Abstract—The critical roles of integrins in thrombosis have enabled the successful development and clinical use of the first generation of integrin antagonists as represented by abciximab (Reopro), eptifibatide (Integrilin), and tirofiban (Aggrastat). These integrin α\textsubscript{IIb}β\textsubscript{3} antagonists are not only potent antithrombetics but also have significant side effects. In particular, their induction of ligand-induced integrin conformational changes is associated with thrombocytopenia. Increased bleeding risk prevents integrin antagonists from being used at higher doses and in patients at risk for bleeding. To address the ligand-induced conformational changes caused by current integrin antagonists, compounds that minimally induce conformational changes in integrin α\textsubscript{IIb}β\textsubscript{3} have been developed. Recent studies on the mechanisms of integrin signaling suggest that selectively targeting integrin outside-in signaling mechanisms allows for potent inhibition of thrombosis, while maintaining hemostasis in animal models. (Arterioscler Thromb Vasc Biol. 2015;35:24-29. DOI: 10.1161/ATVBHA.114.303411.)

Key Words: blood platelets ▪ integrins ▪ platelet aggregation inhibitors ▪ thrombosis

Integrins, a family of cell adhesion receptors, play important roles in cell adhesion, spreading, retraction, migration, anchorage-dependent survival, and proliferation. Integrins exist as an αβ heterodimeric complex of transmembrane proteins. In blood platelets, the most abundant integrin is integrin α\textsubscript{IIb}β\textsubscript{3}. Integrin α\textsubscript{IIb}β\textsubscript{3} binds to fibrinogen through the HHLGGAKQAV sequence in the C terminus of the fibrinogen γ chain and RGD sequences in the α chain. RGD-like sequences are also present in several other integrin-binding adhesive proteins, including vitronectin, fibronectin, and von Willebrand factor. In addition, platelets express integrins α\textsubscript{IIb}β\textsubscript{1}, α\textsubscript{IIb}β\textsubscript{3}, α\textsubscript{IIb}β\textsubscript{1}, and α\textsubscript{IIb}β\textsubscript{1}, among which α\textsubscript{IIb}β\textsubscript{1} and α\textsubscript{IIb}β\textsubscript{1} also recognize the RGD sequence. Integrin α\textsubscript{IIb}β\textsubscript{1} and α\textsubscript{IIb}β\textsubscript{1} bind to collagen and laminin. 1 By binding to adhesive proteins, the integrins mediate platelet adhesion to injured vascular walls and platelet aggregation, which is important for the maintenance of hemostasis, preventing excessive bleeding. The importance of integrin α\textsubscript{IIb}β\textsubscript{3} in hemostasis is exemplified in patients having Glanzmann thrombasthenia, in which genetic deficiencies in integrin α\textsubscript{IIb}β\textsubscript{3} causes bleeding diathesis. 2 Integrin α\textsubscript{IIb}β\textsubscript{3} is critical for arterial thrombosis, 3 which is evident by the protective effects seen in experimental models of thrombosis using either pharmacological inhibition or genetic deletion/mutation of integrin α\textsubscript{IIb}β\textsubscript{3} 4,5 and by the clinical efficacy of α\textsubscript{IIb}β\textsubscript{3} antagonists 5,6. However, despite successful clinical use of integrin antagonists as potent antithrombotics, their use is primarily limited to patients undergoing percutaneous coronary intervention, mainly because of significant bleeding risk. In fact, increased bleeding risks are a major problem shared by all currently available antithrombotic drugs. In this review, we briefly discuss the major problems associated with the currently used integrin antagonists, and new advances in developing the next generation of integrin antagonists.

Current α\textsubscript{IIb}β\textsubscript{3} Integrin Antagonists

The 3 current Food and Drug Administration–approved platelet integrin antagonists are designed to block the ligand-binding function of integrin α\textsubscript{IIb}β\textsubscript{3}. Among these drugs, abciximab (Reopro) is a 48 kDa mouse/human chimeric antibody fragment that binds to an epitope near the ligand-binding site of β\textsubscript{3}, 6-12; eptifibatide (Integrilin) is a 832-Da synthetic disulfide-linked cyclic heptapeptide ligand mimetic, containing an integrin-binding sequence, KGĐ, based on a snake venom peptide, barbourin 9,12–14; tirofiban (Aggrastat) is a 495-Da synthetic compound, engineered to mimic the RGD sequence. 9,12,14–16 Both eptifibatide and tirofiban are integrin ligand mimetics, which interact with the ligand-binding site of integrin α\textsubscript{IIb}β\textsubscript{3}. 1,2 Tirofiban seems to be specific for α\textsubscript{IIb}β\textsubscript{3}, eptifibatide inhibits α\textsubscript{IIb}β\textsubscript{3} and α\textsubscript{IIb}β\textsubscript{3}, and abciximab inhibits α\textsubscript{IIb}β\textsubscript{3}, α\textsubscript{IIb}β\textsubscript{3}, and α\textsubscript{IIb}β\textsubscript{3}. 12,17,18 All 3 integrin antagonists are administered intravenously. Orally active integrin antagonists were also developed. However, clinical trials of oral integrin antagonists suggested increased mortality instead of beneficial effects. 19,20

