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# Hemostatic effects of recombinant DisBa-01, a disintegrin from *Bothrops alternatus*

**Alexandre Kauskot[1,2], Marcia R Cominetti[3], Oscar HP Ramos[3], Iga Bechyne[1,2], Jean-Marie Renard[1,2], Marc F Hoylaerts[4], Michel Crepin[5], Chantal Legrand[1,2], Heloisa S Selistre-de-Araujo[3], Arnaud Bonnefoy[6,7]**

*[1]INSERM U553, Paris, F-75475 France, [2] Universite Paris 7, IFR 105, Paris, F-75475, France, [3]Department of Ciencias Fisiologicas, Universidade Federal de Sao Carlos, Sao Carlos, Brasil, [4]Center for Molecular and Vascular Biology, University of Leuven, B-3000, Leuven, Belgium [5] Universite Paris 13, CNRS UMR 7033, Bobigny, F-93000, France, [6]INSERM U743, Centre de Recherche du Centre Hospitalier de l'Universite de Montreal-Saint-Luc, Montreal QC, H2X1P1, Canada, [7] Centre de Recherche de l'Institut de Cardiologie de Montreal, Montreal QC, H1T1C8, Canada*

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## 1. ABSTRACT

A monomeric RGD-disintegrin was recently identified from a cDNA library from the venom gland of *Bothrops alternatus*. The corresponding 12 kDa-recombinant protein, DisBa-01, specifically interacted with alpha$_v$beta$_3$ integrin and displayed potent anti-metastatic and anti-angiogenic properties. Here, the interaction of DisBa-01 with platelet alpha$_{IIb}$beta$_3$ integrin and its effects on hemostasis and thrombosis were investigated. DisBa-01 bound to Chinese Hamster Ovary (CHO) cells expressing beta$_3$ or alpha$_{IIb}$beta$_3$ and promoted their adhesion and the adhesion of resting platelets onto glass coverslips. The disintegrin inhibited the binding of FITC-fibrinogen and FITC-PAC-1 to ADP-stimulated platelets and inhibited ADP-, TRAP- and collagen-induced aggregation of murine, rabbit or human platelets. In a flow chamber assay, DisBa-01 inhibited and reverted platelet adhesion to immobilized fibrinogen. DisBa-01 inhibited the phosphorylation of FAK following platelet activation. The intravenous injection of DisBa-01 in C57Bl6/j mice, prolonged tail bleeding time as well as thrombotic occlusion time in mesenteric venules and arterioles following vessel injury with FeCl3. In conclusion, DisBa-01 antagonizes the platelet alphaIIbbeta3 integrin and potently inhibits thrombosis.

## 2. INTRODUCTION

The snake *Bothrops alternatus* belongs to the Viperidae family and is primarily present in Northeast Argentina and the South region of Brazil. Victims of *B. alternatus* envenomation suffer from a complex series of pathophysiological alterations including local necrosis, oedema and blisters and severe systemic hemostasis disturbances (hemorrhage and coagulation disorders) that could lead to acute renal failure, cardiovascular shock and death (1). In the search for venom components responsible for the pathophysiology and lethality of the bothropic envenomation, several molecules have been identified. Among these, phospholipase A2 is largely accountable for the lethality of the envenomation (2) but other venom components were isolated and found responsible for bleeding and the prominent local and systemic effects such as the thrombin-like enzyme balterobin (3); the C-Type lectin thrombin inhibitor bothroalternin (4), and alternagin, an hemorrhagic metalloproteinase and its non-RGD disintegrin-like and cysteine-rich domains, alternagin-C (5).

Disintegrins are non-enzymatic, cysteine-rich, small molecular weight proteins (7 to 10 kDa) mainly present in the haemorrhagic venom of crotalidae and viperidae snake (6). Disintegrins can be categorized into at least five sub-families according to their polypeptide length and disulfide-bond framework (medium, short, dimeric, disintegrin/cysteine-rich, ADAM (disintegrin and metalloproteinase)-like character (7). The majority of disintegrins are derived from the C-terminal domain of class P-II snake venom metalloproteinases by proteolysis (SVMP) (8) and found in a soluble form in the snake venom.

Disintegrins have the ability to bind integrins, which are heterodimeric receptors present on the cell surface. Integrins interact with components of the basement membrane such as collagen IV and laminin; with adhesive proteins present in the extracellular matrix (vitronectin, fibronectin, collagen I and III), or in the blood (fibrinogen, von Willebrand factor); or with membrane-bound counter-receptors on other cells. Integrins are involved in many physiological or pathological processes including hemostasis, metastasis formation and inflammation (for review see 9). The interaction of specific ligands with integrins, modifications in the integrin activation state and their expression levels are frequently associated with metastatic progression (10). Therefore, integrin antagonists such as the disintegrins could be particularly useful in targeting drugs against thrombosis and cancer. Based on their high integrin affinity and selectivity, some disintegrins have been investigated in pharmaceutical development (11-12).

Several lines of evidence have demonstrated a potential role for the $alpha_v beta_3$ integrin in invasive tumors and metastasis in bone (10-13). $alpha_v beta_3$ antagonists have been described to reduce the adhesion of breast tumor cells to the vascular subendothelium (14). In addition, platelets have also been described as important factors in facilitating the metastatic process (15). Platelet actions include the protection from immune cells (16), increase in tumor cell adhesion to the endothelium to facilitate their extravazation (17) and the release of angiogenic factors from activated platelets at the metastatic site (18). Therefore, integrin antagonists that bind to receptors in both platelet and tumor cells could be very helpful in anticancer therapy.

We recently reported the identification and production of a novel P-II SVMP-derived disintegrin, DisBa-01 (Genbank accession no. AY259516) produced by recombinant DNA techniques from the venom gland RNAs of a *B. alternatus* specimen (19). DisBa-01 is a 78-residue protein with an RGD adhesive motif (amino acids 56-58). The recombinant protein is fused with a His Tag and has a molecular weight of 11,780 Da deduced by mass spectrometry. *In silico* studies predicted a preferential interaction of the toxin with $beta_3$ integrins and biacore analysis confirmed the specific binding of DisBa-01 to immobilized purified $alpha_v beta_3$ integrin (19). Dis*Ba*-01 inhibited $alpha_v beta_3$-dependent cell adhesion *in vitro* and potently inhibited angiogenesis and metastasis *in vivo*.

In the present study, we addressed the ability of Dis*Ba*-01 to interact and interfere with the other $beta_3$ integrin, $alpha_{IIb} beta_3$ (fibrinogen receptor), a key player in hemostasis and thrombosis mechanisms expressed on megakaryocytes and blood platelets. We demonstrate that Dis*Ba*-01 antagonizes platelet $alpha_{IIb} beta_3$ and interferes with the outside/-in phosphorylation of the focal adhesion kinase (FAK) downstream of the integrin. Dis*Ba*-01 strongly inhibits platelet aggregation in response to several agonists, abolishes and reverses dynamic platelet recruitment to immobilized fibrinogen *in vitro*, and has a strong antithombotic activity *in vivo*.

## 3. MATERIALS AND METHODS

### 3.1. Reagents

Thrombin receptor activating peptide-6 (TRAP-6), bovine alpha-thrombin and rhodamine 6G were from Sigma Chemical Co (St-Louis, Mo). Native type I collagen fibrils from equine tendon (collagen reagent Horm) were from Nycomed (Munich, Germany) and protease inhibitor cocktail Complete tablets from Hoffman-La Roche (Basel, Switzerland). Human fibrinogen (Fg) depleted of von Willebrand factor (vWF) and fibronectin were purchased from Enzyme Research Laboratories Inc. (South Bend, IN). D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was from Bachem (Voisins-le-bretonneux, France). Hirudin and fluorescein isothiocyanate (FITC) were from Calbiochem (Nottingham, UK). FITC-labeling of fibrinogen and DisBa-01 was performed as previously described (20). FITC-labeled PAC-1, an IgM recognizing the active form of $alpha_{IIb} beta_3$ was from Becton Dickinson (Franklin Lakes, NJ, USA). Lamifiban, a nonpeptide analogue of the RGD peptide but 1000 times more potent and selective for $alpha_{IIb} beta_3$ than for $alpha_v beta_3$ (21), was kindly provided by Dr. T. Weller (F. Hoffmann-La Roche, Basel, Switzerland) and the 4A5 monoclonal antibody (MoAb) directed against the carboxy terminus of the

gamma chain of Fg was a kind gift of Gary Matsueda, (Princeton University, NJ).

### 3.2. Animals

Experimental procedures performed on animals were done according to the French legislation on protection of animals. C57BL6/J mice were obtained from Janvier Laboratories (Le Genest-St-Isle, France) and kept in animal facilities (Departement d'experimentation animale, Institut d'Hematologie, Hopital Saint-Louis, Paris, France). Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg) and xylocaine 0.5% was used as local analgesic. Following blood collection or thrombosis experimentation, mice were euthanized by cervical dislocation.

### 3.3. Dis*Ba*-01 expression, purification and characterization

Dis*Ba*-01 was identified and produced from the mRNA fraction purified from the venom gland of a BA specimen as recently described (19). The coding region corresponds to a medium disintegrin (78 amino acids, ~8 kDa) with an RGD adhesive motif at position 56-58. The His-Tag fusion protein produced in *E. coli* pDis*Ba*-01 is a 12 kDa protein as estimated by mass spectrometry and SDS-PAGE and shows sequence similarities with a variety of members of the disintegrin family (19).

