P2Y12 Receptor Blockade Augments Glycoprotein IIb#IIIa Antagonist Inhibition of Platelet Activation, Aggregation, and Procoagulant Activity

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation


Published Version
doi:10.1161/JAHA.113.000026

Citable link

http://nrs.harvard.edu/urn-3:HUL.InstRepos:11877097

Terms of Use

This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
P2Y₁₂ Receptor Blockade Augments Glycoprotein IIb-IIIa Antagonist Inhibition of Platelet Activation, Aggregation, and Procoagulant Activity

Michelle A. Berny-Lang, PhD; Joseph A. Jakubowski, PhD; Atsuhiro Sugidachi, PhD; Marc R. Barnard, MS; Alan D. Michelson, MD; Andrew L. Frelinger III, PhD

Background—New antiplatelet agents that provide greater, more consistent inhibition of the platelet ADP receptor P2Y₁₂ may be used in combination with glycoprotein (GP) IIb-IIIa antagonists, but their combined effect on platelet function and procoagulant activity is not well studied. Therefore, the objective of this study was to evaluate the independent and complementary effects of P2Y₁₂ and GPIIb-IIIa inhibition on platelet function and procoagulant activity.

Methods and Results—Healthy donor blood was treated with the active metabolite of prasugrel (R-138727 5 μmol/L), GPIIb-IIIa antagonists (abciximab 3 μg/mL or eptifibatide 0.9 μg/mL), and combinations thereof, exposed to physiologically relevant agonists (collagen and ADP) and then evaluated for markers of platelet activation and procoagulant activity. Significant interactions between R-138727 and GPIIb-IIIa antagonists were observed. R-138727 and the GPIIb-IIIa antagonists had additive inhibitory effects on collagen-stimulated platelet aggregation and on the collagen plus ADP–stimulated level of activated platelet surface GPIIb-IIIa. R-138727 and abciximab each inhibited collagen plus ADP–stimulated platelet phosphatidylserine expression and prothrombin cleavage, and the combination produced greater inhibition than achieved with abciximab alone. In contrast, eptifibatide did not inhibit, but instead enhanced, collagen plus ADP–stimulated prothrombin cleavage. Addition of R-138727 reduced prothrombin cleavage in eptifibatide-treated samples, suggesting a novel mechanism for potential benefit from combined prasugrel and eptifibatide treatment.

Conclusions—The complementary effects of abciximab and R-138727 on platelet activation, aggregation, and procoagulant activity suggest their combined use may, to a greater degree than with either agent alone, reduce thrombus formation in vivo. (J Am Heart Assoc. 2013;2:e000026 doi: 10.1161/JAHA.113.000026)

Key Words: glycoprotein IIb-IIIa receptor antagonists • platelets • prasugrel

In addition to the most commonly used antiplatelet agent, aspirin, which inhibits platelet cyclooxygenase-1, two distinct classes of antiplatelet agents with distinct mechanisms of action, glycoprotein (GP) IIb-IIIa antagonists (eg, abciximab, eptifibatide) and antagonists of the platelet ADP receptor P2Y₁₂ (eg, clopidogrel, prasugrel), are used for the prevention of cardiac ischemic complications in the setting of percutaneous coronary intervention (PCI).¹ ² Abciximab is the Fab fragment of the chimeric human–murine monoclonal antibody 7E3, whereas eptifibatide is a cyclic hexapeptide, small-molecule inhibitor.³ Abciximab and eptifibatide bind to the GPIIb-IIIa (integrin αIIbβ₃) receptor of human platelets, inhibiting the final common pathway of platelet aggregation in response to all agonists. Clopidogrel and prasagrel are thienopyridine prodrugs, each with an active metabolite that irreversibly inhibits the platelet ADP receptor P2Y₁₂ and, thereby, inhibits ADP-induced platelet activation and aggregation.¹

While aspirin plus clopidogrel is the most frequently orally administered antiplatelet combination in PCI,⁴ novel P2Y₁₂ inhibitors such as ticagrelor and prasugrel are also used in the setting of acute coronary syndrome PCI.⁵ As GPIIb-IIIa and P2Y₁₂ antagonists target distinct steps of the thrombus formation process, supplementing aspirin plus P2Y₁₂ antagonist therapy with a GPIIb-IIIa antagonist enhances platelet...
inhibition and may have clinical benefits in patients, particularly patients with large thrombus burden or poor response to oral antiplatelet agents. Although combination GPIb-IIIa and P2Y12 inhibition on an aspirin background is used in the clinical setting, the combined effect of these inhibitors on platelet responses has not been well studied, particularly in the case of the newer P2Y12 agents.

