Review

Radiolabeled Cyclic RGD Peptides as Radiotracers for Imaging Tumors and Thrombosis by SPECT

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Abstract

The integrin family is a group of transmembrane glycoprotein comprised of 19 α- and 8 β-subunits that are expressed in 25 different α/β heterodimeric combinations on the cell surface. Integrins play critical roles in many physiological processes, including cell attachment, proliferation, bone remodelling, and wound healing. Integrins also contribute to pathological events such as thrombosis, atherosclerosis, tumor invasion, angiogenesis and metastasis, infection by pathogenic microorganisms, and immune dysfunction. Among 25 members of the integrin family, the αvβ3 is studied most extensively for its role of tumor growth, progression and angiogenesis. In contrast, the αIIbβ3 is expressed exclusively on platelets, facilitates the intercellular bidirectional signaling (“inside-out” and “outside-in”) and allows the aggregation of platelets during vascular injury. The αIIbβ3 plays an important role in thrombosis by its activation and binding to fibrinogen especially in arterial thrombosis due to the high blood flow rate. In the resting state, the αIIbβ3 on platelets does not bind to fibrinogen; on activation, the conformation of platelet is altered and the binding sites of αIIbβ3 are exposed for fibrinogen to crosslink platelets. Over the last two decades, integrins have been proposed as the molecular targets for diagnosis and therapy of cancer, thrombosis and other diseases. Several excellent review articles have appeared recently to cover a broad range of topics related to the integrin-targeted radiotracers and their nuclear medicine applications in tumor imaging by single photon emission computed tomography (SPECT) or a positron-emitting radionuclide for positron emission tomography (PET). This review will focus on recent developments of αvβ3-targeted radiotracers for imaging tumors and the use of αIIbβ3-targeted radiotracers for thrombosis imaging, and discuss different approaches to maximize the targeting capability of cyclic RGD peptides and improve the radiotracer excretion kinetics from non-cancerous organs. Improvement of target uptake and target-to-background ratios is critically important for target-specific radiotracers.

Key words: Integrin αvβ3; Integrin αIIbβ3; cyclic RGD peptides; tumor; thrombosis; SPECT.

1. INTRODUCTION

Radionuclide pharmaceuticals, which are also called radiotracers, are drugs containing a radionuclide. Radiotracers are used routinely in nuclear medicine for diagnosis or therapy of diseases, such as cancer, inflammation and myocardial infarction [1-6]. Radiotracers can be classified according to the biodistribution characteristics: those whose biodistribution is determined exclusively by their chemical and physical properties; and those whose biological properties are determined by the receptor binding capability of
radiolabeled biomolecules. The latter class is often called target-specific radiotracers [3, 4]. Diagnostic radiotracers are molecules labeled with either a γ-emitting isotope for single photon emission computed tomography (SPECT) or a positron-emitting radionuclide for positron emission tomography (PET), and provide a method of assessing the disease or disease states by SPECT or PET. They are also useful for monitoring the treatment efficacy of a specific therapeutic regimen in a noninvasive fashion.

![Figure 1](http://www.thno.org)

**Figure 1.** Schematic presentation of the target-specific radiotracer. Radionuclide is the radiation source. BM is the targeting biomolecule for receptor binding. A multidentate bifunctional chelator is used for chelation of metallic radionuclides. A spacer is used to bridge the radiometal chelate and targeting biomolecule.

Fig. 1 shows the schematic illustration of the target-specific radiotracers, which are often radio-metal complexes of a chelator-biomolecule conjugate. In some cases, they can be biomolecules attached with a non-metallic radionuclide, such as 18F and 125I. A target-specific radiotracer is based on the receptor binding of the radiolabeled receptor ligand in the diseased tissue [7-20]. The metal-containing target-specific radiotracer can be divided into four parts: targeting biomolecule (BM), spacer, bifunctional chelating agent (BFC), and radionuclide. The targeting biomolecule serves as a “carrier” for target-specific delivery of radionuclide to the diseased tissue with many targeted receptors. The radiolabeled receptor ligand binds to these receptors with high affinity and specificity, resulting in selective uptake of the radiotracer. The choice of a radionuclide depends on the clinical utility of the radiotracer. Table 1 lists several selected radionuclides useful for planar imaging and SPECT, along with their nuclear characteristics. For SPECT, more than 80% of radiotracers used in nuclear medicine departments are 99mTc compounds mainly due to the optimal nuclear properties of 99mTc and its easy availability at low cost [1-5]. The 6 h half-life is long enough to allow radiopharmacists to carry out radiosynthesis and for physicians to collect clinically useful images. It is also short enough to permit administration of 20 – 30 mCi of 99mTc radiotracer without imposing a significant radiation dose to the patient. 111In is also widely used in gamma scintigraphy (only second to 99mTc in clinical applications). It decays by electron capture and emits two γ-photons of 173 and 247 keV (90% and 94% abundance, respectively). 111In radiotracers are often used as the imaging surrogates for biodistribution and dosimetry determination of their corresponding therapeutic 90Y analogs, which might be useful for treatment of cancer. 67Ga is a cyclotron-produced radionuclide, and has a half-life of 78 h. 67Ga has little use in the development of target-specific radiotracers since 68Ga radiotracers offer significant advantages because of the high spatial resolution of PET as compared to that of SPECT. Due to the low solution stability of 201Tl(I) complexes, 201Tl is used exclusively as its chloride salt for myocardial perfusion imaging in the patients with cardiovascular diseases.

| Radionuclide | Half-life | Mode of decay | Principal γ emission in keV (% abundance) |
|--------------|-----------|---------------|-----------------------------------------|
| 99mTc        | 6.01 h    | γ             | 140.5 (87.2)                            |
| 123I         | 13.27 h   | EC            | 159.0 (83.3)                            |
| 131I         | 8.02 d    | β- & γ        | 364.5 (81.2)                            |
| 67Ga         | 3.261 d   | EC            | 93.3 (37.0), 184.6 (20.4)               |
| 111In        | 2.805 d   | EC            | 171.3 (90.2), 245.4 (94.0)              |
| 201Tl        | 3.038 d   | EC            | 167.4 (9.4)                             |

Nuclear imaging techniques are widely used for clinical applications because of their high sensitivity. Nuclear imaging modalities (PET and SPECT) are able to determine concentrations of specific molecules in the human body in the picomolar range and provide enough sensitivity needed to visualize most interactions between physiological targets and receptor ligands. Many biomolecules (monoclonal antibodies, peptides, or non-peptide receptor ligands) have been successfully used for target-specific delivery of radionuclides. Among them, small peptides with less than 30 amino acids or molecular weight less than 3500 Daltons are of particular interest. Compared to monoclonal antibodies and antibody fragments, small peptides offer several advantages. Peptides are necessary elements in more fundamental biological processes than any other class of molecule. They can also
tolerate harsher conditions for chemical modification or radiolabeling. Small peptides are easy to synthesize and modify, less likely to be immunogenic, and can have rapid blood clearance. The faster blood clearance results in adequate T/B ratios earlier so that it is practical to use $^{99m}$Tc, which is the preferred radionuclide for diagnostic nuclear medicine. In most cases, the primary sites of interactions of peptides are receptors on the outer surface of cell membranes (extracellular). All these factors make small bioactive peptides excellent candidates for development of target-specific radiotracers. The peptide-based radiotracers have been reviewed extensively [7-20].

