A New Model of Dual Interacting Ligand Binding Sites on Integrin αIIbβ3

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Dana D. Hu§, Carol A. White§, Susan Panzer-Knollet§, James D. Page§, Nancy Nicholson¶, and Jeffrey W. Smith§

From the §Program on Cell Adhesion, Cancer Research Center, The Burnham Institute, La Jolla, California 92037 and the ¶Department of Cardiovascular Disease Research, Searle, Skokie, Illinois 60077

The platelet integrin αIIbβ3 mediates platelet aggregation and platelet adhesion. This integrin is the key to hemostasis and also to pathologic vascular occlusion. A key domain on αIIbβ3 is the ligand binding site, which can bind to plasma fibrinogen and to a number of Arg-Gly-Asp (RGD)-type ligands. However, the nature and function of the ligand binding pocket on αIIbβ3 remains controversial. Some studies suggest the presence of two ligand binding pockets, whereas others report a single binding pocket. Here we use surface plasmon resonance to show that αIIbβ3 contains two distinct ligand binding pockets. One site binds to fibrinogen, and a separate site binds to RGD-type ligands. More importantly, however, the two ligand binding pockets are interactive. RGD-type ligands are capable of binding to αIIbβ3 even when it is already occupied by fibrinogen. Once bound, RGD-type ligands induce the dissociation of fibrinogen from αIIbβ3. This allosteric cross-talk has important implications for anti-platelet therapy because it suggests a novel approach for the dissolution of existing platelet thrombi.

Integrins are noncovalently associated αβ heterodimers that serve as a primary link between the extracellular matrix and the cytoplasm (1–4). Integrins contribute to the structural integrity of cells and tissues by providing the physical contact between a cell and the matrix. However, integrins are also involved in bidirectional signaling events that greatly influence development, angiogenesis, wound repair, and a variety of pathological conditions. Although considerable progress has been made toward identifying the members in the integrin protein family and assigning their physiological ligands, there are several biochemical properties of integrins for which the underlying principles are not well understood.

First, many integrins exhibit a very broad ligand binding specificity (4, 5). Some integrins, even those that bind to the RGD tripeptide adhesion motif, can bind to ligands that lack an RGD sequence. For example, the two β3 integrins, αIIbβ3 and αβ1, can bind to as many as 13 different ligands representing several protein families. Furthermore, some integrins can bind to adhesive proteins and to proteases, two classes of ligands with seemingly opposing functions (6–9). It is not known how binding to ligands with such disparate functions is coordinated or regulated.

Second, ligand binding to integrins can depend on activation. Signals transmitted from the cytoplasm can activate the integrin ligand binding function in the ectodomain, resulting in a proadhesive phenotype (2, 10–12). However, integrins can bind to some ligands in the absence of cellular stimulation. Such binding is often referred to as “activation-independent.” Clearly then, there are two separable mechanisms of ligand binding.

Third, when integrins bind their ligands, signals are transmitted in the opposite direction, or “outside-in.” These signals can ultimately alter cellular physiology and gene expression (13–16). Recent work suggests that outside-in signaling can depend upon the type of ligand bound to integrin (17). The biochemical basis for these ligand-dependent differences in signaling is not understood.

One hypothesis that would have a major bearing on each of these issues suggests that integrins contain multiple ligand binding pockets (18). In fact, there is considerable circumstantial evidence to support this hypothesis. However, the existence of two ligand binding sites remains uncertain, particularly because there is little information to explain mechanistically how two such sites would interact, if at all.

To test the two-site hypothesis, we examined the ligand binding function of the platelet integrin αIIbβ3, an integrin that binds to a number of different ligands. The ligands for αIIbβ3 can be grouped into two general categories. One class of ligands contains the well known RGD integrin binding motif (19, 20). The other class of ligands is represented solely by fibrinogen. Fibrinogen binds to αIIbβ3 through a non-RGD sequence present in its γ-chain (21–23). Both types of ligands have physiologic importance for regulating platelet adhesion to the subendothelial matrix (24, 25) and in mediating aggregation with other platelets to form a thrombus (26).

