Engineering pro-angiogenic peptides using stable disulfide-rich cyclic scaffolds

Lai Y. Chan,1 Sunithi Gunasekera,1 Sonia T. Henriques,1,2 Nathalie F. Worth,3 Sarah-Jane Le,3 Richard J. Clark,1,4 Julie H. Campbell,3 David J. Craik,1 and Norelle L. Daly1

1The University of Queensland, Institute for Molecular Bioscience, Brisbane, Australia; 2Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal; 3the Australian Institute for Bioengineering and Nanotechnology, Brisbane, Australia, and 4The University of Queensland, School of Biomedical Sciences, Brisbane, Australia.

Corresponding author: Norelle L. Daly
Address: The University of Queensland, Institute for Molecular Bioscience, Brisbane, QLD 4072, Australia
Email: n.daly@uq.edu.au
Phone: +61 7 3346 2021
Fax: +61 7 3346 2101

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Abstract

Fragments from the extracellular matrix proteins laminin and osteopontin, and a sequence from VEGF, have potent proangiogenic activity despite their small size (< 10 residues). However, these linear peptides have limited potential as drug candidates for therapeutic angiogenesis because of poor stability. Here we show that the therapeutic potential of these peptides can be significantly improved by “grafting” them into cyclic peptide scaffolds. MCoTI-II and SFTI-1, which are naturally occurring plant-derived cyclic peptides of 34 and 14-residues respectively, were used as scaffolds in this study. Using this approach we have designed a peptide that is stable in human serum, in contrast to the small peptide fragments, and at nanomolar concentration induces angiogenesis in vivo. This is the first report of using these scaffolds to improve the activity and stability of angiogenic peptide sequences and is a promising approach for promoting angiogenesis for therapeutic uses.
Introduction

Several small peptides have been reported to promote potent angiogenic activity, including peptides derived from the extracellular matrix proteins laminin and osteopontin. A hexapeptide (LAM) from the laminin α1 chain (residues 2105-2110) promotes angiogenesis by inducing endothelial cell adhesion, migration, and invasion, whereas a heptapeptide (OPN) from osteopontin (residues 162-168) interacts with α9β1 and α4β1 integrins for regulating hemopoietic stem cells and progenitor cells to promote the recovery of the damaged bone marrow and induces angiogenesis in a mouse dorsal air sac assay. Mimics of vascular endothelial growth factor (VEGF) have also been shown to promote angiogenesis. For example, a peptide that mimics the VEGF helix region (residues 17-25), referred to as the QK peptide, promotes proliferation of endothelial cells derived from bovine aorta and induces capillary formation in matrigel in vitro.

Pro-angiogenic agents have significant potential in the treatment of a range of conditions, including wound healing, cardiac ischemia, diabetic retinopathy, and rheumatoid arthritis. Despite this potential, clinical trials carried out with protein therapy and plasmid DNA gene therapy have not produced significant results, primarily because of stability issues. Peptides such as LAM, OPN and QK offer an alternative approach for the design of novel drug leads if the intrinsic instability associated with small unconstrained peptides can be overcome. An exciting approach to improve the stability of peptides is to take advantage of the stability of disulfide-rich cyclic peptides. This approach has recently been exemplified by backbone cyclization of a cone snail peptide to produce an orally active peptide with analgesic activity. In addition to cyclizing naturally occurring peptides, grafting small bioactive sequences into scaffolds that are highly stable has the potential to improve stability and bioavailability. For example the cyclotide, kalata B1, has recently been used as a scaffold to stabilize a six-residue peptide sequence having anti-angiogenic activity. Cyclotides are a large family of plant-derived peptides that contain approximately 30 amino acids and are characterized by a head-to-tail cyclic backbone and cystine knot arrangement of three disulfide bonds. This structural motif is referred to as the cyclic cystine knot (CCK) and provides exceptional stability to cyclotides.
The small size of the LAM, OPN and QK peptides makes them ideal candidates for grafting into disulfide-rich cyclic peptide scaffolds. In the current study we have used the plant-derived peptides MCoTI-II (Momordica cochinensis trypsin inhibitor-II) and SFTI-1 (sunflower trypsin inhibitor-1) as stable scaffolds. Both MCoTI-II and SFTI-1 are potent trypsin inhibitors, but are structurally quite different. MCoTI-II is a 34 amino acid peptide and is a member of the cyclotide family by virtue of its CCK structural motif, but has no sequence similarity to kalata B1 apart from the six cysteine residues. SFTI-1 contains only a single disulfide bond and 14 amino acids. The structures of these frameworks and the targeted grafting sequences are shown in Figure 1. Grafting the bioactive peptide sequences into the cyclic peptide scaffolds resulted in stable and potent angiogenic agents with promising potential in the design of drug leads for therapeutic angiogenesis.
Materials and methods