### 3.4. Blood collection and platelet isolation

Mouse platelets. The blood from C57BL6/J anesthetized mice (8-10 weeks-old), anticoagulated with 0.02 IU/mL Hirudin and 80 micro M PPACK was collected by intracardiac puncture and centrifuged at 150 x g for 5 min. Platelet Rich Plasma (PRP) from three mice was pooled, diluted in pH 6.5 ACD buffer (93 mM citric acid, 7 mM sodium citrate, 0,14 M dextrose) (1v/2v) and centrifuged at 800 x g for 5 min. Platelets were washed once in pH 6.5 Patcheke's buffer (36 mM citric acid, 5 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 103 mM NaCl) containing 0.1 micro M PGE1 and resuspended in pH 7.5 Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.2 mM $NaHCO_3$, 0.36 mM $NaH_2PO_4$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 5.5 mM glucose).

Human platelets. Blood was taken from the antecubital vein of healthy volunteers not taking any medication, and directly added into 3.8% sodium citrate (1:9 vol/vol blood). Blood was centrifuged for 15 min at 150 x g and PRP was harvested. For platelet isolation, PRP was centrifuged for 15 min at 900 x g. Platelet pellets were then resuspended in Patcheke buffer containing 0.1 microM PGE1, washed once and resuspended in Tyrode's buffer.

Rabbit platelets. Rabbit blood was drawn from the central ear vein into tubes containing 12 mM citrate and centrifuged for 10 min at 150 x g to obtain PRP.

### 3.5. Binding assays

#### 3.5.1. FITC-DisBa-01 binding to transfected CHO cells

Chinese Hamster Ovary (CHO) cells, non-transfected (control) or transfected with $beta_3$ (CHO-$beta_3$) or $alpha_{IIb}beta_3$ integrin (CHO-$alpha_{IIb}beta_3$) were selected, upon transfection with a Sal I digest of vectors pBJ1GPIIbWT and pBJ1GPIIIaWT, which were a kind gift by Dr. N. Kieffer (Luxemburg). Stable clones expressing the $alpha_{IIb}$ and $beta_3$ chains were obtained via co-transfection with a 10-fold excess of plasmid pND1 and pNT, encoding resistance to neomycin and hygromycin respectively. Stable clones expressing $alpha_{IIb}beta_3$ resulted from transfection of CHO-$\beta_3$ cells with pBJ1GPIIbWT and double selection with neomycin and hygromycin respectively. Cells were maintained in MCDB-131 medium (Invitrogen), containing 10% foetal bovine serum, penicillin (100UI/ml), streptomycin (100microg/ml) and L-glutamine (2mM). For transfected cells culture, 0.3mg/ml G418 was added to the medium. Cells were incubated for 20 min at room temperature (RT) in the dark with increasing concentrations of FITC-DisBa-01 (0.2 microM to 3.2 microM) and the bound fluorescence was measured by flow cytometry (FACScalibur – BD Biosciences) using a single laser emitting excitation light at 488nm.

#### 3.5.2. Adhesion of CHO-transfected cells

Vitronectin (1microg/100microl of DPBS/well), fibrinogen (1microg), DisBa-01 (10microg) or BSA (1 mg/ml), were immobilized in a 96 well plate overnight at 4°C. Wells were blocked with 1% BSA and $5x10^4$ CHO cells, non-transfected (control) or transfected with $beta_3$ (CHO-$beta_3$) or $alpha_{IIb}beta_3$ integrin (CHO-$alpha_{IIb}beta_3$) were added to each well and the plate was incubated for 1 hour at 37°C. Non-adhered cells were washed with DPBS and bound cells were stained by incubation with crystal violet (0.5%) dissolved in methanol (20%) (50microl/well) for 1h at RT, and extensively washed. Wells were then incubated for 30 min at RT with a solution of ethanol: citrate 0.1M (1:1) pH 4.2. Plates were read at 540 nm in an ELISA reader (Labsystems).

#### 3.5.3. Competition assays

Human washed platelets (WP) (30,000/microL), activated by 50 microM ADP, were incubated for 30 min at 37°C with 200 nM FITC-Fg or 12.5 microg/mL PAC-1-FITC, respectively, in the presence of increasing concentrations of DisBa-01 (0 to 1 microM) in 100 microL of Tyrode's buffer, after which platelet bound FITC was measured by flow cytometry.

### 3.6. Static platelet deposition

Glass coverslips were coated with DisBa-01 (10 microg/ml), Fg (200 microg/ml), RGDS (1 mM), echistatin (1 microg/ml) or the anti-Fg 4A5 MoAb (30 microg/ml), in six well plates overnight at 4°C, washed three times with DPBS and incubated with human WP (5,000 platelets/microL) in Tyrode's buffer containing $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM) for 1 h at RT. Coverslips were washed with PBS, fixed with 4% paraformaldehyde during 15 minutes and permeabilized during 30 min with a solution of Tris-HCl, (100mM) EGTA (10 mM), NaCl (150 mM) , $MgCl_2$ (5 mM) and Triton X-100 (0.2%) (v/v), pH 7.4. Coverslips were saturated with 5% BSA in DPBS and labelled with phalloidin (1 microg/ml) diluted in 0.5% DPBS/BSA for 30 min at RT in the dark. Coverslips were mounted and analysed on an epifluorescent microscope (Nikon Eclipse TE 300, Champigny-sur-Marne, France).

Pictures were taken with an attached 1308C CCD camera (Scion Corp. Frederick, Maryland, USA) coupled to a computer. The public domain software ImageJ 1.34s (National Institute of Health. USA) was used to measure the number of adhered platelets, the surface coverage and the mean single-platelet area.

### 3.7. Blood perfusion assays

The effect of DisBa-01 on the dynamic recruitment of blood platelets onto immobilized fibrinogen was analyzed in a parallel-plate perfusion chamber. Glass coverslips coated overnight at 4°C with either Fg (200 microg/ml) or BSA (1%) constituted the bottom of the chamber and the actual chamber was formed by a plexiglass flow path. Heparin-anticoagulated human whole blood was labelled with rhodamine 6G (15 microg/ml; 5 min at RT) and perfused at a wall shear rate of 300 $s^{-1}$ by maintaining a constant flow rate of 0.6 ml/min with an inverted syringe pump (Harvard Instruments, South Natick, MA). Tyrode's buffer containing 2 mM $CaCl_2$, 1 mM $MgCl_2$) and 1 % BSA was aspirated at 37°C for 5 min to warm the chamber and perfuse the coverslip, after which blood at 37°C was perfused in the absence or presence of 1 microM DisBa-01 at 37°C. The flow chamber was mounted on the table of an inverted epifluorescence microscope (Nikon Eclipse TE 300) coupled to a 1308C CCD camera. Five minutes after the beginning of the perfusion, 5 images of 205 x 330 mm fields were captured randomly in the flow path. To measure platelet detachment by DisBa-01, blood was first perfused during 5 min on immobilized Fg followed by an additional 5-minute run with Tyrode's buffer (with 2 mM $CaCl_2$, and 1 mM $MgCl_2$) containing DisBa-01 (1microM) or saline. Platelet surface coverage was measured with the ImageJ software by applying a grey level-threshold to distinguish platelets from background fluorescence.

### 3.8. Platelet aggregation assays

PRP from human, mouse and rabbit blood, and WP adjusted to 200,000 platelets/microL in Tyrode's buffer completed with $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM), were preincubated for 1 min with varying concentrations of DisBa-01 (0 to 1 microM) and activated by 50 microM ADP (50 or 100 microM) thrombin (0.1 U/mL), TRAP-6 (10 microM) or equine tendon collagen (2 microg/ml). Platelets were transferred in the cuvette of an aggregometer (Beckman Chrono-Log) and aggregation was monitored during 5 min by measuring light transmission through the stirred suspension (1,000 rpm).

### 3.9. Tyrosine phosphorylation assays

Human WP (400,000/microL in 250 µL) were treated with ADP (100 microM), collagen type I (10 microg/ml), thrombin (0.2 U/ml), Abciximab (50 microg/ml), Lamifiban (1 microM), Dis*Ba*-01 (1 microM) or PBS (control), either alone or in combination, and incubated for 5 min at 37°C under agitation in an aggregometer. Samples were lyzed in a solution composed of Complete® Protease Inhibitor Cocktail, supplemented with 15 mM NaF, 3.5 mM $Na_3VO_4$, 0.1 mM phenylarsine oxyde and 1% SDS. The mixture was incubated under agitation for 30 min at 4°C and insoluble material was removed by centrifugation (10,000 × g; 15 min). The supernatant was collected and total protein concentration was measured using the BCA protein assay (Pierce). Ten micrograms of each sample, solubilized in boiling SDS sample buffer (240 mM Tris-HCl, 10% SDS, 50% glycerol, 0.1 mg/ml bromophenol blue, pH 6.8), was separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were blocked with 5% non-fat milk dissolved in PBST (Phosphate-buffered saline; 0.1% Tween 20) during 1 h at RT (or, alternatively, overnight at 4°C). Blots were probed with the Py-Plus-HRP mouse anti-phosphotyrosine cocktail (1:1000) (Zymed laboratories). Primary antibody incubations were performed in blocking buffer for 2 h at RT. After washing in PBST, immunorecognitions were evidenced on membrane using Super Signal West Pico Chemiluminescent Substrate (Pierce). To monitor the loading of gel lanes, the membranes were stripped by incubation with a solution of 2 M glycine (pH 2.5) for 10 min under agitation and then re-probed with a rabbit anti-SYK antibody (2 microg/ml) (Chemicon).

