Published in final edited form as:
Bioorg Med Chem Lett. 2012 September 1; 22(17): 5517–5522. doi:10.1016/j.bmcl.2012.07.024.

Synthesis and Characterization of $\alpha_v\beta_3$-Targeting Peptidomimetic Chelate Conjugates for PET and SPECT Imaging

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Abstract

There is growing interest in small peptidomimetic $\alpha_v\beta_3$ integrin antagonists that are readily synthesized and characterized and can be easily handled using physiological conditions. Peptidomimetic 4-[2-(3,4,5,6-tetrahydropyrimidine-2-ylamino)ethyloxy]benzoyl-2-[N-(3-amino-neopenta-1-carbamyl)]-aminoethylsulfonyl-amino-β-alanine (**IAC**) was successfully conjugated to 1-(1-carboxy-3-carbo-t-butoxypropyl)-4,7-(carbo-*tert*-butoxymethyl)-1,4,7-triazacyclononane (NODAGA(*t*Bu)$_3$) and 1-(1-carboxy-3-carbo*tert*butoxymethyl)-1,4,7,10-tetraazacyclododecane (DOTAGA(*t*Bu)$_4$) and radiolabeled with ^{111}In, ^{67}Ga and ^{203}Pb. Results of a radioimmunoassay demonstrated binding to purified $\alpha_v\beta_3$ integrin when one to four equivalents of integrin were added to the reaction. Based on this promising result, investigations are moving forward to evaluate the NODA-GA-**IAC** and DOTA-GA-**IAC** conjugates for the targeting tumor associated angiogenesis and $\alpha_v\beta_3$ integrin positive tumors to define their PET and SPECT imaging qualities as well as their potential for delivery of therapeutic radionuclides.

Keywords

Integrin $\alpha_v\beta_3$; NODAGA; DOTAGA; Peptidomimetics; antagonist; ^{68}Ga; ^{203}Pb; PET imaging; SPECT imaging

Integrins are a family of transmembrane glycoproteins with associated α and β subunits forming 25 unique heterodimers that facilitate adhesion and migration of cells on the extracellular matrix proteins found in intercellular spaces and basement membranes.[1] One of these integrins, $\alpha_v\beta_3$ integrin, interacts with vitronectin, fibronectin, fibrinogen, thrombospondin, collagen, laminin and von Willebrand factor. This integrin is over-expressed in tumor induced angiogenic vessels and in various human tumors, but is found at low levels on epithelial and endothelial cells. It is therefore a widely recognized target for the development of molecular probes for imaging angiogenesis and cancer therapy. Towards this end, the tumor imaging capability of several RGD peptides that act as $\alpha_v\beta_3$ integrin

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Supplementary data Materials and methods, general synthesis and radiolabeling conditions are provided. Supplementary data associated with this article can be found, in the online version.

antagonists has been demonstrated by several research groups. Additionally, several of these peptides have been shown to inhibit tumor angiogenesis and interrupt metastasis in many models.[2–4]

There is growing interest in peptidomimetic $\alpha_v\beta_3$ integrin antagonists composed of a stable core scaffold with basic and acidic groups that mimic the guanidine and carboxylate pharmacophore of RGD peptides. Peptidomimetics tend to have higher activity, specificity and longer duration of action compared to the peptides. One such peptidomimetic $\alpha_v\beta_3$ integrin antagonist, 4-[2-(3,4,5,6-tetrahydropyrimidine-2-ylamino)ethyloxy]benzoyl-2-aminoethylsulfonyl-amino-β-alanine (**IA**) was synthesized by Hood et al.[5] Subsequent, modification of **IA** to the corresponding carbamate derivatives by the Danthi group resulted in 4-[2-(3,4,5,6-tetrahydropyrimidine-2-ylamino)ethyloxy]benzoyl-2-[N-(3-amino-neopenta-1-carbamyl)]-aminoethylsulfonyl-amino-β-alanine (**IAC**), with a binding affinity 20 times greater than that of **IA**.[6] A SPECT (single photon emission computed tomography) imaging study with ^{111}In-DOTA-Bz-SCN- **IAC** was also performed and tumor was clearly visualized at 4 h p.i.[7]^{111}In-DOTA-Bz-SCN- **IAC** was prepared using the bifunctional chelate DOTA-Bz-SCN which differs from the DOTA-GA described in this study.

