Pretargeted dual-modality immuno-SPECT and near-infrared fluorescence imaging for image-guided surgery of prostate cancer

Susanne Lütje\textsuperscript{1}, Mark Rijpkema\textsuperscript{1}, David M. Goldenberg\textsuperscript{2}, Catharina M. van Rij\textsuperscript{1}, Robert M. Sharkey\textsuperscript{2}, William J. McBride\textsuperscript{2}, Gerben M. Franssen\textsuperscript{1}, Cathelijne Frieling\textsuperscript{1}, Wijnand Helfrich\textsuperscript{3}, Wim J. G. Oyen\textsuperscript{1}, Otto C. Boerman\textsuperscript{1}

\textsuperscript{1}Department of Radiology and Nuclear Medicine, Radboud university medical center, Nijmegen, The Netherlands
\textsuperscript{2}Immunomedics, Morris Plains, NJ, USA
\textsuperscript{3}University of Groningen, University Medical Center Groningen, Department of Surgery, Laboratory for Translation Surgical Oncology, Groningen, The Netherlands

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Corresponding author: Susanne Lütje
Dept. Radiology and Nuclear Medicine
Radboud University Medical Center
PO Box 9101
6500 HB Nijmegen
The Netherlands
Phone: +31-24-3615054
Fax: +31-24-3618942
e-mail: Susanne.Lutje@radboudumc.nl

Conflicts of interest
D.M. Goldenberg, R.M. Sharkey, and W.J. McBride are employed by and have stock in Immunomedics, Inc., which has developed and patented the pretargeting methodology described herein.

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ABSTRACT

Radical removal of malignant lesions may be improved using tumor targeted dual-modality probes that contain both a radiotracer and a fluorescent label, to allow for enhanced intra-operative delineation of tumor resection margins. Since pretargeting strategies yield high signal-to-background ratios, we evaluated the feasibility of a pretargeting strategy for intra-operative imaging in prostate cancer using an anti-TROP-2 x anti-HSG bispecific antibody (TF12), in conjunction with the dual-labeled diHSG peptide (RDC018) equipped with both a DOTA chelate for radiolabeling purposes and a fluorophore (IRdye800CW) to allow near-infrared optical imaging.

Nude mice implanted s.c. with TROP-2-expressing PC3 human prostate tumor cells or with PC3 metastases in the scapular and suprarenal region were injected i.v. with 1 mg of TF12 and, after 16h of tumor accumulation and blood clearance, were subsequently injected with 10 MBq, 0.2 nmol/mouse of either $^{111}$In-RDC018 or $^{111}$In-IMP288 as a control. Two hours post injection, both microSPECT/CT and fluorescence images were acquired, both before and after resection of the tumor nodules. After image acquisition, the biodistribution of $^{111}$In-RDC018 and $^{111}$In-IMP288 was determined and tumors were analyzed immunohistochemically.

The biodistribution of the dual-label RDC018 showed specific accumulation in the TROP-2 expressing PC3 tumors (12.4±3.7% ID/g at 2h p.i.), comparable to $^{111}$In-IMP288 (9.1±2.8% ID/g at 2h p.i.). MicroSPECT/CT and near-infrared fluorescence (NIRF) imaging confirmed this TROP-2-specific uptake of the dual-label $^{111}$In-RDC018 in both the s.c. and metastatic growing tumor model. In addition, PC3 metastases could be visualized preoperatively with SPECT/CT and could subsequently be resected by image-guided surgery using intra-operative NIRF imaging, showing the preclinical feasibility of pretargeted dual-modality imaging approach in prostate cancer.
INTRODUCTION

Despite advances in diagnostic procedures and clinical management prostate cancer remains associated with significant morbidity and is the second leading cause of cancer-related deaths in men in the Western world.