There is a considerable discrepancy regarding the nature of the ligand binding pocket on αIIbβ3. Some studies imply that the two types of ligands could bind to distinct sites on αIIbβ3 (27, 28), whereas other reports indicate the existence of a common, or overlapping, binding pocket (29, 30). A third hypothesis argues that the receptor contains a single binding pocket that can have different depths (31). Most of the ligand binding studies on αIIbβ3 have been performed under equilibrium conditions and show that the two types of ligands competitively inhibit the binding of each other. However, these prior measurements failed to distinguish between the two

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§ Supported in part by a fellowship from the California Affiliate of the American Heart Association and by a fellowship from the U. S. Army Breast Cancer Program. Present address: Monsanto Co., Discovery Pharmacology, Mail Code AA3C, 700 Chesterfield Village Parkway N., St. Louis, MO 63198.

¶ Established investigator of the American Heart Association and Genentech. To whom correspondence should be addressed: Program on Cell Adhesion, Cancer Research Center, The Burnham Inst., 10901 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 619-646-3121; Fax: 619-646-3192; E-mail: jsmith@ljcrf.edu.
mechanisms of competitive inhibition, same site competitive inhibition versus allosteric competitive inhibition (32). Here we apply real time kinetic analysis to show that $\alpha_{IIb}\beta_3$ contains distinct and interacting ligand binding sites.

**MATERIALS AND METHODS**

**Purified Ligand Receptor Binding Measurements—**Competition binding experiments were performed with purified $\alpha_{IIb}\beta_3$ according to methods we have previously published (33). Briefly, $\alpha_{IIb}\beta_3$ purified from platelet lysates by RGD affinity chromatography (34) was immobilized in the wells of microtiter plates. Radiolabeled ligand was added to the immobilized $\alpha_{IIb}\beta_3$ along with competing ligand until the binding had reached equilibrium (empirically determined to be 90 min). Free ligand was removed by extensive washing, and bound ligand was solubilized using boiling 1 x NaOH. Each sample was transferred to a glass 12 x 77-mm tube, and the bound ligand was quantified by $\gamma$ counting. Each experiment was performed at least three times, and all points are the average of triplicate measurements. Data were analyzed and plotted using SigmaPlot™. Double-reciprocal plots were generated using the 95% confidence interval of a least squares analysis.

**Platelet Ligand Binding Studies—**Equilibrium binding studies were performed on freshly isolated human platelets (35). Platelets were purified from platelet-rich plasma by gel filtration on Sepharose CL-4B equilibrated in Tyrode’s buffer containing 2 mM MgCl$_2$, 0.1% bovine serum albumin, and 0.1% glucose, pH 7.2. For binding studies, platelets were diluted in Tyrode’s buffer containing 0.2 mM CaCl$_2$, 1.8 mM MgCl$_2$, 0.1% bovine serum albumin, and 0.1% glucose to a concentration of 1 x $10^8$ platelets/ml. The binding of radiolabeled ligands was measured on platelets stimulated with 20 $\mu$M ADP at ambient temperature and under equilibrium binding conditions (30 min). Bound and free ligand were separated by centrifugation of the platelets through a cushion of 20% sucrose. The platelet pellet was collected into tubes for $\gamma$ counting or scintillation counting by excising the bottom of the centrifuge tube. Nonspecific binding was typically less than 10% of the total binding of the integrin or the ligand was immobilized to the SPR sensor chip via the amine coupling kit. Only minor differences in dissociation rates were observed when comparing the configuration of the assay was switched. Each sensorgram represents at least five similar repetitions, encompassing at least four batches of purified $\alpha_{IIb}\beta_3$ and three batches of human fibrinogen (Enzyme Research Laboratories). The association ($k_1$) and dissociation ($k_2$) rate constants were calculated from sensorgrams as described (36).

**Platelet Aggregation Experiments—**Turbidimetric aggregometry was performed as described (37) with slight modifications. ADP was used as an agonist at 5 $\mu$M. Three minutes after the addition of agonist to the aggregometer tube, SC 52012 or buffer control was added to the reaction. The disaggregation of the platelets was monitored for an additional 6 min. The ability of Fab-9 to induce disaggregation was measured by adding fibrinogen-coated blue polystyrene beads (6 $\mu$m in diameter) to platelet-rich plasma (38). Following stimulation of a suspension of beads and platelets with 20 $\mu$M ADP, platelets adhered to the beads and simultaneously aggregated. The coupled process was monitored in a microtiter plate reader at 562 nm, which detects the light scattering properties of the blue polystyrene beads.

