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Platelet GPIIb/IIIa binding characteristics of small molecule RGD mimetic: distinct binding profile for Roxifiban

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1 A number of non-peptide orally active RGD mimetic prodrug such as Orbofiban, Sibrafiban, SR121566, Roxifiban and others entered into the clinical evaluation stage. Some of these agents were terminated and some are still in clinical trials.

2 The present study examined the platelet GPIIb/IIIa binding profiles for the active form of Roxifiban, Sibrafiban, SR121566 and Orbofiban using ^{3}H-Roxifiban active form (XV459), ^{3}H-DMP728, ^{125}I-Echistatin, and ^{125}I-Fibrinogen.

3 Either DMP728, Orbofiban, Sibrafiban, SR121566 or Roxifiban active form as well as other RGD mimetic bind to the same binding site (s) on human platelets as evident from the competitive inhibition of binding of each other to human platelet. Additionally, Roxifiban active form competed with FITC labeled GPIIb/IIIa antagonist cyclic RGD peptidomimetic (XL086) as demonstrated using confocal microscopy technique.

4 Roxifiban active form (XV459) demonstrated the highest potency in inhibiting ^{3}H-XV459, ^{3}H-DMP728, ^{125}I-Echistatin, and ^{125}I-Fibrinogen binding to human platelets as compared to the others.

5 Structure activity relationship within the isoxazoline Roxifiban series showed that substituent at the α-carbon next to the carboxy terminal represents an exosite for the affinity binding to human platelets leading to slow platelet dissociation rate.

6 These data indicated a distinct binding profile for Roxifiban (high affinity to both activated and resting platelets associated with a relatively slow K_{off}) as compared to others. These differences might determine the pharmacodynamics and pharmackokinetics of the different GPIIb/IIIa antagonists.

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Abbreviations: ADP, adenosine diphosphate; GPIIb/IIIa, glycoprotein αIIb/β3 integrin; IC_{50}, concentration resulting in 50% inhibition; PRP, platelet rich plasma; RGD, Arg-Gly-Asp

Introduction

The platelet glycoprotein IIb/IIIa complex (GPIIb/IIIa), a membrane protein mediating fibrinogen binding, has been identified as the final common pathway for agonist-induced platelet aggregation (D'Souza *et al*., 1990; Pytela *et al*., 1986; Andrieux *et al*., 1989; Mousa & Bennett, 1996). GPIIb/IIIa in activated platelets is known to bind four soluble adhesive proteins: fibrinogen, von Willebrand factor (vWF), fibronectin, and vitronectin. The binding of fibrinogen and vWF to GPIIb/IIIa causes platelets to aggregate (Bennett & Vilaire, 1979). The binding of fibrinogen is mediated in part by the Arg-Gly-Asp (RGD) recognition sequence. Several RGD-containing peptides have been shown to block fibrinogen binding and prevent the formation of platelet thrombi (Bennett & Vilaire, 1979; D'Souza *et al*., 1990; Mousa *et al*., 1994; Mousa & Bennett, 1996).

Several studies have identified a pivotal role for the platelet GPIIb/IIIa receptor in coronary thrombosis in the management of acute coronary syndromes (The EPIC investigators, 1994; Kleiman *et al*., 1993; Simoons *et al*., 1994; Tcheng, 1997; Topol, 1995; Topol *et al*., 1994). Intravenous administration of c7E3 Fab antibody (Abciximab, ReoPro™) in high-risk patients undergoing angioplasty has been shown to reduce the composite incidence of major ischemic events (The EPIC investigators, 1994). Current intravenous GPIIb/IIIa antagonists in clinical trials such as Tirofiban or Integrilin have a faster rate of dissociation from human platelets reflecting their short duration of antiplatelet effects as compared to that of Abciximab (PRISM-PLUS Investigators, 1998; Hamm *et al*., 1999).

