Imaging integrin alpha-v-beta-3 expression in tumors with an $^{18}$F-labeled dimeric RGD peptide

Ingrid Dijkgraaf$^{1,§}$, Samantha Terry$^{2,§,*}$, William J. McBride$^{3}$, David M. Goldenberg$^{3}$, Peter Laverman$^{2}$, Gerben M. Franssen$^{2}$, Wim J. G. Oyen$^{2}$, and Otto C. Boerman$^{2}$

$^{1}$Maastricht University, Maastricht, The Netherlands $^{2}$Department of Nuclear Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands $^{3}$Immunomedics, Inc., Morris Plains, New Jersey, USA

Abstract

Integrin $\alpha_\text{v}\beta_3$ receptors are expressed on activated endothelial cells during neovascularization to maintain tumor growth. Many radiolabeled probes utilize the tight and specific association between the arginine-glycine-aspartic acid (RGD) peptide and integrin $\alpha_\text{v}\beta_3$, but one main obstacle for any clinical application of these probes is the laborious multistep radiosynthesis of $^{18}$F. In this study, the dimeric RGD peptide, E-[c(RGDfK)]$_2$, was conjugated with NODAGA and radiolabeled with $^{18}$F in a simple one-pot process with a radiolabeling yield of 20%; the whole process lasting only 45 min. NODAGA-E-[c(RGDfK)]$_2$ labeled with $^{18}$F at a specific activity of 1.8 MBq/nmol and a radiocational purity of 100% could be achieved. Log P value of $^{18}$F-labeled NODAGA-E-[c(RGDfK)]$_2$ was $-4.26 \pm 0.02$. In biodistribution studies, $^{18}$F-NODAGA-E-[c(RGDfK)]$_2$ cleared rapidly from the blood with 0.03 $\pm$ 0.01 %ID/g in the blood at 2 h p.i., mainly via the kidneys and showed good in vivo stability. Tumor uptake of $^{18}$F-NODAGA-E-[c(RGDfK)]$_2$ (3.44 $\pm$ 0.20 %ID/g, 2 h p.i.) was significantly lower than that of reference compounds $^{68}$Ga-labeled NODAGA-E-[c(RGDfK)]$_2$ (6.26 $\pm$ 0.76 %ID/g; $P < 0.001$) and $^{111}$In-labeled NODAGA-E-[c(RGDfK)]$_2$ (4.99 $\pm$ 0.64 %ID/g; $P < 0.01$). Co-injection of an excess of unlabeled NODAGA-E-[c(RGDfK)]$_2$ along with $^{18}$F-NODAGA-E-[c(RGDfK)]$_2$ resulted in significantly reduced radioactivity concentrations in the tumor (0.85 $\pm$ 0.13 %ID/g). The $\alpha_\text{v}\beta_3$ integrin-expressing SK-RC-52 tumor could be successfully visualized by microPET with $^{18}$F-labeled NODAGA-E-[c(RGDfK)]$_2$. In conclusion, NODAGA-E-[c(RGDfK)]$_2$ could be labeled rapidly with $^{18}$F using a direct aqueous, one-pot method and it accumulated specifically in $\alpha_\text{v}\beta_3$ integrin-expressing SK-RC-52 tumors, allowing for visualization by microPET.

Keywords

Integrin alpha-v-beta-3; PET; radiofluorination; aluminum fluoride; RGD; NODAGA

*Correspondence to: Samantha Terry, Department of Nuclear Medicine, Radboud University Medical Centre Nijmegen, PO Box 9101, 6500 HB Nijmegen, the Netherlands, S.Terry@nuclmed.umcn.nl.

§Both authors contributed equally to this work.

Potential Conflicts

Drs. McBride and Goldenberg have employment and stock ownership with Immunomedics, Inc.
1. INTRODUCTION

Integrin αvβ3 is overexpressed on activated endothelial cells during tumor-induced angiogenesis, but is absent on quiescent endothelial cells. Ruoslahti and co-workers found that this integrin interacts with the arginine–glycine–aspartic acid (RGD) amino acid sequence present on extracellular matrix proteins, such as vitronectin, fibrinogen and laminin (1). Based on the RGD peptide sequence, series of small cyclic peptides, showing high and specific affinity for the αvβ3 integrin, have been developed (2).

