Anticoagulants (Thrombin Inhibitors) and Aspirin Synergize With P2Y\textsubscript{12} Receptor Antagonism in Thrombosis

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**Background**—This study was designed to determine whether (1) P2Y\textsubscript{12} antagonism synergizes with other antithrombotics and (2) anticoagulants (thrombin inhibitors) affect the antithrombotic activity elicited by P2Y\textsubscript{12} antagonism.

**Methods and Results**—Thrombosis was achieved by perfusion of human and murine blood through type III collagen-coated capillaries at arterial shear rate. CT50547, a direct-acting P2Y\textsubscript{12} antagonist, inhibited thrombosis in PPACK- but not heparin-anticoagulated human blood. In contrast, CT50547 inhibited thrombosis in aspirin-treated individuals independently of the anticoagulant. Thrombin and TXA\textsubscript{2} also synergized with P2Y\textsubscript{12} in the absence of anticoagulation, because combined treatment of aspirin or C921-78 (a factor Xa inhibitor) with CT50547 or 2-MeSAMP (a P2Y\textsubscript{12} antagonist) inhibited the thrombotic process, whereas all treatments failed to inhibit thrombosis when used individually. Synergism was also observed ex vivo when P2Y\textsubscript{12}-deficient (P2Y\textsubscript{12}\textsuperscript{−/−}) mice were administered aspirin or coagulation inhibitors (C921-78 and bivalirudin). Finally, using intravital microscopy, we found that both C921-78 and bivalirudin abrogated the thrombotic process in P2Y\textsubscript{12}\textsuperscript{−/−} mice, whereas each showed only partial efficacy in P2Y\textsubscript{12}\textsuperscript{+/+} animals.

**Conclusions**—Our study indicates that (1) thrombin inhibitors and aspirin have a demonstrable synergy of antithrombotic activity with P2Y\textsubscript{12} antagonism and (2) the in vitro analysis of the antithrombotic activity of P2Y\textsubscript{12} antagonists is affected by the anticoagulant used for blood collection. This suggests that the antithrombotic potential of P2Y\textsubscript{12} antagonists in vitro may be overestimated in anticoagulated samples of blood and best achieved in vivo by the inclusion of aspirin and/or a thrombin inhibitor. (Circulation. 2003;108:2697-2703.)

**Key Words:** anticoagulants ■ thrombosis ■ receptors, purinergic P2 ■ synergism

Definitive studies on monitoring the antithrombotic activities of antiplatelet agents used in clinical settings have proved difficult to obtain. Indeed, analyses of samples from patients receiving these drugs are typically performed in anticoagulated blood. Given the uncertainties of the effects of anticoagulants on the activities of antiplatelet agents,\textsuperscript{1-3} it is not clear how such in vitro analysis relates to in vivo activities.

An example of the problems encountered in the pharmacodynamic measurement of antiplatelet agents occurs with antagonists of P2Y\textsubscript{12}, the ADP receptor on platelets targeted by clopidogrel and ticlopidine. Results from the Clopidogrel in Unstable angina to prevent Recurrent ischemic Events (CURE)\textsuperscript{4} and Randomized Evaluation in PCI Linking Angio-max to reduced Clinical Events (REPLACE)-2 trials\textsuperscript{5} demonstrate that clopidogrel, combined with additional antiplatelet agents, provides a therapeutic benefit. It is not known, however, whether the antithrombotic activities of each of these agents is additive or synergistic to achieve the observed outcomes.\textsuperscript{6} In addition, the contribution of P2Y\textsubscript{12} antagonism to the overall antithrombotic activity is controversial.\textsuperscript{7,8}

Recent in vitro attempts were equivocal in assessing the antithrombotic activity of AR-C69931MX, a titratable P2Y\textsubscript{12} antagonist.\textsuperscript{9,10} The present study uses a novel thrombosis assay to determine whether P2Y\textsubscript{12} antagonism synergizes with other antithrombotics, including aspirin and thrombin inhibitors. In addition, we determined whether anticoagulants affected the in vitro analysis of antithrombotic activity achieved by P2Y\textsubscript{12} antagonism.