Clinical studies with orally active prodrug GPIIb/IIIa antagonists including Orbofiban, Sibrafiban and LeFradafiban demonstrated variable antiplatelet activity in man upon their administration 2–3 times per day (Simpfendorfer *et al*., 1997; Muller *et al*., 1997; Cannon *et al*., 1998; Keriakes *et al*., 1998; SYMPHONY Investigators, 2000). Clinical experiences with oral platelet GPIIb/IIIa antagonists such as Xemilofiban (EXCITE), Orbofiban (OPUS), and Sibrafiban (SYMPHONY) and more recently Lotrafiban were disappointing. The lack of clinical benefit for the oral agents (Xemilofiban, Orbofiban, Sibrafiban, and Lotrafiban) as compared to the

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documented benefit with the intravenous agents (Abciximab, Integrilin, and Tirofiban) is puzzling. A number of explanations for the discrepancy between the intravenous and oral agents have been suggested. A likely explanation is the lack of a significant and sustained *in vivo* platelet GPIIb/IIIa blockade.

These factors prompted us to continue the clinical development of a potent GPIIb/IIIa antagonist, with a slow platelet dissociation rate for the treatment of the different thromboembolic disorders. Roxifiban (DMP 754), a methyl ester prodrug, has been shown to be 100% converted into its free acid active form (XV459) upon exposure to blood and liver esterases (Mousa *et al*., 1996). The active form of Roxifiban demonstrated potent antiplatelet efficacy and optimal antiplatelet duration as compared to other isoxazoline analogues (Mousa & Wityak, 1998; Mousa *et al*., 1998a–e; 1999; 2000).

The present study was undertaken to determine the platelet GPIIb/IIIa receptor binding profiles of Roxifiban active form (XV459) as compared to other known GPIIb/IIIa antagonists.

Methods

Reagents

Adenosine 5′-diphosphate (ADP) and other reagents used but not specifically mentioned were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). ^{125}I-Fibrinogen was obtained from DuPont NEN (Boston, MA, U.S.A.). Chimeric 7E3 (c7E3) and ^{125} I-c7E3 were obtained from Centocor (Malvern, PA, U.S.A.). ^{125}I-Echistatin was obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, U.S.A.). All free acid forms of Roxifiban, other isoxazoline analogues (XR299, DMP802 and XV454), and other GPIIb/IIIa antagonists radiolabeled form were synthesized at DuPont Pharmaceuticals, (Wilmington, DE, U.S.A.) (Mousa and Wityak, 1998; Olson *et al*., 1997; Wityak *et al*., 1997; Xue *et al*., 1997). FITC-labelled cyclic RGD (XL086) was synthesized at DuPont Pharmaceuticals Co. (Wilmington, DE, U.S.A.).

Preparation of human platelet rich plasma (PRP)

Citrated whole blood (5 ml draw, Vacutainer tubes) was collected from healthy, aspirin free, human subjects and centrifuged for 10 min at $150 \times g$ (22°C) for the separation of PRP (Mousa *et al*., 1994). PRP was removed, pooled, and platelets were counted using a Coulter T540 Hematology Analyser.

Dissociation rates

For platelet/GPIIb/IIIa ligand dissociation rate ($t_{\frac{1}{2}}$), platelet rich plasma samples were treated for 60 min with 0.04 μM of ^{3}H-Roxifiban or the various Roxifiban isoxazoline analogues including XR299, DMP802, and XV454. Following this 60-min incubation period, the tubes were centrifuged for 10 min ($150 \times g$). The resulting ^{3}H-radioligand/PRP complex was carefully removed and centrifuged for an additional 10 min ($1500 \times g$) at room temperature. The resulting PPP was removed and the platelet pellet re-suspended (1.6×10^{8}/ml) in fresh PPP. Five hundred microlitres of this suspension was transferred to wells of a 24-well plate (blocked with 5% BSA). To initiate dissociation, dilution with 1.0 ml Tris buffer, pH 7.4 containing 100 μM non-radiolabelled ligand was added to the wells. At designated time points (0–60 min), the ^{3}H-GPIIb/IIIa antagonist / PRP complex was removed from the wells. For GPIIb/IIIa antagonists with fast platelet dissociation rate the (min) for the dissociation of platelet bound ^{3}H-GPIIb/IIIa antagonists was carried out at short intervals. The resulting platelet pellet was counted using a liquid scintillation counter. CPMs recovered are compared to the control (t = 0) and presented as per cent bound per 0.8×10^{8} platelets over time.

