Tirofiban Blocks Platelet Adhesion to Fibrin with Minimal Perturbation of GpIIb/IIIa Structure

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Summary

A biophysical approach tested the hypothesis that tirofiban, like eptifibatide, perturbs GpIIb/IIIa structure. Tirofiban bound tightly to platelet GpIIb/IIIa (EC50 ~ 24 nmol/L) and effectively inhibited platelet aggregation (IC50 ~ 37 nmol/L) but blocked platelet adhesion to clotted fibrin only at much higher doses (IC50 ~ 580 nmol/L). Electroscopic analyses demonstrated that tirofiban protected GpIIb/IIIa from SDS-induced subunit dissociation. However, saturating tirofiban concentrations had little or no effect on GpIIb/IIIa secondary or tertiary structure, as determined by circular dichroic spectroscopy, dynamic light scattering, and sedimentation velocity measurements performed with purified receptors in octyl glucoside. Moderate dose-dependent effects on GpIIb/IIIa quaternary structure were detected by sedimentation equilibrium. Transmission electron microscopy showed minimal tirofiban-induced receptor activation or oligomerization. Thus, even at the increased concentrations needed to block platelet:fibrin adhesive interactions, tirofiban exhibited only limited effects on GpIIb/IIIa conformation and clustering. Our results provide new insights into the mechanisms and potential prothrombotic complications of integrin antagonists.

Introduction

Tirofiban (Aggrastat) is a potent, selective Glycoprotein IIb/IIIa (GpIIb/IIIa) inhibitor (1, 2) whose effectiveness in treating acute coronary syndromes has been established in three large-scale clinical trials: RESTORE, PRISM, and PRISM-PLUS (3-5). However, tirofiban and the other integrin antagonists abciximab and eptifibatide have small increased risks of hemorrhage and thrombocytopenia (4-7). The extent to which receptor activation contributes to the prothrombotic effects of GpIIb/IIIa inhibitors remains controversial. Formation of neo-antigenic sites, termed Ligand-Induced Binding Sites (LIBS) (10, 11), has been demonstrated for tirofiban, eptifibatide, and abciximab, suggesting an integrin-antagonist class-effect (1, 12, 13). While Peter et al. (14) reported that abciximab and the RGD analog fradifiban induced fibrinogen binding and platelet aggregation, a recent report challenges this conclusion and attributes the apparent receptor-activating effects of integrin antagonists to artifactual thrombin generation (15).

Given this dilemma, there is a critical need for a better understanding of the molecular mechanisms by which integrin antagonists function. We have developed an integrated biophysical and electron microscopy strategy to detect and to describe the effects of low-molecular weight integrin antagonists on the conformation and oligomerization state of the purified GpIIb/IIIa complex (16, 17). Our unique approach uses spectroscopic “nanometersticks” to avoid the steric hindrance that limits the resolution of immunoprobes. We have recently shown that eptifibatide, at near-stoichiometric concentrations, shifts a conformational equilibrium toward an “open” receptor with increased frictional drag, a distinct subunit separation, and enhanced self-association, or clustering (17). Here, we will demonstrate that while increased tirofiban concentrations are needed to block the strong multisite interactions that stabilize a platelet:fibrin thrombus, these concentrations had minimal effects on the secondary and tertiary structure of the GpIIb/IIIa complex and induced only moderate GpIIb/IIIa receptor clustering.

Materials and Methods

Reagents

AGGRASTAT (tirofiban hydrochloride, L-tyrosine-N-(butylsulfonyl)-O-[-4-(piperidinebutyl)] monohydrochloride) was provided (Merck, West Point, PA) as a dry powder and stored in the dark at room temperature for up to 18 months with no loss in activity. Tyrosine was obtained from Sigma Chemical Co. (St. Louis, MO). Tirofiban and tyrosine concentrations were determined spectrally using experimentally determined molar extinction coefficients (ε280) of 1099 ± 122 L/mol-cm and 1126 ± 106 L/mol-cm, respectively. Highly purified human fibrinogen (free of plasminogen and Factor XIII) was purchased from American Diagnostica (Greenwich, CT) and highly purified human α-thrombin from Sigma Chemical Co. (St. Louis, MO).

