RESEARCH COMMUNICATION

Synthetic RGD peptides derived from the adhesive domains of snake-venom proteins: evaluation as inhibitors of platelet aggregation

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Synthetic peptides based on the RGD domains of the potent platelet aggregation inhibitors kistrin and dendroaspin were generated. The 13-amino-acid peptides were synthesized as di-cysteinyllinear and disulphide cyclic forms. In platelet-aggregation studies, the cyclic peptides showed 3-fold better inhibition than their linear equivalents and approx. 100-fold greater potency

than synthetic linear RGDS peptides derived from fibronectin. An amino acid substitution, Asp¹⁰→Ala, in the kistrin-based peptide gave a 4-fold decrease in potency in the linear peptide, but produced a 2-fold elevation in the inhibitory activity of the cyclic form, generating a peptide of potency comparable with that of the parent protein.

INTRODUCTION

The deformation of occlusive thrombi at sites of vascular injury depends on platelet adhesion to the sub-endothelial matrix and subsequent platelet aggregation [1,2]. These events are regulated by the integrin $\alpha_{IIb}\beta_3$, the major receptor on platelets for fibrinogen [3], fibronectin [4], von Willebrand factor [5], vitronectin [6] and thrombospondin [7]. The adhesive interactions of the $\alpha_{IIb}\beta_3$ complex with these glycoprotein ligands operates predominantly through the ubiquitous adhesive tripeptide Arg-Gly-Asp (RGD) [8]. Consequently, attempts to develop $\alpha_{IIb}\beta_3$ -directed antagonists have focused mainly on amino acid sequences flanking the RGD tripeptide in the fibrinogen A α -chain and fibronectin, but in most cases these reagents have decreased potency and display poor receptor specificity [9].

A further development in the search for $\alpha_{IIb}\beta_3$ antagonists was the discovery in viper venoms of a family of small, highly disulphide-bonded, RGD proteins called disintegrins that inhibit platelet aggregation at nanomolar concentration [10]. The approx. 1000-fold greater potency of disintegrins than of linear RGD peptides in disrupting platelet aggregation has been postulated to reflect an optimal conformation of the RGD tripeptide supported within their protein structure. Consistent with this hypothesis are the observations that the inhibitory activity of disintegrins is greatly decreased upon their treatment with reducing agents [11,12]. Although the pattern of disulphide bonds [13] and the two-dimensional n.m.r. analysis [14,15] of several disintegrins have been reported, the structural features of their RGD domains still remain unclear. In addition to the disintegrins, a natural variant of the short neurotoxin family containing a disintegrin-like RGD domain was isolated from the venom of the mamba *Dendroaspis jamesonii* by Joubert and Taijard [16] and has recently been shown to function as a potent adhesion antagonist [17,18]. The three-dimensional structure of this modified neurotoxin is thought to bear little resemblance to the structure of the disintegrins, and the organization of its RGD domain is predicted to adopt a simple β -hairpin loop [17].

In the present study, we have assessed the efficacy of linear and cyclized synthetic peptides based on amino acid sequences from the RGD domains of the disintegrin kistrin and the neurotoxin homologue to which we refer as dendroaspin [18], as inhibitors of platelet aggregation in comparison with their parent proteins. These peptides, not previously reported, encompass the entire 13 amino acids comprising the looped structure of the RGD domain predicted to occur in dendroaspin. The potential of these RGD snake-venom proteins as models for the design of anti-adhesive reagents is discussed.

MATERIALS AND METHODS

Materials

Freeze-dried snake venoms were obtained from either Latoxan, (Rosans, France) or Sigma. All other chemicals and reagents were obtained from Sigma and Milligen (Watford, U.K.).