**Methods**

**Blood Collection and Human In Vitro Perfusion Chamber**

Whole blood, obtained from healthy volunteers who denied having taken aspirin or other platelet function inhibitors in the preceding week, was anticoagulated with either 300 µmol/L PPACK or 10 U/mL heparin. In some experiments, blood was incubated for 15 minutes at 37°C with various concentrations of CT50547 (C1330-7, a specific reversible P2Y\textsubscript{12} inhibitor)\textsuperscript{11,12} or eptifibatide (Millennium Pharmaceuticals Inc). To reveal a synergistic effect of a P2Y\textsubscript{12} antagonist/aspirin combination, some patients were prescribed 2 chewable tablets of 325 mg aspirin 2 hours before blood collection. Blood was then perfused for 4 minutes through human type III
fibrillar collagen–coated capillaries (557 μm diameter) at 750 s⁻¹ (750 μL/min flow rate).13,14

Platelet Aggregation in Platelet-Rich Plasma
For platelet-rich plasma (PRP) turbidimetry, a Kowa AG-10E aggregation analyzer (Kowa Company Ltd) was calibrated with platelet-poor plasma, and the stirring speed was set at 1000 rpm. Aliquots (300 μL) of PRP were placed in cuvettes containing magnetic stirrer bars. Aggregation of HEPEPS-Tyrode’s P2Y₁₂ antagonist–treated (100 μmol/L 2-MeSAMP, Sigma) or P2Y₁₂ (100 μmol/L 2-MeSAMP)+P2Y₁, antagonist–treated (100 μmol/L MRS2179, Sigma) PRP was induced by either ADP (10 μmol/L) or type III collagen (1 and 10 μg/mL).

Human Ex Vivo Perfusion Chamber
The human capillary perfusion chamber was described recently.14 Nonanticoagulated blood was immediately incubated with various concentrations of CT50547, 2-MeSAMP, or C921-78 (a factor Xa inhibitor)15 and perfused for 4 minutes through human type III collagen–coated capillaries (557 μm in diameter) at 1000 s⁻¹. In 2 sets of experiments, blood donors were prescribed either 325 mg chewable aspirin on the day before and 2 hours before perfusion chamber or just 2 hours before perfusion. Then, nonanticoagulated blood was immediately incubated with or without 100 μmol/mL 2-MeSAMP and perfused through the chambers. All subjects gave written informed consent to the protocol, which was approved by the local Human Subjects Committee (Millennium Pharmaceuticals Inc).

Determination of TAT and Fₐ+₂ Levels in Whole Blood
Nonanticoagulated blood was drawn from healthy male or female volunteers (T=0 minutes). Aliquots were collected at T=1 minute (beginning of the perfusion) and T=5 minutes (end of the perfusion), and immediately treated with a quench solution (citric acid, 7.3 mg/mL; sodium citrate, 22 mg/mL; dextrose, 24.5 mg/mL; EDTA, 2.23 mg/mL; adenosine, 1.60 mg/mL; heparin, 22 U/mL; and hirudin, 25 U/mL). Plasma fractions were obtained by centrifugation at 4°C for 15 minutes at 1600g and stored at −80°C before use. Determination of human thrombin–antithrombin III complex (TAT) and Fₐ+₂ fragment was made by use of immunoenzymoassays according to the manufacturer’s instructions (Enzygnost-TAT micro and Enzygnost F₁, micro, Dade Behring).

