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Synthesis and Characterization of Gadolinium - Peptidomimetic Complex as an $\alpha_v\beta_3$ Integrin Targeted MR Contrast Agent

Young-Seung Kim^a, Yang Zhou^a, Henry Bryant Jr.^b, Diane E. Milenic^a, Kwamena E. Baidoo^a, Bobbi K. Lewis^c, Joseph A. Frank^{c,d}, and Martin W. Brechbiel^{a,*}

^aRadioimmune & Inorganic Chemistry Section, ROB, NCI, NIH, Bethesda, Maryland, USA

^bLaboratory of Diagnostic Radiology Research (CC), NIH, Bethesda, Maryland, USA

^cFrank Laboratory, Radiology and Imaging Sciences, Clinical Center, NIH, Bethesda, Maryland, USA

^dNational Institute of Biomedical Imaging and Bioengineering, NIH, Bethesda, Maryland, USA

Abstract

There is growing interest in small and rigid peptidomimetic $\alpha_v\beta_3$ integrin antagonists that are readily synthesized and characterized and amenable to physiological conditions. Peptidomimetic 4-[2-(3,4,5,6-tetrahydropyrimidine-2-ylamino)ethoxy]benzoyl-2-[N-(3-amino-neopenta-1-carbamyl)]-aminoethylsulfonyl-amino- β -alanine (**IAC**) was successfully conjugated to DOTA, complexed with Gd(III) and radiolabeled with ¹⁵³Gd. Radioassay results demonstrated specificity of the labeled conjugate by blocking ~95% binding with the addition of a 50-fold molar excess of cold **IAC** to the reaction solution. Relaxometry was used to support the hypothesis that the specificity of the Gd-peptidomimetic targeting $\alpha_v\beta_3$ integrin would increase the contrast and therefore enhance the sensitivity of an MRI scan of $\alpha_v\beta_3$ integrin positive tissues. Magnetic resonance imaging of cell pellets (M21 human melanoma) was also performed, and the images clearly show that cells reacted with Gd(III)-DOTA-**IAC** display a brighter image than cells without the Gd(III)-DOTA-**IAC** contrast agent. In addition, Gd(III)-DOTA-**IAC** and **IAC**, with IC₅₀ of 300 nM and 230 nM, respectively, are 2.1 and 2.7 times more potent than c(RGDfK) whose IC₅₀ is 625 nM. This promising preliminary data fuels further investigation of DOTA-**IAC** conjugates for targeting tumor associated angiogenesis and $\alpha_v\beta_3$ integrin positive tumors using magnetic resonance imaging.

Keywords

Integrin $\alpha_v\beta_3$; Gadolinium; DOTA; Peptidomimetics; antagonist; Gd-153; MR imaging

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*To whom correspondence should be addressed: Martin W. Brechbiel, Radioimmune & Inorganic Chemistry Section, Radiation Oncology Branch, NCI, NIH, 10 Center Drive, Building 10, Rm B3B69, Bethesda, Maryland 20892-1002, USA. Phone: 301-496-0591; Fax 301-402-1923; martinwb@mail.nih.gov.

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Magnetic resonance imaging (MRI) has become a widely used imaging modality for not only diagnostic purpose, but also for biomedical research due to its non-invasive nature and higher spatial resolution at the sub-millimeter range.^{1–3} During the development of MRI, the need for enhancing contrast and increasing sensitivity of MRI rapidly became apparent.^{1–3} The increased usage and expanding applications has propelled the pursuit of developing new contrast agents to accommodate this ever diversifying usage. Gadolinium (III) complexes are routinely used in MRI to shorten longitudinal relaxation times (T1) of surrounding water molecules and rendering an increase in signal intensity in MR imaging.⁴ According to Caravan¹, a concentration of 30 μM – 125 μM of biological target is required to observe MR contrast enhancement with gadolinium-based agents in vivo, thus limiting the number of targets that can reasonably be exploited for contrast enhancement. Without the use of a contrast agent, MRI relies heavily on varying tissue density to differentiate between diseased tissues and normal tissues. However, commercially used low molecular weight extracellular contrast agents such as Magnevist suffer from rapid extravasation from blood vessels into the interstitial spaces with a concomitant rapid decrease in concentration in blood vessels, and a rapid whole body clearance. One possible solution to improve MRI for cancer diagnosis is to implement the use of a target-specific molecular contrast agent that would bind to specific receptors or cell surface antigens in order to increase sensitivity and specificity.^{5–8}

