Improved targeting of the $\alpha_v\beta_3$ integrin by multimerisation of RGD peptides

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Abstract. *Purpose:* The integrin $\alpha_v\beta_3$ is expressed on sprouting endothelial cells and on various tumour cell types. 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 an ^{111}In-labelled monomeric, dimeric and tetrameric RGD analogue were compared.
Methods: A monomeric (E-c(RGDfK)), dimeric (E-[c(RGDfK)]$_2$), and tetrameric (E{E[c(RGDfK)]$_2$}$_2$) RGD peptide were synthesised, conjugated with DOTA and radiolabelled with ^{111}In. 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 SK-RC-52 xenografts.
Results: The IC_{50} values for DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$, and DOTA-E{E[c(RGDfK)]$_2$}$_2$were 120 n*M*, 69.9 n*M* and 19.6 n*M*, respectively. At all time points, the tumour uptake of the dimer was significantly higher as compared to that of the monomer. At 8 h p.i., tumour uptake of the tetramer (7.40±1.12%ID/g) was significantly higher than that of the monomer (2.30±0.34%ID/g), $p<0.001$, and the dimer (5.17±1.22%ID/g), $p<0.05$. At 24 h p.i., the tumour uptake was significantly higher for the tetramer (6.82±1.41%ID/g) than for the dimer (4.22±0.96%ID/g), $p<0.01$, and the monomer (1.90±0.29%ID/g), $p<0.001$.
Conclusion: Multimerisation of c(RGDfK) resulted in enhanced affinity for $\alpha_v\beta_3$ as determined in vitro. Tumour uptake of a tetrameric RGD peptide was significantly higher than that of the monomeric and dimeric analogues, presumably owing to the enhanced statistical likelihood for rebinding to $\alpha_v\beta_3$.

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Introduction

The $\alpha_v\beta_3$ integrin is a transmembrane protein consisting of two non-covalently bound subunits, α and β. Integrin $\alpha_v\beta_3$ is preferentially expressed on proliferating endothelial cells [1], whereas it is absent on quiescent endothelial cells. For growth beyond the size of 1–2 mm in diameter, tumours require the formation of new blood vessels. Consequently, $\alpha_v\beta_3$ expression on tumour vasculature is considered as a marker of tumour-induced angiogenesis [2–4]. In addition, $\alpha_v\beta_3$ is expressed on the cell membrane of various tumour cell types, including ovarian cancer, neuroblastoma, breast cancer and melanoma. Due to this restricted expression of $\alpha_v\beta_3$ in tumours, $\alpha_v\beta_3$ is considered a suitable target for tumour targeting [5]. 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. A series of RGD sequence-containing peptides has been tested for their ability to bind the $\alpha_v\beta_3$ integrin. It was found that the cyclic derivative cyclic (Arg-Gly-Asp-D-Phe-Val) was a 100-fold better inhibitor of cell adhesion to vitronectin than the linear variant, having an IC_{50} value in the nanomolar range [8, 9].

In previous studies we showed that radiolabelled c(RGDfK) peptides specifically accumulated in $\alpha_v\beta_3$-positive ovarian cancer xenografts in athymic mice [10]. The aim of this study was to develop modified ligands for improved $\alpha_v\beta_3$

integrin targeting by preparing multimers of cyclic RGD peptides. A multimeric RGD peptide could theoretically bind multivalently and thus more avidly to the target cell. However, the distance between the RGD units of the multivalent RGD peptides is too short to allow simultaneous binding to multiple $\alpha_v\beta_3$ integrins on the cell surface. Therefore, in this manuscript we avoid use of the term "avidity", and only use "affinity".

Multivalent interactions are frequently used in nature to increase the affinity of weak ligand–receptor interactions [11, 12]. For example, the attachment of an influenza virus to its target cell occurs through multiple simultaneous interactions between haemagglutinin and sialic acid [12]. Multivalent structures for the design of drugs and research agents have become a new focus of investigation. Several research groups, including ours, have applied multivalent compounds to enhance the interaction between the ligand and the target cell [13]. Goel et al. radiolabelled divalent and tetravalent scFv's of mAb CC49 with ^{99m}Tc and analysed the imaging potential of these novel radioimmunoconjugates. Biodistribution studies demonstrated that ^{99m}Tc-[sc(Fv)$_2$]$_2$ had approximately threefold higher tumour localisation than ^{99m}Tc-sv(Fv)$_2$ [14]. Recently, a new multivalent synthetic inhibitor of epidemic keratoconjunctivitis-causing adenovirus was evaluated by Johansson et al. [11]. Their results clearly indicated that the inhibition is significantly increased with higher orders of valency. Similarly, Kok et al. and Maheshwari et al. showed increased affinity of RGD ligands with the target cell owing to multivalent interactions [15, 16].

