Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

SUPPORTING INFORMATION

Conotoxin engineering: Dual pharmacophoric noradrenaline transport inhibitor / integrin binding peptide with improved stability

Zoltan Dekan[a], *Ching-I Anderson Wang*[a], *Robert K. Andrews*[b], *Richard J. Lewis*[a], *Paul F. Alewood*[a]

[a]*Institute for Molecular Bioscience, The University of Queensland, St Lucia 4072, Queensland, Australia*

[b]*Australian Centre for Blood Diseases, Monash University, Alfred Medical Research & Education Precinct (AMREP), Melbourne 3004, Victoria, Australia*

Author for correspondence:

Professor Paul Alewood

Institute for Molecular Bioscience, The University of Queensland, St Lucia, Brisbane 4072, Queensland, Australia

Ph: 3346 2982
Email: p.alewood@imb.uq.edu.au

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABBREVIATIONS

Boc, *tert*-butyloxycarbonyl; BrZ, 2-bromobenzyloxycarbonyl; BSA, bovine serum albumin; CL, plasma clearance; ClZ, 2-chlorobenzyloxycarbonyl; DIEA, diisopropylethylamine; DMEM, Dulbecco's Modified Eagle's Medium; DMF, N,N-dimethylformamide; Dnp, dinitrophenyl; HBTU, N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide; HF, anhydrous hydrogen fluoride; Hyp (O), 4-hydroxyproline; IV, intravenous; LC-MS, liquid chromatography-mass spectrometry; LLQ, lower limit of quantitation; MeBzl, 4-methylbenzyl; MESNA, sodium 2-mercaptoethanesulfonate; NE, noradrenaline; NET, noradrenaline transporter; Chxl, cyclohexyl; PAM, phenylacetamidomethyl; PRP, platelet-rich plasma; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; Tos, toluenesulfonyl; TRAP, thrombin receptor agonist peptide; V_D, volume of distribution

EXPERIMENTAL PROCEDURES

General

RP-HPLC solvents consisted of solvent A (0.05% TFA in water) and solvent B (0.043% TFA/90% acetonitrile in water). Analytical HPLC was performed on a Vydac C18 4.6 x 150 mm column running at 1 mL/min with a linear gradient of 0-50% B in 50 min using a Waters 600 solvent delivery system. Absorbance was recorded at 214 nm on Waters 484 UV detector. Purification of peptides was performed on a Vydac C18 22 x 250 mm column running at 10 mL/min with a linear gradient of 0-50% B in 50 min.

Peptide Assembly

The linear precursor of cMrIA(RGD) was assembled on solid-phase using Boc chemistry on a 0.25 mmol scale on Boc-Gly-OCH_2-PAM resin (substitution value 0.6 mmol/g). N-Boc-amino acids with the following side chain protecting groups were used: Arg(Tos), Asp(Chxl), Cys(MeBzl), His(Dnp), Hyp(Bzl), Lys(ClZ) and Tyr(BrZ). The peptide was synthesised using standard HBTU activation protocols with *in situ* neutralisation as outlined by Schnolzer et al.[1] Briefly, the Boc group was removed with 2 x 1 min TFA treatments, the next protected amino acid (4 eq, 1 mmol) was activated using HBTU (4 eq, 1 mmol) and DIEA (5.5 eq, 1.375 mmol) before addition to the resin, which was treated with a 1 min DMF flow wash between deprotection and coupling steps. S-trityl-β-mercaptopropionic acid was coupled to Gly-OCH_2-PAM resin, the S-trityl group removed with 2 x 1 min treatments of 5% TIPS in TFA, then the first amino acid in the sequence (Leu) was coupled to form a thioester.

Following the removal of the final N-terminal Boc group, the peptide was cleaved from the resin and side chain protecting groups (except Dnp) removed by treatment with 9:1 HF : *p*-

cresol for 1 h at 0° C. The peptide was then precipitated and washed with cold Et_2O, dissolved in 50% acetonitrile/0.05% TFA in water then filtered away from the resin and lyophilised.

