Reversible Conformational Changes Induced in Glycoprotein IIb-IIIa by a Potent and Selective Peptidomimetic Inhibitor

By William C. Kouns, Daniel Kirchofer, Paul Hadvary, Albrecht Edenhofer, Thomas Weller, Gabriella Pfenninger, Hans R. Baumgartner, Lisa K. Jennings, and Beat Steiner

Platelet glycoprotein (GP) IIb-IIIa inhibitors may become useful antithrombotic agents. Ro 43-5054 is a low molecular weight, noncyclic, peptidomimetic inhibitor that is three orders of magnitude more potent than RGDS in inhibiting fibrinogen binding to purified GPIIb-IIIa and in preventing platelet aggregation. Comparisons of RGDS and Ro 43-5054 in cell adhesion assays showed that, in contrast to RGDS, Ro 43-5054 was a highly selective GPIIb-IIIa inhibitor. Effects of RGDV and Ro 43-5054 on the conformation and activation state of GPIIb-IIIa were also examined. RGDV and Ro 43-5054 induced conformational changes in purified inactive GPIIb-IIIa as determined by binding of the monoclonal antibody D3GP3 (D3). These conformational alterations were not reversed after inhibitor removal, as indicated by the continued exposure of the D3 epitope and a newly acquired ability to bind fibrinogen. Similarly, RGDV and Ro 43-5054 induced conformational changes in GPllb-IIIa on the intact platelet. However, after removal of the inhibitors, exposure of the D3 epitope was fully reversed and the platelets did not aggregate in the absence of agonist. Thus, while RGD(X) peptides and Ro 43-5054 transformed purified inactive GPIIb-IIIa into an irreversibly activated conformer, the effects of these inhibitors were reversible on the intact platelet. This suggests that factors present in the platelet membrane or cytoplasm may regulate in part the ability of the complex to shift between active and inactive conformers.

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HUMAN BLOOD PLATELETS play a vital role in thrombosis and hemostasis. These small discoid cells patrol the vascular network and are programmed to adhere to extracellular matrix proteins exposed in the subendothelium of a damaged vessel. This adherence generates activation signals that stimulate cell spreading, granular secretion, procoagulant activity, and conformational alterations in the glycoprotein (GP) IIb-IIIa complex.1-3 The conformational changes in GPIIb-IIIa result in the exposure of a fibrinogen-binding site on the receptor complex.1,2,4 Fibrinogen then binds to GPIIb-IIIa and forms a bridgework that supports platelet aggregation.6 Cumulatively, these processes lead to the generation and growth of a thrombus.

The regulation of platelet function is critical because untimely occlusion of vessels results in the development of pathologic conditions such as myocardial infarction, unstable angina, and stroke. To regulate thrombus growth in pathologic conditions, inhibitors of platelet activation pathways have been widely used. However, many of these inhibitors selectively inhibit particular activation pathways with the result that the platelets remain responsive to various stimuli. Because one common end point of all platelet activation pathways is the exposure of fibrinogen receptors and subsequent platelet aggregation, new strategies have been developed that aim at directly blocking GPIIb-IIIa-fibrinogen interactions.

Molecular characterization of GPIIb-IIIa and GPIIb-IIIa-ligand interactions has provided key information required for the development of GPIIb-IIIa inhibitors. The GPIIb-IIIa complex was found to be a member of the integrin family of adhesive protein receptors5 and to recognize ligands, at least in part, via an RGD(X) sequence specificity.9-11 RGD(X) peptides were found to bind to GPIIb-IIIa,12,13 induce conformational changes in the complex,14-17 and inhibit agonist-induced platelet aggregation.11 However, studies on the binding of [125I]-YNRGDS to purified GPIIb-IIIa showed that the affinity of these peptides was low.18 Experiments also showed that the RGDS peptides were not selective for the GPIIb-IIIa complex.7,19