Murine Ex Vivo Perfusion Chamber
Nonanticoagulated blood was collected from the vena cava of anesthetized mice and perfused for 2.5 minutes directly through either type III collagen–coated (Sigma) or tissue factor–coated (Thromborel, Behring) capillary chambers (282 or 345 μm diameter for 1700 and 871 s⁻¹, respectively).13 P2Y₁₂−/− mice were generated as described.16 In some experiments, mice were infused with aspirin (10 mg/kg, Aspegic, Sanofi-Synthelabo), C921-78 at 0.5 mg/kg, CT51464 (a GP IIb/IIIa inhibitor, 100 mg/kg per oral gavage, Millennium Pharmaceuticals Inc), and/or bivalirudin (2 mg/kg; The Medicines Company) 1 hour before perfusion. All procedures conformed to institutional guidelines and to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, Md). A minimum of 6 mice were used for each group studied.

Establishment of the Thrombosis Profile in the Perfusion Chamber Model
Nonanticoagulated or PPACK-anticoagulated human blood was perfused through type III collagen–coated capillary chambers at 1500 or 750 s⁻¹ for 4 minutes. After staining of the thrombotic deposits with toluidine blue for 45 seconds, an En Face picture located 5 mm downstream of the proximal part of the capillary was taken. Measurement of the gray level of each thrombus or platelets located in a window 400 μm long × 250 μm wide was performed, and results were expressed as mean±SEM using the Simple PCI software (Compix Inc Imaging System). Measurement of the mean thrombus volume (μm³/μm²) was performed at the same location on sections as described by Hainaud et al (Figure 1). Mean thrombus volume was expressed by use of Simple PCI software and plotted against the corresponding mean gray level. By using data from both human ex vivo and in vitro (by titrating with the GP IIb/IIIa antagonist epifibatide) experiments at different shear rates in capillaries of similar diameter (557 μm), we established a thrombotic profile that was then used for a rapid measurement of the thrombus volume in subsequent experiments.

For murine perfusion chamber experiments, thrombus volume was analyzed on semithin cross sections and by mean gray level measurements using a thrombotic profile specific for the diameter of the capillary (282 and 345 μm) and established with nonanticoagulated blood from untreated P2Y₁₂−/−, P2Y₁₂−/−, and aspirin-treated P2Y₁₂−/− mice.

Intravital Microscopy
Intravital microscopy on mouse mesenteric arteries (with a shear rate of 1000 to 1400 s⁻¹) was performed as described,5,16 except that rhodamine 6G (0.2 mg/mL) was used to directly label platelets in vivo. Rhodamine 6G was administered through the tail vein 10 minutes before visualization of the arteries. Vessel-wall injury was generated (for 7 minutes) by a 2×1-mm filter paper saturated with a 12.5% FeCl₃ solution. Platelet vessel-wall interactions were recorded for 40 additional minutes or until full occlusion occurred and lasted for more than 20 seconds. P2Y₁₂−/− and P2Y₁₂−/− mice were injected with saline, bivalirudin (2 mg/kg), or C921-78 (3 μmol/L) 15 minutes before FeCl₃-induced injury.

Video Analysis
Platelet vessel-wall interactions and thrombus formation were analyzed in real time with Simple PCI software.16 In summary, the mean
fluorescence intensity at the site of vessel-wall injury parallels the kinetics of the thrombotic process, because all platelets are labeled by rhodamine 6G. The mean fluorescence intensity is recorded at 2 frames per second for 50 minutes and plotted versus time. The kinetics of the thrombotic process are expressed as a curve representing the mean±SEM of all experiments performed for each group (a minimum of 6 animals per group).

Statistical Analysis
Analysis was performed using the standard Student’s t test. For analysis of the synergism in the nonanticoagulated human blood and murine collagen–coated perfusion chamber experiments, 1-way ANOVA analysis was performed with Tukey’s and Dunnett’s multiple comparison tests, respectively. All values were expressed as mean±SEM.

Results
Thrombosis Measurements
Preliminary studies were performed using incremental doses of eptifibatide, a GP IIb/IIIa antagonist, to establish a rapid, reliable quantification of thrombus volume using mean gray levels in a capillary perfusion chamber (Figure 1).

