G-Protein–Coupled Receptors as Signaling Targets for Antiplatelet Therapy


Abstract—Platelet G protein–coupled receptors (GPCRs) initiate and reinforce platelet activation and thrombus formation. The clinical utility of antagonists of the P2Y12 receptor for ADP suggests that other GPCRs and their intracellular signaling pathways may represent viable targets for novel antiplatelet agents. For example, thrombin stimulation of platelets is mediated by 2 protease-activated receptors (PARs), PAR-1 and PAR-4. Signaling downstream of PAR-1 or PAR-4 activates phospholipase C and protein kinase C and causes autoamplification by production of thromboxane A2, release of ADP, and generation of more thrombin. In addition to ADP receptors, thrombin and thromboxane A2 receptors and their downstream effectors—including phosphoinositol-3 kinase, Rap1b, talin, and kindlin—are promising targets for new antiplatelet agents. The mechanistic rationale and available clinical data for drugs targeting disruption of these signaling pathways are discussed. The identification and development of new agents directed against specific platelet signaling pathways may offer an advantage in preventing thrombotic events while minimizing bleeding risk. (Arterioscler Thromb Vasc Biol. 2009;29:449-457.)

Key Words: platelets ■ signaling ■ G proteins ■ receptors ■ thrombosis

Platelets are required for normal hemostasis. They are also an essential component of arterial thrombosis occurring after atherosclerotic plaque rupture, the pathological trigger of most acute coronary syndromes (ACS). Since the first observations of agonist-induced platelet aggregation in 1962,1 remarkable progress has been made in identifying cell surface receptors and intracellular signaling pathways that regulate platelet function. These discoveries have translated into established, new, and emerging therapeutics to treat and prevent acute ischemic events by targeting platelet signal transduction. Indeed, antiplatelet therapy is a mainstay of initial management of patients with ACS and those undergoing percutaneous coronary intervention (PCI). Evidence-based refinements in anticoagulant and antiplatelet therapies have played an important role in the progressive decline in the death rate from coronary disease observed from 1994 to 2004.2 Despite these therapeutic advances, however, ACS patients receiving “optimal” antithrombotic therapy still suffer cardiovascular events.

Given the benefits of agents targeting specific platelet surface receptors (Table 1),3-18 substantial basic and translational investigation is underway to identify new targets and agents for disrupting platelet-associated signaling pathways. Many new agents targeting platelet G protein–coupled receptors (GPCRs) are in clinical trials, and intracellular pathways mediating GPCR signaling will likely be the next generation of targets. This article represents an integrated summary of presentations given at the Third Annual Platelet Colloquium, held in San Antonio, Texas on January 25 to 26, 2008, which focused on the current state of platelet receptor-related research with particular emphasis on GPCR signaling (Table 2).19-25 For additional information about approved and experimental antiplatelet agents, please see supplemental Tables I through III (available online at http://atvb.ahajournals.org).

Platelet Signaling Pathways

Vascular injury—whether caused by spontaneous rupture of atherosclerotic plaque, plaque erosion, or PCI-related or other trauma—exposes adhesive proteins, tissue factor, and lipids promoting platelet tethering, adsorption, and activation. Once bound and activated, platelets release soluble mediators such as thrombin and substances that promote further platelet aggregation.

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*Participants in the 2008 Platelet Colloquium are listed in the Appendix.
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as ADP, thromboxane A₂, and serotonin and facilitate thrombin generation. These mediators, in turn, stimulate GPCRs on the platelet surface (Figure 1)²⁶–²⁸ that are critical to initiation of intracellular signaling pathways, including activation of phospholipase C (PLC), protein kinase C (PKC), and phosphoinositide (PI)-3 kinase. Both calcium and PKC contribute to activation of the small G protein, Rap1b, which, through interactions with the Rap1-GTP interacting adapter molecule (RIAM) and talin, are important for binding of fibrinogen and other multimeric ligands to integrin αIIbβ3, an essential event for platelet aggregation. Recently, members of the kindlin family of focal adhesion proteins have been identified as integrin activators,²⁹–³¹ perhaps functioning to facilitate talin–integrin interactions.

