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ORIGINAL ARTICLE

PET imaging of $\alpha_v\beta_3$ integrin expression in tumours with ^{68}Ga-labelled mono-, di- and tetrameric RGD peptides

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Abstract
Purpose Due to the restricted expression of $\alpha_v\beta_3$ in tumours, $\alpha_v\beta_3$ is considered a suitable receptor for tumour targeting. In this study the $\alpha_v\beta_3$-binding characteristics of ^{68}Ga-labelled monomeric, dimeric and tetrameric RGD peptides were determined and compared with their ^{111}In-labelled counterparts.
Methods A monomeric (E-c(RGDfK)), a dimeric (E-[c(RGDfK)]$_2$) and a tetrameric (E{E[c(RGDfK)]$_2$}$_2$) RGD peptide were synthesised, conjugated with DOTA and radiolabelled with ^{68}Ga. In vitro $\alpha_v\beta_3$-binding characteristics were determined in a competitive binding assay. In vivo $\alpha_v\beta_3$-targeting characteristics of the compounds were assessed in mice with subcutaneously growing SK-RC-52 xenografts. In addition, microPET images were acquired using a microPET/CT scanner.
Results The IC_{50} values for the Ga(III)-labelled DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ were 23.9±1.22, 8.99±1.20 and 1.74±1.18 nM, respectively, and were similar to those of the In(III)-labelled mono-, di- and tetrameric RGD peptides (26.6±1.15, 3.34±1.16 and 1.80±1.37 nM, respectively). At 2 h post-injection, tumour uptake of the ^{68}Ga-labelled mono-, di- and tetrameric RGD peptides (3.30±0.30, 5.24±0.27 and 7.11±0.67%ID/g, respectively) was comparable to that of their ^{111}In-labelled counterparts (2.70±0.29, 5.61±0.85 and 7.32±2.45%ID/g, respectively). PET scans were in line with the biodistribution data. On all PET scans, the tumour could be clearly visualised.
Conclusion The integrin affinity and the tumour uptake followed the order of DOTA-tetramer > DOTA-dimer > DOTA-monomer. The ^{68}Ga-labelled tetrameric RGD peptide has excellent characteristics for imaging of $\alpha_v\beta_3$ expression with PET.

Keywords $\alpha_v\beta_3$ integrin receptor · MicroPET · Multimers · Angiogenesis · ^{68}Ga

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Introduction

Angiogenesis, the formation of new blood vessels from existing ones, is an essential process if solid tumours are to grow beyond 2–3 mm^3, since diffusion is no longer

sufficient to supply the tissue with oxygen and nutrients [1]. Tumour-induced angiogenesis is a complex multistep process that follows a characteristic cascade of events mediated and controlled by growth factors, cellular receptors and adhesion molecules [2–4].

Activated endothelial cells express the integrin $\alpha_v\beta_3$ receptor, whereas this integrin receptor is absent on quiescent endothelial cells. In addition, $\alpha_v\beta_3$ is expressed on the cell membrane of various tumour cell types such as ovarian cancer, neuroblastoma, breast cancer and melanoma. $\alpha_v\beta_3$ Integrin expressed on endothelial cells modulates cell migration and survival during angiogenesis, whereas $\alpha_v\beta_3$ integrin expressed on carcinoma cells potentiates metastasis by facilitating invasion and movement across blood vessels. Due to this restricted expression of $\alpha_v\beta_3$ in tumours, $\alpha_v\beta_3$ is considered a suitable candidate for tumour targeting [5]. Radiolabelled ligands for this integrin could be used as tracers to noninvasively visualise $\alpha_v\beta_3$ expression in tumours. Noninvasive visualisation of $\alpha_v\beta_3$ expression might provide information about the angiogenic process and the responsiveness of a tumour to antiangiogenic drugs. Furthermore, noninvasive determination of $\alpha_v\beta_3$ expression potentially can be used to monitor the effect of antiangiogenic drugs in patients.

The $\alpha_v\beta_3$ integrin is a transmembrane protein consisting of two noncovalently bound subunits, α and β. This integrin can bind to the arginine-glycine-aspartic acid (RGD) amino acid sequence present in extracellular matrix proteins such as vitronectin, fibrinogen and laminin [6]. Based on the RGD tripeptide sequence a series of small peptides have been designed to antagonise the function of the $\alpha_v\beta_3$ integrin [7]. Especially the cyclic peptide derivatives have a relatively high affinity for the $\alpha_v\beta_3$ integrin. Radiolabelled cyclic RGD peptides have the potential for early detection of rapidly growing tumours and noninvasive visualisation of tumour metastasis and therapeutic response in cancer patients.

Over the last several years, significant progress has been made in the development of $\alpha_v\beta_3$-targeting radiotracers for the visualisation of $\alpha_v\beta_3$ expression in tumours by single photon emission computed tomography (SPECT) and positron emission tomography (PET).

Haubner and coworkers developed the first $\alpha_v\beta_3$-specific PET tracer [^{18}F]Galacto-RGD [8], a glycosylated cyclic pentapeptide, which demonstrated that PET with [^{18}F]Galacto-RGD enables receptor-specific monitoring of $\alpha_v\beta_3$ expression in murine tumour models. It was the first PET tracer applied in patients with cancer which could successfully image $\alpha_v\beta_3$ expression with good tumour to background ratios [9]. In addition, a strong correlation between tracer uptake and $\alpha_v\beta_3$ expression was observed [10]. In the mean time, another RGD-based PET tracer, [^{18}F]AH111585, has been developed and evaluated in breast cancer patients [11]. [^{18}F]AH111585 was demonstrated to be safe and metabolically stable and could visualise tumours in breast cancer patients. Although both ^{18}F-labelled RGD monomers bind specifically to the $\alpha_v\beta_3$ integrin, their clinical translation is partly hampered by the time-consuming multistep ^{18}F-labelling procedure and the necessity of a cyclotron facility to produce this PET isotope.

An interesting alternative is the use of the generator-produced radionuclide ^{68}Ga. The application of ^{68}Ga-labelled peptides has attracted considerable interest for cancer imaging, because of its physical characteristics [12]. ^{68}Ga decays at 89% through positron emission of 1.92 MeV (max. energy) and can be eluted from an in-house ^{68}Ge/^{68}Ga generator (^{68}Ge, $T_{1/2}$=270.8 days) which renders it independent of an on-site cyclotron. Furthermore, with a half-life of 68 min, ^{68}Ga is also compatible with the pharmacokinetics of many peptides.

