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ORIGINAL ARTICLE

Targeted radionuclide therapy with RAFT-RGD radiolabelled with ^{90}Y or ^{177}Lu in a mouse model of αvβ3-expressing tumours

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Abstract
Purpose The αvβ3 integrin plays an important role in tumour-induced angiogenesis, tumour proliferation, survival and metastasis. The tetrameric RGD-based peptide, regioselectively addressable functionalized template-(cyclo-[RGDfK])$_4$ (RAFT-RGD), specifically targets the αvβ3 integrin in vitro and in vivo. The aim of this study was to evaluate the therapeutic potential of RAFT-RGD radiolabelled with β^- emitters in a nude mouse model of αvβ3 integrin-expressing tumours.
Methods Biodistribution and SPECT/CT imaging studies were performed after injection of ^{90}Y-RAFT-RGD or ^{177}Lu-RAFT-RGD in nude mice subcutaneously xenografted with αvβ3 integrin-expressing U-87 MG cells. Experimental targeted radionuclide therapy with ^{90}Y-RAFT-RGD or ^{177}Lu-RAFT-RGD and ^{90}Y-RAFT-RAD or ^{177}Lu-RAFT-RAD (nonspecific controls) was evaluated by intravenous injection of the radionuclides into mice bearing αvβ3 integrin-expressing U-87 MG tumours of different sizes (small or large) or bearing TS/A-pc tumours that do not express αvβ3. Tumour volume doubling time was used to evaluate the efficacy of each treatment.
Results Injection of 37 MBq of ^{90}Y-RAFT-RGD into mice with large αvβ3-positive tumours or 37 MBq of ^{177}Lu-RAFT-RGD into mice with small αvβ3-positive tumours caused significant growth delays compared to mice treated with 37 MBq of ^{90}Y-RAFT-RAD or 37 MBq of ^{177}Lu-RAFT-RAD or untreated mice. In contrast, injection of 30 MBq of ^{90}Y-RAFT-RGD had no effect on the growth of αvβ3-negative tumours.
Conclusion ^{90}Y-RAFT-RGD and ^{177}Lu-RAFT-RGD are potent agents targeting αvβ3-expressing tumours for internal targeted radiotherapy.

Keywords RGD peptide · αvβ3 integrin · Internal targeted radiotherapy · ^{90}Y · ^{177}Lu

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Introduction

Integrins belong to a family of αβ heterodimeric transmembrane glycoproteins involved in cell–cell and cell–matrix interactions. Integrins are major adhesion receptors and they also trigger a large variety of signal transduction events that modulate many aspects of cell behaviour such as proliferation, survival/apoptosis, shape, polarity, motility, gene expression and differentiation [1]. One set of the integrin family, including the αvβ3 integrin, recognizes the specific sequence of amino acids Arg-Gly-Asp (RGD) on their extracellular matrix

ligands. The αvβ3 integrin is known to play an important role in tumour-induced angiogenesis, tumour proliferation, survival and metastasis [2]. This integrin is overexpressed in endothelial cells from tumour neovessels, as well as in tumour cells from various origins such as melanomas, gliomas, and breast, ovarian and pancreatic cancers [3–7]. The αvβ3 integrin is an attractive molecular target for diagnosis and therapy of rapidly growing and metastatic tumours. Radiolabelled receptor-binding peptides have been shown to be remarkably specific and effective in the diagnosis and therapy of malignant disease [8]. A tetrameric RGD-based peptide of 5 kDa, regioselectively addressable functionalized template-(cyclo-[RGDfK])$_4$ (RAFT-RGD), specifically targets the αvβ3 integrin in vitro and in vivo. The RAFT-RGD molecule is rapidly internalized following αvβ3 integrin binding, which facilitates preclinical tumour detection using nuclear or optical imaging [9–13]. Recently, RAFT-RGD has been efficiently used for drug delivery [14].

In most radionuclide therapies, bone marrow toxicity is dose-limiting. In addition, peptide receptor radiation therapy (PRRT) also represents a potential risk to the kidney since it is the main excretory organ for peptides which are retained at relatively high levels in the cortex [15]. Therefore, the amount of radioactivity that may be administered in PRRT is currently limited by potential kidney toxicity [16]. We have previously reported the biodistribution of RAFT-RGD radiolabelled with ^{99m}Tc or ^{111}In and found that the compounds were mainly eliminated via the kidneys as expected with approximately 30 – 50 % of the injected dose retained in the kidneys 24 h after injection (p.i.) [11, 12]. This high delivered dose to the kidneys is an important limitation to the amount of radioactivity that can be administered safely when using RAFT-RGD radiolabelled with destructive β$^-$ emitters for internal targeted radiotherapy (ITRT). Several kidney protection methods are available to prevent damage from high absorbed doses. The use of positively charged basic amino acid solutions of L-lysine and L-arginine reduces renal uptake of radiopeptides at the expense of significant side effects [17]. The use of the gelatin-based plasma expander Gelofusine® leads to a reduction of more than 50 % in the renal reabsorption of labelled RAFT-RGD in tumour-bearing mice while increasing the tumour-to-healthy tissue ratio [18]. Gelofusine reduces the renal reabsorption of radiolabelled peptides by interfering with the megalin/cubulin system, a receptor-mediated endocytosis pathway at the membrane of proximal tubular cells. Such strategies might allow the development of therapeutic approaches.

The purpose of the present study was to assess the efficacy and toxicity of ITRT using the tetrameric RGD peptide RAFT-RGD radiolabelled with β$^-$ emitters in a nude mouse model of αvβ3-expressing tumour. Two β$^-$ emitters were evaluated. First, ^{90}Y is a high-energy β$^-$ emitter (E_{max} 2.28 MeV) with a physical half-life ($T_{1/2}$ 2.7 days) compatible with the pharmacokinetics of peptides and with a long penetration range in tissues (R_{max} 11 mm). Second, ^{177}Lu is a β$^-$ emitter (78.7 %, E_{max} 0.497 MeV) and γ emitter (11 %, E_{max} 0.208 MeV; 6.4 %, E_{max} 0.113 MeV), with a short penetration range in tissues (R_{max} 1.8 mm) and a longer half-life ($T_{1/2}$ 6.7 days). The shorter β$^-$ range of ^{177}Lu provides better irradiation of small tumours, in contrast to the longer β$^-$ range of ^{90}Y which allows more uniform irradiation in large tumours that may show heterogeneous uptake [19]. Therefore, we evaluated the efficacy of tumour targeting of RAFT-RGD radiolabelled with ^{90}Y or ^{177}Lu in mice bearing small or large αvβ3-positive tumours.