Platelet Isolation and Characterization Procedures

Blood was obtained by venipuncture from healthy, adult volunteer donors into 1/10 vol of sodium citrate (110 mmol/L) anticoagulant. The Clinical Research Practices Committee of Wake Forest University School of Medicine fully examined and approved these procedures. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were isolated by differential centrifugation. Gel-filtered platelets were isolated from PRP in HEPES-Tyrode’s-Albumin buffer.
(HTA) as previously described (18). Platelet counts were determined with a Coulter MDII Cell Counter (Beckman-Coulter, Miami, FL). Platelet aggregation profiles were obtained in a Chrono-Log Model 500 Aggregometer, following addition of Ca²⁺ (2 mMol/L) and ADP (45 μmol/L) to stirred samples of PRP in the presence/absence of tirofiban or tyrosine. Platelet GpIIb/IIIa occupancy was determined with flow cytometric analysis using a Biocytex Kit BX7001 (Marseille, France) with PRP samples incubated with either tirofiban or tyrosine (19).

Platelet Adhesion to Fibrin

Platelet adhesion to clotted fibrin was determined by a microtiter plate colorimetric assay that monitored the release of acid phosphatase from adherent, lysed platelets (20). Fibrin clots (50 μL) were prepared in 300 μL-wells of a microtiter plate by addition of thrombin (to 0.5 NIH U/mL) to fibrinogen (1.0 mg/mL) in HEPES-Tyrode’s buffer with 2 mM Ca²⁺ followed by a 30-min incubation in a moist chamber at 37°C. Samples of gel-filtered platelets (100 μL of 189,000 ± 10,000 μL (n = 5) in HTA buffer), preincubated for 30 min in the presence/absence of tirofiban or tyrosine, were carefully layered on fibrin clots and incubated for 10 min at 37°C in a humid chamber with modest agitation (Jitterbug Model 130000, Boekel Industries, Philadelphia, PA). Nonadherent platelets were removed by aspiration followed by a 200 μL wash/aspiration step with HTA buffer. Nonspecific adhesion was determined with formaldehyde-fixed, gel-filtered platelets (21). Adherent platelets were lysed in a pH 5.4 buffer containing 0.1% Triton X-100 and pH 8.0 nitrophenylphosphate (5 mmol/L). Following a 30-min incubation at room temperature with moderate agitation, 100 μL of 0.1 mol/L NaOH was added to each well and absorbance at 405 mmol/L measured in a Vmax Kinetic Microtiter Plate reader (Molecular Devices, San Francisco, CA). Calibration profiles obtained with gel-filtered platelets showed color development exhibited a linear dependence on platelet count up to 200,000 pls/μL.

Scanning Electron Microscopy

Platelet adhesion to fibrin clots was visualized by scanning electron microscopy in a physical format similar to that used for the microtiter plate assay described above. Fibrin clots were prepared in wells (6-8 mm diameter × 2 mm deep) that had been machined in the center of carbon planchetttes (22). Gel-filtered platelets, preincubated for 30 min in the presence/absence of tirofiban or tyrosine, were allowed to adhere to the fibrin wells for 30 min at 37°C followed by aspiration and washing with HTA buffer. Nonspecific adhesion was examined with formaldehyde-fixed, gel-filtered platelets. After these treatments, the platelet-fibrin samples were dehydrated through a graded series of ethanol and dried from CO₂ by the critical-point method (22). The samples were sputter-coated with 50 A of gold-palladium and observed at 15 keV in a Phillips 515 scanning electron microscope (Phillips Electronic Instruments, Mahwah, NJ).