Recently, Decristoforo et al. compared the in vitro and in vivo properties of [^{68}Ga]DOTA-RGD with that of the corresponding [^{111}In]DOTA-RGD [13]. They found that especially in the blood and also in tumour the uptake of the ^{68}Ga-labelled peptide was higher than the ^{111}In-labelled counterpart which could be explained by different complex stabilities for the Ga-DOTA and the In-DOTA complexes, resulting in transmetallation of gallium to transferrin. The group in Stanford conjugated RGD monomers and multimers to *p*-SCN-Bn-NOTA and labelled them with ^{68}Ga for imaging integrin expression in a U87MG glioblastoma xenograft model [14, 15]. They clearly observed by increasing RGD units an increase in $\alpha_v\beta_3$ affinity and tumour uptake. In addition, it was possible to increase the $\alpha_v\beta_3$ receptor-binding affinity of the RGD dimer by coupling the two RGD peptide units via Gly_3 and PEG_4 linkers.

Here, we radiolabelled mono-, di- and tetrameric RGD peptides with ^{68}Ga and studied the tumour targeting potential of these peptides in vitro and in vivo. This is the first study in which mono-, di- and tetrameric RGD peptides labelled with ^{68}Ga, for PET imaging of $\alpha_v\beta_3$ expression, are directly compared with their ^{111}In-labelled counterparts.

Materials and methods

Synthesis of DOTA-conjugated RGD peptides

The mono-, di- and tetrameric RGD peptides were synthesised using Fmoc-based solid-phase peptide synthesis (SPPS) as described previously [16–19]. The structural formulas of DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ are shown in Fig. 1.

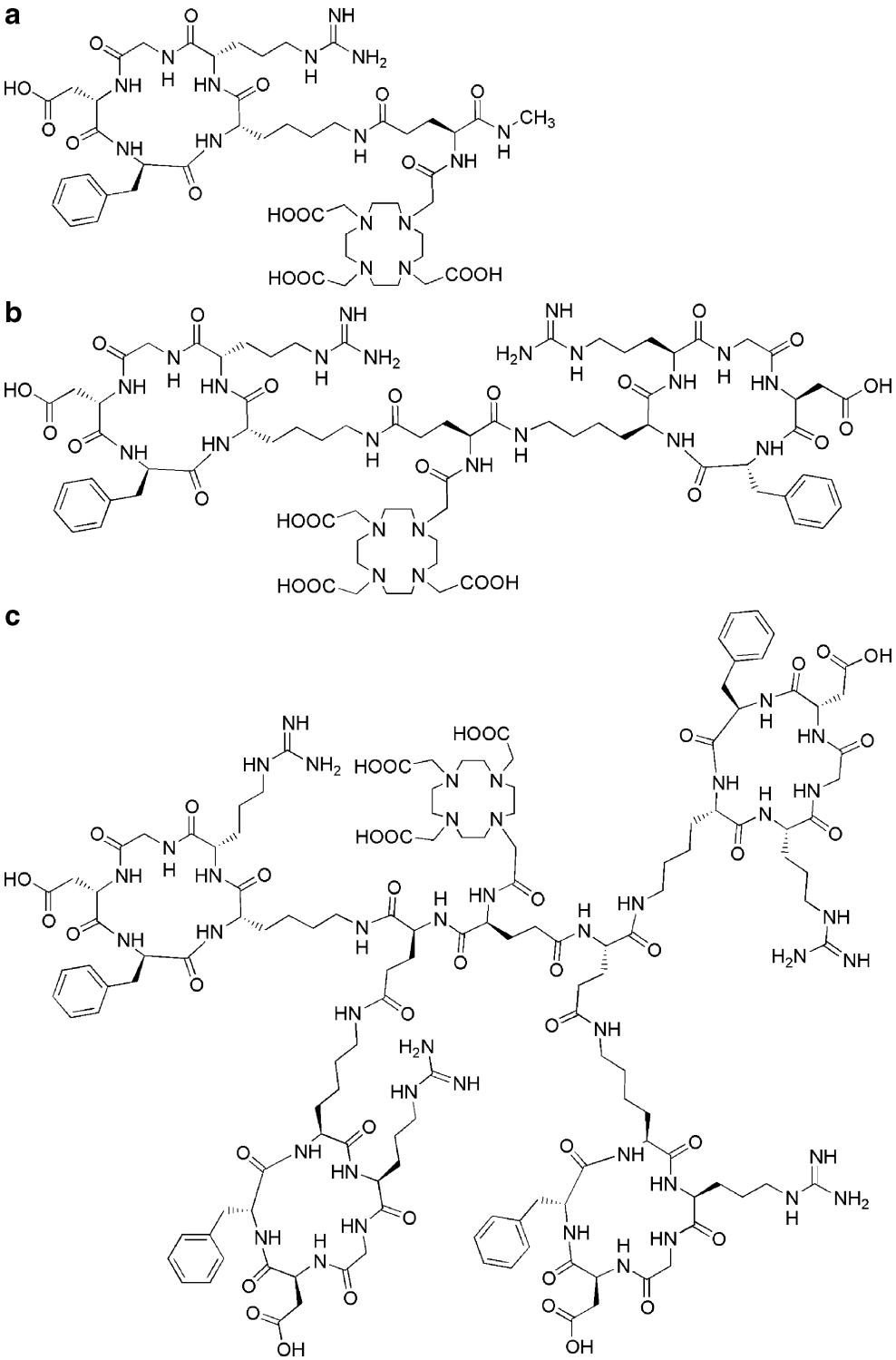

Fig. 1 **a** Structural formula of the DOTA-conjugated monomeric RGD peptide, DOTA-E-c(RGDfK). **b** Structural formula of the DOTA-conjugated dimeric RGD peptide, DOTA-E-[c(RGDfK)]$_2$. **c** Structural formula of the DOTA-conjugated tetrameric RGD peptide DOTA-E{E[c(RGDfK)]$_2$}$_2$

Radiolabelling of the RGD peptides

^{111}*In labelling*

DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ were radiolabelled with $^{111}InCl_3$ as described previously [17]. Briefly, 18.5 MBq $^{111}InCl_3$ (Mallinckrodt, Petten, The Netherlands) was added to 5–20 nmol DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ dissolved in 300 or 500 µl ammonium acetate buffer, pH 6.0, containing 0.6 mg/ml gentisic acid. The mixtures were heated at 100°C for 15 min. For in vitro and in vivo studies, the reaction mixtures were diluted in phosphate-buffered saline (PBS).