At present, extensive research is focused on the development of new molecular imaging techniques to improve detection and staging of this disease. In this regard, radiolabeled antibodies that target the prostate cancer-associated cell surface antigens appear to be particularly promising. This is supported by the clinical application of the PSMA-directed antibody capromab pendetide, also known as Prostascint (7E11-C53) or the antibody J591 [1]. However, antibodies typically show slow clearance from the circulation (t½ = 2-3 days), which requires a relatively long interval between time of injection of the radiolabeled antibody and subsequent image acquisition in order to achieve adequate contrast. In addition, the slow blood clearance results in limited target-to-background ratios.

To overcome these limitations and to improve targeting of tumors, various pretargeting techniques have been developed. Previously, we reported a study in which we evaluated a pretargeting approach that targets TROP-2-expressing prostate cancer [2]. TROP-2, also known as EGP-1 (epithelial glycoprotein-1), is a 46-kDa transmembrane glycoprotein expressed on diverse carcinomas, such as of the lung, bladder, breast, cervix, ovary, stomach and prostate [3]. We previously applied the trivalent bispecific Ab (bsAb) TF12, which consists of two anti-TROP-2 Fab fragments and one anti-HSG (histamine-succinyl-glycine) Fab fragment. In this strategy, an unlabeled bsAb with affinity for both the tumor cell and a radiolabeled diHSG peptide was injected. After TF12 had accumulated in the tumor and was cleared from the blood, the 111In-labeled diHSG peptide IMP288 was administered, which was rapidly and selectively trapped at the tumor. This method allowed for imaging within 1-2 h after injection of the radiolabeled peptide and higher target-to-background ratios than with the directly labeled anti-TROP2 antibody hRS7 [2, 4].

At present, surgical removal of prostate cancer lesions is the treatment of choice for patients with low- and intermediate-risk localized disease, and in selected patients with high-
risk localized disease [5]. The use of intra-operative imaging might guide the surgeon in the
detection of malignant tissue which might improve the outcome, and reduce morbidity and
treatment-related side effects. However, the application of nuclear imaging-based
pretargeting strategies appears to be limited for intra-operative imaging purposes as tumor
tissue cannot be delineated precisely by radionuclide detection in the intra-operative setting.

Near-infrared fluorescence (NIRF) imaging with fluorophores conjugated to tumor
targeting agents is rapidly emerging as a new sensitive intra-operative imaging technique for
improved and real-time detection of malignant lesions during surgery [6 - 9]. However, the
penetration depth of both excitation and emission light in tissues is usually limited to several
millimeters [6]. One strategy to deal with this limited penetration depth is combining NIRF
and radionuclide imaging techniques. With this dual-modality approach, the burden of the
disease could be assessed pre-operatively, followed by localization of the tumor lesions
intra-operatively using a gamma probe, and subsequent intra-operative guidance for surgical
removal of the tumor lesions based on the fluorescent component of the dual-label agent.

Here, we evaluated a bispecific antibody-based pretargeting strategy for prostate
cancer preclinically, using a novel hapten, designated RDC018, a peptide that is based on
the extensively characterized tetrapeptide IMP288 with two HSG haptens and a 1,4,7,10-
tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelate. RDC018 is an IMP288
analog that is C-terminally conjugated with the fluorophore IRDye800CW (Figure 1). The
DOTA chelate of RDC018 was labeled with $^{111}$In. The pretargeting characteristics of the
TF12/RDC018 approach were evaluated in a mouse tumor model bearing TROP-2-
expressing prostate cancer metastases. In addition, the feasibility of this dual-label
pretargeting strategy for image-guided surgery based on microSPECT/CT and NIRF imaging
was investigated.
MATERIALS AND METHODS

Pretargeting reagents TF12 and RDC018

BsAb TF12, having both TROP-2- and HSG-binding specificities, was produced using the Dock-and-Lock® technology as described previously by Rossi et al. [10]. The NIR fluorescent peptide RDC018 is a peptide-hapten derived from IMP288, a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-conjugated D-Tyr-D-Lys-D-Glu-D-Lys tetrapeptide, in which both lysine residues are substituted with an HSG moiety via their ε-amino group. In addition to the DOTA chelator for radiolabeling, RDC018 also contains a NIR fluorescent IRDye800CW moiety (Figure 1).