In the last few years, imaging of angiogenic processes using positron emission tomography (PET) or single photon emission computed tomography (SPECT) with radiolabeled RGD peptides has attracted considerable interest. These tracers have the potential to select patients who might benefit from treatment with antiangiogenic drugs as well as to monitor the therapeutic efficacy of integrin αvβ3-targeted drugs.

The glycosylated cyclo(RGDfK) analog [18F]galacto-RGD is of the αvβ3 integrin ligands the most intensely evaluated and studied in cancer patients (3–5). Recently, another [18F]-labeled RGD peptide, [18F]-AH111585, has also been tested in preclinical studies and clinical trials (6–8). Both tracers showed good pharmacokinetics and receptor-specific uptake, allowing non-invasive imaging of αvβ3 integrin expression. However, the synthesis of these tracers is complex and time-consuming with moderate yield, which hampers routine use in the clinical setting.

[18F] is the most widely used radionuclide in PET and has excellent characteristics for peptide-based imaging, namely a half-life of 110 min that matches the pharmacokinetics of most peptides, and the low positron energy of 635 keV results in short ranges in tissue and excellent preclinical imaging resolution (<2 mm) (9).

For radiofluorination of RGD peptides, a wide range of methods has been investigated. Chemoselective oxime formation allows high-yield two-step radiosynthesis of [18F]-labeled peptides via conjugation of [18F]-labeled aldehydes (e.g. 4-[18F]fluorobenzaldehyde) with an aminooxy functionality in the peptide (10). However, using 4-[18F]fluorobenzaldehyde as the prosthetic group, invariably increased ligand lipophilicity, which often leads to unfavorable characteristics in vivo. It has since been shown that carbohydration is a powerful method to reduce lipophilicity of small radiolabeled peptides, resulting in improved pharmacokinetics of the radioligand (11–14). Recently, the direct conjugation of [18F]FDG as a strategy for simultaneous carbohydration and labeling of a RGD peptide was investigated and resulted in good pharmacokinetics (15,16). However, this approach requires the use of carrier-free [18F]-FDG, necessitating reversed phase high-performance liquid chromatography (RP-HPLC) purification of [18F]-FDG before conjugation with the functionalized peptide, further increasing the length of time of preparation.

The Huisgen 1,3-dipolar cycloaddition reaction for radiofluorination of the dimeric RGD peptide E[c(RGDyK)] was explored and reported by Li and co-workers (17). Although, click-labeled [18F]-FPTA-RGD2 could be obtained with 27% non-decay-corrected labeling yield and exhibited favorable in vivo pharmacokinetics, this method requires azeotropic drying of the fluoride, resulting in a time-consuming multistep procedure. In addition,
new $^{18}$F-labeling strategies based on fluorine-silicon (18–24), fluorine-boron (25), and fluorine-phosphorus (26) have been developed.

In contrast to these laborious radiofluorination methods, McBride et al. developed a direct aqueous $^{18}$F-labeling method wherein $^{18}$F is first attached to Al$^{3+}$. The Al$^{18}$F$^{2+}$ is then bound to a chelator attached to a peptide, forming a stable Al$^{18}$F-chelate peptide complex in an efficient 1-pot process (27).

Here, we describe the $^{18}$F-labeling of 1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid (NODAGA)-E-[c(RGDfK)]$_2$, using this new Al$^{18}$F labeling method. The tumor targeting characteristics of $^{18}$F-NODAGA-E-[c(RGDfK)]$_2$ are compared to those of $^{68}$Ga- and $^{111}$In-labeled NODAGA-E-[c(RGDfK)]$_2$ both in vitro and in vivo.