Platelet activation is also associated with coagulation protease assembly, microparticle production, platelet secretion, fibronectin matrix assembly, leukocyte recruitment, platelet protein translation, and clot retraction.³² The benefits of aspirin, which irreversibly inhibits the cyclooxygenase (COX)-1–mediated production of thromboxane A₂ by platelets,³³ and of antagonists of the platelet P2Y₁₂ receptor for ADP¹⁵–¹⁸ strongly support the clinical utility of strategies designed to target platelet GPCR signaling systems (Table 1).

### Platelet-Directed Therapies Targeting ADP Signaling

#### Purinergic Signaling in Platelets

ADP was the first molecule shown to stimulate platelet aggregation.³ It is contained within platelet granules, and its release after initial platelet activation at local sites of injury further activates nearby circulating platelets and stabilizes aggregates as they form.³³ Thus, ADP may function primarily in an autocrine loop to perpetuate platelet responses.

Extracellular receptors for purine and pyrimidine nucleotides (P₂ purinergic receptors) include the P₂X ion channel family and the P₂Y GPCR family. Platelets possess both types of receptors. The P₂Y family is activated by several ligands, including ATP, ADP, UTP, UDP, and UDP-glucose,³⁴ and 8 subtypes have been cloned. In the cardiovascular system, these receptors are prevalent in vascular endothelial cells, smooth muscle cells, leukocytes, and platelets and are respectively associated with vasodilation, vasoconstriction, inflammation, and hemostasis.³⁵ Two of the cloned receptors appear to be responsible for platelet ADP responses: P₂Y₁, which is coupled to Gq and mediates the calcium response, and P₂Y₁₂, which is coupled to Gi and lowers intraplatelet cAMP levels.³⁶,³⁷ ADP-mediated P₂Y₁₂ receptor activation plays a critical role in facilitating responses to other agonists,³³ partly through alleviating suppression by high concentrations of cAMP and partly through stimulation of phosphoinositol kinase-3 (PI-3K)-dependent pathways.³⁸ Signaling through P₂Y₁₂ and, to a lesser extent, P₂Y₁ receptors, contributes to activation of Rap1b, a critical regulator of integrin affinity for fibrinogen.³⁹,⁴⁰

#### Preclinical Data Supporting the Use of P₂Y₁₂ Inhibitors as Antithrombotic Therapy

Mice lacking the P₂Y₁₂ receptor were generated by 2 groups.⁴¹,⁴² These mice display a prolonged bleeding time and delayed and unstable thrombus formation in a mesenteric artery injury model. The absence of an effect of oral clopidogrel and prasugrel in mice lacking P₂Y₁₂ provided confirmatory evidence that receptor was the target for thienopyridine drugs.⁴¹,⁴³

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### Table 1. Current Therapies Targeting Various Platelet Signaling Pathways: Clinical Outcomes

<table>
<thead>
<tr>
<th>Target</th>
<th>Signaling Pathways</th>
<th>Existing Agents</th>
<th>Outcomes (Relative Risk Reduction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclooxygenase</td>
<td>Production of thromboxane A₂</td>
<td>Aspirin</td>
<td>20%–25% RRR vs no treatment in acute MI⁴ and vs placebo for secondary prevention⁴</td>
</tr>
<tr>
<td>Glycoprotein Ibb/IIa</td>
<td>Final mediator of platelet aggregation; elicits “outside-in” signaling</td>
<td>Abciximab</td>
<td>~30% RRR vs placebo for 30-day MI, death, or urgent revascularization after PCI⁵–⁷</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eptifibatide</td>
<td>~15% RRR vs placebo for 30-day death, MI, or other MACE after ACS⁹,⁹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tirofiban</td>
<td>~15% RRR vs placebo for 30-day death, MI, or revascularization after planned PCI for ACS¹⁰</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prasugrel</td>
<td>~30% RRR vs heparin for death, MI, or refractory ischemia at 48 hours¹¹ and 7 days¹² after ACS</td>
</tr>
<tr>
<td>Phosphodiesterase</td>
<td>Degradation of cyclic nucleotides (cGMP, cAMP)</td>
<td>Dipyridamole</td>
<td>Improvement in claudication at 16 weeks¹³</td>
</tr>
<tr>
<td>P₂Y₁₂ receptor</td>
<td>G₁-mediated signaling</td>
<td>Clopidogrel</td>
<td>Improvement in 24-week maximum walking distance¹⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ticlopidine</td>
<td>~9% RRR vs aspirin for stroke, MI, or vascular death in secondary prevention¹⁵</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>~18% RRR vs placebo for 12-month CV death, nonfatal MI, or stroke after ACS¹⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>~7% RRR vs placebo for 28-day mortality¹⁷</td>
</tr>
</tbody>
</table>

