Journal of Nuclear Medicine, published on February 23, 2017 as doi:10.2967/jnumed.116.187021

Alpha- versus beta-emitting radionuclides for pretargeted radioimmunotherapy of CEA-expressing human colon cancer xenografts

Sandra Heskamp[1], Reinier Hernandez[2], Janneke D.M. Molkenboer-Kuenen[1], Markus Essler[3], Frank Bruchertseifer[4], Alfred Morgenstern[4], Erik J. Steenbergen[5], Weibo Cai[2], Christof Seidl[6,7], William J. McBride[8], David M. Goldenberg[8], Otto C. Boerman[1]

[1] Department of Radiology and Nuclear Medicine, Radboud university medical center, Nijmegen, The Netherlands

[2] Medical Physics Department, University of Wisconsin-Madison, Madison, WI, USA

[3] Klinik und Poliklinik fur Nuklearmedizin, University of Bonn, Bonn, Germany

[4] European Commission, Joint Research Centre – Directorate for Nuclear Safety and Security, Karlsruhe, Germany

[5] Department of Pathology, Radboud university medical center, Nijmegen, The Netherlands

[6] Department of Nuclear Medicine, Technische Universität München, Munich, Germany,

[7] Department of Obstetrics and Gynecology, Technische Universität München, Munich, Germany

[8] Immunomedics, Inc., Morris Plains, NJ, USA

Corresponding author

Sandra Heskamp

Radboud university medical center

Department of Radiology and Nuclear Medicine

P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

Phone: +31-24-36-19097, Fax: +31-24-36-18942

E-mail: Sandra.Heskamp@radboudumc.nl

Word count: 4,995

Running title: Pretargeted alpha therapy

Abstract

Rational: Pretargeted radionuclide therapy (PRIT) with the beta-emitting radionuclide ^{177}Lu is an attractive approach to treat CEA-expressing tumors. The therapeutic efficacy of PRIT could be improved by using alpha-emitting radionuclides such as ^{213}Bi. Herein, we report and compare the tumor targeting properties and therapeutic efficacy of ^{213}Bi and ^{177}Lu for PRIT of CEA-expressing xenografts, using the bispecific antibody TF2 (anti-CEA x anti-HSG) and the di-HSG-DOTA peptide IMP288.

Methods: The in vitro binding characteristics of ^{213}Bi-IMP288 were compared with those of ^{177}Lu-IMP288. Tumor targeting of ^{213}Bi-IMP288 and ^{177}Lu-IMP288 was studied in mice bearing subcutaneous (s.c.) LS174T tumors that were pretargeted with the bispecific antibody TF2. Finally, the effect of ^{213}Bi-IMP288 (6, 12, or 17 MBq) and ^{177}Lu-IMP288 (60 MBq) on tumor growth and survival was assessed. Toxicity was determined by monitoring body weight, analyzing blood samples for haematological and renal toxicity (haemoglobin, leucocytes, platelets, creatinine), and by immunohistochemical analysis of the kidneys.

Results: The in vitro binding characteristics of ^{213}Bi-IMP288 (K_d = 0.45 ± 0.20 nM) to TF-2 pretargeted LS174T cells were similar to those of ^{177}Lu-IMP288 (K_d = 0.53 ± 0.12 nM). In vivo accumulation of ^{213}Bi-IMP288 in LS174T tumors was observed as early as 15 min post injection (9.2 ± 2.0 %ID/g). ^{213}Bi-IMP288 cleared rapidly from the circulation; at 30 min post injection the blood levels were 0.44 ± 0.28 %ID/g. Uptake in normal tissues was very low, except for the kidneys where uptake was 1.8 ± 1.1 %ID/g, at 30 min p.i. The biodistribution of ^{213}Bi-IMP288 was comparable to that of ^{177}Lu-IMP288. Mice treated with a single dose of ^{213}Bi-IMP288 or ^{177}Lu-IMP288 showed significant inhibition of tumor growth. Median survival for the PBS, 6 MBq ^{213}Bi-IMP288, 12 MBq ^{213}Bi-IMP288, and 60 MBq ^{177}Lu-IMP288 treated groups was 22, 31, 45, and 42 days, respectively. Mice receiving 17 MBq ^{213}Bi-IMP288 showed significant weight loss, resulting in a median survival of only 24 days. No changes in haemoglobin, platelets, and leucocytes

were observed in the treatment groups. However, immunohistochemical analysis of the kidneys of mice treated with 17 or 12 MBq ^{213}Bi-IMP288 showed signs of tubular damage, indicating nephrotoxicity.

Conclusion: This study showed, for the first time, that PRIT with TF2 and ^{213}Bi-IMP288 is feasible and comparable to ^{177}Lu-IMP288 in terms of effectiveness. However, at the higher doses, kidney toxicity was observed. Future studies are warranted to determine the optimal dosing schedule to improve the therapeutic efficacy while reducing renal toxicity.

Introduction

Targeted radiotherapy with alpha-emitting radionuclides is a promising strategy for the treatment of cancer. Currently, beta-emitting radionuclides such as lutetium-177 (^{177}Lu), yttrium-90 (^{90}Y), and iodine-131 (^{131}I) are most commonly used. However, alpha-particles present significantly higher energies than beta-particles (4-9 MeV versus 0.1 – 2.2 MeV), which combined with very short path lengths results in high linear energy transfer (LET) and a greater probability of generating DNA double strand breaks (DBS) upon interaction with cell nuclei. This occurs independently of tissue oxygenation, dose rate and cellular resistance to photon irradiation and chemotherapy *(1-5)*. Therefore, alpha particles are highly cytotoxic and promising candidates for targeted radiotherapy.

Colorectal cancer is the third most common type of cancer in both men and women in the Western world *(6)*. Radioimmunotherapy (RIT) is an attractive new treatment option. Unfortunately, for colorectal cancer, RIT has not been very effective due to the radioresistance of these tumors and the limited radionuclide activities that can be administered safely. Myelotoxicity as a result of continuous radiation exposure of red bone marrow by long circulating antibodies is the dose-limiting factor *(7, 8)*. Pretargeted radioimmunotherapy (PRIT) has been developed to reduce the radiation dose to normal tissues while delivering lethal doses to the tumor. In this approach, a bispecific monoclonal antibody (bsMAb) is administered intravenously and at the time it has accumulated in the tumor and cleared from the circulation, a radiolabeled hapten is administered that clears rapidly from the blood, but is trapped in the tumor by the anti–hapten-binding arm of the bsMAb *(9)*.

Previously, we have shown that PRIT with the anti-CEA x anti-HSG bsMAb TF2, and ^{177}Lu-labeled di-HSG-DOTA peptide IMP288, inhibited the growth of CEA-positive human colonic tumors xenografts in mice with limited toxicity *(10, 11)*. Moreover, a phase I study in patients with metastatic colorectal cancer

showed that this treatment is feasible and safe *(12)*. However, in both the preclinical and clinical studies, progressive tumor growth was observed after the ^{177}Lu-IMP288 treatment.

To date, no studies have been performed using alpha-emitting particles for PRIT with TF2 and IMP288. Because of the high cytotoxicity of alpha-particles, they are potentially more therapeutically effective compared with ^{177}Lu. Moreover, the extremely rapid tumor targeting kinetics of IMP288 matches the short half-life of the alpha-emitter ^{213}Bi ($t_{1/2}$: 46 min). Therefore, the aim of our study was to compare the tumor targeting properties and therapeutic efficacy of ^{213}Bi- and ^{177}Lu-labeled IMP288 for PRIT with TF2 in mice bearing CEA-expressing colorectal cancer xenografts.

Material and methods

Cell culture and pretargeting reagents

The CEA-positive human colorectal cancer cell line LS174T was cultured in RPMI1640 (GIBCO, BRL Life Sciences Technologies, The Netherlands), supplemented with 2 mM glutamine (GIBCO) and 10% FCS (Sigma-Aldrich Chemie BV, The Netherlands) at 37 °C in a humidified atmosphere with 5% CO_2.