In addition to the problems of potency and selectivity in the usage of RGD(X) peptides as antithrombotics, Du et al reported that RGD-containing peptides could activate the fibrinogen-binding function of purified GPIIb-IIIa.20 Furthermore, when intact platelets were treated with GRGDSP, fixed with paraformaldehyde, and then washed to remove bound inhibitors, the platelets bound fibrinogen and aggregated. They concluded that such peptides may also function as partial agonists, and that their capacity to generate a high-affinity ligand-binding state should now be considered in efforts to design competitive inhibitors of integrin function.20

The purpose of the present study was to compare the potency, specificity, and selectivity of RGD(X) peptides and a structurally dissimilar peptidomimetic inhibitor of GPIIb-IIIa-ligand interactions, Ro 43-5054. Additionally, we compared the effects of RGD(X) peptides and Ro 43-5054 on the conformation and activation state of GPIIb-IIIa, both in a purified system and on the surface of intact platelets.

MATERIALS AND METHODS

Monoclonal antibodies (MoAbs) and polyclonal antibodies. The MoAbs and polyclonal antibodies used in this study were produced by standard techniques21 and their specificity previously reported.22 The MoAbs pl-36 and pl-79 are directed against GPIIb and GPIIIa, respectively. Kan-1635 is a polyclonal antibody directed against the GPIIb-IIIa complex. The production and characteriza-

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Submitted May 4, 1992; accepted July 13, 1992.

Supported in part by National Institutes of Health Grant No. HL38171 to L.K.J. L.K.J. is an Established Investigator of the American Heart Association.

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tion of the anti-GPIIb MoAb, D3GP3 (D3), has also been previously reported.13 D3 binds preferentially to ligand-occupied GPIIb-IIIa and the epitope has been localized to a 51-kD chymotryptic fragment of GPIIIa.17,23

RGD peptides and peptidomimetic inhibitors. RGDs (Arg-Gly-Asp-Ser) (Fig 1) and RGDV (Arg-Gly-Asp-Val) were synthesized by the classical technique using various coupling procedures and a combination of acid-labile protecting groups.24 The peptides were purified by preparative high performance liquid chromatography (HPLC) using a LiChrosorb RP18 column (Merck, Darmstadt, Germany). The purity of each peptide exceeded 95% as assessed by thin layer chromatography, analytical HPLC, mass spectrometry, and amino acid analysis using a Liquimat III analyzer (Labotron, Munich, Germany). The peptidomimetic, Ro 43-5054 (N-[N-[N-(p-amidinobenzoyl)-β-alanyl]-L-aspartyl]-3-phenyl-L-alanine) (Fig 1), was prepared by coupling of H-L-Asp(Bz1)-Phe-Obzl with Boc-P-ala-OH, followed by removal of the Boc-group, acylation with p-amidinobenzoylchloride, and hydrogenolytic cleavage of the benzyl ester groups. An analytically pure sample was obtained by liquid chromatography of LiChroprep RP18 (40 to 63 μm; Merck) using 0.04% aqueous trifluoroacetic acid/hetrahydrofuran 0% to 30% as a gradient system.

Purification of GPIIb-IIIa (αIIb-β3). Outdated, washed human platelets were lysed with 1% Triton X-100, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, 1 mmol/L CaCl2, 1 mmol/L MgcCl, 0.02% NaN3, 10 μmol/L leupeptin, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 2 mmol/L N-ethylmaleimide, pH 7.3, at 4°C for 15 hours. The glycoproteins were isolated using a concanavalin A-Sepharose 4B column and the bound and eluted proteins were further purified on a Sephacryl S-300 gel filtration column according to the procedure described above for platelet GPIIb-IIIa. Briefly, the glycoproteins were first isolated on a concanavalin A-Sepharose 4B column and the bound and eluted proteins were then applied on an Aeg-RGDS affinity column. After extensive washing, the bound vitronectin receptors were eluted with buffer A, containing 3 mmol/L RGDS.

