Differential Effects of Glycoprotein IIb/IIIa Antagonists on Platelet Microaggregate and Macroaggregate Formation and Effect of Anticoagulant on Antagonist Potency

Implications for Assay Methodology and Comparison of Different Antagonists

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Background—Citrated platelet-rich plasma (PRP) turbidimetry is used for assessing pharmacodynamic effects of glycoprotein (GP) IIb/IIIa antagonists in clinical trials. However, citrate can enhance the potency of at least eptifibatide (Integrilin), and turbidimetry is insensitive to microaggregate formation. We compared PRP turbidimetry, as a measure of macroaggregate formation, with single-platelet counting in both whole blood and PRP as a measure of microaggregate formation, using both citrate and hirudin anticoagulation.

Methods and Results—Three GP IIb/IIIa antagonists, eptifibatide, MK-0852, and GR144053, were compared in PRP (turbidimetry) and whole blood (platelet counting with an Ultra-Flo 100 Platelet Counter), with ADP and collagen used as agonists. Compared with hirudin, citrate enhanced the potency of eptifibatide by up to 4-fold in both PRP and whole blood (P<0.0005), modestly enhanced MK-0852 potency (P=0.001), and had no effect on GR144053. Potency measured in PRP was 2- to 3-fold greater compared with whole blood for MK-0852 and GR144053 but 3- to 4-fold greater for eptifibatide. Simultaneous turbidimetry and platelet counting performed in PRP indicated that this is because GP IIb/IIIa antagonists are more potent inhibitors of in vitro macroaggregation than microaggregation, this effect being greater for eptifibatide in hirudinized PRP compared with GR144053 (P=0.032).

Conclusions—GP IIb/IIIa antagonist potency is variably enhanced by citrate. Macroaggregation is inhibited more effectively than microaggregation, most markedly in the case of eptifibatide in hirudinized blood. These observations have implications for the interpretation and comparison of pharmacodynamic assays and possibly for the risk/benefit ratio of different agents. (Circulation. 1998;98:1616-1621.)

Key Words: glycoproteins • calcium • platelet aggregation inhibitors

Platelet glycoprotein (GP) IIb/IIIa receptors bind fibrinogen and other proteins and mediate the final common pathway of platelet aggregation. Agents that interfere with fibrinogen binding to these receptors (GP IIb/IIIa antagonists) have been developed extensively as antithrombotic agents and are gaining a place in the management of patients with acute coronary syndromes. Trials of these drugs in patients undergoing percutaneous transluminal coronary angioplasty and in patients suffering acute non–Q-wave myocardial infarction or unstable angina have assessed platelet aggregation response in platelet-rich plasma (PRP) by turbidimetry, most often with citrate anticoagulation, however, the techniques required for this are time-consuming, may require large volumes of blood, and require particular skill and specialized equipment. Preparation of PRP creates changes to the milieu and density of platelets, and it may not be obvious how this affects ex vivo assays of different platelet antagonists or how the results obtained may be compared. The choice of anticoagulant may be crucial: it is now realized that citrate anticoagulation led to falsely high estimates of the efficacy of eptifibatide due to lowering of ionized calcium levels in vitro to 40 to 50 μmol/L. Hirudin is a direct thrombin inhibitor that maintains physiological calcium ion levels at 1.1 to 1.2 mmol/L.

Currently, there is no widely available method for rapidly measuring the extent of GP IIb/IIIa receptor blockade ex vivo, although a fibrinogen-coated bead assay has been described. Platelet aggregation can be measured in whole blood by counting the number of single platelets remaining after aggregation relative to a fixed red blood cell count and therefore is highly sensitive to formation of small aggregates, unlike PRP turbidimetry, which fails to detect formation of aggregates with fewer than $\approx$100 platelets. It offers the advantages of rapid results without imposing too many changes on the in vivo platelet environment.