2. RESULTS AND DISCUSSION

2.1. Radiolabeling

Al$^{18}$F-labeled NODAGA-E-[c(RGDfK)]$_2$ was obtained with an overall labeling efficiency of 20%. A radiochemical purity of >98% was obtained after solid phase extraction on a hydrophilic-lipophilic balance (HLB) cartridge (Figure 1A). NODAGA-E-[c(RGDfK)]$_2$ was labeled with $^{68}$Ga and $^{111}$In with a labeling efficiency of 82% and 91% respectively. HLB cartridge purification of these increased the radiochemical purity to 85% and 95% respectively (Figures 1B–C). Specific activities for Al$^{18}$F-, $^{68}$Ga- and $^{111}$In-NODAGA-E-[c(RGDfK)]$_2$ were 1.8 MBq/nmol, 5.5 MBq/nmol and 2.2 MBq/nmol respectively. For SPECT imaging a higher specific activity was used and $^{111}$In-NODAGA-E-[c(RGDfK)]$_2$ was prepared with a specific activity of 40 MBq/nmol.

2.2. Octanol/Water partition coefficient

The lipophilicity of the Al$^{18}$F-, $^{68}$Ga- and $^{111}$In-labeled NODAGA-E-[c(RGDfK)]$_2$ was determined using the octanol-water partition coefficients. The Log P$_{octanol/PBS}$ values of the lipophilic radiolabeled dimeric RGD peptides are given in Table 1.

2.3. Competitive Binding Assay

The affinity of NODAGA-E-[c(RGDfK)]$_2$ and DOTA-E-[c(RGDfK)]$_2$ for integrin $\alpha_v\beta_3$ were determined in a solid-phase competitive binding assay (Figure 2). Binding of $^{111}$In-labeled DOTA-E-[c(RGDfK)]$_2$ to integrin $\alpha_v\beta_3$ was displaced by NODAGA-E-[c(RGDfK)]$_2$ and DOTA-E-[c(RGDfK)]$_2$ in a concentration-dependent manner. The IC$_{50}$ values were in the sub-nanomolar range (0.043 ± 0.037 and 0.037 ± 0.085 nM, respectively), indicating equally high binding affinity as well as specific binding of both NODAGA-E-[c(RGDfK)]$_2$ and DOTA-E-[c(RGDfK)]$_2$ to integrin $\alpha_v\beta_3$.

2.4. Biodistribution Studies

The results of the biodistribution studies of Al$^{18}$F-, $^{68}$Ga- and $^{111}$In-labeled NODAGA-E-[c(RGDfK)]$_2$ are summarized in Figures 3A–C. Al$^{18}$F-NODAGA-E-[c(RGDfK)]$_2$ cleared rapidly from the blood with 0.03 ± 0.01 percentage injected dose/gram (%ID/g) in the blood at 2 h p.i.. A similarly low blood level at 2 h p.i. was found for $^{68}$Ga- and $^{111}$In-labeled...
NODAGA-E-[c(RGDfK)]_2. Tumor uptake of $^{18}$F-labeled NODAGA-E-[c(RGDfK)]_2 ($3.44 \pm 0.20 \% \text{ID/g}, 2 \text{ h p.i.}$) was significantly lower than that of $^{68}$Ga-labeled NODAGA-E-[c(RGDfK)]_2 ($6.26 \pm 0.76 \% \text{ID/g}; P < 0.001$) or $^{111}$In-labeled NODAGA-E-[c(RGDfK)]_2 ($4.99 \pm 0.64 \% \text{ID/g}; P < 0.01$). Coinjection of an excess of unlabeled NODAGA-E-[c(RGDfK)]_2 (100 μg) along with $^{18}$F-labeled NODAGA-E-[c(RGDfK)]_2 resulted in a significantly reduced radioactivity concentration in the tumor ($0.85 \pm 0.13 \% \text{ID/g}; P < 0.001$), indicating that uptake of the major fraction of radiolabeled NODAGA-E-[c(RGDfK)]_2 preparation in the tumor was integrin $\alpha_v \beta_3$-mediated. Uptake of $^{18}$F-NODAGA-E-[c(RGDfK)]_2 in non-target organs like intestine, spleen, stomach and liver was also reduced in the presence of an excess of non-labeled RGD peptide ($P < 0.001$), indicating that the uptake in these tissues was also at least partly $\alpha_v \beta_3$-mediated. This was similar for the $^{68}$Ga- and $^{111}$In-labeled analogues. Kidney uptake of $^{18}$F-NODAGA-E-[c(RGDfK)]_2 ($2.87 \pm 0.96 \% \text{ID/g}$) was not blocked by an excess of non-radiolabeled RGD peptide ($2.58 \pm 0.40 \% \text{ID/g}; P=0.094$). Femur uptake of $^{18}$F-NODAGA-E-[c(RGDfK)]_2 was negligible, indicating good in vivo stability of $^{18}$F-NODAGA-E-[c(RGDfK)]_2.