Results are shown for the highest dose of antiplatelet agent tested vs the control. ACS indicates acute coronary syndromes; CV, cardiovascular; MACE, major adverse cardiac events; MI, myocardial infarction; PCI, percutaneous coronary intervention; RRR, relative risk reduction.
Current Options for P2Y<sub>12</sub> Inhibition
The 2 P2Y<sub>12</sub> antagonists approved for use in the United States, clopidogrel and ticlopidine, are of proven benefit in ACS patients, including those undergoing PCI (Table 1). Both are members of the thienopyridine class and irreversibly inhibit P2Y<sub>12</sub> receptors. Depending on the definition and analytic method, up to 33% of patients receiving clopidogrel or ticlopidine may show “resistance” or “partial responsiveness” to these drugs, although the clinical implications of this phenomenon remain poorly understood. As prodrugs, they require metabolism by the cytochrome P450 system, and activity of this hepatic enzyme system may partly account for observed variability in inhibition of platelet aggregation.

P2Y<sub>12</sub> Inhibitors in Development
Prasugrel, a third-generation thienopyridine compound, has a faster onset of action and inhibits ADP-induced platelet aggregation more uniformly and robustly compared with clopidogrel. The TRial to assess Improvement in Therapeutic Outcomes by optimizing platelet inhibition with prasugrel – Thrombolysis In Myocardial Infarction (TRITON-TIMI) 38 trial, which randomized 13 608 patients with moderate- to high-risk ACS scheduled to undergo PCI to receive either prasugrel or clopidogrel, provided clinical evidence that greater inhibition of platelet aggregation with prasugrel reduces thrombotic events, albeit with an increased risk of bleeding. In a prespecified analysis of net clinical benefit, the findings favored prasugrel, with 138 ischemic events prevented for every 1000 patients treated with prasugrel versus clopidogrel (hazard ratio [HR], 0.81; 95% confidence interval [CI], 0.73–0.90; \( P<0.001 \)), at a cost of 35 additional major bleeding events (HR, 1.32; 95% CI, 1.03–1.68; \( P=0.03 \)). The “optimal” doses of prasugrel and clopidogrel are now being assessed in additional Phase 3 trials.

Cangrelor, a rapidly acting, reversible ATP analog, is a direct P2Y<sub>12</sub> receptor inhibitor available only in intravenous formulation. The addition of cangrelor to ex vivo blood samples from patients receiving clopidogrel resulted in near-complete inhibition of platelet aggregation. In 2 small Phase 2 studies of ACS patients not undergoing PCI, cangrelor given with aspirin and heparin was safe and well tolerated at doses achieving \( 95\% \) inhibition of platelet aggregation in response to ADP. The “optimal” doses of prasugrel and clopidogrel are now being assessed in additional Phase 3 trials.

AZD6140 is the first direct, reversible P2Y<sub>12</sub> inhibitor developed for oral administration. Although AZD6140 requires no metabolism to achieve inhibition of platelet aggregation, its active metabolite may contribute to its pharmacodynamic effects. Two Phase 2 studies of AZD6140 have been completed.

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Table 2. Clinical Outcomes With New Antiplatelet Agents