Peptide synthesis and isolation of MCoTI-II from *M. cochinchinensis* seeds

All grafted peptides (MCo-LAM, MCo-OPN, SFTI-LAM, SFTI-OPN, SFTI-1 and SFTI-QK) were synthesized using manual solid-phase peptide synthesis with Boc-chemistry. Peptides were constructed on PAM-Gly-Boc resin with S-tritylmercaptopropionic acid as a linker using HBTU to activate the amino acids. Grafted peptides and linear peptides were synthesized on a 0.5 mmole scale. Linear peptides (linear LAM, linear OPN, and linear QK) were synthesized using Fmoc chemistry on a microwave synthesizer using chlorotrityl chloride resin. Peptides made using Boc-chemistry were cleaved from the resin using hydrogen fluoride (HF) with *p*-cresol as a scavenger at -5 to 0 °C for 1 h. Fmoc synthesized peptides were cleaved using a mixture of 95% TFA/2.5% TIPS/2.5% H₂O. The TFA was removed by rotary evaporation and the residue partitioned between 50% acetonitrile in water containing 0.1% TFA and cold diethyl ether. The aqueous layer was lyophilized and the resulting crude peptides purified using RP-HPLC. Native MCoTI-II peptide was isolated from a *M. cochinchinensis* seed extract as described by Chan et al.²⁴

Purification of peptides

Crude peptides either obtained from plant extracts or chemical synthesis were purified using a series of Phenomenex C18 columns on RP-HPLC. Gradients of 1% min⁻¹ of 0-80% solvent B (90% MeCN in 0.045% TFA in H₂O) and solvent A (aqueous 0.05% TFA in H₂O) were employed and the eluant was monitored at 215 nm and 280 nm. The purity of the peptides was examined by analytical RP-HPLC on Phenomenex Jupiter 5μ C18 300 Å 150 x 2.0 mm column and masses were determined by electrospray mass spectrometry.

Cyclization and oxidation conditions

Grafted and native SFTI-1 peptides were folded in solution at 0.1 mg/mL using a range of buffer conditions. Synthetic native SFTI-1 peptide was cyclized and oxidized in a two step process. Cyclization was achieved in the presence of 0.1 M tris(2-carboxyethyl)phosphine (TCEP) incubated for 24 h and the cyclic product then
purified by RP-HPLC. The cyclic reduced peptide was oxidized in 0.1 M ammonium bicarbonate (pH 8.0). By contrast, cyclization and oxidation of MCoTI-II grafted peptides were done in a single step reaction with 0.1 M ammonium bicarbonate (pH 8.5) for 24 h. SFTI-I grafted peptides were oxidized in 0.1 M ammonium bicarbonate (pH 8.5).

NMR characterization

Peptides were dissolved at a concentration of approximately 1 mM in 90% H$_2$O/10% D$_2$O (v/v). D$_2$O (99.9%) was obtained from Cambridge Isotope Laboratories, Woburn, MA for $^1$H NMR measurements. Spectra were recorded at 290 K for linear and SFTI-1 grafted peptides, whereas spectra for MCoTI-II grafted peptides were recorded at 298 K. Spectra were recorded on Bruker Avance 500 MHz or 600 MHz spectrometers. NOESY mixing times for linear and SFTI-1 grafted peptides were 300 ms, whereas spectra recorded on MCoTI-II grafted peptides had mixing times of 200 ms. Two-dimensional spectra included TOCSY, NOESY, COSY and ECOSY. The latter two spectra were only recorded for SFTI-OPN to facilitate structure determination. In addition, SFTI-OPN was dissolved in 100% D$_2$O to determine slow exchanging amide protons. Spectra were analyzed using SPARKY. The sequential assignment procedure pioneered by Wüthrich was used to sequence specifically assign the amino acids, using TOCSY and NOESY spectra. The three-dimensional structure of SFTI-OPN was calculated by deriving distance and angle restraints from the NOESY and COSY spectra, respectively. A family of structures that are consistent with the experimental restraints was calculated using the programs CYANA and CNS. A set of 100 structures was calculated and the 20 lowest energy structures selected for further analysis. Structures were analyzed using the programs PROCHECK_NMR and PROMOTIF to generate statistical analyses and a Ramachandran plot. MolMol and PyMol were used to display the structural ensembles and surfaces of the peptides, respectively.

Serum stability assay

Human serum from male AB plasma (Sigma-Aldrich) was centrifuged at 17000 g for 10 min for the removal of the lipid component. All peptides were tested at a final concentration of 6.6 μM after dilution from a stock solution of 200 μM. Supernatant
was taken out and incubated for 15 min at 37 °C before the assay. Each test peptide was diluted in 1:10 dilution with human serum except for controls in PBS. The incubation time points were 0, 3, 8 and 24 h at 37 °C. Controls and test peptides were incubated in parallel at each time point. Serum proteins were denatured by quenching with 40 μL of 6 M urea (10 min, 4 °C), followed by the precipitation of proteins with an addition of 40 μL of 20% trichloroacetic acid (TCA) (10 min, 4 °C). These solutions were then centrifuged at 17000 g for 10 min. 100 μL of supernatant was taken out in triplicate from the serum treated and PBS control peptides at each time point and were run on the RP-HPLC on a 0.3 mL/min Phenomenex column using a linear 1% min⁻¹ gradient of 0-50% solvent B. The elution time for each peptide was determined by the PBS control at zero time point. The stability at each time point was calculated as the area of the serum treated peptide peak on RP-HPLC at 215 nm as percentage of the area of the 0 h PBS treated control peptides. Each experiment was done in triplicate.