Cell Culture

The TROP-2-expressing human prostate cancer cell line PC3, originally derived from a PC bone metastasis was obtained from American Type Culture Collection (CRL 1435, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (Life technologies, Carlsbad, CA) and 2 mM glutamine.

Mouse Models

Male BALB/c nude mice (Janvier, Le Genest Saint Isle, France), 8-9 weeks old, housed in individual ventilated cages (5 mice per cage) under nonsterile standard conditions with free access to standard animal chow and water, were adapted to laboratory conditions for one week before experimental use. Mice were subcutaneously inoculated with 3 x 10^5 PC3 cells (right flank) suspended in 200 µL of 67% complete RPMI 1640 medium with 33% matrigel (BD Biosciences, Franklin Lakes, NJ). PC3 xenografts grew to approximately 0.1 g in 7 days after tumor cell inoculation. To induce metastatic prostate cancer growth, mice were anesthetized with isoflurane and 5 x 10^5 PC3 cells in 50 µl of PBS were inoculated with a 30-gauge needle through the diaphragm into the left cardiac ventricle after midline incision [11]. PC3 metastases were allowed to grow for 10 - 12 weeks after tumor cell inoculation.
All experiments have been approved by the institutional Animal Welfare Committee of the Radboud University Medical Center and were conducted in accordance to the guidelines of the Revised Dutch Act on Animal Experimentation.

**Radiolabeling of IMP288 and RDC018**

IMP288 was labeled with $^{111}$In (Covidien, Petten, The Netherlands) at a specific activity of 60.5 MBq/nmol under strict metal-free conditions. Briefly, 60 MBq of $^{111}$In was added to 2.4 µg of IMP288 in 0.1 M MES buffer pH 5.4 (three times the volume of $^{111}$In-chloride) during 20 min of incubation at 95°C. Following incubation, 50 mM ethylenediaminetetraacetic acid (EDTA) was added to the labeling reaction to a final concentration of 1mM EDTA to chelate unincorporated $^{111}$In. The labeling efficiency, determined by instant thin layer chromatography on silicagel strips (ITLC-SG; Gelman Sciences, Ann Arbor, MI, USA) using 0.1 M ammonium acetate (NH$_4$Ac) buffer with 0.1 M EDTA (pH 5.5) as the mobile phase, reached 97%.

RDC018 was radiolabeled under metal-free conditions at a specific activity of 25.5 MBq/nmol. For this reaction, 127 MBq of $^{111}$In was added to 7.2 µg of RDC018 in 0.1 M MES buffer pH 5.4 (three times the volume of $^{111}$In-chloride) for 20 min of incubation at 95°C. For imaging and image-guided surgery, 2.5 µg of RDC018 was radiolabeled with 32 MBq (specific activity 18.5 MBq/nmol). After incubation, 50 mM ethylenediaminetetraacetic acid (EDTA) was added to the labeling reaction to a final concentration of 1mM EDTA. The labeling efficiency for the RDC018 labeling reaction ranged between 87 and 97%.

**Immunoreactivity**

To demonstrate bispecific immunoreactivity of TF12, a serial dilution of PC3 cells in RPMI medium containing 0.5% BSA (1.6 x 10$^6$ to 2.6 x 10$^7$ cells in 0.5 ml) was incubated with TF12 (50 µg/ml) for 30 min at 37°C. Cells were washed twice in RPMI medium containing 0.5% BSA followed by incubation with $^{111}$In-RDC018 (250 Bq). To determine nonspecific binding, a duplicate of the lowest cell concentration was incubated without TF12. After incubation, cells...
were centrifuged and washed with 500 µl RPMI medium containing 0.5% BSA. The activity in the vials and in the cell pellet was determined in the gamma counter (Wizard 3" 1480, LKB-Wallac, Oy, Finland).

