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NC-100717: A versatile RGD peptide scaffold for angiogenesis imaging

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Abstract—Targeting the molecular pathways associated with angiogenesis offers great potential in detecting disease pathology using in vivo imaging technologies. Initiation of angiogenesis requires activation and migration of endothelial cells in order for neovascularization to proceed. Endothelial cells associate with the extracellular matrix through specific interactions with a variety of cell adhesion receptors known as integrins. Peptides containing the tripeptide sequence RGD are known to bind with high affinity to the αvβ3 and αvβ5 integrins associated with angiogenesis. We present herein the synthesis and in vitro binding affinity of the RGD-containing peptide NC-100717 and a range of molecular probes derived from this intermediate.

The targeting of biological pathways using molecular imaging probes holds great promise as a means of detecting and diagnosing disease at a much earlier stage than is the norm today. Angiogenesis, the growth of new blood vessels, is a common pathology associated with a wide range of diseases requiring activation and migration of endothelial cells before neovascularization can proceed.[1] Endothelial cell interactions with the extracellular matrix are facilitated by cell adhesion receptors known as integrins.[2,3] Studies have demonstrated upregulation of αvβ3/αvβ5 integrins in the new blood vessels associated with several diseases including wound healing, tumour growth, diabetic retinopathy, and macular degeneration. Peptide ligands containing the tripeptide sequence RGD are known to possess high affinity for these integrins and several reports have been published relating to their utility in molecular imaging.[4]

In our search for a robust RGD pharmacophore we were drawn to the peptide ACDCRGDCFCG derived from a phage display library.[5] This peptide was reported to be a potent and selective binder to both αvβ3 and αvβ5 integrins. With four cysteine residues three possible disulfide isomers exist. Our initial efforts focussed on determining the binding affinities of each isomer in order to select the optimal pairing to generate further SAR. In an in vitro assay using membranes derived from the human endothelial adenocarcinoma cell line EA-Hy 926 we found that the nested configuration, comprising the Cys[2–10]; Cys[4–8] pairing, peptide **1**, had highest affinity for αvβ3/αvβ5. The observed K_i was calculated to be 1.6 nM in competition with radioactive [^{125}I]-Echistatin, an RGD-containing peptide isolated from snake venom. This configuration was therefore selected as starting point for more extensive synthetic work. The large-scale synthesis of peptides with multiple disulfide bridges however still remains a significant synthetic challenge. In addition, the possibility of disulfide scrambling led us to select a strategy directed at reducing the complexity of peptide **1** by replacing the outer disulfide bond with a chemically stable bridge. We postulated that replacement of the N-terminal Ala[1] and Cys[2] residues with the chloroacetyl moiety followed by selective cyclization with the thiol group of Cys[10] would yield a stable thioether bond with minimal disruption to the RGD pharmacophore. This was also considered a way of stabilizing the N-terminus to aminopeptidase activity in vivo. Substitution of Asp[3] with Lys introduced a side-chain amino group as an option for further derivitization with imaging reporters. Finally a short PEG-like spacer at the C-terminus had the dual function of biomodifier and contributed to further stabilize against carboxypeptidase attack.