Here we describe the synthesis of a monomeric, E-c(RGDfK), a dimeric, E-[c(RGDfK)]$_2$, and a tetrameric, E{E[c(RGDfK)]$_2$}$_2$, RGD peptide. The RGD peptides were conjugated with 1,4,7,10-tetraazadodecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) and radiolabelled with ^{111}In. Subsequently, the in vitro affinity and the in vivo tumour targeting characteristics of the peptides to $\alpha_v\beta_3$-expressing tumours were determined.

Materials and methods

Synthesis of DOTA-conjugated RGD peptides

The monomeric RGD peptide was synthesised using Fmoc-based solid phase peptide synthesis, as described previously [17]. The structural formula of DOTA-E-c(RGDfK) is shown in Fig. 1a.

The synthesis of the dimeric cyclic RGD peptide E-[c(RGDfK)]$_2$ conjugated with DOTA was described previously [18]. The structural formula of DOTA-E-[c(RGDfK)]$_2$ is shown in Fig. 1b.

The synthesis of the tetrameric cyclic RGD peptide E{E[c(RGDfK)]$_2$}$_2$ conjugated with DOTA was described previously [19]. The structural formula of DOTA-E{E[c(RGDfK)]$_2$}$_2$ is shown in Fig. 1c.

Radiolabelling of the RGD peptides

^{111}In-DOTA-E-c(RGDfK) was prepared by adding 14 MBq ^{111}InCl$_3$ (Mallinckrodt, Petten, The Netherlands) to 15 μg (13.3 nmol) DOTA-E-c(RGDfK) dissolved in 300 μl 0.5 *M* ammonium acetate buffer, pH 6.0, containing 0.6 mg/ml gentisic acid. DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ were radiolabelled with ^{111}InCl$_3$ analogously with minor modifications. DOTA-E-[c(RGDfK)]$_2$ (28 μg, 16.4 nmol) and DOTA-E{E[c(RGDfK)]$_2$}$_2$ (15 μg, 4.79 nmol) were dissolved in 500 μl 0.5 *M* ammonium acetate buffer pH 6.0, containing 0.6 mg/ml gentisic acid. DOTA-E-[c(RGDfK)]$_2$ was radiolabelled with 17 MBq ^{111}InCl$_3$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ was radiolabelled with 13 MBq ^{111}InCl$_3$. The reaction mixtures were degassed and subsequently the mixtures were heated at 100°C for 15 min. ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ was further purified on a C-18 SepPak cartridge (Waters, Milford, MA, USA). After applying the sample on the methanol-activated cartridge, the cartridge was washed with 5 ml 25 m*M* ammonium acetate and eluted with 25% acetonitrile in 25 m*M* ammonium acetate. The radiochemical purity was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) (HP 1100 series, Hewlett Packard, Palo Alto, CA, USA) using a C18 column (RX-C18, 4.6 mm×250 mm, Zorbax) eluted with a gradient mobile phase (8–20% B over 25 min or 8–100% B over 30 min, solvent A=25 m*M* ammonium acetate buffer, solvent B=acetonitrile) at 1 ml/min. The radioactivity of the eluate was monitored using an in-line radiodetector (Flo-One Beta series, Radiomatic, Meriden, CT, USA).

Solid-phase $\alpha_v\beta_3$ binding assay

The affinity of DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ for $\alpha_v\beta_3$ was determined using a solid-phase competitive binding assay. ^{111}In-labelled DOTA-E-[c(RGDfK)]$_2$ (3 MBq/μg) was prepared as described above and 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 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1 m*M* CaCl$_2$, 0.5 m*M* MgCl$_2$ and 1 m*M* 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 11.1 kBq of ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and appropriate dilutions of non-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 radioactivity in each well was determined in a γ-counter (1480 Wizard, Wallac, Turku, Finland). IC$_{50}$ values of the RGD peptides were calculated by non-linear regression using GraphPad Prism (GraphPad Prism 4.0 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 15×10^6 cells/ml SK-RC-52 cells was injected subcutaneously (s.c.). Two weeks after inoculation of the tumour cells, mice were randomly divided into three groups (15 mice/group).