**Enzyme stability assay**

Thrombin from human plasma (Sigma-Aldrich) 25 μg/mL (0.7 μM) was prepared in 100 mM sodium citrate buffer (pH 6.5). Peptide stock solutions were prepared in the same buffer at a concentration of 200 μM. An equal amount of enzyme to peptide was added in an eppendorf tube and incubated at 37 °C in a water bath. 5 μL aliquots were taken at 0, 1, 5, 8, 24 h. To each aliquot 95 μL of 5% formic acid was added to quench the reaction. A blank was prepared by placing 95 μL of 5% formic acid in an analytical vial with 2.5 μL of enzyme added followed by 2.5 μL of peptide. A control was also prepared by placing 95 μL of 5% formic acid in an analytical vial with only 5 μL of enzyme added. Samples run on RP-HPLC on a 0.3 mL/min Phenomenex column using a linear 1% min⁻¹ gradient of 0-50% solvent B. The elution time for each peptide was determined by the blank at the zero time point. The stability at each time point was calculated as the area of the test peptide peak on RP-HPLC at 215 nm as percentage of the area of the 0 h blank. Each experiment was done in duplicate. The same method was applied to metalloproteinase-9 (MMP-9) except using 50 mM Tris in 10 mM CaCl₂ and 150 mM NaCl (pH 7.5) as the buffer.
Hemolytic assay

Human red blood cells (RBCs) were used to assess the toxicity of the peptides against human cells. Peptide samples were prepared by two-serial dilutions with PBS as previously described.24

Cell culture

Rat aortic endothelial cells (RAECs) were seeded at a moderate density (2-3 x 10^4 cells/cm^2) in RPMI (Gibco BRL) containing 10% FCS (Trace Biosciences, Australia), 25 μg/mL endothelial cell growth promoter (Starrate, Australia) and 25 μg/mL heparin in T75 flasks and maintained at 37 °C in an atmosphere of 5% CO_2 in air. Cells were allowed to reach approximately 80% confluency before passaging. Cells were used between passage one and five.

Angiogenesis sprouting assay

RAECs were allowed to form three-dimensional spheroids in 0.5% methylcellulose (Sigma-Aldrich) in RPMI with 10% FCS, within hanging drop wells overnight (1500-2000 cells per well in a 60-well plate). The base gel (1.35 mg/mL rat-tail collagen, 0.25% methylcellulose in RPMI) was then prepared by adding 500 μL into a 12-well plate and allowed to set at 37 °C for 30 min. Spheroids were collected and washed in serum-free medium, then spun at 500 rpm for 5 min. Another 500 μL of a second collagen gel was then added to the spheroids (with or without 10% FCS or variable of interest); and then the gel containing spheroids, was layered on top of the base gel and allowed to set at 37 °C for 30 min. Spheroid images were taken using an Olympus BX60 light microscope with a digital camera for baseline measurements under x 100 magnification. After 24 h, cell sprouting was observed in the cell culture and images were taken systematically for every single spheroid. Only independent spheroids were imaged. Cell sprouting was calculated by measuring 30 sprouts at evenly-spaced intervals per spheroid using Image J. Results were represented as mean sprout length ± SD and statistical comparisons were made by a Kruskal-Wallis test, using Analyse-it. P ≤ .05 was considered significant.
Chorioallantoic membrane (CAM) assay using quail egg

This assay was established based on previous studies on chicken and quail eggs as the model for testing angiogenic compounds.33-37 Fertilized quail (*Cortunix courage*) eggs from a local farm in Adelaide (Australia) were incubated horizontally at 37 °C for 3.5 days in a humidified incubator. On day five eggs were sterilized with 70% EtOH/H<sub>2</sub>O spray and air-dried before opening a 0.5 cm x 0.5 cm window at the centre of the egg shell. The embryo dropped by ~0.5 cm and the CAM with visible blood vessels were gently pulled down using tweezers to a visible area. The window was sealed with invisible tape, followed by overnight incubation at 37 °C. On day six, embryos that survived were recorded by observing the color and different vasculature of the blood vessels. Only embryos that survived were implanted with a filter paper pre-soaked in a test solution. All peptides were initially tested at 50 μg/mL and those that gave the highest activity were subsequently tested at a range of lower concentrations. VEGF solution (10 ng/mL) and RPMI 1640 medium were used as positive and negative controls, respectively. The window was re-sealed after the grafting procedure and further incubated at 37 °C for another 24 h. On the next day, eggs were collected from the incubator and chilled on ice for 30 min, followed by fixation with 25% gluteraldehyde on the implanted area for 2 h. Finally, the implanted filter papers were trimmed off and washed with PBS in a 12 well tissue culture plate and quantification of blood vessels was observed on an Olympus SZX12 dissecting microscope with a light box under x 32 magnification for blood vessel count using a hand tally counter and photographed under x 16 magnification. DP controller software (Olympus) was used to view real-time images. All images were captured using DP manager software. One-way ANOVA and unpaired t-test were applied for the statistical comparison analysis using GraphPad Prism. Each test peptide was compared to the negative control and results were represented as mean blood vessels count ± SD and P ≤ .05 was considered to be significant.