Clinically, SPECT and PET (positron emission tomography) play significant roles allowing noninvasive imaging of internal physiological and biochemical function and pathologies *in vivo*. While PET is more expensive, it has significant advantages over SPECT with respect to its ability to better quantify images. Of metallic radionuclides currently being investigated for PET applications, gallium-68 (^{68}Ga) has grown in popularity.[8, 9] The popularity of ^{68}Ga stems from the ease of on site production from a long lived generator system (^{68}Ge/^{68}Ga) rather than a cyclotron, and automation for incorporation into radiolabeled compounds.[10] The 67.7 min half-life of ^{68}Ga is an appropriate match to the biological half-lives of peptides. Gallium(III) typically binds with chelates possessing multiple anionic oxygen donors preferring a coordination number of six in an octahedral geometry. Fitting these preferences, the macrocyclic ligand, 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), is well established as forming very stable complexes with a wide variety of metals, Ga(III) being one of them.[11] Several NOTA derivatives have been reported (Figure 1) for use in the radiolabeling of proteins and peptides. Recently, Knetsch et al reported a ^{68}Ga-labeled NODA-GA-conjugated RGD peptide ([^{68}Ga]NODAGA-RGD) that showed better tumor to blood ratio *in vivo* than the corresponding [^{68}Ga]DOTA-RGD derivative.[12]

While interest in ^{68}Ga for PET imaging is currently significant, there are Pb(II) isotopes that have also been of interest for biomedical applications. Specifically, ^{203}Pb and ^{212}Pb are radiometals possessing favorable properties for use in nuclear medicine for potential diagnostic and therapeutic applications, respectively.[13–21]^{203}Lead ($t_{1/2}$ = 51.9 h) emits a γ-ray (279 keV) that is ideal for single photon emission computed tomography (SPECT) imaging and is suitable for pharmacokinetic and pharmacodynamic tracer studies. In addition, ^{203}Pb can serve as one half of a potential matched-pair of radioisotopes when combined with ^{212}Pb for therapeutic applications.[212] Lead ($t_{1/2}$ = 10.6 h) has been studied as an `*in vivo* generator' of ^{212}Bi ($t_{1/2}$ = 60 min) to overcome the short half-life of that daughter isotope. The macrocyclic polyaminocarboxylate chelate DOTA, (1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetraacetic acid labeled with ^{212}Pb provides a complex that is adequately stable *in vivo* to sequester the radionuclide.[17]

The Mäcke group in Switzerland have synthesized a DOTA derivative analogous to NOTA-GA, , 1-(1-carboxy-3-carbo*tert*butoxymethyl)-1,4,7,10-tetraazacyclododecane (DOTA-GA(*t*Bu)$_4$).[22] The DOTA-GA(*t*Bu)$_4$ affords four intact carboxylic acid functional groups with a free carboxylate side chain ready for conjugation to the *N*-terminus of peptides which makes it useful for biomedical applications.