The integrin family is comprised of 25 identified members, which are heterodimers of 19 α- and 8 β-subunits imbedded non-covalently into the cell membrane [21]. The member of this family is still expanding as observed from human genome studies [22]. The cell-cell and cell-matrix adhesion processes through binding of integrins to their ligands play critical roles in physiological processes, including cell attachment, proliferation [23-25], bone remodeling [26], and wound healing [27]. Besides, integrins also contribute to pathological events such as thrombosis, atherosclerosis [28, 29], tumor invasion, angiogenesis and metastasis [30-33], infection by pathogenic microorganisms [34, 35], and immune dysfunction [36]. Therefore, the integrins have been proposed as the molecular targets for the treatment of cancer [37-42], thrombosis [43, 44] and other diseases [45, 46] in the last two decades. The role of integrins has been reviewed extensively [21, 47-50].

Many integrin family members are crucial to the initiation, progression and metastasis of solid tumors. Epithelial-derived tumor cells generally retain integrins expressed by epithelial cells including α5β1, α6β1, αvβ5, αvβ6, and αvβ3, and mediate the adhesion, migration, proliferation and survival of tumor cells. Different integrins can promote or suppress the tumor development. For example, integrin αvβ3 is down-regulated in tumor cells, the phenomenon associated with increased tumor cell dissemination [51]. This suggests that αvβ3 could function as a tumor suppressor [52]. On the other hand, the expression of αvβ3, αvβ5, αvβ6, αvβ1, and αvβ5 on tumor cells is correlated with disease progression in various tumor types [53-58]. More importantly, the expression of integrins αvβ5, αvβ1 and αvβ6 are usually at low or undetectable levels in most adult epithelia. Among 25 members of the integrin family, integrin αvβ3 is studied most extensively for its role in the tumor growth and angiogenesis. While the αvβ3 plays pivotal role in the tumor growth and progression, the αvβ3 is critical for platelet aggregation during thrombosis. It is believed that the interaction between the tumor αvβ3 and platelet αvβ3 is also related to the increased tumor metastasis via a bridge such as fibrinogen, von Willebrand factor or thrombospondin [59]. This interaction is believed to facilitate the tumor cell adhesion to the vasculature, and often leads to metastasis to various secondary sites, including bone marrow [60].

Integrin αvβ3 is exclusively expressed on platelets, although α5β1, α6β1, αvβ1 and αvβ1 can also mediate platelet adhesion functions [61]. On the surface of platelet, there are 70–90 thousand copies of αvβ3, which facilitate the intercellular bidirectional signaling (“inside-out” and “outside-in”) and allow the aggregation of platelets during the vascular injury. The αvβ3 plays an important role in thrombosis formation by its activation and binding to fibrinogen especially in arterial thrombi due to the high blood flow rate. In the resting state, the αvβ3 on platelets does not bind to fibrinogen. On activation, the conformation of platelet is altered and the binding sites of αvβ3 are exposed for fibrinogen to crosslink with the activated platelets. Integrin αvβ3 antagonists have been widely used in the antithrombotic therapy in the patients with percutaneous coronary interventions and unstable angina [47, 48, 62-65].

The αvβ3 and αvβ3 receptor ligands share a common RGD tripeptide binding sequence. Generally, linear RGD peptides, such as GRGDS (Gly-Arg-Gly-Asp-Ser), often have low affinity (IC50 > 100 nM) and selectivity for αvβ3 and αvβ3 [66], and undergo rapid degradation in serum by a variety of proteases [67, 68]. It has been shown that cyclization of RGD peptides via linkers, such as S-S disulfide, thioether and rigid aromatic rings, often leads to the increased receptor binding affinity and selectivity [67-77]. It has been reported that the αvβ3 is less sensitive to variations in the RGD backbone structure and can accommodate a larger distance or spacer than αvβ3 and αvβ3 [66]. On the basis of extensive structure-activity-relationship studies, it was found that incorporation of the RGD unit into a cyclic the pentapeptide framework (Fig. 2: top) increases binding affinity and selectivity for αvβ3 over αvβ3 [66, 68-77], while addition of a rigid aromatic ring (Fig. 2: DMP728 and DMP757) into the cyclic hexapeptide structure enhance the receptor binding affinity and selectivity for αvβ3 over both αvβ3 and αvβ3 [66, 79, 80]. It was also found that the valine residue in c(RGDfV) could be readily replaced by lysine (K) or glutamic acid (E) to afford c(RGDfK) or c(RGDfE), without significantly changing the αvβ3 binding affinity [69-71]. Similar behavior was also seen for αvβ3-selective hexapeptides [66].
Figure 2. Examples of monomeric cyclic RGD peptides. Incorporation of the RGD sequence into a cyclic pentapeptide framework increases the binding affinity and selectivity for αvβ3 over αvβ5 and αIIbβ3, while the addition of one or two rigid aromatic rings into cyclic hexapeptide structure enhance the binding affinity and selectivity for the αIIbβ3 over αvβ3 and αvβ5.

Several excellent review articles have appeared recently to cover a broad range of topics related to integrin-targeted radiotracers and their nuclear medicine applications in tumor imaging by SPECT and PET [81-97]. This review is not intended to be an exhaustive review on all radiolabeled cyclic RGD peptides. Instead, it will focus on recent development of αvβ3-targeted SPECT radiotracers for imaging tumor angiogenesis and the use of the αIIbβ3-targeted radiotracers for thrombosis imaging by SPECT. Because of the limited space, authors would apologize to those whose work has not been presented in detail, and for the omission of 123I-labeled cyclic RGD peptides as radiotracers in this review.

2. αvβ3–Targeted Radiotracers for Tumor Imaging

Integrin αvβ3 and tumor angiogenesis. Tumor cells produce many angiogenic factors, which are able to activate endothelial cells on the established blood vessels and induce endothelial proliferation, migration, and new vessel formation (angiogenesis) through a series of sequential but partially overlapping steps [98-103]. Angiogenesis is a key requirement for both the tumor growth and metastasis. Without the formation of the new blood vessels which provide oxygen and nutrients, tumors cannot grow beyond 1–2 mm in size [98, 103]. Angiogenesis is regulated by many proteins, such as vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptors (VEGFR), G-protein coupled receptors for angiogenesis modulating proteins, endogenous angiogenesis inhibitors and integrins [102-105]. Among the angiogenesis factors, integrins are responsible for the cellular adhesion to extracellular matrix proteins in the intercellular spaces and basement membranes and subsequent migration of cells, and regulate cellular entry and withdraw from cell cycle [100, 107-110]. Among the integrins identified so far, the αvβ3 is studied most extensively since serves as a receptor for a variety of extracellular matrix proteins with the exposed RGD tripeptide sequence. These
include vitronectin, fibronectin, fibrinogen, laminin, collagen, von Willebrand factor, and osteopontin [111-119]. The \( \alpha_v\beta_3 \) is usually expressed in relatively low levels on epithelial cells and mature endothelial cells, but is highly expressed in the tumors including osteosarcomas, neuroblastosmas, glioblastomas, melanomas, breast, lung and prostate carcinomas [112-120]. Recently, it has been reported that the \( \alpha_v\beta_3 \) is overexpressed on not only tumor cells but also endothelial cells of the tumor neovascularity [121]. The \( \alpha_v\beta_3 \) expressed on the activated endothelial cells can modulate cell adhesion and migration during tumor angiogenesis, and its expression on carcinoma cells potentiates metastasis by facilitating invasion and movement of tumor cells across blood vessels [121]. It has also been demonstrated that the \( \alpha_v\beta_3 \) expression level correlates well with the potential for metastasis and the aggressiveness of many tumors including glioblastomas, melanoma, ovarian, breast and lung cancers [113, 119-121]. Therefore, the \( \alpha_v\beta_3 \) has been identified as an interesting molecular target for the early diagnosis of rapidly growing and metastatic tumors [81-97].

