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Synthesis of novel cyclic NGR/RGD peptide analogs via on resin click chemistry

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ABSTRACT

Targeted drug deliveries as well as high resolution imaging of cancerous tissues and organs via specific cancer cell markers have become important in chemotherapeutic interventions of cancer treatment. Short peptides such as RGD and NGR are showing promising results for targeted drug delivery and in vivo imaging. We have applied on resin Huisgen's 1,3-dipolar cycloaddition to synthesize new cyclic RGD and NGR peptide analogs. Preliminary binding assays of these new analogs by fluorescence polarization indicates specific binding to purified CD13 (Aminopeptidase N) and cell lysates from MCF-7 and SKOV-3 cancer cell lines.

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The majority of anticancer chemotherapeutic interventions suffer serious drawbacks from poor bioavailability of drugs, cell membrane impermeability, and collateral damage caused by the drugs to normal cells at therapeutic doses. Non-specific cytotoxicity can be minimized or controlled by a targeted drug delivery strategy. Development of targeted delivery of drugs depends on the identification of cellular or vascular epitopes that distinguish cancer cells or tumors from normal cells or vasculatures. Similarly, non-invasive and accurate early diagnosis or prognosis of cancerous diseases depends on a robust imaging that has a low background noise as well as high specificity.[1–3]

Cell surface biomolecules such as integrins (αvβ3, αvβ5, αvβ8, α6β4), folate receptors and CD13 (aminopeptidase N) are highly expressed in cancer cells and are involved in angiogenesis, invasion and metastasis. The differential expression of these cancer cell biomarkers make them attractive targets for delivery of drugs that can bind to these cellular biomolecules selectively.[4–8]

The identification of short peptide sequences such as Arg-Gly-Asp (RGD) and Asn-Gly-Arg (NGR) motifs from phage display peptide libraries has made important contribution in the field of targeted drug delivery and medical imaging research. These short peptide sequences are attractive because they are amenable to large scale synthesis, chemical modification and are non-immunogenic. However, linear RGD peptides are prone to protease degradation making them much less stable than their cyclic counterparts, and limiting their use in in vivo application.[9–12] It has been shown that the constraint imparted by cyclization improves the binding of cyclic peptides to their cellular targets by minimizing the entropic cost of binding.[13]

Colombo and coworkers have reported the use of dithio linked cyclic analogs of Cys-Asn-Gly-Arg-Cys and this cyclic peptide showed a tenfold increase in the targeting of tumor necrosis factor-α (TNF-α) to tumor sites over its linear analog.[14] A recent report on a cyclic analog of Lys-Asp-Gly-Arg-Glu containing an amide linkage showed about a fourfold increase in binding affinity over its open chain analog.[15] The development of robust and enzymatically stable cyclic analogs of NGR and RGD would further improve half life of these targeting agents in circulation as well as in tissues for in vivo applications.[16–19]

Here we demonstrate the synthesis of new cyclic NGR and RGD pentapeptide analogs via on resin click chemistry (Fig. 1).[20–22] These cyclic peptides contain a triazole unit that would be less likely to be attacked by hydrolytic enzymes and esterases; consequently these constructs could have important applications for in vivo targeted drug delivery and clinical imaging.

Synthesis of these analogs started from assembling the resin bound propargyl-glycine-Arg-Gly-Asp-α-azido-Lys open chain substrate **1**. However, since the α-azido lysine monomer was not commercially available, it was prepared following a literature report by azido transfer reaction.[22] Briefly, free α-amino Boc-protected ω-amino-lysine was dissolved in MeOH/water in the presence of 1 mol % $CuSO_4 \cdot 5H_2O$ and 1.5 equiv of K_2CO_3. To this solution freshly prepared trifilic azide in dichloromethane was

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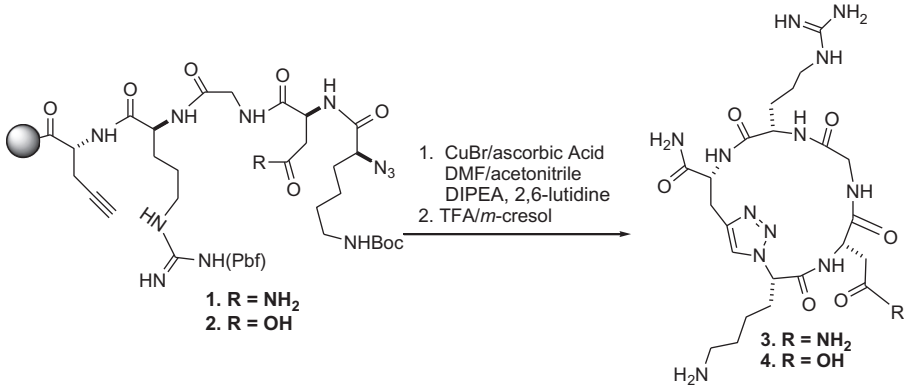