Alternatively, human WP (250,000/microL in 1.5 mL) were incubated for 15 min at 37°C onto six-well plastic plates uncoated or coated overnight at 4°C with Dis*Ba*-01 (10 microg/ml). Adherent platelets were washed and processed for total tyrosine-phosphorylation measurement by western blotting as described above.

#### 3.9.1. Inhibition of focal adhesion kinase (FAK) phosphorylation

Human WP preincubated 1 min with DisBa-01 (5 microM) or echistatin (1 microM) were activated with ADP (100 microM) and stirred during 5 min in the cuvette of an aggregometer. Samples were collected in lysis buffer and processed as above for SDS-PAGE and western blot analysis. Nitrocellulose membranes were probed with rabbit polyclonal anti-FAK (pSer722) phosphospecific antibody (Santa Cruz Biotechnology) (1:100). Membranes were washed in PBST and incubated with peroxidase-conjugated affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch) (1:20,000) in blocking buffer for 2 h at RT. To monitor the loading of gel lanes, the membranes were stripped by incubation with a solution of 2 M glycine (pH 2.5) for 10 min under agitation and then re-probed with the rabbit anti-FAK (A-17) antibody (2 microg/ml) (Santa Cruz Biotechnology).

### 3.10. Bleeding time experiments

One hundred microL of NaCl (0.9%; n=5) or DisBa-01 (2 mg/kg; n=5) in saline buffer was administrated via retro-orbital sinus injection to C57BL6/J anesthetized male mice (8-10 weeks-old, 20 g). Five minutes after the injection, 3 mm of the tail tip was severed with a scalpel, the bleeding tail was immerged in a 0.9% NaCl solution kept at 37°C and the bleeding time was measured. If no cessation of blood flow occurred after 20 min, bleeding was stopped by compression and 1,200 s was recorded as the bleeding time.

### 3.11. Ferric chloride-induced thrombosis model

C57BL6/J male mice (4-5 weeks-old, 15 g) were anesthetized with pentobarbital (60 mg/kg) via

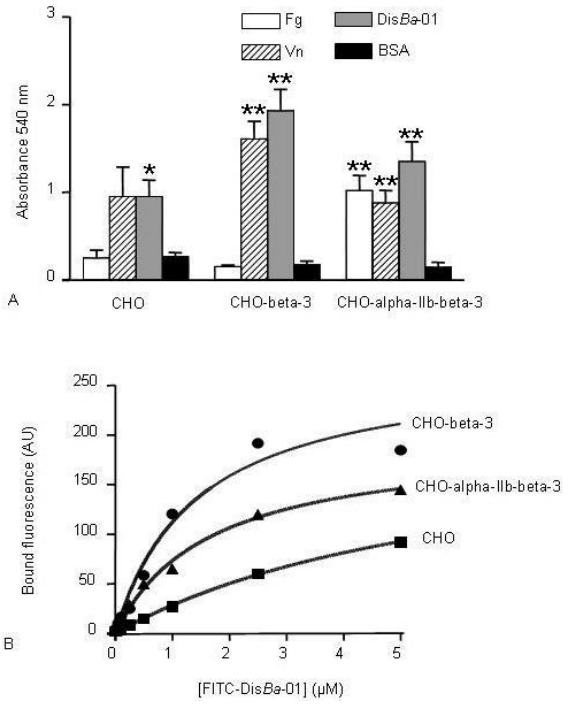


**Figure 1.** DisBa-01 interacts with CHO cells transfected with $beta_3$ or $alpha_{IIb}beta_3$. (A) Promotion of cell adhesion by DisBa-01. CHO cells (wild type) or CHO cells transfected with $beta_3$ (CHO-$beta_3$) or $alpha_{IIb}beta_3$ (CHO-$alpha_{IIb}beta_3$) integrin were added ($5x10^4$) to ELISA plates previously coated with fibrinogen (1microg/well), vitronectin (1microg/well), DisBa-01 (10microg/well) or BSA (1%) as a negative control, and incubated for 1 h at 37°C. Non-adhered cells were washed and adhered cells were stained as described in Materials and Methods. Results are expressed as the mean absorbance at 540 nm (+/- SEM) of triplicate experiments. *p< 0.03; **p<0.009, unpaired t test. (B) CHO, CHO-$beta_3$ or CHO-$alpha_{IIb}beta_3$ cells were incubated for 20 min at RT in the dark with increasing concentrations of FITC-DisBa-01 (0.2 to 3.2microM) and the bound fluorescence was measured by flow cytometry. Results are expressed as the mean of duplicate experiments.

intraperitoneal injection, 0.5 % xylocaine being used for local analgesia. The mesentery was exteriorized and localized thrombosis of mesenteric arterioles and venules was induced by deposition of 10 microL of agarose gel (1%) containing 500 mM $FeCl_3$ on top of an arteriole/venule couple. Ten minutes before the onset of the injury, DisBa-01 (0, 2 or 4 mg/kg) was injected in the blood circulation through the retro-orbital sinus together with 3.3 mg/kg Rhodamine 6G in saline buffer (100 µL). Platelet deposition and thrombus growth were observed in real-time with a Nikon Eclipse TE 300 epifluorescent microscope connected to an attached digital camera Nikon DXM1200, and recorded in the memory of an attached computer for later analysis with the Act-1 program (Nikon). The parameter analyzed was the vessel occlusion times, defined as blood flow arrest for at least 1 minute. Up to 3 lesions were performed on the same animal over a period of 60 min, a single injury being made on each vessel.

### 3.12 Statistical analysis

The statistical significance between means was assessed via non-parametric Mann-Whitney test with two-tailed P-value. Data are expressed as mean +/- SEM.

## 4. RESULTS

### 4.1. Dis*Ba*-01 binding to CHO cells

The interaction of Dis*Ba*-01 with CHO cells expressing human $alpha_{IIb}beta_3$ integrin or the $beta_3$ chain solely was assessed both in adhesion and flow cytometry assays (Figure 1). Consistent with the constitutive expression of $alpha_v beta_3$ by CHO cells that specifically adhere onto surface-adsorbed monoclonal anti-$alpha_v beta_3$ LM609 antibody (not shown), and with the recently reported affinity of Dis*Ba*-01 for $alpha_v beta_3$ (19), untransfected CHO cells and CHO-$beta_3$ were adherent to Dis*Ba*-01 and to vitronectin, but not to Fg and BSA. CHO-$alpha_{IIb}beta_3$ cells adhered to Dis*Ba*-01, vitronectin and Fg, but not to BSA, demonstrating a proper expression and folding of the $alpha_{IIb}beta_3$ complex on the cell surface (Figure 1a). In flow cytometry assays, FITC-Dis*Ba*-01 bound to CHO, CHO-$beta_3$ and CHO-$alpha_{IIb}beta_3$ cells with respective Kds of 6.8 microM, 3.1 microM and 2.1 microM, indicative for a gain of affinity of DisBa-01 for CHO cells expressing human $beta_3$ and $alpha_{IIb}beta_3$ (Figure 1b).

### 4.2. Dis*Ba*-01 antagonizes $alpha_{IIb}beta_3$ on platelets

The ability of Dis*Ba*-01 to antagonize $alpha_{IIb}beta_3$ on human platelets was evaluated in competition assays monitored by flow cytometry. Dis*Ba*-01 dose-dependently inhibited the binding of FITC-Fg to ADP-activated human platelets with an $IC_{50}$ of 100 nM (Figure 2a). Maximal inhibitory effect was achieved with 1 microM Dis*Ba*-01, leaving a basal fluorescent signal identical to the value obtained with saturating concentration of Lamifiban (10 microM), an RGD analogue specific for $alpha_{IIb}beta_3$ (not shown). In addition, Dis*Ba*-01 dose-dependently inhibited the binding of FITC-PAC-1, an IgM specific to the active form of $alpha_{IIb}beta_3$, to ADP-activated human platelets, with an $IC_{50}$ of 70 nM (Figure 2b).

### 4.3. Platelet adhesion studies

The ability of Dis*Ba*-01 to support platelet adhesion in static conditions was assessed by incubating human WP (5,000/microL) on glass coverslips coated with Dis*Ba*-01, Fg, RGDS peptide, echistatin or the 4A5 MoAb, for 1 hour at RT. WP adhered to immobilized Dis*Ba*-01 (145 +/- 29 platelets per field) to the same extent as to Fg (164 +/- 6), RGDS (149 +/- 14) and echistatin (123 +/- 9). By comparison, only 78 +/- 19 platelets adhered to the immobilized 4A5 monoclonal antibody used here as a negative control (p<0.008 versus DisBa-01) (Figure 3a). The total surface covered by platelets adhered onto Dis*Ba*-01 ($10.1x10^4$ +/- $3.2x10^4$ arbitrary units) was much higher than on the 4A5 MoAb ($2.7x10^4$ +/- $0.4x10^4$ AU) and comparable to RGDS ($9.7x10^4$ +/- $1.9\times10^4$ AU) and

**Table 1.** DisBa-01 inhibits the aggregation of human, mouse or rabbit PRP or WP induced by several agonists

| | | $IC_{50}$ (nm) | | |
|---|---|---|---|---|
| | | **Human** | **Rabbit** | **Mouse C56Bl6/j** |
| WP | ADP (100 microM) | 241 +/- 62 | ND | 25[1] |
| | Thrombin (0.1 U/ml) | 1430 +/- 734 | ND | ND |
| PRP | Collagen (2 microg/ml) | 701 +/- 244 | 235[1] | ND |
| | TRAP-6 (10 microM) | 594 +/- 226 | ND | ND |
| | ADP (50 microM) | 203 +/- 12 | 124[1] | 81[1] |

PRP or WP transferred in the cuvette of an aggregometer was preincubated for 1 min with increasing concentrations of DisBa-01 (0 to 1 microM) and stimulated with 50 microM (PRP) or 100 microM (WP) ADP, thrombin (0.1 U/mL), TRAP-6 (10 microM) or equine tendon collagen (2 microg/ml). $IC_{50}$ were calculated from the percentage of light transmission measured after 5 min of stirring. [1] mean $IC_{50}$ of duplicate experiments.