Integrin \( \alpha_v\beta_3 \)-targeted radiotracers under clinical investigation. Many radiolabeled cyclic RGD peptides have been evaluated as the \( \alpha_v\beta_3 \)-targeted radiotracers [122-158]. Significant progress has been made on their use in tumor imaging by either SPECT or PET. Among the radiotracers evaluated in many preclinical tumor-bearing animal models, \([^{18}\text{F}]\text{Galacto-RGD (Fig. 3: top) and }[^{18}\text{F}]\text{AH111585 (Fig. 3: middle) are currently under clinical investigation for non-invasive imaging of the } \alpha_v\beta_3 \text{ expression in cancer patients [159-164]. Imaging studies clearly showed that the accumulation of }[^{18}\text{F}]\text{labeled RGD peptide radiotracers correlated well with the tumor } \alpha_v\beta_3 \text{ expression levels in cancer patients [159-164]. However, their relatively low tumor uptake, high cost and lack of preparative modules for routine radiosynthesis will limit their continued clinical utilities. In addition, several steps of manual radiosynthesis and post-labeling purification can cause significant radiation exposure to radio-pharmacists in the clinics. }[^{99}\text{Tc-NC100692 (Fig. 3: bottom) is a }[^{99}\text{Tc]-labeled cyclic RGD peptide monomer reportedly to have high integrin } \alpha_v\beta_3 \text{ binding affinity [165]. In breast cancer patients, 19 of 22 malignant lesions (86%) were detected by SPECT [165]. However, its intensive liver uptake and hepatobiliary excretion due to its lipophilic Tc-chelate (Fig. 3) will limit its continued clinical applications. Thus, there is a continuing need for more efficient } \alpha_v\beta_3 \text{-specific }[^{99}\text{Tc]-radiotracers that can be readily prepared from a kit formulation at low cost.}

\textbf{Multimer concept.} Since interactions between the \( \alpha_v\beta_3 \) and RGD-containing proteins (e.g. vitronectin, fibronectin and fibrinogen) may involve multiple binding sites, the idea to use multimeric cyclic RGD peptides might provide more effective \( \alpha_v\beta_3 \) antagonists with tumor targeting capability and hence higher cellular uptake for their corresponding radiotracers [166]. Multivalent interactions are used in such a way that weak ligand-receptor interactions may become biologically relevant. The multimer concept has been used for enhancing the radiotracer tumor-targeting capability. For example, biodistribution studies showed that the divalent \( ^{99}\text{Tc-[sc(Fv)]}_2 \) had approximately 3-fold higher tumor uptake than \( ^{99}\text{Tc-sv(Fv)}_2 \) [167]. The increased binding affinity and tumor targeting capability were also reported for the \( ^{125}\text{Tl}-\text{labeled divalent recombinant antibody fragment [168].}

\textbf{Multimeric cyclic RGD peptides.} To improve \( \alpha_v\beta_3 \) binding affinity, dimeric RGD peptides, such as \( \text{E[c(RGDfK)]}_2 \) (Fig. 4: RGD\(_2\)), have been used to develop the \( \alpha_v\beta_3 \)-targeted radiotracers. Rajopadhye et al were the first to use \( \text{E[c(RGDfK)]}_2 \) to develop diagnostic \( ^{99}\text{Tc and }^{64}\text{Cu and therapeutic (}^{90}\text{Y and }^{177}\text{Lu) radiotracers [146-157, 169, 170]. Dijkstra et al found that the tumor uptake of }^{111}\text{In-labeled E[c(RGDfK)]}_2 \text{ was >2x of that for its corresponding monomeric analog in athymic mice with xenografted SK-RC-52 tumors [154]. The same group also reported the DOTA-conjugated cyclic RGD dimers and tetrarsers [154, 155], but no in vivo data was presented. Recently, Chen and coworkers reported }^{64}\text{Cu and }^{18}\text{F-labeled E[c(RGDyK)]}_2 \text{ as PET radiotracers [140, 141]. Poethko et al also found that the RGDfE dimer [c(RGDfE)-HEG]_2-K (Fig. 4) had much better targeting capability than the monomer c(RGDfE)-HEG [128-130]. The multimer concept was also used to prepare cyclic RGD tetrarsers [142, 144, 153, 155, 171-173] and octamers [173]. For example, Botury et al reported a series of cyclic RGDfK tetrarsers [172], and found that increasing the peptide multiplicity significantly enhanced the \( \alpha_v\beta_3 \) binding affinity and internalization. Kessler et al reported a cyclic RGDfE tetramer (Fig. 5) that had better \( \alpha_v\beta_3 \) binding affinity than its corresponding dimer counterpart [128-130]. Liu et al used \( \text{E[E[c(RGDfK)]}_2) \) (Fig. 5: RGD\(_4\)) for the development of \( \alpha_v\beta_3 \)-targeted diagnostic \( ^{99}\text{Tc and }^{64}\text{Cu} \text{ radiotracers [142, 153]. Chen et al also reported the use of }^{64}\text{Cu and }^{18}\text{F-labeled cyclic RGD peptide tetramer E[E[c(RGDyK)]}_2) \) and octamer E[E[E[c(RGDyK)]}_2) for tumor imaging by PET [173]. Both the in vitro assays and the ex vivo biodistribution studies showed that the radiolabeled multimeric cyclic RGD peptides had better tumor uptake with}
longer tumor retention time than their dimeric analogs. However, their T/B ratios were not substantially better due to their high uptake in the normal organs [173]. It remains unclear if the multimeric cyclic RGD peptides, such as \(E[E[c(RGDyK)]_2]_2\), are really multivalent. Moreover, the cost for synthesis of the RGD octamer \(E[E[c(RGDyK)]_2]_2\) is prohibitively high for future development of the \(\alpha_v\beta_3\)-targeted diagnostic radiotracers. Thus, an alternate approach is needed to improve the \(\alpha_v\beta_3\)-targeting capability of the radiotracer and minimize its accumulation in normal organs.

**Figure 3.** Examples of radiolabeled cyclic RGD peptide monomers as radiotracers (\([^{18}F]\)Galacto-RGD, \([^{18}F]\)AH11585 and \(99m\)Tc-NC100692) for imaging tumor angiogenesis. They are currently under clinical investigation for noninvasive visualization of the \(\alpha_v\beta_3\) expression in cancer patients.
Figure 4. Examples of RGD dimers \( E[c(RGDfK)]_2 \), \( E[c(RGDyK)]_2 \) and \( [c(RGDfE)HEG]_2-K \) for \( \alpha_v\beta_3 \)-targeting.

Figure 5. Structure of a cyclic RGD tetramer \( [[c(RGDfE)HEG]_2K]_2-K \).
Improve $\alpha_\beta_3$ binding affinity via bivalency. Fig. 6 illustrates the interaction between $\alpha_\beta_3$ and a cyclic RGD tetramer. The target moiety is c(RGDfK). The spacer is glutamic acid (E) or its derivatives. Two factors contribute to the high $\alpha_\beta_3$ binding affinity of multimeric cyclic RGD peptides: bivalency and the enhanced local RGD concentration. The key for bivalency is the distance between two adjacent cyclic RGD motifs. If this distance is long enough for simultaneous $\alpha_\beta_3$ binding, the cyclic RGD multimer will bind to $\alpha_\beta_3$ in a bivalent fashion. If this distance is too short, the local cyclic RGD peptide concentration is still “enriched” in the vicinity of neighboring $\alpha_\beta_3$ sites once the first RGD motif is bound. The combination of simultaneous $\alpha_\beta_3$ binding (bivalency factor) and the locally enriched RGD concentration (concentration factor) will result in higher $\alpha_\beta_3$ binding affinity for cyclic RGD multimers and better tumor uptake with longer tumor retention for their corresponding radiotracers.