Keywords: Angiogenesis; RGD peptide; Molecular imaging; Integrins; Targeting.
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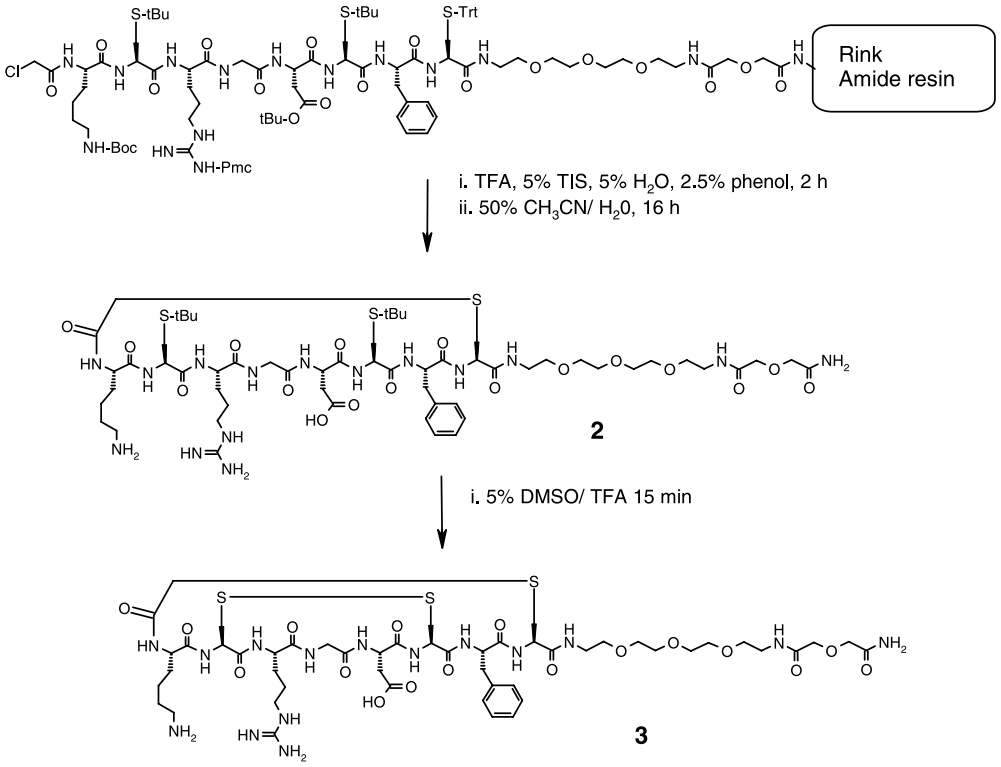
Scheme 1 shows the synthetic route chosen for the synthesis of the key intermediate NC-100717, **3**. The peptide, Lys(Boc)-Cys(*t*-Bu)-Arg(Pmc)-Gly-Asp(O-*t*-Bu)-Cys(*t*-Bu)-Phe-Cys(Trt), was assembled using Fmoc chemistry on Rink Amide AM resin (0.25 mmol scale) preloaded with 17-(Fmoc-amino)-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid using 40 min HBTU/HOBt couplings on an ABI 433A automated peptide synthesizer.[6] The amino-peptidyl resin was N-chloroacetylated by treatment with chloroacetic acid anhydride (1 mmol) in DMF for 30 min followed by extensive washing with DMF, DCM and diethyl ether. Partially protected peptide was liberated from the solid support by acidolysis (2 h) in trifluoroacetic acid (TFA) containing 5% triisopropylsilane, 5% water and 2.5% phenol. Following removal of excess TFA in vacuo the crude peptide was triturated with diethyl ether and dried.

In a typical experiment peptide **2** was obtained by dissolving 322 mg of the crude partially protected peptide in 50% acetonitrile/water (1 mg/mL) and adjusting the pH to 8 by addition of dilute ammonia solution. Thioether bridge formation was complete after 16 h at room temperature as evidenced by LC–MS. The reaction mixture was then dried by lyophilization and conversion to peptide **3** carried out directly on the crude product by addition of TFA containing 5% DMSO (3 mg/mL) with stirring for 15 min at room temperature. The excess TFA was removed in vacuo and crude final product precipitated by addition of cold diethyl ether. We have previously reported this method as a useful procedure to facilitate the rapid cleavage with simultaneous oxidation to cystine of two *t*-butyl-protected cysteine residues in peptide synthesis.[7] Purification of the final product was carried out by preparative HPLC using a Phenomenex Luna C18 250 × 50 mm column and a 0–30% water/acetonitrile gradient over 60 min at a flow rate of 50 ml/min. Fractions were analysed by LC–MS and those with a purity of >95% combined and lyophilized. 150 mg of peptide was obtained, in a yield of 52% from the crude starting product.

Molecular imaging of protein receptor systems in vivo requires the administration by intravenous injection of an imaging agent with properties that render the agent visible when placed in an imaging system. There are many types of reporter groups available to do this including fluorescent dyes, radioactive and paramagnetic metal-chelate systems and short-lived radioisotopes such as 18F-Fluorine and 11-Carbon. Versatile strategies for the conjugation of ligand to reporter in a way which does not drastically alter the binding affinity for the receptor are a prerequisite for successful targeting. In addition, the high cost of developing new pharmaceutical agents means that it is desirable to identify intermediates which can be used to produce a wide variety of imaging probes. To this end we chose the lysine residue as attachment point for the reporter group. By reaction of the ε-amino function of NC-100717 with the reporter using amide bond coupling chemistries conjugates could be prepared readily in good yield and purity.

Figure 1 shows the structures of several conjugates derived from NC-100717. Peptide **4** was formed in good yield by the reaction of **3** with the commercially available



Scheme 1. Synthesis of NC-100717 (**3**) using a 'one pot' protocol for the formation of the two bridges.