In the present study, the objective was to move beyond the use of RGD peptides as delivery vectors to the various integrin targets and explore the utility of **IAC** for such applications. ^{68}Gallium labeling was investigated for PET applications using NODA-GA and ^{67}Ga as a surrogate for ^{68}Ga, and ^{203}Pb for SPECT imaging using DOTA-GA. To this end, **IAC** was successfully conjugated to NODAGA (Scheme 1) and DOTA-GA (Scheme 2) and the conjugates were radiolabeled with ^{111}In or ^{67}Ga for the NODA-GA conjugate and ^{203}Pb for the DOTA-GA conjugate. In brief, NODA-GA(*t*Bu)$_3$ or DOTAGA(*t*Bu)$_4$, *N*-hydroxysuccinimide, and EDC were dissolved in dichloromethane and the reaction mixture was stirred for 24 h. The mixture was extracted with saturated NaCl solution, 5% $NaHCO_3$, and saturated NaCl again. The organic layer was dried over $MgSO_4$, filtered, and dried under vacuum resulting in the formation of yellowish oils **2** or **5**. The **IAC** and **2** or **5** were combined in anhydrous DMF and diisopropylethylamine was added to the mixture which was then stirred overnight at room temperature. Reverse-phase HPLC purification followed by TFA deprotection yielded **1** or **4**, respectively.[23, 24] To evaluate the radiolabeling efficiency of the NODA-GA and DOTA-GA conjugates,^{111}In, ^{67}Ga and ^{203}Pb were employed to demonstrate facile formation of complexes with these radionuclides. The NODA-GA conjugate **1** was efficiently radiolabeled (> 90 %) with ^{111}In and ^{67}Ga within 30 min (Fig. 2A and 2B, respectively). The radiolabeling of the DOTA-GA conjugate **4** with ^{203}Pb was equally efficient (Fig. 2C). Non-radioactive Ga(III)-**1** and Pb(II)-**4** were also synthesized in order to characterize the radiolabeled ^{67}Ga and ^{203}Pb complexes.[25, 26] Figures 3 and 4 demonstrate HPLC profiles of the mixture containing both Ga(III)-**1** and ^{67}Ga-**1**; and Pb(II)-**4** and ^{203}Pb-**4**, respectively.

A radioimmunoassay was performed to assess the binding ability of the radiolabeled NODA-GA and DOTA-GA conjugates with $\alpha_v\beta_3$ integrin. The ^{111}In-labeled **1** (2×10^6 cpm, 0.47 μM), ^{67}Ga-labeled **1** (5×10^5 cpm, 0.45 μM) or ^{203}Pb-labeled **4** (3×10^5 cpm, 0.5 μM) was incubated with 0, 0.5, 1.0 and 2.0 μM of purified human $\alpha_v\beta_3$ integrin (MW 237,000) in a total volume of 25 μL PBS for 3 h at 37 °C. For non-specific binding, excess **IAC** (20 μM) was added to the reaction mixture to block binding. The reaction mixture was then separated on a 10 mL Sephadex G50 column using PBS as eluent. Fractions (0.5 mL) were collected and subsequently counted in a γ-counter. As indicated in Table 1, the labeled conjugates bound the integrin to varying degrees. The binding of ^{111}In-**1** was greatest followed by ^{67}Ga-**1** and then ^{203}Pb-**4**. In addition, binding was blocked ~95% by the addition of a 10 to 20-fold molar excess of the cold **IAC** to the reaction solution indicating specific binding of the labeled conjugates. Furthermore, it is worth noting that the reactivity of the ^{111}In-**1** with $\alpha_v\beta_3$ integrin (88 %) is higher than that reported for ^{111}In-DOTA-**IAC** (72 %).[7]

In conclusion, the peptidomimetic $\alpha_v\beta_3$ integrin antagonist (**IAC**) was conjugated to NODA-GA and DOTA-GA and successfully radiolabeled with ^{111}In, ^{67}Ga and ^{203}Pb. This promising preliminary data is fueling further investigation of NODA-GA-**IAC** and DOTA-GA-**IAC** conjugates for targeting tumor associated angiogenesis and $\alpha_v\beta_3$ integrin positive tumors using PET and SPECT imaging. Other potential applications include the use of radionuclides such as ^{90}Y, ^{177}Lu and ^{212}Pb for radiotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