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.⁹ One of these integrins, $\alpha_v\beta_3$ integrin, interacts with vitronectin, fibronectin, fibrinogen, thrombospondin, collagen, laminin and von Willebrand factor. This integrin is normally over-expressed in tumor induced angiogenic vessels and in various human tumors.^{10–13} The $\alpha_v\beta_3$ integrin is also expressed at low levels on epithelial and endothelial cells. This $\alpha_v\beta_3$ integrin has become a widely recognized target for the development of molecular probes for imaging angiogenesis and cancer therapy. Park et al.^{14,15} has demonstrated that a gadolinium based agent clearly can enhance the contrast of the MR imaging capability while targeting the $\alpha_v\beta_3$ receptor in tumor. Towards this end, the tumor imaging capability of various RGD peptides that act as $\alpha_v\beta_3$ integrin antagonists has been demonstrated by several research groups, and many of these peptides have been shown to inhibit tumor angiogenesis and interrupt metastasis in *in vitro* and *in vivo* models.^{16–18}

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 peptides. One such peptidomimetic $\alpha_v\beta_3$ integrin antagonist, 4-[2-(3,4,5,6-tetrahydropyrimidine-2-ylamino)ethyloxy]benzoyl-2-aminoethylsulfonylethyl-L-alanine (**IA**) was synthesized by Hood et al.¹⁹ Subsequently, 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)]-aminoethylsulfonylethyl-L-alanine (**IAC**), with a binding affinity 20 times greater than that of **IA**.²⁰

The peptidomimetic $\alpha_v\beta_3$ integrin antagonist (**IAC**) has previously shown promising preliminary data for targeting tumor associated angiogenesis and $\alpha_v\beta_3$ integrin positive tumors using PET and SPECT imaging.²¹ In the present study, our objective was to move the use of **IAC** forward as a delivery vector targeting the various integrin molecules and explore the utility of **IAC** for MR imaging. To this end, **IAC** was successfully conjugated to DOTA-Bz-SCN and the subsequent gadolinium(III) (Gd(III)) complex was synthesized as well (Scheme 1). In brief, **IAC** and (*t*Bu)₄DOTA-Bz-SCN 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 DOTA-**IAC** (Fig. 1).²⁴ The conjugate was then added to the solution of Gd(III) acetate (0.1M ammonium acetate buffer, pH 5), and stirred at room temperature for 12 h.²⁵ The Gd(III) complex was separated from unreacted DOTA-**IAC** by ion-exchange HPLC (weak anion exchange, WAX). Figure 2 indicates the separation between Gd(III)-DOTA-**IAC** and DOTA-**IAC**. Gadolinium-153 radiolabeling was also conducted and the DOTA-**IAC** conjugate efficiently radiolabeled (> 90%) within 60 min (Fig. 3).

A radioassay was performed to assess the reactivity of the ¹⁵³Gd radiolabeled DOTA-**IAC** conjugate with $\alpha_v\beta_3$ integrin. This was accomplished by incubating ¹⁵³Gd-labeled DOTA-**IAC** (0.52 μ M) with 0 and 2.0 μ M of purified human $\alpha_v\beta_3$ integrin (MW 237,000) in a total volume of 25 μ L PBS for 3 h at 37 °C. Specificity of the reaction was confirmed by the addition of excess **IAC** (50 μ M) to the reaction mixture. The reaction mixture was then separated on a 10 mL Sephadex G50 column, by gravity, using PBS as eluent. Fractions (0.5 mL) were collected and subsequently counted in a γ -counter. Reactivity of the ¹⁵³Gd-DOTA-**IAC** with $\alpha_v\beta_3$ integrin was indicated by a shift in the retention time (shorter time) of the ¹⁵³Gd-DOTA-**IAC**: $\alpha_v\beta_3$ integrin complex on the sizing column. As indicated in Table 1, the labeled conjugate bound the integrin. Again, specificity of the labeled conjugate was demonstrated by blocking ~95% binding with the addition of a 50-fold molar excess of cold **IAC** to the reaction solution.

M21 human melanoma cells ($\alpha_v\beta_3$ positive) were selected to quantitate the amount of Gd(III)-DOTA-**IAC** to cells; relaxometry was used to determine the mean Gd(III) concentration per cell.²² Cells were harvested by trypsinization, washed with PBS and counted. Ten million cells, in suspension, were then incubated with various concentrations (0.58 to 9.3 mM) of Gd(III)-DOTA-**IAC** in serum-free media at 37 °C for 18 h. For comparison, another set of cells were treated with an equivalent amount of Gd(III)-DOTA. Samples were then completely digested in a mixture (500 μ L) of perchloric and nitric acid (3:1) for 3 h at 60°C using a heating block. NMRD relaxation rate 1/T1 was then measured at room temperature at 1.0 T. Gadolinium concentration in the sample was calculated from a standard curve that was derived from calibration standards of Gd(III) in the same acid mixture. The Gd(III) concentration was expressed as an average fg Gd/cell. In Table 2, Gd(III)-DOTA-**IAC** exhibits a 2-fold increase in Gd(III) content per cell compared to Gd(III)-DOTA. Such data supports the hypothesis that the specificity of the Gd-peptidomimetic targeting $\alpha_v\beta_3$ integrin would increase the contrast and therefore enhance the sensitivity of an MRI scan of $\alpha_v\beta_3$ integrin positive tissues. To this end, magnetic resonance imaging of cell pellets was also performed.