The bsMAb TF2 (anti-CEA x anti-HSG), control bsMAb TF12 (anti-TROP-2 x anti-HSG) and the peptide IMP288, were provided by Immunomedics, Inc. and IBC Pharmaceuticals, Inc. (Morris Plains, NJ, US). The preparation and binding properties of TF2 and TF12 have been described previously *(13-15)*. IMP288 is a 7,10-tetra-azacyclododecane-N,N',N'',N'''-tetraacetic (DOTA)-conjugated D-Tyr-D-Lys-D-Glu-D-Lys-NH_2 tetrapeptide in which both lysine residues are derivatized with a HSG-moiety (Molecular weight: 1456 Da). The synthesis and purification have been described by McBride et al *(16)*.

Radiolabeling and stability

Radiolabeling with ^{213}Bi

IMP288 (0.7 – 7.0 nmol) was added to 150 µl 2 M TRIS and 50 µl 20% ascorbic acid. ^{213}Bi was eluted from a ^{225}Ac/^{213}Bi generator (Institute for Transuranium Elements, Karlsruhe, Germany) using 600 µl 0.1 M HCl, 0.1 M NaI. The eluate was immediately added to the peptide (final pH 9.0) and was incubated for 10 min at 95 °C. Labeling efficiency was determined by instant thin-layer chromatography (ITLC), on silica gel chromatography strips (Agilent Technologies, Palo Alto, CA), using 0.1 M ammonium acetate, 0.1 M EDTA as the mobile phase, and by RP-HPLC on an Agilent 1200 system (Agilent Technologies). A monolithic C18 column (Onyx, 4.6 x 100 mm; Phenomenex, Torrance, CA) was used at a flow rate of 1 ml/min with the following buffer system: buffer A, 0.1% v/v TFA in water; buffer B, 0.1% v/v TFA in acetonitrile; and a gradient of 97% buffer A to 0% buffer A at 5 – 15 min. The radioactivity of the eluate

was monitored using an in-line NaI radiodetector (Raytest GmbH, Straubenhardt, Germany). Elution profiles were analyzed using Gina-star software (version 2.18; Raytest GmbH). For therapy studies, 3.8 µM DTPA was added to a final concentration of 0.14 µM to complex non-incorporated ^{213}Bi. Subsequently, the labeling mixture was trapped on an Oasis HLB Cartridge (30 mg, Waters, Milford, MA, USA) and eluted with 500 µl ethanol. The ethanol was evaporated until < 50 µl and ^{213}Bi-IMP288 was diluted in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Radiochemical purity exceeded 95% in all experiments.

Radiolabeling with ^{177}Lu

No-carrier-added ^{177}Lu (specific activity > 3,000 GBq/mg) was obtained from Isotope Technologies Garching GmbH (Garching, Germany). Radiolabeling of IMP288 was performed in 0.5 M MES buffer, pH 5.5, for 15 min at 95 °C. After the labeling reaction was completed, 50 mM ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 5 mM to complex non-incorporated ^{177}Lu. Labeling efficiency was determined as described for the ^{213}Bi-conjugates and exceeded 95% in all experiments.

Stability

Stability of ^{213}Bi- and ^{177}Lu-labeled IMP288 was tested in PBS at 37 °C. At 30, 60, 90, and 120 min after start of incubation, radiochemical purity was determined with ITLC as described above.

In vitro studies

Binding and internalization

Binding and internalization was determined as described previously.*(17)* In short, 200,000 – 300,000 LS174T cells/well were cultured to confluency in six-well plates (2-3 days). Subsequently, cells were pre-incubated for 1 hour in 1 ml binding buffer (RPMI1640, 0.5% BSA) containing 64 nM TF2 or TF12 at 37 °C in a humidified atmosphere with 5% CO_2. After pre-incubation, cells were incubated for 1, 2, and 3 h with

22 nM of ^{177}Lu-IMP288 or ^{213}Bi-IMP288. Acidic wash buffer (0.1 M HAc, 0.15 M NaCl, pH 2.8) was used to remove the membrane-bound fraction of the cell-associated ^{177}Lu-IMP288 or ^{213}Bi-IMP288 and cells were harvested using 0.1 M NaOH. Activities were measured in a shielded well-type gamma counter, by measuring the γ-emission of ^{213}Bi (440 keV, Perkin-Elmer, Boston, MA, US). Specific binding and internalization were calculated by subtracting the non-specific binding and internalization from the total binding and internalization.

Scatchard analysis

The affinity of ^{177}Lu-IMP288 and ^{213}Bi-IMP288 was determined with Scatchard analysis. Cells were pre-incubated with TF2 or TF12 as described above. Subsequently, cells were incubated with increasing concentrations of radiolabeled IMP288 (0.03 – 100 nM). After 45 min of incubation on ice, the unbound radiolabeled IMP288 was removed and the cell-associated activity was measured in a gamma counter. Dissociation constant (K_d) and maximum binding (B_{max}) were determined using a one-site specific binding equation in GraphPad.

Animal studies

Animal studies were performed using female BALB/c nude mice (Janvier, le Genest-Saint-Isle, France) and were conducted in accordance with the principles established by the revised Dutch Act on Animal Experimentation (1997) and approved by the institutional Animal Welfare Committee of the Radboud University Nijmegen. At 6-8 weeks of age, mice were inoculated subcutaneously with 1 x 10^6 LS174T cells. Experiments started when tumors reached a size of approximately 0.1 cm^3 (8 – 10 days post tumor cell inoculation). Tumor size was determined by caliper measurements in three dimensions (radius x, y, and z), using the following formula: $4/3 \cdot \pi \cdot x \cdot y \cdot z$.

Biodistribution

LS174T tumors in mice were pretargeted by intravenous injection of 5 nmol TF2 or the non-specific bsMAb TF12. After 20 h, mice received an intravenous injection of different doses ^{213}Bi-labeled IMP288 (group 1: 0.14 nmol and 0.98 MBq; group 2: 0.28 nmol and 1.68 MBq; group 3: 0.56 nmol and 3.12 MBq). One hour post injection, mice were euthanized and the biodistribution of the radiolabeled peptide was determined ex vivo. Tumor, blood, muscle, lung, spleen, pancreas, intestine, kidney, and liver were dissected, collected, weighed, and the activities were measured in a gamma counter. To calculate the uptake of radiolabeled peptide in each sample as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously.

Subsequently the pharmacokinetics of ^{213}Bi-IMP288 were determined. Mice with TF2-pretargeted tumors received an intravenous injection of 2.26 MBq ^{213}Bi-labeled IMP288 (0.28 nmol). At 15, 30, 45, and 60 min after injection of ^{213}Bi-labeled IMP288, mice were euthanized and the biodistribution of the radiolabeled peptide was determined ex vivo.

Finally, the biodistribution of 0.28 nmol ^{213}Bi-IMP288 was compared to 0.28 nmol ^{177}Lu-IMP288. Mice with TF2-pretargeted tumors were injected intravenously with 13 MBq ^{213}Bi-labeled IMP288 or 10 MBq ^{177}Lu-IMP288. At 60 min after injection of the radiolabeled IMP288, the mice were euthanized and the biodistribution of the radiolabeled peptide was determined ex vivo. Tumors of the ^{177}Lu-IMP288 group were frozen at -80 °C for ex vivo autoradiography (tumor weight 88 ± 72 mg).

Autoradiography

Frozen tumor sections (5 µm) from the mice injected with TF2 and 10 MBq ^{177}Lu-IMP288 (0.28 nmol) were exposed to a Fujifilm BAS cassette 2025 overnight (Fuji Photo Film). Phospholuminescence plates were scanned using a Typhoon FLA 7000 laser scanner (GE Healthcare Life Sciences) at a pixel size of 25 × 25 µm. Images were analyzed with Aida Image Analyzer software (Raytest).

PRIT study

Six groups of eight mice each bearing LS174T tumors were pretargeted with 5 nmol TF2. Twenty hours later, mice received a single intravenous injection of radiolabeled IMP288. The first three groups received 0.28 nmol of 6 MBq ^{213}Bi-IMP288, 12 MBq ^{213}Bi-IMP288 or 17 MBq ^{213}Bi-IMP288. In order to investigate whether a lower peptide dose would improve the therapeutic efficacy, the fourth group received 0.14 nmol IMP288 labeled with 12 MBq ^{213}Bi. The fifth group received 60 MBq of 0.28 nmol ^{177}Lu-IMP28, which is the maximum tolerable dose as determined previously *(11)*. Mice in the control group received vehicle only (PBS 0.5% BSA).