In in vitro studies, the active metabolite of prasugrel, R-138727, in the absence or presence of aspirin, inhibits platelet activation as well as platelet procoagulant and proinflammatory responses; however, the effect of concomitant prasugrel and GPIb-IIIa antagonist treatment on these end points remains unknown. Therefore, the objective of this study was to evaluate the independent and complementary effects of P2Y12 and GPIb-IIIa inhibition on platelet function. On a background of aspirin, we evaluated the combined effect of in vitro treatment of whole blood with the active metabolite of prasugrel, R-138727, plus abciximab or eptifibatide on platelet activation, aggregation, and procoagulant activity.

Methods

Materials

ADP was purchased from Bio/Data Corporation, and fibrillar type I collagen was purchased from Chrono-log. Gly-Pro-Arg-Pro (GPRP) was purchased from Bachem. The fluorogenic activated factor XIII (FXIIIa) substrate was from Zedira. Fluorescein isothiocyanate (FITC)-conjugated annexin V, FITC-labeled PAC1 (an IgM monoclonal antibody [mAb] specific for the activated conformation of GPIb-IIIa), phycoerythrin (PE)-Cy5–conjugated CD42b-specific mAb, and PE-conjugated CD42a- and CD62P-specific mAbs were from Becton Dickinson. PE-Cy5–labeled CD14-specific mAb was from Beckman Coulter. FITC-conjugated anti–tissue factor mAb (clone VD8) was from American Diagnostica. Purified human coagulation factors Va and Xa (fVa and FXa, respectively) were purchased from Haematologic Technologies. The prothrombin fragment 1.2 (F1.2) ELISA was from Dade Behring.

Blood Collection and Treatment

Institutional review board–approved written informed consent was obtained from all subjects. Two hours after healthy volunteers ingested aspirin 325 mg (Bayer HealthCare), blood was collected into a final concentration of sodium citrate 0.32%, hirudin 25 μg/mL, or δ-Pho-Pro-Arg-chloromethylketone 300 μmol/L (PPACK; EMD Biosciences). Platelet counts for all donors were within the normal range of 150 to 400 × 10⁹/µL. R-138727 (provided by Daiichi Sankyo Company Ltd, Tokyo, Japan) was dissolved in DMSO and kept at −80°C in sealed vials. Immediately before use, R-138727 was diluted in HEPES 10 mmol/L, NaCl 0.15 mol/L, pH 7.4 (HEPES-saline). For all subsequent assays, whole blood was incubated for 30 minutes at 37°C with R-138727 5 μmol/L, abciximab 3.0 μg/mL (ReoPro; Centocor), eptifibatide 0.9 μg/mL (Integrilin; Millennium Pharmaceuticals), R-138727 5 μmol/L plus abciximab 3.0 μg/mL, R-138727 5 μmol/L plus eptifibatide 0.9 μg/mL, or vehicle (DMSO diluted identically to R-138727). R-138727, abciximab, and eptifibatide concentrations used for these experiments were selected to correspond with the level of platelet inhibition achieved in patients treated with the current recommended dosing regimens: prasugrel at 60 mg loading dose and 10 mg/day maintenance dose; abciximab at 0.25 mg/kg intravenous bolus followed by continuous infusion of 0.125 μg/kg per minute; eptifibatide at 180 μg/kg bolus followed immediately by a continuous infusion of 2.0 μg/kg per minute and a second eptifibatide bolus of 180 μg/kg administered 10 minutes later. Concentrations of R-138727, abciximab, and eptifibatide were calculated with respect to the plasma compartment of the blood to account for differences in hematocrit between donors. For selected experiments, platelet-rich plasma (PRP) was prepared by centrifugation of treated whole blood for 10 minutes at 150g. For each assay subsequently described, 6 independent experiments were performed using blood collected from 6 different donors.

Platelet Aggregometry

Light transmission platelet aggregation was evaluated in a Chrono-log aggregometer in PPACK-anticoagulated PRP in response to ADP 20 μmol/L or collagen 20 μg/mL. The aggregation response was recorded for a total of 6 minutes using Aggro/Link software (Chrono-log). Platelet counts were not adjusted before the use of PRP in aggregation.