Surface plasmon resonance

The interaction between the grafted peptides, MCo-OPN and SFTI-OPN, and the integrin α<sub>9</sub>β<sub>1</sub> was evaluated using a Biacore 3000 surface plasmon resonance (SPR) system. The binding of the scaffolds MCoTI-II and SFTI-1 for the integrin was also
evaluated for comparison. The integrin $\alpha_9\beta_1$ was immobilized on the CM5 chip surface by the amine coupling method. Briefly, the dextran surface was activated by injection of a 1:1 (v:v) mixture of 0.4 M EDC ($N$-ethyl-$N'$-(3-dimethylaminopropyl)carbodiimide) and 0.1 M of NHS ($N$-hydroxysuccinimide) (flow rate 5 $\mu$L/min, 10 min). 50 $\mu$g/mL integrin $\alpha_9\beta_1$ (recombinant human integrin $\alpha_9\beta_1$, R&D systems, USA), solubilized in 10 mM acetate buffer pH 4.5, was injected over the activated surface (flow rate 5 $\mu$L/min, 20 min). Unreacted carboxyl groups were blocked by two 35 $\mu$L injections of 1 M ethanolamine, pH 8.5. A reference channel underwent the same treatment without the integrin step. The integrin immobilization led to a signal of approximately 21500 response units (RU). The antibody anti-integrin $\alpha_9\beta_1$ (mouse monoclonal antibody, clone Y9A2, Chemicon, Australia), that identifies the $\alpha_9$ subunit as a heterodimer in association with $\beta_1$, was injected over the immobilized $\alpha_9\beta_1$ integrin to evaluate if the protein was correctly folded (100 $\mu$g/mL anti-integrin $\alpha_9\beta_1$ dialysed and solubilized in running buffer). Anti-integrin $\alpha_9\beta_1$ bound to immobilized integrin confirming that at least a portion of the immobilized $\alpha_9\beta_1$ integrin has a correct conformation. The binding of linear OPN, MCo-OPN, SFTI-OPN, MCoTI-II and SFTI-1 was followed by peptide injection (25, 50, 100 and 200 $\mu$M) for 3 min, followed by 600 s of dissociation. The sensor surface was regenerated with a short pulse of 10 mM HCl, pH 2, injected for 60 s at 5 $\mu$L/min. All the measurements were performed at 25 °C, with a flow rate of 5 $\mu$L/min. 10 mM HEPES, 150 mM NaCl, pH 7.4 was used as running buffer and to prepare all the solutions. All solutions were freshly prepared and filtered (0.22 $\mu$m pore size). Response signals were referenced by subtracting the reference flow channel.
Results

Synthesis and characterization of grafted peptides

The sequences of the grafted peptides are given in Figure 2, labeled with the scaffold name first followed by the code for the linear sequence (e.g. SFTI-OPN). The linear peptides were synthesized by Fmoc chemistry and the cyclic peptides by Boc chemistry using a thioester-based chemistry for backbone cyclization. For the cyclic peptides the cyclization and oxidation were generally carried out in a single step. Peptides were purified by preparative RP-HPLC and the purity determined by analytical RP-HPLC and mass spectrometry.

As the SFTI-1 grafted peptides only contain a single disulfide bond, a single isomer was observed after cyclization and oxidation. However, there are 15 possible disulfide connectivities for the MCoTI-II grafted peptides deriving from the six cysteine residues. Despite this potential heterogeneity MCo-LAM and MCo-OPN folded into one major isomer. Analysis of the peptides with NMR spectroscopy indicated that the native fold was present for all purified peptides (Document 1: Figures S1 and S2, available on the Blood website; see the Supplemental Materials link at the top of the online article).

In contrast to the other grafted peptides, the MCo-QK peptide was poorly soluble in the buffer used for the oxidation and cyclization of MCo-LAM and MCo-OPN. A range of different solution conditions was used for MCo-QK, including varying the pH, and adding organic solvents, but these solution conditions did not result in cyclization and oxidation into the native-like conformation. Therefore, this peptide was not tested in subsequent assays. However, the peptide with the QK grafted into the SFTI-1 scaffold was highly soluble and was efficiently cyclized and oxidized.

Sprouting activity of grafted peptides on rat and human endothelial cells

Gel-embedded endothelial cell spheroids were used to measure the neovascular response of the linear sequences and grafted peptides. Rat aortic endothelial cells
(RAECs) were used and spheroid growth induced by the grafted peptides was monitored for 24 h. In vitro RAECs sprouting assays showed that all RAECs treated with grafted peptides displayed significant sprouting compared to the negative control and corresponding scaffolds with $P \leq 0.05$ (Figure 3A-C) except for SFTI-QK, which did not display significant sprouting compared to the negative control. Although SFTI-OPN showed a slightly lower activity compared to its linear sequence, it has a comparable activity to SFTI-LAM and with an improved activity compared to its corresponding scaffold, linear LAM and the negative control. Details of the sprouting assay results are provided in supplemental Figures S3 and S4.

