Evaluation of the role of proline residues flanking the RGD motif of dendroaspin, an inhibitor of platelet aggregation and cell adhesion

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The effect of a panel of proline mutants of dendroaspin, an inhibitor of platelet aggregation and cell adhesion, including A⁴²-dendroaspin, A⁴⁷-dendroaspin, A⁴⁹-dendroaspin, A^{42,47}-dendroaspin and A^{47,49}-dendroaspin, was investigated using platelet-aggregation and cell-adhesion assays. Here we show that a single alanine-for-proline substitution did not affect potency when measured as the ability either to inhibit platelet aggregation induced by ADP (IC₅₀ ≈ 170 nM) or to block transfected A375-SM cell adhesion to fibrinogen in the presence of Mn²⁺ as compared with wild-type dendroaspin. By comparison, double

proline substitution with alanines significantly reduced the potency in both assays by approx. 5–8-fold. These observations, therefore, suggest that proline residues flanking the RGD motif in dendroaspin and other RGD-containing venom proteins, e.g. disintegrins, may contribute to maintaining a favourable conformation for the solvent-exposed RGD site for its recognition by integrin receptors.

Key words: disintegrin, integrin antagonist, integrin–ligand interaction, neurotoxin.

INTRODUCTION

Snake venoms contain a variety of proteins, some of which contain an Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) sequence and can act as potent inhibitors of platelet aggregation and integrin-mediated platelet adhesion [1–3]. These molecules include the disintegrin family [4], derived from viper venoms, and a neurotoxin-related molecule, dendroaspin, isolated from the venom of the Elapidae family member *Dendroaspis jamesonii* (Jameson's mamba) [5].

The binding of platelets to fibrinogen is an essential step in the formation of a platelet-rich blood clot [6]. This process is mediated by integrin $\alpha_{IIb}\beta_3$ found on the surface of activated platelets. Upon cell activation, the $\alpha_{IIb}\beta_3$ integrin binds several glycoproteins, predominantly through the RGD tripeptide sequence [7–9] present in fibronectin [10], von Willebrand factor [11], vitronectin [12], thrombospondin [13] and tenascin [14]. Potentially, an $\alpha_{IIb}\beta_3$ integrin antagonist could be used as an anti-platelet aggregation agent for the treatment of thrombotic diseases [15].

The structural and functional study of the RGD-containing snake-venom proteins provides important insights into understanding the interaction between integrins and their ligands and may, in turn, aid in the design of clinically useful anti-thrombotic agents. Consequently, the structures of a number of RGD-containing venom proteins have been determined by crystallography, including the disintegrins, apilagin [16], kistrin [17–19], echistatin [20–23], flavoridin [24], albolabrin [25], decorsin [26] and disintegrin-like dendroaspin [27,28].

Structural studies of the RGD-containing peptide dendroaspin showed that its fold is similar to that in both short-chain neurotoxins and cardiotoxins, but that loop III of dendroaspin is in a completely different orientation from that observed in either short-chain neurotoxins or cardiotoxins. This difference can be attributed, at least in part, to the proline residues in the flanking regions of the RGD motif. The Pro⁴⁷-Gly-Pro⁴⁹ sequence is

thought to cause the loop to diverge from the β -sheet [28], preventing the extension of neighbouring secondary structure and permitting the correct conformation for integrin–ligand interactions. The proline residues may contribute in other, as yet uncharacterized, ways since they also occur in the flanking regions of RGD motifs from other venom proteins (Figure 1) [29]. To evaluate the role of proline residues in positions 42, 47 and 49, which flank the RGD motif of dendroaspin, we have examined the effect of mutagenesis of these proline residues from dendroaspin on the potency of inhibition of platelet aggregation and cell adhesion. Our results suggest that these residues may play a crucial role in binding to the integrin.