Effect of Anticoagulants and Aspirin on P2Y12 Antagonism
As shown in Figure 2, 20 μmol/L of the P2Y12 antagonist CT50547 had minimal effect on thrombus formation in human blood anticoagulated with heparin but markedly inhibited thrombosis in blood from the same donor anticoagulated with PPACK. Aspirin alone or in combination with CT50547 decreased the mean thrombus volume in blood anticoagulated with either PPACK or heparin (Figure 2).

Modulation of Platelet Aggregation by a P2Y12 Antagonist in Heparin- or PPACK-Anticoagulated Human Blood
2-MeSAMP 100 μmol/L exhibited a greater efficacy in inhibiting platelet aggregation in PPACK-anticoagulated PRP than in heparin-anticoagulated PRP (Figure 3A). A similar extent of inhibition was achieved with 50 μmol/L 2-MeSAMP (data not shown). We also found that the combination of P2Y12/P2Y1 antagonism totally abrogated the aggregation induced by 10 μmol/L ADP. Platelet aggregation induced by type III fibrillar collagen (10 μg/mL) was partially inhibited by 100 μmol/L 2-MeSAMP in both anticoagulated groups, whereas the same concentration of P2Y12 antagonist fully inhibited collagen (1 μg/mL)–induced aggregation in the PPACK-PRP and to a lower extent in the heparin-PRP (Figure 3, B and C).

Synergism Between P2Y12 Antagonism and Factor Xa Inhibition in Nonanticoagulated Human Blood
To study the effects of factor Xa on thrombosis in human blood, the perfusion system was modified to use nonanticoagulated blood. Because this perfusion was initiated 1 minute after blood collection and the time of perfusion was 4...
minutes, we first determined whether thrombin was generated during this time by measuring the TAT and F_{1/2} levels in whole blood. F_{1/2} levels did not increase between the first and fifth minutes (0.402 ± 0.027 versus 0.382 ± 0.05 nmol/L, respectively) and were within the normal range of values cited by the manufacturer. There was a slight but not significant increase in TAT levels at 5 minutes (0.0255 ± 0.0058 nmol/L) versus 1 minute (0.013 ± 0.0023 nmol/L), and these values were within the normal range of 0.01 and 0.04 nmol/L. This allowed us to study the antithrombotic activities of P2Y_{12} antagonists and factor Xa inhibitors under nonanticoagulated conditions without a significant activation of the coagulation cascade.

Neither the P2Y_{12} antagonists nor the factor Xa inhibitor prevented thrombus formation over type III collagen under arterial shear rate (Figure 4) when used separately. However, arterial thrombogenesis was dramatically affected by their combination (Figure 4). As reported previously with similar ex vivo perfusion chambers, a single dose of aspirin administration (650 mg taken 2 hours before perfusion chamber) did not affect the thrombotic process (Aspirin A, Figure 4). However, when aspirin was administered for 2 consecutive days (Aspirin B, Figure 4), a significant decrease in mean thrombus volume was revealed (P<0.05 versus untreated).

**Effect of Thrombin Inhibition and Aspirin on Thrombosis in P2Y_{12}−/− Mice**

The mean thrombus volume formed on collagen-coated surfaces by P2Y_{12}−/− and P2Y_{12}+/− mice was similar at 1700 s⁻¹ (Figure 5A), although thrombi formed from P2Y_{12}−/− mice appeared loosely packed (Figure 5B). Similar results were observed when the thrombotic process was studied under normal arterial shear rate (871 s⁻¹). We next examined the effects of aspirin, C921-78, and bivalirudin on thrombosis in both mouse genotypes. Each agent resulted in a significant inhibition of thrombus volume in P2Y_{12}−/− mice (Figure 5A), whereas none had any significant effect in wild-type mice. Bivalirudin inhibited the thrombus volume in both WT and P2Y_{12}−/− mice, but aspirin was without effect on tissue factor at 871 s⁻¹ (Figure 6). The GP IIb/IIIa antagonist CT51464 inhibited thrombosis on both collagen and tissue factor surfaces (Figures 5A and 6).