**Immunohistochemical analysis of TROP-2-expressing tumors**

Expression of TROP-2 in subcutaneous PC3 tumors was determined immunohistochemically on 5-µm frozen tissue sections. The tissue sections were fixed with aceton (100%) for 10 min at -20°C. After overnight drying, the sections were blocked with 20% normal goat serum (Bodinco) for 30 minutes and stained with the anti-TROP-2 humanized monoclonal antibody hRS7 (4 µg/ml antibody in PBS 1% BSA) for 1 hour at room temperature under light-protected conditions. Subsequently, the tissue sections were washed in PBS and stained with a secondary goat-anti-human HRP (Abcam, Cambridge, UK) at a dilution of 1:100 in PBS, 1% BSA, for 30 min at room temperature. After incubation, the tissue sections were washed with PBS three times. Finally, tumor sections were incubated with 0.019 g of 3,3-diaminobenzidine tetrahydro-chloride (DAB) substrate (Bright DAB, Immunologic, Duiven, Netherlands) in 25 ml PBS and 62.5 µl H₂O₂ for 10 min for development, followed by a hematoxylin counterstaining. Immunohistochemical analysis of the resected s.c. PC3 tumors revealed TROP-2 expression in all tumors (not shown).

**Pretargeted immuno-SPECT/CT and NIRF imaging**

In the first experiment, male BALB/c nude mice (n=4) with subcutaneous PC3 xenografts in the right flank were intravenously injected into the tail vein with 1 mg of TF12 (6.4 nmol) in 200 µl of PBS followed by i.v. injections of either \(^{111}\)In-RDC018 (10 MBq, 0.6 µg (0.2 nmol)) or \(^{111}\)In-IMP288 (10 MBq, 0.4 µg (0.2 nmol)) 16 h later. Two additional groups of mice (n=2) did not receive TF12 prior to injection of the radiolabeled peptide to determine the non-TF12-mediated localization of the peptide. One of these groups received only \(^{111}\)In-RDC018 (10 MBq, 0.6 µg/mouse) and one group received only \(^{111}\)In-IMP288 (10 MBq, 0.4 µg/mouse).
Two hours after injection of $^{111}$In-RDC018, mice were anesthetized with isoflurane and imaged on a small-animal microSPECT/CT scanner (U-SPECT II, MILabs, Utrecht, Netherlands) with a 1.0 mm diameter pinhole collimator tube (acquisition time 30 min) in prone position. After microSPECT/CT imaging, the mice were euthanized by $O_2/CO_2$ asphyxiation and NIRF images were acquired on the IVIS imaging system (acquisition time 5 min, binning: medium, Fstop: 2, excitation: 745 nm, excitation autofluorescence: 675 nm, emission: ICG, lamp level: high, FOV: D). MicroSPECT/CT scans were reconstructed with MILabs reconstruction software, which uses an ordered-subset expectation maximalization algorithm.

**Biodistribution of pretargeted $^{111}$In-RDC018 and $^{111}$In-IMP288**

Subsequently, the biodistribution in mice ($n=4$) with s.c. PC3 tumors of $^{111}$In-RDC018 was compared to that of $^{111}$In-IMP288. Tissues of interest (tumor, muscle, lung, spleen, kidney, liver, pancreas, stomach, duodenum and prostate) were dissected, weighed and the radioactivity was measured in a $\gamma$-counter to determine the biodistribution of $^{111}$In-IMP288 and $^{111}$In-RDC018. Blood samples were obtained by heart puncture. For calculation of the uptake of radioactivity in each tissue as a fraction of the injected dose, an aliquot of the injection dose was counted simultaneously.