<table>
<thead>
<tr>
<th>Phase</th>
<th>n</th>
<th>Population</th>
<th>Comparator</th>
<th>Outcomes with Antiplatelet Agent vs Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P2Y&lt;sub&gt;12&lt;/sub&gt; Inhibitors</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prasugrel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JUMBO-TIMI</td>
<td>2</td>
<td>904</td>
<td>Elective or urgent PCI</td>
<td>Clopidogrel</td>
</tr>
<tr>
<td>TRITON-TIMI</td>
<td>3</td>
<td>13 608</td>
<td>Moderate-to-high-risk ACS, planned PCI</td>
<td>Clopidogrel</td>
</tr>
<tr>
<td><strong>Cangrelor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Storey</td>
<td>2</td>
<td>39</td>
<td>NSTE ACS</td>
<td>(Dose-ranging)</td>
</tr>
<tr>
<td>Jacobsson</td>
<td>2</td>
<td>94</td>
<td>NSTE ACS</td>
<td>Placebo</td>
</tr>
<tr>
<td><strong>AZD6140</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Husted</td>
<td>2</td>
<td>201</td>
<td>Atherosclerosis</td>
<td>Clopidogrel</td>
</tr>
<tr>
<td><strong>DISPERSE-2</strong></td>
<td>2</td>
<td>990</td>
<td>NSTE ACS not undergoing PCI</td>
<td>Clopidogrel</td>
</tr>
<tr>
<td><strong>PAR-1 Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCH 530348</td>
<td>2</td>
<td>1031</td>
<td>ACS scheduled for angiography, possible PCI</td>
<td>Placebo</td>
</tr>
</tbody>
</table>

*Major or minor bleeding not related to bypass surgery, assessed by the Thrombolysis In Myocardial Infarction (TIMI) scale.

Data are shown for the highest dose of antiplatelet agent tested vs control unless otherwise indicated. CI indicates confidence interval; HR, hazard ratio; NSTE, non-ST-segment elevation; UA, unstable angina; other abbreviations as in Table 1.

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Smyth et al. Signaling Targets for Antiplatelet Agents

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AZD6140 showed greater inhibition of platelet aggregation, less variability in response, and no increase in bleeding compared with clopidogrel\textsuperscript{23}; however, dyspnea and ventricular pauses occurred more frequently with high-dose AZD6140.\textsuperscript{24} Results of an ongoing Phase 3 study of AZD6140, (PLATO; ClinicalTrials.gov identifier: NCT00391872) should clarify the overall clinical efficacy and safety of this drug in patients with ACS.

PRT060128, a direct reversible P2Y\textsubscript{12} receptor inhibitor with a novel structure,\textsuperscript{48} is the first P2Y\textsubscript{12} antagonist developed in both oral and intravenous formulations. The intravenous form was assessed in a recently completed randomized, double-blind, placebo-controlled Phase 2 trial (ERASE-MI; ClinicalTrials.gov identifier: NCT00546260).

**Future Directions: P2Y1 and P2X Inhibition**

Given the clinical success of the P2Y\textsubscript{12} antagonists, it is worthwhile to investigate other purinergic signaling pathways in platelets. Although platelets have 2 P2Y receptors acting synergistically through different signaling pathways, the overall platelet response to ADP is relatively modest. For example, ADP alone elicits only reversible responses and does not promote platelet secretion.\textsuperscript{33} The low number of ADP receptors on the platelet surface also may limit signaling. A platelet contains only 500 to 1000 binding sites for 2-methylthio-ADP (1 third P2Y\textsubscript{1}; 2 thirds P2Y\textsubscript{12}).\textsuperscript{49} This is about half the number of receptors for potent agonists such as thrombin and thromboxane A\textsubscript{2}. Transgenic mice over-expressing the P2Y\textsubscript{1} receptor have shorter bleeding times, and their platelets show heightened aggregation in response to ADP and collagen costimulation and increased ADP-induced granule secretion.\textsuperscript{50} Whether variable expression levels of the P2Y\textsubscript{1} receptor on platelets might contribute to differing thrombotic phenotypes among humans has not yet been explored. In mice, the selective P2Y\textsubscript{1} antagonist MRS2500 reduces laser-induced thrombosis in the mesenteric artery and intravascular platelet thrombosis in response to a collagen-adrenaline infusion, with only moderately prolonged bleeding times.\textsuperscript{51}

The P2X receptors are cation channels—their structure includes 2 transmembrane domains, and, to form a channel, they associate in trimers. When activated by ATP, the P2X\textsubscript{i} receptor triggers calcium entry into platelets and induces minor morphological changes. The hallmark of this receptor is its contribution to shear-induced platelet aggregation.\textsuperscript{52} In a murine model of systemic thromboembolism, intravenous injection of NF449, an experimental P2X\textsubscript{i} receptor inhibitor, was associated with reduced intravascular platelet aggregation without prolongation of the bleeding time.\textsuperscript{53}