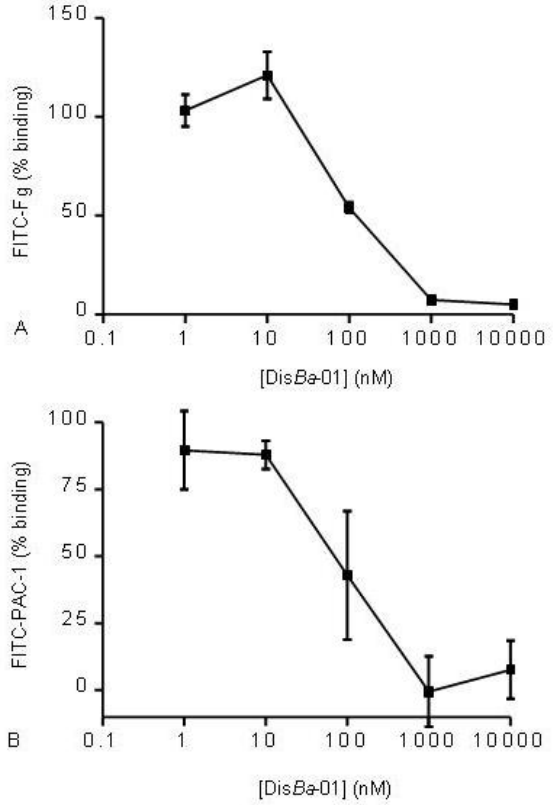


**Figure 2.** DisBa-01 antagonizes $alpha_{IIb}beta_3$ on platelets. FITC-Fg (A) or FITC-PAC-1 (B) binding to human washed platelets (WP) activated by ADP (50 microM), in the presence of increasing concentrations of DisBa-01 (0 to 1 microM) was measured by flow cytometry. Results are expressed as the mean +/- SEM percentage of bound fluorescence measured in the absence of DisBa-01 (triplicate experiments).

echistatin ($6.7x10^4$ +/- $2.2x10^4$ AU), but was about half the value measured on Fg ($18.3x10^4$ +/- $1.9x10^4$ AU) (Figure 3b). These variations are due to differences in the extent of platelet spreading measured on Dis*Ba*-01, RGDS and echistatin (mean platelet area of 690 +/- 136, 648 +/- 79 and 543 +/- 144 AU, respectively) versus Fg (1123 +/- 125 AU, P<0.008) and 4A5 MoAb (353 +/- 63 AU, P<0.008) (Figure 3c). Representative pictures of adhered platelets are shown for each condition on Fig.3d.

### 4.4. Blood Perfusion assays

The anti-platelet activity of Dis*Ba*-01 was further investigated in flow conditions. Heparin-anticoagulated whole blood was labeled with rhodamine 6G and perfused over immobilized fibrinogen at a wall shear rate of 300 $s^{-1}$. The disintegrin added 1 min before the onset of the perfusion inhibited by 80.7 +/- 6.3 % (P<0.0001) platelet recruitment measured after 5 min (Figure 4a). To test whether Dis*Ba*-01 could revert platelet recruitment on Fg, blood was first perfused during 5 min, followed by an additional 5-minute perfusion of Tyrode's buffer containing saline or 1 microM of Dis*Ba*-01 (Figure 4b). Under these conditions the disintegrin substantially reverted platelet recruitment by 40.6 +/- 17.3 % (P<0.0001).

### 4.5. DisBa-01 inhibits platelet aggregation

The preincubation of human WP with 1 microM Dis*Ba*-01 during 1 min fully inhibited aggregation to ADP (100 microM), similar to other $alpha_{IIb}beta_3$ antagonists Abciximab (0.4 microM), Lamifiban (1 microM) and echistatin (1 microM) (Figure 5a). In another set of experiments each antagonist was added 1 min after ADP stimulation. In contrast to Lamifiban (1 microM) but similarly to echistatin (1 microM) and Abciximab (0.4 microM), Dis*Ba*-01 (1 microM) stopped the aggregation process but failed to de-aggregate preformed platelet aggregates (Figure 5b). Dis*Ba*-01 on its own did not trigger platelet aggregation.

The inhibitory effect of Dis*Ba*-01 on aggregation of PRP or WP was species-dependent (Table 1). The disintegrin dose dependently inhibited collagen-, TRAP-6- and ADP-induced aggregation of human PRP with $IC_{50}$ values of 701 +/- 244, 594 +/- 226 and 203 +/- 12 nM respectively, and ADP- and thrombin-induced aggregation of human washed platelets with $IC_{50}$ values of 241 +/- 62 and 1430 +/- 734 nM, respectively. DisBa-01 more potently inhibited collagen-, and ADP-induced aggregation of rabbit PRP with $IC_{50}$ of 235 and 124 nM respectively, and ADP-induced aggregation of mouse PRP ($IC_{50}$ = 81 nM) and washed platelets ($IC_{50}$ = 25 nM).

### 4.6 Phosphorylation assays

#### 4.6.1 Total tyrosine phosphorylation

To investigate the effects of Dis*Ba*-01 on early signaling events during platelet activation, the total tyrosine phosphorylations were examined by western blotting in human WP after a 5-minute period of stirring in the cuvette of an aggregometer, in the absence or presence of several platelet agonists, Dis*Ba*-01 or antagonists. As expected, the content of phosphorylated proteins increased following platelet stimulation with ADP, collagen or thrombin (Figure 6a, lanes 7, 8, 9) concomitantly to platelet aggregation (not shown). The incubation of WP with Dis*Ba*-01 (lane 2), or the platelet antagonists Abciximab,

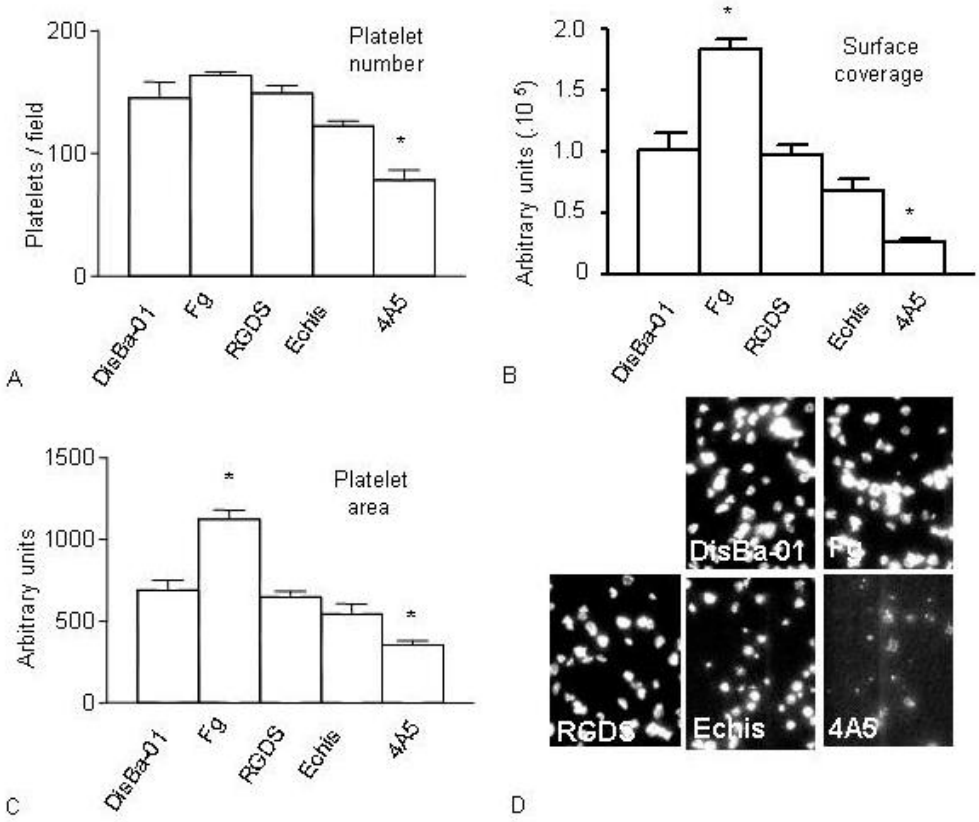


**Figure 3.** Platelet adhesion onto immobilized DisBa-01. The adhesion of human WP to DisBa-01 (1 microg/ml), Fg (200 microg/ml), RGDS (10 microg/ml), echistatin (1 microg/ml) or the anti Fg 4A5 MoAb (10 microg/ml) immobilized onto glass coverslips was performed as described in Materials and Methods. The number of adhered platelets (A), the surface coverage (B) and the mean single-platelet area (C) were quantified by image analysis. Results are the mean +/- SEM of triplicate experiments in which 5 fields per coverslip were acquired randomly. Representative pictures of adhered platelets taken at magnification x40 are shown for each condition (D).

