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Comparative Efficacy Between the Glycoprotein IIb/IIIa Antagonists Roxifiban and Orbofiban in Inhibiting Platelet Responses in Flow Models of Thrombosis

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Summary: This study was undertaken to compare the platelet binding characteristics and anti-platelet efficacy of a nonpeptide glycoprotein IIb/IIIa antagonist roxifiban with orbofiban in static and dynamic adhesion and aggregation assays. The results indicate that roxifiban binds with higher affinity to glycoprotein IIb/IIIa receptors and exhibits slower dissociation rates than orbofiban. Furthermore, the platelet inhibitory effects of roxifiban, but not orbofiban, were unaffected by changes in plasma calcium concentrations. Both agents reduced, in a concentration-dependent manner, the size of platelet thrombi deposited onto collagen I upon perfusion of heparinized blood at a shear rate of 1,500/s. At a clinically achievable concentration of 60 n*M*, roxifiban abrogated the formation of thrombi containing > 20 platelets per thrombus, thereby displaying comparable in vitro efficacy to that achieved by the theoretical maximal abciximab blood concentration (3.5 μg/ml) produced after standard treatment. In contrast, orbofiban, even at 500 n*M*, was only effective in inhibiting the formation of larger platelet thrombi (≥ 150 platelets per thrombus). Pretreatment of surface-anchored platelets with roxifiban (100 n*M*), but not orbofiban (500 n*M*), inhibited monocytic THP-1 cell attachment under flow. However, this heterotypic adhesion process was also suppressed when orbofiban (500 n*M*) was maintained in the perfusion buffer during the entire course of flow experiment. These findings demonstrate roxifiban (unlike orbofiban) is a potent glycoprotein IIb/IIIa antagonist with a long receptor-bound lifetime and prolonged anti-platelet efficacy and may thus be beneficial for the treatment and prevention of acute ischemic syndromes. **Key Words:** Adhesion—Aggregation—Platelets—Roxifiban—Shear stress—THP-1 cells.

Platelet adhesion and subsequent thrombus formation at the site of a ruptured atherosclerotic plaque or vascular injury are recognized as important causative factors in the development of cardiovascular and cerebrovascular thromboembolic disorders such as unstable angina, myocardial infarction, and stroke (1–4). The adhesion of

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platelets to exposed subendothelial surfaces of atherosclerotic or injured vessels presenting collagen and von Willebrand factor (vWF) is mediated primarily by the platelet glycoprotein (GP)Ib/IX/V complex under conditions of elevated fluid shear stress (5,6). This primary adhesion to the matrix activates platelets, leading ultimately to platelet aggregation mediated predominantly by the binding of adhesive proteins such as fibrinogen and vWF to GPIIb/IIIa (5–8).

Several studies have identified the pivotal role of GPIIb/IIIa in coronary thrombosis. Hence, this platelet integrin receptor has emerged as a rational therapeutic target in the management of acute coronary syndromes. The first platelet GPIIb/IIIa antagonist developed was abciximab, a chimeric Fab fragment of the monoclonal antibody 7E3 (9). Intravenous administration of abciximab has been shown to reduce the composite incidence of death and ischemic events in patients undergoing percutaneous transluminal coronary angioplasty (9,10), elective stent implantations (11), as well as in refractory unstable angina patients before and after percutaneous transluminal coronary angioplasty (12). However, the use of monoclonal antibodies as therapeutic agents might present certain limitations, such as immunogenicity, lack of oral bioavailability, and lack of reversibility after i.v. administration. Thus, several groups have developed small-molecule selective GPIIb/IIIa antagonists for i.v. use in percutaneous coronary interventions eptifibatide) and acute coronary syndromes (eptifibatide, tirofiban) (13–15). Eptifibatide and tirofiban have faster rates of dissociation from human platelets, reflecting their short duration of anti-platelet effects as compared with abciximab (7,16,17).

Sustained anti-platelet efficacy levels can be achieved with i.v. GPIIb/IIIa antagonists via i.v. bolus and infusion regimens. However, ex vivo monitoring must reflect the in vivo anti-platelet efficacy to achieve clinical benefit.