Radiolabelled ligand competitive platelet binding studies

Using ^{3}H-Roxifiban, ^{3}H-DMP728, ^{125}I-Fibrinogen or ^{125}I-Echistatin, the relative binding affinity for various platelet GPIIb/IIIa antagonists such as Roxifiban, Sibrafiban, SR121566 and Orbofiban active forms in inhibiting the binding of the various radiolabelled ligand used was determined.

Platelet ^{125}I-Fibrinogen binding assay

Human PRP was applied to a sepharose column to prepare gel filtered platelets (GFP) as previously described (Bennett & Vilaire, 1979). Aliquots of GFP (2×10^{8} platelets ml^{-1}) along with 1.0 mM calcium chloride with or without the test agent were added to removable 96-well plates. ^{125}I-fibrinogen (26.5 μCi mg^{-1}) was added for 10 min, and the h-GFP was activated by addition of ADP at 20 μM for another 10 min. The ^{125}I-fibrinogen bound to the activated platelet was separated from the free form by centrifugation, and then counted on a gamma counter. Non-specific binding (due to entrapment of ^{125}I-fibrinogen) either in the presence or absence of the inhibitors was shown (in the absence of agonist) to be in the range of 4–6% of total ^{125}I-fibrinogen binding to agonist-activated platelets. Per cent inhibition of ^{125}I-fibrinogen binding to activated platelets was calculated by dividing the specific binding in the presence by that of the absence of the inhibitors. For IC_{50} determination, GPIIb/IIIa antagonists were added at various concentrations 10 min prior to platelet activation.

FITC-ligand competitive platelet binding studies–confocal microscopy

Platelet isolation for confocal microscopy Platelet rich plasma (PRP) was diluted 1 : 1 with acid citrate dextrose and centrifuged at $250 \times g$ to isolate the platelets. The platelet pellet was resuspended in Tyrode's buffer (pH 7.4) containing (mM) NaCl 137, KCl 2.7, $MgCl_2$ 1, NaH_2PO_4 0.36, $NaHCO_3$ 12, $CaCl_2$ 2, glucose 5.5 and 0.35% albumin. Isolated platelets were kept at 37°C and used within 1 h of isolation. The 8-chambered coverglass (Nunc, Naperville, II, U.S.A.) was coated overnight at 4°C with fibrinogen (20 μg ml^{-1}) or collagen (30 μg ml^{-1}). The chambers were blocked with 5% BSA for 1 h at room temperature. The platelet suspension was added to the chambers and then platelets were allowed to attach for 30 min at room temperature. After 30 min of

incubation the Tyrode's buffer was replaced by the solution of FITC-labelled XL086 (cyclic RGD analogue) at 100 nM in Tyrode's buffer (Tsao *et al.*, 1995). RGDS peptide (1 mg ml^{-1}) and active form of Roxifiban (10 μM) were used as competitors for binding. Live images were taken at 5 min intervals using LSM 510 Zeiss Laser confocal microscope with 100 × oil immersion objective.

Results

Platelet dissociation rate of roxifiban analogues

XR299, an isoxazoline without substitution on the α-carbon next to the carboxy terminal, showed a relatively fast (0.1–0.2 min) platelet dissociation rate (Figure 1). In contrast, α-carbamate substitution on the α-carbon (Roxifiban) extended the platelet dissociation rate to 8 min (Figure 1). Furthermore, sulfonamide substitutents on the α-carbon resulted in further extension of the dissociation rates to 32 and 110 min with DMP802 and XV454, respectively (Figure 1).