Inhibition of fibrinogen binding to immobilized GPIIb-IIIa and αIIb-β3 by RGDs. The wells of plastic microtiter plates (Nunc Immunoplate MaxiSorp; Nunc Inc, Naperville, IL) were coated overnight at 4°C with purified active GPIIb-IIIa or αIIb-β3 at 0.5 μg/mL (100 μL/well) in a buffer containing 150 mmol/L NaCl, 1 mmol/L CaCl2, 1 mmol/L MgCl2, 0.005% Triton X-100, and 20 mmol/L Tris-HCl, pH 7.4. Blocking of nonspecific binding sites was achieved by incubating the wells with 3.5% bovine serum albumin (Fluka, Buchs, Switzerland) for at least 1 hour at 20°C. Before initiation of the binding assay, the plates were washed once with 150 mmol/L NaCl, 1 mmol/L CaCl2, 1 mmol/L MgCl2, and 20 mmol/L Tris-HCl, pH 7.4 (buffer B). The coated plates can be stored in the presence of 0.05% NaN3 (in buffer B) at 4°C in a humid chamber for at least 2 months without any loss in binding activity.

Fibrinogen (fibrinogen free; IMCO, Stockholm, Sweden) was diluted in buffer B containing 1% bovine serum albumin (BSA) to 0.5 μg/mL for the binding to GPIIb-IIIa and to 1.5 μg/mL for the binding to αIIb-β3. The GPIIb-IIIa-coated wells were incubated with fibrinogen (100 μL/well) for 4 hours or overnight at room temperature. Nonbound fibrinogen was removed by three washes with buffer B and bound fibrinogen was detected by enzyme-linked immunosorbent assay (ELISA). Rabbit antihuman fibrinogen antibodies (Dakopatts, Copenhagen, Denmark) diluted in buffer B containing 0.1% BSA were incubated for 1 hour at room temperature, followed by incubation with biotinylated antirabbit Ig antibodies (Amersham, Amersham, UK) for 30 minutes. The nonbound antibodies were removed by three washes with buffer B. The preformed streptavidin-biotinylated peroxidase complex (Amersham) was then added for an additional 30 minutes. The wells were washed three times with buffer B and, after the addition of the peroxidase substrate ABTS [2,2′-azino-di-(3-ethylbenzthiazoline sulfonate); Boehringer Mannheim, Mannheim, Germany], the enzyme activity in the wells was measured by a multichannel photometer (UVmax, Molecular Devices, Menlo Park, CA). In inhibition experiments, fibrinogen was incubated together with increasing concentrations of RGDs or Ro 43-5054 in the receptor-coated wells. Bound fibrinogen was detected as described above.

For the reversibility studies (see below), the concentration of the inhibitor after gel-filtration was determined in a competition assay. GPIIb-IIIa was treated with RGDV or Ro 43-5054 and then gel-filtered to remove the inhibitor. The gel-filtered GPIIb-IIIa (2 μg/mL) was incubated with fibrinogen (0.5 μg/mL) overnight in
GPIIb-IIIa-coated wells. The residual inhibitor concentration of the sample was read off a standard curve generated by measuring fibrinogen binding to the immobilized GPIIb-IIIa in the presence of 2 μg/mL GPIIb-IIIa and various concentrations of RGDV or Ro 43-5054.

Cell culture. Human endothelial cells were isolated from umbilical veins using a 0.1% collagenase solution (CLL; Worthington, Freehold, NJ) according to the method of Jaffe et al.27 Cells were cultured in gelatine-coated flasks in medium M199 (Sigma, St Louis, MO) supplemented with 20% newborn calf serum (GIBCO, Grand Island, NY), penicillin, streptomycin, glutamine, 50 μg/mL of endothelial cell growth factor (ECGF; Collaborative Research, Bedford, MA), and 100 μg/mL of heparin (Sigma). Confluent cultures acquired the typical cobblestone morphology and immunofluorescence examination showed the presence of von Willebrand factor (vWF) antigen (polyclonal antisera against factor VIII-related antigen was from Dakopatts).