### 2.5. Micro-PET/CT and -SPECT/CT

Fused PET/CT (Figure 4A, C) and SPECT/CT (Figure 4E) scans show images that were in line with the biodistribution data. On these scans, SK-RC-52 tumors were clearly visualized with $^{18}$F- and $^{68}$Ga-labeled NODAGA-E-[c(RGDfK)]_2 and $^{111}$In-NODAGA-E-[c(RGDfK)]_2 respectively. The integrin $\alpha_v \beta_3$ specificity of all three radiolabeled NODAGA-E-[c(RGDfK)]_2 tracers in vivo was demonstrated in a blocking experiment where the tracer was coinjected with an excess of nonradiolabeled NODAGA-E-[c(RGDfK)]_2. Addition of cold excess of NODAGA-E-[c(RGDfK)]_2 resulted in decreased tumor accumulation of the tracer compared to radiolabeled NODAGA-E-[c(RGDfK)]_2 (Figures 4B, D and F). $^{18}$F-, $^{68}$Ga- and $^{111}$In-NODAGA-E-[c(RGDfK)]_2 also showed high retention in the kidneys, from which could be deduced that $^{18}$F was stably chelated by NODAGA due to low uptake in the skeleton.

### 2.6. Discussion

In this study, we used the optimized $^{18}$F-labeling method (27,28) for the radiofluorination of NODAGA-E-[c(RGDfK)]_2 and evaluated this radiotracer in in vitro and in vivo studies. The in vitro and in vivo characteristics of this $^{18}$F-labeled dimeric RGD peptide were directly compared with its $^{68}$Ga-and $^{111}$In-labeled counterparts.

Here, it was shown that $^{18}$F-radiolabeled E-[c(RGDfK)]_2 via NODAGA increased the lipophilicity of the peptide. This was tested as it often leads to unfavorable characteristics in vivo. However the degree of lipophilicity did not alter the preferred route of excretion, which as determined from the biodistribution studies, was renal.

Using NODAGA allowed for the labeling of multiple isotopes and subsequent comparison of images obtained from PET and SPECT. For the labeling with $^{68}$Ga (29) and $^{111}$In, NODAGA was deemed a suitable chelator. However as shown in a study investigating the use of different chelators, NODAGA turned out not to be an adequate chelator for $^{18}$F (30), although labeling efficiencies were similar to those found for $^{18}$F-1,4,7-
triazacyclononane-1,4,7-triacetic acid (NOTA)-PRGD₂ (31). The relatively low radiolabeling yields obtained with Al¹⁸F-NODAGA-E-[c(RGDfK)]₂ might result from the N₃O₃ donor set of NODAGA. This N₃O₃ donor set (hexadentate) provides a suitable environment for the formation of highly stable aluminum chelates (32). However, as the coordination sphere of aluminum is saturated, access for fluorine was limited. D’Souza and colleagues demonstrated that N₃O₂ donor set (pentadentate) ligands possess a single coordination site for binding fluorine and thus might provide a more suitable chelator for all three radionuclides, resulting in a more efficient Al¹⁸F-labeling procedure (33).

