestine was exteriorized. The jejunum was exposed, held in place with clamps, and superfused with Ringer’s solution maintained at 37°C. Small arteries that branch perpendicular to the mesenteric artery and course over the surface of the jejunum were observed through an SZH10 stereomicroscope (Olympus Corp., Lake Success, NY). These vessels were punctured with a 30-gauge hypodermic needle, and the time in seconds from puncturing until bleeding stopped and remained stopped for 30 s was recorded. The maximal bleeding time recorded was 10 min. Bleeding times were determined in three to five animals. In this and the other two bleeding models, replicate bleeding times were averaged to single values for statistical comparison, and the number of bleeds that exceeded the maximal cut-off were also recorded.

Treatment groups consisted of vehicle (n = 12) and clopidogrel doses of 1 (n = 7), 3 (n = 5), 10 (n = 7), or 20 (n = 7) mg/kg. Aspirin (10 mg/kg, n = 6) or vehicle (n = 6) were tested in separate experiments.

Cuticle Bleeding Time. Toenails were cut with a single-edged razor blade at the location where the quick meets the nail. The cuticle was immediately superfused with Ringer’s solution maintained at 37°C, and the time until bleeding stopped and remained stopped for 30 s was recorded. The maximal bleeding time recorded was 15 min. Three replicate bleeding times were determined on the left hind paw.

Treatment groups consisted of vehicle (n = 12) and clopidogrel doses of 1 (n = 8), 3 (n = 8), 10 (n = 10), 20 (n = 10), or 30 (n = 6) mg/kg. Aspirin (10 mg/kg, n = 6) or vehicle (n = 6) were also tested.

Renal Cortex Bleeding Time. The left kidney was exposed by a midline abdominal incision, and the renal capsule was removed to expose the bare renal cortex. The cortex was superfused with Ringer’s solution maintained at 37°C, and controlled incisions were made with a Surgicutt adult template device (International Technidyne Corp., Edison, NJ). This produced a 5-mm-long × 1-mm-deep cut with the tip of a spring-loaded surgical blade (25, sharp tip). While observing under 3× binocular magnification, the time in seconds from injury until bleeding stopped and remained stopped for 30 s was recorded. The maximal bleeding time recorded was 12 min.

Treatment groups consisted of vehicle (n = 5) and clopidogrel doses of 1 (n = 5), 3 (n = 5), 10 (n = 5), 20 (n = 5), or 50 (n = 6) mg/kg. Aspirin (10 mg/kg, n = 7) or vehicle (n = 6) were also tested.

When the bleeding effect of clopidogrel appeared limited in this model, the incision was increased to 3 mm deep × 10-mm long using the Surgicutt blade adapted to a manual template device. Treatment groups for this deep cut included 20 mg/kg clopidogrel (n = 6) and its vehicle (n = 6), and thrombin inhibitor BMS-189664 (2 mg/kg loading plus 6 mg/kg/h sustaining i.v. infusion, n = 7) and its vehicle (n = 7). BMS-189664 was also tested at the same dose with the standard incision (n = 5). BMS-189664 was found previously effective in the primate version of this model (Das et al., 2002), and the dose was selected to produce a 5.6-fold increase in the activated partial thromboplastin time. This represents a near-maximal systemic anticoagulant effect.

Preparation for Ex Vivo Biomarkers. A 5- to 8-ml blood sample was drawn from the carotid artery or in some cases from the abdominal vena cava into a plastic syringe containing a 1/10 volume of 3.8% sodium citrate. Whole-blood samples were used for VASP assay, whereas platelet-rich plasma (PRP) was prepared for platelet aggregation and receptor binding assays. Whole blood sampled for the receptor binding assay also contained apyrase (0.25 U/ml final concentration; Sigma catalog no. A-6132).