^{68}Ga labelling

DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ were labelled with $^{68}GaCl_3$ eluted from a TiO_2-based 1,110 MBq ^{68}Ge/^{68}Ga generator (Cyclotron Co. Ltd., Obninsk, Russia) using 0.1 M HCl (Ultrapure, J.T. Baker, Deventer, The Netherlands). Five 1-ml fractions were collected and an aliquot of the second fraction was used for labelling the peptides.

^{68}Ga-labelled DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ were prepared by adding 250 μl 1 M HEPES, pH 7.0, solution to 10–28 μl of the peptide dissolved (1 μg/μl) in 0.25 M ammonium acetate, pH 5.5. Then, the second millilitre eluted from the generator (315–365 MBq) was added. After 20 min at 95°C, the ^{68}Ga-labelled peptides were further purified on an Oasis® HLB (1 cm^3, 30 mg) cartridge (Waters, Milford, MA, USA). After applying the sample on the cartridge, the cartridge was washed with 3 × 1 ml H_2O and eluted with 200 μl 25% EtOH in H_2O (v/v). For in vitro and in vivo studies, the eluate was diluted to <5% EtOH in PBS.

Analysis

The radiochemical purity was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) on an Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) using a C18 column (RX-C18, 4.6×250 mm, Zorbax) eluted with a gradient mobile phase [0–5 min 97% buffer A, 5-15 min 97% buffer A to 0% buffer A, buffer A=0.1% trifluoroacetic acid (TFA) in H_2O, buffer B=0.1% TFA in acetonitrile] at 1 ml/min. The radioactivity of the eluate was monitored using an in-line NaI radiodetector (Raytest GmbH, Straubenhardt, Germany). Elution profiles were analysed using Gina Star software (version 2.18, Raytest GmbH, Straubenhardt, Germany). An additional quality control after purification on an HLB cartridge was performed by instant thin-layer chromatography (ITLC) using TEC-Control™ chromatography strips (Biodex Medical Systems, Shirley, NY, USA). The strips were developed using two different mobile phases. Mobile phase I was 0.1 M CH_3COONH_4/0.1 M EDTA (1:1 v/v) and mobile phase II was 0.25 M CH_3COONH_4/MeOH (1:1 v/v). The strips were analysed using a Fujifilm BAS-1800II Scanner (Fuji Photo Film Co., Tokyo, Japan).

Octanol/water partition coefficient

To an Eppendorf tube filled with 0.5 ml of the radiolabelled peptide in PBS, pH 7.4, 0.5 ml octanol was added. After the tube was vigorously vortexed for 2 min at room temperature, the two layers were separated by centrifugation (100 *g*, 5 min). Then, 100-μl samples were taken from each layer and radioactivity was measured in a well-type gamma counter (Wallac Wizard 3", PerkinElmer, Waltham, MA, USA) and Log P values were calculated (n=3).

In vitro stability

The stability of the ^{111}In- and ^{68}Ga-labelled RGD peptides was determined by incubating the compounds in both PBS and human serum for 2 h at 37°C. Before analysis of the serum samples the serum proteins were precipitated by adding an equal volume of MeCN to the samples. Subsequently, serum samples were centrifuged for 5 min at 13,500 *g*. The PBS samples were analysed without any sample preparation. An aliquot of the serum and the PBS sample were injected onto HPLC.

Protein binding

To determine their serum protein-binding properties, the ^{68}Ga- and ^{111}In-labelled peptides were incubated in fresh human serum at 37°C. After 2 h, the samples were analysed with fast protein liquid chromatography (FPLC), using a BioSep-Sec-S 3000 column (300×4.60 mm, Phenomenex, Utrecht, The Netherlands) with an isocratic mobile phase (PBS, 1 ml/min).

Solid-phase $\alpha_v\beta_3$ binding assay

The affinity of Ga(III)/In(III)-DOTA-E-c(RGDfK), Ga(III)/In(III)-DOTA-E-[c(RGDfK)]$_2$ and Ga(III)/In(III)-DOTA-E{E[c(RGDfK)]$_2$}$_2$ for $\alpha_v\beta_3$ was determined using a solid-phase competitive binding assay.

For the "cold" labelling of DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ with either Ga(III) or In(III), each of the peptides was dissolved in an aqueous solution. Subsequently, a 3 M excess of $InCl_3$ (Aldrich Chemical Company, Inc., Milwaukee, WI, USA) or $Ga(NO_3)_3$ (Sigma-Aldrich Chemie, Steinheim, Germany) was added. The Ga(III) or In(III) complexation was performed at room temperature overnight or at 40°C for 2 h, respectively.

^{111}In-labelled DOTA-E-[c(RGDfK)]$_2$ (3 MBq/μg) was used as the tracer in this assay. Microtiter 96-well vinyl assay plates (Corning B.V., Schiphol-Rijk, The Netherlands) were coated with 100 μl/well of a solution of purified human integrin $\alpha_v\beta_3$ (150 ng/ml) in Triton X-100 Formulation (Chemicon International, Temecula, CA, USA) in coating buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mM $MnCl_2$) for 17 h at 4°C. The plates were washed twice with binding buffer [0.1% bovine serum albumin (BSA) in coating buffer]. The wells were blocked for 2 h with 200 μl blocking buffer (1% BSA in coating buffer). The plates were washed twice with binding buffer. Then, 100 μl binding buffer containing

10 kBq of ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and appropriate dilutions (2×10^{-6}–8×10^{-11} M) of Ga(III)- or In(III)-labelled DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ in binding buffer were incubated in the wells at 37°C for 1 h. After incubation, the plates were washed three times with binding buffer. The wells were cut out and counted in a gamma counter. IC_{50} values of the RGD peptides were calculated by nonlinear regression using GraphPad Prism (GraphPad Prism 4.0, GraphPad Software, San Diego, CA, USA). Each data point is the average of three determinations.

Biodistribution studies

In the right flank of 6- to 8-week-old female nude BALB/c mice, 0.2 ml of a cell suspension of 2×10^6 cells/ml SK-RC-52 cells was injected subcutaneously (s.c.). Two weeks after inoculation of the tumour cells, mice were injected intravenously (i.v.) with the ^{111}In- or ^{68}Ga-labelled RGD peptides (0.2–0.89 nmol) in 0.2 ml PBS+0.5% BSA. Mice were killed by CO_2 asphyxiation 2 h post-injection (p.i.) (five mice/group). Blood, tumour and the major organs and tissues were collected, weighed and counted in a gamma counter. The percentage injected dose per gram (%ID/g) was determined for each sample.