Platelet Surface Expression of Activated GPIb-IIIa and P-selectin

PPACK-anticoagulated whole blood was incubated with FITC-PAC1, PE anti-CD62P, and PE-Cy5 anti-CD42a (as a platelet identifier) and either collagen 20 μg/mL plus ADP 20 μmol/L or no agonist (HEPES-Tyrode’s buffer) for 15 minutes at room temperature. Samples were then fixed by addition of 1% formaldehyde in HEPES-saline. Flow cytometric analysis was performed in a calibrated Becton Dickinson FACSCalibur as previously described. Platelet Surface Binding of Annexin V

PPACK-anticoagulated whole blood was diluted 1:10 in HEPES-Tyrode’s buffer and incubated with collagen 20 μg/mL plus...
ADP 20 μmol/L or no agonist (HEPES-Tyrode’s buffer) for 20 minutes at 37°C. Phosphatidylserine expression on the platelet surface was determined by annexin V binding as previously described. Briefly, after incubation with collagen plus ADP or buffer, samples were mixed with FITC-conjugated annexin V and a PE-Cy5 anti-CD42a antibody (as a platelet identifier) in the presence of CaCl₂ 4 mmol/L, incubated for 15 minutes at room temperature, and fixed with 1% formaldehyde in HEPES-saline. Flow cytometric analysis was performed in a calibrated Becton Dickinson FACSCalibur.

**Tissue Factor Expression on Monocyte–Platelet Aggregates and Single Platelets**

The presence of monocyte–platelet aggregates and the expression of tissue factor on monocyte–platelet aggregates and single platelets were detected, as previously described, in PPACK-anticoagulated whole blood stimulated with collagen 20 μg/mL plus ADP 20 μmol/L or no agonist (HEPES-saline). In brief, whole blood was incubated with agonists for 15 minutes at room temperature and then incubated with a mixture of PE anti-CD42a 1 μg/mL for platelet identification, PE-Cy5 anti-CD14 for monocyte identification, and FITC-conjugated anti–tissue factor mAb 10 μg/mL for 20 minutes at room temperature. After incubation, FACS Lysing solution (Becton Dickinson) was added to samples, and flow cytometric analysis was performed in a Becton Dickinson FACSCalibur.

**Thrombin Generation**

Hirudin-anticoagulated whole blood was mixed for 15 minutes at 37°C with shaking with FXa 600 pmol/L and FVa 300 pmol/L combined with either collagen 20 μg/mL plus ADP 20 μmol/L or no agonist (HEPES-Tyrode’s buffer) in a 96-well microtiter plate (total volume 100 μL, all concentrations are final concentrations in whole blood). The assay was stopped by the addition of 260 μL of EDTA 2 mmol/L in HEPES-saline. Samples were centrifuged and thrombin generation was measured by the formation of F1.2 in the supernatant using the Enzygnost F1.2 ELISA.

**Activated FXIII Generation**

For FXIII assays, blood was collected from healthy volunteers into sodium citrate, 24 hours after ingestion of aspirin 325 mg. As described earlier, blood was treated with R-138727, abciximab, and eptiabatide, and PRP was prepared via centrifugation. Sodium citrate–anticoagulated PRP was combined with fluorogenic FXIIIa substrate 56 μmol/L, CaCl₂ 20 mmol/L, FXa 60 pmol/L, FVa 30 pmol/L, and GPRP 10 mmol/L and collagen 20 μg/mL plus ADP 20 μmol/L or no agonist (HEPES-saline) in a 96-well microtiter plate (all concentrations are final concentrations in PRP). Fluorescence was monitored (excitation at 313 nm and emission at 418 nm) in a microtiter plate reader for 45 minutes at 37°C with shaking. PPACK-treated PRP was used as the plate blank.

**Statistical Methods**

Data analyses were performed by the authors using GraphPad Prism Version 5.0 and VassarStats: Website for Statistical Computation. Data are presented as mean±SEM from 6 independent experiments. The effects of R-138727, the GPIIb-IIIa antagonists, and the interactions between R-138727 and the GPIIb-IIIa antagonists were determined by 2-factor repeated measures ANOVA (RM-ANOVA) with P<0.05 considered statistically significant. Posttests to compare individual treatment conditions assumed samples were from normal distributions and were by Student’s paired t test or by 1-sample t test (for comparison with a normalized baseline result). To account for multiple comparisons, only posttest P values <0.0071 (Bonferroni correction) were considered significant.