There were two separate studies. In the first study ex vivo platelet aggregation responses to 1, 3, and 10 µM ADP were determined in rats treated with either vehicle (n = 9) or clopidogrel at doses of 1 (n = 6), 3 (n = 5), 10 (n = 5), 20 (n = 5), or 30 (n = 5) mg/kg. In the second study, additional biomarkers of VASP phosphorylation and ligand binding were added to the measurement of 10 µM ADP-induced platelet aggregation using an expanded range of clopidogrel doses (0.3, 1, 3, 10, 20, 30, and 50 mg/kg with n = 6 per dose, including six vehicle-treated rats).
Platelet Aggregation. Platelet count in the PRP was determined on a System 9000 cell counter (Seronco-Baker Diagnostics, Allentown, PA) and adjusted with platelet-poor plasma to obtain $-4.0 \times 10^5$ platelet/$\mu$L. Aggregation responses of PRP were measured by standard photometric technique at 37°C in a model 540 or 490 aggre-gometer (Chrono-Log Corp.). Light transmission through PRP and platelet-poor plasma was set at 0 and 100%, respectively. In the first study, maximal percent aggregation (peak response) observed within 5 min after addition of 1, 3, or 10 $\mu$M ADP (Chrono-Log Corp.) was recorded on a 707 chart recorder (Chrono-Log Corp.). In a second study, the optical signal was digitized and analyzed using aggregor/link software version 4.75 (Chrono-Log Corp.) with ADP tested at 10 $\mu$M concentration. In addition to the peak aggregation re-
sponse, AUC, and percent aggregation measured at 5 min after ADP addition (late response) were measured to capture the ability of clopidogrel to enhance the reversibility of platelet aggregation.

Ligand Binding. Approximately 5 to 7 ml of PRP obtained from citrate-anticoagulated blood containing low-level apprarse (0.25 U/ml; Sigma catalog no. A-6132) was incubated with gentle agitation at 37°C with an additional 2.5 U/ml apprise for 10 min. The sample was then diluted to 15 ml with buffer A (145 mM NaCl, 5.5 mM dextrose, 0.1 mM MgCl$_2$, 5 mM KCl, 15 mM HEPES, pH 7.4, 5 mM EDTA) containing a final concentration of 0.5 $\mu$M prostaglandin E$_1$ (Sigma catalog no. P-5515). The diluted PRP sample was then centrifuged at room temperature (1000g for 10 min), supernatant was discarded, and the pelleted platelets were washed once in 15 ml of assay buffer (145 mM NaCl, 0.1 mM MgCl$_2$, 5 mM KCl, 15 mM HEPES, pH 7.4, 5 mM EDTA) containing 0.25 U/ml apprise and 0.5 $\mu$M prostaglan-
din E$_1$. The sample was then centrifuged (1000g, room temperature, 10 min), the supernatant was again discarded, and the pellet was re-
suspended in 5 ml of assay buffer. Platelet counts were then deter-
mined on a System 9000 cell counter (Seronco-Baker Diagnostics) and were generally 0.2 to 0.4 $\times 10^5$/µl.

Binding reactions were conducted in 96-well filter plates (Milli-
pore Corporation, Billerica, MA; catalog no. MABVN0B50) prewet with 200 µl of assay buffer. Reactions consisted of 50 $\mu$l of the platelet preparation ($-10^7$ platelets/reaction), 12.5 $\mu$M MRS-2179 (Sigma catalog no. M-3808) (an amount that had been previously shown to provide complete P2Y1 receptor block under these condi-
tions), and 1 $\mu$M 2MeS-ADP ($-2000$ Ci/mol; Perkin-Elmer cus-
tom synthesis) in 200 $\mu$l of assay buffer. Binding was allowed to progress for 1 h at room temperature, and the platelets were then separated from the reaction by filtration and washed three times with 200 $\mu$l of ice-cold phosphate-buffered saline. Plates were al-
lowed to air dry, 100 $\mu$l of scintillation fluid was added (Ultima Gold; PerkinElmer Life Sciences, Boston, MA; catalog no. 6013329), and the residual 2MeS-ADP binding was determined by scintillation counting. Total binding (0% receptor occupancy) was determined from vehicle-treated rats and generally corresponded to $-10,000$ cpm/$10^7$ platelets. Nonspecific binding (100% occupancy) was deter-
mained from the same vehicle-treated samples containing 12.5 $\mu$M cold 2MeS-ADP (Sigma catalog no. M-152) and generally corre-
sponded to $-200$ cpm/$10^7$ platelets.