In a competitive binding assay using isolated integrin αᵥβ₃ receptors, the IC₅₀ values of the NODAGA-conjugated and 1,4,7,10-tetra-azacyclododecane-N,N’,N”,N””-tetraacetic acid (DOTA)-conjugated RGD peptide were determined to be within the subnanomolar range and were not significantly different from each other. Recently, Knetsch and coworkers compared the IC₅₀ value of a NODAGA- and DOTA-conjugated RGD peptide (29). In their study, the affinity of both tracers for αᵥβ₃ integrin was also similar, showing that the replacement of the DOTA chelator with the NODAGA chelator has no influence on binding affinity and receptor-specific binding. It had already been demonstrated in previous studies that DOTA-conjugation to a monomeric RGD peptide had no influence on the affinity for integrin αᵥβ₃ (34), which is in line with our observation that NODAGA-E-[c(RGDfK)]₂ has a similar affinity to integrin αᵥβ₃ as the DOTA-conjugated analogue.

In the subcutaneous SK-RC-52 xenograft model, Al¹⁸F-NODAGA-E-[c(RGDfK)]₂ showed specific tumor uptake (3.44 ± 0.20 %ID/g) at 2 h p.i. and good in vivo stability. This has not previously been determined for Al¹⁸F-labeled compounds via the NODAGA chelator. Tumor uptake of this Al¹⁸F-labeled RGD peptide was significantly lower than that of the ⁶⁸Ga- and ¹¹¹In-labeled analogs (5.78 ± 0.76 %ID/g and 4.99 ± 0.64 %ID/g, respectively). Nonetheless, at 2 h p.i. the tumor/muscle ratio uptake of Al¹⁸F-NODAGA-E-[c(RGDfK)]₂ obtained here was at least as high as those reported by Liu et al. and Lang et al. who determined the uptake of Al¹⁸F-NOTA-PRGD₂ in integrin αᵥβ₃-expressing U87MG glioma tumor model (35,36). Whether the fast radiofluorination outweighs the relatively low tumor uptake of Al¹⁸F-NODAGA-E-[c(RGDfK)]₂ depends on the situation and requirements for the use of a radiotracer in general. This study does however show that a vector molecule can be radiofluorinated with Al¹⁸F via NODAGA in a quick fashion and still maintain specific in vivo binding capabilities to its target.

Non-tumor tissues also showed specific uptake of Al¹⁸F-NODAGA-E-[c(RGDfK)]₂, suggesting integrin αᵥβ₃ expression in these tissues. Indeed, β₃ expression in colon, pancreas and lung tissues has previously been described for rodents as well as for humans (37). Al¹⁸F-labeled NODAGA-E-[c(RGDfK)]₂ cleared rapidly from the blood, resulting in high tumor-to-blood ratios at 2 h p.i., namely 120 ± 30.

The Al¹⁸F method is a fast (45 min) radiofluorination strategy that does not affect the pharmacokinetics of the dimeric RGD peptide and the NODAGA chelator allows that the peptide can be labeled with ⁶⁸Ga and ¹¹¹In too. The commonly used method for efficient ¹⁸F-labeling of peptides consists of two steps: first the preparation of ¹⁸F-labeled synths (prosthetic groups), followed by subsequent conjugation to a peptide or protein. In
general, this fluorination is based on a nucleophilic substitution that requires time-consuming azeotropic drying of the $^{18}$F-fluoride-kryptofix complex. The total synthesis and formulation time for these methods ranges from 1 to 3 h. The Al$^{18}$F-method used here however is based on a chelator-derivatized peptide making this labeling method easy and versatile.

3. CONCLUSIONS

The Al$^{18}$F-method combines the ease of chelator-based radiolabeling methods with the advantages of $^{18}$F, e.g., half-life, availability and low positron energy. The Al$^{18}$F-labeled NODAGA-E-[c(RGDfK)]$_2$ could be synthesized in less than 45 min without the need for azeotropic drying nor the need to synthesize a synthon. The results of the biodistribution study of the Al$^{18}$F-, $^{68}$Ga- and $^{111}$In-labeled NODAGA-E-[c(RGDfK)]$_2$ peptide are comparable, despite the low radiolabeling yield of Al$^{18}$F-NODAGA-E-[c(RGDfK)]$_2$, and we hereby conclude that the Al$^{18}$F-labeled NODAGA-E-[c(RGDfK)]$_2$ peptide is a suitable radiotracer for the non-invasive in vivo visualization of integrin $\alpha_v\beta_3$ expression in SK-RC-52 xenografts.