The current integrin antagonists have each demonstrated clear therapeutic benefits in high-risk patients undergoing percutaneous coronary intervention, as indicated by significant reductions
in death and reoccurrence of myocardial infarction. There have also been clinical trials studying the effect of integrin antagonist treatment on patients having acute ischemic stroke. Although, these trials to date have been mainly designed for the purpose of determining safety, and thus the therapeutic efficacy in patients with stroke is yet to be conclusively established. In these trials, α<sub>IIb</sub>β<sub>3</sub> antagonist treatment alone showed no beneficial effect on mortality or debilitating stroke-related outcomes<sup>21,22</sup> but increased the incidence of symptomatic or fatal intracranial hemorrhage.<sup>21,23</sup> with the exception of a trial of tirofiban.<sup>24</sup> In the tirofiban trial, no significant difference in hemorrhage was found between placebo and tirofiban groups although the placebo group had significantly more patients also treated with aspirin, which may influence the outcome. Some clinical trials tested a combination of fibrinolytic therapy, using recombinant tissue-type plasminogen activator (<0.6 g/kg) alone. Treatment of patients with reduced recombinant tissue-type plasminogen activator doses in combination with an integrin antagonist implicates the investigators’ consideration of potential hemorrhagic risk of the combination therapy.

The benefit of current integrin antagonists over other antiplatelet agents for general antimicrobial therapy is their rapid onset of action, potency, and low interpatient variability.<sup>7,9,14</sup> By contrast, there is significant interpatient variability in response to aspirin (irreversible COX-1 inhibitor) or clopidogrel (P2Y12 inhibitor), mainly because of drug resistance.<sup>7,12,16</sup> However, the potent effects of current integrin antagonists are associated with increased bleeding risk,<sup>25–31</sup> which can be potentially life-threatening. Bleeding risk limits the use and dose of integrin antagonists, and thus also limits their effectiveness.<sup>11,31</sup> Abciximab, tirofiban, and eptifibatide all cause thrombocytopenia, which may be associated with conformational changes of integrins after the binding of these drugs.<sup>32</sup>

### New Inhibitors That Minimally Induce Conformational Changes

**Integrin Structure and Conformations**

Both α and β chains of the α<sub>IIb</sub>β<sub>3</sub> complex contain a long extracellular region, a single-pass transmembrane region, and a short cytoplasmic tail. The amino terminal region of the α and β chains interact to form what is known as the head, which contains the ligand-binding pocket where a conserved structural motif, known as the metal ion-dependent adhesion site, is critical.<sup>33</sup> In α<sub>IIb</sub>β<sub>3</sub>, the metal ion-dependent adhesion site is on the β<sub>3</sub>-subunit and thought to stabilize ligand binding by coordinating a metal ion with the aspartic acid on RGD-containing ligands.<sup>34</sup> Some other integrin α-subunits contain an additional domain called the interactive domain (I-domain), which also contain a metal ion-dependent adhesion site.<sup>12</sup> Below the head region are 2 long legs: in α<sub>IIb</sub>, 2 calf domains and a thigh domain constitute a leg; whereas in β<sub>3</sub>, 4 integrin epidermal growth factor–like domains, 2 hybrid domains, and a plexin semaphorin integrin domain form the other leg.