VASP Assay. The VASP-239 phosphorylation state was determined in citrate-anticoagulated blood samples using the PLT VASP/ P2Y$_12$ kit (Biocytec, Marseille, France). Samples were treated ac-
cording to the protocol included with the kit. In brief, whole blood was incubated with either the PGE$_1$ or PGE$_2$ plus 10 $\mu$M ADP solutions provided with the kit for 10 min at room temperature. The exact composition of these reagents was not available from the manu-
ufacturer. Reacted blood samples were treated with fixative contain-
ing paraformaldehyde. Platelets were permeabilized and treated with either an anti-VASP-P mouse monoclonal antibody (16C2) or the negative isotypic control provided with the kit, and samples were then treated with a polyclonal antibody (anti-mouse IgG-FITC) and a platelet counterstaining reagent-PE. The platelet counterstain did not react efficiently with rat platelets. Reactions were immediately analyzed on a Becton Dickinson (San Jose, CA) FACSCalibur Flow Cytometer. The platelet population was identified by the forward and side scatter distributions, and 5000 platelets were gated and collected. Data from the negative isotypic control was used to correct the mean fluorescent intensities (MFIs) of PGE$_1$ (MFIcPGE$_1$) and PGE$_2$ combined with ADP (MFIcPGE$_2$-ADP) samples containing the anti-VASP-P antibody. All blood samples were treated and ana-
alyzed within 4 h of sampling. A platelet reactivity index was calcu-
lated as outlined in the kit using the corrected mean fluorescence intensities in the presence of either PGE$_1$ alone or PGE$_2$ plus ADP using the following formula: platelet reactivity index $= [\text{MFIcPGE}_1 - \text{MFIcPGE}_2 - \text{MFIcPGE}_2^{\text{ADP}}]/\text{MFIcPGE}_2^{\text{ADP}}] \times 100$.  

Statistical Analysis and Potency Calculation. Differences in bleeding time between aspirin or BMS-189664 and respective vehicle were determined by Student’s $t$ test. Clopidogrel effects on thrombus weight, baseline carotid blood flow, ex vivo biomarkers, and renal cortex bleeding time were determined by analysis of variance with Dunnett’s test for comparison with the vehicle group. Due to vari-
ability in baseline carotid blood flows, this parameter was added as a covariate in the analysis of variance for integrated carotid blood flow during thrombosis. Contrasts were used for comparisons be-
tween clopidogrel doses and in the covariate analysis. Mesenteric and cuticle bleeding time data were analyzed by Kruskal-Wallis nonparametric analysis of variance and the Mann-Whitney $U$ test to detect differences in vehicle and clopidogrel bleeding times and rel-
ative change in bleeding time. This nonparametric analysis was applied to mesenteric and cuticle bleeding times because off-scale bleeds in clopidogrel-treated rats rendered the data noncontinuous. The Fisher’s exact test was used to compare frequency of occlusion data between treatment groups in arterial thrombosis and the pro-
portion of animals that exceeded maximal recorded bleeding time. Computations were performed using Systat Version 11.00.01 (Systat Software, Inc., San Jose, CA). Data are presented as mean ± S.E.M. $p < 0.05$ was considered significant.

The clopidogrel dose calculated to produce a half-maximal re-
sponse in each experimental model (ED$_{50}$) was determined by this four-parameter logistic fit employing the Levenburg Marquardt al-
gorithm: $y = A + (B - A)/(1 + ((C/x)D))$, where $x$ is clopidogrel dose, $y$ is percentage of maximal response, $A$ and $B$ are minimal and maximal $y$ value set to 0 ($A$) and 100 ($B$), $C$ is log ED$_{50}$, and $D$ is slope factor. The nonlinear curve fitting calculations were performed using XLfit (ID Business Solutions Ltd., Guildford, UK). Significant differ-
ences in ED$_{50}$ potency values were determined by lack of overlap in the 95% confidence intervals.

Results

Inhibition of Thrombus Formation. Clopidogrel pro-
duced a dose-dependent inhibition of arterial thrombosis (Fig. 1) with a threshold-detectable 45% decrease in throm-
bus weight at the 3 mg/kg dose. The ED$_{50}$ of 2.4 mg/kg was close to this threshold effect as shown in Table 1. The max-
imal 78% decrease in thrombus weight was achieved at the highest clopidogrel dose of 30 mg/kg. This level of efficacy is near maximal for antiplatelet or anticoagulant drugs in our standard FeCl$_2$-induced injury model. The 10 mg/kg dose of clopidogrel decreased arterial thrombus weight by 60% and was the lowest dose capable of maintaining carotid blood flow at 100% of the baseline level throughout the experiment. This 10 mg/kg dose also prevented occlusion in every rat. Higher clopidogrel doses did not produce significantly greater improvement in any of these parameters; therefore, the 10 mg/kg dose was considered the threshold maximally effective dose for the arterial thrombosis model.