**Dual-modality microSPECT/CT and NIRF image-guided surgery**

In the second experiment, male BALB/c nude mice ($n=20$) with PC3 metastases in the submandibular, shoulder, and adrenal gland region that developed after injection of PC3 cells into the right cardiac ventricle were injected intravenously with the bsAb TF12 (1 mg/mouse), followed by i.v. injection of $^{111}$In-RDC018 (0.6 µg/mouse, 20 MBq/µg) at 16 h after injection of TF12. Mice were imaged 2 hours later on both the IVIS imaging system and a small-animal microSPECT/CT scanner with the same settings as in the first experiment. MicroSPECT/CT scans were performed pre-operatively, followed by NIRF imaging of the mice in the supine position after surgical removal of skin, abdominal muscle layers, and
peritoneum. After NIRF image acquisition, the visualized tumor lesions were resected, followed by NIRF imaging to check whether residual tumor tissue was left *in situ*. In addition, microSPECT/CT scans were performed after resection of the tumor lesions, using the same scanning parameters that were used for pre-operative evaluation.

**Statistical analyses**

Statistical analyses were performed with Graphpad Prism, version 5.03 (GraphPad, La Jolla, CA, USA). Results are presented as mean ± standard deviation (SD). To determine statistical differences, unpaired *t*-tests were used.
RESULTS

Tumor growth and development of metastases

Subcutaneous PC3 tumors in the right flank reached a tumor size of approximately 0.1 g at 7 days after tumor cell inoculation. To induce metastases, mice (n=20) received injections of PC3 cells into the left cardiac ventricle. The mice were dissected after 10-12 weeks and soft-tissue PC3 metastases were most frequently found in the submandibular, shoulder, and adrenal gland region. In addition, several PC3 metastases were found in the rectovesical pouch. Besides the formation of soft-tissue PC3 metastases at different anatomical sites, microCT imaging showed the development of PC3 bone metastases in the vertebral column (n=3) and in the ribs (n=2) of the mice.

Biodistribution of $^{111}$In-RDC018 and $^{111}$In-IMP288

Analysis of the bispecific immunoreactivity of TF12 revealed that in the in vitro assay up to 82% of the added peptide specifically bound to the PC3 cells (results not shown).

$^{111}$In-RDC018 and $^{111}$In-IMP288 specifically accumulated in the s.c. TF12-pretargeted TROP-2-expressing PC3 tumors (right flank). Uptake of $^{111}$In-RDC018 and $^{111}$In-IMP288 in the PC3 tumor was comparable at 2 h after injection (12.4 ± 3.7% ID/g (n=4) and 9.1 ± 2.8% ID/g (n=4), respectively), Figure 2a. Tumor uptake of $^{111}$In-RDC018 and $^{111}$In-IMP288 in non-pretargeted tumors was significantly lower, reaching 2.1 ± 0.3% ID/g (p=0.020) and 2.2 ± 0.0% ID/g (p=0.029) for $^{111}$In-RDC018 and $^{111}$In-IMP288, respectively. Overall, the biodistribution profiles of $^{111}$In-RDC018 and $^{111}$In-IMP288 show similar patterns (Figure 2a). One essential difference in the biodistribution profiles of $^{111}$In-RDC018 and $^{111}$In-IMP288 after pretargeting with TF12 is renal accumulation. While the accumulation of $^{111}$In-IMP288 in the kidneys was relatively low (2.4 ± 0.6% ID/g), renal uptake of $^{111}$In-RDC018 was significantly higher, reaching 7.6 ± 0.9% ID/g at 2 h after injection (p<0.0001). In addition, blood levels of $^{111}$In-RDC018 were significantly higher compared to $^{111}$In-IMP288 (4.7 ± 0.6% ID/g and 1.7 ± 0.9% ID/g (p=0.0016), respectively), indicating slower blood clearance. Moreover, liver
uptake of $^{111}$In-RDC018 and $^{111}$In-IMP288 differed significantly, reaching 2.8 ± 0.3% ID/g for $^{111}$In-RDC018 and 1.1 ± 0.4% ID/g for $^{111}$In-IMP288 (p=0.0006).