Figure 1. Azide alkyne cyclization of NGR and RGD analogs.

added and stirred at ambient temperature for 24 h. After removing the organic solvent and standard work up the α-azido lysine was obtained in 70% yield as clear oil without further purification. Commercially available *Fmoc*-D-propargylglycine (Chem-Impex Int. Cat #07424) was conjugated to a Rink amide MBHA resin (Peptide International) using a standard HBTU/HOBt activation. Sequential coupling of the Arg, Gly, Asp and α-azido-Lys, respectively, was carried out on an AB 431 peptide synthesizer. Prior to cyclization, efficiency of the synthesis was determined by cleaving an aliquot of the open chain pentapeptide from the resin and analyzing by HPLC.

Cyclization of the resin-bound substrates **1** and **2** was accomplished using a Huisgen's 1,3-dipolar cycloaddition reaction catalyzed by Cu (I) in the presence of sodium ascorbate.[23,24] The reaction was carried out at room temperature for 8 h. The desired cyclic NGR product was cleaved from the resin by the treatment of 95% trifluoroacetic acid/*m*-cresol and was purified by reverse phase HPLC. The final product was isolated in an overall yield of 22%. The reaction produced trace amounts of oligomerized side products that were not characterized further. Characterization of the major pure products was performed by 1D and 2D NMR spectroscopy as well as high resolution mass spectrometry.[24]

The success of the cyclization of the target pentapeptide **3** was confirmed by the newly generated olefinic proton signal at 7.86 ppm and olefinic carbon signal at 125.4 ppm attributed to the triazole moiety. In addition, HMBC correlations were observed between the carbon at δ_C 125.4 and to the α-lysine proton (δ_H 5.32 ppm) as well as to the propargyl methylene hydrogens (δ_H 3.06, 3.32 ppm). In the ESI/MS/MS spectrum, the parent molecular ion $(M+H)^+$ at *m*/*z* 594 gave no significant fragmentation products other than an ion at *m*/*z* = 560 suggesting the presence of the cyclic pentapeptide while the open chain gave fragment peaks consistent with sequential losses of *N*- and *C*-terminus residues. In similar fashion the new cyclic RGD analog **4** was also synthesized and characterized (see Supplementary data).[25]

Some portion of the new cyclic NGR analog **3** was reacted with Fluorescein-5(6)-carboxamidocaproic acid *N*-succinimidyl ester (Sigma Aldrich Co.) to produce a fluorescently labeled compound **5** for fluorescence polarization assay (Fig. 2). The reaction was carried out by dissolving the NHS activated FITC label in DMSO at 10 mM concentration and to this solution, the cyclic NGR peptide **3** dissolved in a buffered aqueous solution (pH 7.4) was mixed and stirred for 1 h at room temperature. After removing the organic solvent the crude product was concentrated, dissolved in water, lyophilized and purified by reverse phase HPLC. The identity of compound **5** was confirmed by HRESI/MS [$(M+H)^+$ ion at *m*/*z* 1065.4561 (Δ = 1.8 ppm)]. The preparation of such labeled analogs with various fluorescent tags and different ring sizes for near IR in vivo imaging and targeted drug delivery applications is in progress.

Our preliminary evaluation of these peptides focused on the binding of these new analogs to aminopeptidase N (ANPEP/CD13), a 150 kDa metalloprotease that is highly expressed in various tumors and angiogenic cells and has become an attractive molecular tumor marker and potential therapeutic target.[5–7]

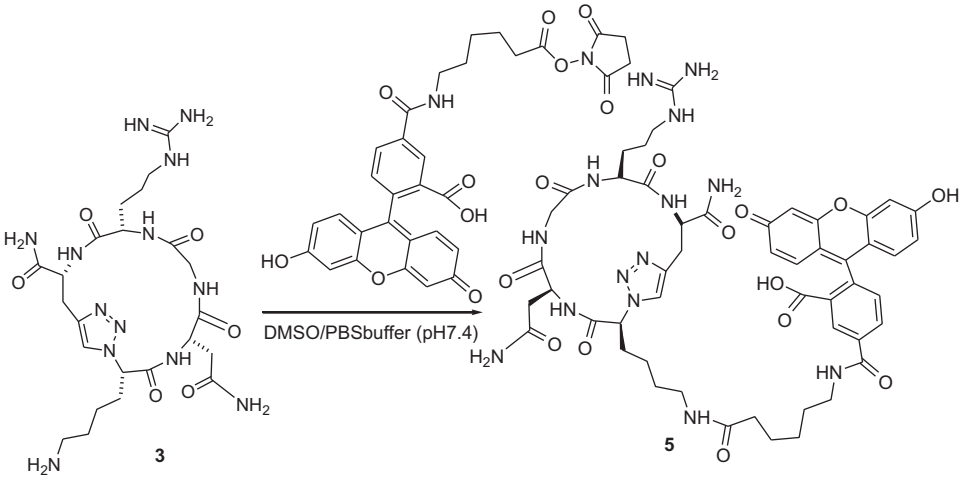

Figure 2. FITC labeled analog of the cyclic NGR.

