University of Groningen

Targeting human prostate cancer with In-111-labeled D2B IgG, F(ab')(2) and Fab fragments in nude mice with PSMA-expressing xenografts

Lutje, Susanne; van Rij, Catharina M.; Franssen, Gerben M.; Fracasso, Giulio; Helfrich, Wijnand; Eek, Annemarie; Oyen, Wim J.; Colombatti, Marco; Boerman, Otto C.

Published in:
Contrast Media & Molecular Imaging

DOI:
[10.1002/cmml.1596](https://doi.org/10.1002/cmml.1596)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Lutje, S., van Rij, C. M., Franssen, G. M., Fracasso, G., Helfrich, W., Eek, A., Oyen, W. J., Colombatti, M., & Boerman, O. C. (2014). Targeting human prostate cancer with In-111-labeled D2B IgG, F(ab')(2) and Fab fragments in nude mice with PSMA-expressing xenografts. *Contrast Media & Molecular Imaging*, 10(1), 28-36. <https://doi.org/10.1002/cmml.1596>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Targeting human prostate cancer with ^{111}In -labeled D2B IgG, F(ab')_2 and Fab fragments in nude mice with PSMA-expressing xenografts

Susanne Lütje^{a*}, Catharina M. van Rij^a, Gerben M. Franssen^a, Giulio Fracasso^b, Wijnand Helfrich^c, Annemarie Eek^a, Wim J. Oyen^a, Marco Colombatti^b and Otto C. Boerman^a

D2B is a new monoclonal antibody directed against an extracellular domain of prostate-specific membrane antigen (PSMA), which is overexpressed in prostate cancer. The potential of D2B IgG, and F(ab')_2 and Fab fragments of this antibody for targeting prostate cancer was determined in mice bearing subcutaneous prostate cancer xenografts. The optimal time point for imaging was determined in biodistribution and microSPECT imaging studies with ^{111}In -D2B IgG, ^{111}In -capromab pendetide, ^{111}In -D2B F(ab')_2 and ^{111}In -D2B Fab fragments in mice with PSMA-expressing LNCaP and PSMA-negative PC3 tumors at several time points after injection. All ^{111}In -labeled antibody formats specifically accumulated in the LNCaP tumors, with highest uptake of ^{111}In -D2B IgG and ^{111}In -capromab pendetide at 168 h p.i. ($94.8 \pm 19.2\%$ injected dose per gram (ID/g) and $16.7 \pm 2.2\%$ ID/g, respectively), whereas uptake of ^{111}In -D2B F(ab')_2 and ^{111}In -D2B Fab fragments peaked at 24 h p.i. ($12.1 \pm 3.0\%$ ID/g and $15.1 \pm 2.9\%$ ID/g, respectively). Maximum LNCaP tumor-to-blood ratios were 13.0 ± 2.3 (168 h p.i.), 6.2 ± 0.7 (24 h p.i.), 23.0 ± 4.0 (24 h p.i.) and 4.5 ± 0.6 (168 h p.i.) for ^{111}In -D2B IgG, ^{111}In - F(ab')_2 , ^{111}In -Fab and ^{111}In -capromab pendetide, respectively. LNCaP tumors were clearly visualized with microSPECT with all antibody formats. This study demonstrates the feasibility of D2B IgG, F(ab')_2 and Fab fragments for targeting PSMA-expressing prostate cancer xenografts. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: prostate cancer imaging; D2B IgG; Fab fragments; capromab pendetide

1. INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths in men in the Western world, and since metastatic prostate cancer is incurable, early diagnosis represents the most effective strategy with a change towards curative treatment options. Additionally, differentiation between malignant and nonmalignant prostate tissue is essential for the initiation of adequate treatment and the evaluation of response to therapy and relapse.