Human melanoma (M21) cells were collected and placed in 3 vials, 1×10^7 cells per vial. Two vials were incubated with Gd(III)-DOTA-**IAC** (10 mg/mL) in serum-free media at 37°C for 18 h. For the third vial, cells were incubated in serum-free media without Gd(III)-DOTA-**IAC**. After incubation, one of the treated groups was washed with PBS while the other was unwashed as a positive control. Cell pellets were kept on ice for MRI imaging. Figure 4 illustrates the efficacy of the new compound as a targeting vector of $\alpha_v\beta_3$ integrin positive tumors. The images clearly show that cells binding with Gd(III)-DOTA-**IAC** display a brighter image than cells without the Gd(III)-DOTA-**IAC** contrast agent.

To determine the binding potency of the compound, M21 human melanoma cells were used to attach to vitronectin²³ in the presence of Gd(III)-DOTA-**IAC**, **IAC** or positive control c(RGDfK). Briefly, vitronectin was diluted in Dulbecco's PBS (DPBS with Ca^{2+} , Mg^{2+}) and was coated onto 96-well flat-bottom plates (NUNC) overnight at 4°C. After aspiration of the buffer, non-specific adherence to plastic was blocked by incubation with DPBS containing 1% BSA at room temperature for 30 min. Cells were collected and washed in DPBS and re-suspended at 1×10^6 cells mL^{-1} in serum-free RPMI1640 containing 0.1% BSA. Aliquots of cells (50 μL) were added to each well containing 50 μL of serial dilution of c(RGDfK), **IAC** or Gd(III)-DOTA-**IAC** (0.04 to 5000 nM) diluted in serum-free RPMI1640 containing 0.1% BSA. The plate was incubated at 37°C for 45 min to allow the cells attach to the plate. After the incubation, medium was discarded and non-adherent cells were removed by washing the plate with DPBS (without Ca^{2+} , Mg^{2+}). The number of adherent cells was quantified using colorimetric hexosaminidase assay. As shown in Table 3, Gd(III)-DOTA-**IAC** and **IAC**, with IC₅₀ of 300 nM and 230 nM, respectively, are 2.1 and 2.7 times more potent than c(RGDfK) whose IC₅₀ is 625 nM. The inhibitory activities of Gd(III)-DOTA-**IAC** and **IAC** were comparable ($p < 0.05$).

In summary, the peptidomimetic $\alpha_v\beta_3$ integrin antagonist (**IAC**) was conjugated to DOTA-Bz-SCN and Gd(III) complex was successfully prepared. Three assay formats were utilized to demonstrate reactivity of the Gd(III)-DOTA-**IAC** with its target molecule, $\alpha_v\beta_3$ integrin. In addition, this study also shows that the target specific MR image with **IAC** is significantly more advantageous in terms of sensitivity and specificity in MR imaging. This promising preliminary data is fueling further investigation of DOTA-**IAC** conjugates for targeting tumor associated angiogenesis and $\alpha_v\beta_3$ integrin positive tumors using magnetic resonance imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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24. ESI-MS: $m/z = 1138.4$ for $[M + H]^+$ (1138.3 calcd. for $C_{48}H_{72}N_{12}O_{16}S_2$).
25. ESI-MS: $m/z = 1291.2$ for $[M + H]^+$ (1291.4 calcd. for $C_{48}H_{68}N_{12}O_{16}S_2Gd$).

Figure 1.
Reverse Phase -HPLC profile of DOTA-IAC.

Figure 2.
Ion Exchange-HPLC (WAX) profile of the mixture of Gd(III)-DOTA-IAC and DOTA-IAC.

Figure 3.
Radio-HPLC profile of ^{153}Gd -DOTA-IAC

Figure 4.
T₁-weighted MR images of M21 cells pellet incubated with Gd(III)-DOTA pellet (left),
Gd(III)-DOTA-IAC (middle), and cell DOTA-IAC (right).

Table 1Reactivity of ^{153}Gd -DOTA-IAC with purified $\alpha_v\beta_3$ integrin.

	Bound (%)
No Integrin (^{153}Gd -DOTA-IAC Only)	1.3 ± 0.2
2 μM Integrin + ^{153}Gd -DOTA-IAC (0.52 μM)	42.5 ± 5
2 μM Integrin + ^{153}Gd -DOTA-IAC + IAC (50 μM)	2.4 ± 0.6

Table 2

The number of Gd(III) per cell and molar relaxivity values.

	Molecular r_1 (mM ⁻¹ s ⁻¹)	fg Gd(III)/cell
Gd(III)-DOTA- IAC	0.79	0.48
Gd(III)-DOTA (control)	0.37	0.22

Table 3Normalized IC₅₀ (nM) data from M21 cell adhesion assay

	IC ₅₀ (nM)
c(RGDfK)	625 ± 45
IAC	230 ± 33
Gd(III)-DOTA- IAC	300 ± 28

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