The general health of the animals was measured by monitoring body weight three times per week. Tumor size was measured three times per week by measuring tumor diameter in three dimensions with a caliper. Mice were removed from the experiment based on the following criteria: weight loss >20% compared with baseline or >15% within 2 days, tumor size ≥ 2 cm^3, or ulcerative tumor growth. Removed animals were inspected for any macroscopical evidence of abnormalities and the kidneys were harvested, stored in 4% formalin, and embedded in paraffin for further analysis.

Four separate groups of non-tumor bearing mice (n=4 per group) received 0.28 nmol of 6 MBq ^{213}Bi-IMP288, 12 MBq ^{213}Bi-IMP288, 17 MBq ^{213}Bi-IMP288, or 60 MBq ^{177}Lu-IMP88. Blood samples were collected from these mice before treatment and at weeks 2, 4, 6, and 8 after treatment to measure hematological toxicity (hemoglobin, leucocytes, thrombocytes). Renal toxicity was analyzed by measuring plasma creatinine levels before therapy and at week 1, 3, and 5 after treatment. Furthermore, kidney sections were stained with hematoxylin-eosin (HE) and periodic acid-Schiff reagent (PAS). Renal damage was microscopically graded from 0 (no damage) to 4 (severe damage) by an experienced pathologist (ES), according to Table 1 *(18)*.

Statistical analyses

Statistical analyses were performed using PASW Statistics version 18.0 (Chicago, IL) and GraphPad Prism version 5.03 (San Diego, CA) for Windows. Continuous data were described with the mean and standard deviation (sd), unless stated otherwise. Comparisons were performed using nonparametric Kruskal-Wallis and Mann-Whitney U test. Survival was described with the median survival, and survival curves were compared with the log-rank test. A p-value below 0.05 was considered statistically significant.

Results

Radiolabeling and stability

IMP288 was labeled with more than 95% efficiency at maximum specific activities of 320 and 214 MBq/nmol for ^{213}Bi and ^{177}Lu, respectively. After 2 h in PBS at 37 °C, no significant loss of the radionuclide was observed. Radiochemical purity was 99.96% and 99.75%, for ^{213}Bi-IMP288 and ^{177}Lu-IMP288, respectively.

In vitro

^{177}Lu-IMP288 and ^{213}Bi-IMP288 showed similar binding to LS174T cells pretreated with TF2 and only a small fraction of the cell-associated activity (<20%) was internalized (Table 2). The internalization rate did not differ significantly between the two tracers. Furthermore, both tracers showed high affinity for binding to TF2 on LS174T cells: K_d = 0.45 ± 0.20 nM and 0.53 ± 0.13 nM for ^{213}Bi- and ^{177}Lu-IMP288, respectively (Figure 1).

Biodistribution

Tumor uptake of ^{213}Bi-IMP288 was the highest in mice receiving 0.14 nmol of ^{213}Bi-IMP288 (17.1 ± 3.4 %ID/g), followed by the groups receiving 0.28 nmol (9.7 ± 1.6 %ID/g) and 0.56 nmol (7.5 ± 0.7 %ID/g, p = 0.005, Figure 2A). Uptake in LS174T tumors pretargeted with the control bsMAb TF12 was significantly lower (0.7 ± 0.5 %ID/g, p = 0.008). Uptake of ^{213}Bi-IMP288 in healthy tissue was very low, except for the kidneys (1.4 ± 0.3 %ID/g in 0.28 nmol group). Subsequently, the biodistribution of 0.28 nmol ^{213}Bi-IMP288 was determined at several time points post injection). Tumor uptake remained stable between 15 min and 60 min post injection (9.2 ± 2.0 %ID/g, 6.6 ± 3.0 %ID/g, 8.9 ± 1.7 %ID/g, and 9.7 ± 1.6 %IDg, at 15, 30, 45, and 60 min post injection, respectively) (Figure 2B). The radiolabeled peptide cleared rapidly from the circulation, 30 min post injection ^{213}Bi-IMP288 concentration in blood was 0.44 ± 0.28 %ID/g.

Kidney uptake at 30 min post injection was 1.8 ± 1.1 %ID/g. Overall, the uptake of ^{213}Bi-IMP288 in tumor and normal tissue was similar to that of ^{177}Lu-IMP288 (Figure 2C).

Autoradiography

Autoradiographical analysis of tumor sections showed that ^{177}Lu-MP288 was distributed heterogeneously within the tumor. HE staining of the same tumor sections showed that low uptake of radiolabeled IMP288 was found both in areas of necrosis, as well as in viable tumor areas (Figure 3).

PRIT study

Body weight of non-tumor-bearing animals after radionuclide-IMP288 treatment

Mice treated with 6 MBq of ^{213}Bi-IMP288 or 60 MBq of ^{177}Lu-IMP288 showed almost no effect on body weight compared with control animals (Figure 4). Mice administered 12 MBq ^{213}Bi-IMP288 showed an initial drop in body weight which slowly recovered. Treatment with 17 MBq ^{213}Bi-IMP288 significantly reduced body weight during the first weeks, resulting in the removal of all animals from the experiment within 10 to 39 days after treatment.

Hematological and renal toxicity of non-tumor-bearing animals after radionuclide-IMP288 treatment

None of the treatment groups showed significant changes in hemoglobin, leucocytes, thrombocytes, and creatinine levels (Supplemental Figure S1). Immunohistochemical analysis of the kidneys treated with PBS, 60 MBq ^{177}Lu-IMP288, or 6 MBq ^{213}Bi-IMP288 showed normal morphology or only mild abnormalities (grade 0-1). Kidney damage in mice treated with 12 or 17 MBq ^{213}Bi-IMP288 varied from grade 1 to 3, as indicated by the observed tubular dilation, flat tubule epithelium, and inflammatory infiltrate in the glomeruli (Figure 5).

Tumor growth and survival of tumor-bearing mice after radionuclide-IMP288 treatment

Tumor growth was significantly inhibited by ^{213}Bi-IMP288 treatment (Figure 6). Mice treated with 17 MBq ^{213}Bi-IMP288 showed a significant decrease in body weight (≥20% compared to baseline) and had to be removed from the experiment within 23 to 41 days after therapy. The delay in tumor growth did not differ between mice that received 0.28 or 0.11 nmol of 12 MBq ^{213}Bi-IMP288. Tumor growth inhibition in these groups was similar to that of mice receiving 60 MBq ^{177}Lu-IMP288 (0.28 nmol). Mean tumor doubling time for PBS, 6 MBq ^{213}Bi-IMP288, 12 MBq ^{213}Bi-IMP288 (0.28 nmol), 12 MBq ^{213}Bi-IMP288 (0.14 nmol), and 60 MBq ^{177}Lu-IMP288 was 3.2 ± 0.6, 6.5 ± 1.2, 9.6 ± 2.7, 10.7 ± 7.8, and 10.6 ± 9.2 days, respectively. The overall survival significantly increased for mice treated with 6 MBq ^{213}Bi-IMP288 ($p = 0.037$), 12 MBq ^{213}Bi-IMP288 (0.28 nmol, $p = 0.003$), 12 MBq ^{213}Bi-IMP288 (0.14 nmol, $p = 0.001$), and 60 MBq ^{177}Lu-IMP288 ($p < 0.001$), as compared to the PBS group. Overall survival of the mice that received 17 MBq ^{213}Bi-IMP288 did not increase significantly. Kaplan Meier curves are presented in Figure 7. Median survival for the PBS, 6 MBq ^{213}Bi-IMP288, 12 MBq ^{213}Bi-IMP288 (0.28 nmol), 12 MBq ^{213}Bi-IMP288 (0.14 nmol), and 60 MBq ^{177}Lu-IMP288 treated groups was 22, 31, 45, 45, and 42 days, respectively. Median survival for 17 MBq ^{213}Bi-IMP288 was 24 days. Supplemental Table 1 summarizes the criteria on which animals were removed from the experiment (weight loss or tumor growth).

Discussion

Pretargeting with the bsMAb TF2 and radiolabeled IMP288 allows rapid and specific targeting of CEA-expressing tumors. Therapy with ^{177}Lu-IMP288 has been shown to be safe and feasible *(10, 11, 19, 20)*. However, eventually tumors showed progressive growth. Because of their high LET, alpha-emitting radionuclides deliver a significantly higher dose to tumors compared with beta-emitting radionuclides and they may improve the therapeutic efficacy of PRIT. To our knowledge, this is first study showing the potential of PRIT with TF2 and IMP288, using the alpha-emitting radionuclide ^{213}Bi.