At present, prostate cancer is usually diagnosed by a combination of digital rectal examination, evaluation of prostate-specific antigen serum concentrations and transrectal ultrasound (TRUS)-guided biopsy (1). However, the ability of TRUS to delineate small cancer foci and differentiate between benign prostatic hyperplasia, atrophy, inflammatory processes and malignancies is limited. Magnetic resonance imaging (MRI) can be used for primary diagnostic purposes of prostate cancer, for instance if clinical suspicion for prostate cancer persists despite negative prostate biopsies, or for local tumor staging purposes (1). Positron emission tomography (PET) imaging is a useful imaging modality to detect metastatic disease in several cancers (2). However, ^{18}F -FDG-PET is not suited for staging patients with prostate cancer, owing to the relatively low avidity of prostate cancer for ^{18}F -FDG (3). The most commonly used approach to identify bone metastases in prostate cancer patients are bone scans. In equivocal cases, ^{11}C -choline- or ^{18}F -fluoride-PET/CT can be an option (1). Recent developments in

molecular imaging include radiolabeled monoclonal antibodies (mAb) used as tracers for single-photon emission tomography (SPECT) and PET.

The prostate-specific membrane antigen (PSMA) is a type II membrane glycoprotein (4) that provides an excellent target for prostate cancer imaging owing to its selective overexpression on the surface of prostate cancer cells (5). Initial validation of PSMA as an *in vivo* target resulted from imaging with the ^{111}In -labeled mAb capromab pendetide (7E11/CYT-356, Prostascint®) (6), which is approved by the US Food and Drug Administration for diagnostic imaging in patients with biopsy-proven prostate cancer, who are at high risk for pelvic lymph node metastases (7). However,

* Correspondence to: S. Lütje, Department of Radiology and Nuclear Medicine, Radboud University Medical Center, PO Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail: Susanne.Lutje@radboudumc.nl

a S. Lütje, C. M. Rij, G. M. Franssen, A. Eek, W. J. Oyen, O. C. Boerman
Department of Radiology and Nuclear Medicine, Radboud University Medical Center, Nijmegen, The Netherlands

b G. Fracasso, M. Colombatti
Department of Pathology and Diagnostics, University of Verona, Verona, Italy

c W. Helfrich
Department of Surgery, University Medical Center Groningen, Groningen, The Netherlands

capromab pendetide is directed against an epitope on an intracellular domain of PSMA (8). Therefore, binding of this antibody to tumor cells requires uptake of the antibody into the cells. It has been postulated that capromab pendetide may only bind to dying or necrotic cells with cellular membranes that are permeable for IgG. The mAb J591, which is directed against an extracellular epitope of PSMA, has shown great potential for diagnostic radionuclide imaging in prostate cancer patients (9–11), especially when labeled with ^{111}In , showing excellent tumor targeting of metastases (12).

In the present study, we use a new mAb D2B IgG, a murine IgG1 mAb directed against an epitope on the extracellular domain of PSMA (13). We characterized the tumor targeting capacity of D2B IgG and compared it with those of F(ab')_2 and Fab fragments of D2B IgG to evaluate possible limitations such as low tumor-to-blood ratios that can occur owing to relatively slow clearance of intact antibodies compared to fragments (14). In these experiments, capromab pendetide was used as a reference.

2. MATERIALS AND METHODS

2.1. Production and purification of antibodies and fragments

The monoclonal antibody D2B (IgG1) was purified from hybridoma culture supernatant by Protein A affinity chromatography. The antibody secreting cells were obtained according to the hybridoma technology from mice that were immunized with a cell lysate of membranes of LNCaP cells. Mice were injected three times (days 0, 14 and 28) i.p. with 800 μg of lysate and Freund's adjuvant (Sigma-Aldrich, St Louis, MO, USA). On the last three days before the spleen of the mice was harvested, mice were boosted with 10 μg of recombinant PSMA protein (Novagen, Milan, Italy) intravenously.

F(ab')_2 fragments were produced by pepsin digestion essentially as described earlier (15). Briefly, after incubation of D2B IgG for 3 h at 37 °C with pepsin in 0.15 M sodium citrate (pH 3.8), digestion was stopped by adjusting the pH of the solution to pH 7.0 with 1.0 M Tris. F(ab')_2 fragments were purified using a cation exchange column (Mono-S/HR 5/5, Pharmacia, Uppsala, Sweden) that was eluted with 40 mM sodium acetate buffer (pH 6.0) with a 0–400 mM lithium chloride gradient. The F(ab')_2 fragment containing fractions were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and pooled.