Studies with orally active, rapidly reversible small-molecule GPIIb/IIIa antagonists including orbofiban, xemilofiban, sibrafiban, lotrafiban, and lefradafiban have demonstrated variable oral anti-platelet activity in humans on their administration two to three times per day (18–21). In particular, clinical development of orbofiban in the large-scale OPUS (Orbofiban In-Patients with Unstable coronary Syndromes) trial was stopped because of lack of clinical benefit and perhaps promotion of thrombotic events. One partial explanation might be the lack of significant and sustained in vivo platelet GPIIb/IIIa blockade due to a possible overestimation of the ex vivo anti-platelet efficacy of orbofiban based on evaluations in citrate anti-coagulated specimens (22). Therefore, it is likely that at trough periods, low plasma concentrations of the rapidly reversible GPIIb/IIIa inhibitor induced a prothrombotic state (19). These factors prompted us to develop a potent small-molecule GPIIb/IIIa antagonist, roxifiban, with a relatively slow platelet dissociation rate for the treatment/prevention of different thromboembolic disorders. Roxifiban, a methyl ester prodrug, has been shown to be 100% converted into its free acid active form on exposure to esterases (23–25).

The present study was undertaken to compare the platelet binding kinetic profiles and anti-platelet efficacy of the nonpeptide GPIIb/IIIa antagonists roxifiban and orbofiban in response to changes in plasma calcium levels in static assays as well as in an in vitro flow model of platelet thrombus formation. To this end, a perfusion chamber coupled with a computerized epifluorescence videomicroscopy system was used to visualize in real time and quantify the adhesion and subsequent aggregation of platelets in anti-coagulated whole blood flowing over type I fibrillar collagen at a wall shear rate of 1,500/s (7,26,27). Furthermore, we used an in vitro flow assay in which surface-anchored platelets were preincubated with the GPIIb/IIIa antagonists, and unbound drug was washed away prior to the perfusion of THP-1 monocytic cells, thereby enabling us to distinguish agents with markedly distinct affinities and receptor-bound lifetimes (26).

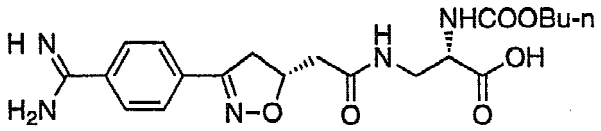
MATERIALS AND METHODS

Reagents

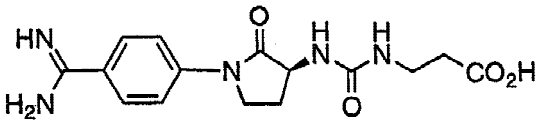
Adenosine 5′-diphosphate (ADP), collagen, and other reagents used but not specifically mentioned were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Thrombin receptor agonist peptide (TRAP) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). Roxifiban, orbofiban, and their respective free acid active forms (Fig. 1) were synthesized at DuPont Pharmaceuticals Co. (Wilmington, DE. U.S.A.). The free acid forms of roxifiban and orbofiban were used in all in vitro studies herein.

Blood collection and preparation

Blood was collected from healthy, aspirin-free donors into polypropylene syringes containing either porcine heparin (Elkins-Sinn, Inc., Cherry Hill, NJ, U.S.A.; heparin sodium, 10 U/ml final concentration) or sodium citrate (0.38% wt/vol final concentration). Specimens were transferred to plastic tubes, stored at room temperature, and used within 4 h of collection (26). For the blood perfusion experiments, the fluorescent dye quinacrine di-



Roxifiban



Orbofiban

FIG. 1. Structure of the free acid forms of roxifiban and orbofiban.

hydrochloride was added to blood samples at a final concentration of 10 μ*M* immediately after drawing to visualize platelets by epifluorescent videomicroscopy (26). In selected experiments, platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 160 *g* for 15 min.

Platelet binding kinetics

In vitro assays using the radiolabeled ^{3}H-active forms of roxifiban and orbofiban were used to determine the equilibrium binding affinity and dissociation rates of these agents from activated (100 μ*M* ADP) and resting human platelets in PRP, as previously described in detail (22,24).

Anti-platelet efficacy: platelet aggregation using light transmittance aggregometry

Citrated PRP (cPRP) and heparinized PRP (hPRP) samples were assayed on a Platelet Aggregation Profiler PAP-4 (Bio/Data Corp., Horsham, PA, U.S.A.). A total of 200 μl of PRP (2×10^8 platelets/ml) were added to each micro test tube, and light transmittance was set to 0%. Then 20 μl of roxifiban, orbofiban, or vehicle (control) was added at different concentrations for 8 min prior to the addition of ADP (10 μ*M*) or TRAP (10 μ*M*). Platelet aggregation response was then measured as percent change in light transmission of either cPRP or hPRP, using platelet-poor plasma to establish 100% light transmission. Results were expressed as mean IC_{50} ± SEM (μ*M*).