4. EXPERIMENTAL

4.1. Synthesis of NODAGA-conjugated dimeric RGD peptide

NODAGA(tBu)$_3$ (CheMatech, Dijon, France) was activated with O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and the pH adjusted to >8 with diisopropylethylamine (DIPEA). Subsequently, the dimeric RGD peptide E-[c(RGDfK)]$_2$ (Peptides International, Inc., Louisville, KY, USA) was added and the reaction was performed in dimethylformamide (DMF) at room temperature for at least 4.5 h. The tBu-protecting groups were cleaved in 95% trifluoroacetic acid (TFA) for 5 h at room temperature. NODAGA-E-[c(RGDfK)]$_2$ was ultimately purified by RP-HPLC. The structural formula of NODAGA-E-[c(RGDfK)]$_2$ is shown in Figure 5.

4.2. Radiolabeling

$^{18}$F labeling—NODAGA-E-[c(RGDfK)]$_2$ was radiolabeled with $^{18}$F essentially as described by Laverman et al (38). Briefly, a Chromafix cartridge with 2–6 GBq $^{18}$F (BV Cyclotron VU, Amsterdam, The Netherlands) was washed with 3 mL of metal-free H$_2$O. $^{18}$F was eluted from the cartridge with 100 µL of 0.9% NaCl. Subsequently, 8.5 µL 2 mM AlCl$_3$ in 0.1 M sodium acetate buffer (pH 4) per GBq of $^{18}$F was added to $^{18}$F. Finally, 80 µL of this Al$^{18}$F solution was added to 400 µL MeCN and 20 µL NODAGA-E-[c(RGDfK)]$_2$ in 0.5 M sodium acetate (pH 4.1; 5 µg/µL). The reaction mixture was heated at 100 °C for 15 min. The radiolabeled peptide was purified by RP-HPLC. Al$^{18}$F-NODAGA-E-[c(RGDfK)]$_2$-containing fractions were collected and diluted 10-fold with H$_2$O and purified on an Oasis$^\text{®}$ HLB cartridge to remove acetonitrile and TFA. In brief, the fraction was applied on the cartridge and the cartridge was washed with 3 × 1 mL H$_2$O. The radiolabeled peptide was then eluted with 500 µL 50% EtOH. Before injection into mice, the EtOH was evaporated at 95 °C and the peptide was diluted with 0.5% bovine serum albumin (BSA) in phosphate-
buffered saline (PBS). Finally, 0.4 MBq (0.4 µg, 0.24 nmol) or 3–10 MBq (2.7–9.1 µg, 1.6–5.5 nmol) was used per mouse for the biodistribution or imaging studies, respectively.

**68Ga labeling**—68Ga was obtained from an 1850 MBq 68Ge/68Ga generator (IGG-100, Eckert & Ziegler, Berlin, Germany). The 68Ga was eluted with 0.1 M HCl (Ultrapure, J.T. Baker, Deventer, The Netherlands) using an Econo Pump (Bio-Rad, Hercules, CA, USA) at 1 mL/min. Five 1 mL fractions were collected and an aliquot of the fraction containing the highest activity was used for radiolabeling. 68Ga-labeled NODAGA-E-[c(RGDfK)]2 was prepared by adding 40 µL of 2.5 M HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) to 10 µL of the peptide dissolved in 2.5 M HEPES (2 µg/µL). Then, 500 µL of the 68Ga-eluate from the generator (200 MBq) was added. After 10 min at 100 °C, the 68Ga-labeled peptide was purified on an Oasis® HLB 1 cc (10 mg) cartridge as described above. Before injection into mice, the EtOH was evaporated at 95 °C and the peptide was diluted with 0.5% BSA/PBS. Finally, 0.4 MBq (0.1 µg, 0.06 nmol) or 3–10 MBq (0.9–3 µg, 0.54–1.8 nmol) was used per mouse for the biodistribution or imaging studies, respectively.