Materials and methods

RGD peptide synthesis and radioactive labelling

DOTA-RAFT(c[-RGDfK-])$_4$ (or RAFT-RGD) and DOTA-RAFT(c[-RADfK-])$_4$ (or RAFT-RAD) were synthesized according to previously reported procedures [20]. Both peptides were radiolabelled with ^{90}Y or ^{177}Lu using the method of Liu et al. [21, 22] and optimized as follows: 100 μg of RAFT-RGD or RAFT-RAD and 185 MBq of $^{90}YCl_3$ (specific activity about 1,600 GBq/μmol) or $^{177}LuCl_3$ (specific activity about 185 GBq/μmol) ($^{90}YCl_3$ and $^{177}LuCl_3$ purchased from Perkin Elmer) were added to 10 mg sodium gentisate in ammonium acetate buffer (0.5 M; pH 6.8) and were then incubated at 90 °C for 7 min. The radiolabelled solutions were diluted 20-fold with 2 mM DTPA (diethylenetriaminepentaacetic acid). Radiochemical purity (RCP) was determined by radio-HPLC using an analytical reverse-phase column (Lichrosorb C18, 5 μm, 4.6×250 mm) and the following gradient mobile phase: 0 – 18 min 13 % B, 18 – 19 min from 13 % to 60 % B, 19 – 25 min 60 % B, 25 – 26 min from 60 % to 13 % B and return to initial condition (total run 30 min); solvent A (0.1 % trifluoroacetic acid in water) and solvent B (0.1 % trifluoroacetic acid in 90 % acetonitrile) at a flow rate of 1 ml/min. The specific activities of all radiolabelled RAFT peptides were about 10 GBq/μmol when radiolabelled with ^{90}Y and about 8 GBq/μmol when radiolabelled with ^{177}Lu.

Cell culture

The human glioblastoma cell line U-87 MG was purchased from the American Type Culture Collection and was cultured in Minimal Essential Medium (MEM) with Earle's salts and stable glutamine (PAA laboratories, Les Mureaux, France) with 10 % fetal calf serum (PAA Laboratories). The mouse mammary carcinoma cell line TS/A-pc from Balb/c mice was kindly provided by L. Sancey (INSERM U823, France). TS/A-pc cells were cultured in RPMI 1640 medium with stable glutamine (PAA laboratories) supplemented with 10 % fetal

calf serum (PAA Laboratories). Cells were cultured at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2. U-87 MG cells have previously been shown to be αvβ3 integrin-positive [23, 24]. The αvβ3 integrin expression profiles of U-87 MG and TS/A-pc cells were confirmed or determined by western blotting and flow cytometry analysis. Both procedures are described in Online Resource 1.

Animal models and in vivo studies

Mice were housed at the animal core facility of our laboratory. All the experiments were approved by the Animal Care and Use Committee of the University of Grenoble (ComEth). Female Swiss Nude mice (Charles River, France; Janvier, France) at 5 – weeks of age were subcutaneously xenografted in the left posterior upper leg with 5×10^6 U-87 MG cells in 0.1 mL of nonsupplemented MEM or with 10^6 TS/A-pc cells in 0.1 mL of nonsupplemented RPMI 1640. Tumour length, width, and thickness were measured daily with a digital calliper. Tumour volume was calculated according to the ellipsoid volume formula: $V(T) = (\text{length}\times\text{width}\times\text{thickness})\times\pi/6$. Tumour growth beyond 1,500 mm^3 was used as a progression point at which animals were killed.

Biodistribution study

Mice with U-87 MG tumours were preinjected via the tail vein with 100 μL of Gelofusine® 4 %, and injected 5 min later with 364.9±50.2 kBq of ^{90}Y-RAFT-RGD. Preinjection of Gelofusine® reduces renal retention of the tracer by more than 50 % without affecting tumour uptake [18]. The mice were killed at 1, 4, 24 and 48 h p.i. (five mice per time point) with an overdose of intraperitoneal sodium pentobarbital, and tissue samples were excised. The tissue samples were weighed and radioactivity (Bremsstrahlung from the β decay of ^{90}Y) was measured with a γ-counter (Cobra II; Packard). Uptake in organs was expressed as percentage of the injected dose per gram of tissue (%ID/g).

SPECT/CT imaging study

Mice with subcutaneous U-87 MG tumours were preinjected via the tail vein with 100 μL of Gelofusine® 4 %, and injected 5 min later with 37 MBq of ^{177}Lu-RAFT-RGD (two mice) or 37 MBq of ^{177}Lu-RAFT-RAD (two mice). Mice were then anaesthetized with isoflurane in an oxygen-supplemented gas mixture (Aerrane; Baxter, France) 4 % for induction and 1.5 % thereafter. At 1, 4, 24 and 48 h p.i., anaesthetized animals were placed on a temperature-controlled bed and whole-body SPECT/CT scans were acquired. SPECT/CT experiments were performed with a four-head multiplexing multipinhole camera (NanoSPECT/CT; Bioscan/Mediso) using collimators with nine pinholes of 1.4 mm in diameter. The scans were acquired sequentially using Nucline software (Mediso). The CT scan was performed using 180 projections (500 ms each) at 45 kV. The SPECT parameters were: 24 projections and 60 – 100 s per projection. The scans were reconstructed using HiSPECT NG (Bioscan). CT and SPECT acquisitions were reconstructed, fused and quantified using dedicated software (InVivoScope; Bioscan). The scale of SPECT images was initially expressed as kilobecquerels per voxel and was converted into percent injected dose per gram (%ID/g) and set to similar levels to allow direct visual comparison.

Experimental radionuclide therapy

All animals including controls received a preinjection of 100 μL Gelofusine® 4 % via the tail vein. The therapeutic potential of ^{90}Y-RAFT-RGD was evaluated on large αvβ3-positive and small tumours. Mice bearing large U-87 MG tumours (800±240 mm^3) were injected via the tail vein with 37 MBq of ^{90}Y-RAFT-RGD (seven mice) or 37 MBq of ^{90}Y-RAFT-RAD (seven mice). The control group received saline solution (six mice). Mice bearing small U-87 MG tumours (230±170 mm^3) were injected via the tail vein with 37 MBq of ^{90}Y-RAFT-RGD (five mice) or saline solution (six mice). The therapeutic potential of ^{177}Lu-RAFT-RGD was evaluated on small αvβ3-positive tumours. Mice bearing small U-87 MG tumours (170±80 mm^3) were injected via the tail vein with 37 MBq of ^{177}Lu -RAFT-RGD (seven mice) or 37 MBq of ^{177}Lu -RAFT-RAD (seven mice). The control group received saline solution (seven mice). The therapeutic dose of 37 MBq was determined on the basis of the results of previously published studies on murine models [25–27].

The relationship between the therapeutic potential of ^{90}Y-RAFT-RGD and the tumoral expression of the αvβ3 integrin was assessed using a control treatment in a αvβ3-negative tumour model. Mice bearing large TS/A-pc tumours (540±190 mm^3) were injected via the tail vein with 30 MBq of ^{90}Y-RAFT-RGD (seven mice) or 30 MBq of ^{90}Y-RAFT-RAD (seven mice). The control group received saline solution (six mice).