Integrin molecules undergo conformational changes on receptor activation and ligand binding.<sup>34,35</sup> Integrin α<sub>IIb</sub>β<sub>3</sub> is kept in a resting (low-affinity) state in normal circulation, preventing undesirable thrombus formation. This state is maintained by interactions between the α and β chains within the transmembrane and membrane proximal cytoplasmic domains, which constrain the ectodomain.<sup>35</sup> The resting state has been suggested to correspond to the bent conformation as revealed in crystal structure and electron microscopy.<sup>33,38–40</sup> Integrin activation induces the separation of α and β transmembrane and cytoplasmic domains and unbending of the ectodomain, resulting in an extended active conformation.<sup>41,42</sup> The extended conformation with a closed configuration, wherein the β head and hybrid domain form an acute angle, represents an active intermediate affinity state, which is recognized by the RGD sequence or HHLGGAKQAGV sequence in fibrinogen. Binding of ligand recognition sequences induces further conformational changes, resulting in an open head piece conformation, which is the high-affinity state<sup>33,38,39,41–44</sup> (Figure 1A–C). Between these major conformational states, 6 different intermediate states have also been suggested, based on crystal structures of the ectodomain of α<sub>IIb</sub>β<sub>3</sub>.<sup>43</sup> Electron microscopy studies using intact α<sub>IIb</sub>β<sub>3</sub> in a nanodisc suggest different pictures of bent and extended conformations.<sup>40,44</sup> Different from models obtained from crystal structures of intact ectodomains, electron microscopy analyses of intact α<sub>IIb</sub>β<sub>3</sub> show that the resting integrin headpiece points away from the membrane and that the intermediate extended integrin conformation contains crossed legs.<sup>40,43</sup> The differences in models of resting and activated integrin structure are possibly because of the contribution of the transmembrane/cytoplasmic domains to integrin conformation.<sup>39</sup> The ligand-induced

### Figure 1. Conformational states during integrin activation and ligand binding. A, The resting (low-affinity) state is maintained by interactions between the α and β chains within the transmembrane and membrane proximal cytoplasmic domains, which constrain the ectodomain. B, The activated (intermediate-affinity) state has an extended conformation with a closed configuration. C, Binding of ligand recognition sequences induces further conformational changes, resulting in an open high-affinity state. Curved black arrow indicates the swing-out motion of the β<sub>3</sub>-subunit hybrid domain on full integrin activation. TM indicates transmembrane domain.

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**Nonstandard Abbreviation and Acronym**

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<td>SFK</td>
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conformational changes are physiologically important because they (1) expose new epitopes and binding sites on integrins (ligand-induced binding sites) ⁴⁶,⁴⁷; (2) enable the initial interaction of resting integrins with the exposed RGD-like sequence in certain ligands (such as immobilized fibrinogen) to transform integrins into a high-affinity form (ligand-induced integrin activation), bypassing the need for inside-out signaling ⁴⁷; and (3) are important for integrin clustering and outside-in signaling. ⁴⁸,⁴⁹

**Integrin Antagonists That Minimally Affect Integrin Conformation**

Tirofiban and epifibatide are RGD mimetics and thus cause ligand-induced conformational changes, ⁴⁹,⁵⁰ resulting in exposure of ligand-induced binding sites and ligand-induced integrin activation ⁵⁰ although these monomeric RGD-like peptides or compounds in general do not seem to induce integrin outside-in signaling directly. ⁴⁷,⁴⁹,⁵² The conformational changes induced by ligand mimetic antagonists are thought to be important for the adverse effect of thrombocytopenia. ⁵³ Abciximab also induces ligand-binding induced binding sites and thrombocytopenia. ⁵³,⁵⁴ The activation ⁵¹ although these monomeric RGD-like peptides or compounds in general do not seem to induce integrin outside-in signaling. ⁴⁷,⁴⁹,⁵² The conformational changes induced by ligand mimetic antagonists are thought to be important for the adverse effect of thrombocytopenia. ⁵³ Abciximab also induces ligand-binding induced binding sites and thrombocytopenia. ⁵³,⁵⁴ The ability of these antagonists to induce an active conformation of integrins carries the risk of possible prothrombotic effects after antagonist dissociation. ⁵⁰,⁵² There were some reports of such antagonist-induced prothrombotic effects. ⁵⁰,⁵²,⁵⁵ Recently, new small-molecule integrin antagonists have been developed that exhibit increased specificity and potency without exposing β₃ ligand-induced binding sites epitopes. ⁵⁶,⁵⁷ RUC-1 and its more potent derivative RUC-2 inhibit the ligand-binding function of integrins, platelet aggregation, and in vivo thrombus formation, and importantly they do not induce integrin activation. ⁵⁶,⁵⁷ RUC-1 interacts with α₃β₃, whereas RUC-2 seems to interact with β₃Mg²⁺-coordinating sites. Interestingly, unlike RUC-1 and current integrin antagonists, RUC-2 competes with Mg²⁺ for binding to the β₃-subunit, and its inhibitory effects are attenuated by adding exogenous Mg²⁺. ⁵⁷