Preservation of blood flow was an important component of antithrombotic activity, and all clopidogrel doses that sig-
ficantly decreased thrombus weight also improved integrated
blood flow and the proportion of patent vessels (Fig. 1). However, these flow-related parameters offered no clear advantage over thrombus weight reduction in dose-response analyses. Thrombus weight reduction was therefore selected as our primary measure of antithrombotic activity.

Aspirin produced a near-threshold antithrombotic effect with a 32 ± 12% reduction in thrombus weight comparing the vehicle (5.6 ± 0.5 mg, n = 5) and aspirin (3.8 ± 0.7 mg, n = 6; p = 0.068) groups. This result was reminiscent of three previous studies where decreases in thrombus weight of 9, 26, and 36% were observed comparing the same aspirin dose with vehicle. None of these thrombus reductions were significant. In view of this repeated and limited response to aspirin, we combined the four studies into a single meta-analysis achieving n = 30 to 33 per treatment group (Fig. 2). In the analysis of this combined study, aspirin treatment decreased thrombus weight by 24 ± 6%, improved average flow during thrombosis by 61 ± 16%, and maintained patency in 33% of the vessels compared with a 100% occlusion rate in vehicle-treated rats. These antithrombotic effects of aspirin were all significant.

Bleeding Time Prolongation. The 10 mg/kg dose of clopidogrel increased both mesenteric and cuticle bleeding times by a moderate ~3-fold (Fig. 3). Renal cortex incisions were practically unaffected by all but the highest 50 mg/kg dose of clopidogrel, where a 40% (i.e., 1.4-fold) increase in bleeding time was observed. The ED50 for prolonging bleeding time was 10 mg/kg (Table 1). Higher doses, which provided little additional benefit in preventing arterial thrombosis, resulted in off-scale bleeding upon severance of cuticle blood vessels at 30 mg/kg. The off-scale bleeding times were established in each model based on the Fisher’s exact test, by contrasts. The Fisher’s exact test was used for vessel patency. Thrombus weight reductions averaged 45 ± 17, 60 ± 12, 67 ± 5, and 78 ± 5% at clopidogrel doses of 3, 10, 20, and 30 mg/kg, respectively. Data are mean ± S.E.M.* p < 0.05 compared with vehicle.

Fig. 1. Clopidogrel inhibits arterial thrombosis. Vehicle or clopidogrel was dosed orally once daily for 3 days, and thrombosis was induced by topical application of FeCl3 to the carotid artery ~1 h after the last dose. Significant differences in baseline flow, thrombus weight, and integrated average blood flow (area under flow curve) were determined by analysis of variance. Although baseline flow did not differ between any clopidogrel dose and vehicle, the overall variation in this parameter among treatments was nearly significant (p = 0.072); therefore, baseline flow was included as a covariate in the analysis of integrated blood flow. Mean differences were detected by either Dunnett’s test, or in the case of the covariate analysis, by contrasts. The thrombin inhibitor was used as a positive control for this model. The thrombin inhibitor BMS-189664 produced similar relative increases for both

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Clopidogrel Potency ED50 (mg/kg) ± S.E.M.</th>
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</thead>
<tbody>
<tr>
<td>Arterial thrombosis (thrombus weight reduction)</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Platelet P2Y12 receptor (ligand displacement)</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Platelet aggregation (10 μM ADP, 5-min late response)</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>Platelet aggregation (10 μM ADP, AUC)</td>
<td>9.4 ± 0.1*</td>
</tr>
<tr>
<td>Platelet aggregation (10 μM ADP, peak response)</td>
<td>11.9 ± 0.4*</td>
</tr>
<tr>
<td>Platelet VASP (phosphorylation index)</td>
<td>16.4 ± 1.3*</td>
</tr>
<tr>
<td>Mesenteric bleeding time (~fold increase)</td>
<td>10.4*</td>
</tr>
<tr>
<td>Cuticle bleeding time (~fold increase)</td>
<td>10.5 ± 3.4*</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with ED50 for arterial thrombosis.
* Responses for each parameter (data from Figs. 1, 3, and 6) were converted to percentage of maximal effect in relation to the vehicle response. The clopidogrel dose projected to produce half maximal response was calculated from these normalized data using the curve fitting algorithm described under Materials and Methods.