To demonstrate the proof-of-principle for the bivalency concept, Shi et al recently reported a series of cyclic RGD dimers (Fig. 7) with G3 (Gly-Gly-Gly) and PEG4 (15-amino-4,7,10,13-tetraoxapentadecanoic acid) linkers [174-181]. The G3 and PEG4 linkers were used to increase the distance between two RGD motifs from 6 bonds in RGD2 to 24 bonds in 3G-RGD2 and 38 bonds in 3P-RGD2 [174, 175]. The $\alpha_\beta_3$ binding affinities (Table 2) against $^{125}$I-echistatin bound to U87MG human glioma cells follow the order of HYNIC-RGD1 (IC$_{50}$ = 7 ± 2 nM) > HYNIC-2P-RGD2 (IC$_{50}$ = 52 ± 7 nM) > HYNIC-3P-RGD2 (IC$_{50}$ = 60 ± 4 nM) > HYNIC-3G-RGD2 (IC$_{50}$ = 61 ± 2 nM) > HYNIC-P-RGD2 (IC$_{50}$ = 84 ± 7 nM) > HYNIC-RGD2 (IC$_{50}$ = 112 ± 21 nM) > HYNIC-G-RGD (IC$_{50}$ = 358 ± 8 nM) > HYNIC-P-RGD (IC$_{50}$ = 452 ± 11 nM). A similar trend was observed for their DOTA-conjugates against $^{125}$I-c(RGDyK) bound to U87MG glioma cells [176]: DOTA-RGD1 (IC$_{50}$ = 1.3 ± 0.3 nM) ~ DOTA-3P-RGD2 (IC$_{50}$ = 1.1 ± 0.2 nM) > DOTAP-RGD2 (IC$_{50}$ = 8.0 ± 2.8 nM) > DOTAP-P-RGD (IC$_{50}$ = 42.1 ± 3.5 nM) ~ c(RGDfK) (IC$_{50}$ = 38.5 ± 4.5 nM) > DOTA-3P-RGK2 (IC$_{50}$ = 452 ± 11 nM). These data suggest that the G3 and PEG4 linkers between two RGD motifs are responsible for the improved $\alpha_\beta_3$ binding affinity of HYNIC-3P-RGD2 and HYNIC-3G-RGD2 as compared to HYNIC-P-RGD2 [174, 175]. The higher $\alpha_\beta_3$ binding affinity of HYNIC-RGD4 is likely due to the presence of its two extra RGD motifs in RGD4 as compared to those in HYNIC-3P-RGD2 and HYNIC-3G-RGD2 [174].

It is important to note that the IC$_{50}$ values of cyclic RGD peptides are largely dependent on the type of assay (the immobilized $\alpha_\beta_3$-binding assay vs whole-cell $\alpha_\beta_3$ competition assay), the radioligand ($^{125}$I-c(RGDyK) vs $^{125}$I-echistatin) and tumor cell lines (U87MG vs MDA-MB-435). Caution should be taken when comparing their IC$_{50}$ values. Whenever possible, a “control compound”, such as c(RGDfK) and c(RGDyK), should be used in each experiment. In addition, the IC$_{50}$ values obtained from the in vitro assays cannot be used as the “absolute proof” to support the concept of bivalency. They must be used in combination with the biodistribution data of their corresponding radiotracers.

To prove the bivalency of cyclic RGD dimers (Fig. 7: 3P-RGD2 and 3G-RGD2), complexes $^{99m}$Tc-3P-RGD2 and $^{99m}$Tc-3G-RGD2 (Fig. 8) were evaluated in the athymic nude mice bearing U87MG human glioma and MDA-MB-435 human breast tumor xenografts [174, 175]. For comparison purposes, $^{99m}$Tc-P-RGD2 and $^{99m}$Tc-RGD4 (Fig. 8) were also evaluated using the same tumor-bearing animal models [174, 175]. As expected, the breast tumor uptake of $^{99m}$Tc-3P-RGD2 and $^{99m}$Tc-3G-RGD2 was comparable to that of $^{99m}$Tc-RGD4 (Fig. 8), and was >2x higher than that of $^{99m}$Tc-P-RGD2 [174]. These data strongly suggest that RGD, 3P-RGD2 and 3G-RGD2 are bivalent and P-RGD2 is only monodentate in binding to the integrin $\alpha_\beta_3$ even though it has two RGD motifs. Similar conclusion was also made for 3P-RGD2 in $^{64}$Cu(DOTA-3P-RGD2) [176], 3G-RGD2 in $^{64}$Cu(DOTA-3G-RGD2) [176], $^{18}$F-2P-RGD2 in $^{99m}$Tc-G3-2P-RG2 [177], and 2P-RGD2 in their $^{68}$Ga and $^{18}$F radiotracers [178, 179]. If P-RGD2 were bivalent, HYNIC-P-RGD2 would have had the same $\alpha_\beta_3$ binding affinity as HYNIC-3P-RGD2 and HYNIC-3G-RGD2 while $^{99m}$Tc-P-RGD2 would have shared the same tumor uptake with $^{99m}$Tc-3P-RGD2 and $^{99m}$Tc-3G-RGD2.
Figure 7. Examples of cyclic RGD dimers with PEG$_4$ and G$_3$ linkers, which are used to increase the distance between two RGD motifs and to improve radiotracer excretion kinetics from normal organs.
Figure 8. Comparison of the tumor uptake for $^{99m}$Tc$^\text{-P}^\text{-RGD}_2$, $^{99m}$Tc$^\text{-3G}^\text{-RGD}_2$, $^{99m}$Tc$^\text{-3P}^\text{-RGD}_2$ and $^{99m}$Tc$^\text{-RGD}_4$ in the athymic nude mice bearing MDA-MB-435 breast cancer xenografts.

Table 2. Integrin $\alpha_v\beta_3$ binding data for cyclic RGD peptides and their corresponding HYNIC and DOTA conjugates against $^{125}$I-echistatin bound to the $\alpha_v\beta_3$-positive U87MG human glioma cells.