Blood perfusion experiments

Platelet deposition onto collagen was quantified under dynamic flow conditions by using a parallel-plate flow chamber. A coverslip coated with type I collagen (2 μg/cm^2) (26,28) was assembled to a chamber (205-μm channel depth, 1.26-cm channel width) and mounted on the stage of an inverted microscope (Nikon TE300, Melville, NY, U.S.A.) equipped with an epifluorescence illumination attachment, a X60 FLUOR objective, a X1 projection lens, and a silicon-intensified target video camera (VE-1000, Dage, Michigan City, IN, U.S.A.) connected to a videocassette recorder and a television monitor. Heparinized or citrated whole blood was perfused through the chamber for 1 min at a wall shear rate of 1,500/s, thereby mimicking the fluid mechanical environment encountered in partially constricted arteries (29). The accumulation of quinacrine-labeled platelets onto collagen was visualized in real time and recorded on videotapes. Prior to the perfusion experiments, blood was incubated with either roxifiban or orbofiban or vehicle (control) at 37°C for 10 min. The microscope stage, flow chamber, and blood were maintained at 37°C during the course of the experiment (26).

Evaluation of platelet adhesion and subsequent platelet aggregation

Videotape images were digitized and computer analyzed at 5, 15, and 60 s for each perfusion experiment as previously described (26). In brief, the number of adherent individual platelets in the microscopic field of view (3.2×10^4 μm^2) during the initial 15 s of flow was used as the measurement of platelet adhesion that initiates platelet thrombus formation (7,26,27). The number of platelets in each individual thrombus was calculated as the total thrombus intensity (area × average intensity) divided by the average intensity of single platelets determined in the 5-s images (26). The extent of platelet aggregation that occurred subsequent to platelet adhesion was represented by the number of platelets in each individual thrombus over the perfusion period of 1 min. Three-dimensional representations were generated using Inovision software (Inovision Inc., Durham, NC, U.S.A.) on a Spark workstation (San Microsystems, Mountain View, CA, U.S.A.) (7,27).

THP-1 monocytic cell-platelet adhesion assays

The monocytic THP-1 cell line was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.), and cultured in a CO_2-independent medium (Life Technologies, Rockville, MD, U.S.A.) supplemented with 10% fetal bovine serum, 4 m*M* L-glutamine and 2.5 g/l glucose. THP-1 cells were washed once, resuspended in serum-free media containing 0.1% bovine serum albumin at a concentration of 10^6 cells/ml, stored at 37°C, and used within 4 h in the adhesion flow assay.

Platelets from cPRP were bound to 3-aminopropyltriethoxysilane-treated glass slides and then incubated with thrombin (1 U/ml) for 10 min at 37°C, as previously described (26,30). After washing the platelet layer with serum-free media/0.1% bovine serum albumin for 4 min, THP-1 cells were perfused through the chamber for 3 min at a wall shear stress of 1.5 dyn/cm^2, thereby simulating flow conditions in postcapillary venules (29). THP-1 cell adhesion to immobilized platelets was visualized with phase-contrast videomicroscopy (Nikon TE300 microscope [Nikon Corp., Melville, NY, U.S.A.], a CCD100 camera [Dage-MTI, Michigan City, IN, U.S.A.]). A single field of view (×10; 0.55 mm^2) was monitored during the 3 min of the experiment, and at the end five additional fields of view were monitored for 15 s each. Three parameters were quantified in the analysis: the number of total interacting cells during the entire 3-min experiment; the number of firmly adherent cells after 3 min of shear flow; and the average rolling velocity (26,30). Platelet P-selectin and GPIIb/IIIa are essential for optimal THP-1 cell adhesion to immobilized, activated platelets at a wall shear rate of 1.5 dyn/cm^2 (unpublished observations). In particular, binding of P-selectin-ligand-1 (PSGL-1) on THP-1 cells to platelet P-selectin is required for efficient tethering/rolling of free-flowing THP-1 cells in shear flow, whereas platelet GPIIb/IIIa is at least partially responsible for mediating the stable adhesion of previously tethered THP-1 cells.