**Angiogenic activity of grafted peptides in an in vivo CAM assay**

The linear sequences and grafted peptides were tested in an in vivo CAM assay using *Cortunix cortunix* eggs. The extent of angiogenic activity was determined by quantifying the vessels that grew towards the implant area using a dissecting microscope. Peptides were initially tested at 50 $\mu$g/mL, with VEGF used as a positive control. Among the linear sequences, linear OPN had the highest angiogenic effect followed by linear LAM and linear QK (Figure 4A-B). SFTI-1 had no angiogenic effect in the CAM assay but interestingly MCoTI-II has a significantly higher effect than the negative control, indicating intrinsic angiogenic activity for this scaffold. Grafting the LAM sequence into MCoTI-II resulted in a similar level of activity to the native peptide, but grafting the OPN sequence significantly enhanced the angiogenic activity. The SFTI-OPN graft also had the highest activity for the SFTI-1 grafted peptides (Figure 4B). A 10-fold lower concentration of SFTI-LAM and SFTI-OPN was also used in this assay. Although the blood vessel count for SFTI-LAM was significantly reduced at the lower concentration, the SFTI-OPN blood vessel count was only slightly reduced when compared to its activity at the higher concentration. A further 10-fold lower concentration of SFTI-OPN (320 nM) also resulted in significant angiogenic activity. A comparison of SFTI-OPN and the linear OPN sequence at the same concentration indicated a significant increase in activity upon grafting into the cyclic peptide scaffold (Figure 4C). Overall, the OPN sequence was the most effective sequence studied with SFTI-OPN having the highest and MCo-
OPN the second highest angiogenic effect. Further details of the CAM assays are given in the supplemental Figures S5 and S6.

**Stability of native, linear and grafted peptides**

The native, linear and grafted peptides were incubated in human serum to investigate their stability. All grafted peptides were stable in human serum with 100% peptide remaining after 24 h, except for SFTI-OPN which degraded to approximately 40% of the initial peptide concentration within the first 4 h and then remained unchanged for the rest of the assay (Figure 5A). The results observed for SFTI-OPN might be related to binding to serum proteins, resulting in an apparent decrease in peptide recovery. By contrast all linear peptides were significantly degraded within the first 3 hours. Although SFTI-OPN was not as stable as the other grafted peptides, it is more stable than its linear counterpart. Given that the SFTI-OPN has the highest angiogenic activity among the grafted peptides, its stability of this peptide was further tested against thrombin and metalloproteinase-9 (MMP-9), enzymes involved in angiogenesis. MCoTI-II was also tested in these enzyme stability assays. It was found that SFTI-1, SFTI-OPN and linear OPN were stable to thrombin over 24 h whereas N-benzoyl-Phe-Val-Arg-p-nitroanilide (FVR), a known substrate for thrombin, was cleaved. However, in the MMP-9 stability assay, linear OPN was gradually cleaved by the enzyme whereas SFTI-1, SFTI-OPN and MCoTI-II were found to be stable over 24 h (supplemental Figure S7).

**Hemolytic activity of all native, linear analogues and grafted peptides**

The hemolytic activity of all peptides was tested in human blood. PBS and Triton-X were used in the assay as positive and negative controls respectively. Melittin, a highly hemolytic honey bee venom peptide, was used as a positive control. None of the grafted peptides exhibited hemolytic activity, as shown in Figure 5B.

**Solution structural analysis of SFTI-OPN**

To gain an insight into structure activity relationships of the grafted angiogenic peptides, the three-dimensional structure of SFTI-OPN, the most potent grafted
peptide was determined. Two conformations were observed for this peptide, most likely as a result of cis or trans isomerization of Pro 13. The $\alpha$H secondary shifts of the two conformations are given in the supporting information (supplemental Figure S1). The $\alpha$H chemical shift of the trans isomer of SFTI-OPN indicated that the non-grafted region has a similar structure to the native peptide. The ratio of the trans and cis conformations is 70:30 in the NMR spectra and the structure was determined for the predominant trans conformation of SFTI-OPN.

A set of 100 structures was calculated using 98 distance restraints comprising 39 intra-residue, 43 sequential, eight medium range and eight long range restraints, and five dihedral restraints which were derived from the NMR data (Table 1). The 15 lowest energy structures, shown in Figure 6A, are in good agreement with the experimental data, show no dihedral angle violations exceeding 3° and no distance violations more than 0.3 Å (Table 1). The RMSD over the well-defined region, residues 1-3 and 11-14, is 0.55 ± 0.33 Å for the backbone atoms. The secondary structure of SFTI-OPN was analyzed by PROMOTIF and revealed that a $\beta$-hairpin is the major element of secondary structure. The residues comprising the two strands are 2-5 and 8-12. A type IV $\beta$-turn connects the two strands. These structural findings are consistent with the $\alpha$H secondary shifts analysis, which suggests the grafted region between Ser4-Arg10 is disordered (Figure 6A). D$_2$O slow exchange experiments indicated there were no slowly exchanging amide protons and therefore no hydrogen bonds to stabilize the structure. A comparison of SFTI-1 with SFTI-OPN is given in Figure 6A-B, indicating that the overall fold is similar between the two peptides. A surface representation highlights the distribution of hydrophobic residues in SFTI-OPN and native SFTI-1. SFTI-OPN has a larger hydrophobic surface area in the grafted region. The structural data of SFTI-OPN have been deposited in the Biological Magnetic Resonance Data Bank with the accession number 17426 (http://www.bmr.b.wisc.edu/).