Cell attachment assays. For all experiments, endothelial cells were used at confluency between passages 2 and 5. The attachment assays were performed as described by Ruoslahti et al.28 Briefly, 96-well microtiter plates (Flow Laboratories, Allschwit, Switzerland) were coated overnight at 4°C with 5 μg/mL of human fibrinogen, laminin, vitronectin, fibronectin (Telios Pharmaceuticals, San Diego, CA), and BSA. Unoccupied sites were blocked with 0.25% BSA in phosphate-buffered saline (PBS) for 2 hours at room temperature. Each well received 40,000 cells in 200 μL M199 containing 0.25% BSA and the cells were allowed to adhere in the presence of RGDS peptide and Ro 43-5054 for 45 minutes (laminin, fibronectin, and vitronectin) or 90 minutes (fibrinogen) at 37°C. After two washes with PBS, the attached cells were fixed for 10 minutes in 3% paraformaldehyde and stained with 0.5% toluidine blue in 3.7% formaldehyde. The cells were solubilized in 2% sodium dodecyl sulfate (SDS) and the absorbance at 650 nm was measured in a vertical pathway spectrophotometer.

Exposure of a ligand-induced binding site (LIBS) on purified GPIIb-IIIa by RGDV and Ro 43-5054. The wells of the microtiter plates were coated with the MoAb D3 at 2 μg/mL (100 μL/well) overnight at 4°C in buffer B. The wells were then filled with buffer B containing 3.5% BSA for an additional overnight incubation. GPIIb-IIIa (0.01 to 0.1 μg/mL) in buffer B containing 0.035% Triton X-100 and 1% BSA was incubated for 4 hours or overnight at room temperature in the fibrinogen-coated wells. Nonspecific binding was determined in the presence of 100 μmol/L RGDS. The wells were washed three times with buffer B, followed by 1 hour of incubation at room temperature with rabbit anti–GPIIb-IIIa antibodies (Kan-1635) that were diluted in buffer B containing 1% BSA. The bound antibodies were detected as described above.

Platelet preparation. Whole blood (45 mL) was drawn from the antecubital vein of aspirin-free adult donors into anticoagulant (5 mL) consisting of 98 mmol/L sodium citrate, 161 mmol/L dextrose, 50 mmol/L citric acid, and 50 ng/mL prostacyclin. Platelet-rich plasma (PRP) was isolated by centrifugation of the whole blood for 20 minutes at 160g. Platelets were isolated from PRP by gel-filtration on a Sepharose CL-2B (Pharmacia, Uppsala, Sweden) column preequilibrated in modified Tyrode’s column buffer containing 133 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L Na2CO3, 0.4 mmol/L NaH2PO4, 0.4 mmol/L MgCl2, 5 mmol/L glucose, prostacyclin (50 ng/mL), 0.2% BSA, and 10 mmol/L HEPEs, pH 7.4. CaCl2 was added to a concentration of 1 mmol/L and the platelets were allowed to rest for 30 minutes at 37°C before use.

Flow cytometric measurement of induction and reversibility of the D3 epitope by RGDS and Ro 43-5054. To determine the effect of RGDS and Ro 43-5054 on the exposure of the D3 epitope, flow cytometry was performed according to the method of Ginsberg et al.29 Briefly, PRP was isolated as described above. PRP (5 μL) was added to 45 μL of modified Tyrode’s buffer containing 2 μg of the primary antibody and RGDS (1 mmol/L), Ro 43-5054 (10 μmol/L), or buffer alone. After 20 minutes of incubation at room temperature, the diluted PRP was mixed with an equal volume of modified Tyrode’s buffer containing the appropriate inhibitor and 24 μL of fluorescein isothiocyanate (FITC)-goat antirabbit IgG (Becton Dickinson Immunochemistry Systems). The light scatter and fluorescence intensity of 10,000 platelets was collected using a logarithmic gain.

To determine the reversibility of the exposure of the D3 epitope, PRP was incubated with 10 μmol/L Ro 43-5054 for 60 minutes at room temperature. An aliquot of the Ro 43-5054–treated PRP was then gel-filtered through Sepharose CL-2B as described above to remove the bound inhibitor. An aliquot of the gel-filtered platelets was retreated with Ro 43-5054 (10 μmol/L). After 30 minutes of incubation, the Ro 43-5054–treated PRP, the gel-filtered platelets, and the gel-filtered platelets that had been reincubated with Ro 43-5054 were analyzed as described above for the exposure of the D3 epitope.