References and notes

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23. ^{1}H NMR (D_2O) δ 0.90 (s, 6H), 1.82 (br, 2H), 2.00 (m, 2H), 2.39 (br t, 2H), 2.9 – 3.4 (m, 20H), 349 (m, 6H), 3.53 (m, 2H), 3.80 (m, 3H), 3.88 (br s, 2H), 4.15 (br, 2H), 4.34 (br t, J = 6.0 Hz, 1H), 6.95 (d, J = 8.1 Hz, 2H), 7.74 (d, J = 8.1 Hz, 2H). ^{13}C NMR (D_2O) δ 14.2, 19.8, 35.1, 39.0, 40.4, 51.7, 66.2, 67.1, 114.5, 121.2, 129.5, 153.8. ESI-MS: *m/z* = 943.3 for $[M + H]^+$, 472.2 for $[M + 2H]^{2+}$ (943.03 calcd. for $C_{39}H_{62}N_{10}O_{15}S$). Anal. Calcd. for $C_{39}H_{62}N_{10}O_{15}S(TFA)_2(H_2O)$: C 43.43; H 5.59; N 11.77; S 2.61; Found: C 43.56; H 5.69; N 11.08; S 2.61.
24. ESI-MS: *m/z* = 1042.3 for $[M - H]^-$ (1043.46 calcd. for $C_{43}H_{69}O_{17}S$).
25. ESI-MS: *m/z* = 1009.2 for $[M + H]^+$, 506.0 for $[M + 2H]^{2+}$ (1008.31 calcd. for $C_{39}H_{59}N_{10}O_{15}SGa$)

26. ESI-MS: m/z = 1248.3 for $[M + H]^+$ (1247.50 calcd. for $C_{43}H_{65}N_{11}O_{17}SPb$)

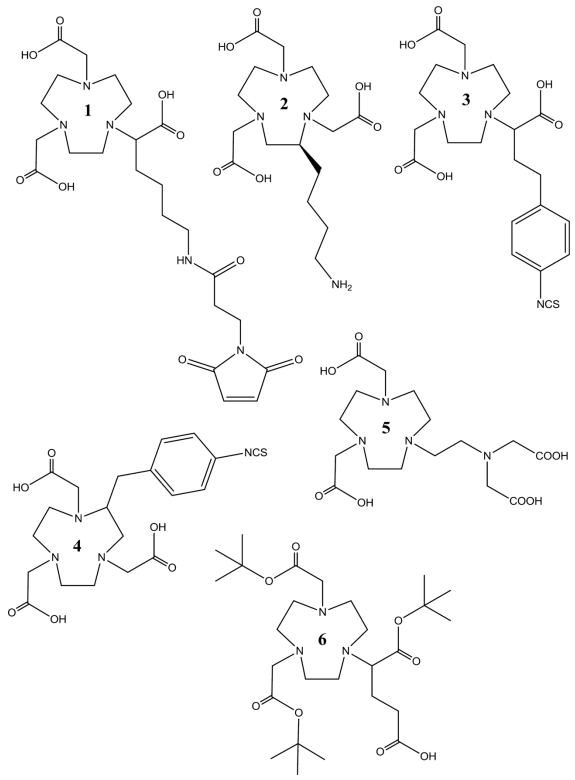


Figure 1.
Structures of NOTA derivatives. 7-(5-Maleimido-1-ethoxycarbonylphenyl)-1,4,7-triazacyclononane-1,4-diylacetic acid (**1**), 2-(4-Aminobutyl)-1,4,7-triazacyclononane-1,4,7-triyltriacetic acid (**2**), nNOTA (**3**), *p*-SCN-Bn-NOTA (**4**), NETA (**5**), and NODA-GA(tBu)$_3$ (**6**).