Finally, the efficacy of fractionated treatment with ^{90}Y-RAFT-RGD in αvβ3-positive tumours was evaluated in comparison with unfractionated treatment. Mice bearing large U-87 MG tumours (550±240 mm^3 at the time of injection) were injected via the tail vein with 2×18.5 MBq of ^{90}Y-RAFT-RGD at 3-day intervals (seven mice) or with 2×18.5 MBq of ^{90}Y-RAFT-RAD at 3-day intervals (six mice). The control group received saline solution (seven mice). The main parameter used to evaluate the efficacy of each type of treatment was the tumour volume doubling time (TVDT) given by the formula: $TVDT = \ln2/(\ln(V2/V1)/(t2-t1))$ where $V1$ and $V2$ are the tumour volumes on days $t1$ and $t2$, respectively, $t1$ being the day of treatment and $t2$ the day of the animal was killed [28].

Toxicity evaluation

Blood cell count was determined in samples drawn from the tail vein. Variations in the numbers of erythrocytes, leucocytes and platelets were used as a parameter to evaluate medullary toxicity of the treatments. Blood cell concentrations were determined by microscopic counting using single test kits (Leuko-TIC®, Ery-TIC® and Thrombo-TIC®; Bioanalytic GmbH, Umkirch, Germany) the day before implantation, treatment and death. Data are expressed as the ratio (%) of the blood cells measured the day before treatment or death to the value measured the day before implantation.

Renal toxicity of the treatments was assessed by measuring creatinine concentrations in serum with a creatinine assay kit (BioVision, Mountain View, CA) [29]. Renal toxicity was evaluated the day of death by comparison of serum the creatinine levels in the treated animals and the values in the control animals. Blood samples were collected by intracardiac puncture under deep ketamine/xylazine anaesthesia (intraperitoneal injection of 100 mg/kg and 10 mg/kg body weight, respectively).

Radiation dosimetry extrapolation to humans

Human dosimetry was estimated from ^{90}Y-RAFT-RGD biodistribution data obtained in female Swiss nude mice bearing U-87 MG tumours, assuming that the biodistribution of the radiotracer in mice is similar to that in adult humans. The radiotracer was administered to five mice which were then killed at 1, 4, 24 and 48 h. The organs were collected and the radioactivity counted. One method of extrapolating animal data is the percent kilogram per gram method [30]. Residence times for ^{90}Y-RAFT-RGD were calculated as the area under the curve of the organ time–activity data determined by monoexponential fitting, divided by the injected dose. The residence time for the red marrow radioactivity was estimated by assuming a red marrow radioactivity concentration of 30 % of the whole blood activity concentration [31] or using the bone activity. Absorbed doses were calculated using the standard dedicated software OLINDA/EXM® (Vanderbilt University) with an adult male model input [32].

Statistical analysis

Mean values were compared using nonparametric tests, i.e. the Mann-Whitney *U* test for comparison of two groups and the Kruskal-Wallis test for comparison of more than two groups. Survival curves were compared using a Wilcoxon rank sum test in accordance with the lack of censored observations. *P* values of 0.05 or less were considered significant. BioEstat statistical software (version 5.0) was used.

Results

Radiolabelling of RAFT-RGD

HPLC analyses indicated the presence of a single major radioactive compound for all radiolabelled peptides as shown in Online Resource 2. The RCP of ^{90}Y-RAFT-RGD and ^{90}Y-RAFT-RAD were higher than 90 % immediately after labelling. Both radioactive compounds remained stable in the solution to be injected at room temperature for up to 24 h after labelling (RCP>88 %). ^{177}Lu-RAFT-RGD and ^{177}Lu-RAFT-RAD also displayed high RCP (>97 %) and were stable in the solution to be injected at room temperature for up to 24 h after labelling (RCP>95 %).

Expression of αvβ3 integrin by U-87 MG and TS/A-pc cells

The results of the western blot analysis of αvβ3 integrin expression are presented in Fig. 1a. Western blotting revealed that U-87 MG cells and TS/A-pc cells expressed the αv subunit while only U-87 MG cells expressed the β3 subunit. The αvβ3 integrin was therefore expressed by U-87 MG cells and not by the TS/A-pc cells. The flow cytometry results (Fig. 1b) confirmed this pattern of expression.

Biodistribution of ^{90}Y-RAFT-RGD in U-87 MG tumour-bearing mice

After injection of ^{90}Y-RAFT-RGD, the radioactivity cleared rapidly from the blood (Fig. 2). ^{90}Y-RAFT-RGD was eliminated via the urinary route as indicated by kidney activities (13.9±3.5 %ID/g at 1 h after injection, 13.3±1.4 %ID/g at 4 h p.i., 5.9±2.7 %ID/g at 24 h p.i. and 5.8±2.5 %ID/g at 48 h p.i.). The tumour uptake of ^{90}Y-RAFT-RGD was rapid and high (9.0±4.3 %ID/g at 1 h p.i. and 9.0±3.1 %ID/g at 4 h p.i.) and remained at 2.6±0.9 %ID/g and 1.8±0.7 %ID/g at 24 h and 48 h p.i., respectively. Tissues including muscle, liver and pancreas had relatively lower uptake (Fig. 2), resulting in high tumour-to-blood and tumour-to-muscle ratios of 27 and 15, respectively, at 24 h p.i.

SPECT/CT imaging of U-87 MG tumour-bearing mice after ^{177}Lu-RAFT-RGD or ^{177}Lu-RAFT-RAD injection

Noninvasive SPECT/CT imaging was performed to visualize and quantify the biodistribution of ^{177}Lu-RAFT-RGD and ^{177}Lu-RAFT-RAD in U-87 MG tumour-bearing mice over time. The kidneys and the bladder were the organs with the highest activity levels (about 6 %ID/g in the kidney for both) on SPECT/CT images acquired at 1 h and 4 h p.i. for either ^{177}Lu-RAFT-RGD or ^{177}Lu-RAFT-RAD, confirming the renal excretion pattern of the radiolabelled peptides (Fig. 3). ^{177}Lu-RAFT-RGD activity was readily observed in U-87 MG