Platelet aggregation. To determine the inhibitory potency of RGDS and Ro 43-5054, platelet aggregation in PRP was performed in the presence of increasing concentrations of the two inhibitors. Aggregation was initiated by addition of 10 μmol/L ADP and measured in a dual-channel aggregometer (ELVI, Milan,
RESULTS

Comparison of the potency and selectivity of RGDS and Ro 43-5054. Figure 2A shows a representative experiment in which the potency of RGDS peptides and Ro 43-5054 in inhibiting fibrinogen binding to purified active GPIIb-IIIa (αIβ3) coated onto microtiter plate was compared. RGDS was found to inhibit fibrinogen binding to GPIIb-IIIa with an IC50 of 6 μmol/L, whereas Ro 43-5054 inhibited the fibrinogen–GPIIb-IIIa interaction with an IC50 of 1.5 nmmol/L. Cumulatively, RGDS inhibited fibrinogen binding to GPIIb-IIIa, with an IC50 of 3.6 ± 0.4 μmol/L (mean ± SEM, n = 165), as compared with an IC50 of 0.6 ± 0.1 nmmol/L (mean ± SEM, n = 12) for Ro 43-5054. In a similar assay using vWF and fibronectin as ligands, Ro 43-5054 was also three orders of magnitude more potent than RGDS in inhibiting ligand binding to GPIIb-IIIa (data not shown). The increased potency of Ro 43-5054 versus RGDS observed in the solid-phase assay was also apparent on the cellular level. Ro 43-5054 and RGDS inhibited ADP-induced aggregation of platelets in PRP with IC50s of 50 nmmol/L and 100 μmol/L, respectively.

To determine the selectivity of RGDS and Ro 43-5054, the ability of the two compounds to inhibit fibrinogen binding to the VnR (α5β1) was compared. Figure 2B shows that RGDS inhibited fibrinogen binding to the VnR with an IC50 of 12 nmmol/L. In contrast, Ro 43-5054 was relatively ineffective in inhibiting the fibrinogen-α5β1 interaction (IC50 = 23 μmol/L). Cumulatively, RGDS inhibited fibrinogen binding to VnR with an IC50 of 9.4 ± 1.8 nmmol/L (mean ± SEM, n = 9), while Ro 43-5054 inhibited with an IC50 of 3.6 ± 3.8 μmol/L (mean ± SEM, n = 3). Selectivity of the inhibitors was also investigated at the cellular level. The ability of RGDS and Ro 43-5054 to inhibit endothelial cell adhesion to fibrinogen, vitronectin, fibronectin, and laminin was examined. Endothelial cells have been reported to express the RGD-dependent integrins, α5β1,30,31 and the fibronectin receptor (α5β1),32 in addition to non–RGD-dependent integrins such as α6β4,33 α6β1,34,36 and α6β3.37 As shown in Table 1, RGDS inhibited endothelial cell adhesion to fibrinogen, vitronectin, and laminin, and the difference between the total signal obtained in the absence of the inhibitors (ODwater = 2.6) and nonspecific signal obtained in the presence of 10 μmol/L Ro 43-5054 (ODwater 0.2) was designated as specific binding and set equal to 100%.

Effects of RGDV and Ro 43-5054 on the conformation of GPIIb-IIIa. To compare the effects of RGDV and Ro 43-5054 on the conformation of GPIIb-IIIa, we used purified inactive GPIIb-IIIa as a model of GPIIb-IIIa on the resting platelet and the MoAb D3, which was previously shown to bind preferentially to the ligand-occupied con-
form of GPIIb-IIIa on intact platelets. Figure 3 shows that both inhibitors were capable of inducing GPIIb-IIIa binding to D3. Micromolar concentrations of RGDV were required to change the conformation of GPIIb-IIIa. In contrast, nanomolar concentrations of Ro 43-5054 were needed to induce a comparable extent of D3 epitope exposure. The difference in the potency of Ro 43-5054 and RGDV in inducing conformational changes in purified GPIIb-IIIa was similar to the difference in the inhibitory potency of the two compounds as described above.