**RESULTS AND DISCUSSION**

**Modes of Competition between Ligands for a Common Receptor—**Two distinct classes of competitive inhibition between ligands for a common receptor are illustrated in Fig. 1. Simple competitive inhibition, or same-site competitive inhibition (Scheme 1), is a competition between ligands A and B for the same binding pocket. In such a case, one ligand alters the affinity of a receptor for the other ligand. A separate type of competitive inhibition is allosteric, and it is more difficult to identify (Scheme 2). Allosteric inhibitors bind at physically separate sites. Like same-site inhibition, an allosteric inhibitor will change the overall binding affinity of a receptor for its ligand. However, allosteric inhibition differs because the two ligands bind at separate sites, and it may be possible for one ligand to interact with the receptor when the other ligand is already bound. Hence, ligand B could still interact with its binding pocket, even when ligand A is bound at a separate site. Here we show that $\alpha_{IIb}\beta_3$ has two separate binding pockets that interact in an allosteric manner (Scheme 3). The evidence in support of this conclusion is presented below.

**RGD Ligands Competitively Inhibit Fibrinogen Binding to $\alpha_{IIb}\beta_3$—**A series of competition binding experiments were conducted to determine whether $\alpha_{IIb}\beta_3$ contains two ligand binding pockets. Such measurements cannot be performed properly with natural RGD-type ligands like vitronectin and fibronectin because, in comparison with fibrinogen, they exhibit very slow association rates (39) and because they are multivalent. Both properties invalidate many types of kinetic analysis, a method that must be used to distinguish simple competitive inhibition from allosteric competitive inhibition. Therefore, we employed two model RGD ligands; Fab-9 is a human antibody engineered by phage display to contain an RGD in the antigen binding site (36, 41), and SC 52012 is a small molecule mimic of RGD (33). These two model ligands have several advantages. First, the rate at which they bind to $\alpha_{IIb}\beta_3$ (the association rate) is comparable with that of fibrinogen (39, 41). Second, both ligands are monovalent and bind to $\alpha_{IIb}\beta_3$ in a reversible manner, allowing for meaningful kinetic measurements and comparisons.

As a first step, we measured the ability of the RGD mimetic SC 52012 to block the binding between purified $\alpha_{IIb}\beta_3$ and the
two macromolecular ligands Fab-9 and fibrinogen. The binding isotherms are shown in Fig. 2 along with double-reciprocal transformations of the data. The shape of these plots enables one to distinguish noncompetitive inhibition from competitive inhibition. Increasing concentrations of competing SC 52012 shift the binding isotherms for both $^{125}$I-fibrinogen (Fig. 2A) and $^{125}$I-Fab-9 (Fig. 2C) to the right, indicating a change in their overall binding affinity. The double-reciprocal transformations of both sets of binding data intersect on the y axis (Fig. 2, B and D), a hallmark of competitive inhibition (32). Based on

**Fig. 2.** The RGD mimetic SC 52012 is a competitive inhibitor of the binding of Fab-9 and fibrinogen to $\alpha_{IIb}\beta_3$. Competition binding studies were performed using purified $\alpha_{IIb}\beta_3$ as described previously (39). $^{125}$I-Fibrinogen ($Fg$) (A, B) or $^{125}$I-Fab-9 (C, D) were used as ligands for $\alpha_{IIb}\beta_3$. The ability of 0 (●), $3 \times 10^{-11}$ M (●), $6 \times 10^{-11}$ M (▲), and $1 \times 10^{-10}$ M (▼) SC 52012 to interfere with the binding of each ligand was assessed under equilibrium conditions. At the end of the binding reactions, microtiter plates were washed three times and the bound ligand was harvested and quantified by γ counting. Binding isotherms are shown in A and C. The data were transformed to double-reciprocal plots (32) by replotting the inverse of each value. The double-reciprocal plots were generated with SigmaPlot using a 95% confidence interval for the construction of lines for each data series. The character of the double-reciprocal plots indicates the type of inhibition. A plot in which the series of fitted lines intersect on the y axis indicates competitive inhibition (see plots B and D). Each plot represents an experiment that was repeated at least three times. All points are the average of triplicate points in which the S.E. was less than 12%.
In the absence of competitor ( ), in the presence of 1 μM SC 52012 ( ), or in the presence of 2.3 μM fibrinogen ( ). All experiments were performed with gel-filtered human platelets stimulated with 20 μM ADP. Each point is the average of triplicate data points in which the S.E. was typically less than 12%. Each plot is representative of at least three experiments that yielded nearly identical results.