**Results**

**Inhibition of Platelet Aggregation by P2Y₁₂ and GPIIb-IIIa Antagonists**

The P2Y₁₂ antagonist R-138727 has been previously shown to dose-dependently inhibit ADP-induced platelet aggregation. To investigate the combined effect of P2Y₁₂ and GPIIb-IIIa inhibition on a background of aspirin, platelet aggregation was studied in PRP from aspirin-treated subjects, treated in vitro with R-138727 alone or in combination with the GPIIb-IIIa antagonists, abciximab or eptiabatide. Consistent with previous studies, when platelets were stimulated with ADP, aggregation was significantly inhibited in the presence of R-138727 (Figure 1). Likewise, as expected, treatment with either GPIIb-IIIa antagonist resulted in a marked decrease in ADP-induced aggregation. However, the addition of either abciximab or eptiabatide to R-138727 completely abrogated platelet aggregation (Figure 1). Two-factor RM-ANOVA of ADP-induced platelet aggregation (Table) showed a significant effect of both R-138727 (P<0.0001) and the GPIIb-IIIa antagonists (P<0.0001) and a significant interaction between R-138727 and the GPIIb-IIIa antagonists (P=0.0009). When collagen was used as the agonist, the presence of R-138727 resulted in a smaller, but still significant, decrease in maximal platelet aggregation (Figure 1). As with ADP-induced aggregation, the GPIIb-IIIa antagonists significantly inhibited collagen-induced platelet aggregation compared with vehicle. Combined treatment with R-138727 plus abciximab or
epitibatide resulted in a markedly reduced aggregation response compared with R-138727 treatment alone (Figure 1). Furthermore, when R-138727 was added to the GPIIb-IIIa antagonists, platelet aggregation was decreased beyond the level of aggregation with GPIIb-IIIa inhibition alone.

Platelet Surface Activated GPIIb-IIIa and P-selectin

Because platelet aggregation is dependent on the activation of GPIIb-IIIa, the combined effect of P2Y12 and GPIIb-IIIa inhibition on ADP- or collagen-induced platelet aggregation. Platelet-rich plasma (PRP) was prepared from PPACK-anticoagulated blood treated with vehicle or R-138727 5 μmol/L in the absence or presence of abcximab 3 μg/mL or epitibatide 0.9 μg/mL. PRP was stimulated with ADP 20 μmol/L or collagen 20 μg/mL, and the extent of platelet aggregation was determined. Data are reported as mean ± SEM from 6 experiments. *P<0.0071 vs no R-138727, vehicle treatment; †P<0.0071 vs corresponding treatment in the absence of R-138727; ‡P<0.0071 vs R-138727, vehicle treatment.

Table. Statistical analysis of platelet activation, aggregation, and procoagulant responses to ADP 20 μmol/L and collagen 20 μg/mL in the presence of R-138727 5 μmol/L, abcximab 3 μg/mL, or epitibatide 0.9 μg/mL, and combinations thereof

<table>
<thead>
<tr>
<th>Factor</th>
<th>Interaction</th>
<th>R-138727</th>
<th>GP IIb-IIIa antagonists</th>
<th>&lt;P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet aggregation—ADP (maximum % aggregation)</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelet aggregation—collagen (maximum % aggregation)</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Activated GP IIb-IIIa—ADP (PAC1 MFI)</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelet surface P-selectin—collagen—ADP (MFI)</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>0.131</td>
</tr>
<tr>
<td>% MPAs—collagen—ADP</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>0.0008</td>
</tr>
<tr>
<td>Platelet fluorescence in MPAs—collagen—ADP (MFI)</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>0.0002</td>
</tr>
<tr>
<td>% Annexin V—positive platelets—collagen—ADP</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>0.0010</td>
</tr>
<tr>
<td>MPA tissue factor—collagen—ADP (MFI)</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>0.0007</td>
</tr>
<tr>
<td>Platelet tissue factor—collagen—ADP (MFI)</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>0.0001</td>
</tr>
<tr>
<td>Plasma F1.2—collagen—ADP (normalized)</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>0.0102</td>
</tr>
<tr>
<td>Relative FXIIIa activity—collagen—ADP (1/time to Vmax, normalized)</td>
<td></td>
<td>R-138727</td>
<td>GP IIb-IIIa antagonists</td>
<td>0.3542</td>
</tr>
</tbody>
</table>