Lamifiban and echistatin (not shown) did not trigger higher phosphorylations than saline (lane 1). Although Dis*Ba*-01 was less prone to inhibit phosphorylations induced by ADP stimulation than abciximab (lane 3), it inhibited the phosphorylation of a 60 kDa and a 100 kDa band protein (lane 4). Interestingly, Dis*Ba*-01 induced the phosphorylation of a 80Kda band that was absent with ADP stimulation alone (lane 7) and barely detectable with Dis*Ba*-01 alone (lane 2). The disintegrin had no effect on the phosphorylation of a 120kDa protein induced by ADP. Dis*Ba*-01 inhibited the strong collagen-induced phosphorylation of the 60kDa and the 100kDa protein and, conversely, strongly induced the phosphorylation of proteins of MW 31, 88 and 93 kDa (lane 5) as compared with collagen (lane 8) or Dis*Ba*-01 stimulation alone. Upon activation with thrombin Dis*Ba*-01 inhibited the strong phosphorylation of the 60kDa and the 100 kDa band protein and, conversely, induced the phosphorylation of the 31 kDa protein as compared with thrombin (lane 9) or Dis*Ba*-01 alone.

Adsorption of resting platelets to immobilized Dis*Ba*-01 induced the phosphorylation of several bands including the 88/93 kDa band doublet, the 60kDa, and the 31 kDa protein, compared to adsorption on plastic (Figure 6b).

#### 4.6.2. Focal adhesion kinase (FAK) phosphorylation

Because FAK plays a key role in mediating signaling downstream of platelet integrins, the inhibition of FAK phosphorylation by DisBa-01 was tested. We performed ADP-induced aggregation of human WP preincubated with saline, DisBa-01 (5 microM) or echistatin (1 microM). Platelets were then lysed and immunoblotted with an antibody specific to the phosphorylated serine residue (722) on FAK (Figure 6c). As shown in Figure 6c, FAK phosphorylation triggered by ADP stimulation was fully prevented by soluble DisBa-01 or echistatin.

### 4.7. *In vivo* effects

#### 4.7.1. Prolongation of mice tail bleeding time

We evaluated the effect of DisBa-01 on the tail bleeding time in C57BL6/J mice. A 2 mg/kg bolus of DisBa-01 injected 5 min before severing the tail induced a dramatic prolongation of the bleeding, from 202 +/- 22 s to 989 +/- 140 s in treated mice (Figure 7). Three out of 5 mice didn't show any bleeding arrest after the 20 min

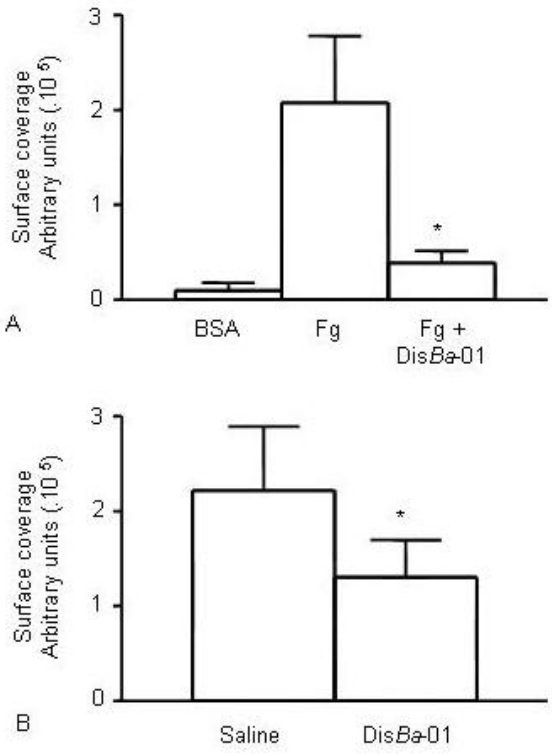


**Figure 4.** DisBa-01 inhibits and reverses platelet recruitment onto immobilized fibrinogen. (A) Heparin-anticoagulated whole blood was perfused during 5 min on BSA (control) or fibrinogen-coated glass coverslips at a shear rate of 300 $s^{-1}$ in the absence or presence of 1 microM DisBa-01. (B) Heparin-anticoagulated whole blood was perfused during 5 min on fibrinogen-coated glass coverslips at 300 $s^{-1}$, then Tyrode's buffer containing either saline or 1 microM DisBa-01 was perfused over the surface-bound platelets for an additional 5 min. Results are the mean +/- SEM of platelet surface coverage of three perfusion assays. In each assay 5 fields of the glass coverslips were randomly analysed. * indicates P<0.0001.

measurement. All mice survived the bolus injection of DisBa-01.

#### 4.7.2. Inhibition of thrombus formation *in vivo*

The anti-thrombotic activity of DisBa-01 was evaluated in a mouse model of mesenteric venules and arterioles injury induced by local exposure to FeCl3 and monitored by fluorescence videomicroscopy. Upon the chemical injury, thrombi progressively developed in the injured blood vessels of control mice, until complete occlusion occurring after 27.2 +/- 1.1 min in venules and 22.5 +/- 2.4 min in arterioles (Figure 8a). The distribution of occlusion times was larger in arterioles than in venules. The injection of DisBa-01 by the retro-orbital sinus 10 min before the thrombogenic lesion prolonged occlusion times in a concentration-dependent manner in both arterioles (2 mg/kg: 34.6 +/- 2.8 min, p<0.02; 4 mg/kg: 58.4 +/- 1.6 min, p<0.003) and venules (34.7 +/- 2.7 min, p<0.03; 46.6 +/- 6.8 min, p<0.02, respectively). Inspection of the thrombotic process in real time revealed that prolonged occlusion times in Dis*Ba*-01 treated-mice resulted from defective thrombus formation (Figure 8b).

## 5. DISCUSSION

Intramuscular inoculation of *Bothrops alternatus* venom provokes severe hemostatic alterations, including spontaneous and sustained bleeding, intense hemorrhage, hemolysis, thrombosis in lung arterioles and arteries, prolongation of activated partial thromboplastin time and prothrombin time (22). The present study demonstrates that Dis*Ba*-01 potently inhibits platelet aggregation *in vitro*, dramatically prolongs mouse tail-bleeding time and impairs thrombus formation following vascular injury, suggesting that the disintegrin may significantly contribute to the local and systemic hemorrhagic effect of *Bothrops alternatus* envenomation.

In a recent paper (19), using surface plasmon resonance analysis and cell adhesion assays, we demonstrated that DisBa-01 binds to $alpha_v beta_3$ integrin and antagonizes its cell adhesive functions. *In silico* studies of DisBa-01 in complex with the $alpha_v beta_3$ integrin, generated by homology modeling using the crystal structure of trimestatin and the solution structure of flavoridin as templates, predicted that the disintegrin largely engages the $beta_3$ subunit. The aspartic acids D58 (comprised in the RGD motif) and D61 were found to point directly to metal ions coordinated at MIDAS and ADMIDAS (adjacent to MIDAS) of $beta_3$ subunit, respectively. Also in the C-terminal part of the modeled DisBa-01, F76 and H77 were predicted to engage hydrophobic contacts with R214 and R216, for the former, and Y166 of the $beta_3$ subunit for the latter.

In the present study, we provide further experimental evidences for the interaction of DisBa-01 with the $\beta_3$ subunit by demonstrating that FITC-labelled DisBa-01 binds to CHO cells expressing human $beta_3$ with a higher affinity than to untransfected cells. Furthermore, we demonstrate that DisBa-01 directly interacts with the activated $alpha_{IIb} beta_3$ as evidenced by its capacity to displace FITC-PAC-1 bound to ADP-activated platelets. Integrin $alpha_{IIb} beta_3$ plays a critical role in hemostasis by triggering platelet-to-platelet aggregation via the binding of divalent fibrinogen to two adjacent cells. The finding that immobilized DisBa-01 promotes static adhesion of resting washed platelets suggests that the disintegrin interacts with both non-activated and activated forms of $alpha_{IIb} beta_3$. Flow cytometry data also indicate that FITC-DisBa-01 binds to resting platelets (unpublished observation). Furtheremore, Dis*Ba*-01 antagonizes platelet $alpha_{IIb} beta_3$ integrin functions as demonstrated by its capacity to inhibit FITC-Fg binding to platelets and to impair and partially reverse the recruitment of flowing platelets to immobilized Fg.