**111In labeling**—For 111In-labeling, NODAGA-E-[c(RGDfK)]2 was dissolved 0.1 M 2-(N-morpholino)ethanesulfonic acid, pH 5.5 (MES). Subsequently, 1–20 MBq 111InCl3 (Covidien, Petten, the Netherlands) per µg peptide was added. After 15 min at 100 °C, the 111In-labeled peptide was further purified on an Oasis® HLB 1 cc (10 mg) cartridge as described above. Before injection into mice, the EtOH was evaporated at 95 °C and the peptide was diluted with 0.5% BSA/PBS. Finally, 0.4 MBq (0.3 µg, 0.18 nmol) or 10–20 MBq (0.5–0.8 µg, 0.24–0.48 nmol) was used per mouse for the biodistribution or imaging studies, respectively.

**HPLC analysis**—Quality control was performed using RP-HPLC on a C-18 (Onyx monolithic, 4.6 mm × 100 mm; Phenomenex) column. The column was eluted at a flow rate of 3 mL/min with a gradient of 97% buffer A at 0–5 min and 80% buffer A to 75% buffer A at 5–20 min (buffer A, 0.1% v/v TFA in H2O; buffer B, 0.1% v/v TFA in acetonitrile). The preparations were analyzed on an Agilent 1200 system (Agilent Technologies, Palo Alto, CA, USA). Radioactivity was monitored using an in-line NaI radiodetector (Raytest GmbH, Straubenhardt, Germany). Elution profiles were analyzed using Gina-star software (Raytest GmbH, Straubenhardt, Germany). To obtain a radiochemical purity of >95%, Al18F-NODAGA-E-[c(RGDfK)]2 was purified with the same HPLC system using the same conditions.

**4.3. Octanol/Water partition coefficient**

To 0.5 mL of the radiolabeled peptide (1 kBq) in PBS, pH 7.4, 0.5 mL octanol was added. After vigorous vortexing for 2 min at room temperature, the two layers were separated by centrifugation (100 × g, 5 min). 100 µL samples were taken from each layer and radioactivity was measured in a well-type gamma counter (Wallac Wizard 3”, Perkin-Elmer, Waltham, MA). Log P values were calculated (n=3).
4.4. Cell culture

SK-RC-52 human renal carcinoma cells, stably expressing integrin αvβ3, were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, 1% penicillin/streptomycin and 1% glutamine (Invitrogen, Paisley, UK). Cells were maintained at 37 °C in a humidified 5% CO2 atmosphere and routinely passaged using a 0.25% trypsin/EDTA solution (Invitrogen).

4.5. Solid-phase αvβ3 integrin binding assay

The affinity of DOTA-E-[c(RGDfK)]2 and NODAGA-E-[c(RGDfK)]2 for integrin αvβ3 was determined using a solid-phase competitive binding assay as described previously (39). Briefly, 111In-labeled DOTA-E-[c(RGDfK)]2 was prepared as described above at 5 MBq/nmol. Plates coated with purified human integrin αvβ3 (150 ng/mL, Chemicon International, Temecula, CA, USA) were blocked with 1% BSA in PBS, before incubation with 1 kBq of 111In-DOTA-E-[c(RGDfK)]2 and appropriate dilutions (2×10^{-6} – 8×10^{-11} M) of NODAGA-E-[c(RGDfK)]2 or DOTA-E-[c(RGDfK)]2 at 37°C for 1 h. The amount of bound radioactivity was counted in a gamma counter. IC_{50} values of the E-[c(RGDfK)]2 peptides were calculated by nonlinear regression using GraphPad Prism software (version 5.03 for Windows). Each data point is the average of three determinations.