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The purpose of this study was to compare the standard platelet aggregation methodology used in clinical trials of GP IIb/IIIa antagonists, which provides information on the effects of the drugs on platelet macroaggregation in PRP, with a method of aggregometry based on platelet counting. We studied 3 different antagonists, eptifibatide (Integrilin, a cyclic heptapeptide based on the KGD sequence), MK-0852 (a cyclic heptapeptide based on the RGD sequence), and GR144053 (a nonpeptide high-affinity antagonist), with ADP and collagen as agonists and sodium citrate and hirudin as anticoagulants.

Methods

Part 1: In Vitro Platelet Aggregation Assessments
Venous blood was obtained from healthy volunteers via a 19-gauge needle and syringe, and aliquots (9 mL) were immediately transferred to tubes containing either 1 mL of 3.13% (wt/vol) sodium citrate dihydrate or 1 mL hirudin (Revasc, Novartis) 500 mg/L in normal saline. Twenty milliliters of blood was incubated in a water bath at 37°C for 30 minutes for whole-blood aggregation studies, and 60 mL of blood was centrifuged at 180g for 10 minutes to prepare PRP, which was separated into clean tubes. PPP was prepared by centrifuging the remaining blood at 1500g for 10 minutes. Platelet counts were performed on the PRP, and the latter was diluted with platelet-poor plasma (PPP) to obtain a final platelet count of 300 000 platelets/μL.

Eptifibatide (COR Therapeutics), MK-0852 (Merck Sharp and Dohme), and GR144053 (Glaxo) were prepared to obtain a range of doubling concentrations in normal saline previously determined to give a full range of inhibition.

For PRP turbidimetry, a PAP-4 aggregometer (Biodata) was calibrated with PPP, and the stirring speed was set at 1000 rpm. Aliquots (460 μL) of PRP were placed in cuvettes containing magnetic stirrer bars, and 20-μL aliquots of normal saline (control) or antagonist were added to each cuvette. The samples were warmed at 37°C for 1 minute, then stirred for 2 minutes to obtain a stable baseline. Twenty microliters of either ADP 750 μmol/L (final concentration, 30 μmol/L; Sigma) or collagen 50 μg/mL (final concentration, 2 μg/mL; Nycomed) was then added, and change in light transmittance was recorded for 4 minutes (for ADP) or 6 minutes (for collagen). All assays were complete within 2 hours of venesection.

For whole-blood aggregation studies, 460-μL aliquots of incubated whole blood were placed into test tubes containing stirrer bars, and 20 μL of normal saline (control) or antagonist was added. Blood
(460 μL) was also fixed with 920 μL fixing solution saline with 4.6 mmol/L Na₂EDTA, 4.5 mmol/L Na₂HPO₄, 1.6 mmol/L KH₂PO₄, and 0.16% wt/vol formaldehyde, pH 7.4) for a baseline platelet count. The samples were stirred for 2 minutes in a water bath at 37°C, and 20 μL of either ADP or collagen was then added. Aggregation was terminated by addition of 880 μL fixing solution after stimulation for 4 minutes (for ADP samples) or 6 minutes (for collagen samples). Platelet counts were then performed on an Ultra-Flo 100 platelet counter (Becton Dickinson), and percentage aggregation was calculated from single-platelet counts before and after stimulation with agonist. All assays were complete within 90 minutes of venesection.

Percentage inhibition was calculated for each stimulated sample as percentage of control aggregation for both turbidimetry and whole-blood aggregometry, and results were plotted on a scale of log(agonist concentration) against inhibition. From these graphs, values for 20%, 50%, and 80% inhibition were obtained (IC values).

Part 2: Simultaneous Assessment of Single-Platelet Counts and Turbidimetry in PRP
Venous blood was obtained from healthy volunteers as above, with only hirudin used as anticoagulant, and PRP and PPP were prepared as above. After preparation of PPP, the remaining blood was diluted to the initial volume with PBS and centrifuged at 180g for 10 minutes. The supernatant was removed, this process was repeated, then 1 final dilution was made to the initial volume to produce a washed red-cell preparation. Dilutions of eptifibatide and GR144053 were made as above, and ADP was used as the agonist.