Two-factor ANOVA with repeated measures on both factors. GP indicates glycoprotein; MFI, mean fluorescence intensity; MPA, monocyte–platelet aggregate; Vmax, maximum velocity.
Monocyte–Platelet Aggregates

As an additional marker of the level of platelet activation with combined P2Y12 and GPIIb-IIIa inhibition, monocyte–platelet aggregates were measured with and without collagen plus ADP stimulation. In the absence of antiplatelet agents, as expected, collagen plus ADP increased the percentage of monocytes bound to platelets (monocyte–platelet aggregates) and the platelet fluorescence in monocyte–platelet aggregates (Figure 3A and 3B). By 2-factor RM-ANOVA, R-138727, GPIIb-IIIa antagonists, and the interaction between R-138727 and GPIIb-IIIa antagonists were highly significant for the collagen plus ADP–stimulated percentage of monocyte–platelet aggregates and the platelet fluorescence in monocyte–platelet aggregates (Table). In collagen plus ADP–stimulated samples, R-138727 reduced the percentage of monocyte–platelet aggregates and the level of platelet fluorescence in the aggregates, indicating a reduced number of platelets in the aggregates (Figure 3). Although abciximab and eptifibatide each resulted in numerical increases in the percentage of monocyte–platelet aggregates and platelet fluorescence in monocyte–platelet aggregates, in posttests, only the abciximab-induced increase in platelet fluorescence remained statistically significant. Addition of R-138727 to abciximab abrogated this increase, reducing platelet fluorescence in monocyte–platelet aggregates to the level observed with R-138727 treatment alone (Figure 3).

Platelet Surface Expression of Phosphatidylserine

In addition to their role in aggregate formation, activated platelets promote coagulation through surface exposure of procoagulant phosphatidylserine.17 As expected, stimulation with collagen plus ADP increased the percentage of annexin V–positive platelets (Figure 4). Two-factor RM-ANOVA analysis (Table) showed a significant effect of both R-138727 (P=0.0010) and the GPIIb-IIIa antagonists (P=0.0001) and a significant interaction between R-138727 and the GPIIb-IIIa antagonists (P=0.0001). Individual treatment with R-138727 or abciximab reduced annexin V binding in collagen plus ADP–stimulated samples (annexin V–positive platelets 22.9 ± 2.7% with vehicle versus 9.5 ± 1.6% for R-138727, P=0.0004, and 14.7 ± 1.9% for abciximab, P=0.0004), but no significant effect was observed with eptifibatide (P=0.0247 versus vehicle; nonsignificant with Bonferroni correction). No further inhibi-

Figure 2. Inhibition of platelet surface expression of activated GPIIb-IIIa and P-selectin by R-138727 plus abciximab or eptifibatide. PPACK-anticoagulated whole blood treated with R-138727, abciximab, or eptifibatide, and combinations thereof, was incubated with collagen 20 μg/mL plus ADP 20 μmol/L or no agonist and labeled with fluorescently-conjugated antibodies for analysis by whole blood flow cytometry. A, Platelet surface activated GPIIb-IIIa, as determined by platelet binding of the monoclonal antibody PAC1 (mean fluorescence intensity). B, Platelet surface expression of P-selectin (mean fluorescence intensity). Data are shown as mean ± SEM, n=6. *P<0.0071 vs no R-138727, vehicle treatment; †P<0.0071 vs corresponding treatment in the absence of R-138727; ‡P<0.0071 vs R-138727, vehicle treatment. GP indicates glycoprotein; PPACK, D-Phe-Pro-Arg-chloromethylketone.
tion was seen when abciximab or eptifibatide was added to R-138727 treatment (Figure 4).

Tissue Factor Expression on Monocyte–Platelet Aggregates and Individual Platelets