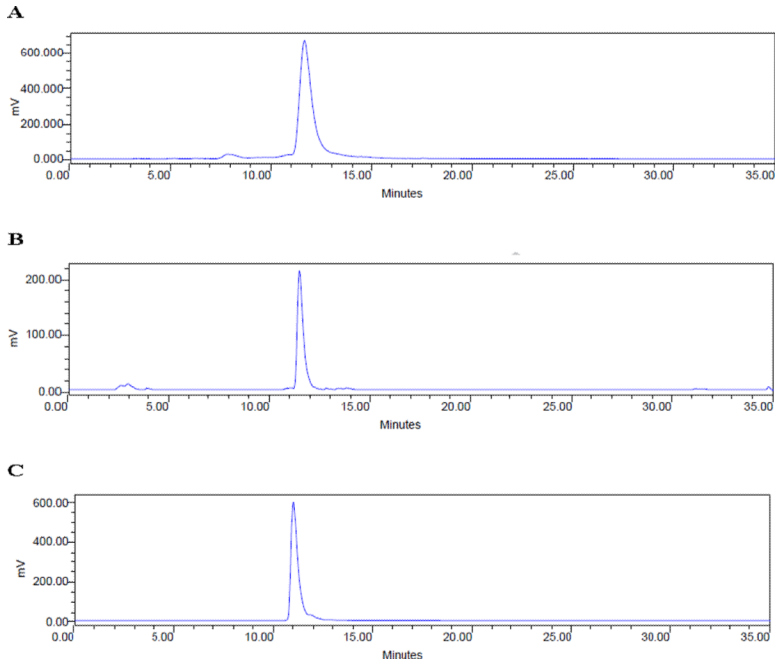


Figure 2.
Radio-HPLC profiles of ^{111}In-**1**, **A**; ^{67}Ga-**1**, **B**; and ^{203}Pb-**4**, **C** and

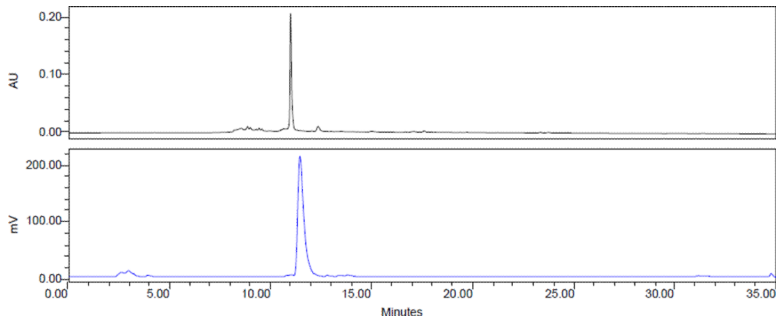


Figure 3.
HPLC profiles of Ga(III)-**1** (top) and ^{67}Ga-**1** (bottom)