**Determination of the reversibility of inhibitor-induced conformational changes in GPIIb-IIIa.** We next examined whether inhibitor-induced conformational changes in purified GPIIb-IIIa were reversible and whether Ro 43-5054 activated the purified inactive complex. Figure 4 shows that little inactive gel-filtered GPIIb-IIIa bound to the immobilized D3. In contrast, a large increase in binding was observed when inactive GPIIb-IIIa was incubated with Ro 43-5054 (10 μmol/L) before the gel-filtration. The increased binding to D3 was not due to residual Ro 43-5054 because the concentration of Ro 43-5054 in the gel-filtered sample was less than 5% of the GPIIb-IIIa concentration, as determined in a competition assay. Further, readdition of Ro 43-5054 (10 μmol/L) to the inhibitor-treated, gel-filtered GPIIb-IIIa only slightly increased the binding to D3. Similar data were obtained by pretreating inactive GPIIb-IIIa with 1 mmol/L RGDV followed by gel-filtration (data not shown). Thus, it was apparent that the binding of RGDV and Ro 43-5054 to purified GPIIb-IIIa induced the D3 epitope and that the removal of the inhibitor did not significantly reverse this conformational alteration.

We have previously shown that inactive GPIIb-IIIa fails to bind to immobilized fibrinogen. Because the binding of the inhibitors irreversibly altered the conformation of the inactive GPIIb-IIIa, we next determined if it had been transformed into an active conformer. Figure 5 shows that little inactive GPIIb-IIIa bound to the immobilized fibrinogen. However, a large increase in specific binding was observed when inactive GPIIb-IIIa was incubated with Ro 43-5054 before gel-filtration. Furthermore, after gel-filtration, we applied untreated and Ro 43-5054-pretreated GPIIb-IIIa onto an Aeg-RGDS affinity column.

### Table 1. Inhibition of Endothelial Cell Adhesion: Comparison of the Effects of RGDV and Ro 43-5054

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Fibrinogen</th>
<th>Vitronectin</th>
<th>Fibronectin</th>
<th>Laminin</th>
</tr>
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<tbody>
<tr>
<td>RGDS</td>
<td>21</td>
<td>29</td>
<td>&gt;1,000</td>
<td>ND</td>
</tr>
<tr>
<td>Ro 43-5054</td>
<td>&gt;2,000</td>
<td>&gt;2,000</td>
<td>&gt;2,000</td>
<td>&gt;2,000</td>
</tr>
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</table>

Each value represents the IC50 (μmol/L) and is the mean of at least two independent experiments. The figure is representative of the absence of varying concentrations of RGDV or Ro 43-5054 was added to the wells and incubated overnight. Bound GPIIb-IIIa was detected by ELISA as stated in Materials and Methods. Each data point is the mean of a duplicate determination. The figure is representative of three independent experiments.
on intact platelets. If Ro 43-5054 treatment of intact platelets irreversibly transformed GPIIb-IIIa into an active receptor, as had been observed with purified GPIIb-IIIa, then after removal of the inhibitor, the platelets should spontaneously aggregate in the presence of fibrinogen. Therefore, PRP was incubated with buffer or a concentration of Ro 43-5054 that completely blocked platelet aggregation (Table 2A). After gel-filtration, the untreated and Ro 43-5054-treated platelets did not spontaneously aggregate (Table 2B). The addition of ADP induced a comparable extent of platelet aggregation in both gel-filtered samples. These data indicated that, after removal of the inhibitor by gel-filtration, GPIIb-IIIa on the platelet surface was not present in an activated state.