The receptor-mediated localisation of the radiolabelled RGD peptides was investigated by determining the biodistribution of the ^{111}In- or ^{68}Ga-labelled compounds in the presence of an excess (100-fold excess) unlabelled DOTA-E-[c(RGDfK)]$_2$ (n=3). DOTA-E-[c(RGDfK)]$_2$ was used for these "blocking studies" as in our previous studies this compound was demonstrated to be $\alpha_v\beta_3$ specific [20, 21]. All animal experiments were approved by the local Animal Welfare Committee in accordance with the Dutch legislation and carried out in accordance with their guidelines.

MicroPET imaging

Mice with s.c. SK-RC-52 tumours were injected i.v. with 10 MBq ^{68}Ga-labelled mono-, di- or tetrameric RGD peptide per mouse (0.89 nmol). Two hours after the injection of the peptide, mice were scanned on an animal PET/CT scanner (Inveon®, Siemens Preclinical Solutions, Knoxville, TN, USA) with an intrinsic spatial resolution of 1.5 mm [22]. The animals were placed in a supine position in the scanner. PET emission scans were acquired over 15 min. CT images were acquired for anatomical correlation directly after PET imaging (spatial resolution 113 μm, 80 kV, 500 μA, exposure time 300 ms).

Scans were reconstructed using Inveon Acquisition Workplace software version 1.2 (Siemens Preclinical Solutions, Knoxville, TN, USA), using an ordered subset expectation maximisation 3-D/maximum a posteriori (OSEM3D/MAP) algorithm with the following parameters: matrix 256×256×159, pixel size 0.43×0.43×0.8 mm^3 and a beta value of 0.1.

Statistical analysis

All mean values are given ± standard deviation (SD). Statistical analysis was performed using the one-way analysis of variance. Bonferroni corrections for multiple comparisons were applied. The level of significance was set at $p<0.05$.

Results

Radiolabelling

RP-HPLC analysis indicated that the radiochemical purity of the ^{68}Ga- or ^{111}In-labelled DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ preparations used in these experiments ranged from 93 to 97%.

The HPLC chromatograms of ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ showed a single peak for each of the compounds with a retention time of 14, 26 and 15 min, respectively. Note that different gradients were used.

The ITLC profiles of ^{68}Ga-DOTA-E-c(RGDfK) (R_f=0.35), ^{68}Ga-DOTA-E-[c(RGDfK)]$_2$ (R_f=0.53) and ^{68}Ga-DOTA-E{E[c(RGDfK)]$_2$}$_2$ (R_f=0.08) after HLB purification demonstrated a purity of 97, 98 and 99%, respectively. The maximum specific activity of the ^{68}Ga-labelled mono-, di- and tetramer was 11.2 MBq/nmol.

Octanol/water partition coefficient

To determine the lipophilicity of the ^{68}Ga- and ^{111}In-labelled DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$, the octanol/water partition coefficients were determined. The Log $P_{octanol/water}$ values for the ^{68}Ga-labelled RGD mono-, di- and tetramer were −4.37±0.13, −4.04±0.15 and −3.76±0.07, respectively. The Log $P_{octanol/water}$ values of the ^{111}In-labelled RGD mono-, di- and tetramer were −4.38±0.25, −3.95±0.05 and −4.15±0.07, respectively.

In vitro stability

Determination of the stability of the ^{111}In- and ^{68}Ga-labelled RGD peptides indicated high stability of the compounds. There was no evidence of release of ^{68}Ga or ^{111}In from the peptides or radiolysis of any of the compounds in both PBS and human serum (data not shown). After 2 h incubation at 37°C more than 95% of the activity was still associated with the DOTA-conjugated cyclic peptides and no significant reduction was observed.

Protein binding

No differences in the protein-binding properties between the ^{68}Ga- and ^{111}In-labelled RGD peptides were observed by FPLC (data not shown). The protein-bound activity was negligible (<5%) after 2 h incubation in human serum for the ^{68}Ga- as well as for the ^{111}In-labelled peptides. For each of the peptides >95% of the activity eluted as a monomeric peak at 13 min.

Solid-phase $\alpha_v\beta_3$ binding assay

We determined the affinity of Ga(III)-DOTA-E-c(RGDfK), Ga(III)-DOTA-E-[c(RGDfK)]$_2$ and Ga(III)-DOTA-E{E[c(RGDfK)]$_2$}$_2$ and their In(III)-labelled analogues for integrin $\alpha_v\beta_3$ in a competitive binding assay. The results of these assays are summarised in Fig. 2. Binding of ^{111}In-labelled dimeric peptide, ^{111}In-DOTA-E-[c(RGDfK)]$_2$, to $\alpha_v\beta_3$ was competed by Ga(III)- or In(III)-labelled DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ in a concentration-dependent manner. The IC_{50} values were 23.9±1.22 nM, 8.99±1.20 nM and 1.74±1.18 nM for Ga(III)-labelled DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$, respectively (Table 1). The affinities of the In(III)-labelled mono-, di- and tetrameric RGD peptides were similar: 26.6±1.15 nM, 3.34±1.16 nM and 1.80±1.37 nM, respectively.

Biodistribution studies

The results of the biodistribution studies of both ^{111}In- and ^{68}Ga-labelled DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ are summarised in Fig. 3. DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ radiolabelled with either ^{68}Ga or ^{111}In all cleared rapidly from the blood. At 2 h p.i. the blood level of all compounds was below 0.4%ID/g. Tumour uptakes of the ^{68}Ga-labelled mono-, di- and tetrameric RGD peptides (3.30±0.30, 5.24±0.27 and 7.11±0.67%ID/g, respectively) were comparable to those of their ^{111}In-labelled counterparts (2.70±0.29, 5.61±0.85 and 7.32±2.45%ID/g, respectively). At 2 h p.i., the tumour uptake was significantly higher for the ^{68}Ga-labelled tetramer (7.11±0.67%ID/g), compared to that of the dimer (5.24±0.27%ID/g) and that of the monomer (3.30±0.30%ID/g). For the ^{111}In-labelled analogues, there was no difference in tumour uptake between the tetramer (7.32±2.45%ID/g) and dimer (5.61±0.85%ID/g), whereas the tumour uptake of the ^{111}In-labelled dimer was significantly higher than that of the ^{111}In-labelled monomer (2.70±0.29%ID/g).