Biophysical Measurements

Milligram quantities of highly purified GpIIb/IIIa were isolated from outdated human blood platelets (American Red Cross, Triad Blood Center, Winston-Salem, NC) as previously described (16, 23). Biophysical measurements were performed on peak integrin fractions obtained by size exclusion chromatography at 4°C on a 0.9 × 85 cm column of Sephacryl S-300 equilibrated in a pH 7.4 buffer (HISC-OG) containing 0.13 mol/L NaCl, 0.01 mol/L HEPES, 0.002 mol/L CaCl₂, 3 × 10⁻⁶ mol/L basic trypsin inhibitor, 10⁻⁶ mol/L leupeptin, and 0.03 mol/L n-octyl-B-D-glucopyranoside. Circular dichroic spectroscopy was performed in a Jasco Model 720 Spectropolarimeter (Japan Spectroscopic Co., Tokyo) in a 0.1 cm path-length cuvette in a chamber flushed with nitrogen. Static and dynamic light scattering measurements were performed in a Brookhaven Instruments BI-2030 AT correlator operated in conjunction with a BI-200 SM light scattering photometer/photon counting detector and a Spectra Physics 127 He-Ne laser as previously described (16). Sedimentation velocity and equilibrium measurements were performed as previously described (16) in a Beckman Optima XL-A analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA) equipped with absorbance optics and an An60 Ti rotor.

Transmission Electron Microscopy

Rotary-shadowed GpIIb/IIIa samples (in the presence/absence of tirofiban (10 μmol/L) or tyrosine (10 μmol/L) were prepared by spraying a dilute solution (final concentration ~ 20-25 μg/mL) of molecules in a buffer containing 0.05 mol/L ammonium formate at pH 7.4, 30 mmol/L octyl glucoside, and 30% (v/v) glycerol onto freshly cleaved mica and shadowing with tungsten in a vacuum evaporator (Denton Vacuum Co., Cherry Hill, NJ) (24–26). These samples were examined in a Philips 400 electron microscope (FEI Co., Hillsboro, OR) operating at 60 kV and a magnification of 60,000×. Counts of molecules with different conformations or different amounts of oligomers were made from prints of the micrographs, using images from many different areas of several different preparations to get a random sample.

Results

Tirofiban’s Effects on Platelet-Platelet and Platelet-Fibrin Interactions

Tirofiban is clearly an effective inhibitor of platelet aggregation, a fibrinogen-mediated process, but our results indicate that much higher doses were required to block platelet adhesion to fibrin. As shown in Figure 1, tirofiban inhibited ADP-stimulated platelet aggregation with an IC₅₀ = 37 ± 9 nmol/L. Tirofiban’s dose-response profile for inhibition of aggregation (filled circles) closely followed that for its occupancy of...
the GpIIb/IIIa receptor (filled triangles), as determined by an antibody-based flow cytometric assay that yielded EC50 = 24 ± 16 nmol/L. A calculation that considers these binding and inhibition parameters as well as the average GpIIb/IIIa receptor density (68,000 ± 14,000) and platelet count (336,000 ± 81,000) obtained in our studies indicates that a 4-fold molar excess of tirofiban over GpIIb/IIIa is sufficient to cause 80% inhibition of platelet aggregation.

In contrast, blocking platelet adhesion to fibrin by 50% required ~15-fold higher tirofiban doses (IC50 = 580 ± 190 nmol/L) as quantified as described above. Note the persistence of the ~180 kDa species up to 5000 min after drug removal.

Fig. 2  Tirofiban’s Effects on Platelet:Fibrin Interactions – Scanning Electron Microscopy. Gel-filtered platelets adherent to the surface of clotted fibrin in the absence (A) and presence (B) of a saturating tirofiban concentration (1 μmol/L). Formaldehyde-fixed platelets nonspecifically adherent to fibrin are shown in C. Samples were examined in a Philips Model 515 scanning electron microscope, and micrographs were taken at a magnification of 2250 × (Bar = 10 μm).