To ensure that the mice in each group received equal amounts of RGD units, 0.5 μg per mouse of ^{111}In-DOTA-E-c(RGDfK) (0.52 MBq), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ (0.43 MBq) or ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ (0.34 MBq) was administered via a tail vein. Mice were killed by CO_2 asphyxiation 2, 8 and 24 h post

injection (p.i.) (five mice/group). Blood, tumour and the major organs and tissues were collected, weighed and counted in a γ-counter. The percentage injected dose per gram (%ID/g) was determined for each sample.

Statistical analysis

All mean values are given ±standard deviation. Statistical analysis was performed using one-way analysis of variance. Bonferroni

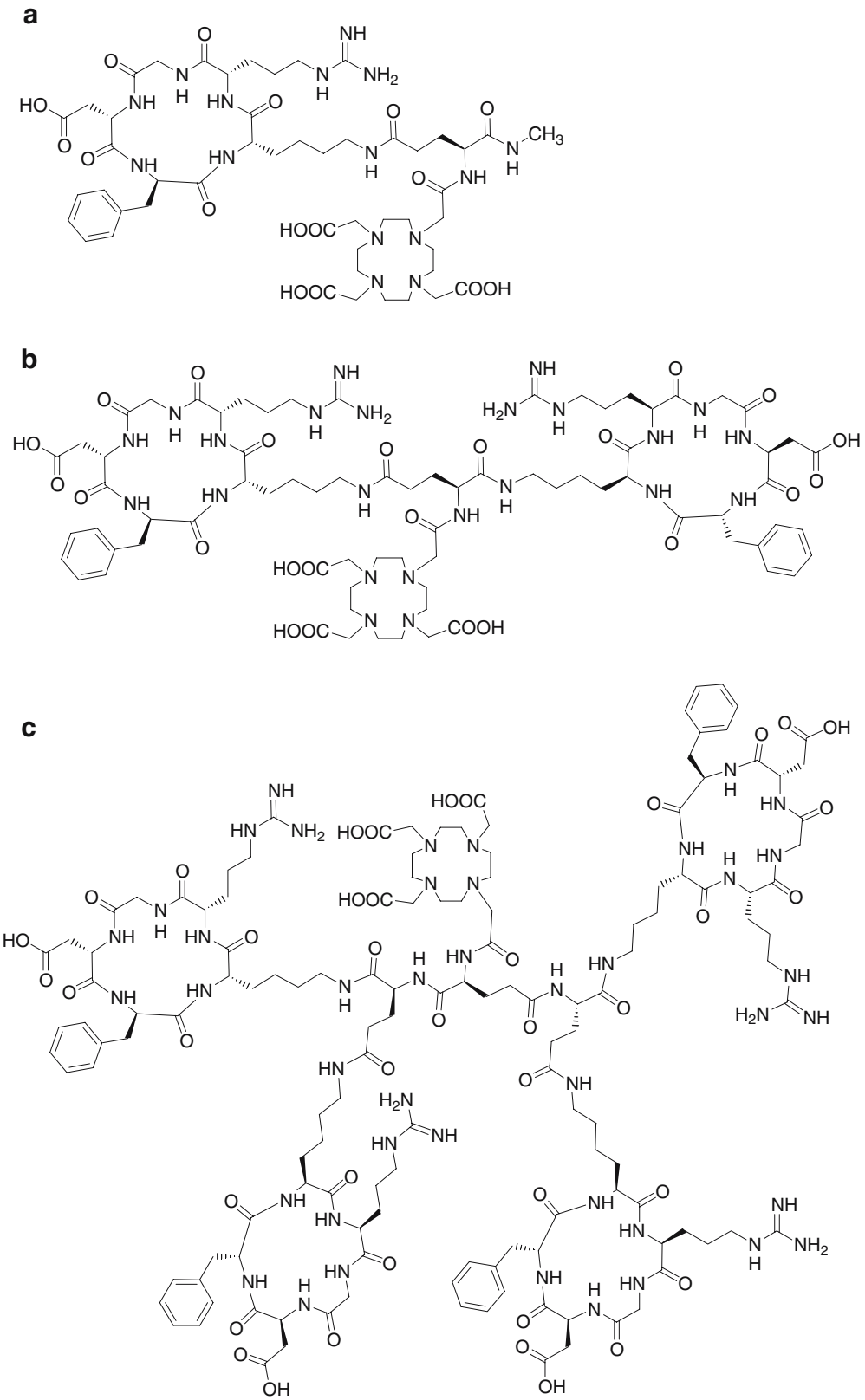

Fig. 1. a Structural formula of the DOTA-conjugated monomeric RGD peptide, DOTA-E-c(RGDfK). **b** Structural formula of the DOTA-conjugated dimeric RGD peptide, DOTA-E-$[c(RGDfK)]_2$. **c** Structural formula of the DOTA-conjugated tetrameric RGD peptide DOTA-$E\{E[c(RGDfK)]_2\}_2$

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 ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ preparations used in these experiments was at least 93%. The elution profile 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 three compounds with an elution time of 14 min and 26 min for ^{111}In-DOTA-E-c(RGDfK) and ^{111}In-DOTA-E-[c(RGDfK)]$_2$, respectively. The retention time of ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ was 15 min. Note that different gradients were used for elutions.

Solid-phase $\alpha_v\beta_3$ binding assay