For inhibition studies, surface-adherent platelets were preincubated with roxifiban (100 n*M*), orbofiban (500 n*M*), or vehicle (control) for 10 min during the thrombin incubation. Unbound GPIIb/IIIa antagonist was removed by a brief washing step (4 min) prior to the perfusion of THP-1 cells over the platelet layers. However, the orbofiban concentration (500 n*M*) was continuously maintained in the perfusion buffer during the entire course of selected experimental runs (orbofiban + in-flow).

Statistical analysis

The data are expressed as mean ± SEM). Statistical significance of differences between means was determined by single-factor analysis of variance. If means were shown to be significantly different, multiple comparisons by pairs were performed by Tukey test. Probability values < 0.05 were selected to indicate statistical significance.

RESULTS

Platelet glycoprotein IIb/IIIa binding affinity and dissociation rates

Roxifiban binds with high affinity to unactivated and activated human platelets with K_d = 2.5 ± 1.0 n*M* and 0.8 ± 0.2 n*M*, respectively. In marked contrast, orbofiban binds to resting human platelets with a K_d of 427 ± 85 n*M* and to activated platelets with a K_d of 52 ± 15 n*M*.

Roxifiban demonstrated a relatively slow dissociation rate from either resting ($t_{1/2}$ = 8 min) or activated ($t_{1/2}$ = 10 min) human platelets. In contrast, orbofiban exhibited a relatively faster dissociation rate from either resting ($t_{1/2}$ = 0.20 min) or activated ($t_{1/2}$ = 0.75 min) human platelets. These in vitro dissociation rates of roxifiban and orbofiban may reflect their in vivo duration of anti-platelet efficacy.

Anti-platelet efficacy studies

Platelet aggregation: light transmittance aggregometry. The effect of anti-coagulating blood with citrate versus heparin on the anti-platelet efficacy (IC_{50}) of GPIIb/IIIa

TABLE 1.

Comparative antiplatelet efficacy between roxifiban and orbofiban in inhibiting human platelet aggregation in citrate-versus heparin-anticoagulated platelet-rich plasma

	IC_{50} (μ*M*)					
	ADP			TRAP		
	Citrate	Heparin	Ratio*	Citrate	Heparin	Ratio*
GPIIb/IIIa antagonists						
Roxifiban	0.031 ± 0.019	0.038 ± 0.007	1.2	0.063 ± 0.01	0.084 ± 0.033	1.3
Orbofiban	0.067 ± 0.013	0.140 ± 0.034	2.1†	0.200 ± 0.07	0.400 ± 0.036	2.0†

ADP, Adenosine 5′-diphosphate; GP, glycoprotein; TRAP, thrombin receptor agonist peptide.

Data represent mean ± SEM, n = 4–5. *Mean IC_{50} ratio in hPRP/cPRP; †$p < 0.01$.

Platelet aggregation (light transmittance aggregometry) was induced by adding ADP (10 μ*M*) or TRAP (10 μ*M*) to platelet-rich plasma obtained from either citrated (0.11 m*M* free Ca^{2+}) or heparinized (1.2 m*M* free Ca^{2+}) human blood. The free acid active forms of roxifiban and orbofiban were used in these experiments.

TABLE 2.
Effects of GPIIb/IIIa antagonists roxifiban and orbofiban on platelet adhesion and deposition to a collagen I/vWF surface

GPIIb/IIIa Antagonists	Platelet Adhesion to Collagen I (% of control)	Platelet Deposition/SA
Heparinized Blood		
Control	100	16,289 ± 1,733
Roxifiban		
40 n*M*	111.2 ± 4.5	733 ± 66†
60 n*M*	115.7 ± 11.3	202 ± 10†‡
100 n*M*	91.5 ± 9.2	291 ± 6†‡
Orbofiban		
100 n*M*	108.3 ± 11.7	2,102 ± 274
300 n*M*	97.5 ± 9.3	1,534 ± 238
500 n*M*	120.5 ± 4.8*	916 ± 127*
Citrated Blood		
Control	100	15,873 ± 843
Orbofiban		
100 n*M*	85.7 ± 6.2	737 ± 206†

GP, glycoprotein; SA, surface area; vWF, von Willebrand factor.