Beyond platelet surface expression of phosphatidylinerine, platelets and monocyte–platelet aggregates can stimulate coagulation through surface expression of tissue factor. In previous studies, treatment with R-138727 decreased the level of tissue factor on monocyte–platelet aggregates. To extend these studies, we investigated the effect of R-138727 combined with abciximab or eptifibatide on procoagulant surface expression of tissue factor. Stimulation with collagen plus ADP increased the level of tissue factor fluorescence on both monocyte–platelet aggregates and single platelets (Figure 5A and 5B). Two-factor RM-ANOVA analysis of tissue factor on monocyte–platelet aggregates (Table) showed significant effects of R-138727 (P<0.0007), GPIIb-IIIa antagonists (P<0.0001), and the interaction between R-138727 and GPIIb-IIIa antagonists (P=0.0001). Tissue factor on monocyte–platelet aggregates induced by collagen plus ADP was reduced by R-138727 (Figure 5A). In parallel to the increase in monocyte–platelet aggregates observed with abciximab or eptifibatide (Figure 3), individual treatment with either GPIIb-IIIa antagonist caused an increase in monocyte–platelet tissue factor (Figure 5A). The addition of R-138727 to abciximab or eptifibatide reduced tissue factor levels on monocyte–platelet aggregates. When tissue factor on individual platelets stimulated with collagen plus ADP was examined, R-138727 treatment caused significant inhibition, while the GPIIb-IIIa antagonists had no significant effect (2-factor RM-ANOVA R-138727, P=0.0001; GPIIb-IIIa antagonists, P=0.2341; Table) on platelet tissue factor (Figure 5B).

Platelet-Dependent Coagulation Factor Activation: F1.2 Generation and FXIIIa Activity

To determine the effects of combined platelet P2Y12 and GPIIb-IIIa inhibition on coagulation, we investigated platelet-dependent enhancement of thrombin generation and activation of...
FXIII in the presence of R-138727 plus abciximab or eptifibatide. When thrombin generation was measured by the formation of F1.2 in whole blood, addition of collagen plus ADP, in the presence of priming concentrations of FVa and FXa, caused a marked increase in F1.2 compared with blood primed with FVa and FXa but without the addition of collagen plus ADP (Figure 6). Two-factor RM-ANOVA (Table) showed a significant effect of R-138727 (P=0.0002) and the GPIIb-IIIa antagonists (P=0.0102) and a significant interaction between R-138727 and the GPIIb-IIIa antagonists (P=0.0183). Collagen plus ADP–enhanced F1.2 levels were reduced by 38% by R-138727 treatment compared with vehicle (P=0.0064, 1-sample t test, Figure 6). Treatment with abciximab alone reduced the collagen plus ADP–dependent increase in F1.2 (P=0.0250, 1-sample t test), while eptifibatide treatment enhanced collagen plus ADP–stimulated F1.2 (P=0.0482, 1-sample t test), although neither effect reached statistical significance after Bonferroni correction. The combination of R-138727 plus abciximab or eptifibatide reduced F1.2 compared with individual GPIIb-IIIa antagonist treatment and resulted in a level of F1.2 that was comparable to R-138727 treatment alone (Figure 6).

FXIII generation, primed by addition of FVa and FXa, was significantly increased by platelet activation with collagen plus ADP (Figure 7). Relative FXIII activity in collagen plus ADP–activated samples was not significantly affected by treatment with R-138727 or eptifibatide alone (Figure 7). However, the relative FXIII activity was significantly, albeit modestly, decreased in the presence of abciximab (P=0.0015, 1-sample t test, Figure 7).

Discussion

The main findings of this study are as follows. (1) The combination of P2Y12 and GPIIb-IIa antagonists, at whole blood concentrations that demonstrate pharmacological
Combined Platelet P2Y₁₂ and GPIIb-IIIa Inhibition

The presently described additive effects of GPIIb-IIIa antagonists and prasugrel’s active metabolite on platelet activation, aggregation, and procoagulant activities have not previously been reported. While current indications for GPIIb-IIIa antagonist use are limited, broader application of GPIIb-IIIa antagonists in combination with P2Y₁₂ antagonists may have clinical benefit. For example, when prasugrel was administered with or without the GPIIb-IIIa antagonist tirofiban in ST-segment elevation acute myocardial infarction patients on aspirin, platelet inhibition was suboptimal (as measured by ADP-induced aggregation) for at least 2 hours with prasugrel alone, but the addition of tirofiban led to a significantly higher degree of platelet inhibition. Furthermore, a recent meta-analysis of randomized trials of patients undergoing elective PCI with stents and periprocedural thienopyridines, assigned to randomly receive a GPIIb-IIIa antagonist or control, show a clinical benefit of GPIIb-IIIa inhibitors (both abciximab and small-molecule inhibitors). Specifically, GPIIb-IIIa antagonists added on a background of aspirin and a thienopyridine were shown to reduce nonfatal myocardial infarction, without an increase in major bleeding, but with an increase in minor bleeding. These studies combined with the present study suggest that GPIIb-IIIa antagonists are beneficial in inhibiting platelet function when used in combination with P2Y₁₂ antagonists.