| Compound          | IC$_{50}$ (nM) | Radiotracer                                                                 |
|-------------------|----------------|------------------------------------------------------------------------------|
| c(RGDyK)          | 458 ± 45       | $[^{99m}\text{Tc}]$([HYNIC-RGD$_2$](tricine)(TPPTS))                         |
| HYNIC-G-RGD       | 335 ± 11       | $[^{99m}\text{Tc}]$([HYNIC-P-RGD$_2$](tricine)(TPPTS))                      |
| HYNIC-P-RGD       | 452 ± 11       | $[^{99m}\text{Tc}]$([HYNIC-P-RGD$_2$](tricine)(TPPTS))                      |
| HYNIC-RGD$_2$     | 112 ± 21       | $[^{99m}\text{Tc}]$([HYNIC-RGD$_2$](tricine)(TPPTS))                       |
| HYNIC-P-RGD$_2$   | 84 ± 7         | $[^{99m}\text{Tc}]$([HYNIC-P-RGD$_2$](tricine)(TPPTS))                      |
| HYNIC-2G-RGD$_2$  | 60 ± 4         | $[^{99m}\text{Tc}]$([HYNIC-2G-RGD$_2$](tricine)(TPPTS))                    |
| HYNIC-2P-RGD$_2$  | 52 ± 7         | $[^{99m}\text{Tc}]$([HYNIC-2P-RGD$_2$](tricine)(TPPTS))                    |
| HYNIC-3G-RGD$_2$  | 61 ± 2         | $[^{99m}\text{Tc}]$([HYNIC-3G-RGD$_2$](tricine)(TPPTS))                    |
| HYNIC-3P-RGD$_2$  | 62 ± 5         | $[^{99m}\text{Tc}]$([HYNIC-3P-RGD$_2$](tricine)(TPPTS))                    |
| HYNIC-RGD$_4$     | 7 ± 2          | $[^{99m}\text{Tc}]$([HYNIC-RGD$_4$](tricine)(TPPTS))                       |
| DOTA-RGD$_2$      | 102 ± 5        | $[^{64}\text{Cu}]$([DOTA-RGD$_2$])$/[^{111}\text{In}]$([DOTA-RGD$_2$])       |
| DOTA-3G$_2$-RGD$_2$ | 74 ± 3       | $[^{64}\text{Cu}]$([DOTA-3G$_2$-RGD$_2$])$/[^{111}\text{In}]$([DOTA-3G$_2$-RGD$_2$]) |
| DOTA-3PEG$_4$-RGD$_2$ | 62 ± 6   | $[^{64}\text{Cu}]$([DOTA-3PEG$_4$-RGD$_2$])$/[^{111}\text{In}]$([DOTA-3PEG$_4$-RGD$_2$]) |
| DOTA-RGD$_4$      | 10 ± 2         | $[^{64}\text{Cu}]$([DOTA-RGD$_4$])$/[^{111}\text{In}]$([DOTA-RGD$_4$])       |
| NOTA-RGD$_2$      | 100 ± 3        | $[^{68}\text{Ga}]$([NOTA-RGD$_2$])                                          |
| NOTA-2G$_2$-RGD$_2$ | 66 ± 4       | $[^{68}\text{Ga}]$([NOTA-2G$_2$-RGD$_2$])                                   |
| NOTA-2PEG$_4$-RGD$_2$ | 54 ± 2   | $[^{68}\text{Ga}]$([NOTA-2PEG$_4$-RGD$_2$])                                 |
Impact of radiometal chelate on tumor uptake and pharmacokinetics. Shi et al [180, 181] also prepared the cyclic RGD conjugates: MAG2-3P-RGD$_2$ and MAG2-3G-RGD$_2$. It was found that $^{99m}$TcO(MAG2-3P-RGD$_2$) had better tumor uptake than $^{99m}$Tc-3P-RGD$_2$ [180], while their liver and kidney uptake was almost identical at >60 min p.i. On the other hand, $^{99m}$TcO(MAG2-3G-RGD$_2$) had the same tumor uptake as $^{99m}$Tc-3G-RGD$_2$ at <60 min p.i., but its liver and kidney uptake was much lower than that of $^{99m}$Tc-3G-RGD$_2$ [181]. Among $^{99m}$Tc-labeled cyclic RGD dimers evaluated in the U87MG glioma-bearing model, $^{99m}$TcO(MAG2-3P-RGD$_2$) has the highest glioma uptake (~15 %ID/g at 30 min p.i. and 1.87 ± 0.51 %ID/g at 240 min) and 1.61 ± 0.06 %ID/g at 240 min p.i. Obviously, replacing $^{99m}$Tc(HYNIC)(tricine)(TPPTS)] (M.W. = ~1000 Daltons) with $^{99m}$TcO(MAG2) (M.W. = ~350 Daltons) had a significant impact on both tumor uptake and pharmacokinetics of $^{99m}$Tc radiotracers. In contrast, substituting the bulky $^{99m}$Tc(HYNIC)(tricine)(TPPTS)] with a much smaller and more hydrophilic $^{111}$In(DOTA) had little impact on the radiotracer tumor uptake [182, 183]. However, the liver and kidney uptake of $^{111}$In(DOTA-3P-RGD$_2$) is significantly lower than that of $^{99m}$Tc-3P-RGD$_2$, probably due to higher hydrophilicity of $^{111}$In(DOTA) [82]. Similar conclusion could be made by directly comparing $^{111}$In(DOTA-3G-RGD$_2$) and $^{99m}$Tc-3G-RGD$_2$ [181, 183].

$^{111}$In(DOTA-3P-RGD$_2$) and $^{64}$Cu(DOTA-3P-RGD$_2$) share the same DOTA-conjugate. The tumor uptake of $^{111}$In(DOTA-3P-RGD$_2$) was very close to that of $^{64}$Cu(DOTA-3P-RGD$_2$) [176, 182]. They also have a similar uptake in normal organs. For example, the kidney uptake of $^{111}$In(DOTA-3P-RGD$_2$) was compared well with that of $^{64}$Cu(DOTA-3P-RGD$_2$) within the experimental errors. The liver uptake of $^{111}$In(DOTA-3P-RGD$_2$) was 2.52 ± 0.57 %ID/g at 30 min and 1.61 ± 0.06 %ID/g at 240 min p.i., while $^{64}$Cu(DOTA-3P-RGD$_2$) had the liver uptake of 2.80 ± 0.35 %ID/g at 30 min p.i. and 1.87 ± 0.51 %ID/g at 240 min p.i. These data suggest that the radiometal ($^{64}$Cu vs. $^{111}$In) has little impact on the radiotracer tumor uptake and excretion kinetics, probably due to the overwhelmingly large size of the dimeric RGD peptides as compared to that of the radiometal chelate. The same conclusion was also made by directly comparing the uptake in tumor and normal organs for $^{111}$In(DOTA-3G-RGD$_2$) [183] and $^{64}$Cu(DOTA-3G-RGD$_2$) [176].

Integrin $\alpha_\beta_3$ and RGD specificity. The $\alpha_\beta_3$-specificity of $^{99m}$TcO(MAG2-3P-RGD$_2$) and $^{111}$In(DOTA-3P-RGD$_2$) was demonstrated by the “blocking experiment” (Fig. 9).

![Figure 9](http://www.thno.org)
MAG\(_2\)-3P-RGD\(_2\) (IC\(_{50}\) = 3.9 ± 0.4 nM) and DOTA-3P-RGD\(_2\) (IC\(_{50}\) = 1.3±0.3 nM) against \(\text{\(^{125}\)I-c(RGDyK)}\) bound to the U87MG glioma cells. As a result, \(^{99m}\)TcO(MAG\(_2\)-3P-RGK\(_2\)) and \(^{111}\)In(DOTA-3P-RGK\(_2\)) had much lower uptake as compare to that of \(^{99m}\)TcO(MAG\(_2\)-3P-RGD\(_2\)) and \(^{111}\)In(DOTA-3P-RGD\(_2\)) in both tumor and normal organs (Fig. 10). These data strongly suggest that the localization of \(^{99m}\)TcO(MAG\(_2\)-3P-RGD\(_2\)) and \(^{111}\)In(DOTA-3P-RGD\(_2\)) in the tumor is indeed based on interactions between RGD motifs and \(\alpha_v\beta_3\) [181, 182].

Figure 10. Comparison of biodistribution data of \(^{99m}\)TcO(MAG\(_2\)-3P-RGD\(_2\))/\(^{99m}\)TcO(MAG\(_2\)-3P-RGK\(_2\)) and \(^{111}\)In(DOTA-3P-RGD\(_2\))/\(^{111}\)In(DOTA-3P-RGK\(_2\)) in athymic nude mice bearing U87MG glioma xenografts at 60 min post-injection. The low tumor uptake for \(^{99m}\)TcO(MAG\(_2\)-3P-RGK\(_2\)) and \(^{111}\)In(DOTA-3P-RGK\(_2\)) indicates that the radio-labeled cyclic RGD dimers are RGD-specific.