MATERIALS AND METHODS

Materials

Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, isopropyl β -D-thiogalactoside and DH5 α competent cells were purchased from Life Technologies (Paisley, Scotland, U.K.) or Promega (Southampton, Hants., U.K.). Vent (exo-) DNA polymerase was supplied by New England Biolabs (Hitchin, Herts., U.K.). Proteinase Factor Xa was purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). Oligonucleotides were made by Cruachem (Glasgow, U.K.). dNTPs, plasmid pGEX-3X, a vector that expresses a cloned gene as a fusion protein linked to glutathione S-transferase (GST), and glutathione-Sepharose CL-4B were purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Gel-extraction kit and Plasmid maxi kit were purchased from Qiagen (Crawley, West Sussex, U.K.). Human fibrinogen was supplied by Calbiochem (Nottingham, U.K.). P2 monoclonal antibody, recognizing the human α_{IIb} subunit, was purchased from Immunotech (Marseille, France). The other monoclonal antibodies, including LM609, recognizing human integrin $\alpha_v\beta_3$, 25E11, recognizing the β_3

Abbreviations used: PRP, platelet-rich plasma; GST, glutathione S-transferase; A⁴²-Den, A⁴²-dendroaspin.

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Neurotoxin			
Dendroaspin	RICYNHLGTPPTTETCQEDSCYKNIWTFDNIIRRGCG	CFT P RGDM P GPYC	CESDKCNL
Disintegrins			
Kistrin	GKECDCSSPENPCCDAATCKLRPGAQCGEGLCCEQCKFSRAGKI	CRI P RGDM P DDRC	TGQSADCPRNYH
Flavoviridin	GEEDCGSPSNPCCDAATCKLRPGAQCADGLCCDQCRFKKKTGI	CRIARGDF P DDRC	TGLSNDPCRWN DL
Batroxostatin	EAGEECDGTPENPCCDAATCKLRPGAQCAEGLCCDQCRFKGAGKI	CRRARGDN P DDRC	TGQSADCPRNRF
Elegantin	EAGEECDGSPENPCCDAATCKLRPGAQCADGLCCDQCRFKKRTI	CRRARGDN P DDRC	TGQSADCPRNGLYS
Jararacin	EAGEECDGPPGNPCCDAATCKLRPGAQCAEGLCCDQCRFKGAGKI	CRRARGDN P DDRC	TGQSADCPRNRFHA
Lachesin	EAGEECDGAPANPCCDAATCKLRPGAQCAEGLCCDQCRFIKKGKI	CRRARGDN P DDRC	TGQSADCPRNGYYG
Basilicin	AGEECDGSPANPCCDAATCKLRPGAQCAEGLCCDQCRFIKKGKI	CRRARGDN P DDRC	TGQSADCPRNHFHA
Cereberin	EAGEECDGSPANPCCDAATCKLRPGAQCAEGLCCDQCRFIKKGKI	CRRARGDN P DDRC	TGQSADCPRNRFH
Viridin	AGEECDGSPANPCCDAATCKLRPGAQCAEGLCCDQCRFIKKGKI	CRRARGDN P DDRC	TGQSADCPRNRFH
Molossin	EAGIECDGSPENPCCDAATCKLRPGAQCADGLCCDQCRFIKKGKI	CRRARGDN P DDRC	TGQSADCPRNRFHA

Figure 1 Amino acid sequences of RGD loops that contain proline residue(s) flanking the RGD tripeptide

The RGD loops are boxed and proline residues are shown in bold.

subunit, JBS2, recognizing $\alpha_2\beta_1$, PC42, recognizing the α_4 subunit, JBS5, recognizing $\alpha_5\beta_1$, 4F10, recognizing the α_6 subunit, 6S6, recognizing the β_1 subunit, YFC118.3, recognizing the β_2 subunit and P3G8, recognizing the α_v subunit, were obtained from Chemicon (Harrow, Middx, U.K.). The A375-SM human melanoma cell line was provided by M. J. Humphries (University of Manchester, Manchester, U.K.).