^{213}Bi-IMP288 has high binding affinity towards TF2-pretargeted LS174T cells and showed efficient targeting to TF2 pretargeted xenografts, similar to that of ^{177}Lu-IMP288. In vitro, internalization rates were low for both tracers, which is expected since CEA is a slow-internalizing antigen.*(21)* In vivo, tumor uptake was observed as early as 15 min post injection and the peptide and was cleared rapidly from normal tissue via the kidneys. Therefore, the tumor targeting kinetics of the peptide match the short half-life of ^{213}Bi ($t_{1/2}$ = 45.6 min).

A single dose of TF2 and ^{213}Bi-IMP288 significantly delayed tumor growth and prolonged the survival of tumor bearing mice, which is in line with literature on pretargeted-alpha therapy with antibody-streptavidin constructs and ^{213}Bi-labeled biotin *(22-24)*. The therapeutic efficacy of ^{213}Bi-IMP288 was as least as effective as a single injection of ^{177}Lu-IMP288 at the previously reported MTD level. This is contrary to other preclinical studies which have shown that alpha-emitting radionuclides are more effective compared with beta-emitting radionuclides *(23, 25, 26)*. For example, Pagel et al. have shown that pretargeted-alpha-therapy with an anti-CD45 antibody-streptavidin complex and 29.6 MBq ^{213}Bi-labeled was more effective than 4.1 MBq ^{90}Y-labeled biotin.*(23)* The differences between our results and literature can be attributed to several factors, such as the type of alpha- and beta-emitter used, dosing schedule, radiosensitivity of the tumor model, and size of tumor at start of therapy. Furthermore, similar

therapeutic effectiveness of ^{177}Lu and ^{213}Bi has also been demonstrated. For example in radioimmunotherapy in a gastric cancer model using the mutant E-cadherin targeting antibody d9MAb *(27)*.

Recent clinical studies have shown that targeted-alpha therapy is effective in patients who have been treated inefficiently with beta-emitting radionuclides. For example, ^{225}Ac-PSMA-617 and ^{213}Bi-DOTATOC can overcome radioresistance to targeted radionuclide therapy with beta-emitting radionuclides in patients with neuroendocrine tumors and prostate cancer, respectively *(28, 29)*. The therapeutic efficacy of PRIT with ^{213}Bi-IMP288 may be further improved by using a fractionated dosing schedule, as was previously shown for ^{177}Lu-IMP288 and other ^{213}Bi-labeled targeting agents *(10, 26, 30, 31)*. Furthermore, because of the long retention of radiolabeled IMP288 in the tumor, alpha-emitting radionuclides with a longer half-life, such as ^{225}Ac ($t_{1/2}$ = 10 days), may be more effective *(10, 19)*.

The markedly higher specific activity that can be attained with ^{213}Bi-IMP288 compared to ^{177}Lu-IMP288, together with the lower activity doses required to achieve a therapeutic effect, allow for a significant reduction in the administered peptide dose. This is a potential advantage since a lower peptide dose results higher IMP288 tumor uptake, which may enhance the therapeutic efficacy. However, this was not observed in our study. Autoradiography showed heterogeneous uptake of radiolabeled IMP288 in the tumor. Given that the range of the alpha-particles is only 40 – 100 µm (a few cell diameters), tumor cells in areas of low ^{213}Bi-IMP288 uptake are likely to receive sub-lethal radiation doses by the alpha-particles. In contrast, the beta particles emitted by ^{177}Lu have a longer range (1.5 mm) which results in a more homogeneous irradiation of the tumor bed, allowing for effective cell killing in areas with low IMP288 uptake due to a crossfire effect. Pretargeted-alpha-therapy might be more effective for smaller and more homogeneous tumor lesions, such as for example in the LS174T intraperitoneal tumor model, or in more differentiated CEA-positive xenograft models such as SW1222 *(11, 32)* .

^{213}Bi-IMP288 at 12 or 17 MBq caused nephrotoxicity in a subgroup of mice. The retention of radiolabeled IMP288 in the kidney is low, while the peptide is cleared very rapidly from the circulation. Within 30 min, almost all IMP288 has been filtered by the glomeruli and has passed the proximal and distal tubuli. Since ^{213}Bi has a very short half-life of only 45.6 min, within the first 30 min a very high dose is delivered to the kidneys. Several other studies have also observed renal toxicity in mice after the intravenous administration of targeted alpha therapy *(22, 26, 33, 34)*. Potentially, renal toxicity could be reduced by dose fractionation or by using alpha-emitting radionuclides with a longer half life *(26, 30, 31)*. More importantly, renal toxicity profiles have not been reported in clinical studies using targeted radionuclide in patients *(28, 29)*.

Conclusion

This study showed for the first time that PRIT with ^{213}Bi-IMP288 is feasible and at least as effective as ^{177}Lu-IMP288. However, at higher doses, kidney toxicity was observed. Future studies are needed to determine the optimal dosing schedule or using alpha-emitting radionuclides with a longer half-life , to improve the therapeutic efficacy and reduce renal toxicity.

Conflict of interest

DMG is, and WJM was, an employee of Immunomedics, Inc., and IBC Pharmaceuticals, Inc., which have patented this technology and reagents. These authors are also inventors on these patents. DMG has stocks in Immunomedics.

Acknowledgements

We thank Birgit Blechert, Cathelijne Frielink, Bianca Lemmers-van de Weem, Henk Arnts, Iris Lamers-Elemans, and Kitty Lemmens-Hermans for excellent technical assistance. We gratefully acknowledge the support of the National Science Foundation (DGE-1256259), the National Institutes of Health (NIBIB/NCI 1R01CA169365, and T32-GM008349), and the Dutch Cancer Society.

References

1. Milenic DE, Brady ED, Brechbiel MW. Antibody-targeted radiation cancer therapy. *Nature reviews. Drug discovery.* 2004;3:488-499.
2. Franken NA, Hovingh S, Ten Cate R, et al. Relative biological effectiveness of high linear energy transfer alpha-particles for the induction of DNA-double-strand breaks, chromosome aberrations and reproductive cell death in SW-1573 lung tumour cells. *Oncol Rep.* 2012;27:769-774.
3. Seidl C. Radioimmunotherapy with alpha-particle-emitting radionuclides. *Immunotherapy.* 2014;6:431-458.
4. Wulbrand C, Seidl C, Gaertner FC, et al. Alpha-particle emitting 213Bi-anti-EGFR immunoconjugates eradicate tumor cells independent of oxygenation. *PLoS One.* 2013;8:e64730.
5. Friesen C, Glatting G, Koop B, et al. Breaking chemoresistance and radioresistance with [213Bi]anti-CD45 antibodies in leukemia cells. *Cancer Res.* 2007;67:1950-1958.
6. Howlader N NA, Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review, 1975-2012, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2012/, based on November 2014 SEER data submission, posted to the SEER web site, April 2015.
7. Liersch T, Meller J, Kulle B, et al. Phase II trial of carcinoembryonic antigen radioimmunotherapy with 131I-labetuzumab after salvage resection of colorectal metastases in the liver: five-year safety and efficacy results. *J Clin Oncol.* 2005;23:6763-6770.
8. Sharkey RM, Goldenberg DM. Cancer radioimmunotherapy. *Immunotherapy.* 2011;3:349-370.
9. Goldenberg DM, Chang CH, Rossi EA, McBride WJ, Sharkey RM. Pretargeted Molecular Imaging and Radioimmunotherapy. *Theranostics.* 2012;2:523-540.
10. Schoffelen R, van der Graaf WT, Franssen G, et al. Pretargeted 177Lu radioimmunotherapy of carcinoembryonic antigen-expressing human colonic tumors in mice. *J Nucl Med.* 2010;51:1780-1787.
11. Schoffelen R, van der Graaf WT, Sharkey RM, et al. Quantitative Immuno-SPECT Monitoring of Pretargeted Radioimmunotherapy with a Bispecific Antibody in an Intraperitoneal Nude Mouse Model of Human Colon Cancer. *J Nucl Med.* 2012;53:1926-1932.
12. Schoffelen R, van der Graaf WTA, Sharkey R, et al. Phase I clinical study of pretargeted radioimmunotherapy (PRIT) in patients with colorectal cancer (CRC): An update. *J Nucl Med.* 2012;53:(Supplement 1):496.
13. Goldenberg DM, Rossi EA, Sharkey RM, McBride WJ, Chang CH. Multifunctional antibodies by the Dock-and-Lock method for improved cancer imaging and therapy by pretargeting. *J Nucl Med.* 2008;49:158-163.
14. Rossi EA, Goldenberg DM, Cardillo TM, McBride WJ, Sharkey RM, Chang CH. Stably tethered multifunctional structures of defined composition made by the dock and lock method for use in cancer targeting. *Proc Natl Acad Sci U S A.* 2006;103:6841-6846.
15. Sharkey RM, van Rij CM, Karacay H, et al. A new Tri-Fab bispecific antibody for pretargeting Trop-2-expressing epithelial cancers. *J Nucl Med.* 2012;53:1625-1632.
16. McBride WJ, Zanzonico P, Sharkey RM, et al. Bispecific antibody pretargeting PET (immunoPET) with an 124I-labeled hapten-peptide. *J Nucl Med.* 2006;47:1678-1688.
17. Heskamp S, Hobo W, Molkenboer-Kuenen JD, et al. Noninvasive Imaging of Tumor PD-L1 Expression Using Radiolabeled Anti-PD-L1 Antibodies. *Cancer Res.* 2015;75:2928-2936.
18. Rolleman EJ, Krenning EP, Bernard BF, et al. Long-term toxicity of [(177)Lu-DOTA (0),Tyr (3)]octreotate in rats. *Eur J Nucl Med Mol Imaging.* 2007;34:219-227.