**Multimeric ≠ multivalent.** On the basis of the in vitro \(\alpha_v\beta_3\) binding assays and the ex-vivo biodistribution data, it becomes quite clear that 3P-RGD\(_2\), 3G-RGD\(_2\) and RGD\(_4\) are bivalent in binding to the \(\alpha_v\beta_3\). However, it remains unclear if RGD\(_4\) will become tetravalent if a number of G\(_3\) or PEG\(_4\) linkers are incorporated between its four cyclic RGD motifs. To answer this fundamental question, two DOTA-conjugated cyclic peptide RGD tetramers (Fig. 11: 6P-RGD\(_4\) and 6G-RGD\(_4\)) have been successfully prepared [183, 184]. Fig. 12 compares the tumor uptake of \(^{111}\)In-labeled RGD dimers (3P-RGD\(_2\) and 3G-RGD\(_2\)) and tetramers (6P-RGD\(_4\) and 6G-RGD\(_4\)) in the athymic nude mice bearing U87MG glioma xenografts. The fact that \(^{111}\)In(DOTA-3P-RGD\(_2\)) and \(^{111}\)In(DOTA-6P-RGD\(_4\)) shared a very similar initial tumor uptake within the experimental errors suggests that 6P-RGD\(_4\) and 6G-RGD\(_4\) may not be truly tetravalent [183, 184].

As discussed previously, both bivalency and the locally enhanced RGD concentration contribute to the high \(\alpha_v\beta_3\) binding affinity of multimeric RGD peptides. The concentration factor exists in all multimeric RGD peptides regardless of spacers or linkers. The key for bivalency is the distance between two RGD motifs. For example, this distance in 3P-RGD\(_2\) (38 bonds) and 3G-RGD\(_2\) (26 bonds) is long enough for them to achieve bivalency, which leads to higher \(\alpha_v\beta_3\)
binding affinity of DOTA-3P-RGD$_2$ and DOTA-3G-RGD$_2$ than that of DOTA-RGD$_2$ (Table 2), and higher tumor uptake of $^{111}$In(DOTA-3P-RGD$_2$) and $^{111}$In(DOTA-3G-RGD$_2$) than that of $^{111}$In(DOTA-P-RGD$_2$) [139]. In contrast, the concentration factor might be responsible for the longer tumor retention times (Fig. 13) of $^{111}$In(DOTA-6G-RGD$_4$) as compared to that of $^{111}$In(DOTA-3G-RGD$_2$). Even if 6P-RGD$_4$ and 6G-RGD$_4$ are not tetravalent, the mere presence of two extra RGD motifs definitely helps to improve the radiotracer tumor retention time, which may become important for $^{90}$Y and $^{177}$Lu radiotracers, which have great potential for systemic radiotherapy of the $\alpha_\beta_3$-positive tumors.

It must be noted that the ability of a multimeric RGD peptide to achieve bivalency also depends on the $\alpha_\beta_3$ density. If the $\alpha_\beta_3$ density is very high, the distance between two neighboring $\alpha_\beta_3$ sites will be short, which makes it easier for the multimeric cyclic RGD peptide to achieve the bivalency. If the $\alpha_\beta_3$ density is very low, the distance between two neighboring $\alpha_\beta_3$ sites will be very long, and it might be more difficult for the same multimeric cyclic RGD peptide to achieve simultaneous $\alpha_\beta_3$ binding.

Figure 11. DOTA-conjugated cyclic RGD tetramers: DOTA-6P-RGD$_4$ and DOTA-6G-RGD$_4$. 

http://www.thno.org
Figure 12. Direct comparison of the tumor uptake of the $^{111}$In-labeled cyclic RGD dimers (3P-RGD$_2$ and 3G-RGD$_2$) and tetramers (RGD$_4$, 6P-RGD$_4$, and 6G-RGD$_4$) in athymic nude mice bearing U87MG human glioma xenografts.

Figure 13. The whole-body planar images of the tumor-bearing mice administered with ~100 μCi of $^{111}$In(DOTA-6G-RGD$_4$) and $^{111}$In(DOTA-3G-RGD$_2$) at 1, 4, 24 and 72 h p.i. The concentration factor is responsible for the longer tumor retention time of $^{111}$In(DOTA-6G-RGD$_4$) (left) as compared to that of $^{111}$In(DOTA-3G-RGD$_2$) (right).

3. αIIbβ3–TARGETED RADIOTRACERS FOR THROMBOSIS IMAGING

Cardiovascular diseases and vulnerable plaque. Cardiovascular diseases are the most frequent causes of death in the Western world. Atherosclerosis is the main cause of coronary and peripheral arterial diseases [185-189]. Atherosclerosis is a chronic and progressive systemic disease, with a long asymptomatic phase, characterized by accumulation of lipids, inflammatory cells and connective tissue within the intima of arterial wall [187, 189]. The initial pathologic...
abnormality is the fatty streak, due to accumulation of lipoproteins and macrophages, which may develop into a mature atherosclerotic plaque, with a lipid core bounded on its lumen side by a fibrous cap containing vascular smooth muscle cells and connective tissue. Atherosclerosis remains clinically silent until the lesion can expand to the point at which it limits flow, producing symptoms of reversible ischemia, such as angina, during periods of high demand [187-192]. Alternatively, the fibrous plaque can erode or rupture, resulting in the exposure of subendothelial collagen and lipid [187, 190], which leads to activation of platelets and clotting cascade proteins. Platelet activation upregulates αIIbβ3 (or glycoprotein IIb/IIa) on the platelet surface which, when stimulated, promote platelet aggregation [193-199]. Activation of clotting factor proteins VII and XI results in production of thrombin, fibrinogen, and fibrin through the so-called extrinsic and intrinsic coagulation pathways, respectively. The result is the formation of thrombus composed of both fibrin and platelets. The consequences of plaque rupture range from complete lysis of the thrombus by endogenous fibrinolytic pathways with subsequent healing of the fibrous cap and overlying endothelium to the unchecked thrombosis and complete lumen occlusion. Such an event can range from being clinically silent at one extreme through precipitation of an acute vascular event, such as unstable angina, myocardial infarction or stroke, to sudden death at the other extreme [188-190]. It is the rupture of plaque and formation of a thrombus that causes the most serious complications of atherosclerosis, such as acute coronary syndromes and stroke [187, 189, 190].

In fact, the plaque rupture is responsible for 76% of all fatal heart attacks caused by coronary thrombosis worldwide [187-190, 196-204]. Thus, early detection of the processes underlying progressive plaque destabilization for the purpose of identifying the patients in whom rupture of a vulnerable plaque is likely to result in a clinical event, is of the utmost importance [201-205]. Since the disruption of atherosclerotic plaques is known to initiate thrombus formation leading to thrombotic and thromboembolic events, it has been suggested that the thrombogenicity of atherosclerotic plaques is one of the most promising approaches to detecting vulnerable plaques [189, 190, 196-205]. From this point of view, the accurate detection of the intra-arterial thrombus noninvasively could have significant diagnostic and prognostic implications [201-205].

_Deevein thrombosis (DVT)._ DVT is the formation of blood clots in veins and is also known as venous thromboembolism [14, 65, 206]. DVT occurs when a thrombus forms in one of large veins in the lower extremities, leading to partially or completely blocked blood circulation. The condition may result in health complications, such as a pulmonary embolism (PE) or death if not diagnosed and treated effectively. A majority of DVT patients will experience PE (~30% are symptomatic, and 40% are asymptomatic and at high risk) because the blood clot is unstable and can travel to, and lodge in, the lungs. More than 1 million people in the United States suffer from DVT blood clots every year. Complications from DVT blood clots kill almost 300,000 people a year—more than AIDS and breast cancer combined [14, 206]. Thus, accurate early detection of DVT and PE is highly desirable so that various therapeutic regimens can be given.