Construction of a synthetic dendroaspin gene and variant constructs

A synthetic dendroaspin gene was constructed by ligating 10 complementary and overlapping oligonucleotides coding for the protein sequence of dendroaspin, using *Escherichia coli* codon-usage data, and cloned into plasmid pGEX-3X. A detailed description of protein expression, purification and functional characterization of recombinant dendroaspin and comparison with its natural counterpart purified from snake venom has been reported previously [30]. The genes for A⁴²-dendroaspin (A⁴²-Den), A⁴⁷-Den, A⁴⁹-Den, A^{42,47}-Den and A^{47,49}-Den were amplified by PCR using a mixture of 1 μ l of wild-type dendroaspin gene (5 ng) as a template, 1 μ l of two 5'-overlapping deoxy-oligonucleotides as primers (100 ng for each) and 2 units of vent polymerase. The primers used included a forward primer for all mutants, 5'-GGGATCCATATCGAAGGTCGTCGTATCTGCTACAACCATCTTGGTACTAAAC-3', and five distinct reverse primers that contained appropriate mutated codes (underlined) to produce the desired mutants: (i) 5'-AATTCTCAAAGGTTGCATTTGTCAGATTCGCAGCAGTACGGACCCGGCATGTCAGCACGCGGAGTGAAGCA3', (ii) 5'-AATTCTCAAAGGTTGCATTTGTCAGATTCGCAGCAGTACGGACCCAGCCATGTCACACGCGGAGTGAAGCA3', (iii) 5'-AATTCTCAAAGGTTGCATTTGTCAGATTCGCAGCAGTACGGACCCGGCATGTCACACGAGCAGTGAAGCA3', (iv) 5'-AATTCTCAAAGGTTGCATTTGTCAGATTCGCAGCAGTACGGACCCAGCCATGTCACACGAGCAGTGAAGCA 3' and (v) 5'-AATTCTCAAAGGTTGCATTTGTCAGATTCGCAGCAGT-AAGCACCAGCCATGTCACACGCGGAGTGAAGCA-3'. The following programme was applied: one cycle of 3 min at 94 °C and 1 min at 72 °C, followed by 39 cycles of 30 s at 94 °C, 2 min at 72 °C and

1 min at 60 °C. The amplification product was checked and found to be of the expected size, as ascertained on a 2 % agarose gel, and purified further on a 2 % low-melting-point agarose gel.

The A⁴⁹-Den gene was made from the ligation of two fragments, a 105 bp fragment made by digestion of the wild-type gene with *TagI* and *HpaII*, and a 92 bp fragment made by digestion of the T⁵A⁴⁹-Den gene (X. Lu and V. V. Kakkar, unpublished work) with *MboII* and *TaqI*. The purified PCR products and the A⁴⁹-Den gene were digested with *BamHI* and *EcoRI* and then cloned into the restricted vector pGEX-3X.

Transformation and protein expression

Transformation and protein expression were as performed previously [30]. In brief, recombinant plasmid (5 ng) was used to transform 50 μ l of *E. coli* DH5 α competent cells by standard methods [31]. The presence of the correct coding sequence of the constructs was verified by complete DNA sequencing of the inserted fragments using the dideoxy chain-termination method [32]. Bacterial culture conditions were carried out as follows; the culture was inoculated with an overnight seed culture (1 %, v/v), grown in 2 $\times$ YT/ampicillin medium (100 μ g/ml) and shaken at 37 °C until it reached a D_{600} of 0.7. Isopropyl β -D-thiogalactoside was then added to a final concentration of 0.1 mM for induction. The cells were grown for an additional 4 h at a low temperature of 30 °C and harvested by centrifugation.

Purification of mutant dendroaspins

Purification of recombinant dendroaspins was performed as described previously [30]. In brief, recombinant GST-dendroaspin and GST-mutant-dendroaspins from supernatants of sonicated cell debris after centrifugation were purified by affinity chromatography on glutathione-Sepharose CL-4B columns. The recombinant GST-dendroaspin and GST-mutant-dendroaspins were digested with Factor Xa (1:300, w/w, Factor Xa/fusion protein) at 4 °C for 12 h. After cleavage, the fractions were loaded on to a Vydac C₁₈ reversed-phase HPLC analytical column (TP104) and eluted with a gradient of 0–26 % acetonitrile (1.78 % per min) containing 0.1 % trifluoroacetic acid (TFA), followed by 26–36 % acetonitrile in 0.1 % TFA (0.25 % per min). When necessary, further analytical columns were run under the

same conditions. The fractions from HPLC were freeze-dried, dissolved in water and assayed for the inhibition of ADP-induced platelet aggregation.

Platelet-aggregation assay

Platelet aggregation was measured by the increase in light transmission as described previously [33]. Briefly, platelet-rich plasma (PRP) was prepared from citrated human blood obtained from healthy individuals, by centrifugation at 200 *g* for 15 min. Platelet aggregation was induced with 10 μ M ADP and measured using a Payton Dual-Aggregometer linked to a chart recorder.