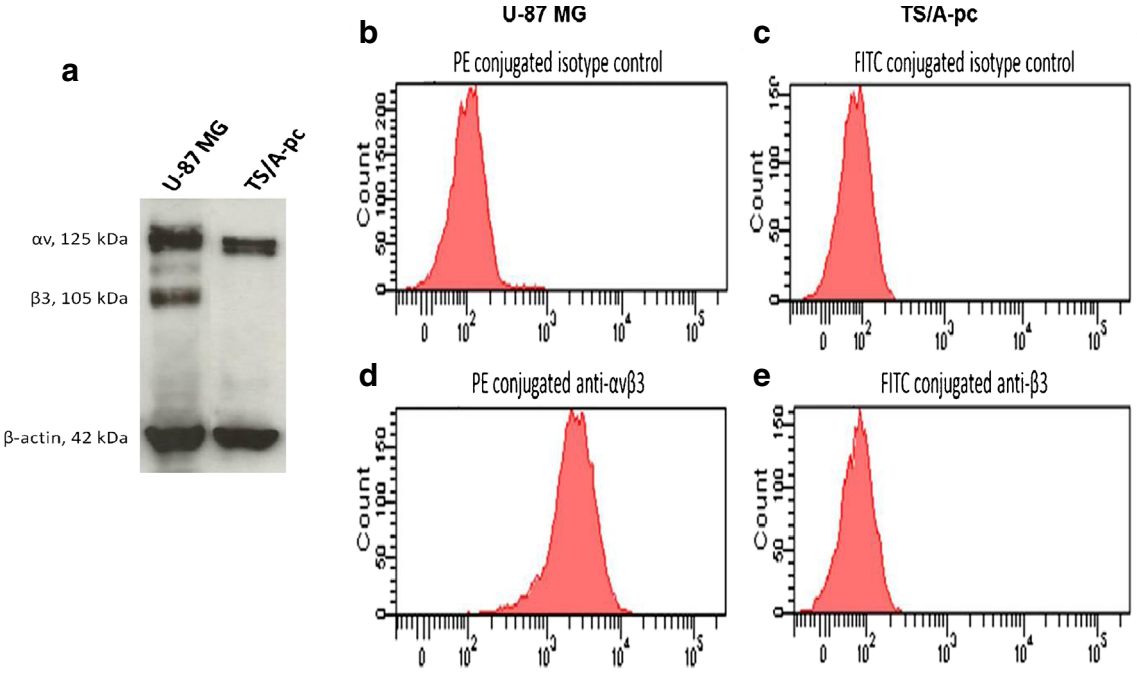

Fig. 1 Evaluation of integrin expression on U-87 MG and TS/A-pc cells by western blot analysis (**a**) and flow cytometry (**b**–**e**, x *axis* arbitrary units of fluorescence intensity on a log scale, y *axis* count of events). **b**, **c** Fluorescence intensity of the isotype controls incubated respectively on U-87 MG and TS/A-pc cells. **d** Fluorescence intensity of phycoerythrin-conjugated mouse anti-human αvβ3 antibody attached to U-87 MG cells (*PE* phycoerythrin). **e** Fluorescence intensity of fluorescein isothiocyanate-conjugated anti-mouse β3 subunit antibody attached to TS/A-pc cells (*FITC* fluorescein isothiocyanate)

subcutaneous tumours 1 h p.i. (3.3±0.5 %ID/g), 4 h p.i. (3.8±0.9 %ID/g) and until 48 h p.i. (1.6±0.0 %ID/g; Fig. 3 and Online Resource 3). On the other hand, ^{177}Lu-RAFT-RAD activity was not detected in tumours at any time after injection. ^{177}Lu-RAFT-RGD therefore specifically accumulated in the tumour with an excellent tumour/muscle ratio of about 10 from 1 h p.i.

Radionuclide therapy with ^{90}Y- or ^{177}Lu- radiolabelled RAFT-RGD

Effect of ^{90}Y-RAFT-RGD in αvβ3-positive tumours The results are presented in Fig. 4. ^{90}Y-RAFT-RGD significantly increased the TVDT of αvβ3-positive U-87 MG tumours, both small (4.8±1.4 days vs. 3.6±0.5 days in the control group, $P<0.05$; Fig. 4a) and large (6.3±1.8 days vs. 2.6±1.2 days in ^{90}Y-RAFT-RAD-treated animals, and 3.0±0.8 days in the control group, $P<0.01$ for both comparisons; Fig. 4c). No significant difference was observed between the TVDT of the control group and that of ^{90}Y-RAFT-RAD-treated animals. Survival was also significantly improved by ^{90}Y-RAFT-RGD treatment in αvβ3-positive U-87 MG tumours, both small (Fig. 4b) and large (Fig. 4d).

Effect of ^{177}Lu-RAFT-RGD in αvβ3-postive tumours The therapeutic potential of ^{177}Lu-RAFT-RGD was evaluated in small αvβ3-positive tumours (Fig. 5). The TVDT in the group that received 37 MBq of ^{177}Lu -RAFT-RGD was significantly (1.4 times) longer than in the control group and 1.3 times longer than in the group that received 37 MBq of ^{177}Lu -RAFT-RAD (4.8±0.7 days vs. 3.4±0.3 days and 3.7±0.8 days, respectively, $P<0.05$). There was no significant difference between the TVDT of the control group and that of the group treated with 37 MBq of ^{177}Lu-RAFT-RAD ($P=0.46$).

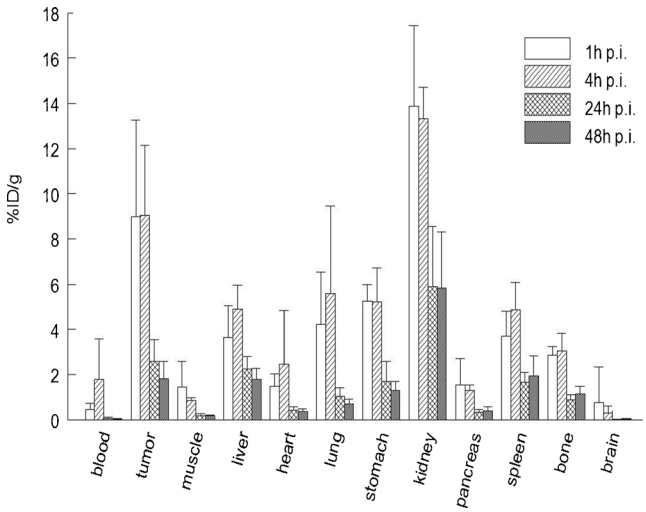

Fig. 2 Biodistribution of ^{90}Y-RAFT-RGD in nude mice bearing subcutaneous U-87 MG tumours expressing the $\alpha v\beta_3$ integrin