**Grafted peptides bind to integrin $\alpha_9\beta_1$**

The integrin $\alpha_9\beta_1$ has been shown to recognize the angiogenic heptapeptide OPN and this binding appears to be correlated with OPN-induced angiogenesis. To evaluate if
the activity of the new pro-angiogenic peptides, grafted with the OPN sequence, can be recognized by $\alpha_9\beta_1$. SPR studies were performed. The binding of SFTI-OPN and MCo-OPN, and their corresponding scaffolds, to immobilize integrin $\alpha_9\beta_1$ was compared. Injection of SFTI-OPN (Figure 7A) or MCo-OPN (Figure 7B) over $\alpha_9\beta_1$ clearly showed binding of the grafted peptides with the immobilized integrin, whereas the corresponding scaffolds displayed no binding in the concentration range tested (Figure 7A-B). The linear OPN also appeared to show no binding under the conditions tested.
Discussion

Stimulation of blood vessel regeneration following cardiac ischemic is a promising therapeutic strategy and clinical studies have started to assess the potential of angiogenic agents.\textsuperscript{11} Although small peptide sequences have been shown to have potent angiogenic activity, these peptides are generally easily degraded \textit{in vivo}. To facilitate the delivery of bioactive peptides to specific target sites, one approach is the use of cyclic peptide scaffolds. This approach is based on the intrinsic stability of cyclic peptide scaffolds,\textsuperscript{13,40} and we have shown in the current study that grafting angiogenic sequences into cyclic peptide scaffolds significantly enhances stability, and in the case of the OPN peptide leads to a grafted peptide with nanomolar angiogenic activity.

The cyclic peptide scaffolds used in this study, MCoTI-II and SFTI-1, have significantly different structures, despite both have a cyclic backbone. The loops in the cyclic peptide scaffolds chosen for grafting were based primarily on structural considerations. Loop 6 of MCoTI-II had previously been shown to be disordered in solution\textsuperscript{21,41} and therefore it was hypothesized that it would be amenable to changes in sequence. The trypsin inhibitory loop of SFTI-1 is larger than the secondary loop and therefore was more likely to accommodate a larger bioactive sequence. Despite the differences between the scaffolds, all the grafted peptides, except MCo-QK, folded with a high yield of the native isomer.

Characterization of the grafted peptides with NMR spectroscopy was valuable for determining if the native fold had been maintained, particularly for the MCoTI-II grafted peptides, which have more complex structures than the SFTI-1 grafted peptides. Given that both MCo-LAM and MCo-OPN were able to maintain the native conformation, it is clear that loop 6 is not critical in maintaining the structure of MCoTI-II and therefore is a useful site for introducing foreign sequences into this cyclic peptide scaffold.

Analysis of the angiogenic activity of peptides using a sprouting assay with RAECs provided insights into the angiogenic responses of native, linear and grafted peptides.
From the RAECs assay, most of the grafted analogues showed significant sprouting compared to the native scaffolds and negative controls. This result shows that once the linear bioactive sequences are grafted into their corresponding cyclic scaffolds, the sequences are able to adopt a conformation in the newly constrained structure that can interact with their receptors on endothelial cells to provoke cell proliferation and movement towards the extracellular matrix. SFTI-QK was found to have a lower neovascular response than its linear counterpart and no significant difference to the negative control, which might be due to distortion of the native helical conformation of linear QK after constraining into the SFTI-1 scaffold. Furthermore, a truncated version of the original full-length sequence of QK peptide was grafted into SFTI-1 scaffold, which might account for the lower angiogenic activity than previously reported for the QK peptide alone.8

Since in vitro assays are only indicative, an in vivo CAM assay was carried out to further characterize the angiogenic potential of the grafted peptides. A clearer differentiation of the angiogenic effect was observed between the linear sequences and grafted peptides in the CAM assay compared to the sprouting assays. All grafted peptides showed a significant increase in activity compared to the linear sequences, with SFTI-OPN exhibiting the highest angiogenic activity, followed by MCo-OPN, SFTI-LAM, MCo-LAM and SFTI-QK. MCoTI-II showed a slight increase in angiogenesis activity relative to the negative control, interestingly thus indicating weak intrinsic angiogenic activity of the scaffold. There is no sequence similarity between MCoTI-II and the linear sequences to account for this activity and detailed structure activity studies would be required to dissect out the features responsible for this activity. However, the intrinsic activity is weak and grafting the OPN sequence into the MCoTI-II framework significantly enhanced the angiogenic effect. Overall, both the SFTI-1 and MCoTI-II grafted peptides were able to maintain or indeed increase the angiogenic activity of all linear sequences. Interestingly, SFTI-OPN has a disordered loop and it is possible that this disorder is related to the enhanced potency of this peptide compared to the other peptides. A similar result was observed when an anti-angiogenic peptide sequence was grafted into kalata B1. The peptide with the greatest bioactivity had significantly more structural disorder than the other grafted peptides.17
Analysis of the binding of SFTI-OPN and MCo-OPN to integrin $\alpha_9\beta_1$ using SPR confirmed that the grafted cyclic peptides bind to this receptor. Linear OPN has previously been shown to interact with integrin $\alpha_9\beta_1$ in cell based assays\(^3\) and the OPN protein shown to interact via SPR.\(^3\) Therefore, it appears that the mechanism of action is maintained in the grafted peptides. Interestingly, in the current study we found that linear OPN peptide does not bind to the integrin $\alpha_9\beta_1$ under the experimental conditions. The response in SPR is molecular mass dependent but given that the linear OPN and SFTI-OPN only differ by a factor of two in molecular mass, the lack of binding observed for linear OPN relative to the strong response observed for SFTI-OPN suggests the binding is significantly enhanced for the grafted cyclic peptide. MCo-OPN displays a slower dissociation rate than SFTI-OPN, indicating stronger binding and consequently that it might be a better candidate for drug development.