**Thrombin Inhibitors Heighten the Phenotype of P2Y_{12}−/− Mice In Vivo**

In a FeCl₃-injury model in mesenteric arteries, previous data have established that P2Y_{12}−/− mice present a cyclic throm-
platelet aggregation. The thrombotic process (with no occlusion), whereas P2Y12−/− mice exhibited an intermediate phenotype. Similar results were obtained when platelets were labeled in vivo with rhodamine 6G (Figure 7). Bivalirudin (2 mg/kg) and C921-78 (3 μmol/L) delayed both the time for appearance of first thrombus and the time for occlusion in P2Y12−/− arteries, although neither prevented vessel occlusion (Figure 7). However, the same treatment with either the thrombin inhibitor or the factor Xa inhibitor abolished thrombus formation in P2Y12−/− mice. We noted that the thrombotic process reappeared 40 minutes after the beginning of the injury on bivalirudin treatment, most likely reflecting the clearance of the drug. The factor Xa inhibitor C921-78 (3 μmol/L) abrogated the thrombotic process in P2Y12−/− arteries for the whole visualization period. Bivalirudin (7.5 mg/kg) injected into P2Y12−/− animals gave a similar profile, with a thrombotic rebound occurring after 45 minutes (data not shown).

Discussion

The present study was designed to model the antithrombotic activities of P2Y12 antagonists, aspirin, anticoagulants, and GP IIb/IIIa antagonists at shear rates designed to cover those encountered within the arterial circulation. To initiate thrombosis, perfusion chambers were coated either with fibrillar collagen, a potent platelet agonist, or tissue factor, the initiator of coagulation. To assess the efficacy of the combination of thrombin inhibition and P2Y12 antagonism in vivo, thrombosis in mesenteric arteries of P2Y12−/− mice was initiated by ferric chloride and monitored using intravital microscopy with or without thrombin inhibitor treatment. Using the perfusion chamber assay with fibrillar collagen as a thrombogenic surface, we demonstrated a marked synergy between P2Y12 antagonism and aspirin in humans. This synergy was expected because of the improved clinical properties of the P2Y12 antagonists, which in combination with aspirin, led to a marked reduction in thrombus formation. However, in vivo, the thrombotic process was more complex, as it was not completely abrogated by either the thrombin inhibitor or the factor Xa inhibitor. Bivalirudin, on the other hand, was effective in abolishing thrombus formation, but the thrombotic rebound was observed after 45 minutes, possibly due to the clearance of the drug. The factor Xa inhibitor C921-78, on the other hand, was able to completely abrogate the thrombotic process in vivo, suggesting that it may be a better candidate for clinical use in this setting.
benefit achieved by including aspirin with clopidogrel therapy.\(^4\,19\) We also demonstrated a strong synergy in antithrombotic activity when either a direct thrombin inhibitor or a factor Xa inhibitor was coupled with inhibition of P2Y\(_{12}\). More importantly, the inhibitors of coagulation abolished the thrombotic process in vivo in the P2Y\(_{12}\) mice, inhibition that was more effective than that reported for aspirin in vivo (the synergism resulting from chronic aspirin therapy in P2Y\(_{12}\) mice remains to be established).\(^16\) Such synergy was not observed when thrombosis was induced by tissue factor, the thrombotic process being modulated by thrombin inhibitors and GP IIb/IIIa antagonism but not by the lack of P2Y\(_{12}\). Tissue factor is known to exist within atherosclerotic plaques and to become exposed on plaque rupture. In addition, an alternatively spliced soluble form of tissue factor may be induced in response to inflammatory stimuli, which may in turn colocalize with growing thrombi and even act as an agonist of the thrombotic process.\(^20\) Because tissue factor is an active component of growing thrombi, the present data suggest that the clinical benefit of P2Y\(_{12}\) antagonism may be more pronounced when used in combination with inhibitors of coagulation or GP IIb/IIIa antagonists than with aspirin.