19. Schoffelen R, Woliner-van der Weg W, Visser EP, et al. Predictive patient-specific dosimetry and individualized dosing of pretargeted radioimmunotherapy in patients with advanced colorectal cancer. *Eur J Nucl Med Mol Imaging.* 2014;41:1593-1602.
20. Schoffelen R, van der Graaf WT, Sharkey RM, et al. Pretargeted immuno-PET of CEA-expressing intraperitoneal human colonic tumor xenografts: a new sensitive detection method. *EJNMMI Res.* 2012;2:5.
21. Schmidt MM, Thurber GM, Wittrup KD. Kinetics of anti-carcinoembryonic antigen antibody internalization: effects of affinity, bivalency, and stability. *Cancer Immunol Immunother.* 2008;57:1879-1890.
22. Yao Z, Zhang M, Garmestani K, et al. Pretargeted alpha emitting radioimmunotherapy using (213)Bi 1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetraacetic acid-biotin. *Clin Cancer Res.* 2004;10:3137-3146.
23. Pagel JM, Kenoyer AL, Back T, et al. Anti-CD45 pretargeted radioimmunotherapy using bismuth-213: high rates of complete remission and long-term survival in a mouse myeloid leukemia xenograft model. *Blood.* 2011;118:703-711.
24. Park SI, Shenoi J, Pagel JM, et al. Conventional and pretargeted radioimmunotherapy using bismuth-213 to target and treat non-Hodgkin lymphomas expressing CD20: a preclinical model toward optimal consolidation therapy to eradicate minimal residual disease. *Blood.* 2010;116:4231-4239.
25. Nayak TK, Norenberg JP, Anderson TL, Prossnitz ER, Stabin MG, Atcher RW. Somatostatin-receptor-targeted alpha-emitting 213Bi is therapeutically more effective than beta(-)-emitting 177Lu in human pancreatic adenocarcinoma cells. *Nucl Med Biol.* 2007;34:185-193.
26. Wild D, Frischknecht M, Zhang H, et al. Alpha- versus beta-particle radiopeptide therapy in a human prostate cancer model (213Bi-DOTA-PESIN and 213Bi-AMBA versus 177Lu-DOTA-PESIN). *Cancer Res.* 2011;71:1009-1018.
27. Seidl C, Zockler C, Beck R, Quintanilla-Martinez L, Bruchertseifer F, Senekowitsch-Schmidtke R. 177Lu-immunotherapy of experimental peritoneal carcinomatosis shows comparable effectiveness to 213Bi-immunotherapy, but causes toxicity not observed with 213Bi. *Eur J Nucl Med Mol Imaging.* 2011;38:312-322.
28. Kratochwil C, Bruchertseifer F, Giesel FL, et al. 225Ac-PSMA-617 for PSMA targeting alpha-radiation therapy of patients with metastatic castration-resistant prostate cancer. *J Nucl Med.* 2016.
29. Kratochwil C, Giesel FL, Bruchertseifer F, et al. (2)(1)(3)Bi-DOTATOC receptor-targeted alpha-radionuclide therapy induces remission in neuroendocrine tumours refractory to beta radiation: a first-in-human experience. *Eur J Nucl Med Mol Imaging.* 2014;41:2106-2119.
30. Chan HS, Konijnenberg MW, de Blois E, et al. Influence of tumour size on the efficacy of targeted alpha therapy with (213)Bi-[DOTA(0),Tyr(3)]-octreotate. *EJNMMI Res.* 2016;6:6.
31. Essler M, Gartner FC, Neff F, et al. Therapeutic efficacy and toxicity of 225Ac-labelled vs. 213Bi-labelled tumour-homing peptides in a preclinical mouse model of peritoneal carcinomatosis. *Eur J Nucl Med Mol Imaging.* 2012;39:602-612.
32. El Emir E, Qureshi U, Dearling JL, et al. Predicting response to radioimmunotherapy from the tumor microenvironment of colorectal carcinomas. *Cancer Res.* 2007;67:11896-11905.
33. Song H, Hobbs RF, Vajravelu R, et al. Radioimmunotherapy of breast cancer metastases with alpha-particle emitter 225Ac: comparing efficacy with 213Bi and 90Y. *Cancer Res.* 2009;69:8941-8948.
34. Beck R, Seidl C, Pfost B, et al. 213Bi-radioimmunotherapy defeats early-stage disseminated gastric cancer in nude mice. *Cancer Sci.* 2007;98:1215-1222.

Figures

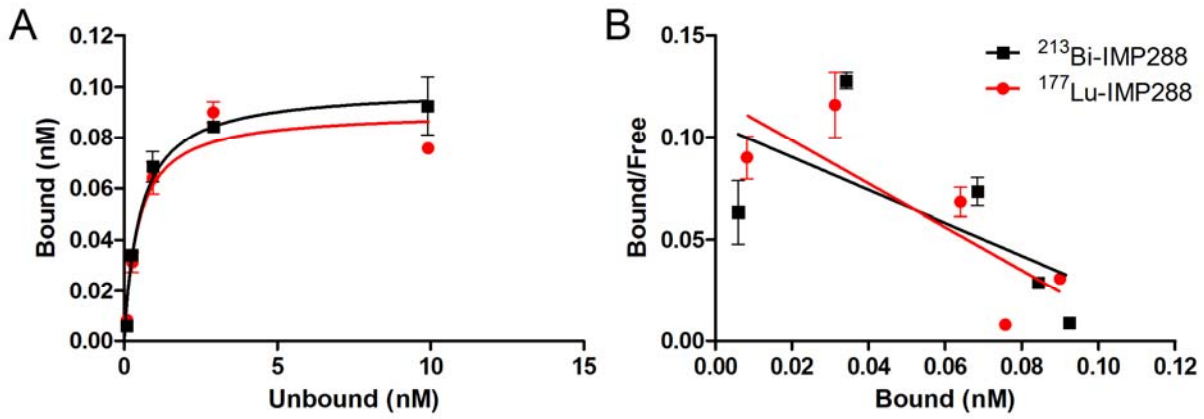


Figure 1. A: Binding plot and **B:** Scatchard plot of ^{177}Lu-IMP288 and ^{213}Bi-IMP288 to TF2-pretargeted LS174T cells.