Cell-adhesion assays

The A375-SM human melanoma cell line was maintained in minimal essential medium supplemented with 10% (v/v) fetal bovine serum, MEM vitamins (Sigma), non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate. 96-well plates were coated overnight at 4 °C with human fibrinogen, reconstituted in PBS (pH 7.4) at a concentration of 10 μ g/ml. Plates were washed twice with 200 μ l of PBS and blocked with 200 μ l of 2 mg/ml BSA in PBS for 2 h at room temperature to prevent non-specific binding. Wells were washed three times with 200 μ l of PBS, pH 7.4. Cells were spun at 1000 *g* for 10 min. The cell pellet was resuspended in an appropriate volume of Tyrodes wash buffer (10 mM Na-Hepes, 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂ and 10 mM glucose, pH 7.35). Cells were spun at 1000 *g* for 10 min and resuspended in Tyrodes resuspension buffer (10 mM Na-Hepes, 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 10 mM glucose, 1 mM MgSO₄ and 3.5 mg/ml BSA, pH 7.35). Cells were treated with either appropriate antibodies (5–10 μ g/ml) or mutants of dendroaspin for 30 min using a blood-tube rotator prior to adding 100 μ l to wells. The plates were incubated for 1 h at room temperature. Wells were washed with Tyrodes wash buffer and the number of adherent cells determined by measurement of endogenous acid phosphatase by adding 130 μ l/well of developing buffer (10 mM *p*-nitrophenyl phosphate, 0.1 M sodium acetate, pH 5.5, and 0.1% Triton X-100) and incubating for 1 h at 37 °C. The absorbance at 405/650 nm was measured using an automated plate reader.

RESULTS

Production of recombinant dendroaspins

In the present study, the role of proline residues flanking the RGD motif of dendroaspin was investigated. Five dendroaspin variants were prepared with either single or double proline-to-

Figure 2 Inhibition of ADP-induced platelet aggregation in PRP by wild-type and mutant dendroaspins

PRP (300 μ l) was incubated with various concentrations of inhibitors at 37 °C for 30 s before addition of ADP (10 μ M). The main graph shows incubations with micromolar inhibitor concentrations, and the insert shows those with nanomolar concentrations. Aggregation was measured over 2 min (maximal aggregation) and the results are expressed as the percentage inhibition relative to aggregation levels observed in the absence of inhibitors. IC₅₀ values are shown in Table 1. ●, PRGDMMPG-Den; ○, ARGDMMPG-Den; ▼, PRGDMAGP-Den; ▽, PRGDMPGA-Den; ■, ARGDMAGP-Den; □, PRGDMAGA-Den. The figure is representative of a number of determinations as indicated in Table 1.

alanine substitutions, namely A⁴²-Den, A⁴⁷-Den, A⁴⁹-Den, A^{42,47}-Den and A^{47,49}-Den (Table 1).

Inhibition of platelet aggregation by mutants of dendroaspin

The efficacy of wild-type and five variants of dendroaspin was assessed using ADP-induced platelet aggregation in PRP. The percentage inhibition of platelet aggregation by these mutant dendroaspins was compared with that of wild-type dendroaspin, as illustrated in Figure 2, and the inhibition of platelet aggregation expressed in terms of IC₅₀ is shown in Table 1. A⁴²-Den, A⁴⁷-Den and A⁴⁹-Den were, approximately, equally potent inhibitors of ADP-induced platelet aggregation in PRP, with IC₅₀ values of $\approx$ 170 nM. They showed similar potency to wild-type dendroaspin. In contrast, both mutants with double alanine substitutions, A^{42,47}-Den and A^{47,49}-Den, were $\approx$ 6–8-fold less-potent inhibitors of platelet aggregation than wild-type dendroaspin, with IC₅₀ values of $\approx$ 1.3 μ M.