Although not significantly higher than on immobilized 4A5 Moab, we observed some platelet spreading on immobilized DisBa-01 suggesting that the disintegrin, similarly to the RGDS peptide or echistatin,

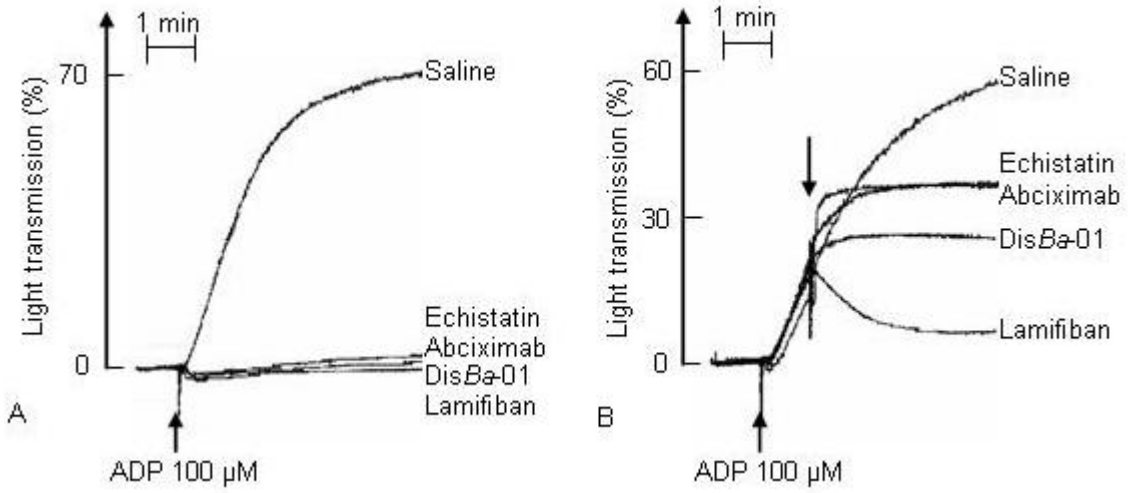


**Figure 5.** DisBa-01 inhibits platelet aggregation. Human WP were stimulated with 100 microM ADP, in the presence of DisBa-01 (1 microM) or the other alpha$_{IIb}$beta$_3$ antagonists Abciximab (0.4 microM), Lamifiban (1 microM) or echistatin (1 microM) added either 1 min before, or 1 min after the addition of ADP. Aggregation curves representative of 3 separate experiments are shown.

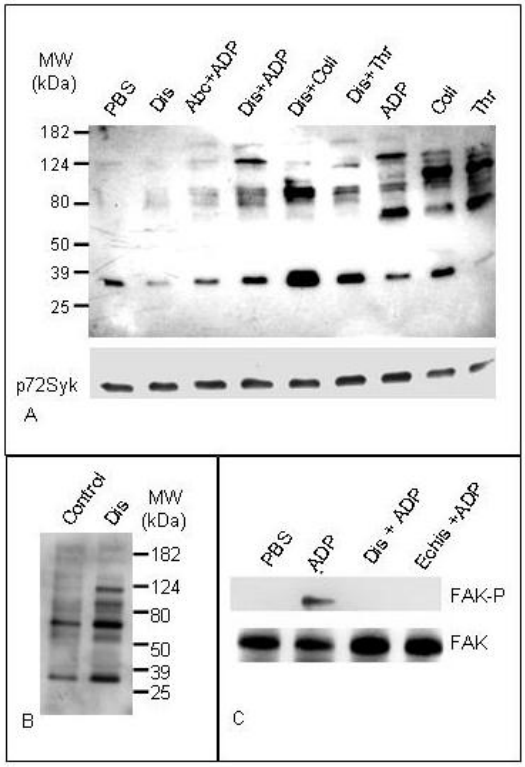


**Figure 6.** Effect of DisBa-01 on platelet protein phosphorylation. (A) Platelet tyrosine phosphorylation. WP pre-treated with PBS, DisBa-01 (1microM) Abciximab (50 microg/ml), ADP (100 microM), equine collagen (10 microg/ml), and/or thrombin (0.2 U) were incubated for 5 minutes at 37°C under agitation in an aggregometer, lysed, and immunoblotted for phosphotyrosine-containing proteins and total p72Syk content as described in Material and Methods. (B) Tyrosine phosphorylation induced by immobilized DisBa-01. WP were incubated for 15 min at 37°C in plastic wells uncoated or coated Dis*Ba*-01 (10 microg/ml). Adherent platelets were washed, lysed and immunoblotted for phosphotyrosine-containing proteins. (C) FAK phosphorylation by DisBa-01. WP pre-treated for 1 min with PBS, DisBa-01 (5microM) or Echistatin (1microM) were activated with ADP (100microM) and aggregation tests were performed for 5 min at 37°C under agitation in an aggregometer. Platelets were lysed and immunoblotted with anti-FAK (pSer$^{722}$) phosphospecific antibody (1:100). For a control of protein loading on gel, membranes were stripped and then reprobed with (A) anti-SYK (4D10-1) antibody or (C) anti-FAK (A-17) antibody.

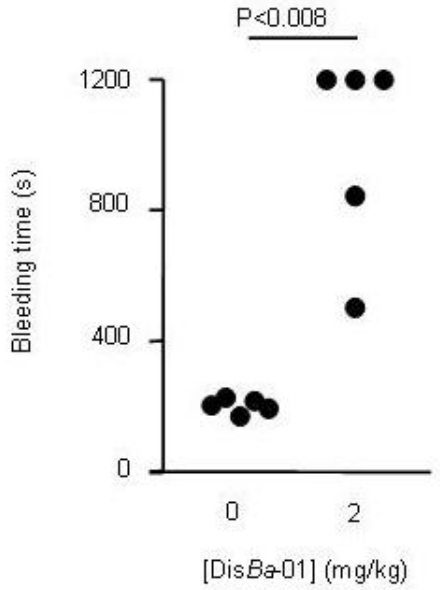


**Figure 7.** DisBa-01 prolongs mice tail bleeding time. Bleeding time was measured in mice 5 min after injection of 2 mg/kg of DisBa-01 (n=5) or vehicle (Saline; n=5) in the caudal vein. When necessary, bleeding was manually stopped at the 20 min time point.

triggers intracellular signaling leading to cytoskeletal reorganization. Immobilized DisBa-01 also induced the spreading of CHO-alpha$_{IIb}$beta$_3$ cells, but not of untransfected CHO cells (not shown). Western blot analyses on platelets adhered onto DisBa-01 further indicated that the immobilized disintegrin increased the tyrosine phosphorylation of several proteins (Figure 6c). It is consistent with the fact that anti-alpha$_{IIb}$beta$_3$ agents, although very potent to block the integrin function, can perturb the conformation of alpha$_{IIb}$beta$_3$ and promote its oligomerization (clustering) leading to platelet activation, which is particularly true for echistatin (23). Despite looking controversial, these results can be explained by ligand density and /or ligand immobilization. In a soluble form, echistatin inhibits alpha$_{IIb}$beta$_3$-mediated platelet aggregation whereas it stimulates platelet adhesion and signaling when immobilized (24). Recently, it has been demonstrated that ligand density may strongly affects alpha$_{IIb}$beta$_3$-mediated platelet signaling and spreading (25). Platelet adhesion to immobilized fibrinogen in high or low concentrations led to distinct patterns of cytosolic $Ca^{2+}$ increase, tyrosine phosphorylation and formation of filopodia and lamellipodia, indicating that ligand density can trigger different signaling mechanisms.

Antagonists of alpha$_{IIb}$beta$_3$ are potent inhibitors of platelet aggregation, several of which having proven clinical efficacy in the treatment of non-ST-segment elevation acute coronary syndrome and in percutaneous coronary interventions. There are several classes of alpha$_{IIb}$beta$_3$ antagonists including antibodies, small peptides, peptidomimetics and disintegrins. Among those, Abciximab (human/mouse chimeric 7E3 Fab, Reopro) binds to the C177–184 loop of the beta$_3$ subunit of alpha$_{IIb}$beta$_3$ integrin on platelets and blocks the binding to fibrinogen (26-27). Abciximab also binds with equivalent affinity to alpha$_v$beta$_3$ integrin (28). Lamifiban is a non-peptide RGD mimetic specific for alpha$_{IIb}$beta$_3$, which interacts with the RGD-binding, sequence in the beta$_3$ integrin subunit, located in residues 118–131 (29) and inhibits ADP-induced platelet aggregation (30).

A recent study demonstrated that Lamifiban, in contrast with Abciximab, can dissociate platelet aggregates preformed *in vitro* either under arterial shear condition or in the stir condition of the aggregometer (31). This property of the RGD mimetic Lamifiban was attributed to its capacity to allosterically reverse Fg binding to activate alpha$_{IIb}$beta$_3$ (32), whereas Abciximab binds at a site on alpha$_{IIb}$beta$_3$ distinct from that of RGD and Fg, with no demonstrated allosteric effect (26). We found that in contrast to Lamifiban but similarly to Abciximab, Dis*Ba*-01 and the short monomeric RGD-containing disintegrin echistatin failed to reverse ADP-induced platelet aggregation.

Circulating platelets have a basal level of protein tyrosine phosphorylation. Upon stimulation by agonists such as ADP, thrombin, or collagen, they undergo a rapid increase in the content of tyrosine phosphorylation (33). Such increase occurs not only as a result of the interaction of the primary agonist with its receptor (inside-out signaling), but also following fibrinogen binding to the activated alpha$_{IIb}$beta$_3$ integrin during platelet aggregation (outside-in signaling).