Tumor-to-blood ratios of $^{111}$In-RDC018 and $^{111}$In-IMP288 in the TF12-pretargeted tumors were 2.6 ± 0.7 and 5.9 ± 2.0 (p=0.021), respectively.

**Pretargeted immuno-SPECT/CT and NIRF imaging**

Five BALB/c nude mice with s.c. TROP-2-expressing PC3 tumors (right flank) were imaged by both fluorescence imaging and microSPECT/CT at 2 h after injection of $^{111}$In-RDC018 (18 h after pretargeting with TF12). A typical set of microSPECT/CT images with corresponding fluorescence images of mice in prone and left lateral positions is shown in Figure 2 b. PC3 xenografts were clearly and specifically visualized with both imaging modalities.

**Dual-modality microSPECT/CT and NIRF image-guided surgery**

BALB/c nude mice with PC3 metastases were imaged by both fluorescence imaging and microSPECT/CT at 2 h after injection of radiolabeled RCD018. A typical example of a microSPECT/CT image with corresponding NIRF image of a mouse with two bone metastases in the vertebral column is shown in Figure 3. Another example of dual-modality microSPECT/CT and NIRF imaging of a mouse with TF12-pretargeted bone metastases originating from the ribs is depicted in Figure 4.

After pre-operative evaluation of tumor lesions by microSPECT/CT, image-guided surgery of the tumor lesions was performed. First, the exact tumor location was identified by NIRF imaging. Subsequently, these tumor lesions were resected and NIRF and microSPECT/CT imaging was repeated to ensure radical surgical resection. In Figure 5, the feasibility of the dual-modality probe for intra-operative image-guided surgical resection of the metastatic tumor nodules is shown.
DISCUSSION

In the present preclinical study, the in vivo tumor targeting characteristics were evaluated of the dual-modality fluorescent $^{111}$In-labeled hapten RDC018 in TROP-2-expressing prostate cancer pretargeted with the trivalent bsAb TF12. The feasibility of this approach for image-guided surgery was demonstrated in mice bearing TROP-2-expressing metastases.

In a previous study, the potential for imaging pretargeted prostate cancer with the bsAb TF12 and the radiolabeled hapten IMP288 was evaluated [2]. In mice with s.c. PC3 tumors it was shown that this pretargeting approach allows for rapid and sensitive imaging of TROP-2-expressing prostate cancer. For intra-operative imaging purposes, radionuclide detection can be used to localize tumor nodules with a gamma probe; however, precise delineation of tumor lesions or assessment of tumor cell-containing surgical margins remains challenging. NIRF imaging is a sensitive technique that may be exploited to improve visualization of small to microscopic malignant lesions and potentially of positive resection margins during surgery. So far, several preclinical studies have evaluated methods to intra-operatively visualize tumor tissue and resection margins in prostate cancer [12 – 14]. Targeted approaches using antibodies or peptides are particularly promising to apply dual-modality imaging. Very recently, the first clinical trial using the anti-VEGF antibody bevacizumab tagged with the NIRF dye IRDye800CW (clinicaltrials.gov, NCT01508572) has begun, with the purpose to show the feasibility of tumor-targeted intra-operative fluorescence imaging in breast cancer patients.

Since the penetration depth of emitted light in tissue is limited to several millimeters, the combination with a radiolabel would allow intra-operative probe-guided tumor detection and image-guided surgery.

In the present study, analysis of biodistribution of $^{111}$In-RDC018 and $^{111}$In-IMP288 showed that the addition of the IRDye800CW fluorophore to the IMP288 diHSG peptide hapten only slightly affected the in vivo behavior of the molecule. The overall biodistribution profile of both peptides was similar, however, RDC018 showed enhanced hepatic uptake.
Previously, this has also been observed for IRDye800CW conjugated antibodies [15] and may be due to the increased lipophilicity of RDC018 compared to IMP288.