Purification of venom proteins

Elegantin (*Trimeresurus elegans*), and dendroaspin (*Dendroaspis jamesonii*) were purified as previously described [18,19]. Kistrin (*Agkistrodon rhodostoma*) and albolabrin (*Trimeresurus albolabris*) were purified from crude venoms by reverse-phase h.p.l.c. using a 0.1% trifluoroacetic acid (TFA)/acetonitrile buffer system. Briefly, freeze-dried venoms were reconstituted in 0.1% TFA for 10 min on ice, centrifuged to remove insoluble material and chromatographed on either Vydac C₁₈ analytical or preparative columns. Active fractions were identified by platelet-aggregation assay and rechromatographed on Pharmacia Pep-S 5 μ m C₂/C₁₈ (for albolabrin) and Vydac C₁₈ (for kistrin) analytical columns. Kistrin and albolabrin were eluted at 22% (v/v) and 28% (v/v) acetonitrile respectively. Recoveries of 2.5–3.0% were obtained for kistrin and 0.4% for albolabrin. Purified proteins were characterized by either N-terminal sequencing or fast-atom-bombardment mass spectrometry.

Abbreviations used: PRP, platelet-rich plasma; TFA, trifluoroacetic acid; kistrin, *Agkistrodon rhodostoma* aggregation inhibitor; albolabrin, *Trimeresurus albolabris* aggregation inhibitor; elegantin, *Trimeresurus elegans* aggregation inhibitor; dendroaspin, *Dendroaspis jamesonii* aggregation inhibitor.

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1	5	10	15	20	25	30	35	40	45	50	55	60	65	70	
EAGEDCDCGSPANPCDDAATCKLLPGAQCGEGLCCDQCSFMKKGTIC CR RARGDDLD DD YCNGISAGCPRNPLHA															A
EAGEECDGCGSPENPCDDAATCKLRPGAQCADGLCCDQCRFKEKRTIC CR RARGDNP DD DRCTGQSADCPRNGLY															E
GKECDCSSPENPCDDAATCKLRPGAQCGEGLCCCEQCKFSRAGKIC CR IPRGDMP DD DRCTGQSADCPRNYH															K
RICYNHLGTPPTTECQED SCYKNIWTFD NIIRRGCG CT TPRGDMP GP YCCESD KCNL															D
RICFNHQSSQPQTTKTCSPESSCYHKQWSDFRGTIIERGCGCPTVKPGIKLS CCESEVCN															E.b

H ₂ N- CR IPRGDMP DD RC-COOH	K(C)	H ₂ N- CR IPRGDMP DD RC-COOH	K(L)
H ₂ N- CR IPRGDMP AD RC-COOH	K _s (C)	H ₂ N- CR IPRGDMP AD RC-COOH	K _s (L)
H ₂ N- CT TPRGDMP GP YC-COOH	D(C)	H ₂ N- CT TPRGDMP GP YC-COOH	D(L)

As illustrated in Figure 1, all four venom proteins were potent inhibitors of ADP-induced platelet aggregation in PRP, with IC_{50} values below 400 nM (Table 2). Kistrin and dendroaspin were the most potent antagonists and consistently produced similar IC_{50} values in the range 155–180 nM. By comparison, the synthetic peptides K(L) and D(L) were approx. 25-fold less

Table 2 Inhibition of platelet aggregation in PRP expressed as IC_{50} values

Abbreviations are the same as in Table 1. Results are means $\pm$ S.D. for the numbers of determinations in parentheses.

	IC_{50} (μ M)
Albolabrin	0.392 ± 0.025 (3)
Elegantin	0.330 ± 0.030 (3)
Kistrin	0.181 ± 0.010 (3)
Dendroaspin	0.155 ± 0.028 (4)
K(C)	0.90 ± 0.010 (2)
K(L)	3.0 ± 0.25 (2)
K_a (C)	0.43 ± 0.050 (2)
K_a (L)	13.0 ± 0.50 (2)
D(C)	1.80 ± 0.12 (2)
D(L)	5.40 ± 0.39 (2)
RGDS	220.0 ± 45.0 (2)
GRGDS	100.0 ± 15.0 (2)

Figure 2 Inhibition of ADP-induced platelet aggregation in PRP by synthetic peptides

The aggregation assays were performed as described in the legend to Figure 1 and the Materials and methods section. Cyclic peptides are denoted by black symbols, and linear peptides by white symbols: ●, ○, peptide K; ▲, △, peptide K_a ; ■, □, peptide D. IC_{50} values are shown in Table 2. The Figure is representative of a number of determinations as indicated in Table 2.