Tyrosine phosphorylations in platelets stimulated with ADP were strongly inhibited by Abciximab indicating that these phosphorylations are mainly induced following alpha$_{IIb}$beta$_3$ engagment, exepted for the 31 kDa and 88/83 kDa doublet bands. DisBa-01 inhibited the ADP-induced tyrosine phosphorylation of a 60 kDa band (that could correspond to p60Src) without affecting the ADP-induced tyrosine phosphorylation of the 120 kDa band, indicating that DisBa-01 interferes with signalling events downstream of alpha$_{IIb}$beta$_3$ differently to Abciximab. DisBa-01 inhibited the tyrosine phosphorylation of a 100 kDa band induced by collagen and thrombin, but not ADP, and strongly increased the collagen-induced tyrosine phosphorylation of a 88/93 kDa doublet and the 31 kDa band. These findings indicate, although binding of DisBa-01 to the alpha$_{IIb}$beta$_3$ integrin of platelets in suspension does not trigger obvious phosphorylation, that DisBa-01 can potently and actively interfere with fibrinogen-mediated outside-in signaling, most strikingly during platelet activation by collagen.

In contrast to the lack of obvious phopshorylation induction by DisBa in suspended platelets, platelet adhesion to coated DisBa resulted in platelet spreading, indicative of platelet activation (Figure 3D). Correspondingly, spreading on DisBa resulted in extensive tyrosine phosphorylation. Focal adhesion kinase (FAK) is a 125kDa cytoplasmic protein (pp125$^{FAK}$) found in structures called focal adhesions, which are formed when cells spread on a substratum (34). In platelets, pp125$^{FAK}$ becomes phosphorylated on tyrosine residues upon aggregation in

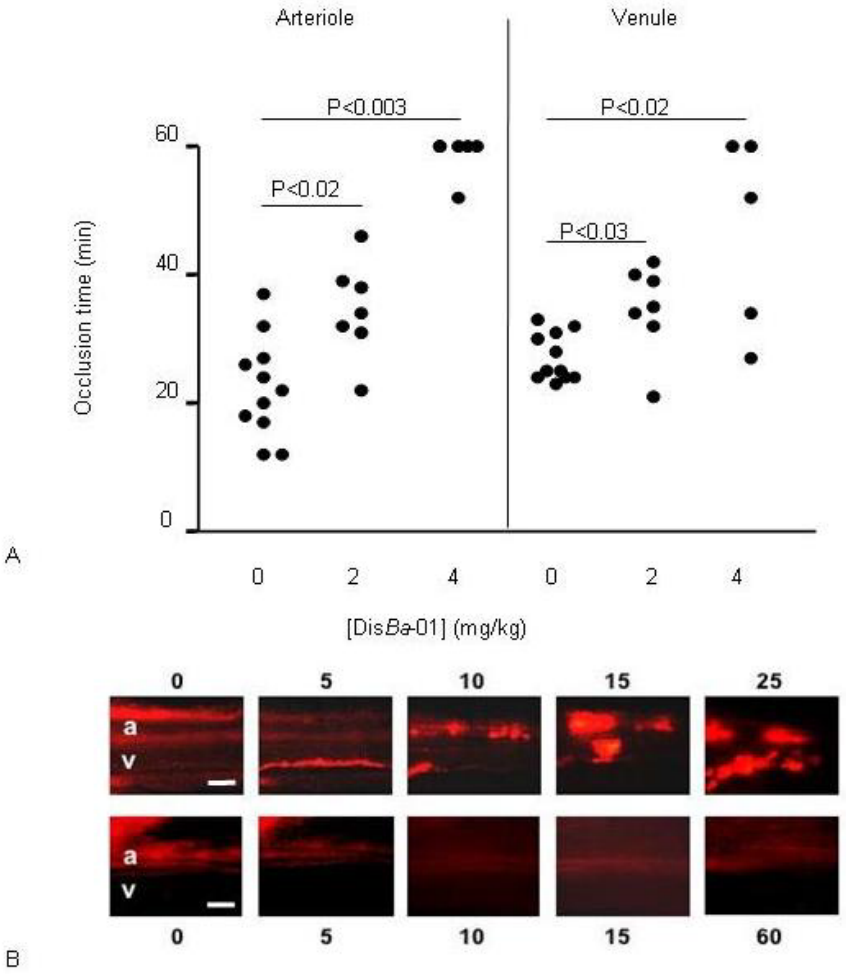


**Figure 8.** DisBa-01 inhibits thrombus formation. (A) Mean time (+/- SEM) to complete thrombotic occlusion following injury induced by $FeCl_3$ (500 mM) in the mesenteric arteries (left panel) and venules (right panel) of C57BL/6 mice injected intravenously with saline (n=8) or DisBa-01 at 2 mg (n=4) or 4 mg (n=5) per kg. Up to 2 vessel injuries per mouse were performed. (B) Representative example of on-line recordings showing adhesion of Rhodamine 6G-labeled platelets and thrombus formation following injury of mesenteric arterioles (a) and venules (v) of mice treated with saline (0 to 25 min-time points, upper panels) or 4 mg/kg DisBa-01 (0 to 60 min-time points, lower panels). White bars represent 100 microm length.

response to agonists (35; 36). Although $pp125^{FAK}$ regulation by tyrosine phosphorylation has been the most studied, $pp125^{FAK}$ is also regulated by phosphorylation on four major serine residues: Ser-722, Ser-842, Ser-845 and Ser-911. Little is known on the role of Ser-722 in FAK regulation. The phosphorylation of Ser-722 was shown to negatively regulate cell spreading and migration, through the competing actions of glycogen synthase kinase 3 and the serine/threonine protein phosphatase type-1 (37). In this study we show that DisBa-01 inhibits $pp125^{FAK}$ phosphorylation on Ser-722 residue upon suspended platelet activation by ADP, probably as a direct result of the blockade of fibrinogen-$alpha_{IIb}beta_3$ integrin interactions. Concordant results were observed for echistatin (38) that also binds to $alpha_{IIb}beta_3$, by using anti-human $pp125^{FAK}$ polyclonal antiserum and platelet adhesion assays (39). In agreement with this previous work, our results show that platelet treatment with soluble echistatin (1 microM) before ADP stimulation provokes an inhibition of Ser-722 phosphorylation. As discussed by these authors (39), echistatin, and presumably DisBa-01, acting as an antagonist of the $alpha_{IIb}beta_3$ integrin in platelets, can cause a conformational change in this receptor, leading to the inactivation of $pp125^{FAK}$ signaling cascades. Similar results were observed for contortrostatin (0.5 microM) and multisquamatin (0.7 microM), a monomeric disintegrin from *Echis multisquamatus*. These disintegrins inhibited thrombin-induced tyrosine phosphorylation of $pp125^{FAK}$ and a set of other platelet proteins after aggregation assays, probably by blocking fibrinogen $alpha_{IIb}beta_3$ interaction (40). The platelet spreading observed on coated DisBa is in agreement with this interpretation: conformational changes in $alpha_{IIb}beta_3$ brought about by the coated DisBa-01 then would lead to $pp125^{FAK}$-independent outside-in signaling, detected via tyrosine phosphorylation of several proteins and platelet spreading.

The intravenous injection of DisBa-01 in mice at doses up to 4 mg/kg was not lethal but induced a dramatic increase in the tail bleeding time, and a striking prolongation of thrombotic occlusion times in both veins and arteries, which are common features of $alpha_{IIb}beta_3$ antagonists. Further investigations will be needed to determine the plasma and platelet half lives of DisBa-01 and to measure possible disintegrin-induced transient thrombocytopenia.

In conclusion, in addition to its recently reported anti- $alpha_vbeta_3$ activity10, the novel recombinant disintegrin DisBa-01 is a potent inhibitor of the platelet integrin $\alpha_{IIb}\beta_3$ functions both *in vitro* and *in vivo*. This study adds to the comprehension of the pathological process of *Bothrops alternatus* envenomation suggesting that the disintegrin contributes to its haemorrhagic manifestations. Although additional investigations are necessary to delineate its integrin specificity, DisBa-01 constitutes a new template molecule for the development of new $\beta_3$ integrin blockers.

## 6. ACKNOWLEDGEMENTS

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## 7. REFERENCES

1. Bauab F.A., G.R. Junqueira, M.C. Corradini, P.V. Silveira & A. Nishioka Sde: Clinical and epidemiological aspects of the 'urutu' lance-headed viper (*Bothrops alternatus*) bite in a Brazilian hospital. *Trop Med Parasitol* 45, 243-245 (1994)

2. Nisenbom H.E., J.C. Perazzo, A.J. Monserrat & J.C. Vidal: Contribution of phospholipase A2 to the lethal potency of *Bothrops alternatus* (vibora de la cruz) venom. *Toxicon* 24, 807-817 (1986)

3. Smolka M.B., S. Marangoni, B. Oliveira, & J.C. Novello: Purification and partial characterization of a thrombin-like enzyme, balterobin, from the venom of *Bothrops alternatus*. *Toxicon* 36, 1059-1063 (1998)

4. Castro H.C., D.L. Dutra, A.L. Oliveira-Carvalho, & R.B. Zingali: Bothroalternin, a thrombin inhibitor from the venom of *Bothrops alternatus*. *Toxicon* 36, 1903-1912 (1998)

5. Souza D.H., M.R. Iemma, L.L. Ferreira, J.P. Faria, M.L. Oliva, R.B. Zingali, S. Niewiarowski & H.S. Selistre-de-Araujo: The disintegrin-like domain of the snake venom metalloprotease alternagin inhibits alpha2beta1 integrin-mediated cell adhesion. *Arch Biochem Biophys* 384, 341-350 (2000)