4.6. Biodistribution studies

In the right flank of 6- to 8-week-old female nude BALB/c mice, 2×10^6 SK-RC-52 cells in 200 µL medium was injected subcutaneously (s.c.). Two weeks after inoculation, when tumor sizes ranged from 60–90 mm^3 when measured by caliper, mice were injected intravenously (i.v.) with the Al^{18}F-, 68Ga- or 111In-labeled RGD peptide (0.4 MBq) in 200 µL 0.5% BSA in PBS. Mice were euthanized by CO2/O2 asphyxiation 2 h post-injection (p.i.) (n=3–5). Blood, tumor and the major organs and tissues were dissected, weighed and counted in a gamma counter. The percentage injected dose per gram (%ID/g) was determined for each sample. The receptor-mediated localization of the radiolabeled peptide was investigated by determining the biodistribution of the Al^{18}F-, 68Ga- or 111In-labeled peptide in the presence of an excess (100-fold) unlabeled peptide (n=3–5).

All animal experiments were approved by the local Animal Welfare Committee in accordance with Dutch legislation and carried out in accordance to their guidelines.

4.7. microPET/CT and microSPECT/CT

In the right flank of 6- to 8-week-old female nude BALB/c mice, 2×10^6 SK-RC-52 cells in 200 µL medium was injected s.c.. Two weeks after inoculation, when tumor sizes ranged from 60–90 mm^3 when measured by caliper, mice were injected i.v. with 3–10 MBq Al^{18}F-, 68Ga- labeled NODAGA-E-[c(RGDfK)]2 or 12–20 MBq of 111In-NODAGA-E-[c(RGDfK)]2. Cold excess NODAGA-E-[c(RGDfK)]2 (100-fold) was also injected. The 24.4 mmol/kg blocking dose of non-radiolabeled NODAGA-E-[c(RGDfK)]2 administered to the control mice to acquire the PET/CT did not induce any side effects. One hour p.i., mice were euthanized by CO2/O2 asphyxiation and scanned on either an animal PET/CT scanner (Inveon®, Siemens Preclinical Solutions, Knoxville, TN, USA) with an intrinsic spatial resolution of 2.5 mm (3) or the U-SPECT-II/CT (MILabs) (40). Mice were sacrificed prior
to imaging, rather than imaged under anaesthesia, to exclude movement artifacts as well as to allow comparison of the SPECT/CT images with the biodistribution data. The animals were then placed in a supine position in the scanner. Static PET or SPECT scans were acquired over 45 m, followed by a CT scan for anatomical reference (PET: spatial resolution 113 µm, 80 kV, 500 µA, SPECT: spatial resolution 160 µm, 65 kV, 615 µA). PET/CT scans were reconstructed using Inveon Acquisition Workplace software version 1.5 (Siemens Preclinical Solutions, Knoxville, TN, USA), using an ordered set expectation maximization-3D/maximum a posteriori (OSEM3D/MAP) algorithm with the following parameters: matrix 256 × 256 × 159, pixel size 0.43 × 0.43 × 0.8 mm³ and a beta-value of 1.5. SPECT/CT scans were reconstructed with software from MII Labs, using an ordered-subset expectation maximization algorithm, with a voxel size of 0.375 mm.

4.8. Statistical analysis

All mean values are given as ± standard deviation (SD). Statistical analysis was performed using a Welch’s corrected unpaired Student t test or one-way ANOVA using GraphPad InStat software (version 3.06; GraphPad Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Table 1

Log P values of radiolabeled NODAGA-E-[c(RGDfK)]$_2$ determined by octanol/water partition assays

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log P$_{octanol/PBS}$ ± SD</th>
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<tbody>
<tr>
<td>$^{18}$F-NODAGA-RGD$_2$</td>
<td>$-4.26 \pm 0.02$</td>
</tr>
<tr>
<td>$^{68}$Ga-NODAGA-RGD$_2$</td>
<td>$-4.50 \pm 0.01$</td>
</tr>
<tr>
<td>$^{111}$In-NODAGA-RGD$_2$</td>
<td>$-4.34 \pm 0.09$</td>
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