Fab fragments were generated by papain digestion (6 h at 37 °C) of D2B IgG using papain immobilized on agarose resin according to the protocol of the manufacturer (Thermo Scientific, Pierce Fab Preparation Kit) followed by purification using protein A affinity chromatography and dialysis.

2.2. Cell culture

The human prostate cancer cell lines PC3 (PSMA[−]) and LNCaP (PSMA⁺) derived from a bone metastasis and a supraclavicular lymph node, respectively, were used. Both cell lines were obtained from the ATCC and grown in RPMI 1640 medium, supplemented with 10% fetal calf serum (Life Technologies, Bleiswijk, The Netherlands) and glutamine 2 mM.

2.3. Mouse models

Male BALB/c nude mice (Janvier, Le Genest Saint Isle, France), 8–9 weeks old, housed in filter-topped cages (five mice per cage) under nonsterile standard conditions with free access

to standard animal chow and water, were adapted to laboratory conditions for 1 week before experimental use. Mice were subcutaneously inoculated with 3×10^6 LNCaP cells (right flank) suspended in 200 μl of 33% complete RPMI 1640 medium with 67% Matrigel (BD Biosciences) and with 3×10^6 PC3 cells (left flank) suspended in 200 μl of 67% complete RPMI 1640 medium with 33% Matrigel. LNCaP cells were inoculated 7 days prior to PC3 cells. LNCaP and PC3 tumors grew to approximately 0.1 g in 14 and 7 days after tumor cell inoculation, respectively.

All experiments have been approved by the institutional Animal Welfare Committee of the Radboud University Medical Center and were conducted in accordance with the principles set forth by the Revised Dutch Act on Animal Experimentation.

2.4. Radiolabeling of D2B

The murine mAb D2B IgG and all fragments were conjugated with *p*-isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (ITC-DTPA; Macrocytics) in 0.1 M NaHCO_3 , pH 9.5, with a 10-fold molar excess of ITC-DTPA [2.5 mg D2B IgG with 110 μg ITC-DTPA, 4.5 mg F(ab')_2 with 250 μg ITC-DTPA and 0.75 mg Fab with 100 μg ITC-DTPA]. After incubation for 1 h at room temperature, the reaction mixture was dialyzed for 3 days in a Slide-A-Lyzer (20 kDa cutoff, Pierce) against 0.25 M NH_4Ac , pH 5.4.

For biodistribution studies, D2B IgG-DTPA (28 μg), F(ab')_2 -DTPA (22 μg), Fab-DTPA (20 μg) and capromab pendetide (30 μg) were radiolabeled with 3.7 MBq ^{111}In -chloride (Covidien, Petten, Netherlands) in 0.1 M MES buffer pH 5.4 (three times the volume of ^{111}In -chloride) and incubated during 30 min at room temperature under metal-free conditions. For SPECT/CT studies, D2B IgG-DTPA (20 μg), F(ab')_2 -DTPA (25 μg), Fab-DTPA (25 μg) and capromab pendetide-DTPA (30 μg) were labeled with ^{111}In in 0.1 M MES buffer pH 5.4 at specific activities of 2.57 GBq/nmol (D2B IgG), 982.3 MBq/nmol (F(ab')_2), 232.4 MBq/nmol (Fab), and 56 MBq/nmol (capromab pendetide). Volumes of the radiolabeling reaction were 1.3 ml (D2B IgG), 1.5 ml [F(ab')_2], 2.0 ml (Fab) and 1.5 ml (capromab pendetide). Following incubation, 50 mM EDTA was added to the final concentration of 5 mM to chelate unincorporated ^{111}In .

The labeling efficiency was determined by instant thin-layer chromatography on silicagel strips (ITLC-SG; Gelman Sciences, Ann Arbor, MI, USA) using 15 mM citrate buffer, pH 6.0, as the mobile phase. The radiochemical purity of the preparations that were used in the biodistribution experiments was 95, 58, 90 and 95%, for ^{111}In -labeled D2B IgG-DTPA, F(ab')_2 -DTPA, Fab-DTPA and capromab-DTPA, respectively. For SPECT-imaging, it was 70, 79, 97 and 95%, respectively.