Cyclisation

Following purification, HPLC fractions containing the linear precursor of cMrIA(RGD) were combined and MESNA was added to concentration of 50 mM. The solution (~30% acetonitrile / 0.05% TFA in water) was mixed with an equal amount of 30% acetonitrile/0.1 M NH_4HCO_3, the final pH adjusted to 7.5 and stirred at room temperature under an argon atmosphere for 60 min. Samples were taken during this time to monitor the reaction by analytical RP-HPLC and LCMS (Figure S1). When cyclisation and Dnp group removal were judged to be complete, the solution was diluted to ~10% acetonitrile and acidified to pH 3. Cyclic reduced cMrIA(RGD) was then purified by RP-HPLC and lyophilised to give 34 mg white powder.

Oxidation

Purified cyclic reduced cMrIA(RGD) was dissolved in 0.1 M NH_4HCO_3 (pH 8.0) at a concentration of ~100 μM and stirred at room temperature in an open flask. When oxidation was judged to be complete (by analytical HPLC and MS, ~6h), the solution was acidified to pH 3 and purified by RP-HPLC. Following lyophilisation, 16 mg of white powder was obtained (~4% yield from initial resin loading) ESI-MS *m/z* $(M+2H)^{2+}$ calcd. 802.8, found 803.1.

Inhibition of [^{3}H]noradrenaline uptake by MrIA and cMrIA(RGD)

COS-7 cell (ATCC, Manassas, VA) were transiently transfected with hNET cDNA using 10 µl FuGENE (Roche, Australia) and 2 µg DNA per 10^6 cells. Twenty-four hours post-transfection, 10,000 cells per well were aliquoted in 96-well plates. Substrate transport efficiency and binding affinity were determined with 30nM of (^{3}H)noradrenaline ((^{3}H)NE; 40.5 Ci/mmol, Perkin Elmer) and increasing concentrations of wild type MrIA and cMrIA(RGD) from 10^{-9} M to 10^{-4} M in a final volume of 50 µl per well in 96-well plates and incubated at 37 °C for 10 min, followed by two gentle washes using 100 µl assay buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 1.2 mM $MgSO_4$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 5.55 mM D-(+)-glucose, 1 mM ascorbic acid) to remove excess (^{3}H)NE. Cells were then lysed using 50 µl of 0.1 M NaOH with gentle shaking for 60 min at room temperature. OptiPhase Supermix scintillant (Perkin Elmer) was added to each well and the specific (^{3}H)NE uptake was measured using Micro-Beta counter (Perkin Elmer). Each experiment was performed in triplicate in three separate experiments.

NMR Spectroscopy

A sample of cMrIA(RGD) was dissolved in 90% H_2O / 10% ^2H_2O at a concentration of ~3 mM at pH 3.5. TOCSY and NOESY spectra were recorded on a Bruker Avance 600 MHz spectrometer at 298 K with mixing times of 80 and 300 ms respectively.

Preparation of Human Citrated Platelet-Rich Plasma

Human platelet-rich plasma (PRP) was prepared from venous blood of normal healthy volunteers collected using a 19-gauge winged-infusion kit into a syringe containing trisodium citrate as anticoagulant (9:1 blood : 3.2% (w/v) trisodium citrate).[2,3] PRP was obtained by spinning the blood at 100*g* for 20 min at room temperature. At this citrate concentration,

there is sufficient free Ca^{2+} to allow $\alpha_{IIb}\beta_3$-dependent platelet aggregation, but not coagulation. Platelet-poor plasma (PPP) is obtained by spinning PRP for 1 min at 15,400*g*.

Measurement of Anti-Platelet Aggregation Activity

Platelet aggregation of human citrated PRP stirred at 900 rpm and 37°C was assayed using a Chrono-Log lumi-aggregometer as previously described.[2,3] The reference cuvette contained 300 μL PPP plus 100 μL TS buffer (0.01 M Tris, 0.15 M NaCl, pH 7.4). The sample cuvette contained PRP (300 μL) pre-incubated with TS buffer alone or peptide (50-250 μM, final concentration) for 3 min at 37°C, prior to addition of the agonists**,** thrombin receptor agonist peptide (TRAP; 10 μM final concentration) or collagen (5 μg/mL, final concentration), in a final volume of 0.4 mL.

