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COMMUNICATION

Conotoxin engineering: dual pharmacophoric noradrenaline transport inhibitor/integrin binding peptide with improved stability†‡

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A dual-pharmacophoric peptide was engineered by grafting the integrin binding RGD motif between the C- and N-termini of a disulfide-rich noradrenaline transporter inhibiting χ-conotoxin resulting in a stable backbone cyclized peptide. The construct maintained two independent biological activities and showed increased plasma stability with no adverse effects observed following administration to rats, highlighting the potential value of pharmacophore grafting into constrained peptide scaffolds.

Venoms of predatory marine snails of the genus *Conus* contain a diverse range of short (10–30 residues), disulfide-rich bioactive peptides known as conotoxins. The high degree of selectivity and potency for a range of receptors and ion-channels make many of the conotoxins important pharmacological tools as well as potential new drug leads.[1] χ-Conotoxin MrIA, a component of the venom of *Conus marmoreus* has the sequence NGVCCGYKLCHOC (O = 4-hydroxyproline) with disulfide bonds between Cys4–Cys13 and Cys5–Cys10.[2] χ-MrIA selectively inhibits the human noradrenaline transporter (NET) and is currently undergoing clinical development for the treatment of neuropathic pain.[2,3]

The integrins constitute a family of receptors that mediate a number of cellular processes including adhesion, migration and proliferation by interacting with extracellular matrix proteins or other ligands. Many of the integrins recognise the Arg-Gly-Asp (RGD) sequence, a common structural element of extracellular matrix proteins.[4] Naturally occurring RGD- or Lys-Gly-Asp (KGD)-containing peptides known as disintegrins are also derived from snake venoms, and cyclic peptides based on disintegrin sequences have been developed as integrin-targeting drugs.[5,6] Relevant features of these compounds are selectivity for particular integrins, as well as pharmacological properties such as stability and potency (IC_{50} values for integrin–ligand interactions range from pM to μM).[5–7] The structural and functional diversity of RGD-based peptides raises the possibility of therapeutic applications not only as integrin inhibitors, but also for targeting other activities to activated integrins at the surface of cells.

Snake venom disintegrins or RGD- or KGD-containing peptides prevent platelet aggregation by blocking RGD-dependent binding of fibrinogen or von Willebrand factor to the platelet-specific integrin, $\alpha_{IIb}\beta_3$. A clinically important example of a $\alpha_{IIb}\beta_3$-targeting anti-thrombotic is the cyclic heptapeptide (Integrilin) developed from barbourin, a disintegrin from the southeast pigmy rattlesnake.[8,9] Integrilin or related compounds have had a major impact on controlling acute thrombotic events[10] when delivered intravenously (IV) to patients undergoing percutaneous coronary interventions, but more widespread clinical use is limited by lack of effective orally available forms and the potential for associated bleeding. Platelet adhesion to collagen or von Willebrand factor in subendothelial matrix or platelet activation in response to agonists such as collagen, ADP or thrombin (or the thrombin receptor agonist peptide, TRAP), leads to activation of $\alpha_{IIb}\beta_3$, that binds fibrinogen or von Willebrand factor and mediates platelet aggregation.

In general, peptides are highly susceptible to degradation by protease enzymes. While most conotoxins including χ-MrIA are highly structured and relatively stable, cyclization of the peptide backbone can confer additional stability,[11] as demonstrated with a previously reported cyclic MrIA analogue (cMrIA(AG)).[12] Grafting of bioactive motifs into more stable peptidic scaffolds is a promising approach of artificially introducing novel activities.[13–15] Merging two pharmacophores into a single molecule allows the simultaneous targeting of multiple biological processes, which may be advantageous in certain pharmacotherapeutic settings.[16,17] In this study, we combined these three drug design approaches to engineer a bifunctional molecule through the extension of one peptidic pharmacophore by another followed by backbone cyclization.

With the aims of producing a stable bifunctional peptide, we synthesized a cyclic χ-MrIA analogue appended with the RGD motif. It was also hypothesized that the RGD sequence could bind to ubiquitously present integrins and prolong the *in vivo* residence time of the parent peptide *via* a depot effect. Since the

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residues essential for interaction of χ-MrIA with the NET are located in the loop formed by Cys5–Cys10,[3] modifications were carried out at the opposite end of the molecule, as performed in our previous studies.[3,12,18] The RGD sequence was inserted between the termini of χ-MrIA and the backbone cyclized through an amide bond, with the Asn residue removed to maintain a two-residue spacer (Fig. 1).