**DISCUSSION**

In this study, we report on the synthesis and characterization of a potent and highly selective peptidomimetic inhibitor of GPIIb-IIIa function. Ro 43-5054 was three orders of magnitude more potent than RGDS in inhibiting fibrinogen binding to purified GPIIb-IIIa and in preventing platelet aggregation. Because GPIIb-IIIa is one of a number of integrins that bind RGD-containing peptides, we also examined the integrin selectivity of Ro 43-5054. Despite the increased potency of Ro 43-5054 versus RGDS in inhibiting fibrinogen binding to GPIIb-IIIa, the peptidomimetic was three orders of magnitude less potent than RGDS in inhibiting fibrinogen binding to the purified VnR. The VnR is closely related to GPIIb-IIIa in that it is composed of the same β subunit, the α subunits share significant homology.
Fig 7. Conformational changes in GPIIb-IIIa on the intact platelet induced by Ro 43-5054 are reversible. PRP (5 μL) was incubated with Tyrode’s buffer (45 μL), Tyrode’s buffer containing D3 (2 μg) or D3 and Ro 43-5054 (10 μmol/L) for 60 minutes at room temperature and subjected to flow cytometric analysis for exposure of the D3 epitope (A), as stated in the legend to Fig 6. An aliquot of the Ro 43-5054-treated PRP was removed and gel-filtered through Sepharose CL-2B to remove the Ro 43-5054 and then retreated with Tyrode’s buffer (45 μL) or Tyrode’s buffer and D3 (2 μg) or Ro 43-5054 (10 μmol/L) and D3 (2 μg) for 15 minutes and then analyzed for D3 epitope exposure (B), as stated in the legend to Fig 6.

It is interesting to consider the structural features of Ro 43-5054 that may account for the increased potency and selectivity of the compound as compared with RGDS. In RGDS, replacement of serine with hydrophobic amino acids (ie, phenylalanine) has been shown to increase the inhibitory potency of the resulting peptide. Furthermore, the basic guanidino function of arginine and the side chain carboxylate function of aspartic acid are crucial for GPIIb-IIIa antagonistic activity. Additionally, the distance between these two functional groups is a critical parameter. Inspection of molecular models shows that, upon superimposing the side chain carboxylate functions of aspartic acid in RGDS and Ro 43-5054, the guanidino group of the former can be perfectly matched to the amidino group of the latter if both molecules are allowed to adopt an extended conformation. However, compared with the highly flexible arginine side chain in RGDS, the 4-amidinobenzoyl group of Ro 43-5054 is conformationally highly constrained. These features might be responsible for the significantly higher potency of Ro 43-5054 versus RGDS as well as the selectivity of Ro 43-5054 for GPIIb-IIIa versus the VnR.

When one considers the extent of the homology between the VnR and GPIIb-IIIa and the similarity of their ligand specificity, it is remarkable that Ro 43-5054 showed such a high degree of selectivity. Two recent reports by D’Souza et al and Gulino et al indicated that segments of GPIIb containing either a portion of the Ca²⁺-binding domains (amino acids [aa] 209 to 306) or all four Ca²⁺-binding repeats (aa 171 to 464) bound directly to fibrinogen via an RGD-inhibitable mechanism. Because RGD peptides bind in a Ca²⁺-dependent manner to a single site on GPIIb-IIIa, these data suggest that at least some coordinates of the RGD binding site are contributed by aa 171 to 464 of GPIIb. Interestingly, earlier cross-linking studies on the binding of RGD peptides to GPIIb-IIIa showed that the peptides cross-linked preferentially to GPIIIa. Furthermore, molecular characterization of GPIIb-IIIa defects in platelets from patients with Glanzmann’s thrombasthenia clearly showed a contribution of GPIIIa (aa 119 and 214) in the high-affinity binding of both GRGDSP peptides and adhesive protein ligands. It is unknown how the binding of Ro 43-5054 to GPIIb-IIIa differs from that of RGDS. Because the most apparent differences in α₂β₁ and GPIIb-IIIa lie in the α subunit, and Ro 43-5054 is a potent inhibitor of the GPIIb-IIIa-fibrinogen interaction (but a