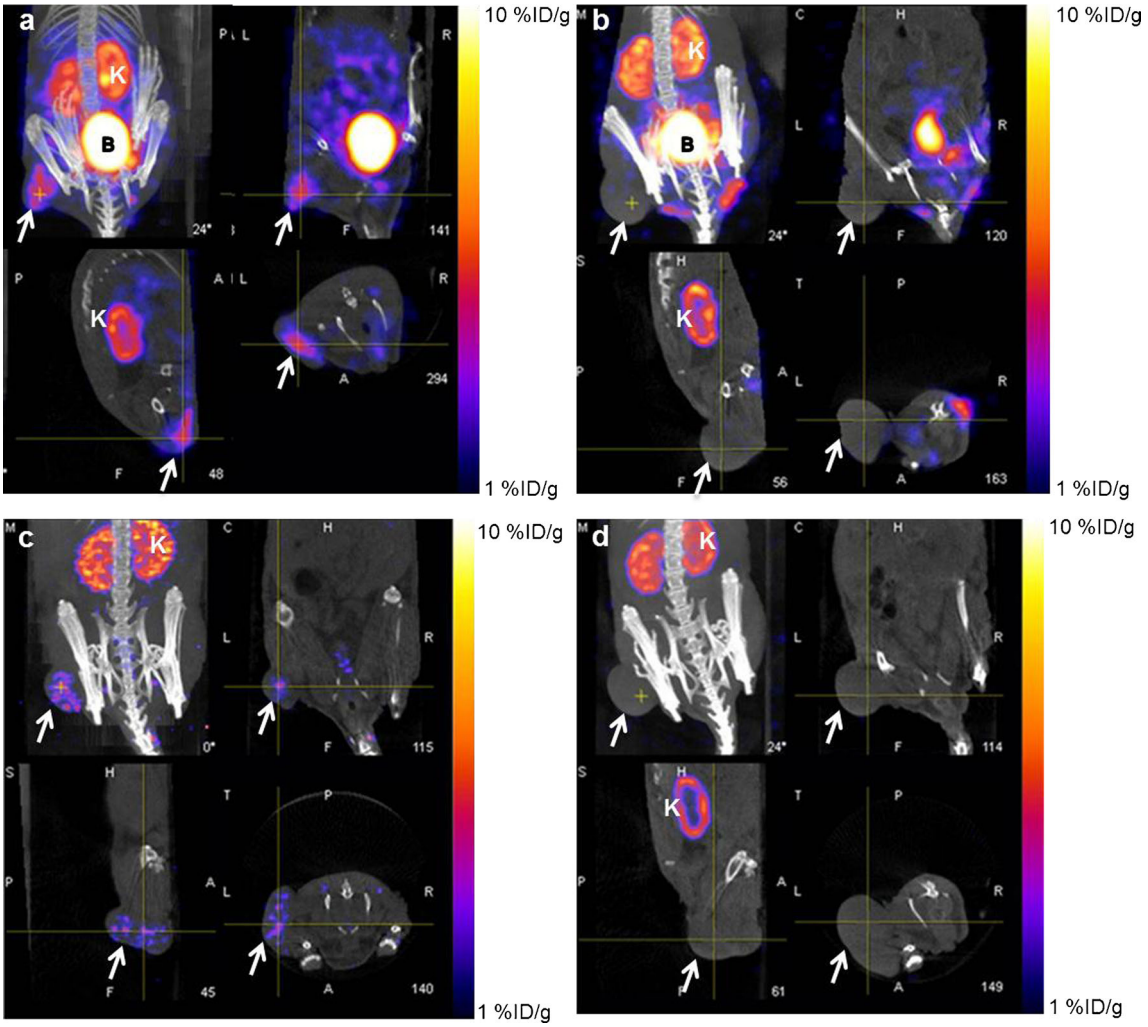

Fig. 3 Representative SPECT/CT images of U-87 MG tumour-bearing mice after preinjection of Gelofusine® and injection of ^{177}Lu-RAFT-RGD (**a**, **c**) or ^{177}Lu-RAFT-RAD (**b**, **d**). The scans were acquired 1 h (**a**, **b**) and 24 h (**c**, **d**) after intravenous injection of the radiolabelled peptides (*B* bladder, *K* kidney, *arrows* tumour)

Effect of ^{90}Y-RAFT-RGD in αvβ3-negative tumours The survival curves of the three groups of mice (the groups bearing large TS/A-pc tumours that received the ^{90}Y-RAFT-RGD or ^{90}Y-RAFT-RAD nonspecific treatment and the control group) are given in the Online Resource 4. There were no significant differences in TVDT among the groups (0.8±0.2 days, 0.8±0.1 days and 0.7±0.3 days, respectively; P=0.66).

Effect of fractionated treatment with ^{90}Y-RAFT-RGD The effect of fractionated treatment with ^{90}Y-RAFT-RGD in αvβ3-postive tumours was compared with that of unfractionated treatment (Fig. 6). The TVDT in the group that received 2×18.5 MBq of ^{90}Y-RAFT-RGD was significantly (2.4 times) longer than in the control group, and 1.7 times longer than in the group that received 2×18.5 MBq of ^{90}Y-RAFT-RAD (8.8±2.3 days, 3.6±1.5 days and 5.1±0.8 days, respectively; P<0.01). Nevertheless, there was no difference in tumour growth between mice receiving the fractionated treatment and those receiving the unfractionated treatment. In both cases, the TVDT was 2.4 times longer in the treated groups than in the control group. As observed with the other treatments, there was no significant difference in TVDT between the control group and the group treated with the radiolabelled nonspecific control RAFT-RAD (P=0.22).

Treatment toxicity

Medullary toxicity In untreated controls, the mean erythrocyte concentration before tumour implantation was 10.3×10^6/mm^3 (range 9.51 – 12.6×10^6/mm^3), the leucocyte concentration was 15,000/mm^3 (range 10,100 – 19,800/mm^3) and the platelet concentration was 9,800/mm^3 (range 7,000 – 14,300/mm^3). Toxicity in erythrocytes, leucocytes and platelets was expressed as the percentage relative to