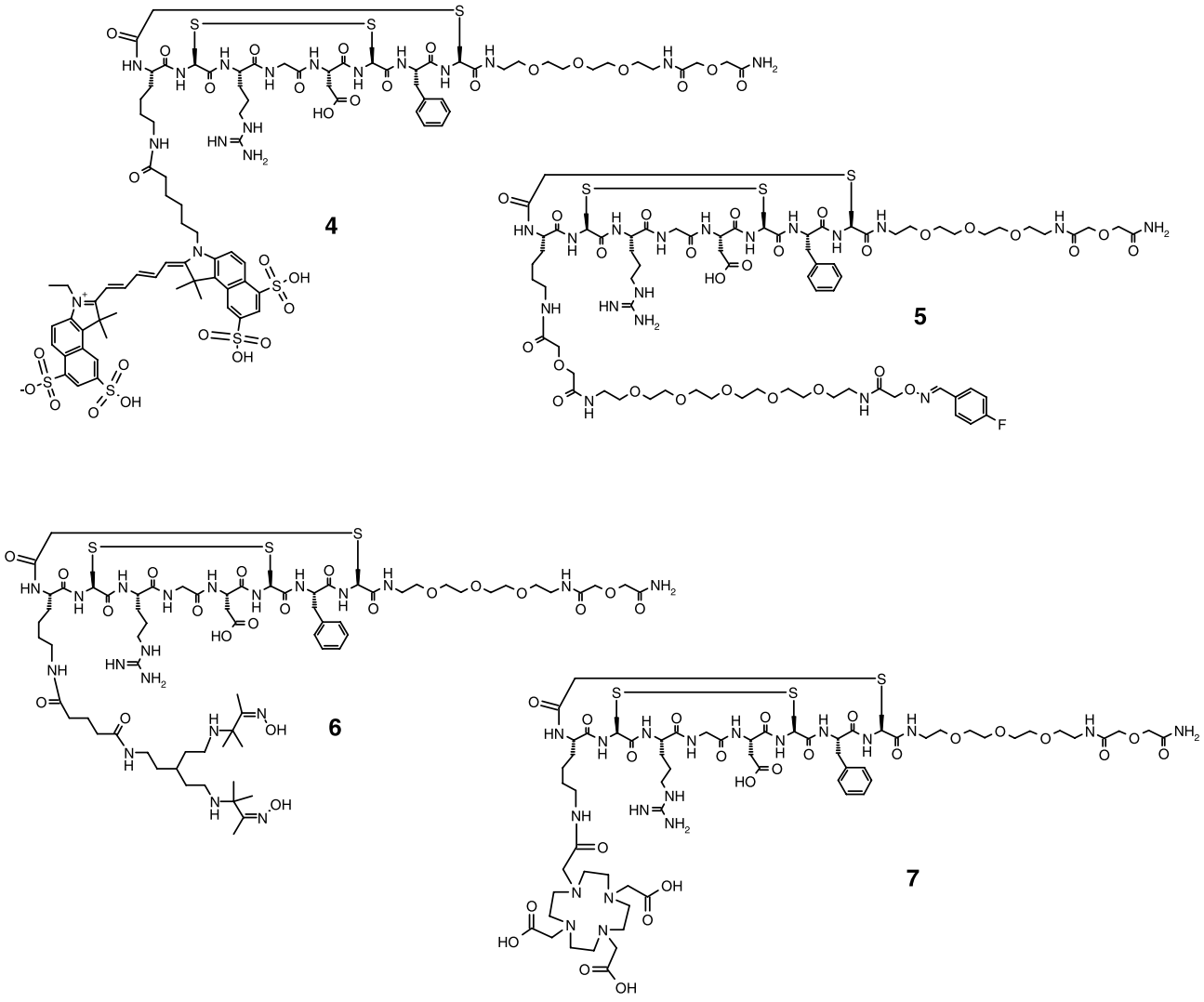


Figure 1. The structures of four imaging probes derived from the core pharmacophore NC-100717.

active ester of the cyanine dye Cy5.5.[8] The dye conjugate has been used in vivo for the fluorescent detection of Lewis Lung carcinoma in mice using an optical imaging camera system. Peptide **5** was prepared in three steps from **3** first by amide bond coupling of the (Boc-aminooxy)-PEG-diglycolic acid unit followed by Boc cleavage in TFA liberating the free aminooxy group and finally hydrazone formation at pH 3.5 following addition of 4-fluorobenzaldehyde.[9] This chemistry is particularly useful for labelling peptides with radioactive 18F-Fluorine for Positron Emission Tomography (PET) Imaging and has shown utility in vivo for angiogenesis imaging.[10] Peptide **6** was prepared from **3** by reaction with the tetrafluorophenol ester of the diamine dioxime chelate.[11] The peptide chelate conjugate was labelled with ^{99m}-Technetium for use in Single Photon Emission Computed Tomographic (SPECT) Imaging. The ^{99m}Tc complex has been used successfully for detection of malignant lesions in patients with breast cancer.[12] Finally, 9.6 mg of the *N*-hydroxysuccinimide ester of tetraazacyclododecanetetraacetic acid (DOTA) was reacted with 20 mg of **3** in 5 ml DMF for 16 h. Following evaporation of DMF in vacuo the crude product was re-dissolved in water and peptide **7** isolated by preparative HPLC. DOTA is a useful chelate for complexation of gallium-68 (68 Ga). Studies using eluate from a Germanium/Gallium generator system have demonstrated labelling yields of >90% and work is on-going to study the utility of this PET radionuclide in angiogenesis imaging. Peptides **1** and **3**–**8** were purified by HPLC and analysed by LC–MS.[13] The core peptide **3** and peptide **1** were further analysed by amino acid analysis.[14]