Table 2. The Effect of Ro 43-5054 on Platelet Aggregation Before and After Removal of the Inhibitor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Agonist</th>
<th>% of Control</th>
</tr>
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<tbody>
<tr>
<td>(A) PRP</td>
<td>None</td>
<td>ADP</td>
</tr>
<tr>
<td>Ro 43-5054 (PRP2)</td>
<td>ADP</td>
<td>0</td>
</tr>
<tr>
<td>(B) GFP</td>
<td>GF1 + FG</td>
<td>None</td>
</tr>
<tr>
<td>GF1 + FG</td>
<td>ADP</td>
<td>100</td>
</tr>
<tr>
<td>GF2 + FG</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>GF2 + FG</td>
<td>ADP</td>
<td>100</td>
</tr>
</tbody>
</table>

The extent of ADP-induced aggregation of the two control samples (PRP1 and GF1, respectively) was set equal to 100%. (A) PRP (2.5 x 10⁴ platelets/mL) was incubated with buffer (PRP1) or 1 μmol/L Ro 43-5054 (PRP2) for 20 minutes at room temperature. Both samples were then gel-filtered through Sepharose CL-2B yielding gel-filtered platelets 1 (GF1) from PRP1 and GF2 from PRP2. (B) After the addition of fibrinogen (to 0.6 μmol/L), the extent of aggregation was determined in the presence and absence of 10 μmol/L ADP.
very weak inhibitor of \( \alpha_{\text{IIb}}\beta_3 \), our data would suggest a primary role of GPIIb\textsubscript{2} in granting receptor selectivity. Whether the selective binding of Ro 43-5054 to GPIIb-IIIa is a direct result derived from the contribution of inhibitor binding regions of GPIIb (\( \alpha_{\text{IIb}} \)) that are not homologous with \( \alpha_{\text{IIb}}\beta_3 \) or an indirect result due to distinct conformational differences in the ligand binding pockets of GPIIb-IIIa and \( \alpha_{\text{IIb}}\beta_3 \) awaits determination of the crystal structure of inhibitor-bound GPIIb-IIIa.

In vivo applications of GPIIb-IIIa inhibitors may involve short-term infusion of an inhibitor and long-term administration of orally active compounds for secondary prophylaxis. Therefore, we addressed the following questions: (1) Does the binding of Ro 43-5054 induce changes in the three-dimensional structure of GPIIb-IIIa and, if so, do these changes lead to activation of the receptor? (2) Are these changes reversible upon removal of the inhibitor? The importance of these considerations became apparent from the studies of Du et al., who showed that RGD peptides could act as a partial agonist of GPIIb-IIIa receptor function. In the present study, using the D3 MoAb and fibrinogen binding assays, we observed that Ro 43-5054 induced irreversible conformational changes in purified inactive GPIIb-IIIa that resulted in the transformation of the receptor into an activated conformer. In contrast, on the intact platelet, the Ro 43-5054-induced conformational alterations in GPIIb-IIIa reported by the D3 MoAb were reversible. A similar finding has been reported for GRGD-SP-induced exposure of a neoantigen on GPIIb\textsubscript{2}.

Furthermore, the Ro 43-5054-treated platelets did not spontaneously aggregate after the removal of the inhibitor by gel-filtration and they were equally as responsive to agonist as untreated, gel-filtered platelets. These data indicated that GPIIb-IIIa was not in the activated conformation after the removal of the inhibitor and that the inhibitory effect of Ro 43-5054 on platelet function could be reversed. The reversibility of the conformational changes in GPIIb-IIIa on the intact platelet as compared with the irreversibility of these changes in the purified system suggests a role for cytoplasmic or membrane components in regulating GPIIb-IIIa conformational transitions in the intact platelet.

In conclusion, it is unlikely that inhibitor-induced structural alterations in GPIIb-IIIa present the threat of inducing a thrombotic episode because the effects of the inhibitors on intact platelet GPIIb-IIIa appeared to be fully reversible. Furthermore, no agonist activity of GPIIb-IIIa inhibitors has ever been reported in animal models. However, when one considers administration of orally active GPIIb-IIIa inhibitors over long periods of time, the potential antigenicity of epitopes exposed by the binding of the inhibitors to GPIIb-IIIa should be evaluated. In this regard, inhibitors with minimal effects on the conformation of GPIIb-IIIa might be advantageous for in vivo use.

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