Coinjection of an excess unlabelled DOTA-E-[c(RGDfK)]$_2$ (50 μg) along with 0.5 μg of ^{68}Ga- or ^{111}In-labelled DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ or DOTA-E{E[c(RGDfK)]$_2$}$_2$ resulted in a significantly reduced radioactivity concentration in the tumour, indicating that uptake of the major fraction of DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ in the tumour was $\alpha_v\beta_3$ mediated. Uptake in nontarget organs such as lung, spleen and intestine was also reduced in the presence of an excess of unlabelled RGD peptide, indicating that the uptake in these tissues was at least partly $\alpha_v\beta_3$ mediated. The kidney uptake of the ^{68}Ga- and ^{111}In-labelled monomer and dimer could partly be blocked. However, renal uptake of the ^{68}Ga- and ^{111}In-labelled tetramer could not be blocked. Kidney uptake was remarkably high for the ^{68}Ga-labelled tetramer compared to its ^{111}In-labelled analogue.

Fused PET and CT scans are shown in Fig. 4. PET scans were in line with the biodistribution data. On all PET scans, the tumour could be clearly visualised. The ^{68}Ga-labelled tetramer showed the highest tumour uptake compared to the monomer and dimer. All three tracers showed some uptake in the kidneys, especially the tetramer. On the other hand, the monomer demonstrated relatively high intestinal uptake. The ^{68}Ga-labelled tetramer showed the highest tumour to background ratio and therefore this tracer is the most suitable for imaging $\alpha_v\beta_3$ expression by PET.

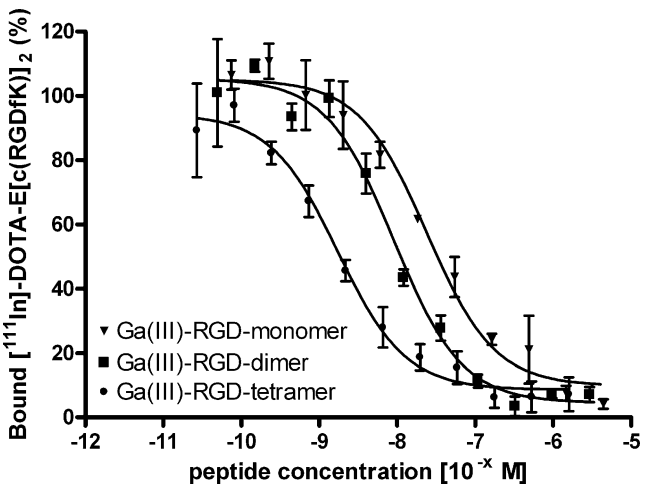

Fig. 2 Competition of specific binding of ^{111}In-DOTA-E-[c(RGDfK)]$_2$ with Ga(III)-DOTA-E-c(RGDfK), Ga(III)-DOTA-E-[c(RGDfK)]$_2$ and Ga(III)-DOTA-E{E[c(RGDfK)]$_2$}$_2$

Discussion

In this study, the feasibility of using ^{68}Ga-labelled multimeric RGD peptides for radionuclide imaging of the $\alpha_v\beta_3$ integrin expression with PET was investigated. The radiolabelled mono-, di and tetrameric RGD peptides were very hydrophilic as demonstrated by their partition coefficients (Log $P_{octanol/water}$). The Log P values varied between −4.38±0.25 for the ^{111}In-labelled monomer and −3.76±0.07 for the ^{68}Ga-

Table 1 IC_{50} values arising from a competitive binding assay of Ga (III)- and In(III)-labelled RGD mono-, di- and tetramer

Compound	Ga(III)-labelled ± SD (nM)	In(III)-labelled ± SD (nM)
Monomer	23.9±1.22	26.6±1.15
Dimer	8.99±1.20	3.34±1.16
Tetramer	1.74±1.18	1.80±1.37

labelled tetramer. These values are even lower than the value found for [^{18}F]Galacto-RGD (Log P=−3.2) which is cleared almost exclusively via the kidneys [23]. The hydrophilicity of the ^{68}Ga-labelled monomer and dimer labelled with either ^{68}Ga or ^{111}In was not different. Only for the tetramer was the Log P value lower for the ^{111}In-labelled variant compared to its ^{68}Ga-labelled counterpart. Heppeler et al. demonstrated that for the complexation of gallium by DOTA four nitrogen atoms of the macrocycle and two oxygen atoms from the carboxylate groups are involved [24]. Thus, one carboxylic acid group of the DOTA chelator is not involved in complexation of ^{68}Ga. However, for ^{111}In it is assumed that the four nitrogen atoms of the DOTA macrocycle and the four oxygen atoms of the carboxylic groups are involved in the complexation. Despite the fact that the ^{68}Ga-DOTA complex has one more carboxylic acid group that is not involved in the complexation compared to the ^{111}In-DOTA complex, the ^{68}Ga-labelled analogue does not have an increased hydrophilicity.

The binding affinity of Ga(III)-DOTA-tetramer (IC_{50}= 1.74±1.18 nM), as determined in a solid-phase competitive binding assay, was about 5 times higher compared to Ga (III)-DOTA-dimer (IC_{50}=8.99±1.20 nM) and about 13 times higher compared to Ga(III)-DOTA-monomer (IC_{50}= 23.9±1.22 nM). The binding affinity of the In(III)-labelled monomeric (26.6±1.15 nM), dimeric (3.34±1.16 nM) and tetrameric RGD peptides (1.80±1.37 nM) was comparable.

In the s.c. SK-RC-25 renal cell carcinoma xenograft model, the tetrameric RGD peptide, labelled with either ^{68}Ga or ^{111}In, showed the highest tumour uptake. Thus, there is a relation between the binding affinity for $\alpha_v\beta_3$ and the accumulation of the compound in $\alpha_v\beta_3$-expressing tumours. All three RGD peptides of this study labelled with either ^{68}Ga or ^{111}In showed specific tumour uptake in