The affinity of DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$ for the $\alpha_v\beta_3$ integrin was determined 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 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 120 n*M* for DOTA-E-c(RGDfK), 69.9 n*M* for DOTA-E-[c(RGDfK)]$_2$ and 19.6 n*M* for DOTA-E{E[c(RGDfK)]$_2$}$_2$.

Biodistribution studies

The results of the biodistribution studies of ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ in athymic mice with s.c. SK-RC-52 tumours are summarised in Table 1 and Fig. 3. ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ all cleared rapidly from the blood. At 8 h p.i., tumour uptake of the tetramer (7.40±1.12%ID/g) was significantly higher than that of the monomer (2.30±0.34%ID/g), $p<0.001$, and the dimer (5.17±1.22%ID/g), $p<0.05$. Furthermore, uptake of the dimer in the tumour was significantly higher than that of the monomer, $p<0.01$ (Fig. 3a). At 24 h p.i., uptake in the tumour was significantly higher for the tetramer (6.82±1.41%ID/g) than for the dimer (4.22±0.96%ID/g), $p<0.01$, and the monomer (1.90±0.29%ID/g), $p<0.001$ (Fig. 3b). Co-injection of an excess unlabelled DOTA-E-[c(RGDfK)]$_2$ (50 μg) along with 0.5 μg of ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ or ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ resulted in a significant decrease in radioactivity concentration in the tumour, indicating that uptake of the major fraction of ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$in the tumour was $\alpha_v\beta_3$ mediated. Uptake in non-target organs like 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 also at least partly $\alpha_v\beta_3$ mediated.

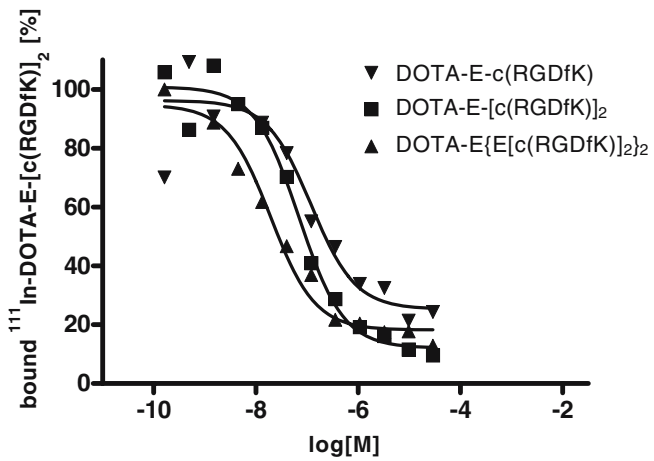

Fig. 2. Competition of specific binding of ^{111}In-DOTA-E-[c(RGDfK)]$_2$ with DOTA-E-c(RGDfK), DOTA-E-[c(RGDfK)]$_2$ and DOTA-E{E[c(RGDfK)]$_2$}$_2$