**Sheepness of dose-response precluded accurate error estimation.

**Table 1**

Potency of ex vivo biomarkers of ADP reactivity compared with in vivo responses in clopidogrel-treated rats
standard incisions (4.49- ± 0.69-fold from 70 ± 3 to 313 ± 47 s, n = 5; p < 0.05) and deep incisions (4.03- ± 0.64-fold from 159 ± 10 to 616 ± 74 s, n = 7; p < 0.05).

In each model, the 10 mg/kg dose of aspirin significantly extended bleeding time, but the effect was less than a doubling. The -fold increases in bleeding time were 1.46 ± 0.28 (n = 6) for mesenteric bleeding, 1.42 ± 0.10 (n = 6) for renal cortex bleeding, and 1.70 ± 0.28 (n = 7) for cuticle bleeding (all p < 0.05). There were no off-scale bleeds in any aspirin-treated rat.

Inhibition of Platelet Aggregation. In the first platelet function study, inhibition of ADP-induced aggregation by clopidogrel was dependent upon ADP concentration over the range of 1, 3, and 10 μM ADP (Fig. 4). Increasing ADP to 20 μM did not produce additional aggregation of rat platelets, so 10 μM ADP was set as the top agonist challenge (not shown). Although clopidogrel was most effective against 1 μM ADP, aggregation evoked by this concentration of agonist was highly reversible (Fig. 4, inset), and repeated studies in our lab have characterized this response as poorly reproducible. For the purpose of tracking in vivo effects, a more intermediate ADP concentration provided both reproducibility and sensitivity to clopidogrel. The optimal selection of agonist concentration was best revealed by plotting the relative antithrombotic and hemostatic effects of clopidogrel as a function of ex vivo inhibition of platelet aggregation at each ADP concentration (Fig. 5). By this analysis, the 10 mg/kg optimal antithrombotic dose of clopidogrel was at the midpoint of
platelet function inhibition against 3 μM ADP. The corresponding bleeding time effect was a 3-fold increase, or about half-maximal. The 3 μM concentration of ADP, therefore, provided the broadest dynamic range of IPA for tracking both efficacy and bleeding liability. Interestingly, a significant antithrombotic effect was still observed with only 18% inhibition of aggregation to 3 μM ADP, and maximal effects on bleeding time were only achieved with near complete inhibition of this aggregation response. For this reason, a second series of experiments were conducted to evaluate additional biomarkers of platelet activation with the intent of obtaining greater sensitivity in tracking in vivo activity.

Effects on VASP and Ligand Binding Compared with Platelet Aggregation. In these experiments, simultaneous ex vivo platelet measurements were made of ADP-induced aggregation, ligand binding to the P2Y<sub>12</sub> receptor, and phosphorylation of VASP. Clopidogrel dosed from 0.3 to 50 mg/kg produced maximal effects of ≥88% inhibition for all indexes of platelet activation (Fig. 6). In the first IPA study, 10 μM ADP had provided the strongest challenge for clopidogrel inhibition; therefore, this stimulus was set as the target for improving IPA. Measuring platelet aggregation by AUC and at 5 min after agonist challenge (late response) were two simple approaches to increase IPA sensitivity. Clopidogrel might be expected to convert stable aggregation to reversible aggregation as is typically seen at lower ADP concentrations (Fig. 4, inset). Both AUC and late aggregation are indicators of reversible aggregation. The resulting ED<sub>50</sub> values (Table 1) revealed a minimal sensitivity advantage of the AUC measure but a ~2-fold improvement by using late aggregation. The VASP index, which has been characterized as a relevant P2Y<sub>12</sub> effector response (Grossmann et al., 2004), offered no advantage over IPA in tracking platelet activation. Receptor occupancy was also a sensitive biomarker of clopidogrel's platelet effects because it matched the ED<sub>50</sub> for antithrombotic activity.

The relative antithrombotic and hemostatic activities of clopidogrel were plotted as a function of platelet biomarker inhibition to better visualize the correspondence with in vivo activity (Fig. 7). In this graphical representation, the preferred biomarker would show equal separation between clopidogrel doses over the full dynamic range of the biomarker response and do so with no plateau in the response. Optimally, there would also be good sensitivity to the 1 and 3 mg/kg doses of clopidogrel that produced threshold antithrombotic effects. The result for the VASP assay and the most sensitive IPA measurement (late aggregation) involved overlapping of responses to clopidogrel doses at the high or low ends of the efficacy dose response. The ligand binding assay showed good separation of clopidogrel doses over the full dose response for antithrombotic activity.