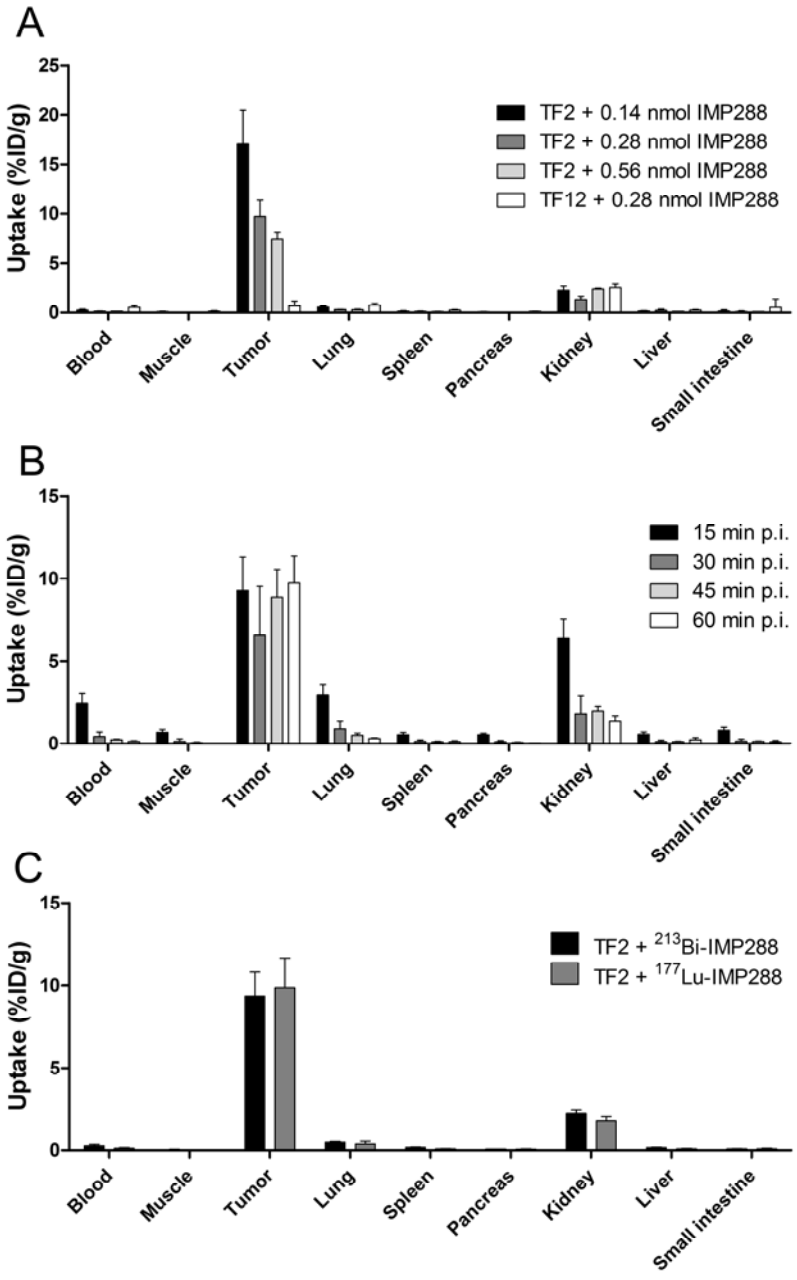


Figure 2. Biodistribution of radiolabeled IMP288. (A) ^{213}Bi-IMP288 biodistribution in TF2 or TF12 pretargeted LS174T tumor bearing mice at, 60 post injection. (B) Biodistribution of ^{213}Bi-IMP288 (0.28 nmol) in mice bearing TF2-pretargeted LS174T xenografts at 15, 30, 45, and 60 min post injection. (C) Biodistribution of ^{213}Bi-IMP288 (0.28 nmol) or ^{177}Lu-IMP288 (0.28 nmol) in mice bearing TF2-pretargeted LS174T xenografts at 60 min post injection.

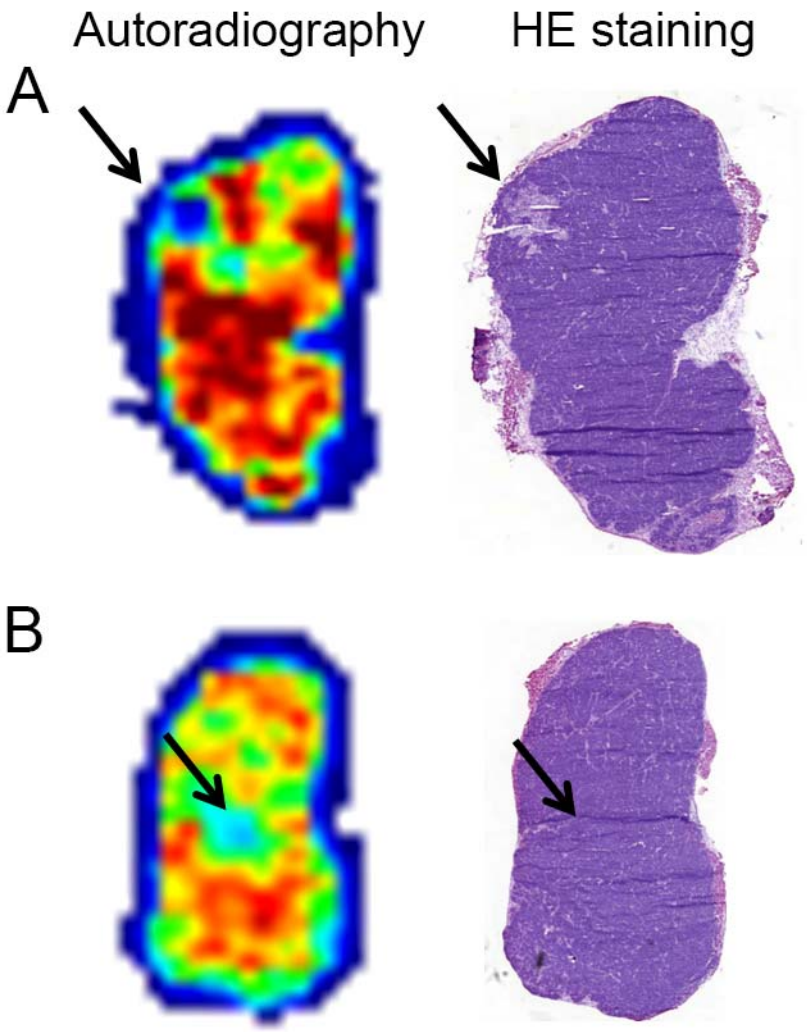


Figure 3. Autoradiography and HE staining of LS174T tumor xenografts of mice injected with TF2 and ^{177}Lu-IMP288. Autoradiography reveals heterogeneous uptake of ^{177}Lu-IMP288. Areas with low uptake were found in both necrotic (**A**) and viable (**B**) tumor tissue (black arrow).