Radiolabelled ligand competitive platelet binding studies:

Using ^{3}H-XV459, ^{3}H-DMP728, ^{125}I-Fibrinogen and ^{125}I-Echistatin, the relative binding affinity for various platelet GPIIb/IIIa antagonists was determined. Either DMP728, active forms of Orbofiban, Sibrafiban, SR121566 or Roxifiban as well as other RGD mimetic bind to the same binding site(s) on human platelets as evident from the competitive inhibition of binding or displacement of each other binding to human platelet. Roxifiban active form demonstrated the highest potency in inhibiting and displacing bound ^{3}H-DMP728 (Figure 2), ^{3}H-XV459 (Figure 3), ^{125}I-Echistatin or ^{125}I-fibrinogen (Table 1) as compared to other GPIIb/IIIa antagonists including active forms of Orbofiban, Sibrafiban or SR121566.

Roxifiban free acid form demonstrated 5–30 fold greater potency in inhibiting the binding of the cyclic RGD analog (^{3}H-DMP728) to activated human platelets as compared to active forms of Orbofiban. Sibrafiban, and SR121566 (Table 1). Additionally, Roxifiban demonstrated 100–800 fold greater potency in inhibiting the binding of ^{3}H-XV459 to activated human platelets as compared to other GPIIb/IIIa antagonists including active forms of Orbofiban, Sibrafiban or SR121566 (Table 1).

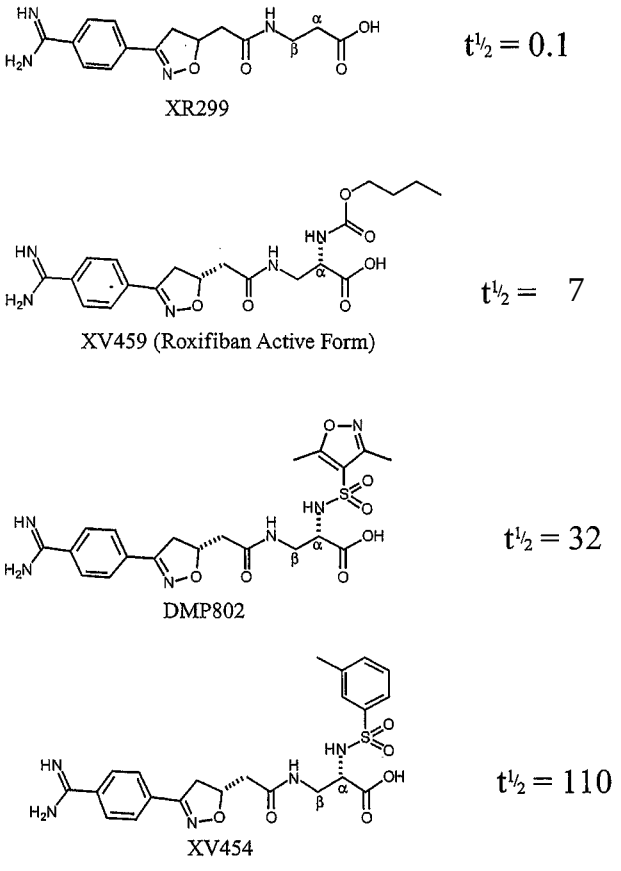

Figure 1 Isoxazoline analogues of Roxifiban and human platelet dissociation rates: Structure activity relationship of a-carbon substituents on the $t_{\frac{1}{2}}$ (minutes) for dissociation from human platelets.

^{125}I-Fibrinogen binding to activated human platelets

Roxifiban free acid form demonstrated 5–10 fold greater potency in inhibiting the binding of ^{125}I-Fibrinogen or ^{125}I-Echistatin to activated human platelets as compared to Orbofiban, Sibrafiban, and SR121566 (Table 1).