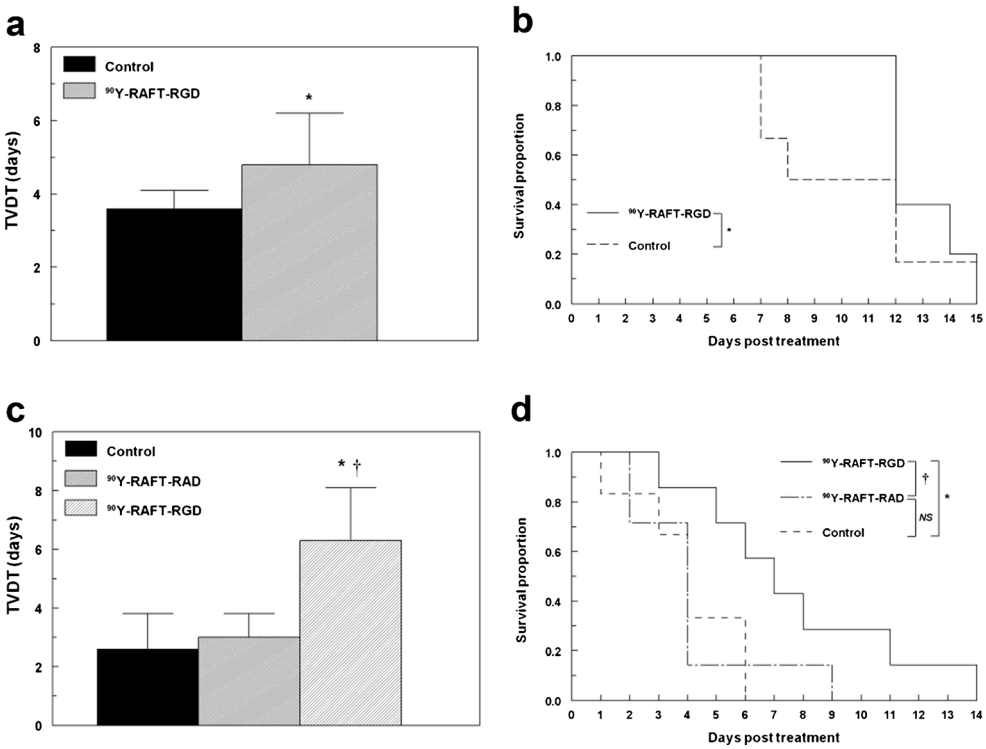

Fig. 4 Effect of 37 MBq ^{90}Y-RAFT-RGD in animals bearing small (**a**, **b**) and large (**c**, **d**) αvβ3-positive U-87 MG tumours (**a**, **c** tumour volume doubling time; **b**, **d** survival. *P<0.05 vs. control, †P<0.05 vs. ^{90}Y-RAFT-RAD

the basal concentration and percentages between 70 % and 130 % were considered normal. No significant erythropenia was observed in any treated or control groups (Table 1). There was an important increase in leucocyte and platelet counts in untreated groups. There was also an important reduction in leucocyte and platelet counts in treated groups compared to control groups by the end of the study, indicating bone marrow suppression. Nevertheless, these reductions were less important in mice treated with 37 MBq of ^{177}Lu-RAFT-RGD (leucocyte count 141± 21 % on the day the animal was killed in relation to the day of tumour implantation in the treated group vs. 195± 28 % in the control group, a difference of 54 %) or with fractionated treatment of 2×18.5 MBq of ^{90}Y-RAFT-RGD (equivalent values: 113±36 % vs. 370±44 %, a difference of 257 %) than in mice treated with 37 MBq of ^{90}Y-RAFT-RGD (equivalent values: 34±7 % vs. 482±183 %, a difference of 448 %). Whatever the treatment, there was no difference between the radiolabelled RAFT-RGD and RAFT-RAD.

Renal toxicity There was an increase in serum creatinine levels in the groups of mice treated with ^{90}Y-RAFT-RGD (Table 2). The fractionated treatment with 2× 18.5 MBq of ^{90}Y-RAFT-RGD raised the creatinine level by 32 % compared with the level in the control group, whereas the unfractionated treatment induced an increase of 64 %. There was no significant difference in serum creatinine levels among the groups of mice treated with 37 MBq of ^{177}Lu-RAFT-RGD or 37 MBq of ^{177}Lu-RAFT-RAD and the control group.

Radiation dosimetry extrapolation to humans

Human absorbed doses to normal organs were estimated from the ^{90}Y-RAFT-RGD biodistribution data obtained in Swiss Nude mice bearing U-87 MG tumours (Table 3). The highest absorbed dose was received by the stomach (0.0482 mGy/MBq) followed by the kidneys (0.0263 mGy/MBq). All organs had a very low level of estimated absorbed radiation doses. The estimated whole-body effective dose was 0.114 mSv/MBq.

Discussion

The present study showed that the radiolabelled RGD peptides ^{90}Y-RAFT-RGD and ^{177}Lu-RAFT-RGD were able to target αvβ3-expressing tumours following intravenous injection into the U-87 MG xenograft mouse model. These results are consistent with those of previous studies using ^{99m}Tc-labelled and ^{111}In-labelled RAFT-RGD which demonstrated selective accumulation of the tracer in αvβ3-positive tumours [11, 12]. The tumoral concentration of ^{90}Y-RAFT-RGD reached 9 %ID/g as early as 1 h p.i. and compared favourably with other RGD peptides in xenograft mouse models [11, 33, 34]. The tumour-to-blood tracer activity ratio reached 27 for ^{90}Y-

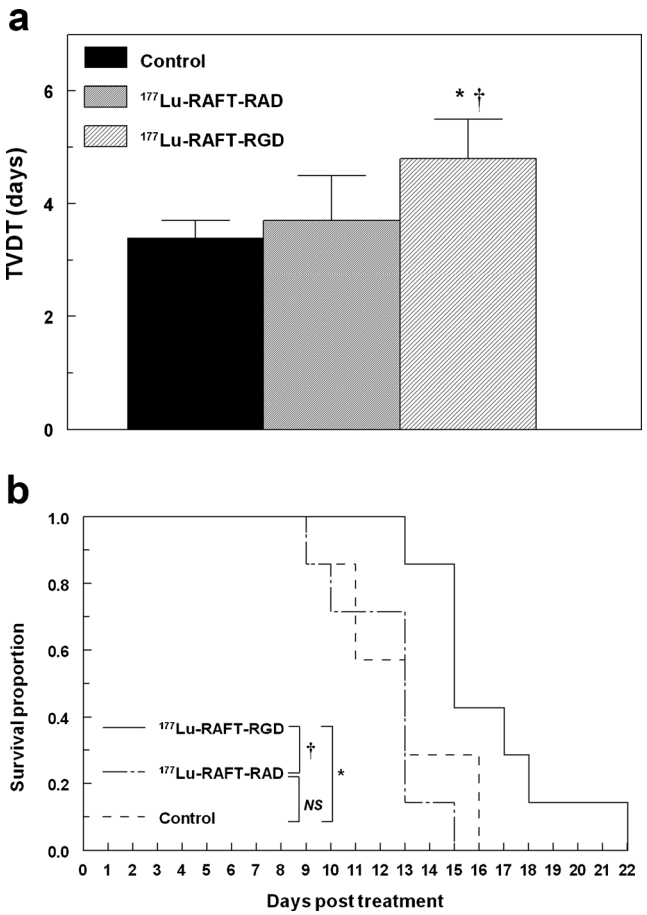

Fig. 5 Effect of 37 MBq ^{177}Lu-RAFT-RGD in animals bearing small αvβ3-positive U-87 MG tumours (**a** tumour volume doubling time; **b** survival). *P<0.05 vs. control, †P<0.05 vs. ^{90}Y-RAFT-RAD