Clinical Evidence Suggesting a Benefit From Combined GPIIb-IIIa and P2Y₁₂ Inhibition

The presently described additive effects of GPIIb-IIIa antagonists and prasugrel’s active metabolite on platelet activation, aggregation, and procoagulant activities have not previously been reported. While current indications for GPIIb-IIIa antagonist use are limited, broader application of GPIIb-IIIa antagonists in combination with P2Y₁₂ antagonists may have clinical benefit. For example, when prasugrel was administered with or without the GPIIb-IIIa antagonist tirofiban in ST-segment elevation acute myocardial infarction patients on aspirin, platelet inhibition was suboptimal (as measured by ADP-induced aggregation) for at least 2 hours with prasugrel alone, but the addition of tirofiban led to a significantly higher degree of platelet inhibition. Furthermore, a recent meta-analysis of randomized trials of patients undergoing elective PCI with stents and periprocedural thienopyridines, assigned to randomly receive a GPIIb-IIIa antagonist or control, show a clinical benefit of GPIIb-IIIa inhibitors (both abciximab and small-molecule inhibitors). Specifically, GPIIb-IIIa antagonists added on a background of aspirin and a thienopyridine were shown to reduce nonfatal myocardial infarction, without an increase in major bleeding, but with an increase in minor bleeding. These studies combined with the present study suggest that GPIIb-IIIa antagonists are beneficial in inhibiting platelet function when used in combination with P2Y₁₂ antagonists.

Augmented Inhibition of Platelet Activation, Aggregation, and Procoagulant Activities by Combined Use of R-138727 and GPIIb-IIIa Antagonists

Because light transmission platelet aggregation has been the de facto gold standard for platelet function testing throughout the development of the GPIIb-IIIa antagonists and newer P2Y₁₂ antagonists, the additive inhibitory effect of R-138727 plus abicipiximab or eptifibatide on collagen– and ADP–stimulated platelet aggregation is not surprising. The additional inhibition of platelet aggregation and platelet surface activated GPIIb-IIIa expression by combined P2Y₁₂ and GPIIb-IIIa antagonists may in part explain the clinical benefit resulting from their combined use in the meta-analysis described earlier. Downstream effects of combined inhibition with abicipiximab and R-138727 observed in the present study [reduced annexin V–positive platelets (Figure 4) and reduced conversion of prothrombin to thrombin with concomitant release of F1.2 (Figure 6)] are also likely to be mechanistically linked to the clinical benefit observed in the meta-analysis. The results of the present study are similar to that reported for the combination of cangrelor, a reversible P2Y₁₂ antagonist and abicipiximab or tirofiban, which resulted in additive inhibition of platelet aggregate formation, dense granule secretion, soluble CD40 ligand release, annexin V binding, and procoagulant microparticle formation, and to a study of in vivo clopidogrel plus in vitro GPIIb-IIIa inhibition that demonstrated additive
inhibition of ADP- or collagen-induced platelet aggregation and fibrinogen binding to GPIIb-IIIa.\textsuperscript{20,21}

Enhanced Procoagulant Status Following Treatment With GPIIb-IIIa Antagonists Alone, and Ablation by R-138727

The results of previous studies on the effect of GPIIb-IIIa inhibitors on monocyte–platelet aggregate formation and tissue factor on monocyte–platelet aggregates are inconsistent, with GPIIb-IIIa inhibitors reported to both augment and inhibit these processes.\textsuperscript{22–25} In agreement with the present results, separate studies have shown that abciximab enhanced ADP-induced platelet fluorescence in monocyte–platelet aggregates,\textsuperscript{22} and eptifibatide enhanced tissue factor expression on monocytes.\textsuperscript{23} The enhanced procoagulant status observed after GPIIb-IIIa antagonist treatment may be relevant to the increased thrombotic complications observed with oral GPIIb-IIIa antagonists.\textsuperscript{26} In the current study, whole blood stimulated with collagen plus ADP in the presence of either abciximab or eptifibatide appears to be in a procoagulant state, as evidenced by increased exposure of tissue factor antigen on monocyte–platelet aggregates (Figure 5A), together with an increase in platelet mass associated with each monocyte (Figure 3B). Indeed, platelet activation–dependent prothrombinase activity is modestly enhanced in the presence of eptifibatide (Figure 6). However, abciximab under the same conditions reduced prothrombinase activity (Figure 6), thus demonstrating a difference in the ability of eptifibatide (a 1000 Da, small molecule, reversible GPIIb-IIIa antagonist) and abciximab (a much larger [50 000 Da], tight-binding GPIIb-IIIa antagonist) to modulate platelet-dependent procoagulant activity.