^{111}In -labeled D2B IgG-DTPA, F(ab')_2 -DTPA, Fab-DTPA, and capromab-DTPA were purified by gel filtration on a PD-10 column. The radiochemical purity was determined by ITLC and exceeded 97% for all preparations used in the studies.

2.5. Immunoreactivity

The immunoreactive fractions of the radiolabeled antibody/fragment preparations were determined using freshly trypsinized LNCaP cells, essentially as described by Lindmo et al. (16,17). The activity in the vials and in the cell pellet after washing with 500 μl binding buffer was determined in the gamma counter (Wizard 3[™] 1480, LKB-Wallac, Oy, Finland) after incubation of 1 h at 37 °C. Immunoreactive fractions of all radiolabeled preparations used in the experiments exceeded 80%. For ^{111}In -capromab pendetide,

the assay was carried out on live and permeabilized LNCaP cells using the Fix & Perm Cell Fixation and Permeabilization Kit (Invitrogen) to permeabilize the cells.

2.6. Competitive binding assay

The IC_{50} of D2B IgG, $F(ab')_2$ and Fab fragments was determined in a competitive binding assay on LNCaP cells using ^{111}In -labeled D2B as a tracer. LNCaP cells were grown to confluency in six-well plates, and incubated on ice for 2 h in 1 ml of binding buffer with 1.85 kBq of ^{111}In -labeled D2B IgG and increasing concentrations (0.005–300 nM) of unlabeled D2B IgG, $F(ab')_2$ and Fab fragments. Cells were washed with binding buffer after incubation and the cell-associated activity was measured in a gamma counter. IC_{50} values were calculated using GraphPad Prism software, version 5.03 (GraphPad, La Jolla, CA, USA).

2.7. Flow cytometric analysis

For cell surface staining, LNCaP cells were incubated with either the mAbs D2B IgG, capromab pendetide or an isotype control murine IgG (Jackson ImmunoResearch) in phosphate-buffered saline (PBS) with 2% of bovine serum albumin (BSA) and 2% human serum (HS) for 30 min on ice. Part of the cells were fixed in 2% paraformaldehyde and incubated in PBS, 2% HS and 0.1% saponin to permeabilize the cells. After the incubation, samples were washed three times in PBS or PBS and 0.1% saponin and incubated for 20 min on ice with the secondary anti-mouse-alexa 488 antibody for staining. Cells were analyzed in a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences).

2.8. Biodistribution studies

The effect of mAb protein dose on the biodistribution of ^{111}In -D2B in nude mice with subcutaneous LNCaP tumors was determined in

groups of five mice that received different protein doses of ^{111}In -D2B IgG (0.1, 1, 3, 10, 30 or 100 μ g of D2B IgG per mouse, 0.4 MBq). Mice were euthanized with CO_2/O_2 asphyxiation 3 days after injection of ^{111}In -D2B IgG. Blood samples were obtained by heart puncture and tissues (LNCaP tumor, muscle, lung, spleen, kidney, liver, pancreas, stomach, duodenum and prostate) were dissected, weighed, and the radioactivity was measured in a gamma counter. 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.

In a subsequent study, ^{111}In -D2B IgG (0.4 MBq, 3.0 μ g/mouse), ^{111}In - $F(ab')_2$ (0.4 MBq, 2.2 μ g/mouse), ^{111}In -Fab fragments (0.4 MBq, 1 μ g/mouse) or ^{111}In -capromab pendetide (0.4 MBq, 5 μ g/mouse) was injected i.v. into groups of five mice with subcutaneous PC3 tumors (left flank) and LNCaP tumors (right flank). In addition, for Fab and $F(ab')_2$, five mice per antibody format received an excess of unlabeled D2B IgG (300 μ g/mouse) two days before injection of the ^{111}In -labeled fragments. For D2B IgG and capromab pendetide, the excess of unlabeled D2B IgG or capromab was injected on the same day as the ^{111}In -labeled antibodies. Mice were euthanized by CO_2/O_2 asphyxiation and the biodistribution of ^{111}In -D2B IgG was determined at 1, 2, 4, 24, 72 and 168 h after injection. Because of their more rapid clearance, biodistribution of the ^{111}In - $F(ab')_2$ and ^{111}In -Fab fragments was determined at 1, 2, 4 and 24 h after injection. Activity concentrations in the tissues (% ID/g) were determined as described above.