Blood specimens were incubated with the indicated concentrations of the free acid active forms of roxifiban, orbofiban or vehicle (control) for 10 min at 37°C before use in perfusion assays. Platelet adhesion is defined as the percentage of the number of adherent platelet thrombi on the collagen I surface (3.2×10^4 μm^2) after 15 sec of flow relative to control samples. Platelet deposition refers to the total number of single platelets accumulated to the collagen I surface after blood perfusion for 1 min at a shear rate of 1,500/sec. Data represent mean ± SEM, n = 3–11, except for *(mean ± range for n = 2). †$p < 0.05$ versus 100 n*M* orbofiban in heparinized blood, ‡$p < 0.05$ versus 40 n*M* roxifiban.

antagonists, roxifiban and orbofiban, was determined. Our data showed that roxifiban demonstrated a high potency (IC_{50} = 0.031–0.084 μM) in inhibiting human platelet aggregation induced by either ADP or TRAP (Table 1). Moreover, an IC_{50} ratio of 1.2–1.3 in hPRP/cPRP (not statistically different) was determined for roxifiban. In contrast, orbofiban showed a two-fold shift in the IC_{50} when using heparin versus citrate, and a ratio of 2.0–2.1 in hPRP/cPRP ($p < 0.01$) was determined (Table 1). Taken together, our data suggest that the GPIIb/IIIa antagonists, roxifiban and orbofiban, demonstrate a differential sensitivity to changes in free plasma calcium concentrations as a result of the calcium chelation by the anti-coagulant citrate (Table 1).

Platelet deposition to type I collagen-coated surfaces. Heparinized blood from healthy donors, treated in vitro with roxifiban, orbofiban, or vehicle, was perfused over collagen I at a wall shear rate of 1,500/s for 1 min. The resulting platelet adhesion, measured by the number of adherent platelet thrombi onto the collagen I surface after 15 s of flow (7,26,27), is shown in Table 2. The data indicate that there was no inhibition in the extent of GPIb/IX/V-mediated platelet adhesion to collagen I/vWF between GPIIb/IIIa antagonist-treated and control (vehicle-treated) blood specimens at all concentrations studied.

At the shear rate of 1,500/s, extensive platelet deposition onto a collagen I surface (16,289 ± 1,733 platelets per 3.2×10^4 μm^2) was detected after 1 min of heparinized blood perfusion (Table 2). Also at this time point, large thrombi containing > 500 platelets accounted for ≥ 75% of all platelets on the surface (Table 3, Fig. 2). Both roxifiban and orbofiban inhibited total platelet accumulation and the number of platelets per thrombus (platelet aggregation) in a concentration-dependent manner (Tables 2 and 3). As a result, the size distribution of platelet thrombi was shifted from large platelet thrombi in control specimens to smaller thrombi in GPIIb/IIIa antagonist-treated blood samples (Table 3). Maximal inhibition of platelet aggregation was achieved by roxifiban at a clinically achievable concentration of 60 n*M*. After 1 min of blood flow in the presence of roxifiban (60 n*M*), the majority of the platelets (≥ 94%; Table 3) were in the form of single platelets, whereas the largest thrombi formed on the collagen surface contained about ≤ 20 platelets accounting for the remaining 3–6% of total platelet accumulation (Table 3). Similar results have been reported with abciximab (26) when used at a concentration of 3.5 μg/ml, which corresponds to maximally achieved concentration observed in vivo after standard patient treatment (31).

Although orbofiban (100 n*M*) significantly reduced total platelet accumulation (Table 2), thrombi containing 21–500 platelets were detected after perfusion of heparinized blood over collagen I (Table 3). An illustration for the contrast between the inhibitory effects of orbofiban (100 n*M*) and roxifiban (100 n*M*) on platelet thrombus formation is shown in Figure 2. It is noteworthy that roxifiban when used at a concentration of 40 n*M* was relatively more effective than orbofiban (100 n*M*) in suppressing platelet deposition onto a collagen I surface in heparinized blood specimens (Tables 2 and 3).

In accordance with the light transmittance aggregometry assays, the anti-platelet efficacy of orbofiban demonstrated a significant sensitivity to changes in plasma calcium levels in the blood perfusion experiments. Our data suggest that orbofiban showed a more than fivefold higher efficacy in inhibiting platelet accumulation onto collagen I on lowering normal plasma calcium levels from about 1 to 0.1 m*M* by using citrate versus heparin as an anti-coagulant (Tables 2 and 3).