these findings, we conclude that the small molecule RGD mimic, SC 52012, is a competitive inhibitor of the binding between αIIbβ3 and Fab-9 and also a competitive inhibitor of the binding between αIIbβ3 and fibrinogen. Although these findings are consistent with previously published reports indicating competitive inhibition between RGD ligands and fibrinogen, they do not distinguish between same site competitive inhibition (Fig. 1, Scheme 1) and allosteric competitive inhibition (Fig. 1, Scheme 2).

**Fibrinogen Fails to Compete for the Binding of RGD Ligands to αIIbβ3**—As a second step, we performed the converse experiment and measured the ability of fibrinogen to block the binding of each of the RGD-type ligands to αIIbβ3. These studies were performed on gel-filtered human platelets, although virtually identical results were obtained with purified αIIbβ3 (not shown). An extensive series of preliminary binding experiments showed that all of the αIIbβ3 molecules on the platelet surface could be saturated at a fibrinogen concentration of 500 nM. Hence, fibrinogen was used in excess of this concentration (2.3 μM) in attempts to compete for the binding of [H]SC 52012 and 125I-Fab-9. Fibrinogen failed to block the binding of [H]SC 52012 to αIIbβ3 (Fig. 3A). In several experiments of this type, we observed no significant effect of fibrinogen on the affinity of αIIbβ3 for [H]SC 52012. In contrast, Fab-9 (an RGD ligand) did block binding of [H]SC 52012 ( ).

A similar binding study showed that fibrinogen had only a minimal effect on the binding of 125I-Fab-9 to αIIbβ3 (Fig. 3B). The presence of saturating levels of fibrinogen (2.3 μM) caused only a 2–3-fold shift in the Kd of whole platelets for Fab-9 (1.9 ± 0.9 nM in the absence of fibrinogen to 5.9 ± 2.9 nM in the presence of fibrinogen (n = 3)). In contrast, SC 52012 was able to block virtually all of the specific binding between 125I-Fab-9 and αIIbβ3 on platelets ( ).

In other studies, we attempted to favor the binding of fibrinogen over both RGD ligands by allowing a 20-min pre-binding step with competing fibrinogen. However, even under these conditions, fibrinogen did not interfere with the binding of [H]SC 52012 or 125I-Fab-9. It is important to emphasize that we have found that Fab-9 and fibrinogen associate with αIIbβ3 at similar rates, so fibrinogen’s inability to block the binding of Fab-9 is not a kinetic artifact. Collectively, the results show that even though RGD-type ligands are competitive inhibitors of fibrinogen binding to αIIbβ3, fibrinogen fails to interfere with the binding of either RGD ligand. Such findings are inconsistent with same site competitive inhibition (Fig. 1, Scheme 1) and strongly hint that the RGD ligands are allosteric inhibitors of fibrinogen binding (Fig. 1, Scheme 2).

The Allosteric Nature of the Two Ligand Binding Pockets on αIIbβ3 Is Revealed by Plasmon Resonance—Because our measurements indicated that RGD ligands are competitive inhibitors of fibrinogen binding, we sought to perform a definitive test that would distinguish between same site competitive inhibition (Fig. 1, Scheme 1) and allosteric competitive inhibition (Fig. 1, Scheme 2). Measuring Kd under equilibrium binding conditions cannot provide such a distinction because both modes of inhibition alter the overall binding affinity between receptor and ligand. Therefore, we used SPR because it allows one to measure the two components of overall affinity, ligand association and ligand dissociation, independently. In essence, SPR allows one to observe the binding reaction in real time, i.e. as it happens. Because fibrinogen failed to block the binding of RGD ligands to αIIbβ3, we reasoned that the RGD binding site is likely to be accessible even when fibrinogen and αIIbβ3 are in a complex. We further suspected that the binding of RGD might induce the dissociation of fibrinogen from the integrin. Such an observation would prove the two-site model. To test this idea, we measured the effects of SC 52012 on the association and dissociation rates between αIIbβ3 and fibrinogen or Fab-9. Particular interest was paid to the effects of the compound on ligand dissociation.