Flow cytometry

Binding of FITC-labelled PAC-1 (BD Biosciences, San Jose, CA, USA), a monoclonal antibody that recognises an activation-dependent epitope on $\alpha_{IIb}\beta_3$, was assessed in untreated or TRAP-stimulated washed platelets as previously described.[4]

Plasma Stability

Heparin treated rat plasma was centrifuged at 12000 rpm for 15 min to separate lipids, then pre-warmed to 37 °C for 15 min before use. 200 μg of each peptide in 25 μL water was mixed with 200 μL plasma and incubated at 37 °C. A sample of 30 μL was taken at every time point and quenched with 70 μL of 2% TFA in water, then cooled to 4 °C for 15 min and centrifuged at 12000 rpm for 5 min. 20 μL of the supernatant was analysed by LC-MS on a Phenomenex 2.1 x 50 mm C18 300 Å column running at 200 μL/min with a gradient of 5 to

35% B in 20 min. Eluting products were detected on a QStar Pulsar mass spectrometer operating in positive ion mode between 400 and 1800 Da. Areas under the peaks corresponding to the starting peptide were measured and plotted as a percentage of the area at t = 0.

Pharmacokinetics

cMrIA(AG) or cMrIA(RGD) was administered to overnight-fasted male Sprague Dawley rats (weighing between 256 and 308 g) intravenously as a 5 min constant rate infusion (1.0 mL per rat, n=2 rats for each compound) and orally by gavage (1.0 mL per rat, n=2 rats for each compound). Rats had access to water *ad libitum* throughout the pre- and post-dose sampling period, and access to food was re-instated 4 h post-dose.

Samples of arterial blood and total urine were collected up to 24 h post-dose. Arterial blood was collected directly into borosilicate vials (at 4°C) containing heparin, Complete® (a protease inhibitor cocktail), potassium fluoride, and EDTA to minimise potential for *ex vivo* degradation of cMrIA(AG) and cMrIA(RGD) in blood/plasma samples. Once collected, blood samples were centrifuged, supernatant plasma was removed, and plasma concentrations of cMrIA(AG) and cMrIA(RGD) were determined by LC-MS (lower limits of quantitation (LLQ) were 0.0033 μM for cMrIA(AG) and 0.0031 μM for cMrIA(RGD)).