Fig. 3  Tirofiban’s Effects on GpIIb/IIIa Subunit Interactions. A) Electrophoretic analyses (SDS-PAGE) of purified GpIIb/IIIa samples incubated for 1 h at 23° C with buffer only (lane 1), 9 μmol/L tirofiban (lane 2), or 9 μmol/L tyrosine (lane 3) prior to the addition of 1% SDS. Note the strongly staining band at ~180 kDa observed only with the tirofiban-containing sample. B) SDS-PAGE of purified GpIIb/IIIa samples incubated for 1 h at 23° C with 9 μmol/L tirofiban followed by a 1 h incubation at 23 C in 1% SDS (lane 1) or 1% SDS/1% DTT (lane 2). Note the stability of the ~180 kDa band to reduction at 23° C. Lanes 3 and 4 denote the effects of a 1 h incubation at 40° C in 1% SDS and 1% SDS/1% DTT, respectively. Note that increased temperature alone is sufficient to cause the virtual disappearance of the ~180 kDa species. C) Effects of temperature on the relative staining intensity of the ~180 kDa tirofiban-induced species. GpIIb/IIIa samples preincubated with 9 μmol/L tirofiban for 1 h at 23° C were then incubated at the indicated temperature for 1h in the presence (solid symbols) or absence (open symbols) of 1% SDS. In each case, SDS-PAGE was performed under nonreducing conditions and the density of the ~180 kDa band expressed as a percent of the total integrin subunit staining intensity. Note the temperature-dependent decreased intensity of the ~180 kDa band only when GpIIb/IIIa + tirofiban samples were heated in the presence of SDS. D) Time-dependent changes in the relative staining intensity of the ~180 kDa tirofiban-induced species following removal of excess ligand from GpIIb/IIIa samples. SDS-PAGE was performed under nonreducing conditions, and band intensities quantified as described above. Note the persistence of the ~180 kDa species up to 5000 min after drug removal.
in a colorimetric microtiter plate assay (Fig. 1, filled squares). However, even at micromolar concentrations, tirofiban’s effects were specific, since tyrosine, a structurally similar control compound (1), had little or no effect on either platelet aggregation, receptor occupancy, or adhesion to fibrin even at a 500-fold molar excess over GpIIb/IIIa (Fig. 1, open symbols).

Scanning electron microscopy (Fig. 2) shows the close contact between (control) platelets adherent to fibrin strands (A) and the substantially reduced adhesion observed in the presence of excess tirofiban (B). Panel C was obtained with inactive, formaldehyde-fixed platelets. The inhibition data obtained in our fibrin adhesion assay, coupled with scanning electron micrographs like these, indicate that tirofiban must overcome strong multisite interactions in order to inhibit platelet adhesion to clotted fibrin (21, 27).

**Tirofiban Stabilizes GpIIb/IIIa Subunit Interactions**

Our results indicate that near-stoichiometric tirofiban concentrations stabilize the GpIIb/IIIa complex against SDS-induced subunit dissociation. As shown in Figure 3A, SDS-PAGE analyses of a (nonreduced) integrin sample showed prominent bands, corresponding to the GpIIb and IIIa subunits (lane 1). However, integrin samples incubated with 9 μmol/L tirofiban for 1 h displayed a major new band at ~180 kDa (lane 2). This band was not observed with integrin samples incubated with tyrosine (9 μmol/L; lane 3). Replicate analyses (n = 20) demonstrated that the tirofiban-induced, high molecular mass species (183 ±12 kDa) accounted for 60 ± 12% of the integrin subunit staining intensity in GpIIb/IIIa samples incubated with 3–23 μmol/L tirofiban (1-7 fold molar excess).

Additional data (Fig. 3B) demonstrated that the tirofiban-induced band was stable to disulfide bond reduction at room temperature but not at 40°C (lanes 1, 2; 23°C, nonreduced and reduced; lanes 3, 4, 40°C, nonreduced & reduced). Note the temperature-dependent disappearance of the ~183 kDa band and the parallel increase in staining intensity of the individual integrin subunits. In fact, these data also show that incubation in 1% SDS at elevated temperature even in the absence of reducing agent was sufficient to disrupt the ~183 kDa band, which “melted” at ~27°C (as shown in Fig. 3C, solid symbols). However, this band was stable in samples heated in the absence of SDS (Fig. 3C, open symbols). These observations suggest that ligation with tirofiban...
stabilizes GpIIb/IIIa subunit interactions but that this stabilization was reversed by the combined effects of SDS denaturation and moderately increased temperatures.

Electrophoretic analyses were also used to monitor the time dependence of tirofiban’s effects on GpIIb/IIIa stabilization. Following a 1-h incubation with 9 μmol/L tirofiban, unbound ligand was removed from an integrin sample by rapid desalting (16, 17). Figure 3D shows the staining intensity of the ~183 kDa species as a function of time, both prior to addition of tirofiban and following removal of excess ligand. Tirofiban’s effects were observed as early as 1 min after ligand addition and persisted for more than 5000 min after the separation step. While tirofiban binds quite rapidly and dissociates with a half-time ~150 s (1), the long-term subunit stabilization observed here suggests that some of the conformational changes in GpIIb/IIIa induced by tirofiban may not be readily reversed.