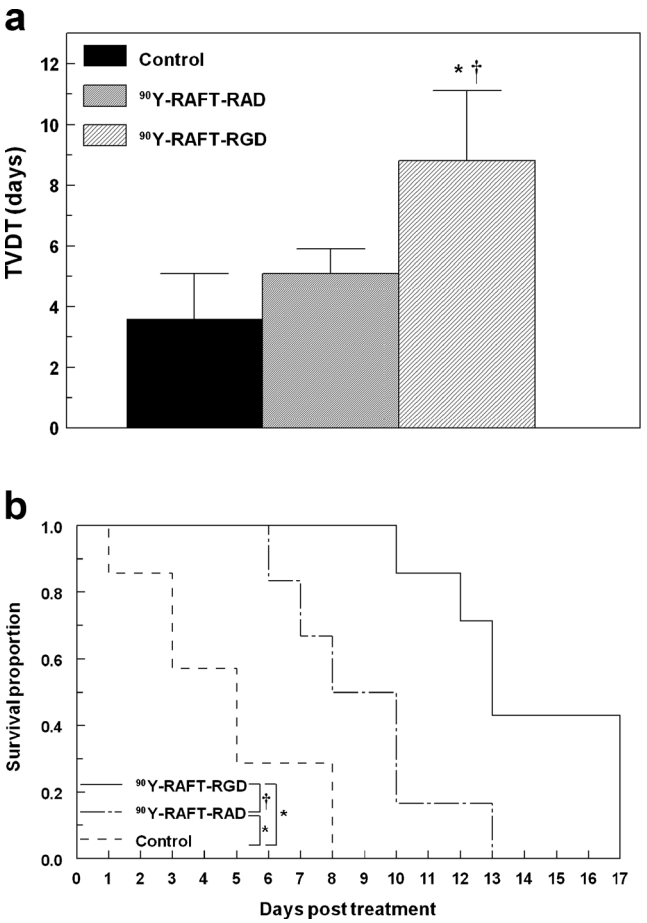

Fig. 6 Effect of fractionated ^{90}Y-RAFT-RGD treatment (2×18.5 MBq) on the tumour volume doubling time (**a**) and survival (**b**) in animals bearing large αvβ3-positive U-87 MG tumours. *P<0.05 vs. control, †P<0.05 vs. ^{90}Y-RAFT-RAD

RAFT-RGD at 24 h p.i. As expected, in vivo ^{177}Lu-RAFT-RGD and ^{177}Lu-RAFT-RAD imaging indicated that radiolabelled peptide accumulation in αvβ3-positive tumour was dependent of the RGD sequence of the peptide.

ITRT using ^{90}Y-RAFT-RGD caused a significant growth delay in both small and large αvβ3-expressing tumours. However, the effectiveness of treatment was better in large rather than small tumours. TVDT was 2.4 and 1.3 times higher than in the control group in large and small tumours, respectively. The improved efficacy in the treatment of large rather than small tumours was consistent with the long tissue penetration of the ^{90}Y β^- emission (R_{max} 11 mm) which is better suited for the irradiation of large tumours [35]. In comparison, the injection of 30 MBq of ^{90}Y-RAFT-RGD was not effective in treating TS/A-pc αvβ3-negative tumours. TVDT were similar in groups of mice treated with 30 MBq of either ^{90}Y-RAFT-RGD or ^{90}Y-RAFT-RAD as well as in the control group. Furthermore, 37 MBq of the scrambled sequence control peptide, ^{90}Y-RAFT-RAD, did not elicit a delay in αvβ3-positive tumour growth in comparison to that in the untreated control groups. These two last-mentioned results indicate that the efficacy of ^{90}Y-RAFT-RGD treatment can be attributed to specific αvβ3 integrin-targeting mediated by the RGD sequence of the peptide.

ITRT using ^{177}Lu-RAFT-RGD also elicited a significant delay in the growth of small αvβ3-expressing tumours with TVDT in the treated group being 1.4 times higher than in the control group. This was slightly higher than the increase in TVDT in mice bearing small αvβ3-positive tumours treated with ^{90}Y-RAFT-RGD which was 1.3 times higher than in the control group. Moreover, the absorbed tumoral dose was probably different despite similar injected doses (37 MBq). Indeed, ^{177}Lu is a less energetic β^- emitter than ^{90}Y and the time required to deliver a given dose of radioactivity with ^{177}Lu is longer than with ^{90}Y due to the 2.5-fold longer half-life of ^{177}Lu.

In order to precisely compare the efficacy of treatments using ^{90}Y-labelled or ^{177}Lu-labelled RAFT-RGD in small tumours, further dosimetry studies should be performed to determine the delivered tumour dose of ^{90}Y-RAFT-RGD and ^{177}Lu-RAFT-RGD, which will enable injections of distinct activities leading to similar tumour doses. Nevertheless, these preliminary results indicated that treatment with ^{177}Lu-RAFT-

Table 1 Blood cell counts relative to basal concentrations

Group	Erythrocytes (%)			Leucocytes (%)			Platelets (%)		
	T0	T1	T2	T0	T1	T2	T0	T1	T2
37 MBq ^{90}Y-RAFT-RGD in small tumours	100±11	111±13	77±4	100±19	175±27	121±56	100±31	140±33	98±38
Control			99±4			266±90			343±135
37 MBq ^{90}Y-RAFT-RGD in large tumours	100±24	124±10	69±9	100±21	177±47	34±7	100±16	194±56	194±147
37 MBq ^{90}Y-RAFT-RAD in large tumours			73±12			31±10			264±48
Control			84±12			482±183			877±591
2×18.5 MBq ^{90}Y-RAFT-RGD in large tumours	100±4	109±14	83±8	100±16	161±30	113±36	100±15	179±34	218±38
2×18.5 MBq ^{90}Y-RAFT-RAD in large tumours			87±4			128±18			201±12
Control			104±15			370±44			366±72
37 MBq ^{177}Lu-RAFT-RGD in small tumours	100±4	103±7	81±4	100±16	127±19	141±21	100±15	123±25	130±14
37 MBq ^{177}Lu-RAFT-RAD in small tumours			78±13			152±57			121±33
Control			97±6			195±28			278±89

The data presented are means±SD

T0 day of tumour implantation, *T1* day before treatment, *T2* day the animal was killed

RGD might be more effective than ^{90}Y-RAFT-RGD in slowing the growth of small αvβ3-positive tumours, even though the absorbed tumour dose from ^{177}Lu-RAFT-RGD was probably lower than that from ^{90}Y-RAFT-RGD for a similar injected activity.

The clinical application of αvβ3-targeted ITRT with RAFT-RGD radiolabelled ^{90}Y or ^{177}Lu might be promising only if toxicity is found to be acceptable. As a consequence of the long path-length of β^- particles in tissues [36], medullary toxicity is a major limitation of radiopeptide therapy and therefore had to be evaluated. Renal toxicity was more specific with peptide accumulation in the kidneys and was a matter of concern as we had previously observed high levels of renal retention of radiolabelled RAFT-RGD [11, 12].