SC 52012 prevented the association of Fab-9 with αIIbβ3 (Fig. 4A) but had no effect on the dissociation rate for this ligand (Fig. 4B). This behavior is consistent with the conclusion that SC 52012 and Fab-9 bind the same binding pocket on αIIbβ3. SC 52012 cannot gain access to the RGD binding pocket and induce dissociation of Fab-9 because that binding site is already occupied by Fab-9 (same site competitive inhibition, Fig. 1, Scheme 1).

SC 52012 had markedly different effects on the interaction between fibrinogen and αIIbβ3. It blocked association between fibrinogen and αIIbβ3 (Fig. 4A), but more importantly, SC 52012 induced the dissociation of prebound fibrinogen from the integrin (Fig. 4C). Saturating levels of SC 52012 increase the off-rate between fibrinogen and αIIbβ3 by 160-fold (from 6.2 × 10 −5 s −1 to 1 × 10 −2 s −1). Because SPR reports ligand binding in real time (as the binding reaction is occurring), the latter observation unequivocally demonstrates that SC 52012 can bind to αIIbβ3 even when fibrinogen is bound. Consequently, SC 52012 and fibrinogen bind to separate sites on the integrin (Fig. 1, Scheme 3).

**RGD Ligands Dissociate Platelet Aggregates**—An important prediction of the two-site model is that integrins that are already in contact with the extracellular matrix could still bind ligands at the second ligand binding pocket and be redirected to

![Fig. 3. Fibrinogen fails to block binding of RGD-type ligands to αIIbβ3](image-url)
other functions. Indeed, the two-site model also has important implications for anti-integrin therapy because it suggests a novel approach toward reversing integrin-mediated matrix contact. To test these predictions, we used platelet aggregates as a physiologic model of the interaction between an integrin and its matrix. We measured the ability of the two RGD ligands to dissociate an existing platelet aggregate, a structure that is formed by the binding of fibrinogen and αIIbβ3. As shown in Fig. 5A, the RGD mimic, SC 52012, enacted the complete dissolution of the aggregate within a period of minutes. When platelet aggregates were allowed to incubate for extended periods of time before the addition of the RGD-type ligand, less dissolution of the platelet aggregate occurred. Yet, even when aggregation was allowed to proceed for 15 min in the presence of maximal platelet stimulation (20 μM ADP), before challenge with SC 52012, 40–60% of the aggregate was consistently dissociated by the compound. Using a slightly different assay of platelet aggregation (38), we found that Fab-9 also dissociated platelets that were aggregated with fibrinogen-coated polystyrene beads (Fig. 5B). In five separate experiments of this type, the molar ratio of Fab-9/αIIbβ3 required to enact 50% disaggregation ranged from 0.5:1 to 1:1. Calculations are based on an estimate of 80,000 αIIbβ3 molecules/platelet. A platelet aggregate is a complex structure, and we cannot exclude the possibility that there are other parameters that influence the dissolution of aggregates when challenged with an RGD compound. However, the simplest interpretation of these findings is that dissolution of the aggregate is enacted by the same mechanism that induces dissociation of complexes between purified αIIbβ3 and fibrinogen.

The Two Ligand Binding Pockets on αIIbβ3 Can Be Regulated Independently by Activation of the Integrin—Consideration

![Graphs and images related to the text content.](Image)
must also be given to the idea that the two ligand binding sites on αIIbβ3 are regulated by different means. The fibrinogen binding function of αIIbβ3 is tightly controlled by platelet stimulation, a process that can be brought about by a host of physiologic stimuli such as ADP, thrombin, or collagen (2). In fact, the binding of fibrinogen to αIIbβ3 on resting platelets is of such low affinity that it cannot be measured (35). Fibronectin, an RGD ligand for αIIbβ3, cannot bind to resting platelets or even to platelets stimulated by ADP. It will only bind αIIbβ3 on the platelet surface when the platelets have been stimulated by thrombin (42). Thus, the activation requirement for ligand binding appears to depend on the type of ligand being examined. In light of the findings in the current report, another interpretation of the observation of Plow and Ginsberg (42) is that the two ligand binding pockets on αIIbβ3 are regulated independently by activation.