**Tirofiban’s Effects on GpIIb/IIIa Secondary Structure**

Circular dichroic (CD) spectroscopy measurements were performed on GpIIb/IIIa in its ligand-free and tirofiban-bound states to probe for possible differences in secondary structure induced by receptor occupancy. As shown in Figure 4, both ligand-free (open circles) and tirofiban-bound GpIIb/IIIa samples (filled circles) exhibited very similar CD spectra in the far UV region. By comparison, integrin denaturation by 4 M guanidinium chloride yielded a 47% decrease in CD signal at 220 nm (gray triangles). Since tirofiban caused less than a 1% difference between free and bound integrins, we conclude that tirofiban binding caused little, if any, change in integrin secondary structure.

**Tirofiban’s Effects on GpIIb/IIIa Tertiary Structure**

Measurements of GpIIb/IIIa’s hydrodynamic properties were performed in the presence/absence of tirofiban to probe for ligand-induced conformational changes, such as those we reported when either RGDX peptides and eptifibatide bind to the receptor (16, 17). As shown in Figure 5, dynamic light scattering measurements of GpIIb/IIIa’s Stokes Radius showed minimal changes at saturating tirofiban concentrations (control 8.34 ± 0.32 nm vs. 8.53 ± 0.32 nm, tirofiban-bound [p = 0.4]). The small decrease observed at 25 μmol/L tyrosine (to 7.58 nm) may indicate some nonspecific subunit dissociation.

**Tirofiban’s Effects on GpIIb/IIIa Tertiary Structure – Sedimentation Velocity.** Distributions of sedimenting species [g(S) vs. S] obtained with purified GpIIb/IIIa alone (3.6 μmol/L, open triangles), + 23 μmol/L tirofiban (black triangles), or 25 μmol/L tyrosine (gray triangles) were determined by time-derivative analyses of data obtained by sedimentation velocity (28). The small but significant shift toward slower sedimenting species seen with tirofiban indicates a ligand-induced transition to an open GpIIb/IIIa conformation. Inset: Similar decreases in Sw were observed over a 7-fold range of tirofiban concentrations. Sw for GpIIb/IIIa (open triangles), + tirofiban (black triangles), + tyrosine (light gray triangle), and a GpIIb/IIIa sample transiently exposed to tirofiban (dark gray triangle).

**Tirofiban’s Effects on GpIIb/IIIa Quaternary Structure – Sedimentation Equilibrium.** Weight-average molecular weights (Mw) were determined by sedimentation equilibrium for GpIIb/IIIa alone (open circles), in the presence of tirofiban (black circles), or in the presence of tyrosine (gray circles). Note the ligand-dependent increases in Mw that reached a plateau in the range 5–12 μmol/L tirofiban, followed by a decline at higher concentrations. These observations coupled with the small decreases in Mw seen with tyrosine may indicate some nonspecific subunit dissociation.

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**Fig. 6** Tirofiban’s Effects on GpIIb/IIIa Tertiary Structure – Sedimentation Velocity. Distributions of sedimenting species [g(S) vs. S] obtained with purified GpIIb/IIIa alone (3.6 μmol/L, open triangles), + 23 μmol/L tirofiban (black triangles), or 25 μmol/L tyrosine (gray triangles) were determined by time-derivative analyses of data obtained by sedimentation velocity (28). The small but significant shift toward slower sedimenting species seen with tirofiban indicates a ligand-induced transition to an open GpIIb/IIIa conformation. Inset: Similar decreases in S are observed over a 7-fold range of tirofiban concentrations. S for GpIIb/IIIa (open triangles), + tirofiban (black triangles), + tyrosine (light gray triangle), and a GpIIb/IIIa sample transiently exposed to tirofiban (dark gray triangle).

**Fig. 7** Tirofiban’s Effects on GpIIb/IIIa Quaternary Structure – Sedimentation Equilibrium. Weight-average molecular weights (Mw) were determined by sedimentation equilibrium for GpIIb/IIIa alone (open circles), in the presence of tirofiban (black circles), or in the presence of tyrosine (gray circles). Note the ligand-dependent increases in Mw that reached a plateau in the range 5–12 μmol/L tirofiban, followed by a decline at higher concentrations. These observations coupled with the small decreases in Mw seen with tyrosine may indicate some nonspecific subunit dissociation.