Confocal microscopy FITC-ligand competitive platelet binding

The platelet preparation had mixed population of unactivated and partially activated platelets. Upon adhesion to fibrinogen the unactivated platelets spread whereas partially activated platelets shape change forming filopodia. The FITC-labelled XL086 (cyclic RGD analogue) bound to the cell surface integrin GPIIb/IIIa and staining is initially localized to the membrane, which then concentrates to the center of the platelets (Figure 4A) as the platelet spreads. Certain unspread platelets show the typical ring-like staining accompanied with round microparticle-like structures. On collagen

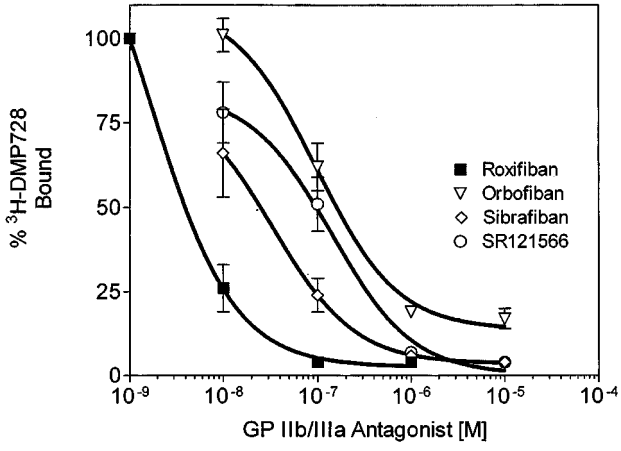

Figure 2 Relative affinity of various GPIIb/IIIa antagonist active forms in inhibiting ^{3}H-DMP728 binding to activated (20 μM ADP) human platelets. Data represent mean per cent ^{3}H-DMP728 bound to activated platelets in the presence of different concentrations of the different GPIIb/IIIa antagonists, $n=3$.

Table 1 Relative binding affinity for various platelet GPIIb/IIIa antagonists to human platelets

	Mean IC_{50} (nM ± s.e.mean)			
GPIIb/IIIa antagonists	*^{3}H-DMP728*	*^{3}H-XV459*	*^{125}I-Echistatin*	*^{125}I-Fibrinogen*
Abciximab	> 10,000	> 10,000	> 10,000	12.0 ± 1.2
Roxifiban	6 ± 1.0	5 ± 0.6	8 ± 1	1.0 ± 0.1
Orbofiban	178 ± 49	4,130 ± 80	91 ± 5	10.0 ± 1.0
Sibrafiban	24 ± 9.0	540 ± 30	40 ± 1	5.0 ± 1.0
SR121566	103 ± 32	1,100 ± 120	–	8.0 ± 2.0
RGD	> 10,000	> 10,000	> 30,000	> 10,000

Data represent mean ± s.e.mean, $n = 3$. The active free acid form of all listed GPIIb/IIIa antagonists were used in these *in vitro* studies. Abciximab did not compete with any of these small molecule GPIIb/IIIa antagonists, but blocked ^{125}I-Fibrinogen binding to activated platelets.

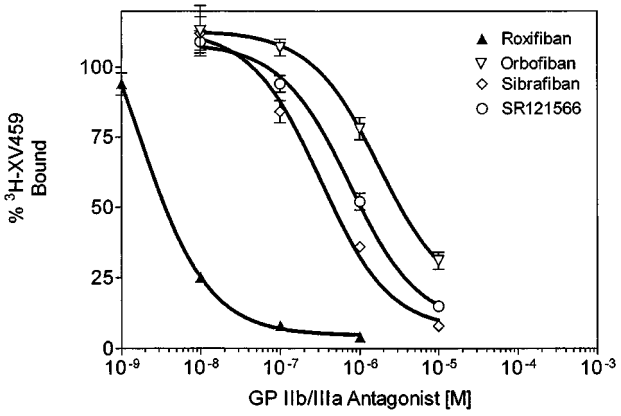

Figure 3 Relative affinity of various GPIIb/IIIa antagonist active forms in inhibiting ^{3}H-XV459 binding to activated (20 μM ADP) human platelets. Data represent mean per cent ^{3}H-Roxifiban free acid form bound to activated platelets in the presence of different concentrations of the different GPIIb/IIIa antagonists, $n = 3$.

the platelets attach but do not spread and the staining is at the periphery which then concentrates at the center of these platelets (Figure 4B). The observed staining is highly specific and could be completely abolished by addition of 100 fold excess active form of Roxifiban (Figure 4C versus A). The binding of FITC-labelled XL086 is specific to platelet surface integrin αIIbβ3, since it could be inhibited by addition of the potent specific GPIIb/IIIa antagonist Roxifiban active form (Figure 4D versus B).