Turbidimetry was performed as above, but 460-μL samples of PRP were fixed as for whole blood at the start of the experiment and aggregation was terminated as for whole blood by the addition of fixing solution 2 minutes after addition of ADP. Counts were performed by first counting precisely 15-μL aliquots of the washed red-cell preparation, adding precisely 36 μL of the fixed PRP samples and recounting, then subtracting the initial count to give an estimate of the single-platelet count in the PRP.

Levels of inhibition were calculated as previously.

In all cases, statistical analysis was performed by ANOVA with SPSS for Windows software.

Results

Part 1: In Vitro Inhibition of Aggregation by Eptifibatide, MK-0852, and GR144053 in PRP and Whole Blood
Mean inhibition of platelet aggregation by eptifibatide, MK-0852, and GR144053 is represented in Figure 1. Figure 2 shows the ratios, according to antagonist and anticoagulant, of IC₅₀ values measured by whole-blood single-platelet counting and by PRP turbidimetry. Table 1 summarizes the concentrations at which antagonists yielded 50% inhibition (IC₅₀) for different methodologies. All the results show that higher concentrations of any GP IIb/IIIa antagonist are required to inhibit platelet aggregation as assessed by platelet counting in whole blood compared with PRP turbidimetry. Eptifibatide was markedly more effective when citrate was used as anticoagulant rather than hirudin, both in whole blood and in PRP (P<0.0005); there was a lesser effect for MK-0852 (P=0.001) and no effect for GR144053. There was a more marked difference in inhibition with eptifibatide, compared with the other agents, between hirudinized PRP and hirudinized whole blood (P<0.01).

Part 2: Single-Platelet Counting in PRP Compared With PRP Turbidimetry
Table 2 provides the IC₅₀ values determined for eptifibatide and GR144053 in hirudinized PRP by both turbidimetry and single-platelet counting and illustrates that when results obtained by whole-blood single-platelet counting are compared with those obtained by PRP turbidimetry, the major factor accounting for the perceived difference in efficacy of antagonists is the differential effect on microaggregation and macroaggregation. A 3-fold higher concentration of eptifibatide was required to inhibit ADP-induced microaggregate formation than was required to inhibit macroaggregate formation.
Moreover, this differential effect was significantly more marked for eptifibatide at physiological calcium ion levels than for GR144053, explaining at least part of the greater difference noted for eptifibatide, compared with MK-0852 and GR144053, between hirudinized whole blood and hirudinized PRP in part 1. For eptifibatide, when inhibition of ADP-induced aggregation in PRP reached 100% assessed by turbidimetry, only 60% to 70% inhibition of aggregation was assessed by single-platelet counting (data not illustrated); this latter figure was higher for GR144053 at 70% to 80%. Similarly, 80% inhibition of the light response correlated with ≈10% and 20% inhibition of the single-platelet count fall by eptifibatide and GR144053, respectively, indicating substantial microaggregation despite high inhibition of macroaggregation.

**Discussion**

This study illustrates the variable effect of citrate anticoagulation on the efficacy of GP IIb/IIIa antagonists compared with direct thrombin inhibition with hirudin, which maintains ionized calcium at physiological levels. The marked effect of citrate on eptifibatide previously demonstrated in PRP by turbidimetry15 is confirmed with whole-blood single-platelet counting. Phillips et al15 reviewed the evidence that lowering divalent cation levels affects the structure of GP IIb/IIIa. Clearly, alterations in the structure of GP IIb/IIIa induced by citrate affect the binding of some ligands but not others, although it is not clear whether antagonists are acting on more than 1 binding site. The effect of citrate on individual GP IIb/IIIa antagonists needs to be taken into account in assessment of a method for assay of GP IIb/IIIa receptor blockade.