In addition to potent activity, biological and chemical stability are important in the design of novel drug leads. Plasma stability is an essential factor that affects the half-life of peptides, and therefore all the grafted peptides were tested in a serum stability assay. All the linear sequences showed improvement in stability after grafting into the cyclic scaffolds. In addition, MCo-OPN and SFTI-OPN are stable against the enzymes thrombin and MMP-9. Thus, it is possible the enhancement in angiogenic activity observed in the CAM assays might be related to this increase in stability. However, enhanced binding of SFTI-OPN and MCo-OPN to integrin $\alpha_9\beta_1$ relative to linear OPN might also contribute to the enhanced potency.

Previous studies have shown that MCoTI-II and SFTI-1 can be used to design peptides with non-native bioactivities, although until now these have been restricted to protease activities. For example, Thongyoo et al\(^4^2\) showed that the active site (loop 1) of MCoTI-II is capable of accommodating a bioactive sequence that leads to the inhibition of a protease from the Foot and Mouth Disease virus (FMDV). In addition, SFTI-1 has been modified to design a peptide that selectively inhibits human kallikrein-related peptidase 4 (KLK4) with potential applications in the treatment of prostate cancer.\(^4^3\) Overall, these peptide scaffolds have significant promise in pharmaceutical applications and the current study has expanded their potential from
simply being involved in protease interactions. Furthermore, we have shown that not only are these scaffolds useful for stabilizing bioactive sequences but that bioactivity can be enhanced as a result of being constrained in the cyclic peptide scaffolds, making this approach extremely valuable for drug design.

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Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.
Correspondence: Norelle L Daly, The University of Queensland, Institute for Molecular Bioscience, Brisbane, QLD 4072, Australia; email: n.daly@uq.edu.au
References


Table

Table 1. NMR data and refinement statistics for SFTI-OPN

<table>
<thead>
<tr>
<th>NMR constraints</th>
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<tr>
<td>Dihedral violations &gt; 3.0°</td>
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</table>

**Energies (kJ mol⁻¹)**

| Overall                                  | -379.24 ± 12.79 |
| Bomds                                    | 3.40 ± 0.56     |
| Angles                                   | 20.47 ± 3.76    |
| Improper                                 | 2.32 ± 0.57     |
| van der Waals                            | -7.75 ± 7.34    |
| NOE                                      | 7.28 ± 4.76     |
| eDIH                                     | 0.17 ± 0.13     |
| Dihedral                                 | 57.09 ± 9.47    |
| Electrostatic                            | -462.23 ± 20.13 |

**r.m.s.d.**

| Bonds (Å)                                | 0.0039 ± 0.00032 |
| Angles (°)                               | 0.58 ± 0.059     |
| Improper (°)                             | 0.35 ± 0.044     |
| NOE                                      | 0.037 ± 0.011    |
| eDIH                                     | 0.78 ± 0.29      |

**Ramachandran analysis (%) (residue 1-3, 11-14)**

| Most favoured                            | 80.0    |
| Additionally allowed                     | 20.0    |
| Generously allowed                       | 0       |
| Disallowed                               | 0       |

**Average pairwise r.m.s.d. (Å)**

| Backbone                                 | 1.59 ± 0.79 |
| Heavy atoms                              | 2.93 ± 0.99 |
| Backbone (residue 1-3, 11-14)            | 0.55 ± 0.33 |
| Heavy atoms (residues 1-3, 11-14)        | 1.44 ± 0.44 |

* Average pairwise r.m.s.d. was calculated among 15 refined structures for mean global backbone and mean global heavy atoms.
† Well defined residues were chosen for analysis.
Figure legends

Figure 1. Sequence comparison of laminin α1, osteopontin and VEGFA in various species and overview diagram of the loops where angiogenic peptides were grafted into MCoTI-II and SFTI-1 scaffolds. (A) Sequence alignment of the laminin α1, osteopontin and QK sequences from different organisms. Active angiogenesis sequences are highlighted in grey. Non-conserved residues in the active region are bolded. * indicates the QK peptide is a chemically designed peptide that mimics the sequence of VEGFA. (B) MCoTI-II (PDB ID: 1HA9) and SFTI-1 (PDB ID: 1JBL) used as scaffolds for grafting three major angiogenic sequences. Loop 6 of MCoTI-II and the trypsin inhibitor loop of SFTI-1 are the regions for introducing pro-angiogenic sequences. These loops are circled in black.

Figure 2. Sequences of the grafted peptides with their corresponding native cyclic peptide scaffolds. This diagram shows the sequences of grafted and native peptides. The highlighted region is the position where angiogenic sequences were grafted. All grafted peptides were successfully folded except the one labeled with an asterisk. Disulfide bond connectivities are I-IV, II-V, III-VI and I-II for MCoTI-II and SFTI-1 grafted peptides, respectively. The bold line indicates that all grafted and native peptides were cyclized.

Figure 3. Rat aortic endothelial cells (RAECs) assay. (A) Grafted analogues of SFTI-1 with linear LAM and linear OPN. (B) Grafted analogues of SFTI-1 with linear QK. (C) Grafted analogues of MCoTI-II with linear LAM and linear OPN. The following peptide concentrations were tested; SFTI-1, linear LAM, SFTI-LAM: 161

25
μM; linear OPN, SFTI-OPN, MCo-OPN: 5 μM; linear QK, SFTI-QK: 0.1μM; FCS only; negative control (medium only). Two asterisks indicate a significant change relative to the native cyclic scaffold; one asterisk indicates significance relative to negative control with \( P \leq .05 \) and \( n \geq 50 \). Error bars indicate the standard error of the mean.