potent inhibitors of platelet aggregation than their parent proteins, producing similar IC_{50} values of 3.0 and 5.4 μ M respectively (Table 2). The modified peptide K_a (L) was approx. 4-fold less potent than the wild-type mimetic K(L), suggesting that the loss of the aspartyl carboxylate group at position 10 had unfavourably altered the solution structure of the RGD tripeptide. Despite the decreased potency compared with the parent proteins, these linear peptides were approx. 30-fold more potent inhibitors of aggregation than linear RGD peptides composed of sequences derived from fibrinogen and fibronectin (Table 2), and 60-fold more potent than the fibrinogen γ -chain dodecapeptide (HHLGGAKQAGDV, amino acids 400–411; results not shown). The natural design of the RGD domains of the snake-venom proteins therefore appears to position the RGD tripeptide in a functionally optimal peptide environment, which serves as suitable templates for the construction of potent platelet-aggregation inhibitors. Both protein and peptide inhibitors had no effect on

agonist-induced platelet shape change, indicating that the mode of antagonism was not operating at the level of cytoskeletal reorganization.

As several studies have reported the increased inhibitory activities of cyclized RGD peptides compared with linear equivalents [20–22], we assessed the effect of the cyclization of peptides K, K_a and D through the oxidation of trityl-protected cysteine groups. The 13-amino-acid cyclic loops were constructed to mimic the RGD-domain looped structure predicted to occur in dendroaspin [17]. The cyclic peptides K(C) and D(C) were approx. 3-fold more potent inhibitors of ADP-induced platelet aggregation than the linear equivalents K(L) and D(L) (Figure 2; Table 2). The improved potency of the peptides engendered through cyclization is interpreted to reflect the promotion of an optimal conformation(s) of the RGD tripeptide, resulting from constraints (i.e. decrease in degrees of freedom) imposed on the peptide backbone by the disulphide linkage. Structural studies of several RGD peptides have suggested that the improved potency due to cyclization may occur with the emergence of predominating β -secondary structure and increased peptide rigidity [22–25].

In contrast with the relative potencies of the linear forms of peptides K, K_a and D, peptide K_a (C) was the most potent inhibitor of platelet aggregation, with an IC_{50} value approaching those observed for disintegrins (Table 2). The cyclization of peptide K_a produced a 30-fold increase in inhibitory activity, resulting in a peptide that was twice as potent as the wild-type cyclic equivalent K(C). The loss of a carboxylate group, resulting from the Asp¹⁰→Ala substitution, appeared to supplement the alterations to the peptide structure induced by cyclization, further optimizing the conformation of the RGD tri-peptide. These observations suggest that constraints imposed on the mobility of the peptide backbone of these inhibitors by cyclization may exert a more significant effect on their functional performance than specific intramolecular side-chain interactions. This notion is consistent with the observations by McDowell and Gadek [22], who suggested that the conformational stability of constrained RGD peptides might relate to the stereochemistry of the cyclic backbone rather than to the interactions of amino acid side chains. The analysis of both linear and cyclic forms of peptides K, K_a and D by n.m.r. and other structure-determining techniques should identify features that dictate the potency of interaction of these RGD peptides with the $\alpha_{IIb}\beta_3$ complex.

The results of the present study highlight the potential of snake-venom RGD proteins as models for the design of platelet-aggregation inhibitors. The peptides generated in this study are among the largest and most potent RGD peptides reported to date and serve as useful templates for further refinement. The data presented show that both cyclization and subtle modifications of the amino acids sequence surrounding the RGD tripeptide can influence the functional properties of these reagents. The design of antagonists of $\alpha_{IIb}\beta_3$ -mediated platelet adhesion will benefit greatly from structural data outlining the organization of the RGD domains within native snake-venom proteins. Recently, a panel of peptides based on the adhesive domain of the $\alpha_{IIb}\beta_3$ specific disintegrin barbourin was described [26], which retains both potency and specificity of interaction with the receptor. The further functional characterization of snake-venom adhesion antagonists should identify suitable candidates as models for the design of a variety of integrin-specific inhibitors, thereby allowing the disruption of selective cell-adhesion events.

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