This study also demonstrates the value of whole-blood single-platelet counting as a sensitive tool for assessing the efficacy of GP IIb/IIIa antagonists, providing a simpler, more rapid alternative to PRP turbidimetry and yielding specific information on macroaggregate formation, which is not obtained with turbidimetry. The degree of inhibition calculated by single-platelet counting is always less than that calculated by turbidimetry, in view of differences between the dynamics of microaggregate and macroaggregate formation; in other words, it is easier to inhibit the formation of large aggregates of platelets than to prevent pairing of platelets and formation of smaller aggregates. However, eptifibatide in the presence of physiological ionized calcium levels is more effective at preventing macroaggregate formation than preventing microaggregate formation compared with GR144053 or MK-0852 (regardless of ionized calcium level) or eptifibatide in citrated blood or plasma. The mechanism for this cannot be explained simply by differences between the antagonists in their affinity for the resting GP IIb/IIIa receptor. A number of possible mechanisms can be hypothesized: First, different antagonists will have different ratios of affinities for the receptor, given the relatively high plasma concentration of fibrinogen, whereas macroaggregate formation occurs progressively over a considerably longer time period, during which competitive antagonism may play a more important role. Second, adhesive proteins other than fibrinogen, such as thrombospondin,23 may play an important role in macroaggregate formation, and there may be differential inhibition of binding of these proteins compared with fibrinogen. Third, there may be a variable inhibition of the release reaction by the antagonists as a result of outside-in signaling, which may have a greater effect on in vitro macroaggregation.

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**TABLE 1. IC50 Values Determined With Whole-Blood Single-Platelet Counting and PRP Turbidimetry and ADP and Collagen as Agonists**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>ADP</th>
<th>Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eptifibatide, ng/mL</td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td>Hirudinized whole blood</td>
<td>1208 (193)</td>
<td>2048 (573)</td>
</tr>
<tr>
<td>Citrated whole blood</td>
<td>331 (122)</td>
<td>505 (118)</td>
</tr>
<tr>
<td>Hirudinized PRP</td>
<td>391 (104)</td>
<td>588 (278)</td>
</tr>
<tr>
<td>Citrated PRP</td>
<td>120 (33)</td>
<td>188 (111)</td>
</tr>
<tr>
<td><strong>Effect of anticoagulant?</strong></td>
<td>P &lt; 0.0005</td>
<td></td>
</tr>
<tr>
<td><strong>Effect of agonist?</strong></td>
<td>P = 0.028</td>
<td></td>
</tr>
<tr>
<td><strong>Ratio single-platelet IC50:light IC50</strong></td>
<td>2.28 (0.36)</td>
<td></td>
</tr>
<tr>
<td><strong>Single-platelet counting</strong></td>
<td>151 (35)</td>
<td></td>
</tr>
<tr>
<td><strong>Turbidimetry (light transmittance)</strong></td>
<td>67 (12)</td>
<td></td>
</tr>
<tr>
<td><strong>Difference between ratios for eptifibatide and GR144053?</strong></td>
<td>P = 0.032</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean (SD).*

**TABLE 2. Average IC50 Values for Simultaneous Turbidimetry and Single-Platelet Counting in Hirudinized PRP With ADP as Agonist**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>IC50 (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eptifibatide, ng/ml</td>
<td>n=6</td>
</tr>
<tr>
<td>Turbidimetry (light transmittance)</td>
<td>398 (77)</td>
</tr>
<tr>
<td>Single-platelet counting</td>
<td>1228 (374)</td>
</tr>
<tr>
<td>Ratio single-platelet IC50:light IC50</td>
<td>3.07 (0.68)</td>
</tr>
<tr>
<td>GR144053, nmol/L</td>
<td></td>
</tr>
<tr>
<td>Turbidimetry (light transmittance)</td>
<td>67 (12)</td>
</tr>
<tr>
<td>Single-platelet counting</td>
<td>151 (35)</td>
</tr>
<tr>
<td>Ratio single-platelet IC50:light IC50</td>
<td>2.28 (0.36)</td>
</tr>
<tr>
<td><strong>Difference between ratios for eptifibatide and GR144053?</strong></td>
<td>P = 0.032</td>
</tr>
</tbody>
</table>