There was a proportionately lesser effect on bleeding than on thrombus formation within the midrange of the dose-response for each biomarker (Fig. 7). However, the effects on both bleeding and thrombosis were maximal at clopidogrel doses that produced full inhibition of each biomarker. Efficacy and bleeding liability were separated at the intermediate 10 mg/kg dose of clopidogrel, but this separation disappeared at the 30 mg/kg dose.

Discussion

In rats, clopidogrel produced near-complete effects on hemostasis, arterial thrombosis, and multiple indicators of
platelet activation. This contrasts to the weaker effects of aspirin, where threshold-detectable increases in bleeding time and thrombus weight reduction were seen. The partial activity of aspirin was not due to limited inhibition of platelet cyclooxygenase, which was completely blocked by the 10 mg/kg dose. The weaker effect of thromboxane A$_2$ receptor activation compared with ADP-induced aggregation in rat platelets compared with human platelets (Schumacher et al., 2005). Nevertheless, the strong effects of P2Y$_{12}$ antagonists in rats make them ideally suited to investigate the relationship between in vivo effects and ex vivo biomarkers. Unlike humans, the risk/benefit of pushing IPA to full effect can be easily studied in rats.

The standard for assessing clopidogrel activity ex vivo remains IPA, using the peak or maximal platelet aggregation to quantify the response (Cattaneo, 2006). Choice of ADP concentration has a direct impact on clopidogrel sensitivity. In rats, reversible aggregation to 1 μM ADP was too variable for reliable use. Full aggregation to 10 μM ADP was the least sensitive to inhibition, whereas 3 μM ADP offered a balance of reproducibility and sensitivity. The 10 mg/kg clopidogrel dose inhibited peak aggregation to 3 μM ADP by 49%, decreased thrombus weight by 60%, and provided maximal preservation of blood flow. Higher clopidogrel doses offered marginal improvements in antithrombotic effect despite having a much greater impact on IPA. Higher doses also revealed a bleeding liability. Mesenteric and cuticle bleeds were half-maximal at 10 mg/kg, whereas higher doses could elicit maximal bleeds. Using an intermediate IPA to 3 μM ADP, we identified the 10 mg/kg clopidogrel of having the most favorable balance between efficacy and safety.

However, low IPA measured in response to 3 μM ADP was also meaningful. The subthreshold 18% IPA achieved with the 3 mg/kg clopidogrel dose decreased thrombus weight by 45% and doubled the bleeding times. This suggests that standard IPA with 3 μM ADP was insensitive for identifying threshold antithrombotic activity. Standard IPA also did not produce a graded response in tracking in vivo activity over the full range of clopidogrel doses, opening the opportunity to improve or replace IPA with more sensitive measures.

We first sought an IPA measure having an improved potency against the strong 10 μM ADP stimulus. It has been observed in human PRP that clopidogrel reduces the stability of platelet aggregates, leading to augmented reversal of optical aggregation (Labarthe et al., 2005). Destabilization of aggregation results in diminished late aggregation, which can be measured as percent light transmission at 5 min after agonist challenge or as an AUC of the light transmission record. The result was a 2-fold decrease in the ED$_{50}$ for late aggregation compared with peak aggregation. It is tempting to speculate that destabilization of platelet aggregates contributes to the antithrombotic and bleeding effects of P2Y$_{12}$ antagonists. In this regard, clopidogrel was found to destabilize thrombi rather than limit initial thrombus formation in an in vitro perfusion system using human blood (Phillips et al., 2005).
Activation of the G1-coupled P2Y12 receptor by ADP leads to both reduced cAMP formation and the associated dephosphorylation of VASP. Platelet fibrinogen receptors required for platelet aggregation are negatively regulated by phosphorylated VASP, and P2Y12 activation augments aggregation by releasing this inhibition. Clopidogrel inhibits platelet aggregation in part by limiting the ADP-mediated dephosphorylation of VASP in response to cAMP-elevating stimuli (especially PGE2). This formed the basis for the original VASP assay (Geiger et al., 1999), which was subsequently modified to track VASP phosphorylation by flow cytometry (Aleli et al., 2005). The VASP assay has been used in small clinical studies to quantify responsiveness of patients to clopidogrel therapy (Grossmann et al., 2004). However, in our experiments, clopidogrel effects on the VASP index were no better and slightly less sensitive to clopidogrel than standard IPA. It is possible that the coupling between receptor and effector responses, such as VASP phosphorylation, differs between rodents and man. In comparing the proportionate effect on VASP and receptor binding (Fig. 6), it would appear that in rats, blockade of ~90% P2Y12 receptors is required to produce a ~50% effect on the VASP index.