A thioester containing linear precursor of cMrIA(RGD) was prepared by solid-phase Boc chemistry using standard protocols, and backbone cyclization was accomplished using cysteine mediated intramolecular native chemical ligation.[19] The Cys–Leu site was selected for optimal ligation based on the synthesis of previously reported cyclic MrIA analogues.[12,18] Cyclization proceeded efficiently with the concurrent removal of the Dnp group of His by thiolysis using an excess of sodium 2-mercaptoethanesulfonate (MESNA). Within 1 h, the only major products observed were cyclic reduced cMrIA(RGD) without the Dnp group and the Dnp-MESNA adduct (ESI, Fig. S1‡). Disulfide bonds were formed by treating the purified cyclic reduced peptide with 30% DMSO (aq, pH 8.2) for 6 h to give cMrIA (RGD).

Consistent with the predicted pharmacophoric model of χ-MrIA,[3] the biological activity of cMrIA(RGD) was not compromised by the introduction of the RGD functionality. Inhibition of [^{3}H]noradrenaline uptake at the human NET expressed in COS-7 cells by cMrIA(RGD) (pIC_{50} = 6.06) was comparable to that of χ-MrIA (pIC_{50} = 5.82) (ESI, Fig. S2‡). The synthetic RGD motif of cMrIA(RGD) was functional, since the peptide blocked TRAP- or collagen-dependent aggregation of human platelet-rich plasma mediated by the platelet integrin, $\alpha_{IIb}\beta_3$ (Fig. 2).

Similar dose dependence for cMrIA(RGD)-mediated inhibition (maximal inhibition at ~250 μM) of aggregation induced by agonists acting at either thrombin or collagen receptors, suggests cMrIA(RGD) is acting at $\alpha_{IIb}\beta_3$, required for aggregation by both agonists.[20,21] This was confirmed since cMrIA(RGD) at 125 μM completely inhibited binding of FITC-labelled PAC-1 to TRAP-activated washed platelets (data not shown); PAC-1 binding to platelet $\alpha_{IIb}\beta_3$ is inhibited by RGD peptides.[22] No inhibition of platelet aggregation was evident by χ-MrIA at 500 μM, confirming that the activity was conferred by the presence of RGD sequence. Inhibition of platelet aggregation by cMrIA(RGD) was more potent than a linear RGD-containing peptide, GRGDSP, previously shown to block $\alpha_{IIb}\beta_3$-dependent platelet aggregation (reported IC_{50} in human PRP, ~270 μM),[23] but less potent than other larger peptides, which inhibit in the nM range.[5,6,14] This lower potency may be attributed to a non-ideal environment for the RGD sequence, as the surrounding residues are critical for high-affinity interactions.[24] Neither peptide inhibited platelet shape change (indicated by the initial decrease in transmittance, prior to platelet aggregation), consistent with blockade of $\alpha_{IIb}\beta_3$, rather than interfering with agonist–receptor interaction.

Structural analysis using two-dimensional ^{1}H NMR (ESI, Table S1‡) revealed no major differences between the structural features of cMrIA(AG)[12] and cMrIA(RGD). Aside from minor deviations at the substituted residues (D1 and R14), the αH

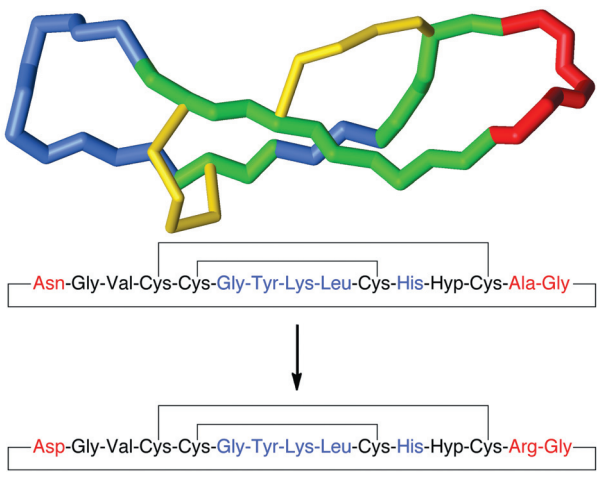


Fig. 1 Backbone structure and sequence of cyclic cMrIA(AG) (PDB ID 2J15) (top). Disulfide bonds are shown in yellow, residues in blue constitute the NET binding pharmacophore (Gly6, Tyr7, Lys8, Leu9 and His11)[3] and highlighted in red are the non-functional Asn1, Ala14 and Gly15 residues that were substituted by the integrin binding Arg-Gly-Asp sequence in this study (bottom).