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**Figure.** Role of G protein–coupled receptors in the thrombotic process. In humans, protease-activated receptors (PAR)-1 and PAR-4 are coupled to intracellular signaling pathways through molecular switches from the \( G_{\alpha} \), \( G_{i12} \), and \( G_{i3} \) protein families. When thrombin (scissors) cleaves the amino-terminal of PAR-1 and PAR-4, several signaling pathways are activated, one result of which is ADP secretion. By binding to its receptor, P2Y\textsubscript{12}, ADP activates additional \( G_{i} \)-mediated pathways. In the absence of wounding, platelet activation is counteracted by signaling from PG \(_{i2}\) (PGI\(_{2}\)). Adapted from references 26–28 with permission. Ca\(^{2+}\) indicates calcium; CalDAG-GEF1, calcium and diacylglcerol-regulated guanine-nucleotide exchange factor 1; GP, glycoprotein; IP, prostacyclin; PKC, protein kinase C; PLC, phospholipase C; RIAM, Rap1-GTP–interacting adapter molecule.
**Antiplatelet Therapies Targeting Thrombin Signaling**

**Thrombin Signaling in Platelets**

Thrombin, the most potent platelet agonist, has diverse effects on various vascular cells. For example, thrombin promotes chemotaxis, adhesion, and inflammation through its effects on neutrophils and monocytes. Thrombin also influences vascular permeability through its effects on endothelial cells and triggers smooth muscle vasconstriction and mitogenesis.54

Thrombin interacts with 2 protease-activated receptors (PARs) on the surface of human platelets—PAR-1 and PAR-4. Signaling through the PARs is triggered by thrombin-mediated cleavage of the extracellular domain of the receptor and exposure of a “tethered ligand” at the new end of the receptor (Figure 1).55 Signaling through either PAR can activate PLC and PKC and cause autoamplification through the production of thromboxane A2, the release of ADP, and generation of more thrombin on the platelet surface.

**Preclinical Data Supporting the Use of PAR-1 Antagonists as Antithrombotic Therapy**

The expression profiles of PARs on platelets differ between humans and nonprimates. Mouse platelets lack PAR-1 and largely signal through PAR-4 in response to thrombin, with PAR-3 serving a cofactor function. Platelets from cynomolgus monkeys contain primarily PAR-1 and PAR-4, and a peptide-mimetic PAR-1 antagonist extends the time to thrombosis after carotid artery injury.56 The nonpeptide antagonist SCH 530348 (described below) inhibits thrombin- and PAR-1 agonist peptide (TRAP)-induced platelet aggregation (inhibitory concentrations of 47 nmol/L and 25 nmol/L, respectively), but it has no effect on ADP, collagen, U46619, or PAR-4 agonist peptide stimulation of platelets.57 SCH 530348 has excellent bioavailability in rodents and monkeys (82%; 1 mg/kg) and completely inhibits ex vivo platelet aggregation in response to TRAP within 1 hour of oral administration in monkeys with no effect on prothrombin or activated partial thromboplastin times.58

**PAR-1 Inhibitors in Development**

Of the PAR-1 antagonists, SCH 530348 and E5555 are the compounds farthest along in development and clinical testing. SCH 530348 is an oral reversible PAR-1 antagonist derived from hirudin, a compound found in the bark of the Australian magnolia tree.58 In clinical trials, 68% of patients showed ≥80% inhibition of platelet aggregation in response to thrombin receptor activating peptide (TRAP; 15 μmol/L) 60 minutes after receiving a 40-mg loading dose of SCH 530348. By 120 minutes, the proportion had risen to 96%.25 In a Phase 2 trial of SCH 530348, 1031 patients scheduled for angiography and possible stenting were randomized to receive SCH 530348 or placebo plus aspirin, clopidogrel, and antithrombin therapy (heparin or bivalirudin).25 Major and minor bleeding did not differ substantially between the placebo and individual or combined SCH 530348 groups (Table 2). Although the study was not powered to detect differences in clinical end points, there was a trend toward a lower incidence of major adverse cardiac events (MACE) with increasing doses of SCH 530348 versus placebo (8.5% for 10 mg, 5.0% for 20 mg, and 4.0% for 40 mg SCH 530348 versus 8.6% for placebo, no probability value given), largely attributable to reductions in nonfatal periprocedural MI. Two Phase 3 trials are underway to determine whether adding a PAR-1 antagonist to standard therapy can prevent ischemic events (TRA•CER; ClinicalTrials.gov identifier: NCT00527943 and (TRA-2°P - TIMI 50; ClinicalTrials.gov identifier: NCT00526474).