Discussion

It has been recognized that the platelet glycoprotein IIb/IIIa complex (GPIIb/IIIa) *via* its binding to circulating fibrinogen is the final common pathway for all agonist-induced platelet aggregate formation (Bennett & Valaire, 1979; Andrieux *et al*., 1989; Pytela *et al*., 1986). The binding of fibrinogen is mediated in part by the RGD recognition sequence which is common to other adhesive proteins that bind to GPIIb/IIIa receptors or other integrins (D'Souza *et al*., 1990; Pytela *et al*., 1986). Various large-scale phase III clinical trials have illustrated the usefulness of intravenous bolus followed by infusion of Abciximab in the treatment of percutaneous coronary interventions (The EPIC Investigators, 1994; Topol, 1995). Additionally, other selective GPIIb/IIIa antagonists, including Integrilin and Tirofiban demonstrated clinical benefits in the treatment and prevention of acute ischaemic heart diseases after intravenous controlled delivery (Peerlinck *et al*., 1993; Tcheng *et al*., 1995; Topol *et al*., 1994). This is not the case with the oral delivery of platelet GPIIb/IIIa antagonists, which might be due to the variability in attaining sustained and controlled levels of platelet aggregation inhibition (Cannon *et al*., 1998: The Symphony Investigators, 2000).

Data showed competitive inhibition or displacement of platelet binding between the different cyclic RGD analogues and RGD non-peptide mimetic. Additionally, using confocal microscopy data showed inhibition of cyclic RGD platelet binding by the non-peptide GPIIb/IIIa antagonist Roxifiban active form. Roxifiban, an α-carbamate substituted isoxazoline analogue demonstrated tight binding and slow dissociation rate from human platelet (Mousa *et al*., 1998a) as compared to non-α carbon substituted analogue, XR299 (Mousa & Wityak, 1998; Wityak *et al*., 1997). Furthermore, α-substitution with sulfonamide resulted in an even slower platelet dissociation rate (Olson *et al*., 1997). Structure activity relationship within the isoxazoline Roxifiban series showed that substituent at the α-carbon next to the carboxy terminal represent an exosite for the tight binding to human platelets leading to slow platelet dissociation rate (Mousa & Wityak, 1998; Xue *et al*., 1997). The difference in platelet dissociation rate appears to be responsible for the duration of *in vivo* antiplatelet efficacy. XR299 demonstrated sustained antiplatelet efficacy when given three times a day in canine platelets (Mousa *et al*., 1998d), Roxifiban when given QD in baboon platelets (Mousa *et al*., 1998b), and DMP802 when given once a week in baboon platelets (Mousa *et al*., 1998e; Olson *et al*., 1997). Roxifiban active form demonstrated high potency in inhibiting platelet aggregation. A lower IC_{50} for Roxifiban active form in inhibiting ^{125}I-fibrinogen binding to purified platelets as compared to other GPIIb/IIIa antagonists was demonstrated. Comparable IC_{50}s for Roxifiban in inhibiting platelet aggregation was demonstrated regardless of the agonist or the anticoagulant used (Mousa *et al*., 2000). This is in contrast to the significant shift in the IC_{50} of Orbofiban in inhibiting platelet aggregation to greater extent in citrate (relatively lower IC_{50}) versus heparin (relatively higher IC_{50}) resulting an artificial enhancement of *ex vivo* or *in vitro* Integrilin antiplatelet efficacy (Mousa *et al*., 2000). The implication is that Orbofiban might be under-dosed and that greater efficacy might be possible with upward dose