Table 1. In vitro assay results for compounds **1**, **3**–**8** in the αvβ3/αvβ5 EA-Hy 926 membrane assay

Peptide numbers	αvβ3/αvβ5 in vitro assay K_i, nM[a]
1	1.6 (±1.6)
3	6.8 (±0.2)
4	0.75 (±0.06)
5	8.2 (±2.4)
6	3.1 (±1.6)
7	3.2 (±2.5)
8	NDA

[a] Values are means of three experiments, standard deviation is given in parentheses; NDA, no detectable activity.

Table 1 summarizes the in vitro K_i data calculated from competition experiments with [^{125}I]-Echistatin on EA-Hy 926 membrane fractions.[15] All peptides tested had binding affinities in the low nanomolar range except for the negative control peptide **8**, a scrambled isomer prepared by replacing the RGDCF sequence of **3** with GDFCR.

In summary, we present herein a robust and flexible RGD-containing peptide NC-100717, with affinity for the integrin receptors αvβ3/αvβ5. We have demonstrated that modification of this flexible scaffold by amide bond formation at the ε-amino group of lysine has limited impact on affinity. We have also shown that a range of molecular imaging probes with application spanning several imaging modalities are readily accessible using this peptide. The ultimate goal is to develop imaging probes with clinical utility for the early detection and diagnosis of disease where angiogenesis plays a significant role.

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13. Peptides **3**–**8** were characterised by LC–MS on a Thermo Finnigan instrument using positive mode ESI and peptide **1** on a Thermo Bioanalysis Lasermat MALDI instrument. The analysis of peptides **3** and **6** was performed using a gradient of 0–30% B over 10 min on a Phenomenex Luna 5 μm, C18, 50 × 4.6 mm column at a flow rate of 2 mL/min; peptides **1**, **4** and **7** were analysed using a gradient of 10–50% B over 20 min on a Vydac218TP54, 5 μm, 250 × 4.6 mm column at a flow of 1 mL/min; peptides **5** and **8** were analysed using a gradient of 5–50% B over 10 min on Phenomenex Luna 5 μm, C18, 50 × 2 mm column at a flow of 0.3 mL/min; where A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile. Peptide **1**: t_R: 16.3 min, expected [MH$^+$]: 1148: found [MH$^+$] 1148; amino acid analysis, Ala 1.0 (1.0) Asp 2.0 (2.0), Arg 1.0 (1.0), half-cystine 3.7 (4.0), Gly 2.0 (2.0), Phe 1.0 (1.0); peptide **3**: t_R: 6.9 min, expected [MH$^+$]: 1258.5; found [MH$^+$]: 1258.8; Amino acid analysis, Asp 1.0 (1.0), Arg 1.0 (1.0), half-cystine 2.1 (2.0), Gly 1.0 (1.0), Lys 1.0 (1.0), Phe 1.0 (1.0); peptide **4**: t_R: 13.9 min, expected [MH$^+$]: 2156.7; found [MH$^+$]: 2156.4; peptide **5**: t_R: 8.4 min, expected [MH$^+$]: 1815.7; found [MH$^+$]: 1815.8; peptide **6**: t_R: 7.9 min, expected [MH$^+$]: 1697.8; found [MH$^+$]: 1698.0; peptide **7**: t_R: 9.7 min, expected [MH$^+$]: 1644.7; found [MH$^+$]: 1645.2; peptide **8**: t_R: 5.3 min, expected [MH$^+$]: 1258.5; found [MH$^+$]: 1258.6.
14. Amino acid analysis was performed by Aminosyraanalyscentralen, University of Uppsala, Sweden.
15. Membrane fractions were prepared from EA-Hy 926 cells using a Polytron homogenizer and isolation by ultracentrifugation. The observed K_d was calculated to be 0.2 nM for [^{125}I]-Echistatin. Peptides were dissolved in phosphate buffer and serial dilutions tested for competition against [^{125}I]-Echistatin. K_is were calculated from the binding curve using Prism Software.