_Imaging arterial thrombi._ To identify healthy subjects at risk for future cardiovascular events, a consensus of experts has recently defined criteria for the diagnosis of vulnerable plaques [190, 192]. Major criteria have been established to represent different aspects of the rupture-prone plaque. These include the calcified nodules, yellow appearance of the plaque, intraplaque hemorrhage, thrombogenicity, active inflammation and plaque injury. Further major criteria include a thin cap, a large lipid core, and luminal stenosis [190, 192, 203]. Although many imaging techniques are now clinically available for diagnosis of luminal narrowing, arterial occlusion and intramural hematoma [201-205], arterial thrombi are not reliably detected by current diagnostic methods. Coronary angiography remains the gold standard to assess vessel lumen narrowing. Other invasive techniques include intravascular coronary ultrasound, coronary angioscopy, intravascular elastography, elastography, thermography, or optical coherence tomography [201-205, 207]. These techniques can provide anatomic details of plaque size and composition, but they have the disadvantage of being invasive. MRI and CT have also been used for diagnosis of arterial thrombi; but these two modalities are anatomical and functional [208-213]. It is difficult to distinguish the “fresh” and “old” thrombi with MRI and CT. In contrast, nuclear imaging by SPECT and PET has the most potential to furnish functional information on biologic events which determine the risk of plaque rupture [201, 203, 205]. Besides their noninvasive nature, nuclear medicine techniques have the potential to evaluate important determinants of plaque vulnerability, taking into account specific cellular or biochemical changes that characterize these lesions. Radiolabeled monoclonal antibodies have been used to target fibrin or platelets on acute thrombi in humans [214-229], but they were expected to have very limited clinical usage due to their long blood circulation time. These limitations can be alleviated by
using synthetic peptides that are much smaller and are cleared quickly from the blood circulation [230-232]. Examples of $^{99m}$Tc-labeled small peptide radiotracers include $^{99m}$Tc-apticide [233-239] and DMP444 [240-255], both of which target $\alpha_{IIb}\beta_3$ receptors on the activated platelets. $^{99m}$Tc-TP850 is a $^{99m}$Tc-labeled linear peptide targeting the fibrin component of thrombus [256]. The peptide-based radiotracers for thrombus imaging have been reviewed extensively [201-205, 257-265].

**Imaging deep vein thrombosis.** Contrast-enhanced venography remains the gold standard for diagnosis of DVT, but compression ultrasonography is the most common technique used to detect DVT in the lower extremities. Pooled analyses showed that ultrasonography has a sensitivity of 96 % and a specificity of 98 % for proximal vein thrombosis. The primary limitation of these diagnostic procedures is that neither technique can distinguish between chronic and unstable thrombi [14, 207, 257]. Both contrast venography and ultrasonography are imaging procedures that detect changes in venous anatomy that are caused by the intraluminal thrombus that is sufficiently formed either to reduce vascular filling with contrast medium or to resist compression. However, these procedures do not reflect the metabolic activity of the clot, and therefore, they may overestimate the presence of active clots. The sensitivity of ultrasonography is also limited by disease-related and technical factors. An alternative approach for diagnosis of acute DVT is to detect a molecular marker that is not present in old, organized DVT.

$^{99m}$Tc-apticide: approved for imaging DVT. One of the important components of clotting process is platelet activation, which leads to the expression of $\alpha_{IIb}\beta_3$ receptors that bind fibrinogen and promote platelet–platelet interaction, resulting in platelet aggregation and the formation of a secure plug. Many synthetic peptides targeting the $\alpha_{IIb}\beta_3$ on activated platelets have been successfully radiolabeled with $^{99m}$Tc. Because of their small size, these radiotracers often have very rapid clearance from the blood circulation. For example, $^{99m}$Tc-P280 (Fig. 14: $^{99m}$Tc-apticide) was the first RGD-mimicking peptide studied in humans [233]. $^{99m}$Tc-apticide was shown to specifically bind the $\alpha_{IIb}\beta_3$ (IC$_{50} = 0.20 \pm 0.11 \mu M$ for dog platelets as compared with $0.056 \pm 0.011 \mu M$ for human platelets), and to selectively accumulate in fresh thrombi [233, 234]. Imaging studies in dogs also showed that the thrombus could be readily detected with $^{99m}$Tc-apticide [234]. A pilot study of 9 patients with carotid atherosclerosis showed the uptake in 11 of 18 carotid arteries after injection of $^{99m}$Tc-apticide [237, 238]. There was only a moderate correlation when compared with ultrasound findings. Bates et al enrolled patients with newly diagnosed first DVT and the patients with previous DVT [235]. It was found that the sensitivity and specificity of $^{99m}$Tc-apticide were 92% and 86%, respectively, for differentiating between the acute and chronic thrombus [235]. $^{99m}$Tc-apticide had a sensitivity and specificity of 87% and 100%, respectively, for the patients with DVT. These data have clearly demonstrated the potential of the $^{99m}$Tc-apticide scintigraphy to address the important issues in terms of identifying the arterial lesions responsible for recent symptoms. However, $^{99m}$Tc-apticide was not particularly useful for detection of pulmonary embolism (PE) in 83% of the patients, most likely due to its low thrombus uptake and prolonged radioactivity accumulation in the blood pool and chest region [234]. $^{99m}$Tc-apticide has been approved by FDA (Food and Drug Administration) for imaging acute venous thrombosis in the lower extremities of patients. Apcitide (AcuTec™) is available commercially as a non-radioactive freeze-dried kit that can be labeled with $^{99m}$Tc for clinical usage [236-239].

![Structure of $^{99m}$Tc-apticide, a cyclic RGD peptide mimetic specifically binding to the $\alpha_{IIb}\beta_3$ expressed on fresh thrombi. $^{99m}$Tc-apticide has been approved by FDA for diagnosis of DVT in patients.](http://www.thno.org)

**Figure 14.** Structure of $^{99m}$Tc-apticide, a cyclic RGD peptide mimetic specifically binding to the $\alpha_{IIb}\beta_3$ expressed on fresh thrombi. $^{99m}$Tc-apticide has been approved by FDA for diagnosis of DVT in patients.

$^{99m}$Tc-P280 ($^{99m}$Tc-apticide)