THP-1 monocytic cell adhesion to surface-adherent platelets under flow. Our results indicate that immobilized platelets supported extensive THP-1 monocytic cell

TABLE 3.
Effects of the glycoprotein IIb/IIIa antagonists roxifiban and orbofiban on platelet thrombus size distribution

	Number of Thrombi Containing These Amounts of Platelets					
Treatment Group	1–5	6–20	21–150	151–500	501–1000	>1000
Heparinized Blood						
Control	20 (0.7)	12 (1.3)	7 (6.6)	2 (5.4)	4 (23.9)	4 (62.1)
Roxifiban						
40 n*M*	116 (33.8)	31 (39.4)	8 (26.8)	0	0	0
60 n*M*	141 (94.0)	1 (6.0)	0	0	0	0
100 n*M*	169 (97.7)	1 (2.3)	0	0	0	0
Orbofiban						
100 n*M*	65 (6.7)	23 (11.5)	22 (61.7)	2 (20.1)	0	0
300 n*M*	97 (10.8)	31 (18.2)	24 (61.8)	1 (9.2)	0	0
500 n*M*	136 (25.7)	41 (42.6)	11 (31.7)	0	0	0
Citrated Blood						
Control	0 (0)	5 (0.3)	3 (2.1)	6 (8.5)	3 (16.1)	7 (73.0)
Orbofiban						
100 n*M*	158 (59.7)	16 (28.6)	3 (11.7)	0	0	0

The number of thrombi in each platelet number category on the collagen I surface (3.2×10^4 μm^2) after anticoagulated blood was perfused at 1,500/sec for 1 min. Values in parentheses indicate the percentage of total accumulated platelets in thrombi of various sizes in the area of the observation. The size distribution of platelet thrombi was shifted from large (>500) platelets towards smaller thrombi (≤500) in samples incubated with GPIIb/IIIa antagonists. These are results from a representative perfusion experiment. The free acid active forms of roxifiban and orbofiban were used in this in vitro study.

adhesion (182 ± 26 firmly adherent cells per mm^2) at a wall shear stress of 1.5 dyn/cm^2. Treatment of platelet layers with roxifiban (100 n*M*), followed by brief washing to remove unbound drug prior to the perfusion of THP-1 cells, resulted in an approximately 55–60% reduction of firm adhesion (Fig. 3). This inhibitory effect was accompanied by a marked increase in both the number of THP-1 rolling cells (data not shown) and their respective average rolling velocity (43.3 ± 3.4 μm/s and 19.4 ± 1.7 μm/s for roxifiban-treated and control platelets, respectively), in accordance with previously published data (26). In distinct contrast, the short-acting GPIIb/IIIa antagonist orbofiban (500 n*M*) failed to suppress the extent of THP-1-platelet adhesive interactions (Fig. 3) and to substantially affect the average rolling velocity of THP-1 cells (22.9 ± 2.7 μm/s) under the same experimental conditions. However, maintaining the orbofiban concentration (500 n*M*) in the perfusion buffer (orbofiban + in-flow) during the entire course of the experiment inhibited THP-1 firm adhesion to immobilized platelets at a level comparable to that of roxifiban (100 n*M*) (Fig. 3). Furthermore, this treatment significantly increased the average rolling velocity of interacting THP-1 cells (44.4 ± 3.6 μm/s). These findings correlate with the markedly distinct affinities and receptor-bound lifetimes of roxifiban and orbofiban to the platelet GPIIb/IIIa receptor and may reflect their respective in vivo duration of anti-platelet efficacy.

DISCUSSION

Various large-scale clinical trials have demonstrated the efficacy of abciximab against major ischemic events in patients undergoing percutaneous coronary interventions (9,10,12). Additionally, studies with the small-molecule GPIIb/IIIa antagonists, eptifibatide and tirofiban, have illustrated the clinical benefits of i.v. use of specific GPIIb/IIIa inhibitors in patients with acute coronary syndromes (13,15). However, clinical development programs with orally active, rapidly reversible GPIIb/IIIa antagonists including xemilofiban (Evaluation of Oral Xemilofiban in Controlling Thrombotic Event [EXCITE] study), orbofiban (Orbofiban in Patients with Unstable Coronary Syndromes [OPUS] study), and sibrafiban (Sibrafiban Versus Aspirin to Yield Maximum Protection from Ischemic Heart Events Post-acute Coronary Syndromes [SYMPHONY] study) were halted because of lack of efficacy, and perhaps increased thrombotic events (32). The lack of clinical benefit for the oral agents is puzzling and might be at least partially attributed to underdosing. GPIIb/IIIa antagonists, when administered at suboptimal levels, may transiently bind to the platelet receptor, induce conformational changes, which then allow fibrinogen binding to occur after the drug dissociates from the receptor (33,34). Along these lines, it was recently shown that oral administration of orbofiban enhanced platelet reactivity as determined by