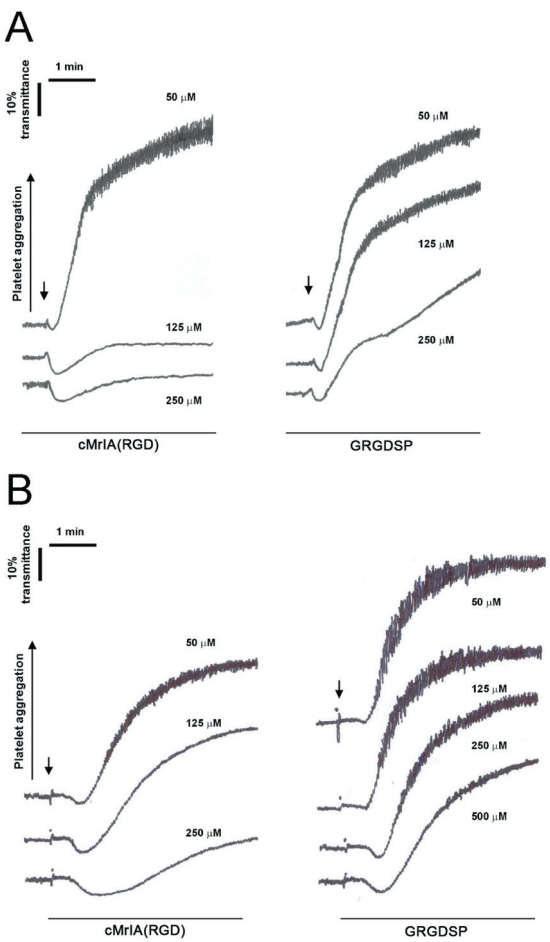


Fig. 2 Inhibition of (A) TRAP- and (B) collagen-induced platelet aggregation in human platelet-rich plasma by cMrIA(RGD) and the linear RGD peptide GRGDSP. Arrow indicates addition of agonist (10 μM TRAP or 5 μM collagen).

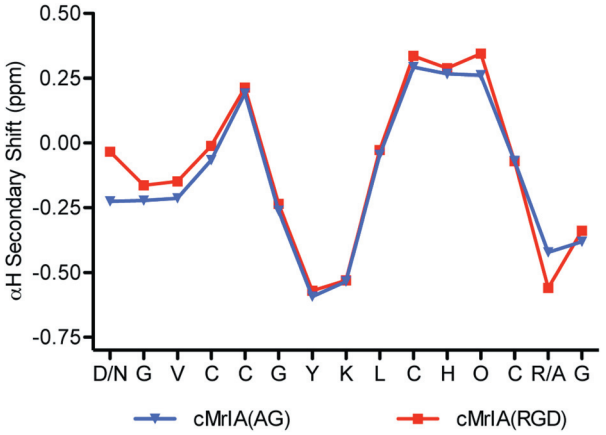


Fig. 3 ^{1}H NMR αH secondary shifts of cMrIA(AG) and cMrIA-(RGD).

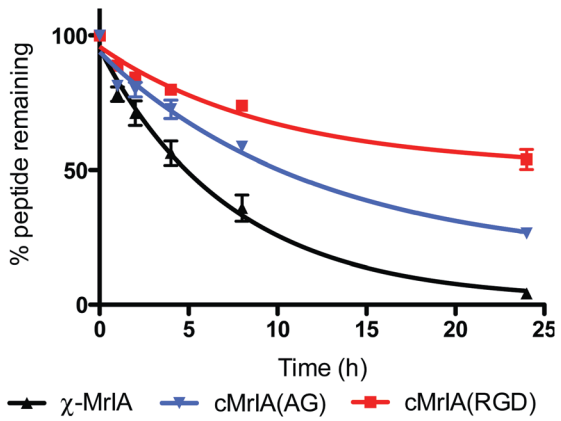
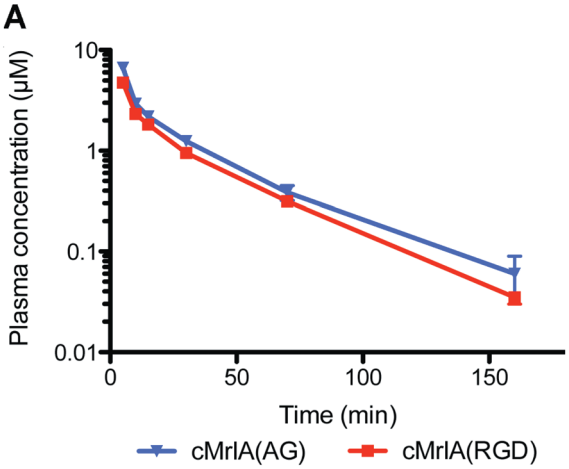