Discussion

In this study the potential advantage of using multimeric RGD peptides for targeting of the $\alpha_v\beta_3$ integrin receptor was investigated. The binding affinity of DOTA-E{E[c(RGDfK)]$_2$}$_2$ (IC_{50}=19.6 n*M*), as determined in a solid phase competitive binding assay, was about four times higher than that of DOTA-E-[c(RGDfK)]$_2$ (IC_{50}=69.9 n*M*) and about six times higher than that of DOTA-E-c(RGDfK) (IC_{50}=120 n*M*). Enhanced affinity of $\alpha_v\beta_3$-expressing cells for multiple RGD sequences on a protein backbone as compared with the monomeric RGD peptides was demonstrated by Kok et al. [15]. They postulated that the enhanced affinity was due to cooperative binding of multiple RGD units. Recently, Thumshirn et al. [20] synthesised multimeric RGD peptides by oxime ligation. These multimeric RGD peptides showed retained affinity for the $\alpha_v\beta_3$ integrin. Their tetrameric RGD compound with a Hegas spacer showed enhanced affinity as compared to the monomeric and the dimeric analogue. However, the tetrameric RGD compound with an aminohexanoic acid spacer showed a lower affinity than the dimeric analogue [20], indicating clearly the importance of the proper choice of spacer unit.

In athymic mice with s.c. SK-RC-52 tumours, all three RGD peptides of this study showed specific uptake in the tumour: 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 peptide was clearly demonstrated. The tumour uptake of ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ beyond 2 h p.i. was significantly higher than that of ^{111}In-DOTA-E-[c(RGDfK)]$_2$. Similarly, the tumour

Table 1. Biodistribution data of ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ in the presence and absence of an excess of unlabelled DOTA-E-[c(RGDfK)]$_2$ in athymic mice with s.c. SK-RC-52 tumours 2 h after injection

The organ uptake is expressed as %ID/g

	Monomer	Monomer + excess cold	Dimer	Dimer + excess cold	Tetramer	Tetramer + excess cold
Blood	0.04±0.01	0.01±0.01	0.10±0.02	0.02±0.01	0.09±0.01	0.03±0.01
Muscle	0.19±0.02	0.17±0.17	0.37±0.06	0.03±0.00	0.49±0.06	0.07±0.02
Tumour	2.70±0.29	0.39±0.05	5.61±0.85	0.47±0.08	7.32±2.45	0.84±0.12
Lung	0.38±0.05	0.08±0.01	1.12±0.18	0.13±0.01	1.37±0.36	0.20±0.05
Spleen	1.43±0.10	0.16±0.01	2.40±0.38	0.22±0.02	2.53±0.31	0.28±0.03
Kidney	1.85±0.15	1.38±0.07	3.56±0.28	1.90±0.33	6.15±0.41	5.30±0.53
Liver	1.32±0.05	0.18±0.01	2.71±0.21	0.24±0.03	2.41±0.49	0.28±0.03
Intestine	3.18±0.68	0.21±0.01	6.65±1.11	0.20±0.04	7.30±0.73	0.49±0.10
Colon	1.71±0.11	0.18±0.02	3.77±0.79	0.16±0.01	3.94±0.54	0.67±0.11

uptake of ^{111}In-DOTA-E-[c(RGDfK)]$_2$ was significantly higher than that of ^{111}In-DOTA-E-c(RGDfK) at all time points (Fig. 4). Thus, the higher the binding affinity for $\alpha_v\beta_3$, the higher the accumulation of the compound in $\alpha_v\beta_3$-expressing tumours. It appears that the enhanced uptake in the tumour of the tetrameric RGD peptide is mainly determined by early uptake in the tumour, while retention in the tumour at later time points of the monomer, dimer and tetramer was similar.