6. Gould R.J., M.A. Polokoff, P.A. Friedman, T.F. Huang, J.C. Holt, J.J. Cook & S. Niewiarowski: Disintegrins: a family of integrin inhibitory proteins from viper venoms. *Proc Soc Exp Biol Med* 195, 168-171 (1990)

7. Calvete J.J., M.P. Moreno-Murciano, R.D. Theakston, D.G. Kisiel & C. Marcinkiewicz: Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulphide bond engineering. *Biochem J* 372, 725-734 (2003)

8. Fox, J.W., & S.M.T. Serrano: Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon* 45, 969-985 (2005)

9. McLane M.A., E.E. Sanchez, A. Wong, C. Paquette-Straub & J.C. Perez: Disintegrins. *Curr Drug Targets Cardiovasc Haematol Disord* 4, 327-355 (2004)

10. Sloan E.K., N. Pouliot, K.L. Stanley, J. Chia, J.M. Moseley, D.K. Hards & R.L. Anderson: Tumor-specific expression of alphavbeta3 integrin promotes spontaneous metastasis of breast cancer to bone. *Breast Cancer Res* 8, 1-14 (2006)

11. Sheu, J.R., M.H. Yen, Y.C. Kan, W.C. Hung, P.T. Chang & H.N. Luk: Inhibition of angiogenesis in vitro and in vivo: comparison of the relative activities of triflavin, an Arg-Gly-Asp-containing peptide and anti-alpha(v)beta3 integrin monoclonal antibody. *Biochim Biophys Acta* 1336, 445-454 (1997)

12. Swenson S., F. Costa, R. Minea, R.P. Sherwin, W. Ernst, G. Fujii, Y. Yang, F.S. Markland: Intravenous liposomal delivery of the snake venom disintegrin contortrostatin limits breast cancer progression. *Mol Cancer Ther* 3, 499-511 (2004)

13. Zhao, Y., R. Bachelier, I. Treilleux, P. Pujuguet, O. Peyruchaud, R. Baron, P. Clement-Lacroix, P. Clezardin: Tumor alphavbeta3 integrin is a therapeutic target for breast cancer bone metastases. *Cancer Res* 67, 5821-5830 (2007)

14. Gomes N., J. Vassy, C. Lebos, B. Arbeille, C. Legrand & F. Fauvel-Lafeve: Breast carcinoma cell adhesion to the vascular subendothelium in whole blood and under flow conditions: effects of alphavbeta3 and alphaIIbbeta3 antagonists. *Clin Exp Metast* 21, 553-561 (2004)

15. Gupta G.P., & J. Massague: Platelets and metastasis revisited: a novel fatty link. *J Clin Invest* 114, 1691-1693 (2004)

16. Nieswandt B., M. Hafner, B. Echtenacher & D.N. Mannel: Lysis of tumor cells by natural killer cells in mice is impeded by platelets. *Cancer Res* 59, 1295-1300 (1999)

17. Karpatkin S. & E. Pearlstein: Role of platelets in tumor cell metastases. *Ann Intern Med* 95, 636-641 (1981)

18. Trikha M & M.T. Nakada: Platelets and cancer: implications for antiangiogenic therapy. *Sem Thromb Hemost* 28, 39-44 (2002)

19. Ramos O.H., A. Kauskot, M.R. Cominetti, I. Bechyne, C.L. Salla Pontes, F. Chareyre, J. Manent, R. Vassy, M. Giovannini, C. Legrand, H.S. Selistre-de-Araujo, M. Crepin & A. Bonnefoy. A novel alpha(v)beta (3)-blocking disintegrin containing the RGD motive, DisBa-01, inhibits bFGF-induced angiogenesis and melanoma metastasis. *Clin Exp Metastasis* (2007)

20. Xia Z., T. Wong, Q. Liu, A. Kasirer-Friede, E. Brown & M.M. Frojmovic: Optimally functional fluorescein

isothiocyanate-labelled fibrinogen for quantitative studies of binding to activated platelets and platelet aggregation. *Br J Haematol* 93, 204-214 (1996)
21. Alig L., A. Edenhofer, P. Hadvary, M. Hürzeler, D. Knopp, M. Muller, B. Steiner, A. Trzeciak & T. Weller: Low molecular weight, non-peptide fibrinogen receptor antagonists. *J Med Chem* 35, 4393-4407 (1992)
22. Jacome D., M.M. Melo, M.M. Santos & L.G. Heneine: Kinetics of venom and antivenom serum and clinical parameters and treatment efficacy in *Bothrops alternatus* envenomed dogs. *Vet Hum Toxicol* 44, 334-338 (2002)
23. Hantgan R.R., M.C. Stahle, J.H. Connor, R.F. Connor & S.A. Mousa: AlphaIIbbeta3 priming and clustering by orally active and intravenous integrin antagonists. *J Thromb Haemost* 5, 542-550 (2007)
24. Belisario M.A, S. Tafuri, C. Di Domenico, R. Della Morte, C. Squillacioti, A. Lucisano & N. Staiano: Immobilised echistatin promotes platelet adhesion and protein tyrosine phosphorylation. *Biochim Biophys Acta* 1497, 227-236 (2000)
25. Jirouskova M., J.K. Jaiswal & B.S. Coller: Ligand density dramatically affects integrin alphaIIbbeta3-mediated platelet signaling and spreading. *Blood* 109, 5260-5269 (2007)
26. Artoni A., J. Li , B. Mitchell, J. Ruan, J. Takagi, T.A. Springer, D.L. French & B.S. Coller: Integrin beta3 regions controlling binding of murine mAb 7E3: implications for the mechanism of integrin alphaIIbbeta3 activation. *Proc Natl Acad Sci USA.* 101, 13114-13120 (2004)
27. Coller B.S., J.D. Folts, S.R. Smith, L.E. Scudder & R. Jordan: Abolition of *in vivo* platelet thrombus formation in primates with monoclonal antibodies to the platelet GPIIb/IIIa receptor. Correlation with bleeding time, platelet aggregation, and blockade of GPIIb/IIIa receptors. *Circulation* 80, 1766-1774 (1989)
28. Tam S.H., P.M. Sassoli, R.E. Jordan & M.T. Nakada: Abciximab (ReoPro, chimeric 7E3 Fab) demonstrates equivalent affinity and functional blockade of glycoprotein IIb/IIIa and alpha(v)beta3 integrins. *Circulation* 98, 1085-1091 (1998)
29. D'Souza S.E., T.A. Haas, R.S. Piotrowicz, V. Byers-Ward, D.E. McGrath, H.R. Soule, C. Cierniewski, E.F. Plow & J.W. Smith: Ligand and cation binding are dual functions of a discrete segment of the integrin beta 3 subunit: cation displacement is involved in ligand binding. *Cell* 79, 659-667 (1994)
30. Coller, B.S: Anti-GPIIb/IIIa drugs: current strategies and future directions. *Thromb Haemost* 86, 427-443 (2001)
31. Frojmovic, M., B. Labarthe & C. Legrand: Inhibition and reversal of platelet aggregation by alphaIIbbeta3 antagonists depends on the anticoagulant and flow conditions: differential effects of Abciximab and Lamifiban. *Br J Haematol* 131, 348-355 (2005)
32. Hu, D.D. C.A. White, S. Panzer-Knodle, J.D. Page, N. Nicholson, & J.W. Smith: A new model of dual interacting ligand binding sites on integrin alphaIIbbeta3. *J Biol Chem* 274, 4633-4639 (1999)
33. Shattil S.J., H. Kashiwagi, N. Pampori: Integrin signaling: the platelet paradigm. *Blood* 91, 2645-2657 (1998)
34. Schaller M.D., C.A. Borgman & J.T. Parsons: Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK. *Mol Cell Biol* 13, 785-791 (1993)
35. Lipfert L., B. Haimovich, M.D. Schaller, B.S. Cobb, J.T. Parsons & J.S. Brugge: Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. *J Cell Biol* 119, 905-912 (1992)
36. Haimovich B., L. Lipfert, J.S. Brugge & S.J. Shattil: Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. *J Biol Chem* 268, 15868-15877 (1993)
37. Bianchi M., S. de Lucchini, O. Marin, D. L. Turner, S. K. Hanks & E. Villa-moruzzi: *Biochem J.* 391, 359-370 (2005)
38. Calvete, J.J., M.A. McLane, G.J. Stewart, & S. Niewiarowski: Characterization of the cross-linking site of disintegrins albolabrin, bitistatin, echistatin, and eristostatin on isolated human platelet integrin GPIIb/IIIa. *Biochem Biophys Res Commun* 202, 135-140 (1994)
39. Staiano N., R. Della Morte, C. Di Domenico, S. Tafuri, C. Squillacioti, M.A. Belisario & P. Di Natale: Echistatin inhibits pp72syk and pp125FAK phosphorylation in fibrinogen-adherent platelets. *Biochimie* 79, 769-773 (1997)
40. Clark, E.A., M. Trikha, F.S. Markland, & J.S. Brugge: Structurally distinct disintegrins contortrostatin and multisquamatin differentially regulate platelet tyrosine phosphorylation. *J Biol Chem* 269, 21940-21943 (1994)

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**Send correspondence to:** Arnaud Bonnefoy, INSERM U743, Centre de Recherche du Centre Hospitalier de l'Universite de Montreal, Campus Saint-Luc, Montreal QC, H2X1P1, Canada, Tel: 514-376 3330 ext. 3017 Fax: 514-376 1076, E-mail: arnaud.bonnefoy@umontreal

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