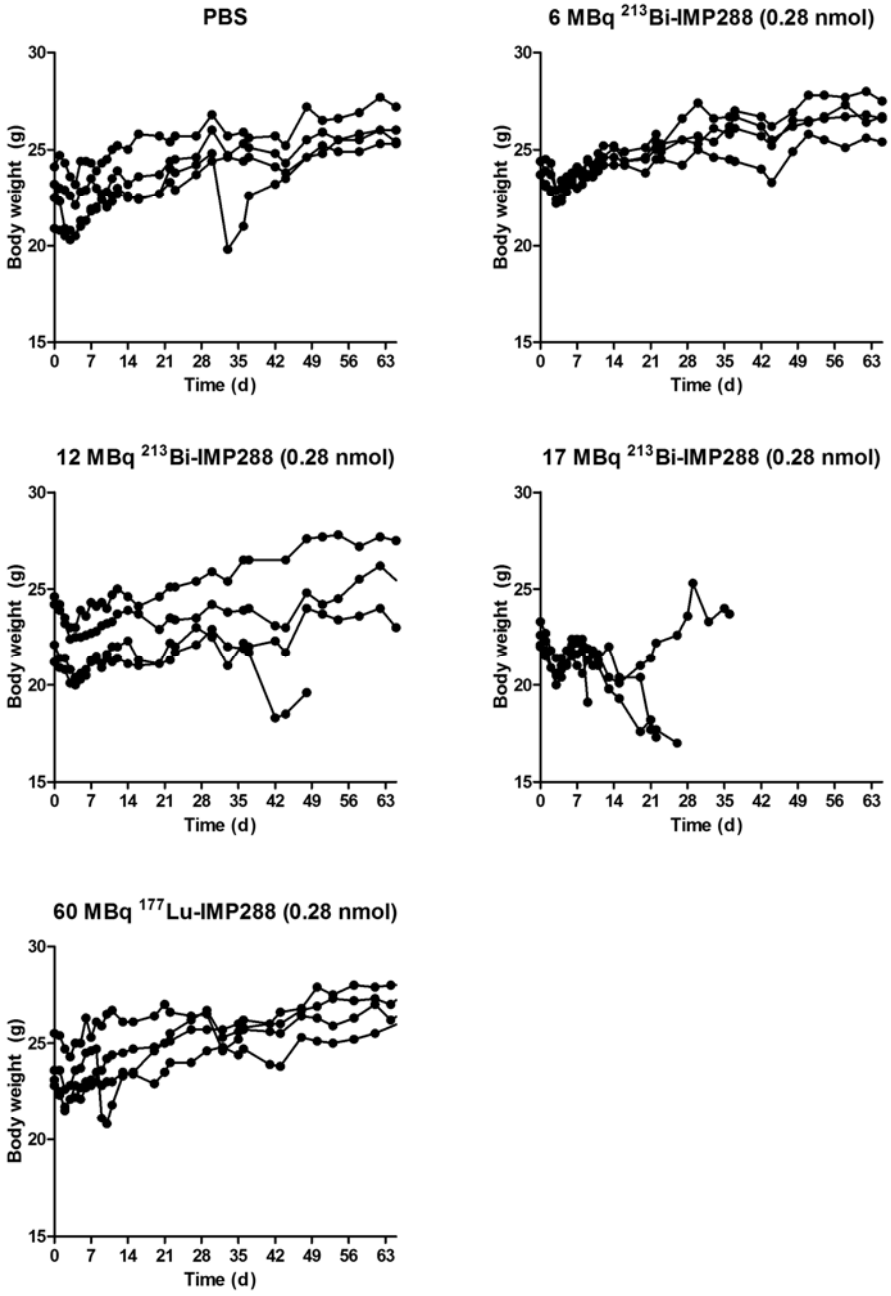


Figure 4. Bodyweight of mice non-tumor bearing mice, treated with TF2 and different activity doses of ^{213}Bi-IMP288 or ^{177}Lu-IMP288.

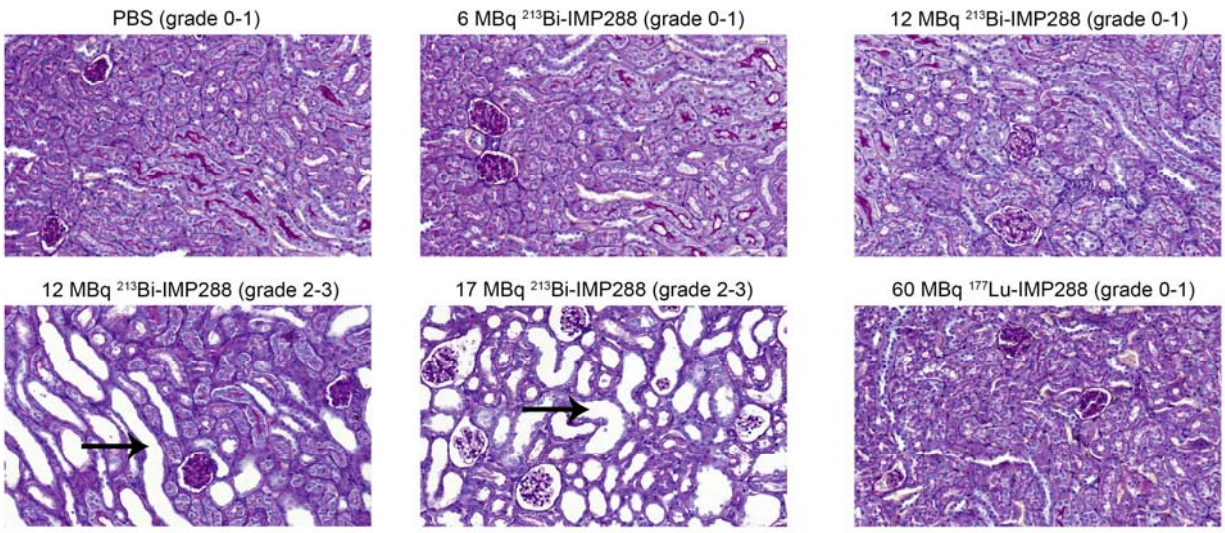


Figure 5. Representative PAS stained kidney slices of mice treated with ^{213}Bi-IMP288 or ^{177}Lu-IMP288. Arrows indicate tubular damage in mice treated with 12 or 17 MBq ^{213}Bi-IMP288.

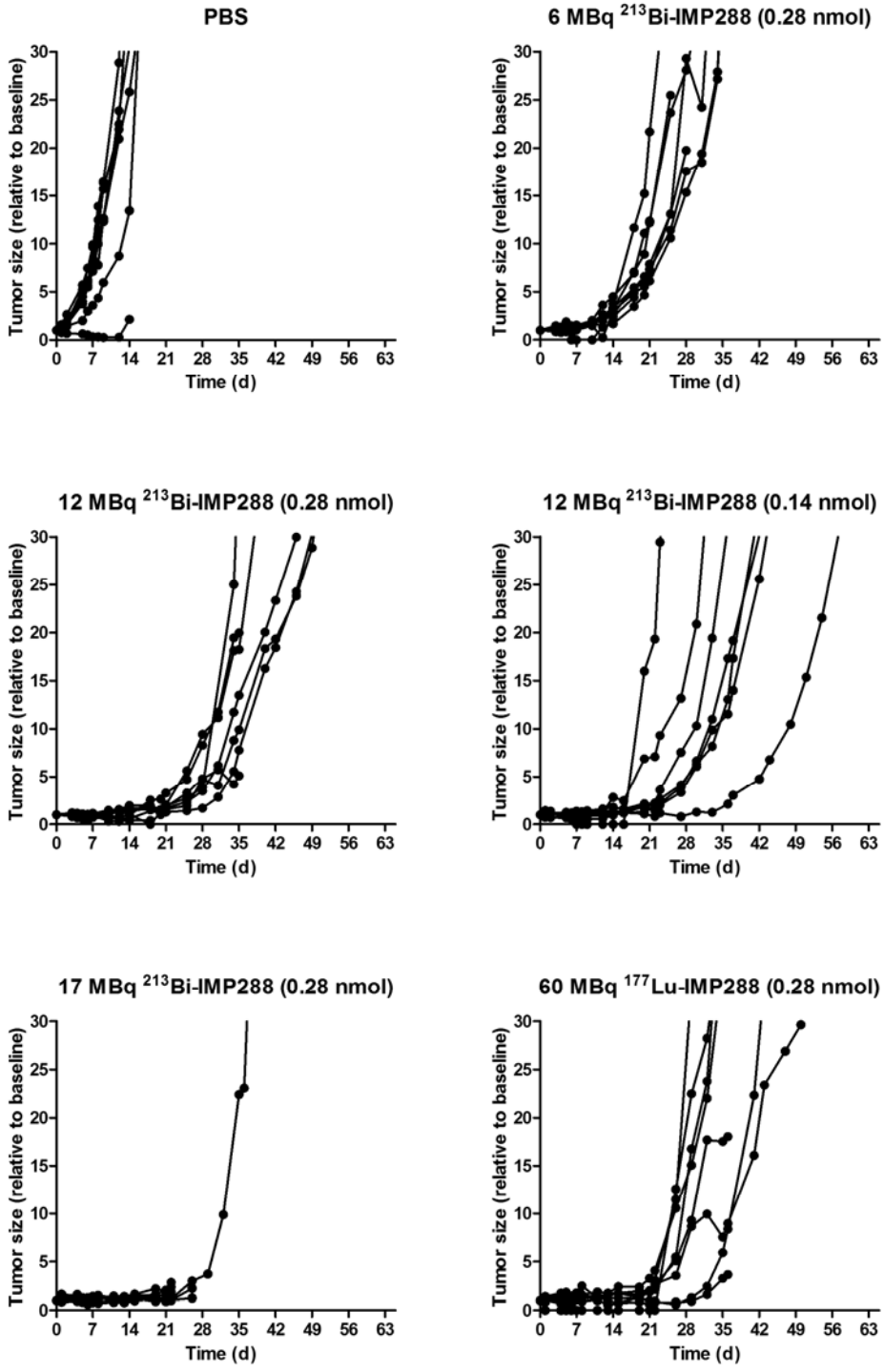


Figure 6. Tumor size of mice treated with TF2 and different activity doses of ^{213}Bi-IMP288 or ^{177}Lu-IMP288.