Biodistribution studies demonstrated high and specific PC3 tumor targeting for both $^{111}$In-RDC018 as well as $^{111}$In-IMP288. Using this subcutaneous tumor model, we provide proof-of-principle that $^{111}$In-RDC018 can be used for rapid dual-modality imaging of TROP-2-expressing tumors. As early as 2 hours after injection, high tumor-to-background ratios were achieved. With both imaging modalities, the fluorescent $^{111}$In-labeled hapten-peptide RDC018 accumulated specifically in the TROP-2-expressing PC3 tumors, whereas no specific tumor targeting with $^{111}$In-RDC018 was observed in mice with subcutaneous tumors that were not pretargeted with TF12. Accordingly, the non-pretargeted PC3 tumors were not visualized by NIRF imaging. These results clearly demonstrate that the accumulation of $^{111}$In-RDC018 is TF12-mediated. While the uptake of $^{111}$In-RDC018 in other tissues remained low, intermediate accumulation in the kidneys was observed, which is most likely caused by renal clearance of the peptide. The enhanced renal retention of RDC018 compared to that of IMP288 suggests enhanced tubular reabsorption of the dual-labeled peptide in the kidneys, which may be due to the presence of the IRDye800CW moiety in RDC018. Similarly, blood levels of RDC018 are higher than those obtained with IMP288 in the same model, most likely because the IRDye800CW moiety of the RDC018 peptide caused the enhanced residence time in the circulation. A clearing agent may not help in this case, as it is unlikely that the enhanced blood levels are due to complex formation of the peptide with TF12 in the circulation, because the affinity of TF12 for the HSG hapten is the same for IMP288 and RDC018.

High accumulation of small-sized fluorescent tracers has been observed previously. Banerjee and colleagues synthesized a dual-label PSMA ligand (based on glutamate urea) which was tagged with both $^{111}$In and IRDye800CW for targeting PSMA-expressing prostate cancer in mice with subcutaneous xenografts [16]. In this study, high tumor uptake in the PSMA-expressing PC3-PIP xenografts was observed, while uptake in the PSMA-negative PC3-FLU tumors remained low. Both tumor cell lines were derived from PC3 human prostate...
cancer cells and only differ in their expression of PSMA (PIP: PSMA^+, FLU: PSMA^-). PSMA-expressing tumors could be visualized specifically on both SPECT/CT and NIRF imaging. However, intense radiotracer uptake was observed in the kidneys, which were attributed to the tracer’s route of excretion and specific uptake by PSMA expressed in the kidneys.

In addition to biodistribution and NIRF imaging experiments we also performed sequential dual-modality microSPECT/CT and NIRF imaging to demonstrate the feasibility of this pretargeting approach using the bsAb TF12 and the dual-label fluorescent hapten-peptide \(^{111}\)In-RDC018. Preferential accumulation of the dual-label hapten-peptide \(^{111}\)In-RDC018 was confirmed with both NIRF and microSPECT/CT imaging in TROP-2-expressing soft-tissue and bone metastases. PC3 metastases could be visualized clearly with both microSPECT/CT and NIRF imaging, showing accurate conformity of single tumor lesions by both imaging modalities. In addition, image-guided surgery based on microSPECT/CT and NIRF images was performed to provide proof-of-principle for feasibility of using this pretargeting approach for intra-operative detection of tumor lesions. It was shown that metastases imaged by microSPECT/CT and NIRF prior to surgery could be resected with image-guidance using this pretargeting approach. Moreover, NIRF imaging could be performed intra-operatively to ensure that the tumor tissue was resected completely without leaving visible tumor-cell containing resection margins in situ. MicroSPECT/CT imaging, which was performed after surgery to exclude false negative observations from intra-operatively conducted NIRF imaging, confirmed complete resection of all metastatic tumor lesions.