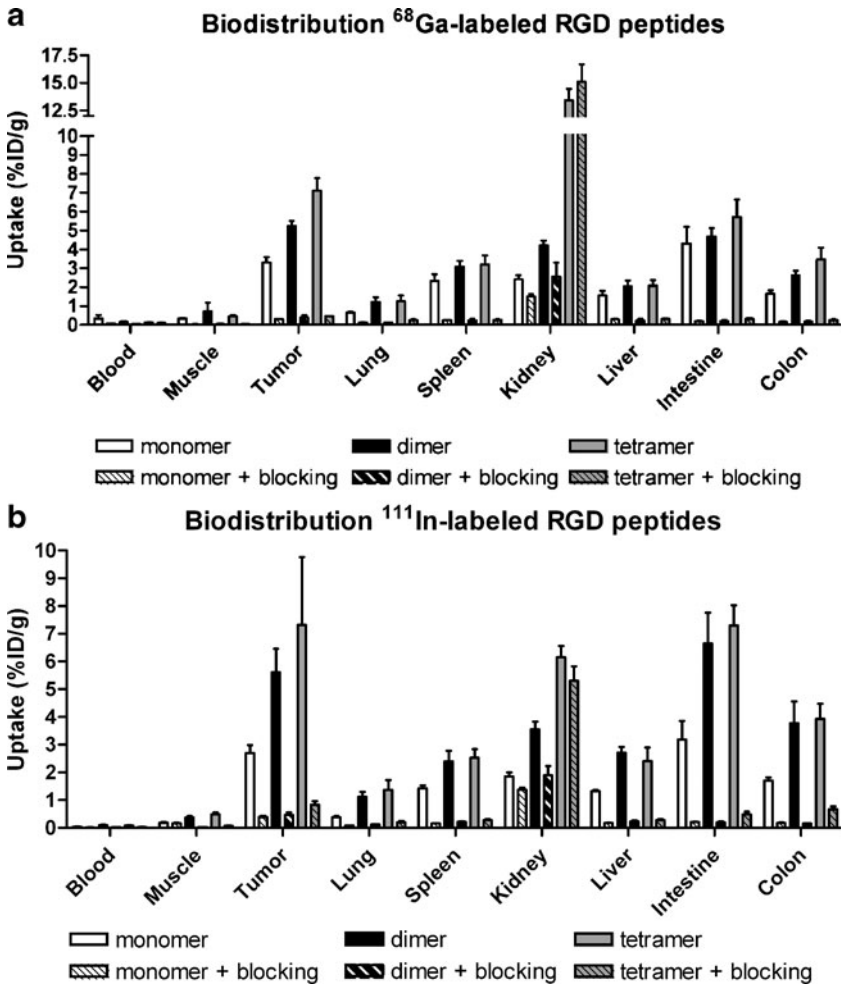

Fig. 3 **a** Biodistribution of [^{68}Ga]DOTA-E-c(RGDfK), [^{68}Ga]DOTA-E-[c(RGDfK)]$_2$ and [^{68}Ga]DOTA-E{E[c(RGDfK)]$_2$}$_2$ at 2 h p.i. in athymic mice with s. c. SK-RC-52 tumours in the absence (five mice/group) or presence (three mice/group) of an excess of DOTA-E-[c(RGDfK)]$_2$. **b** Biodistribution of [^{111}In] DOTA-E-c(RGDfK), [^{111}In] DOTA-E-[c(RGDfK)]$_2$ and [^{111}In]DOTA-E{E[c(RGDfK)]$_2$}$_2$ at 2 h p.i. in athymic mice with s. c. SK-RC-52 tumours in the absence (five mice/group) or presence (three mice/group) of an excess of DOTA-E-[c(RGDfK)]$_2$

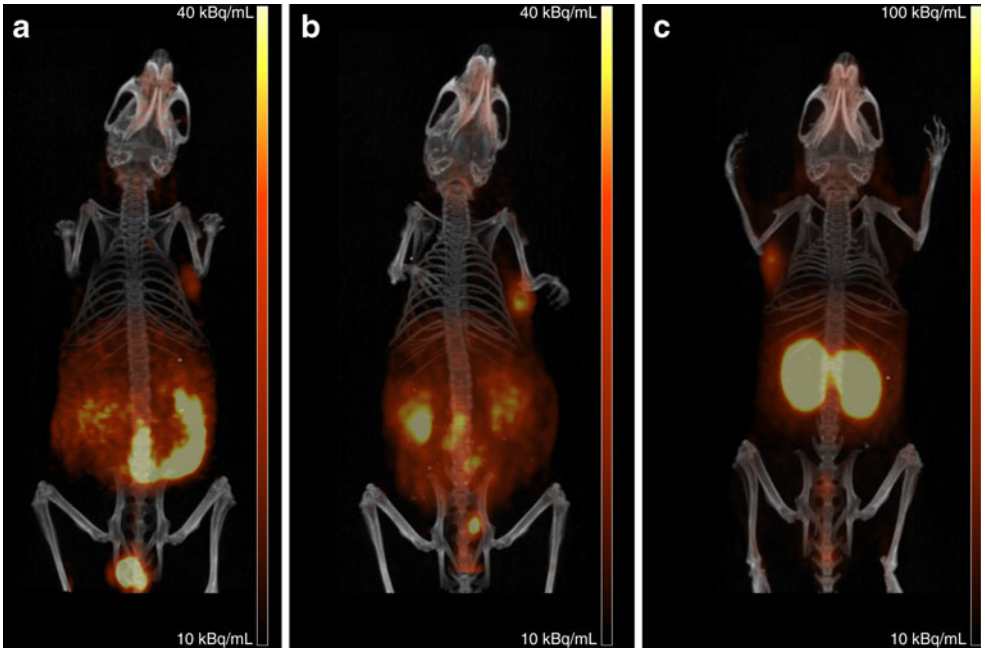

Fig. 4 Anterior 3-D volume rendering projections of fused PET and CT scans of mice with a s.c. growing SK-RC-52 tumour after i.v. injection of [^{68}Ga]DOTA-E-c(RGDfK) (**a**), [^{68}Ga]DOTA-E-[c(RGDfK)]$_2$ (**b**) or [^{68}Ga]DOTA-E{E[c(RGDfK)]$_2$}$_2$ (**c**). Scans were recorded at 2 h p.i.

athymic mice with s.c. SK-RC-52 tumours: in the presence of an excess of unlabelled DOTA-E-[c(RGDfK)]$_2$, the specificity of the tumour targeting of the monomeric, dimeric, and tetrameric RGD peptides was evident.