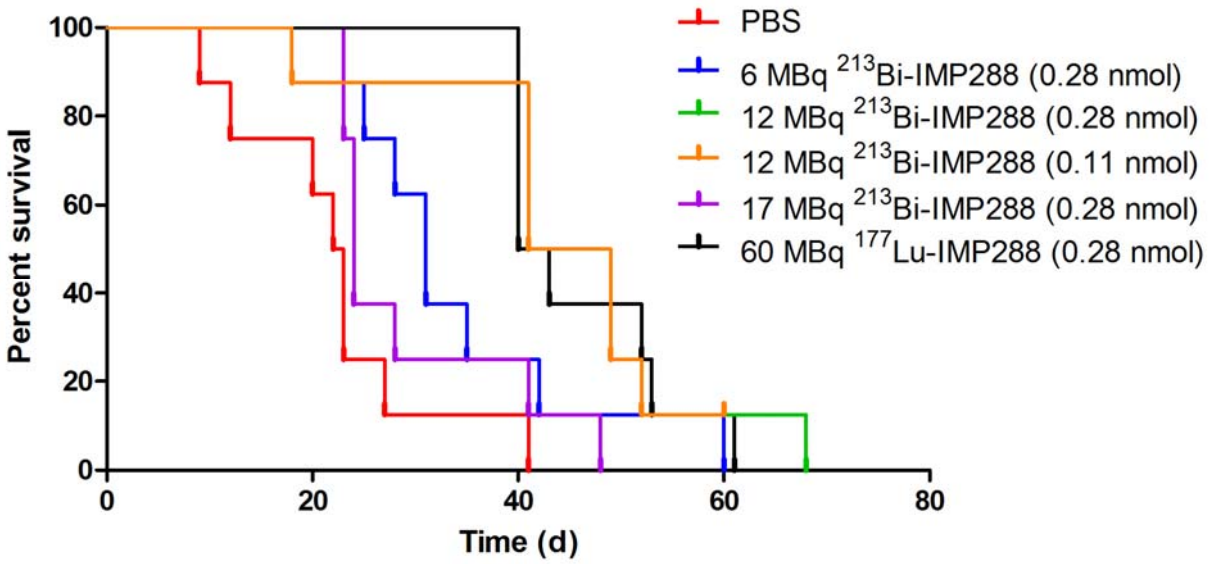


Figure 7. Survival curves of mice treated with TF2 and different activity doses of ^{213}Bi-IMP288 or ^{177}Lu-IMP288.

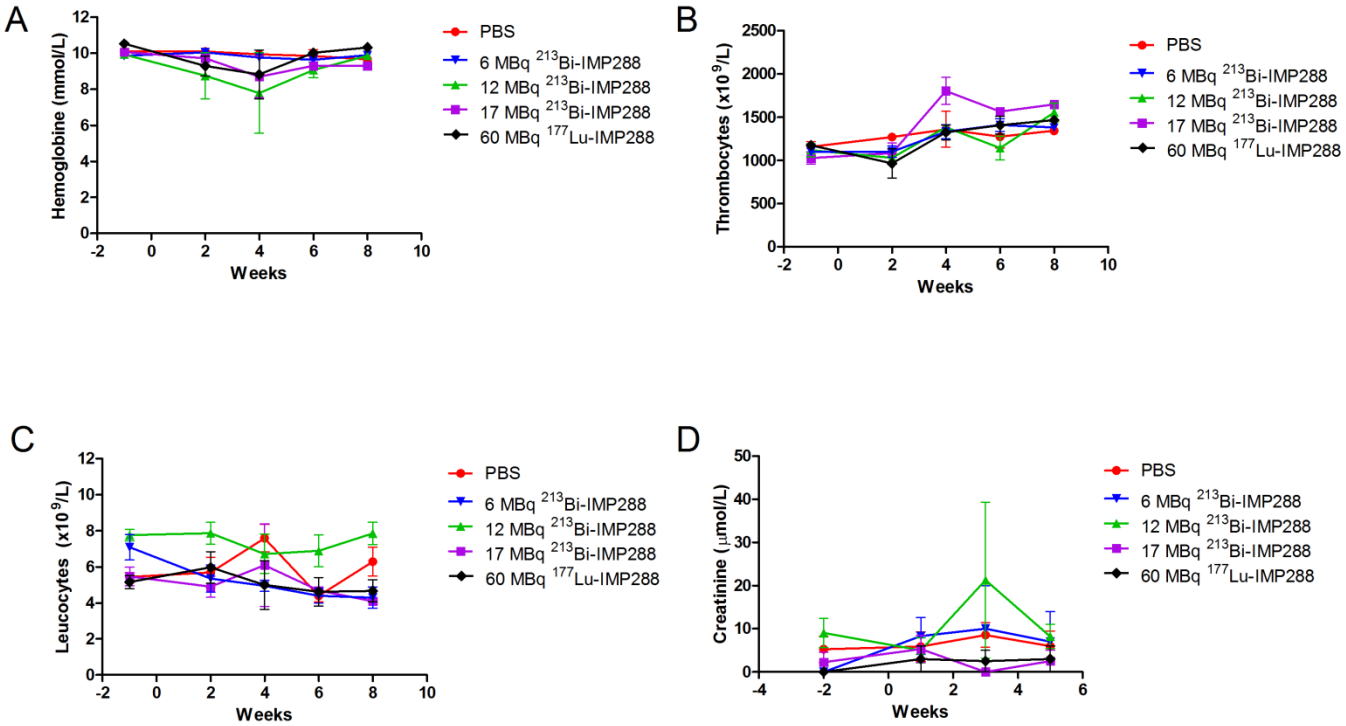
Tables

Table 1. Criteria for the assessment of renal damage

Grade	**Criteria**
Grade 0	No irregularities
Grade 1	Little dilatation of tubules; no basal membrane thickening
Grade 2	Criteria as for grade 1, but more pronounced tubule dilatation, basal membrane thickening and mitotic activity
Grade 3	Shrinkage of a small number of glomeruli; flat or lost tubule epithelium, strong tubule dilatation and more pronounced basal membrane thickening
Grade 4	Increased shrinkage of glomeruli leading to optical emptiness; strongly dilated tubules and signs of peripheral fibrosis

Table 2. Comparison of the in vitro characteristics of ^{213}Bi-IMP288 and ^{177}Lu-IMP288.

	^{213}Bi-IMP288	**^{177}Lu-IMP288**
Internalization (% of cell-associated activity at 3 h that is internalized, mean ± sd)	17.5% ± 5.3%	12.1% ± 0.8%
K_d (95% CI)	0.45 ± 0.20 nM	0.53 ± 0.12 nM
B_{max} (95% CI)	22,182 ± 1,338 molecules/cell	20,114 ± 2,164 molecules/cell



Supplemental Figure S1. Hemoglobine (**A**), thrombocytes (**B**), leucocytes (**C**) and creatinine levels (**D**) in mice treated with TF2 and different activities of ^{213}Bi-IMP288 or ^{177}Lu-IMP288. Treatment was administered in week 0.

Supplemental Table 1. The number of animals taken out of the experiment based on the predefined criteria of weight loss and tumor growth.

Criteria to remove animal from experiment	**PBS**	**6 MBq ^{213}Bi-IMP288**	**12 MBq ^{213}Bi-IMP288 (0.14 nmol)**	**12 MBq ^{213}Bi-IMP288 (0.28 nmol)**	**17 MBq ^{213}Bi-IMP288**	**60 MBq ^{177}Lu-IMP288**
Weight loss (>20% compared with baseline or >15% within 2 days)	5/8	6/8	4/8	3/8	1/8	3/8
Tumor size ≥ 2 cm^3	2/8	2/8	3/8	3/8	0/8	5/8
Tumor ulceration	1/8	0/8	1/8	2/8	7/8	0/8