Prothrombin has been shown to bind to GPIIb-IIIa and this binding accelerates thrombin generation.\textsuperscript{27} With its larger size, abciximab (and other antibody inhibitors) may not only block prothrombin binding to GPIIb-IIIa but may also sterically hinder other binding reactions on the platelet surface that are important for thrombin generation, such as coagulation factor binding to surface-exposed phosphatidylserine. This concept is in agreement with a previous study, showing that abciximab more strongly inhibited annexin V and factor V/Va binding to the platelet surface than eptifibatide.\textsuperscript{28} The molecular size difference and specific molecular interactions between each antagonist and GPIIb-IIIa may explain the variable response in prothrombin activation. Because tissue factor activation is modulated by protein disulfide-isomerase,\textsuperscript{29} it is also tempting to speculate that eptifibatide and abciximab differentially affect this activation.

In the present study, the addition of R-138727 to GPIIb-IIIa antagonists resulted in a significant decrease compared with GPIIb-IIIa inhibitors alone in the percentage of monocyte–platelet aggregates, tissue factor expression on monocyte–platelet aggregates, and thrombin generation. This is in agreement with a previous study on clopidogrel and abciximab which reported that the addition of clopidogrel to abciximab treatment ablated the abciximab-induced increase in monocyte–platelet aggregates.\textsuperscript{22} Taken together, the presently-described ablation by prasugrel’s active metabolite of the GPIIb-IIIa antagonist-induced increase of tissue factor on monocyte–platelet aggregates and the eptifibatide-induced increase in thrombin generation, suggests that inhibition of the platelet P2Y\textsubscript{12} ADP receptor may reduce the prothrombotic effects of GPIIb-IIIa antagonists.

Study Limitations and Strengths

This study used blood from healthy subjects rather than from acute coronary syndrome patients, the population for whom these drugs have an FDA-approved indication, to minimize the variables of comedication and the degree of disease. However, all healthy donors were treated with aspirin to correspond to the clinical conditions where prasugrel and GPIIb-IIIa antagonists might be coadministered. The power to detect treatment differences is limited by the small sample size (n=6); however, significant effects of GPIIb-IIIa and/or P2Y\textsubscript{12} inhibition were detected for each end point, even after application of the conservative Bonferroni correction.

Conclusions

The presently described complementary effects of abciximab and prasugrel’s active metabolite on collagen plus ADP–induced platelet activation, aggregation, and procoagulant activity suggest that the combined use of abciximab and prasugrel may, to a greater degree than with either agent alone, reduce and destabilize thrombus formation in vivo. The GPIIb-IIIa antagonist-induced increase of tissue factor on monocyte–platelet aggregates and the eptifibatide-induced increase in thrombin generation were attenuated by prasugrel’s active metabolite, suggesting a novel mechanism for reducing the prothrombotic effects of these GPIIb-IIIa antagonists.

Acknowledgments

The authors acknowledge the statistical support of Carter Petty, Harvard Catalyst Biostatistics Program, and technical contributions of Youfu Li, Anu Nigam, and Michael Lampa.

Sources of Funding

This study was supported in part by a grant from Eli Lilly and Daiichi Sankyo. Dr. Berny-Lang was supported by an NIH T32 Training Grant (5T32HL007574-31). This work was conducted...
with support from Harvard Catalyst | The Harvard Clinical and Translational Science Center (National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health award 8UL1TR000170-05 and financial contributions from Harvard University and its affiliated academic health care centers). The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, Harvard University and its affiliated academic health care centers, or the National Institutes of Health.

Disclosures

Drs. Berny-Lang and Barnard report no conflicts. Dr. Jakubowski is an employee and shareholder of Eli Lilly and Company. Dr. Sugidachi is an employee of Daiichi Sankyo Co., Ltd. Dr. Michelson received research grants from GLS Synthesis and Lilly/Daichi Sankyo; and is a member of a data monitoring committee for clinical trials of prasugrel sponsored by Lilly/Daichi Sankyo. Dr. Frelinger III received research grants from GLS Synthesis and Lilly/Daichi Sankyo and is a consultant for Eli Lilly.

References