The mechanism responsible for the synergism between either aspirin or anticoagulants and P2Y\(_{12}\) antagonism is of interest. It is now known that most primary platelet agonists, ie, collagen acting on GP VI, von Willebrand factor on GP Ib/IX/V, thrombin on PAR-1 and PAR-4 (on human platelets), or ADP on P2Y\(_1\) and P2Y\(_{12}\), are capable of directly activating the receptor function of GP IIb/IIIa to induce a maximal platelet aggregation response. In addition, signaling by these receptors causes the secretion of ADP, which also participates in GP IIb/IIIa activation. Accordingly, the relatively limited effect on thrombus growth with only P2Y\(_{12}\) antagonism is expected. Likewise, because multiple receptors are capable of inducing full GP IIb/IIIa activation, it is also not surprising that both aspirin and direct thrombin inhibitors, used as single agents, have only moderate antithrombotic activity. What, then, accounts for the strong synergy between P2Y\(_{12}\) signaling and signaling that can be affected by thrombin inhibition and aspirin? Because suboptimal concentrations of many primary platelet agonists (eg, thrombin, collagen) require the activity of secreted ADP to achieve maximal platelet stimulation, one might predict that P2Y\(_{12}\) antagonism would be additive with either thrombin inhibition or aspirin. Synergism was observed in both human and mouse models and may have originated from the complementation of P2Y\(_{12}\), PAR-1, and the thromboxane A2 receptor, all of which are heterotrimeric G protein–coupled. Emerging data suggest that optimal platelet stimulation by such receptors requires simultaneous signaling by G\(_i\) and G\(_a\) pathways. This requirement is satisfied by the 2 ADP receptors: P2Y\(_{12}\), coupled to G\(_{a2}\) and P2Y\(_1\), coupled to G\(_i\). In support of this, Turner et al\(^10\) showed that the simultaneous inhibition of both P2Y\(_1\) and P2Y\(_{12}\) was required to efficiently inhibit thrombosis in heparin-anticoagulated blood. Because both PAR-1 and the thromboxane A2 receptor couple to both G\(_i\) and G\(_{12/13}\), it is likely that the mechanisms responsible for the synergies described here are linked to requirements of the G\(_i\) signaling by P2Y\(_{12}\), which synergizes with G\(_i\) coupling of the thromboxane A2 and thrombin receptors.

The synergy between P2Y\(_{12}\) and anticoagulants also affects pharmacodynamic measurements of the antithrombotic activities by P2Y\(_{12}\) antagonism. Previous studies have established that citrate anticoagulation enhances the platelet aggregation inhibitory activity of GP IIb/IIIa antagonists, a contributing factor in the underdosing of eptifibatide in the initial clinical trials of this drug and possibly other GP IIb/IIIa antagonists.\(^3\) The present study shows that the antithrombotic activity achieved by P2Y\(_{12}\) antagonism is greater in blood anticoagulated with PPACK than with heparin, demonstrating that the ex vivo observations on synergy between anticoagulants and P2Y\(_{12}\) found in the P2Y\(_{12}\) mouse are relevant to in vitro pharmacodynamic measurements. The same synergism might account for the weak thrombotic profile of a patient who lacks the P2Y\(_{12}\) receptor,\(^22\) because blood was doubly PPACK/ pentasaccharide anticoagulated. These observations reveal that the most reliable method of assessing platelet function is to avoid the use of anticoagulants. The imaging system described here, which allows for the rapid (<15 minutes after blood perfusion) quantification of thrombosis of native blood under defined shear rates and defined thrombogenic surfaces (a prerequisite, because antithrombotic activities vary with the thrombogenic surface), may represent a suitable tool to monitor the efficacy and adjust antplatelet therapies used to prevent or treat cardiovascular events.

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References

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