**DMP444: clinically useful for imaging DVT.** Activated platelets express $\alpha_{IIb}\beta_3$ receptors which recognize proteins and small peptides bearing the RGD sequences while non-activated platelets express virtually no $\alpha_{IIb}\beta_3$ receptors in their active conformation [240]. DMP728 and DMP757 (Fig. 2) were originally developed by DuPont Merck Pharmaceuticals as an antithrombotic agents and had very high selectivity and binding affinity for GPIIb/IIIa with IC$_{50}$ values in the nanomolar range against fibrinogen binding to the activated platelets [240]. Therefore, DMP728 and DMP757 are excellent biomolecules to target the fresh
thrombi. Liu and coworkers at DuPont Medical Imaging used the 6-aminocaproic acid linker to connect DMP757 with a Tc-binding group, and to keep the Tc chelate separate from the cyclic RGD motif to minimize the impact of $^{99m}$Tc-labeling on the binding affinity for $\alpha_{\mathrm{IIb}}\beta_3$ [241-247]. The $N_2S_2$ and $N_2S$-type of BFCs were used for $^{99m}$Tc-labeling of DMP757. It was found that BFCs had a significant impact on thrombus uptake and excretion kinetics of radiotracers [240-244]. DMP444 (Fig. 15: top) was prepared using HYNIC as the BFC, tricine and TPPTS as coligands. Among the $^{99m}$Tc radiotracers evaluated in various models, DMP444 had the best thrombus uptake with the highest thrombus/blood and thrombus/muscle ratios [246]. In the AV shunt model, DMP444 was able to detect a growing venous thrombus (Fig. 15: bottom) as early as 15 min p.i. DMP444 has a slow blood clearance (45 % of the injected dose at 2 h) and a high thrombus uptake (9.93 ± 0.52 % ID/g for arterial thrombi; and 2.86 ± 0.37 % ID/g for venous thrombi). Mitchel et al [250] tested the ability of DMP444 to identify platelet-rich thrombus in a canine model, and found that the thrombus radioactivity correlated well with thrombus weights. Kaul’s group found that the microthrombembli can be detected after primary percutaneous transluminal coronary angioplasty (PTCA), and the infarct size was proportional to the magnitude and extent of microthrombembli [255]. Thrombus imaging during reperfusion may provide important information in the patients with acute myocardial infarction that may lead to better adjuvant therapy during PTCA. In the patients suspected with DVT, no clinically significant adverse effects were noted after administration of DMP444 [253]. Most of patients were taking Warfarin® (Coumadin®) and heparin (n = 8) or Heparin® (n = 1) and Warfarin® (n = 1) alone at the time of imaging. The average time from the onset of symptoms to injection of DMP444 was 5 days (range 1 to 18 days). At 10 – 40 min p.i., 8 of 10 patients demonstrated an area of the increased radioactivity that was clearly related to the abnormality as noted by ultrasound methods [253]. These preliminary data lead to the comprehensive Phase II clinical studies. It was concluded that DMP444 is very useful for noninvasive imaging of DVT with high sensitivity and specificity. In addition, it has also shown that the DMP444 SPECT allows in vivo visualization of infective endocarditis if it is performed within 1 to 2 weeks after antibiotic treatment [251]. A non-radioactive freeze-dried kit has been developed, and can be used for routine $^{99m}$Tc-labeling in clinical settings [248, 266].

Figure 15. Top: Structure of DMP444; Bottom: DVT images of a dog administered with DMP444 at 15, 30, 60, and 120 min post-infusion. The bar to the right of the images indicates the scale from 0 (white) to 506 (greatest/black). The phase II clinical studies have demonstrated that DMP444 is clinically useful for imaging DVT.

4. SUMMARY AND OUTLOOK

Radiolabeled cyclic RGD peptides represent a new class of radiotracers for diagnosis of tumor or thrombosis, depending upon their selectivity for $\alpha_{\mathrm{IIb}}\beta_3$ or $\alpha_{\mathrm{IIa}}\beta_3$. While cyclic RGD pentapeptides have high binding affinity and selectivity for $\alpha_{\mathrm{IIa}}\beta_3$, the cyclic hexapeptides with one or more rigid aromatic rings tend to show high binding affinity and selectivity for $\alpha_{\mathrm{IIa}}\beta_3$ over $\alpha_{\mathrm{IIa}}\beta_3/\alpha_{\mathrm{IIb}}\beta_3$. The $\alpha_{\mathrm{III}}\beta_3$-targeted radiotracers have the potential for early detection of rapidly growing and metastatic tumor, and for monitoring the tumor growth, metastasis and therapeutic response.
by PET or SPECT [267, 268]. $[^{18}F]$Galacto-RGD, $[^{18}F]$FAH111585 and $^{99m}$Tc-NC100692 are currently under clinical investigations for noninvasive visualization of the $\alpha_\beta_3$-positive tumors in cancer patients. While the research efforts on $\alpha_\beta_3$-targeted radiotracers have been focused on new RGD peptides with the improved $\alpha_\beta_3$ affinity, the formulation development for routine preparation of radiotracers remains to be strengthened. It must be emphasized that the success of a new $\alpha_\beta_3$-targeted radiotracer relies largely on its clinical availability at reasonable cost and capability to improve the quality of cancer patient’s life. In this respect, the $^{99m}$Tc radiotracers will offer significant advantages because of the nuclear properties of $^{99m}$Tc for SPECT, easy availability of $^{99m}$Mo-$^{99m}$Tc generators, and the kit formulation for routine preparation of $^{99m}$Tc radiotracers at low cost.

Increasing the RGD peptide multiplicity can significantly enhance their $\alpha_\beta_3$ binding affinity, and improve tumor targeting ability of their radiotracers. However, the tumor selectivity is not substantially improved because the uptake of radiolabeled cyclic RGD peptide multimers in the intestine, liver and kidneys is also significantly increased. As a result, there is no significant advantage in using radiolabeled tetramers (such as RGD$_4$, 6G-RGD$_4$ and 6F-RGD$_4$) over their dimeric counterparts (such as 3G-RGD$_2$ and 3P-RGD$_2$) as diagnostic radiotracers with respect to the tumor selectivity or T/B ratios. Among the cyclic RGD dimers evaluated in different preclinical tumor-bearing animal models, 3G-RGD$_2$ and 3P-RGD$_2$ are the best $\alpha_\beta_3$-targeting biomolecules because their corresponding PET and SPECT radiotracers tend to have excellent tumor uptake with very high T/B ratios. Recently, $^{99m}$Tc-3P-RGD$_2$ has been selected as a candidate for clinical evaluations because of its high tumor uptake, long tumor retention and high metabolic stability [174, 175].

It is important to emphasize that $\alpha_\beta_3$ is also over-expressed on the activated endothelial cells during wound healing and post-infarction remodeling, in rheumatoid arthritis and psoriatic plaque [269-271]. Thus, the $\alpha_\beta_3$-targeted radiotracers developed for tumor imaging have been proposed for imaging myocardial angiogenesis. For example, recent studies clearly showed that the $^{111}$In-labeled nonpeptide $\alpha_\beta_3$ antagonist (RF748) was able to image angiogenesis in the heart after myocardial infarction [271], and the radiotracer uptake in the infarct region was associated with the level of $\alpha_\beta_3$ expression. The results from imaging studies also suggest that $[^{18}F]$Galacto-RGD might be a powerful tool to distinguish between acute and chronic phases of T-cell mediated immune responses [272]. These promising results give rise to the possibility of extending applications of the $\alpha_\beta_3$-targeted radiotracers from imaging tumor angiogenesis to detection of inflammatory processes, and to monitoring outcomes of therapeutic interventions in patients with cancer, myocardial infarction, and inflammation.

While the DVT can be detected by contrast-enhanced venography and compression ultrasonography, accurate detection of arterial thrombi and PE remains a significant challenge because of their small size and location. $^{99m}$Tc-apcitide was approved for diagnosis of DVT; but its T/B ratios are low due to its accumulation in the blood pool and chest region [246]. DMP444 has higher thrombus uptake with better T/B ratios than $^{99m}$Tc-apcitide [240, 246]. However, its blood clearance rate is relatively slow, due to the lipophilic 6-aminocaproic linker and/or the highly charged ternary ligand system. Therefore, the focus of future research in this area should be directed towards developing more efficient radiotracers that have faster blood clearance and are useful for accurate detection of small thrombosis lesions in the coronary artery, as well as DVT and PE in patients. The “bivalency concept” developed for $\alpha_\beta_3$-targeted radiotracers may also apply to cyclic RGD hexapeptides to improve the $\alpha_{in}\beta_3$-targeting capability. Since thrombus formation represents the final step in atherosclerosis progression, imaging with the $\alpha_\beta_3$-targeted radiotracers may be able to not only identify those patients at high risk for cardiovascular events (death, myocardial infarction or stroke) not identified by routine clinical evaluation, but also characterize the lesion vulnerability in high-risk areas of the coronary vasculature. Once the lesion is determined to be of particularly high risk, novel local therapies such as intracoronary drug-eluting stents or local drug delivery with suitable drug-delivery balloon catheters could be justified. In addition, molecular imaging of arterial thrombi will help to select the individualized treatment strategies based on the molecular profile of vulnerable plaques identified in a particular patient.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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