For clinical translation of the current approach toxicity tests will have to be carried out. The toxicity of IMP288 was investigated in mice: high doses were administered without any signs of toxicity. We have applied the IMP288 peptide labeled with In-111 in patients (100 microgram/patient), without any toxicity [17]. The toxicity of IRDye800CW has been studied extensively in rats [18]. Also, the first clinical studies with IRDye800CW conjugated antibodies are ongoing and so far no toxicity has been reported.
In conclusion, compared to targeting approaches using dual-labeled antibodies, pretargeting strategies may yield better signal-to-background ratios. Here, we show feasibility of the $^{111}$In-labeled near-infrared fluorescent hapten-peptide RDC018 for dual-modality detection of prostate cancer metastases in a mouse model of TROP-2-expressing soft-tissue and bony prostate cancer metastases pretargeted with the bsAb TF12. Both soft-tissue as well as bony prostate cancer lesions were specifically and sensitively detected in vivo. While radionuclide imaging may allow pre-operative detection and intra-operative localization of tumor lesions, NIRF imaging enables subsequent accurate delineation of tumors and real-time assessment of resection margins. In addition, proof-of-principle for image-guided resection of metastatic lesions using this dual-modality pretargeting approach was provided.
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FIGURE LEGENDS

Figure 1: Structural formula of RDC018.

Figure 2: a) Biodistribution of $^{111}$In-RDC018 and $^{111}$In-IMP288 in BALB/c nude mice with TF12-pretargeted s.c. PC3 xenografts (right flank) measured at 2 h after injection of the radiolabeled haptens (n=4). Two groups of mice did not receive TF12 prior to injection of the radiolabeled haptens to determine non-specific binding. Tumor uptake for both $^{111}$In-RDC018 and $^{111}$In-IMP288 was significantly higher in the TF12-pretargeted tumors compared to non-pretargeted tumors. b) Representative SPECT/CT (panel 1) and NIRF image (panel 2) of a BALB/c nude mouse with a s.c. PC3 xenograft (right flank) pretargeted with TF12 and 2 h after injection of $^{111}$In-RDC018. In addition to tumor uptake, on the SPECT image also rest activity originating from renal excretion of the hapten can be observed.

Figure 3: Sequentially acquired dual-modality microSPECT/CT and NIRF image with $^{111}$In-RDC018 in a mouse with two TF12-pretargeted tumors. PC3 bone metastases located in 2 vertebrae with soft tissue outgrowth were visualized 2 h after injection of $^{111}$In-RDC018. a) CT scan illustrating the alterations in bone structure in the two vertebrae, b) microSPECT/CT scan showing uptake in the bone metastases with soft tissue outgrowth, as well as accumulation in kidneys and bladder due to renal excretion of $^{111}$In-RDC018 c) corresponding NIRF image showing specific uptake in both PC3 metastases and kidneys.

Figure 4: Sequentially acquired dual-modality microSPECT/CT and NIRF image with $^{111}$In-RDC018 (12 MBq, 0.5 µg/mouse) in a mouse with two TF12-pretargeted PC3 bone metastases originating from the ribs. Images were acquired 2 h after injection of $^{111}$In-RDC018. a) MicroSPECT/CT scan showing kidneys, bladder and two tumor manifestations (green and blue arrow), b) photographs of exposed metastases, and c) corresponding NIRF images showing specific uptake of $^{111}$In-RDC018 in both PC3 metastases.
**Figure 5:** MicroSPECT/CT and NIRF image-guided surgery with $^{111}$In-RDC018 in a TF-12 pretargeted PC3 soft-tissue metastasis located below the scapular bone. Images were acquired at 2 h after injection of $^{111}$In-RDC018. a) microSPECT/CT image prior to resection, b) NIRF image before resection illustrating the limited penetration depth of the emitted fluorescent light, since the tumor is located below a bony structure, c) NIRF image after lifting the scapular bone to the left hand-side, exposing the tumor, d) microSPECT/CT image after resection, and e) corresponding NIRF image after resection.
1
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