To explore this possibility further, we compared the binding of Fab-9 and fibrinogen on resting versus activated platelets. The binding studies were done with 250 nM 125I-Fab-9, a concentration we found to just saturate the number of αIIbβ3 molecules on the platelet surface when platelets were stimulated with ADP (see Fig. 3B). 125I-Fab-9 bound the same number of αIIbβ3 molecules on resting and stimulated platelets. In binding studies performed on blood from four separate donors, 125I-Fab-9 bound to between 30,000 and 100,000 sites/platelet, depending on the donor. The number of molecules of Fab-9 bound was equivalent in each case on resting versus ADP-stimulated platelets. The number of binding sites for Fab-9 was also equivalent to the number of αIIbβ3 molecules on the platelet as reported by the binding of 125I-abciximab, an antibody that binds to αIIbβ3 in an activation-independent manner (43). As expected, parallel binding studies performed on the same platelets showed that 125I-fibrinogen is unable to bind specifically to resting platelets. However, upon activation with ADP, 125I-fibrinogen bound to the full complement of αIIbβ3 molecules.

Because Fab-9 and fibrinogen bind to separate sites on αIIbβ3, these observations are consistent with the conclusion that the two ligand binding pockets on αIIbβ3 are regulated independently by activation of the integrin. However, our findings do not resolve all of the discrepancies in binding data relating to activation of αIIbβ3. Although Fab-9 and fibronectin are both RGD-type ligands, Fab-9 binds αIIbβ3 in the absence of platelet stimulation, whereas fibronectin will bind only when platelets are stimulated with thrombin. Nevertheless, in conjunction with prior reports, the results presented here suggest that the distinct ligand binding pockets on αIIbβ3 can be regulated independently by physiologic stimuli.

Do Some Glanzmann’s Thrombasthenics Have a Defect at Only One of the Ligand Binding Pockets on αIIbβ3?—Glanzmann’s thrombasthenia is a series of genetic disorders in which patients either fail to express αIIbβ3 on the platelet surface or express a dysfunctional form of the integrin (44). Glanzmann’s patients suffer from chronic bleeding problems. Interestingly, however, not all Glanzmann’s defects are associated with the complete dysfunction of αIIbβ3. There are reports of defects in which αIIbβ3 fails to mediate platelet aggregation or bind to fibrinogen but retains the ability to bind RGD ligands. The two-site model of αIIbβ3 proposed in Fig. 1, Scheme 3 provides a basis for such observations. The Strasbourg variant of Glanzmann’s thrombasthenia contains a point mutation of arginine to tyrosine at residue 214 in the integrin β3 subunit (45). Interestingly, Strasbourg αIIbβ3 bound to small RGD peptides but not to fibrinogen. It is reasonable to suggest that the Strasbourg variant results from a defect specific to the fibrinogen binding site even though the RGD ligand binding pocket remains functional.

The Impact of a Two-site Model on Anti-integrin Therapy—The knowledge that αIIbβ3 contains two interacting ligand binding pockets also has bearing on the application of small molecule antagonists of αIIbβ3 in anti-platelet therapy. Several such drugs are currently being tested as antithrombotic agents in large clinical trials (46–48). These trials are aimed at eliminating the ischemic complications that often accompany cardiac interventions like balloon angioplasty. During coronary intervention, complications are presumed to arise when platelets aggregate to form a thrombus, occluding a vessel and reducing blood supply. Anti-platelet therapy has been proposed as a solution to this problem. If αIIbβ3 interacts with two classes of ligands in a “mutually exclusive” manner (27, 30), then antagonists of αIIbβ3 could function only by preventing the association of fibrinogen with the integrin. In such cases, drugs
directed toward α_{Ib}β_{3} would be effective only when applied before the formation of thrombi or in a prophylactic manner. The results presented here indicate that the binding of RGD-type antagonists to α_{Ib}β_{3} will occur even when fibrinogen is already bound and when platelets have already aggregated. Thus, such ligands could enact the dissolution of an existing thrombus. Therefore, drugs that bind to site II on α_{Ib}β_{3} (Fig. 1, Scheme 3) may provide an additional benefit to the patient by enacting the dissolution of existing thrombi.

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