Several research groups have applied the multivalent concept to prepare cyclic RGD peptides with an enhanced binding affinity and demonstrated that the multimeric RGD peptides have an enhanced localisation in $\alpha_v\beta_3$-expressing tumours [17, 19, 25–30]. Although the advantages of multimeric RGD peptides as targeting molecules are universally accepted, the cause of the enhanced affinity of the multimeric RGD analogues for integrin $\alpha_v\beta_3$ is still a matter of debate [31]. Cells can form a cluster of many monovalent receptors on the cell surface [32], and particularly multimeric ligands with a spacer between the binding moieties could span the required distance between binding sites and could then bind multiple receptors simultaneously. On the other hand, multimeric compounds could have enhanced affinity due to statistical rebinding: the receptor binding of one RGD unit will significantly enhance the local concentration of the second RGD unit in the vicinity of the receptor. This could lead to an enhanced $\alpha_v\beta_3$-binding rate or a reduced $\alpha_v\beta_3$-dissociation rate of the RGD multimer [19]. The distance between the RGD units of the multimers used in this study is relatively short and therefore statistical rebinding might be the most likely explanation for the increased affinity in the series tetramer > dimer > monomer.

The three ^{68}Ga-labelled RGD peptides showed a remarkable difference in kidney uptake. The uptake of the ^{68}Ga-labelled tetrameric RGD peptide was at 2 h p.i. significantly higher than that of the ^{68}Ga-labelled dimer and monomeric RGD peptides. In the integrin specificity experiment, the excess of nonradiolabelled RGD peptide partly inhibited the kidney uptake of the radiolabelled monomer and dimer, but not the kidney uptake of the radiolabelled tetramer. This indicates that different mechanisms cause the relatively high uptake of the RGD peptides in the kidneys. Wu and coworkers have recently shown by immunohistochemistry that the endothelial cells of the glomeruli vessels in the kidneys express β_3 integrins [33], which could explain the partly specific kidney uptake of the RGD peptide. Furthermore, the difference in charge between the three peptides could cause the difference in tubular reabsorption. A trend has been observed that positively charged peptides are more efficiently reabsorbed by the proximal renal tubular cell than neutral peptides [34]. Due to the presence of more guanidine groups, the tetrameric RGD peptide is more positively charged than the dimeric and monomeric RGD peptides. Remarkably, the ^{68}Ga-labelled tetramer demonstrated a much higher kidney uptake than the ^{111}In-labelled tetramer which may hamper its clinical application.

Other nontumour tissues such as lung, liver and colon also showed specific uptake of the mono-, di- and tetrameric RGD peptides, suggesting $\alpha_v\beta_3$ expression in these tissues. Indeed, β_3 expression in these tissues has been described for rodents as well as for humans [33, 35]. Decristoforo and coworkers compared the biodistribution of [^{68}Ga]DOTA-RGD, [^{111}In]DOTA-RGD and [^{18}F]Galacto-RGD and found that [^{68}Ga]DOTA-RGD had the highest tracer uptake in all organs [13]. Especially, the radioactivity concentration in the blood was significantly higher for [^{68}Ga]DOTA-RGD compared with [^{111}In]DOTA-RGD. The authors hypothesised that the lower complex stability of the

^{68}Ga-DOTA complex could result in transchelation of gallium to transferrin. In our study, the ^{68}Ga-labelled peptides, especially the dimer and tetramer, did not show enhanced blood levels as compared to the ^{111}In-labelled counterparts. In addition, in our in vitro studies no evidence of instability of the ^{68}Ga-DOTA complex or protein-binding activity was observed. This is in line with the recent observation of Haukkala and coworkers who found that there was no evidence of dissociation of ^{68}Ga from DOTA in the blood [36]. Although DOTA has a larger cavity than NOTA and the log stability constants are in favour of the Ga-NOTA complex compared with the Ga-DOTA complex [37, 38], the ^{68}Ga-DOTA complex is stable enough for in vitro and in vivo studies.

In conclusion, the tetrameric RGD peptide demonstrated improved tumour targeting compared to the dimeric RGD peptide. Analogously, the dimeric RGD peptide exhibits improved tumour targeting compared to the monomeric RGD peptide. The results of the biodistribution study of the ^{68}Ga- and ^{111}In-labelled dimer and tetramer are rather comparable. The ^{68}Ga-labelled tetrameric RGD peptide is a suitable ligand for the noninvasive visualisation of $\alpha_v\beta_3$ expression in vivo.

Conflicts of interest None.