**Targeting Integrin Signaling**

**Inside-Out Signaling**

Platelets circulating in blood vessels are normally in a resting state and become activated and adherent only when exposed to the site of vascular injury or platelet agonists. Platelet agonists elicit platelet activation signals via various receptor-mediated intracellular signaling pathways. ⁵⁸ These intracellular signals converge to transform αmβ₃ from a resting state to an activated state. ⁵⁸ This process is called inside-out signaling (Figure 2). A key requirement for integrin inside-out signaling is the induction of the binding of talin to the membrane proximal half of the β₃ cytoplasmic domain, which includes an important NPXY motif. Talin-binding induces unclamping of the transmembrane and cytoplasmic domains of α₃ and β₃ and thus integrin activation. ⁵⁹-⁶² This talin-dependent integrin activation is facilitated by kindlin, which interacts with the C-terminal region of the β₃ cytoplasmic domain. ⁶²,⁶³ It is conceivable that disruption of talin/kindlin binding to integrin α₃β₃ or disruption of the signal responsible for the induction of talin/kindlin binding would also inhibit integrin activation and thus thrombus formation, as evidenced by talin1 gene deletion or mutations. ⁶³,⁶⁴

**Inhibitors of Inside-Out Signaling**

Current platelet inhibitors, including ADP receptor antagonists (eg, clopidogrel), cyclooxygenase inhibitors (eg, aspirin), and thrombin receptor inhibitors (eg, vorapaxar), primarily exert their effects by inhibiting early receptor signaling pathways that initiate inside-out signaling and integrin activation. ¹⁶ Pharmacological inhibition of inside-out signaling was demonstrated with cell-permeable peptides containing talin-binding sequences. ⁶⁵,⁶⁶ A cell-permeable peptide corresponding to α₃β₃ residues 1000 to 1008 important in talin binding and β₃ interaction was also used to inhibit integrin activation. ⁶⁷ Because inhibition of inside-out signaling results in the loss of the activation of the ligand-binding

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**Figure 2.** Inside-out and outside-in signaling of the platelet integrin α₃β₃. A detailed description of individual agonist-induced platelet activation pathways is given by Li et al. ⁹⁹ CalDAG-GEF1, calcium and diacylglycerol-regulated guanine nucleotide exchange factor 1; DAG indicates diacylglycerol; G13, guanosine nucleotide-binding protein α subunit 13; GAP, GTPase-activating protein; GEF, Guanine nucleotide exchange factor; GPCRs, G-protein-coupled receptors; IP3, inositol triphosphate; ITAM, immunoreceptor tyrosine-based activation motif; PKC, protein kinase C; PLC, phospholipase C; PI3K, phosphoinositide 3-kinase; Rho, Rho GTPase; Rap1, Rap1-GTP interacting adapter molecule; SFK, src family kinase; Syk, spleen tyrosine kinase; and TXA₂, thromboxane A₂.
function of integrins, it is expected that the inhibitors of inside-out signaling should show characteristics similar to that of integrin antagonists, which inhibit both thrombosis and hemostasis. Indeed, talin1 deletion or mutational disruption of talin-binding site (β1 L746A) protected mice from thrombosis, but they still displayed impaired hemostasis as shown by prolonged tail bleeding times in these mice. However, one report suggests that partial inhibition of talin binding to the integrin β1 NPYX motif caused defective thrombus formation, with only minor bleeding side effect. It remains to be investigated whether partial inhibition of c-Src–binding RGT sequence in the cytoplasmic domain. Deletion of the c-Src–binding RGT sequence in the β3-subunit abolishes platelet spreading without affecting inside-out signaling.76 Gt13 binding to β3 occurs only during early phase outside-out signaling. Gt13 binds to an ExE motif located near talin-binding sites of β3. However, the ExE motif is not required for talin binding, and Gt13-binding is not involved in integrin activation. Thus, suppression of Gt13 expression or disruption of the Gt13-binding site in β3 selectively inhibits the early phase of outside-in signaling responsible for stabilization and amplification of a thrombus but does not affect inside-out signaling or the ligand-binding function of integrins.

**Outside-In Signaling**

Ligand binding to integrins not only mediates platelet adhesion and primary aggregation but also induces signal transduction into cells that triggers the activation of vast intracellular signaling networks and cytoskeleton reorganization. This process is known as outside-in signaling. Outside-in signaling leads to a series of cellular responses, including platelet spreading, stable adhesion, granule secretion, and clot retraction, which greatly amplify platelet aggregation and thrombus size (Figure 2).