A second PAR-1 antagonist, E5555, is being assessed in 3 randomized, double-blind, placebo-controlled, Phase 2 trials. These studies will assess safety and tolerability of E5555 versus placebo in patients with low- and high-risk ACS (ClinicalTrials.gov identifiers: NCT00619164, NCT00548587, and NCT00312052).

**Future Directions: PAR-4 Inhibition**

Activation and signaling of PAR-1 and PAR-4 provoke a biphasic “spike and prolonged” response, with PAR-1 activated at thrombin concentrations ~50% lower than those required to activate PAR-4.59 A 4-amino acid segment, YEFP, on the extracellular domain of PAR-1 appears to account for the receptor’s high-affinity interactions with thrombin. The YEFP sequence has homology to the COOH-terminal of hirudin and its synthetic GEPF analog, bivalirudin, which can interact with exosite-1 on thrombin. Thus, thrombin may interact in tandem with PAR-1 and PAR-4, with the initial interactions involving exosite-1 and PAR-1, and subsequent docking at PAR-4 via the thrombin active site.56 PAR-1 and PAR-4 may form a stable heterodimer that enables thrombin to act as a bivalent functional agonist, rendering the PAR-1–PAR-4 heterodimer complex a unique target for novel antithrombotic therapies.

Pepducins, or cell-permeable peptides derived from the third intracellular loop of either PAR-1 or PAR-4, disrupt signaling between the receptors and G proteins and inhibit thrombin-induced platelet aggregation. In mice, a PAR-4 pepducin has been shown to prolong bleeding times and attenuate platelet activation.60 Combining bivalirudin with a PAR-4 pepducin (P4pal-i1) inhibited aggregation of human platelets from 15 healthy volunteers, even in response to high concentrations of thrombin.56 In addition, although bivalirudin and P4pal-i1 each delayed the time to carotid artery occlusion after ferric chloride-induced injury in guinea pigs, their combination prolonged the time to occlusion more than did bivalirudin alone. Additional blockade of the PAR-4 receptor may confer a benefit beyond that achieved by inhibition of thrombin activity.

**Antiplatelet Therapies Targeting Thromboxane Signaling**

Thromboxane A2 acts on the thromboxane A2/prostaglandin (PG) H2 (TP) receptor, causing PLC signaling and platelet activation. Several drugs have been tested and developed that prevent thromboxane synthesis—most notably, aspirin. Beyond the documented success of aspirin, however, results have been uniformly disappointing with a wide variety of thromboxane synthase inhibitors.61

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 Likewise, a multitude of TP receptor antagonists have been developed, but few have progressed beyond Phase 2 trials because of safety concerns. More recently, the thromboxane $A_2$ receptor antagonist terutroban (S18886) showed rapid, potent inhibition of platelet aggregation in a porcine model of in-stent thrombosis that was comparable to the combination of aspirin and clopidogrel but with a more favorable bleeding profile. Ramatroban, another TP inhibitor approved in Japan for treatment of allergic rhinitis, has shown antiaggregatory effects in vitro comparable to those of aspirin and cilostazol.

**Novel Downstream Signaling Targets**

Signaling pathways stimulated by GPCR activation are essential for thrombus formation and may represent potential targets for drug development. One pathway involved in platelet activation is signaling through lipid kinases.

PI-3 kinases transduce signals by generating lipid secondary messengers, which then recruit signaling proteins to the plasma membrane. A principal target for PI-3K signaling is the protein kinase Akt (Figure 1). Platelets contain both the Akt1 and Akt2 isoforms. In mice, both Akt1 and Akt2 are required for thrombus formation. Mice lacking Akt2 have aggregation defects in response to low concentrations of thrombin or thromboxane $A_2$ and corresponding defects in dense and $\alpha$-granule secretion. The Akt isoforms have multiple substrates in platelets. Glycogen synthase kinase (GSK)-3$\beta$ is phosphorylated by Akt in platelets and suppresses platelet function and thrombosis in mice. Akt-mediated phosphorylation of GSK-3$\beta$ inhibits the kinase activity of the enzyme, and with it, its suppression of platelet function. Akt activation also stimulates nitric oxide production in platelets, which results in protein kinase G–dependent degranulation. Finally, Akt has been implicated in activation of cAMP-dependent phosphodiesterase (PDE3A), which plays a role in reducing platelet cAMP levels after thrombin stimulation. Each of these Akt-mediated events is expected to contribute to platelet activation.