*Values are mean (SD).*
These differential effects on microaggregation and macroaggregation may have implications for the risk/benefit ratio of different GP IIb/IIIa antagonists. An antagonist that completely inhibits macroaggregate formation in response to ADP and submaximal concentrations of more potent agonists but yet has modest effects on microaggregation may be safer than an antagonist that has a marked effect on microaggregate formation when there is therapeutic inhibition of macroaggregation, assuming that the microaggregates are sufficient to ensure hemostasis. Indeed, such differences may partly explain why there has been variability in the amount of major serious bleeding events between trials despite similar levels of inhibition of ADP-induced platelet aggregation in citrated PRP, although different levels of heparin dosing may explain a large part of this variability. Furthermore, the PURSUIT study with eptifibatide has shown a modest benefit in terms of reduced combined incidence of death and myocardial infarction and only a small increase in atherothrombosis. 24

Higher concentrations of all antagonists were necessary to inhibit collagen-induced aggregation compared with ADP-induced aggregation, and the differences in IC50 were similar for all 3 antagonists by all methods. It is well recognized that more potent agonists, such as collagen, lead to surface expression of an internal pool of GP IIb/IIIa receptors, partially with bound fibrinogen. Under these circumstances, therefore, there is a subpopulation of receptors to which fibrinogen has bound noncompetitively, and more extensive blockade of other receptors is necessary to prevent aggregate formation. If ADP serves as a rapid-acting agonist to sequester platelets in areas of developing intra-arterial thrombus where they may be exposed to more potent agonists, such as thrombin, that can partially overcome the effects of GP IIb/IIIa antagonists at current therapeutic levels, then inhibition of ADP-induced microaggregate formation may be more clinically relevant than inhibition of in vitro macroaggregate formation. This would then contradict the assumption that inhibition of macroaggregation is a reliable guide to in vivo antithrombotic efficacy and would undermine the hypothesis that differences between antagonists in their effects on macroaggregate and microaggregate inhibition may influence the risk/benefit ratio. Further evidence is required to establish the relative importance of inhibition of microaggregation compared with inhibition of macroaggregation with regard to both safety and efficacy.

One limitation of this study in relating the in vitro findings to ex vivo assays was the addition of antagonist in vitro and comparing final plasma concentrations with final whole-blood concentrations. In ex vivo studies, the antagonist is present in whole blood before preparation of PRP. The effect of hematocrit would in fact enhance the differences between whole-blood inhibition and PRP inhibition seen in this study, at least for ADP-induced aggregation, in which ADP was used at a maximal concentration for both methods. Another important reason for using a maximal concentration of ADP was that erythrocytes release ADP on stirring. Citrate anticoagulation may enhance the response to ADP by enabling thromboxane A2 synthesis and granule secretion, and it was therefore interesting that citrate did not increase the IC50 measurements for GR144053, suggesting that citrate does not enhance expression of internal GP IIb/IIIa receptors in response to ADP or collagen. With regard to the use of a submaximal dose of collagen in this study, it is necessary to acknowledge that when the same submaximal dose of any agonist is added to platelet preparations in both the presence and the absence of erythrocytes, the hematocrit effect will lead to a higher plasma level of agonist in the former, and it may artifactualy appear that erythrocytes are enhancing the platelet responses. Overall, the effect of hematocrit on both agonist and antagonist concentrations in this study emphasizes the importance of performing combined turbidimetry and single-platelet counting in PRP to establish the cause of the differences seen between PRP and whole blood.

Further ex vivo comparisons are needed to quantify differences between whole-blood single-platelet counting and turbidimetry. Ideally, future clinical trials of GP IIb/IIIa antagonists should incorporate dose adjustment according to a suitable pharmacodynamic measure, particularly with chronic administration of oral antagonists, and whole-blood single-platelet counting merits assessment as a means for monitoring treatment and for determining the clinical importance of inhibition of microaggregation.

Acknowledgments

We are grateful to Novartis Pharmaceuticals Ltd for providing the hirudin, Revasc. We are also grateful to COR Therapeutics, Merck Sharp and Dohme, and Glaxo for supplies of eptifibatide, MK-0852, and GR144053, respectively, and to P.H. Riley for statistical support.

References


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