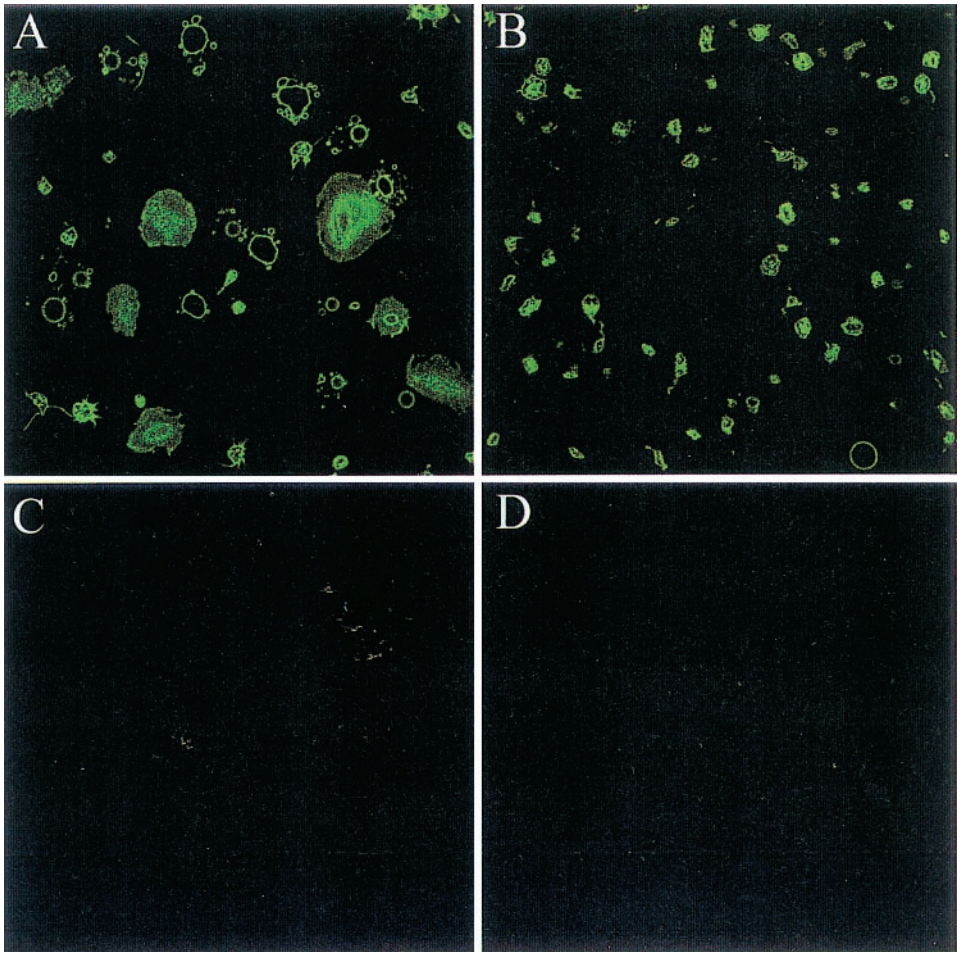

Figure 4 Ligand-binding characteristics of GPIIb/IIIa integrin during platelet spreading: Platelets were isolated as described in the Methods. Isolated platelets were allowed to spread on fibrinogen (A, C) or collagen (B, D). FITC-labelled XL086 (100 nM) was added to all chambers. Active form of Roxifiban (XV459) was added at 10 μM (C, D).

adjustment which might not be the case when using either citrate or heparin as an anticoagulant (Mousa *et al*., 2000).

The active form of Roxifiban demonstrated high affinity for both activated and resting platelets along with relatively slow dissociation rates suggesting a possible prolonged duration of *in vivo* antiplatelet effects (Mousa *et al*., 1998a,b). This is in contrast to the failed oral GPIIb/IIIa antagonists such as Orbofiban and Sibrafiban, which have a short duration of antiplatelet effects associated with their relative fast dissociation rates from human platelets (Mousa *et al*., 2000).

These data indicated a distinct binding profile for Roxifiban as compared to Orbofiban, Sibrafiban, and SR121566. These differences in the binding kinetics might determine the pharmacodynamics and pharmackokinetics of the different GPIIb/IIIa antagonists.

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