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Complementary data were obtained by sedimentation velocity, which in our experience provides an even more sensitive indicator of the receptor’s open conformation, a form with increased frictional drag that sediments more slowly than the ligand-free integrin (16). As shown in Figure 6, time-derivative analysis makes clear that the distribution of GpIIb/IIIa sedimenting species was shifted toward lower S-values in the presence of tirofiban (25 \(\mu\)mol/L, dark triangles), compared to either the ligand-free integrin (open triangles) or integrin in the presence of tyrosine (25 \(\mu\)mol/L, gray triangles). In each case, the solid lines were obtained by fitting the data to a single species model that considers both sedimentation and diffusion effects (28). Analyses with SVEDBERG software demonstrated that similarly decreased sedimentation coefficients were obtained from 4–23 \(\mu\)mol/L tirofiban. Comparison of the mean sedimentation coefficient for the ligand-free integrin, 8.63 ± 0.06 S, to that for the tirofiban-occupied conformer, 8.41 ± 0.12 S, revealed a significant difference (\(p = 0.04\)). In contrast, addition of 25 \(\mu\)mol/L tyrosine yielded an integrin sedimentation coefficient of 8.59 S that was indistinguishable from the ligand-free integrin. These observations are consistent with a small but significant shift in conformational equilibrium toward the open or activated integrin induced by tirofiban binding.

**Tirofiban’s Effects on GpIIb/IIIa Quaternary Structure**

Data obtained by sedimentation equilibrium [analyzed by NONLIN software (30)] showed tirofiban caused dose-dependent changes in GpIIb/IIIa self-association (Fig. 7). The receptor’s weight-average molecular weight (\(M_w\)) increased from 210 ± 8 K to 253 ± 7 K over the tirofiban concentration range 0–2 \(\mu\)mol/L (\(p = 0.003\)). However, GpIIb/IIIa’s molecular weight distribution decreased at increasing tirofiban concentrations, reaching \(M_w = 187\) K at 23 \(\mu\)mol/L. Since similarly decreased molecular weights were obtained at 10 and 25 \(\mu\)mol/L tyrosine (\(M_w = 182 ± 4\) K), there appear to be some nonspecific effects on integrin structure/subunit association at high effector concentrations.

**Electron Microscopy Examination of Tirofiban’s Effects on GpIIb/IIIa Structure**

Transmission electron microscopy (TEM) provides insights into the effects of ligation on GpIIb/IIIa tertiary and quaternary structure, yielding images that complement our biophysical data (16). In the presence of 10 \(\mu\)mol/L tirofiban, most GpIIb/IIIa particles had the same appearance as control preparations; i.e., a globular head with two tails projecting from one side and joined together distally (16, 31, 32). Only 4.2% of particles appeared to be in the open conformation, slightly more than the 2.7% in control without tirofiban or 3.0% in the presence of tyrosine (n = 1000). A relatively small number of GpIIb/IIIa particles were present as aggregates, 11.4%, but this figure was nearly twice the percentage of aggregates present in the control preparations, 5.6%, or GpIIb/IIIa in the presence of tyrosine, 6.0% (n = 1000). Nearly all aggregates were dimers joined at the distal end of their tails.

**Discussion**

This study demonstrates that tirofiban, even at concentrations required to block platelet:fibrin adhesive interactions, causes minimal activation of the GpIIb/IIIa complex. Our results indicate that achieving 80% inhibition of platelet aggregation requires an approximate 4-fold molar excess of tirofiban over platelet GpIIb/IIIa receptors. In contrast, results from our in vitro model indicate that micromolar concentrations (~100-fold molar excess) were required to block platelet adhesion to clotted fibrin. We conclude that strong multisite interactions stabilize platelet:fibrin adhesion (21), an interpretation that is supported by scanning electron microscopy images (Fig. 2) demonstrating the close contact between adherent platelets and multiple fibrin strands.