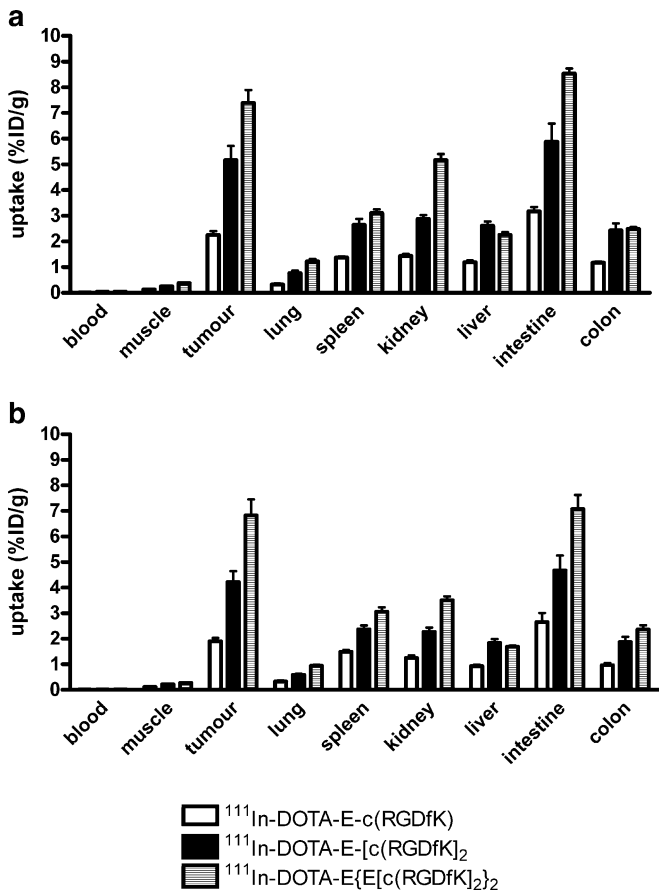

Fig. 3. a Biodistribution of ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ at 8 h p.i. in athymic mice with s.c. SK-RC-52 tumours (five mice/group). **b** Biodistribution of ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ at 24 h p.i. in athymic mice with s.c. SK-RC-52 tumours (five mice/group)

Recently, Wu and co-workers tested ^{64}Cu-DOTA-E{E[c(RGDfK)]$_2$}$_2$ for positron emission tomography imaging of $\alpha_v\beta_3$ expression in athymic nude mice with s.c. U87MG glioma xenografts [19]. ^{64}Cu-DOTA-E{E[c(RGDfK)]$_2$}$_2$ accumulated rapidly and efficiently in the tumour (9.93±1.05%ID/g at 30 min p.i.). At 2 h p.i., the uptake of this ^{64}Cu-labelled compound in the tumour was 7.61±0.68% ID/g, slowly decreasing to 4.56±0.51%ID/g at 24 h p.i. Although our compound was labelled with another radionuclide and tested in another animal model, the uptake in the tumour during 24 h after injection was very similar. At 2 h p.i., the uptake in the tumour of ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ was 7.32±2.45%ID/g and it decreased to 6.82±1.41%ID/g at 24 h p.i.

One may argue that the enhanced tumour uptake of ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ might be due to the

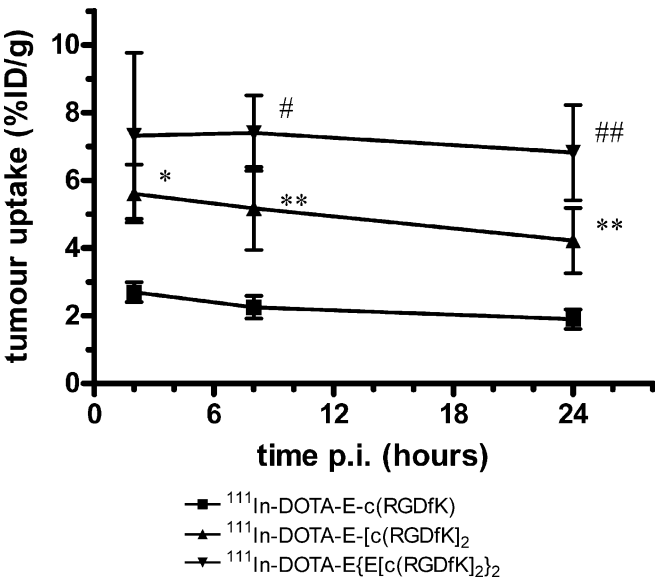

Fig. 4. Tumour uptake of ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTAE{E[c(RGDfK)]$_2$}$_2$ at 2, 8 and 24 h after injection in athymic mice with s.c. SK-RC-52 tumours. Resultsare expressed as mean %ID/g±SD. Values were analysed using one-way analysis of variance; *p<0.05, **p<0.01, $^{\#}p$<0.05, $^{\#\#}p$<0.01. The p values refer to differences in tumour uptake between ^{111}In-DOTA-E-c(RGDfK) and ^{111}In-DOTA-E-[c(RGDfK)]$_2$ or between ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$