Table 2 Serum creatinine concentrations

Group	Serum creatinine (μmol/L)
37 MBq ^{90}Y-RAFT-RGD in large tumours	86.6±23*
37 MBq ^{90}Y-RAFT-RAD in large tumours	81.4±18.3*
Control	52.8±13.6
2×18.5 MBq ^{90}Y-RAFT-RGD in large tumours	79.4±15.2*
2×18.5 MBq ^{90}Y-RAFT-RAD in large tumours	81.3±11.5*
37 MBq ^{177}Lu-RAFT-RGD in small tumours	67.8±12
37 MBq ^{177}Lu-RAFT-RAD in small tumours	74.2±9.9
Control	60±18.1

The data presented are mean±SD

*$P<0.05$ vs. control group

An important reduction in leucocyte and platelet counts was observed in treated groups compared to control groups, probably indicating bone marrow suppression. However, these reductions were less important when mice were treated with RAFT-RGD labelled with ^{177}Lu rather than ^{90}Y or with fractionated treatment instead of a single administration of ^{90}Y-RAFT-RGD, indicating a reduced toxicity induced by ^{177}Lu-RAFT-RGD compared to ^{90}Y-RAFT-RGD and a positive effect of fractionating the treatment. Control groups showed

Table 3 Human absorbed doses estimated from the biodistribution of ^{90}Y-RAFT-RGD in Swiss nude mice bearing U-87 MG tumours

Organ	Absorbed dose (mGy/MBq)
Brain	1.96×10^{-5}
Stomach	4.82×10^{-2}
Kidneys	2.63×10^{-2}
Liver	1.30×10^{-2}
Lungs	2.04×10^{-2}
Muscle	9.66×10^{-6}
Pancreas	1.18×10^{-4}
Bone marrow[a]	
Using bone measured activities	4.52×10^{-3}
Using blood measured activities	1.38×10^{-3}
Bones	4.01×10^{-4}
Spleen	5.97×10^{-4}
Whole-body effective dose (mSv/MBq)[a]	
Using bone measured activities	0.114
Using blood measured activities	0.110

[a] According to the method described by Rizvi et al. [31]

an important elevation in leucocyte and platelet counts caused by the presence and growth of the xenografted tumour. Nude mice lack a thymus and therefore cannot generate mature T lymphocytes, yet all other lymphocyte types are produced, and this could explain the immune elevation of leucocytes that was observed in untreated groups corresponding to their immune response against the xenograft. The elevation in thrombocyte counts is a frequent side effect in different types of cancer and is mainly caused by peritumoral inflammation. The elevations in leucocyte and platelet counts in untreated groups make it difficult to assess the differences in medullary toxicity between treated groups and could be resolved by future evaluation of medullary toxicity in mice without tumour.

In a study evaluating the effect of dimeric or tetrameric ^{90}Y-labelled RGD peptides, medullary toxicity was evaluated by monitoring blood cell counts in mice without tumour injected with escalating doses of ^{90}Y-labelled RGD peptides [27]. A dose-dependent reduction in leucocytes was observed. The leucopenia nadir was observed on day 12 after injection after which the leucocyte concentration recovered gradually to baseline values by 20 days after injection. Similar temporary leucopenia was observed in another study following radioimmunotherapy, with a leucopenia nadir observed on day 15 after injection and the concentration restored by day 30 [37]. In our study, the average survival times did not exceed 15 days, which allowed us to consider that the medullary toxicity observed with the different treatments was also only temporary.

The increase observed in serum creatinine levels in animals treated with ^{90}Y-RAFT-RGD indicated a decrease in glomerular filtration rate and acute nephrotoxicity of ^{90}Y-radiolabelled peptide despite the use of the renoprotective Gelofusine® injection. Nevertheless, this acute nephrotoxicity seemed to be attenuated by fractionated treatment rather than by single dose administration. The increase in serum creatinine levels compared with control groups was twice as high in the group treated with 37 MBq of ^{90}Y-RAFT-RGD (64 %) than in the group treated with 2×18.5 MBq of ^{90}Y-RAFT-RGD (32 %). No acute nephrotoxicity was observed in groups treated with ^{177}Lu-radiolabelled peptide. The difference observed in nephrotoxicity between the groups treated with ^{90}Y-radiolabelled and with ^{177}Lu-radiolabelled peptide could be explained by the difference in their β^- emission path-lengths. The radiosensitive glomeruli are mostly localized in the outer cortical region and the retention of the radiolabelled peptides occurs in the relatively radioresistant tubular proximal cells of the inner cortical region of the kidney. The physical distance separating the glomeruli from the tubular proximal cells is such that the glomeruli are not irradiated by ^{177}Lu β^- emission because of its short path-length, whereas the long path-length of ^{90}Y β^- emission covers the entire mouse kidney [15]. For the same reasons and because of the size difference between a human and a mouse kidney, the glomeruli in a human kidney would probably be less exposed to the radiation from ^{90}Y-RAFT-RGD that is trapped in the farther tubular proximal cells than the glomeruli of the smaller mouse kidney.

Moreover, estimated human absorbed doses to normal organs based on the biodistribution results of the ^{90}Y-RAFT-RGD in nude mice bearing U-87 MG tumours indicated that the absorbed dose of ^{90}Y-RAFT-RGD in human kidneys would be 2.6×10^{-2} mGy/MBq. This low renal dose is unlikely to induce nephrotoxicity. For example, the renal absorbed dose of ^{90}Y-ibritumomab tiuxetan (Zevalin®) evaluated in a clinical study was 0.22 mGy/MBq [38], and Zevalin® has been approved by the Food and Drug Administration since 2002 for the treatment of non-Hodgkin's lymphoma.

Overall, our results indicated that ITRT using ^{90}Y-RAFT-RGD caused a significant growth delay in both large and small αvβ3-expressing tumours and the treatment efficacy was superior when large rather than small tumours were targeted with the TVDT being 2.4 and 1.3 times longer than in the control group in large and small tumours, respectively. ^{90}Y-RAFT-RGD treatment induced acute renal and bone-marrow toxicities in mice. Fractionating the 37 MBq dose of ^{90}Y-RAFT-RGD into two injections of 18.5 MBq did not improve the tumour growth inhibition since the TVDT was increased 2.4-fold compared to the control group in both cases, but reduced haematological and renal toxicity. ITRT using ^{177}Lu-RAFT-RGD elicited a significant growth delay in small αvβ3-expressing tumours with the TVDT in the treated group being 1.4 times longer than in the control group with no acute nephrotoxicity and reduced bone-marrow toxicity than with ^{90}Y-RAFT-RGD. In this last model, the ^{177}Lu-RAFT-RGD treatment appeared to be less toxic in mice than ^{90}Y-RAFT-RGD. Yet the ^{90}Y-RAFT-RGD toxicities that we found in this mouse model might not be transposable to humans.

Conclusion

The present study highlights the potential of ^{90}Y-RAFT-RGD and ^{177}Lu-RAFT-RGD as agents for ITRT targeting αvβ3-expressing tumours. Further optimization of the therapy is required which will involve increasing the number of injections and performing complete dosimetry studies to determine the most effective radiation dose. Such optimization would lead to an improvement in the therapeutic effect while reducing radiation toxicity to normal tissues.

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Conflicts of interest None.

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