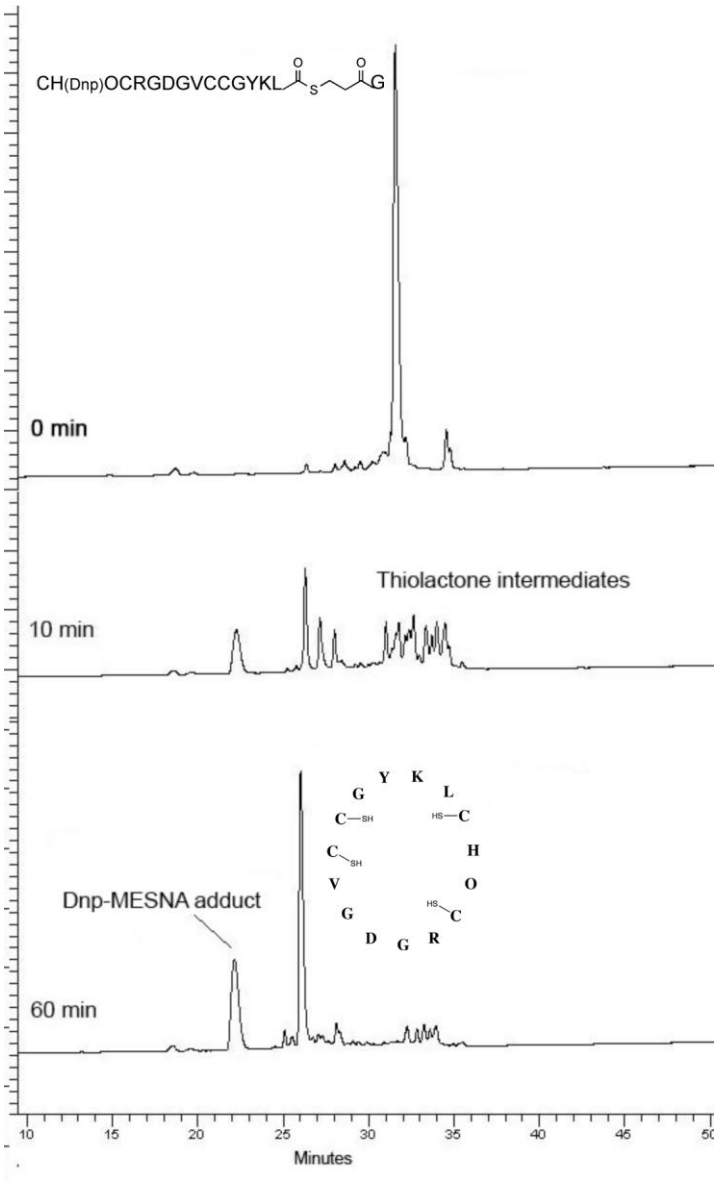

Figure S1. Analytical HPLC traces of the progress of cyclization of cMrIA(RGD)

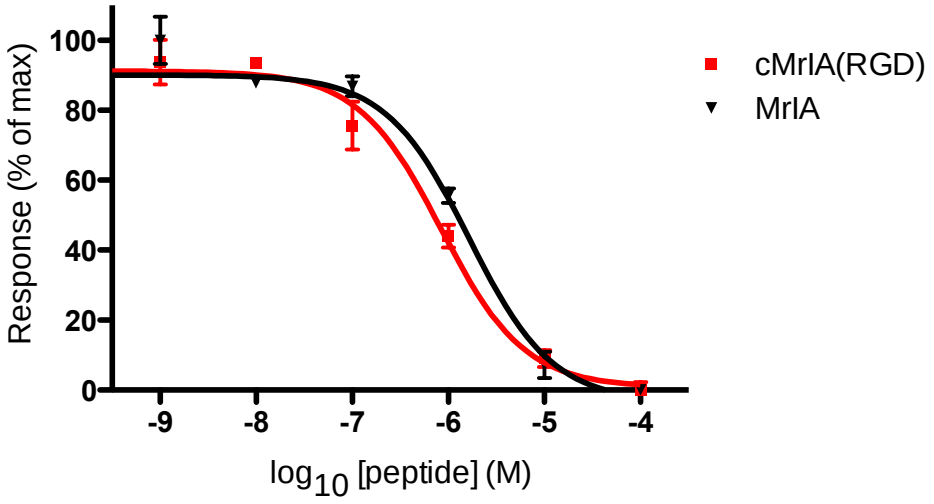

Figure S2. Inhibition of [^{3}H]noradrenaline uptake at the human NET expressed in COS-7 cells by MrIA and cMrIA(RGD).

Table S1. ^{1}H NMR αH and backbone NH chemical shifts of cMrIA(RGD).

Residue	αH shift (ppm)	NH shift (ppm)
D1	4.56	7.814
G2	3.86, 3.67	7.627
V3	4.03	7.896
C4	4.68	8.519
C5	4.90	8.613
G6	3.95, 3.44	8.128
Y7	4.05	8.316
K8	3.74	8.094
L9	4.29	7.611
C10	5.03	8.335
H11	4.91	8.419
O12	4.74	-
C13	4.62	8.047
R14	3.74	8.511
G15	3.88, 3.31	8.298

REFERENCES

1 M. Schnolzer; P. Alewood, A. Jones; D. Alewood, S. B. Kent, *Int. J. Pept. Protein. Res.* 1992, **40**, 180.

2 R. K. Andrews, M. H. Kroll, C. M. Ward, J. W. Rose, R. M. Scarborough, A. I. Smith, J. A. Lopez, M. C. Berndt, *Biochemistry* 1996, **35**, 12629.

3 R. K. Andrews, E. E. Gardiner, N. Asazuma, O. Berlanga, D. Tulasne, B. Nieswand, A. I. Smith, M. C. Berndt, S. P. Watson, *J. Biol. Chem.* 2001, **276**, 28092.

4 S. Dunkley, J. F. Arthur, S. Evans, E. E. Gardiner, Y. Shen, R. K. Andrews, *Br. J. Haematol.* 2007, **137**, 569.