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Synthesis and characterization of gadolinium—Peptidomimetic complex as an $\alpha_v\beta_3$ integrin targeted MR contrast agent

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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)ethyloxy]benzoyl-2-[*N*-(3-amino-neopenta-1-carbamyl)]-aminoethyl-sulfonyl-amino-β-alanine (**IAC**) was successfully conjugated to DOTA, complexed with Gd(III) and radiolabeled with ^{153}Gd. 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 IC_{50} of 300 nM and 230 nM, respectively, are 2.1 and 2.7 times more potent than c(RGDfK) whose IC_{50} 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.

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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 Caravan,[1] a concentration of 30–125 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

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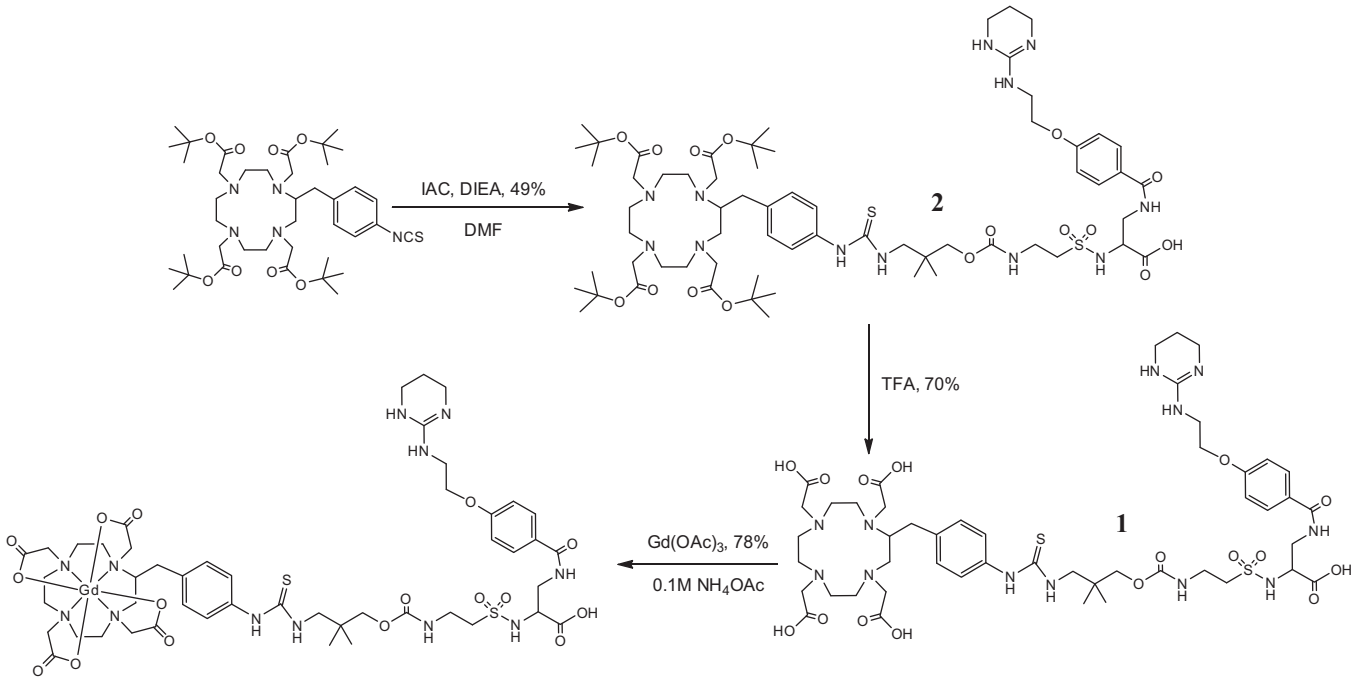
for imaging angiogenesis and cancer therapy. Park et al.[14,15] has demonstrated that a gadolinium based agent clearly can enhance the contrast of the MR imaging capability while targeting the $\alpha_v\beta_3$ receptor in tumor. Towards this end, the tumor imaging capability of various RGD peptides 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-aminoethylsulfonyl-amino-β-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)]-aminoethylsulfonyl-amino-β-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 (tBu)$_4$DOTA-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 (0.1 M 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 ^{153}Gd radiolabeled DOTA-**IAC** conjugate with $\alpha_v\beta_3$ integrin. This was accomplished by incubating ^{153}Gd-labeled DOTA-**IAC** (0.52 μM) with 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. Specificity of the reaction was confirmed by the addition of excess **IAC** (50 μM) 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 ^{153}Gd-DOTA-**IAC** with $\alpha_v\beta_3$ integrin was indicated by a shift in the retention time (shorter time) of the ^{153}Gd-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 (0.58–9.3 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 μL) 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 $\alpha_v\beta_3$



Scheme 1. Synthesis of Gd(III)-DOTA-**IAC**.