Inhibition of A375-SM (β_3 integrins) cell adhesion to fibrinogen

The inhibitory efficacy of wild-type and five variants of dendroaspin for A375-SM cell adhesion was also determined in the presence of Mn²⁺ cations. As shown in Figure 3(A), A375-SM cell adhesion to fibrinogen was maximal at a Mn²⁺ concentration of 500 μ M. In contrast, there was little or no absorbance in the absence of either ligand, fibrinogen or Mn²⁺, and in the presence of either 2 mM Ca²⁺ or 1 mM Mg²⁺ or both. This suggests that A375-SM cell adhesion is dependent upon the presence of fibrinogen and Mn²⁺, and that neither Ca²⁺ nor Mg²⁺ is necessary for this adhesion. To investigate which receptors were involved by A375-SM cell adhesion to fibrinogen under such conditions, the effects of function-blocking anti-integrin antibodies were tested. Anti-integrin antibodies were preincubated with A375-SM cells (which contain integrins) to decrease the binding of A375-SM cells to fibrinogen, thus integrin specificity can be

Table 1 Inhibition of platelet aggregation expressed in terms of IC₅₀ values

Results are means $\pm$ S.E.M. for the number of determinations shown in parentheses. The superscript numbers correspond to the position of the RGD loop in the dendroaspin sequence, with substituted amino acid residues shown in bold.

Mutant	RGD loop	IC ₅₀ (μ M)
Recombinant dendroaspin	³⁹ CFTPRGDMPGPYC ⁵¹	0.17 $\pm$ 0.03 (4)
A ⁴² -Den	³⁹ CFT A RGDMPGPYC ⁵¹	0.15 $\pm$ 0.04 (2)
A ⁴⁷ -Den	³⁹ CFTPRGDM A GPYC ⁵¹	0.18 $\pm$ 0.01 (2)
A ⁴⁹ -Den	³⁹ CFTPRGDMPG A YC ⁵¹	0.19 $\pm$ 0.02 (2)
A ^{42,47} -Den	³⁹ CFT A RGDM A GPYC ⁵¹	1.2 $\pm$ 0.1 (2)
A ^{47,49} -Den	³⁹ CFTPRGDM A GA ⁵¹	1.45 $\pm$ 0.12 (2)

Figure 3 Inhibition of A375-SM cell adhesion to fibrinogen

(A) Effect of Mn^{2+} on A375-SM cell adhesion to fibrinogen. A375-SM cells (10^6 /ml) were resuspended in Tyrode's buffer supplemented with Ca^{2+} , Mg^{2+} , Ca^{2+}/Mg^{2+} or various concentrations of Mn^{2+} as shown and then added (100μ l) to microtitre wells coated with fibrinogen. Cells were treated as described in the Materials and methods section. The results are means $\pm$ S.E.M. for at least three determinations expressed in terms of A_{405} , obtained by measurement of endogenous acid phosphatase. (B) Inhibition of A375-SM cell adhesion to fibrinogen by monoclonal antibodies. A375-SM cells (10^6 /ml) were suspended in Tyrode's buffer supplemented with Mn^{2+} and incubated in the presence of inhibitory monoclonal antibodies (see the text for details; 5–10 μ g/ml) or 10 mM EDTA for 30 min before addition to microtitre wells coated with fibrinogen. Cells were treated as described in the Materials and methods section. (C) Inhibition of A375-SM cell adhesion to fibrinogen by recombinant mutants of dendroaspin. Inhibition by recombinant molecules at the concentration of 10 nM for the molecules with single proline substitution and 20 nM for the molecules with double proline substitutions. A concentration of 10 nM causes 50–90% inhibition of A375-SM cell adhesion to fibrinogen for wild-type (WT) dendroaspin (B. Wattam, D. Shang, S. Egglezou, M. Scully, V. V. Kakkar and X. Lu, unpublished work). A375-SM cells were suspended (10^6 /ml) in an appropriate volume of Tyrodes buffer supplemented with 500 μ M Mn^{2+} and incubated for 30 min before addition to microtitre wells coated with fibrinogen (see the Material and methods section for details). For (B) and (C), results are expressed as the percentage inhibition (means $\pm$ S.D.) with points determined in quadruplicate.

established by the ability of blocking antibodies. The effectiveness of anti-integrin antibodies in blocking A375-SM cell adhesion to fibrinogen is shown in Figure 3(B). LM609 (specific for the $\alpha_v\beta_3$)

and 25E11 (specific for the β_3 subunit) substantially blocked A375-SM cell adhesion to fibrinogen, suggesting that the adhesion to A375-SM cells was predominantly β_3 integrin-dependent. In contrast, little or no inhibition was observed when cells were preincubated with monoclonal antibodies JBS2, PC42, JBS5, 4F10, 6S6, YFC118.3, P2 and P3G8, indicating minimal involvement of the $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ complexes and the β_2 , α_{IIb} and α_v subunits.