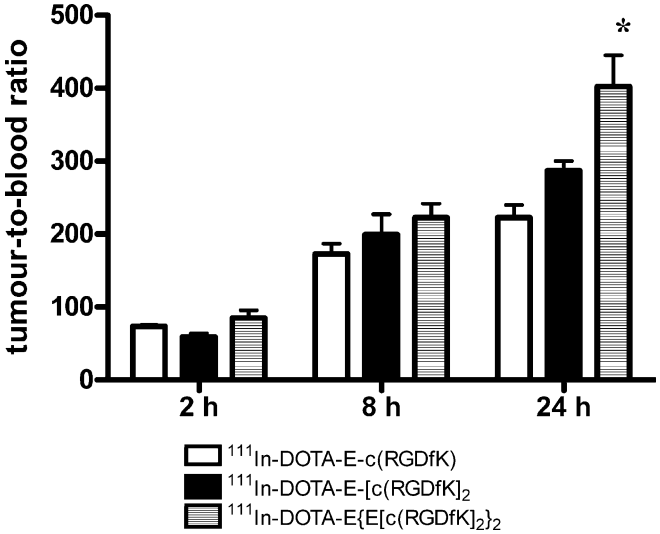

Fig. 5. Tumour-to-blood ratios of ^{111}In-DOTA-E-c(RGDfK), ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ at 2, 8 and 24 h after injection in athymic mice with s.c. SK-RC-52 tumours.Each *bar* represents the mean values ±SD. Values were analysed using one-way analysis of variance, *p<0.05

higher molecular weight of the tetramer, resulting in a longer blood circulation time. However, not only the uptake in the tumour but also the tumour-to-blood ratio of ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ (402±96, 24 h p.i.) was significantly higher than that of ^{111}In-DOTA-E-[c(RGDfK)]$_2$ (287±30) and ^{111}In-DOTA-E-c(RGDfK) (223±39) (p<0.05 and p<0.01, respectively) (Fig. 5).

While the potential benefits of multivalency of targeting vehicles are universally accepted, the cause of the enhanced affinity is not yet clear [21]. Multivalent compounds could have enhanced affinity due to subsite binding, statistical rebinding or receptor clustering [12, 22]. Subsite binding refers to binding of a ligand to sites other than the binding site. It is not likely that this mechanism is responsible for the enhanced uptake of RGD multimers in $\alpha_v\beta_3$-expressing tumours because subsites have not been identified for these peptides.

Cells can form a cluster of many monovalent receptors on a small area of the cell surface [22] and multivalent ligands that can span the required distance between binding sites could then bind multiple receptors simultaneously. However, it is unlikely that the multivalent RGD peptides used in this study could bind multiple $\alpha_v\beta_3$ integrins simultaneously because the distance between the RGD units is very short. Therefore, statistical rebinding might be the most likely explanation for the enhanced affinity of the multimeric RGD peptides. The receptor binding of one RGD unit will significantly enhance the local concentration of the other RGD unit in the vicinity of the receptor. This could lead to a higher "on rate" of receptor binding and/or a lower "off rate" of the RGD multimer [19].

The three RGD peptides showed a remarkable difference in uptake in the kidneys. The uptake of ^{111}In-DOTA-E{E[c(RGDfK)]$_2$}$_2$ is at all time points significantly higher than that of ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and ^{111}In-DOTA-E-c(RGDfK). Uptake in the kidney was not $\alpha_v\beta_3$ mediated; thus the enhanced $\alpha_v\beta_3$ affinity cannot explain the enhanced kidney uptake. Most likely, the difference in charge between the three peptides could cause the difference in renal uptake. It has been shown that positively charged peptides are more efficiently reabsorbed by the proximal renal tubular cell than neutral peptides [23]. Owing to the presence of more guanidine groups, the tetrameric RGD peptide is more positively charged than the dimeric and monomeric RGD peptide.

In conclusion, the present study shows that multimeric RGD peptides have enhanced affinity for the $\alpha_v\beta_3$ integrin, most likely due to statistical rebinding of these RGD peptides. The tetrameric RGD peptide demonstrated improved tumour targeting compared with the dimeric RGD peptide. Analogously, the dimeric RGD peptide exhibited improved tumour targeting compared with the monomeric RGD peptide. The tetrameric RGD peptide is the favourable ligand for $\alpha_v\beta_3$ targeting in vivo. The uptake of this compound in the kidneys and intestines is relatively high and could hamper imaging of tumours in the abdomen.

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