References

1. Ferrara N. Vascular endothelial growth factor and the regulation of angiogenesis. Recent Prog Horm Res 2000;55:15–35.
2. Kuwano M, Fukushi J, Okamoto M, Nishie A, Goto H, Ishibashi T, et al. Angiogenesis factors. Intern Med 2001;40:565–72.
3. Ellis LM, Liu W, Ahmad SA, Fan F, Jung YD, Shaheen RM, et al. Overview of angiogenesis: biologic implications for antiangiogenic therapy. Semin Oncol 2001;28:94–104.
4. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000;407:242–8.
5. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995;1:27–31.
6. Plow EF, Haas TA, Zhang L, Loftus J, Smith JW. Ligands binding to integrins. J Biol Chem 2000;275:21785–8.
7. Haubner R, Finsinger D, Kessler H. Stereoisomeric peptide libraries and peptidomimetics for designing selective inhibitors of the αvβ3 integrin for a new cancer therapy. Angew Chem Int Ed Engl 1997;36:1374–89.
8. Haubner R, Wester HJ, Weber WA, Mang C, Ziegler SI, Goodman SL, et al. Noninvasive imaging of alpha(v)beta3 integrin expression using 18F-labeled RGD-containing glycopeptides and positron emission tomography. Cancer Res 2001;61:1781–5.
9. Haubner R, Weber WA, Beer AJ, Vabuliene E, Reim D, Sarbia M, et al. Noninvasive visualization of the activated alphavbeta3 integrin in cancer patients by positron emission tomography and [18F]Galacto-RGD. PLoS Med 2005;2:e70.
10. Beer AJ, Haubner R, Sarbia M, Goebel M, Luderschmidt S, Grosu AL, et al. Positron emission tomography using [18F]Galacto-RGD identifies the level of integrin alpha(v)beta3 expression in man. Clin Cancer Res 2006;12:3942–9.
11. Kenny LM, Coombes RC, Oulie I, Contractor KB, Miller M, Spinks TJ, et al. Phase I trial of the positron-emitting Arg-Gly-Asp (RGD) peptide radioligand 18F-AH111585 in breast cancer patients. J Nucl Med 2008;49:879–86.
12. Maecke HR, Hofmann M, Haberkorn U. (68)Ga-labeled peptides in tumor imaging. J Nucl Med 2005;46(Suppl 1):172S–8.
13. Decristoforo C, Hernandez Gonzalez I, Carlsen J, Rupprich M, Huisman M, Virgolini I, et al. 68Ga- and 111In-labelled DOTA-RGD peptides for imaging of alphavbeta3 integrin expression. Eur J Nucl Med Mol Imaging 2008;35:1507–15.
14. Li ZB, Chen K, Chen X. (68)Ga-labeled multimeric RGD peptides for microPET imaging of integrin alpha(v)beta(3) expression. Eur J Nucl Med Mol Imaging 2008;35:1100–8.
15. Liu Z, Niu G, Shi J, Liu S, Wang F, Liu S, et al. (68)Ga-labeled cyclic RGD dimers with Gly3 and PEG4 linkers: promising agents for tumor integrin alphavbeta3 PET imaging. Eur J Nucl Med Mol Imaging 2009;36:947–57.
16. Dijkgraaf I, Kruijtzer JA, Frielink C, Soede AC, Hilbers HW, Oyen WJ, et al. Synthesis and biological evaluation of potent alphavbeta3-integrin receptor antagonists. Nucl Med Biol 2006;33:953–61.
17. Dijkgraaf I, Kruijtzer JA, Liu S, Soede AC, Oyen WJ, Corstens FH, et al. Improved targeting of the alpha(v)beta(3) integrin by multimerisation of RGD peptides. Eur J Nucl Med Mol Imaging 2007;34:267–73.
18. Liu S, Cheung E, Ziegler M, Rajopadhye M, Edwards DS. (90)Y and (177)Lu labeling of a DOTA-conjugated vitronectin receptor antagonist useful for tumor therapy. Bioconjug Chem 2001;12:559–68.
19. Wu Y, Zhang X, Xiong Z, Cheng Z, Fisher DR, Liu S, et al. microPET imaging of glioma integrin {alpha}v{beta}3 expression using (64)Cu-labeled tetrameric RGD peptide. J Nucl Med 2005;46:1707–18.
20. Janssen M, Frielink C, Dijkgraaf I, Oyen W, Edwards DS, Liu S, et al. Improved tumor targeting of radiolabeled RGD peptides using rapid dose fractionation. Cancer Biother Radiopharm 2004;19:399–404.
21. Janssen ML, Oyen WJ, Dijkgraaf I, Massuger LF, Frielink C, Edwards DS, et al. Tumor targeting with radiolabeled alpha(v)beta (3) integrin binding peptides in a nude mouse model. Cancer Res 2002;62:6146–51.
22. Visser EP, Disselhorst JA, Brom M, Laverman P, Gotthardt M, Oyen WJ, et al. Spatial resolution and sensitivity of the Inveon small-animal PET scanner. J Nucl Med 2009;50:139–47.
23. Haubner R, Kuhnast B, Mang C, Weber WA, Kessler H, Wester HJ, et al. [18F]Galacto-RGD: synthesis, radiolabeling, metabolic stability, and radiation dose estimates. Bioconjug Chem 2004;15:61–9.
24. Heppeler A, Froidevaux S, Mäcke H, Jermann E, Behe M, Powell P, et al. Radiometal-labelled macrocyclic chelator-derivatised somatostatin analogue with superb tumour-targeting properties and potential for receptor-mediated internal radiotherapy. Chem Eur J 1999;5:1974–81.
25. Boturyn D, Coll JL, Garanger E, Favrot MC, Dumy P. Template assembled cyclopeptides as multimeric system for integrin targeting and endocytosis. J Am Chem Soc 2004;126:5730–9.
26. Poethko T, Thumshirn G, Hersel U, Rau F, Haubner R, Schwaiger M, et al. Improved tumor uptake, tumor retention and tumor/background ratios of pegylated RGD multimers. J Nucl Med 2003;44:46P.
27. Poethko T, Schottelius M, Thumshirn G, Herz M, Haubner R, Henriksen G, et al. Chemoselective pre-conjugate radiohalogenation of unprotected mono- and multimeric peptides via oxime formation. Radiochim Acta 2004;92:317–27.

28. Thumshirn G, Hersel U, Goodman SL, Kessler H. Multimeric cyclic RGD peptides as potential tools for tumor targeting: solid-phase peptide synthesis and chemoselective oxime ligation. Chem Eur J 2003;9:2717–25.
29. Poethko T, Schottelius M, Thumshirn G, Hersel U, Herz M, Henriksen G, et al. Two-step methodology for high-yield routine radiohalogenation of peptides: (18)F-labeled RGD and octreotide analogs. J Nucl Med 2004;45:892–902.
30. Liu S, Hsieh WY, Jiang Y, Kim YS, Sreerama SG, Chen X, et al. Evaluation of a (99m)Tc-labeled cyclic RGD tetramer for noninvasive imaging integrin alpha(v)beta3-positive breast cancer. Bioconjug Chem 2007;18:438–46.
31. Vrasidas I, André S, Valentini P, Böck C, Lensch M, Kaltner H, et al. Rigidified multivalent lactose molecules and their interactions with mammalian galectins: a route to selective inhibitors. Org Biomol Chem 2003;1:803–10.
32. Kiessling LL, Pohl NL. Strength in numbers: non-natural polyvalent carbohydrate derivatives. Chem Biol 1996;3:71–7.
33. Wu Z, Li ZB, Chen K, Cai W, He L, Chin FT, et al. microPET of tumor integrin alphavbeta3 expression using 18F-labeled PEGylated tetrameric RGD peptide (18F-FPRGD4). J Nucl Med 2007;48:1536–44.
34. Behr TM, Goldenberg DM, Becker W. Reducing the renal uptake of radiolabeled antibody fragments and peptides for diagnosis and therapy: present status, future prospects and limitations. Eur J Nucl Med 1998;25:201–12.
35. Max R, Gerritsen RR, Nooijen PT, Goodman SL, Sutter A, Keilholz U, et al. Immunohistochemical analysis of integrin alphavbeta3 expression on tumor-associated vessels of human carcinomas. Int J Cancer 1997;71:320–4.
36. Haukkala J, Laitinen I, Luoto P, Iveson P, Wilson I, Karlsen H, et al. (68)Ga-DOTA-RGD peptide: biodistribution and binding into atherosclerotic plaques in mice. Eur J Nucl Med Mol Imaging 2009;36:2058–67.
37. Clarke ET, Martell AE. Stabilities of trivalent metal ion complexes of the tetraacetate derivatives of 12-, 13- and 14-membered tetraaza-macrocycles. Inorganica Chim Acta 1991;190:37–46.
38. Clarke ET, Martell AE. Stabilities of the Fe(III), Ga(III), and In(III) chelates of N,N′,N″-triazacyclononanetriacetic acid. Inorganica Chim Acta 1991;181:273–80.