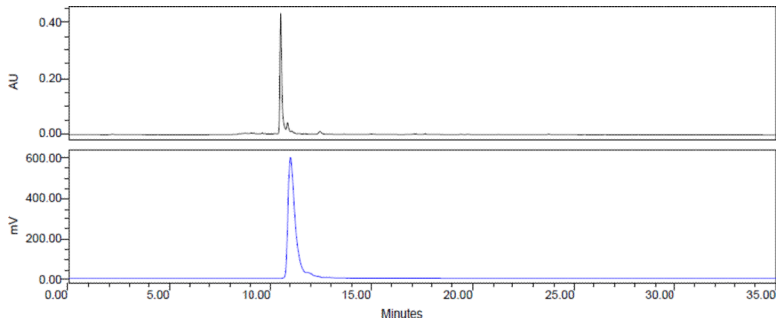
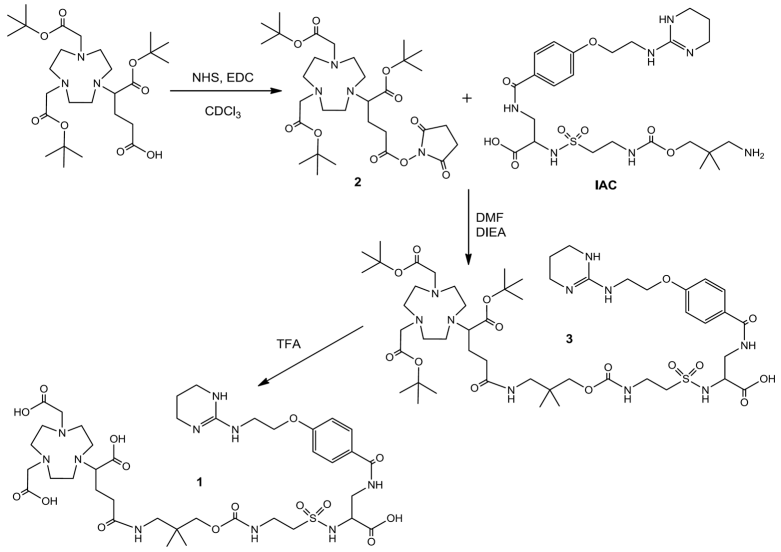
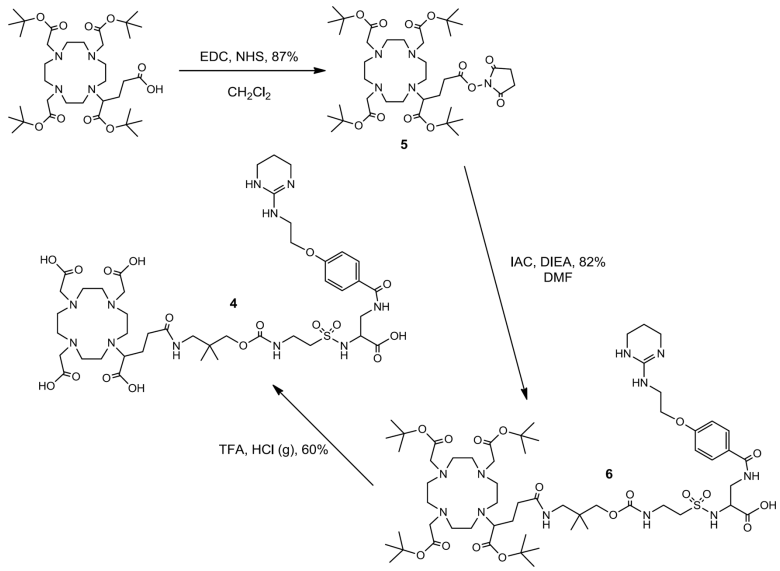


Figure 4.
HPLC profiles of Pb(II)-**4** (top) and ^{203}Pb-**4** (bottom).



Scheme 1.
Synthesis of compound **1**.



Scheme 2.
Synthesis of compound **4**.

Table 1

Binding of ^{111}In-**1**, ^{67}Ga-**1** and ^{203}Pb-**4** to purified $\alpha_v\beta_3$ integrin.

	Bound (%)
No Integrin (^{111}In-**1** Only)	5.3
0.5 μM Integrin + ^{111}In-**1** (0.47 μM)	66.0
1 μM Integrin + ^{111}In-**1** (0.47 μM)	88.1
1 μM Integrin + ^{111}In-**1** + IAC (20 μM)	5.4

	Bound (%)
No Integrin (^{67}Ga-**1** Only)	2.1
0.5 μM Integrin + ^{67}Ga-**1** (0.45 μM)	10.7
1 μM Integrin + ^{67}Ga-**1** (0.45 μM)	25.5
2 μM Integrin + ^{67}Ga-**1** (0.45 μM)	43.6
2 μM Integrin + ^{67}Ga-**1** + IAC (20 μM)	1.6

	Bound (%)
No Integrin (^{203}Pb-**4** Only)	0.3
0.5 μM Integrin + ^{203}Pb-**4** (0.5 μM)	10.9
1 μM Integrin + ^{203}Pb-**4** (0.5 μM)	20.0
2 μM Integrin + ^{203}Pb-**4** (0.5 μM)	33.4
2 μM Integrin + ^{203}Pb-**4** + IAC (20 μM)	0.3