**Figure 4. CAM assay results.** (A) Comparison of blood vessel count of the linear and MCoTI-II grafted peptides. (B) Comparison of blood vessel count of the linear and SFTI-1 grafted peptides. (C) Comparison of blood vessel count with a range of concentrations of linear OPN and SFTI-OPN. Comparison of blood vessel count of the linear OPN and SFTI-OPN Each group of test and control peptides consist are \( n = 12 \) except VEGF is \( n = 9 \) and SFTI-OPN 0.5 μg/mL is \( n = 6 \). Linear angiogenic sequences were grouped as ‘a’ and all grafted peptides were grouped as ‘b’. Different batches of *cortunix cortunix* eggs were used for this assay. The following peptide concentrations were tested; VEGF: 0.26 nM; linear LAM: 81.2 μM; linear OPN: 63.1 μM; linear OPN: 32 μM; linear OPN 3.2 μM; linear QK: 39.4 μM; MCoTI-II: 14.5 μM; MCo-LAM: 14.6 μM; MCo-OPN: 13.9 μM; SFTI-1: 33 μM; SFTI-LAM: 36.4 μM; SFTI-LAM: 3.6 μM; SFTI-OPN: 32 μM; SFTI-OPN: 3.2 μM; SFTI-OPN: 320 nM; SFTI-QK: 24.6 μM. All means are significantly different, with \( P \leq .05 \) versus control (medium) indicates with an *. Error bars indicate the standard error of the mean. A red line in each of the bar graph indicates a base line for a peptide to be considered of having angiogenic activity.

**Figure 5. Hemolytic and serum stability assay.** (A) This graph shows the percentage of peptide remaining in human serum after 24 h. (B) This graph shows the
percentage of hemolysis of peptides tested in this assay. Melittin was used as a positive control and the data represents mean ± S.D.

**Figure 6. Structure analysis of SFTI-1 and SFTI-OPN.** (A) The surface diagram, ribbon representation and 15 lowest energy structures of SFTI-OPN. (B) The surface diagram, ribbon representation and 20 lowest energy structures of native SFTI-1. Hydrophobic residues are labeled and highlighted in dark grey. These diagrams were created in MolMol.31

**Figure 7. Interaction of cyclic scaffolds versus OPN grafted peptides with immobilized α9β1 integrin.** (A) SPR sensorgrams of SFTI-1 and SFTI-OPN. (B) SPR sensorgrams of MCoTI-II and MCo-OPN. SFTI-OPN and MCo-OPN bind to the integrin in contrast to SFTI-1 and MCoTI-II.
Figures

Figure 1

A
LAMININ α1
LAMA1_HUMAN 2113 AASIKVAVSA
LAMA1_MOUSE 2121 AASIKVAVSA
OSTEOPONTIN
OSTP_HUMAN 158 GRGSVYGLRSK 170
OSTP_MOUSE 143 GRGSVAYGLRSK 160
OSTP_BOVIN*E 151 GRGSVAYGLKR 163
OSTP_RAT 142 GRGSVAYGLRSK 155
OSTP_CHICK 137 GRGSVAYGFK 149
OSTP_PIG 153 GRGSVYGLRSK 165
OSTP_RABIT 154 GRGSVAYRKS 166
OSTP_SHEEP 152 GRGSVAYGLRSK 163
VEGFA
VEGFA_HUMAN 40 VVKEMDYQRSYCHPIE 56
VEGFA_MOUSE 39 VVKEMDYQRSYCRPIE 55
VEGFA_BOVIN*E 39 VVKEMDYQRSYCRPIE 55
QK*KLTWQELYQLYKGI

B

MCot-I-II

NH₂—SIKVA—COOH LAM
NH₂—SVYYGLR—COOH OPN
NH₂—WQELYQLKY—COOH QK

SFTI-I
Figure 2

linear LAM  SIKVAV
linear OPN  SVVYGLR
linear QK   WQELYQLKY

MCcTI-II  CPKIKKKRDSDCPGACIRCNGGYCSIKVAV...
MCc-LAM   CPKIKKKRDSDCPGACIRCNGGYCSIKVAV...
MCc-OPN   CPKIKKKRDSDCPGACIRCNGGYCSUVYGLR...
MCc-QK*   CPKIKKKRDSDCPGACIRCNGGYCWQELYQLKY

SFTI-1    CTKSIPPI...CFPDGR
SFTI-LAM  CSIKVAV...CFPDGR
SFTI-OPN  CSVVYGLR...CFPDGR
SFTI-QK   CWQELYQLKY...CFPDGR
Figure 4
Figure 5
Figure 6

A
SFTI-OPN

B
SFTI-1
Figure 7

A

response (RU)

100 μM SFTI-1

100 μM SFTI-OPN

-100

100

300

500

B

response (RU)

100 μM MCoTI-II

100 μM MCo-OPN

0

100

200

300

400

500

600

time (s)
Engineering pro-angiogenic peptides using stable disulfide-rich cyclic scaffolds

Lai Y. Chan, Sunithi Gunasekera, Sonia T. Henriques, Nathalie F. Worth, Sarah-Jane Le, Richard J. Clark, Julie H. Campbell, David J. Craik and Norelle L. Daly

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