Several protein tyrosine kinases have been shown to be important in outside-in signaling, including focal adhesion kinase, ILK, and Syk. Src family kinases (SFK). In particular, integrin β3-bound c-Src is now recognized as a key early signaling molecule. After integrin ligation, c-Src has been shown to phosphorylate 2 NXXY motifs in the β tail and induce activation of the phosphoinositide 3-kinase pathway, inhibition of RhoA, and the activation of the Syk-immune receptor tyrosine-based activation motif pathway. Src-dependent transient inhibition of RhoA and the activation of phosphoinositide 3-kinase is necessary for platelet spreading on integrin ligands. The phosphoinositide 3-kinase pathway and the Syk-immune receptor tyrosine-based activation motif pathway stimulate granule secretion. Tyrosine phosphorylation in β3 may also help recruit phosphorytosine-binding proteins, such as SHC (Src homology 2 domain-containing protein) and myosin heavy chain. Phosphorylation at Y522 also regulates talin binding and thus the direction and dynamics of integrin signaling, and phosphorylation at Y570 protects β3 from calpain cleavage. These events are important for controlling the switch between platelet spreading and retraction. Interestingly, the role of c-Src requires its interaction with the β3 cytoplasmic domain. Deletion of the c-Src–binding RGT sequence in the C terminus of β3 abolished the ability of c-Src to mediate cell spreading even when constitutively active c-Src was expressed. Thus, it seems that targeting c-Src binding to integrins may selectively inhibit integrin outside-in signaling. This notion is supported by a study using β3-RGT-deleted integrin-expressing mice, which are defective in platelet responses associated with outside-in signaling, and protected against arterial thrombosis, but display only a mild defect in inside-out signaling. The most proximal signaling mediator of outside-in signaling identified to date is Gt13. Gt13 directly interacts with an ExE motif in the cytoplasmic domain of integrin β-subunits, and this binding is required for c-Src activation and Src-dependent outside-in signaling. Gt13 binding to β3 occurs only during early phase outside-in signaling. Gt13 binds to an ExE motif located near talin-binding sites of β3. However, the ExE motif is not required for talin binding, and Gt13-binding is not involved in integrin activation. Thus, suppression of Gt13 expression or disruption of the Gt13-binding site in β3 selectively inhibits the early phase of outside-in signaling responsible for stabilization and amplification of a thrombus but does not affect inside-out signaling or the ligand-binding function of integrins.

**Selective Inhibitors of Outside-In Signaling**

Recent conceptual advances in integrin outside-in signaling reveal the potential in developing selective inhibitors of integrin outside-in signaling as new antithrombotic drugs. A major advantage for targeting outside-in signaling is that inhibition of outside-in signaling should not affect primary platelet adhesion and aggregation, which is critical for hemostasis, but should limit the size of a thrombus to prevent vessel occlusion (Figure 3). Our laboratory has recently demonstrated the potential of such an approach with a myristoylated ExE motif peptide that selectively inhibits Gt13-integrin interaction. This inhibitor selectively inhibits outside-in signaling, platelet spreading, and the second wave of platelet aggregation without affecting primary platelet aggregation. Importantly, this inhibitor potently inhibits occlusive thrombosis in mouse models in vivo without affecting bleeding time, unlike eptifibatide, which dramatically prolongs bleeding time. Thus, selective inhibitors of outside-in signaling as a new antithrombotic strategy have the potential to inhibit arterial thrombosis without causing excessive hemorrhage selectively. However, it is still important to consider and investigate potential off-target effects caused by selective targeting of Gt13.

Because SFK is a required signal downstream of Gt13 in outside-in signaling, inhibitors of SFK could potentially be effective inhibitors. However, SFK play multiple roles in platelets and other cells. For example, SFK is important in the immunoreceptor tyrosine-based activation motif pathway and GPIb-IX signaling pathways, and thus is important in inside-out signaling, which limits the value of SFK inhibitors as selective outside-in signaling inhibitors. However, blocking the interaction between SFK and integrins may selectively inhibit outside-in signaling. A myristoylated peptide inhibitor derived from the c-Src–binding sequence of β3 abolished platelet spreading without affecting ADP-induced fibrinogen binding. However, disruption of the...
β, c-Src–binding site in mice not only provided protection from thrombosis but also affected hemostasis mildly.76

Conclusions

All current integrin antagonists function by blocking the binding of integrin ligands.5,12,16 These inhibitors induce conformational changes in integrins, which are associated with thrombocytopenia and possibly other adverse effects. New inhibitors with minimal conformational effects may potentially help resolve this issue. A major problem associated with the current integrin antagonists is that at doses where they exhibit high potency they also increase the risk of hemorrhage. Emerging evidence suggests that selective inhibition of outside-in signaling has the potential to have potent antithrombotic effects without causing bleeding.

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Disclosures

Dr Du, University of Illinois, Chicago, holds patents relevant to the topic of this review. The others authors report no conflicts.

References


**Targeting Integrin Signaling in Thrombosis**

Estevé et al
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Brian Estevez, Bo Shen and Xiaoping Du

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