To assess the binding and specificity of these novel NGR cyclic peptides to CD13, fluorescence polarization assay conditions were optimized with the fluorescently labeled compound **5**. The FITC labeled compound **5** was first reconstituted at various concentrations in a binding buffer (50 mM Tris, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μM $MnCl_2$, 100 mM NaCl) and its total fluorescence (RFU) was measured at 485/525 nm excitation and emission wavelengths, respectively (with a 515 cutoff). Similarly, the inherent background fluorescence polarization of the peptide without any binding protein was measured. From these two experiments, the optimal concentration of compound **5** for the polarization assays was determined to be 10 nM to give optimal signal and a background fluorescence polarization of about 40 mP (Fig. 3). Next, control experiments to measure the binding of **5** to purified CD13 (R&D Systems) were carried out. Compound **5** and CD13 were mixed in a binding buffer to a total volume of 100 μL in a 96 well plate and incubated at 37 °C for 30 min. Measurements were performed with similar conditions and settings as described above. The data shown in Figure 4 using 0, 50 and 100 nM CD13 in the presence of 10 nM of **5** shows an increase in fluorescence polarization with increasing concentrations of CD13. The increase in fluorescence polarization at 100 nM of CD13 is about 25% as compared to the blank background polarization. Encouraged by these results, we sought to evaluate the specific binding of **5** to CD13 in a complex matrix system, and designed polarization experiments using total protein lysates from two human adenocarcinomas, namely SKOV-3 (CD13 positive ovarian cancer) and MCF-7 (CD13 negative breast cancer) cell lines.

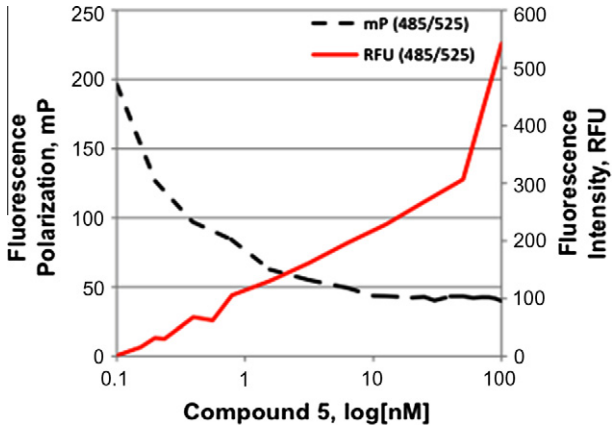

Figure 3. Concentration scouting for compound **5** in binding buffer (10 nM produces low fluorescence polarization and low total fluorescence).

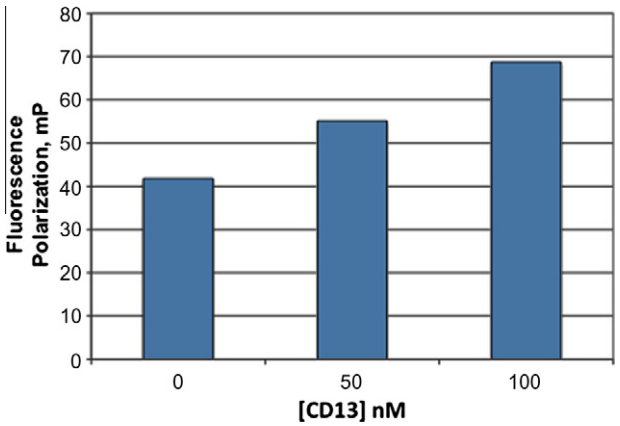

Figure 4. Fluorescence polarization of **5** (10 nM) with purified CD13.

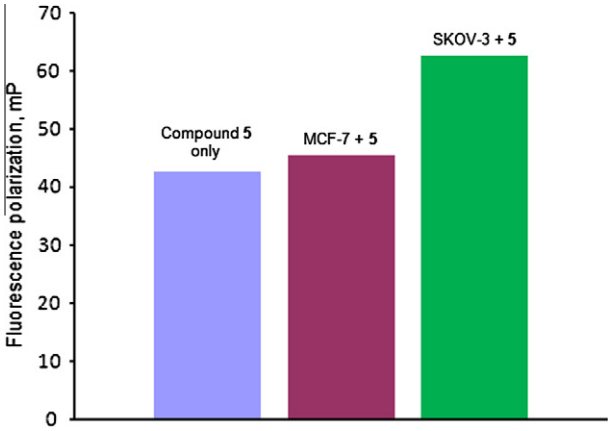

Figure 5. Fluorescence polarization of **5** (10 nM) with 100 μg total cell lysate protein.