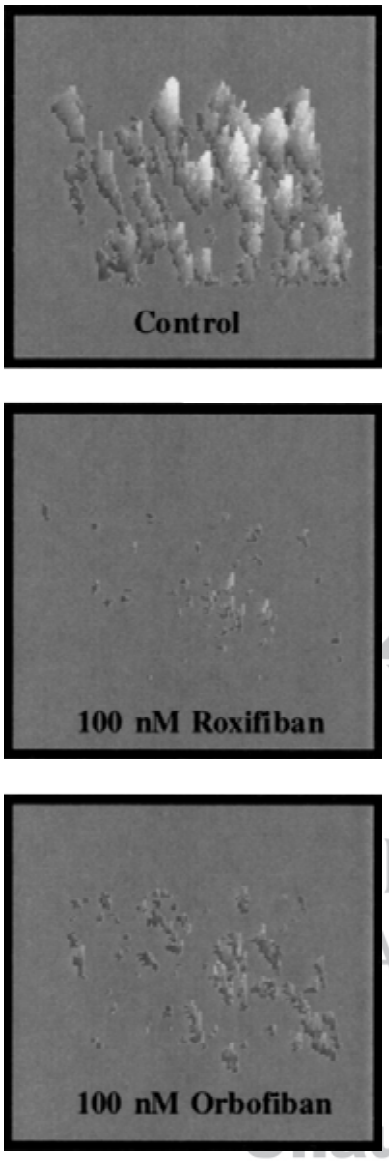


FIG. 2. Three-dimensional computer-generated representations (from videotape of real-time data) of platelet adhesion and subsequent aggregation on collagen I/von Willebrand factor from normal heparinized blood perfused at 37°C for 1 min at 1,500/s. The free acid active forms of roxifiban (100 n*M*) and orbofiban (100 n*M*) were used in this in vitro study.

increased fibrinogen binding to the platelet surface and elevated P-selectin expression levels (33). Therefore, ex vivo monitoring must reflect the in vivo anti-platelet efficacy to optimize therapeutic dose regimens and thus achieve clinical benefit.

In this study, we compared the anti-platelet efficacy of roxifiban to orbofiban in both static and flow assays. Roxifiban exhibited a much higher potency than orbofiban in inhibiting platelet responses regardless of the agonist or the choice of anti-coagulant. Additionally, the anti-platelet efficacy of roxifiban was unaffected by changes in plasma calcium levels. In marked contrast, our data show that there was a significant shift in the IC_{50} of orbofiban in inhibiting platelet aggregation to a greater extent in citrated (relatively lower IC_{50}) than heparinized (relatively higher IC_{50}) PRP specimens. This is attributed to the fact that the anti-coagulant citrate partially chelates calcium, which is necessary for maintaining the integrity of GPIIb/IIIa receptor complex–a calcium-dependent association of the GPIIb and GPIIIa subunits—and for optimal receptor-ligand binding. Consequently, the ex vivo and in vitro potency of certain GPIIb/IIIa antagonists such as orbofiban is artificially enhanced in the presence of citrate as opposed to a non-calcium chelating anti-coagulant (22). This was also the case with eptifibatide with regard to its anti-platelet efficacy in citrate versus heparin or D-Phe-Pro-Arg-chloromethyl ketone hydrochloride (PPACK) anti-coagulated specimens (22,35).

Roxifiban, when used at a clinically achievable concentration of 60 n*M*, was effective in essentially abolishing platelet thrombus formation upon perfusion of heparinized blood over a collagen I/vWF coated surface at a wall shear rate of 1,500/s. Comparable anti-platelet efficacy has been reported with 3.5 μg/ml of abciximab (26), which corresponds to the theoretical maximal blood concentration level achieved after standard patient treat-