Rap1 members of the Ras family of small G proteins have been implicated in GPCR signaling and integrin activation. Rap1b, the most abundant Ras GTPase in platelets, is activated rapidly after GPCR stimulation and plays a key role in the activation of integrin $\alpha_{IIb}\beta_3$. Stimulation of G$_i$-linked receptors, such as PAR-4 or PAR-1, activates PLC and, with consequent increases in intracellular calcium, PKC. These signals in turn activate calcium and diacylglycerol-regulated guanine-nucleotide exchange factor 1 (CalDAG-GEF1), which has been implicated in activation of Rap1 in platelets. Experiments in CalDAG-GEF1-deficient platelets indicate that PKC- and CalDAG-GEF1–dependent events represent independent synergistic pathways leading to Rap1-mediated integrin $\alpha_{IIb}\beta_3$ activation. Consistent with this concept, ADP can stimulate Rap1b activation in a P2Y$_{12}$- and PI-3K-dependent, but calcium-independent, manner.

A final common step in integrin activation involves binding of the cytoskeletal protein talin to the integrin $\beta$-subunit cytoplasmic tail. Rap1 appears to be required to form an activation complex with talin and the Rap effector RIAM, which redistributes to the plasma membrane and unmasksthe talin binding site, resulting in integrin activation. Mice that lack Rap1b or platelet talin have a bleeding disorder with impaired platelet aggregation because of the lack of integrin $\alpha_{IIb}\beta_3$ activation. In contrast, mice with a $\beta_3$ subunit mutation that prevents talin binding have impaired agonist-induced platelet aggregation and are protected from thrombosis, but do not display pathological bleeding, suggesting that this interaction may be an attractive therapeutic target. Recently, members of the kindlin family of focal adhesion proteins, kindlin-2 and kindlin-3, have been identified as coactivators of integrins, required for talin activation of integrins. Kindlin-2 binds and synergistically enhances talin activation of $\alpha_{IIb}\beta_3$. Of note, deficiency in kindlin-3, the predominant kindlin family member found in hematopoietic cells, results in severe bleeding and protection from thrombosis in mice.

**Conclusions**

Antiplatelet therapy targeting thromboxane production, ADP effects, and fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ have proven benefit in preventing or treating acute arterial thrombosis. New agents that provide greater inhibition of ADP signaling and agents that impede thrombin’s actions on platelets are currently in clinical trials. Emerging strategies to inhibit platelet function include blocking alternative platelet GPCRs and their intracellular signaling pathways.

The challenge remains to determine how to best combine the various current and pending antiplatelet therapies to maximize benefit and minimize harm. It is well documented that aspirin therapy increases bleeding compared with placebo; that when clopidogrel is added to aspirin therapy, bleeding increases relative to the use of aspirin therapy alone; and that when even greater P2Y$_{12}$ inhibition with prasugrel is added to aspirin therapy, bleeding is further increased compared with the use of clopidogrel and aspirin combination therapy. Does this mean that improved antiplatelet efficacy is mandated to come at the price of increased bleeding? Not necessarily, but it will require a far better understanding of platelet signaling pathways and what aspects of platelet function must be blocked to minimize arterial thrombosis.

One of the best clinical examples of the disconnect between antiplatelet-related bleeding and antithrombotic efficacy is the case of the oral platelet glycoprotein (GP) IIb/IIIa antagonists. The use of these agents uniformly led to significantly greater bleeding compared with aspirin but no greater efficacy; in fact, mortality was increased among patients receiving the oral glycoprotein IIb/IIIa inhibitors. Through an improved understanding of platelet signaling pathways, antiplatelet therapies likely can be developed not based on their ability to inhibit platelets from aggregating, as current therapies are, but rather based on their ability to prevent the clinically meaningful consequences of platelet activation. What exactly these are remains the greatest obstacle.

**Appendix**

**Participants in the 2008 Platelet Colloquium**

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