Under assay conditions, the percentage of inhibition of A375-SM cell adhesion to fibrinogen by these mutant dendroaspins shows similar patterns to those seen in the platelet-aggregation assay. Inhibition of A375-SM cell adhesion to fibrinogen by A⁴²-Den, A⁴⁷-Den and A⁴⁹-Den was approximately equal to that of wild-type dendroaspin at a concentration of 10 nM. In contrast, both mutants with double alanine substitutions, A^{42,47}-Den and A^{47,49}-Den, were $\approx$ 5-fold less-potent inhibitors of cell adhesion than wild-type dendroaspin, even when their concentration was double that of wild-type dendroaspin (Figure 3C).

DISCUSSION

Dendroaspin is a unique protein belonging structurally to a neurotoxin family [28], and functionally it is a potent inhibitor of platelet aggregation [33]. The RGD motif in dendroaspin, as in RGD-containing disintegrins, is at the apex of a solvent-exposed loop, a characteristic of prime importance to its inhibitory action. Unlike short linear RGD peptides and disintegrins [34], dendroaspin has a well-defined secondary structure, making it a suitable template for structure/function studies. Our previous work demonstrated that the amino acid residues flanking the RGD sequence (in particular P⁴² and M⁴⁶ of dendroaspin) modulate its specificity of interaction with integrins, e.g. $\alpha_{IIb}\beta_3$ [35,36]. The present study has shown the fundamental functional role of the prolines flanking the RGD sequence of dendroaspin.

We used both ADP-induced platelet aggregation and cell-adhesion assays for determining the effect of mutagenesis of proline residues on the potency of inhibition of platelet aggregation and A375-SM cell adhesion. The results showed that a single alanine-for-proline substitution did not affect the potency of inhibiting either platelet aggregation or A375-SM cell adhesion to fibrinogen, producing similar potencies to that of wild-type dendroaspin. By comparison, double proline-to-alanine substitutions significantly reduced the potency in both assays by $\approx$ 5–8-fold. This suggests that neither proline is essential for binding to the integrin, but that loss of both prolines may affect the structure of the loop.

A role for proline has been found previously in controlling processing of signal peptides [37] and in phosphorylation [38,39], but our findings are the first example of a role for this amino acid in determining the activity of RGD proteins (see Figure 1), potent inhibitors of platelet aggregation in which prolines are often located in the vicinity of the RGD recognition site. Our results suggest that presentation of the proline provides a favourable environment for the RGD anti-platelet activity. This property may be due to the nature of the proline residue, the only common imino acid in proteins with a bulky pyrrolidine ring that restricts the conformational range of adjacent residues [40]. The lack of a proton on the imino nitrogen blocks hydrogen-bond formation required for α -helix and β -sheet secondary structure, and thus disrupts the propagation of neighbouring secondary structures through interactive site(s) [41]. Proline also causes kinks and bends in the protein structure that, in dendroaspin, diverge the RGD loop from the β -sheet [28]. Thus proline residues, particularly when either flanking the RGD sequence (P⁴²RGDMP⁴⁷) or neighbouring it (RGDMP⁴⁷GP⁴⁹), provide a

favourable conformation for the RGD site in which it is exposed in solvent and recognized by integrin receptors, such as integrin $\alpha_{IIb}\beta_3$. It is possible that the middle residue glycine in the P⁴⁷GP⁴⁹ sequence may also assist in 'presentation' of the RGD site due to the lack of side chain. Future analyses of these mutants with proline substitutions by NMR and other structure-determining techniques hold considerable promise for elucidating the precise structural features of the proline residues that affect the potency of RGD-protein- $\alpha_{IIb}\beta_3$ integrin interactions.

The results of the present study have highlighted a significant role of the proline residues flanking the RGD sequence of dendroaspin. These studies also further support the other important roles of amino acids beyond prolines around the RGD motif described in our earlier studies [36]. Moreover, the results provide a new approach for the design and development of potent platelet-aggregation and cell-adhesion inhibitors.

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