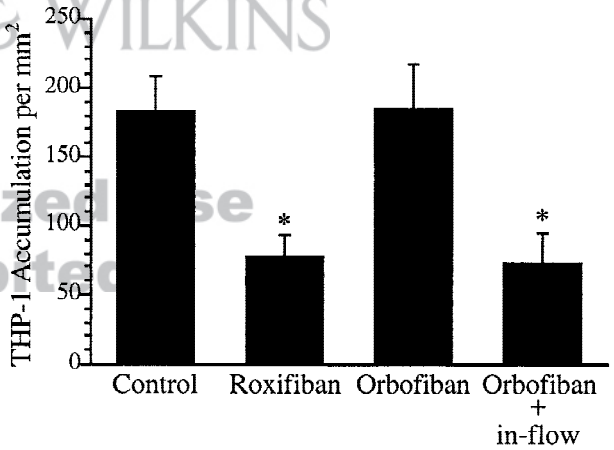


FIG. 3. Effects of the glycoprotein IIb/IIIa antagonists roxifiban and orbofiban on THP-1 cell adhesion to immobilized platelets at a wall shear stress of 1.5 dyn/cm^2. Surface-anchored platelets were treated with roxifiban (100 n*M*), orbofiban (500 n*M*), or vehicle during the 10-min thrombin (1 U/ml) incubation. Unbound drug was removed by brief washing (4 min) prior to perfusion of THP-1 cells, except for selected experimental runs in which orbofiban (500 n*M*) was continuously maintained in the flow buffer (orbofiban + in-flow). Values are expressed as mean ± SEM (n = 3). *$p < 0.05$ versus control, orbofiban.

ment (31). In contrast, orbofiban, even at 500 n*M*, was only effective in inhibiting the formation of larger platelet thrombi containing > 150 platelets per thrombus. Furthermore, a fivefold enhancement in the anti-platelet efficacy of orbofiban was detected on lowering the plasma calcium concentration by using citrate versus heparin as an anti-coagulant. Cumulatively, these results suggest that orbofiban underdosing could be due to the ex vivo anti-platelet measurements in citrated blood, thereby overestimating its anti-platelet efficacy. Consequently, the use of noncalcium chelating anti-coagulants along with a whole blood (rather than PRP) platelet functional assay is recommended to determine the true anti-platelet efficacy of GPIIb/IIIa antagonists (22,35).

Roxifiban binds with higher affinity to both activated and resting platelet GPIIb/IIIa receptors and exhibits relatively slower platelet dissociation rates than orbofiban. GPIIb/IIIa antagonists with slow platelet off-rates such as roxifiban are distributed predominantly as receptor-bound entities, and their anti-platelet efficacy is not dependent on plasma levels (17). In contrast, agents with relatively fast dissociation rates such as orbofiban exist in equilibrium between platelet and plasma compartments, and their anti-platelet efficacy depends on drug plasma (media) levels (17). We therefore used an in vitro flow assay (26) to distinguish agents with markedly distinct affinities and off-rates. In this assay, immobilized platelets were pretreated with the GPIIb/IIIa antagonists, and unbound drug was washed away before the perfusion of monocytic THP-1 cells. We hypothesized that agents with slow platelet off-rates would effectively inhibit THP-1 cell attachment to surface-anchored platelets, whereas rapidly reversible agents would not demonstrate anti-platelet efficacy. Our data indeed showed that roxifiban, but not orbofiban even when used at very high concentrations, significantly reduced the extent of these heterotypic adhesive interactions, a finding that correlates with their respective platelet dissociation rates. It is noteworthy that orbofiban inhibited this adhesion process only when a high concentration level (500 n*M*) was continuously maintained in the perfusion buffer during the entire course of the experiment.

Taken together, our studies suggest that orbofiban might be underdosed and that clinical benefit might be possible with upward dose adjustment and prolonged platelet inhibitory effects. Furthermore, roxifiban, when used in vitro at clinically achievable concentrations, demonstrated a potent anti-platelet efficacy comparable to that achieved by the theoretical maximal abciximab blood concentration detected after standard patient treatment (26,31). In marked contrast to orbofiban, roxifiban exhibits a high affinity binding to both resting and activated GPIIb/IIIa receptors coupled with a long receptor-bound lifetime and thus extended anti-platelet effects. This prolonged anti-platelet efficacy would avoid the possibility of "on-off" proaggregatory effects of the GPIIb/IIIa antagonists that could potentially be observed with oral agents such as orbofiban between doses, thereby providing a more stable anti-platelet activity and eliminating potential prothrombotic effects. Consequently, we could speculate that an oral version of a high-affinity antagonist with slow platelet dissociation rates such as roxifiban might have higher chances for success rather than short-acting GPIIb/IIIa antagonists.

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