Using the aggregation dosing range as a clinically relevant benchmark, we carried out a series of biophysical studies to probe the effects of tirofiban on the secondary, tertiary, and quaternary structure of the GpIIb/IIIa complex.
purified GpIIb/IIIa complex. Our results indicate that receptor-saturating concentrations of tirofiban caused no detectable change in GpIIb/IIIa’s secondary structure; circular dichroic spectroscopy showed no effects of the drug on the receptor’s a-helix or b-sheet content. We employed a hydrodynamic approach to monitor tirofiban’s effects on GpIIb/IIIa’s tertiary structure using both dynamic light scattering and sedimentation velocity to probe for ligand-induced conformational changes, such as those we observed for RGD ligands and eptifibatide (16, 17). Since we observed only small effects on GpIIb/IIIa’s hydrodynamic radius (2.5 ± 1.5% increase) and sedimentation coefficient (2.4 ± 1.1%), we conclude that tirofiban caused minimal receptor activation. In an earlier study, we found that equivalent concentrations of eptifibatide increased the receptor’s Stokes radius by 11.0 ± 1.6% and decreased its sedimentation coefficient by 4.6 ± 0.8% (17). These observations indicate that tirofiban caused less pronounced changes in GpIIb/IIIa solution conformation than eptifibatide (17).

Quaternary structural changes were measured by sedimentation equilibrium, which demonstrated a 19 ± 4% increase in GpIIb/IIIa’s weight-average molecular weight at a 1-4 fold molar ratio of tirofiban:receptor. Higher tirofiban concentrations resulted in a decreased molecular weight distribution, though some nonspecific effects may have contributed to this decline. We previously found that at equivalent concentrations, eptifibatide caused a 33 ± 10% increase in GpIIb/IIIa molecular weight distribution and that this ligand-induced clustering persisted up to a 20-fold molar excess of eptifibatide over GpIIb/IIIa (17). Tirofiban’s effects on GpIIb/IIIa conformation and clustering were also examined by transmission electron microscopy, an approach that complements the data obtained by biophysical studies. Quantitative analyses demonstrated only ~4% receptor activation and 11% clustering by tirofiban, much less than the 55% activation and 65% clustering we previously observed by electron microscopy with eptifibatide (17).

In contrast to these differences in receptor activation, we found that tirofiban shares with eptifibatide (17) the ability to stabilize subunit interactions within the GpIIb/IIIa complex, preventing the SDS-induced subunit dissociation seen in the absence of ligand. In both cases, the combined effects of SDS and moderately increased temperature reversed this stabilization (17). However, tirofiban’s effects differed from those of eptifibatide in that SDS-resistance was observed long after removal of unbound ligand. This finding is somewhat surprising in light of tirofiban’s relatively rapid off-rate (1) and may indicate that the modest perturbation in GpIIb/IIIa structure induced by tirofiban binding is not readily reversed. This concept is supported by our sedimentation velocity studies, which showed that the decrease in GpIIb/IIIa’s sedimentation coefficient induced by tirofiban remained hours after removal of excess ligand.

While tirofiban and eptifibatide are both highly selective, tight-binding inhibitors of GpIIb/IIIa function (1, 33), our results indicate that tirofiban causes considerably smaller integrin-activating effects. Differences in the chemical structure of these antagonists may be responsible for these observations. Eptifibatide is a cyclized peptide based on the Lys-Gly-Asp motif found in bearboulin, a naturally occurring disintegrin (33-35), whereas tirofiban is a non-peptide compound designed to incorporate the spatial distribution of charges found in the classical Arg-Gly-Asp integrin binding sequence (1-2). Tirofiban’s structure also includes an S-butyrolsufonamido substituent that has been proposed to exhibit a unique interaction with an exosite on GpIIb/IIIa (1). Molecular dynamics simulations further suggest that tirofiban adopts a solution conformation quite similar to the Lys-Gln-Ala-Gly-Asp-Val sequence on the carboxy-terminus of fibrinogen’s y-chain that binds to GpIIb/IIIa (36). Thus, we propose that tirofiban’s tight fit into GpIIb/IIIa’s fibrinogen-binding pocket is responsible for its ability to block integrin function with minimal receptor activation.

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References


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33. Phillips DR, Scarborough RM. Clinical pharmacology of epifibatide. Am J Cardiol 1997; 80: 11B-20B.


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