The irreversible binding of clopidogrel to P2Y12 receptors offers the opportunity for an ex vivo binding assay based on the readily available radioligand ([33P]2MeS-ADP). Because this antagonist binds to both P2Y1 and P2Y12 receptors (Baurand et al., 2000), an excess of P2Y1 antagonist was added to confer selectivity to P2Y12 receptors. In rats, clopidogrel (20 mg/kg) achieved 96% inhibition of [33P]2MeS-ADP-specific binding under these conditions. Ligand binding was a sensitive measure of platelet inhibition by clopidogrel with an ED50 equal to the antithrombotic ED50, but it was only marginally superior to the late aggregation response. Although binding showed significant activity over the entire clopidogrel dose-response range for both antithrombotic and bleeding effects, as an ex vivo biomarker, it is only applicable to irreversible antagonists like clopidogrel. Its ability to replace modified IPA assays is therefore limited and would be best suited to comparing compounds of similar mechanism.

Regardless of the biomarker selected, a proportionately greater effect on efficacy over bleeding time prolongation was observed at all but the highest levels of biomarker inhibition. In general, inhibition of arterial thrombosis was well matched to P2Y12 ligand binding, whereas increased cuticle bleeding time tracked well with either VASP index or inhibition of peak (maximal percentage) platelet aggregation (Fig. 8).

ADP-induced platelet activation via the P2Y12 receptor must differentially affect thrombogenesis in the microcirculation (hemostasis) compared with large blood vessels based on our results. Failure of clopidogrel to destabilize thrombi at microcirculatory bleeds might contribute to its decreased potency for affecting hemostasis compared with thrombosis (van Gestel et al., 2003). Off-scale bleeding in mesenteric or cuticle models required clopidogrel doses (10 and 20 mg/kg) that had maximal effects on the most sensitive biomarkers. Other bleed models may be less sensitive. Depending on methodology, a 20 mg/kg dose of clopidogrel produced either off-scale bleeds (Herbert et al., 1993) or no effect (Daykin et al., 2006) on rat tail bleeding time. In the renal cortex bleeding time, only the highest clopidogrel dose (50 mg/kg) had a significant effect in matching the threshold activity of aspirin. When the depth of the renal incision was increased, the 20 mg/kg dose of clopidogrel produced a significant yet modest 44% increase in bleeding time. We have also found renal cortex bleeds insensitive to the combination of a P2Y1 antagonist (MRS-2500) and clopidogrel at maximal IPA doses, whereas anticoagulants (heparin, thrombin inhibitors, and factor Xa inhibitors) have produced bleeding time increases of 2- to 5-fold (W. A. Schumacher, unpublished data). In the current study, the thrombin inhibitor BMS-189664 was highly effective in prolonging bleeding in response to renal injury, suggesting that thrombin generation is a most important contributor to hemostasis in the rat renal cortex. The discrepancy in tail bleeds and the limited effects of clopidogrel on renal bleeds suggest that the microcirculation of different tissues vary in sensitivity to P2Y12 antagonism. Although the applicability of these results in other species remains to be investigated, it is clear that vascular bed selection is crucial when assessing hemostatic liability.

In summary, the study of clopidogrel in these rat models supports paying careful attention to biomarker selection when attempting to predict in vivo activity. These findings strongly suggest that methods more sensitive than standard IPA may need to be developed to detect functional antiplatelet activity of clopidogrel associated with less than 50% P2Y12 blockade. In addition to measurements of P2Y12 receptor binding and postreceptor signaling, alternatives may include modifications of IPA with either simultaneous blockade of P2Y1 receptors or amplification of the cAMP effect (Malini et al., 2007). Regardless of the approach used, any effect on an ex vivo platelet function biomarker becomes validated on the basis of its consistent and predictable association with in vivo efficacy and bleeding safety. A very high standard of...
evidence is required for such a biomarker to guide medical treatment decisions, and establishing the target range for therapeutic biomarker responses will require extensive testing in large clinical event trials.

References


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