Fig. 4 Degradation of χ-MrIA, cMrIA(AG) and cMrIA(RGD) in rat plasma.

secondary shifts are in good agreement, indicating that the overall fold was maintained (Fig. 3).

Stability of the cMrIA(RGD) construct was assessed in rat plasma over a period of 24 h. While χ-MrIA was degraded to <3% of its original concentration, >50% of cMrIA(RGD) remained intact after 24 h (Fig. 4). The major degradation products (as observed by LC-MS, data not shown) were consistent with the hydrolysis of a single amide bond within a loop (+18 Da) and scrambling of the disulfide bonds (changes in retention time without changes in mass). cMrIA(RGD) also appeared to be more stable than cMrIA(AG) due to the absence of the Asn residue, which was observed to be deamidated (+1 Da) in rat plasma.

Pharmacokinetic properties were evaluated in Sprague Dawley rats (n = 2) after both intravenous and oral administrations of cMrIA(AG) and cMrIA(RGD). Plasma concentrations of cMrIA-(AG) and cMrIA(RGD) following IV administration of a dose of 2 mg kg^{-1} are shown in Fig. 5. Both compounds exhibited apparent terminal elimination half-lives of approximately 0.6 h, low volumes of distribution and low plasma clearance. The masses recovered in urine over the 24 h collection period accounted for 30.4 ± 4.0% of the IV dose for cMrIA(AG) and



B

	cMrIA(AG)	cMrIA(RGD)
Measured Dose (mg/kg)	2.1	2.2
Apparent $t_{1/2}$ (h)	0.6 ± 0.1	0.6 ± 0.0
CL_{total} (mL/min/kg)	10.3 ± 0.8	13.2 ± 0.1
V_b (L/kg)	0.6 ± 0.0	0.7 ± 0.0
% Dose in urine	30.4 ± 4.0	16.7 ± 4.5

Fig. 5 (A) Plasma concentrations over time and (B) pharmacokinetic parameters of cMrIA(AG) and cMrIA(RGD) in Sprague Dawley rats following IV administration (n = 2) Mean ± SEM.

16.7 ± 4.5% of the IV dose for cMrIA(RGD), suggesting that direct urinary excretion may contribute significantly to the *in vivo* clearance of both compounds.

Following oral administration of cMrIA(AG), the compound was detected in the plasma of both rats, however levels were above the analytical lower limit of quantitation (up to 1 h post-dose) in one animal only. Given the limited data available, pharmacokinetic parameters were not calculated, however bioavailability was clearly very low. Following oral administration of cMrIA(RGD), measurable plasma concentrations were observed up to 1 h post-dose in one animal and up to 5 h post-dose in the other. Pharmacokinetic parameters were calculated for one animal only, and in that rat, the apparent terminal elimination half-life was broadly comparable to that after IV administration. Absorption of cMrIA(RGD) was relatively rapid, with maximum plasma concentrations being observed 30 min post-dose, and the apparent oral bioavailability was 3.3%. Consistent with the low oral bioavailability of both compounds, there was negligible urinary excretion cMrIA(AG) and cMrIA(RGD) after oral administration. No adverse reactions or compound-related side effects were observed following IV and oral administration of cMrIA(AG) or cMrIA(RGD).

In conclusion, a cyclic bifunctional peptide was synthesized, showing activity at two independent biological targets. While plasma stability was improved, the pharmacokinetic profile did not significantly differ from that of the cyclic non-RGD containing cMrIA(AG) with both peptides showing equally short *in vivo* half-lives. This study demonstrates a feasible approach to the design of stable multiple pharmacophoric peptides that can potentially be applied to other pharmaceutically relevant targets.

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