Prior to performing the polarization assay, the expression levels of CD13 in these two cell lines were validated by Western blot analysis of protein lysates. Next, using 100 μg (as quantified by BCA assay) of total protein per sample and 10 nM of **5**, we conducted polarization assays. Gratifyingly, the lysate from SKOV-3 (CD13+) cell line gave a higher polarization (by about 17%) than the lysate from MCF-7 (CD13−) while the lysate from MCF-7 produced polarization close to the blank control (Fig. 5). The experimental result clearly distinguishes between the expressions levels of CD13 between the cell lines and suggests specificity towards CD13. However, more extensive binding studies using flow cytometry as well as cellular imaging are underway. Furthermore, the effect of the triazole ring on the conformation of these analogs is yet to be determined.

In summary, the application of click chemistry produced novel analogs of cyclic NGR and RGD peptides, and we further anticipate generating libraries of various homing peptides with various sizes and composition and evaluating their biological activities both in vitro and in vivo.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.064.

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24. *General procedure*: The open chain pentapeptide **1** was prepared by *Fmoc*-chemistry using a Rink amide MBHA resin (0.48 meq/g) at 0.1 mmol scale on the AB431 peptide synthesizer following standard protocol. *Click chemistry*: In 20 mL syringe tube fitted with a frit, a resin bound **1** was suspended and swelled in 4.5 mL of argon purged DMF. 2,6-Lutidine (0.6 mmol, 70 μL, 10 equiv) and DIPEA (0.6 mmol, 105 μL, 10 equiv) were added via syringe. A solution of sodium ascorbate (0.24 mmol, 48 mg, 4 equiv) in 5 mL DMF was added followed by addition of 1% solution of CuBr (0.09 mmol, 13 mg) in 1.5 mL acetonitrile. The reaction mixture under argon was agitated for 8 h at room temperature. The mixture was then filtered and the resin was washed three times each with water, methanol, DMF and dichloromethane. The product was cleaved from the resin by the treatment of 95% trifluoroacetic acid and 5% *m*-cresol. The precipitated crude peptide was purified by HPLC with acetonitrile/water 0.1% trifluoroacetic acid (5–60% acetonitrile gradient over 60 min). The overall isolated yield is about 22%. *Characterization data for NGR analog* **3**: ^{1}H NMR (600 MHz, D_2O, δ) 1.37 (m, 2H), 1.41 (m, 1H), 1.48 (m, 1H), 1.50 (m, 1H), 1.65 (m, 2H), 1.89 (m, 1H), 2.14 (m, 1H), 2.29 (m, 1H), 2.72 (dd, J = 8.5, 6.5 Hz, 1H), 2.89 (t, J = 6.5 Hz, 2H), 2.94 (dd, J = 5.2, 9.8 Hz, 1H), 3.06 (m, 1H), 3.09 (m, 2H), 3.32 (dd, J = 5.2, 10.4 Hz, 1H), 3.59 (d, J = 17.0 Hz, 1H), 4.01 (J = 17.6 Hz, 1H), 4.37 (dd, J = 5.0 Hz, 1H), 4.41 (dd, J = 5.9, 4.0 Hz, 1H), 4.77 (dd, J = 5.0, 5.9 Hz, 1H), 5.32 (dt, J = 6.8, 9.0 Hz, 1H), 7.86 (s, 1H), 7.82 (1H), 8.35 (1H); ^{13}C NMR (150 MHz, D_2O, δ) 23.0, 25.0, 27.2, 27.7, 28.4, 31.1, 35.5 (2C), 40.2, 41.4, 52.1(2C), 53.2, 64.2, 125.4, 144.0, 157.4, 171.7 172.8, 173.1 (2C), 175.5, 176.0; HRMS (ES+) m/z: $[M+H]^+$ calcd for $C_{23}H_{40}N_{13}O_6$: 594.3225; found 594.3225 ppm.
25. HRMS data for cyclic RGD analog **4**: (ES+) m/z: $[M+H]^+$ calcd for $C_{23}H_{39}N_{12}O_7$: 595.3065; found 595.3067, Δ = 0.3 ppm. HRMS data for FITC-RGD analog **6**: (ES+) m/z: $[M+H]^+$ calcd for $C_{44}H_{49}H_{12}O_{13}$: 953.3542; found 953.3522, Δ = −2.1 ppm.