2.9. MicroSPECT/CT imaging

Four groups of five mice with subcutaneous LNCaP (right flank) and PC3 (left flank) tumors were injected with 37 MBq of ^{111}In -D2B IgG (3.0 μ g/mouse), ^{111}In - $F(ab')_2$ (1.5 μ g/mouse), ^{111}In -Fab (1 μ g/mouse) and ^{111}In -capromab pendetide (5 μ g/mouse),

Figure 1. Flow cytometric (FACS) analysis of D2B IgG and capromab pendetide on live (black peaks) and saponin-permeabilized (gray lines) LNCaP cells showing intracellular binding of capromab pendetide.

respectively. The mice that received ^{111}In -labeled D2B IgG and capromab pendetide, SPECT/CT scans were imaged 0, 1, 3 and 7 days after injection using a small animal SPECT/CT scanner (U-SPECT II, MILabs, Utrecht, Netherlands; acquisition time 30–90 min, depending on the time point) with a 1.0 mm diameter pinhole collimator tube. Mice injected with ^{111}In -F(ab')₂ and ^{111}In -Fab fragments underwent SPECT/CT at 0, 1 and 2 days after injection. For Fab and F(ab')₂, two additional mice were injected with an excess of unlabeled D2B IgG 2 days before injection of the ^{111}In -labeled antibody fragments. For D2B IgG, the excess of unlabeled antibody formats was coadministered on the same day as ^{111}In -D2B.

Figure 2. Competitive binding curves of IC₅₀ determination of D2B IgG, F(ab')₂ and Fab fragments on prostate-specific membrane antigen (PSMA)-positive LNCaP cells.

Figure 3. Biodistribution results of the dose escalation of ^{111}In -D2B in mice ($n = 5$) with subcutaneous LNCaP xenografts. Data are presented as mean $\pm$ SD.

After the last scan, mice were euthanized and biodistribution of the ^{111}In -D2B, ^{111}In -F(ab')₂, ^{111}In -Fab or ^{111}In -capromab pendetide was determined as described above. Scans were reconstructed with MILabs reconstruction software (MILabs, Utrecht, Netherlands), which uses an ordered-subset expectation maximization algorithm.

2.10. Statistical analysis

Statistical analyses were performed with Graphpad Prism, version 5.03. Results are presented as mean $\pm$ standard deviation (SD). Differences in tumor uptake of the antibody formats were tested for significance using the two-tailed *t*-test. A *p*-value below 0.05 was considered significant.

3. RESULTS

3.1. In vitro characterization

Flow cytometric analysis (FACS) with live and permeabilized LNCaP cells revealed high binding of D2B IgG to live ($95.9 \pm 2.2\%$) as well as to permeabilized cells ($96.9 \pm 1.7\%$). In contrast, for capromab pendetide, binding to live cells was low ($7.6 \pm 0.7\%$), while binding to permeabilized cells was high ($96.1 \pm 1.1\%$), showing that D2B recognizes an epitope on the cell surface, while capromab is directed against an intracellular epitope (Fig. 1).

For ^{111}In -D2B IgG the immunoreactive fraction on live LNCaP cells was 84.1%, and the immunoreactive fractions of ^{111}In -capromab pendetide on permeabilized LNCaP cells were 93.6%, indicating that the immunoreactivity of both antibodies was preserved during the labeling procedure.

The IC₅₀ of D2B IgG and F(ab')₂ fragments was 3.7 nM (95% confidence interval, CI: 2.8–4.8 nM) and 1.9 nM (CI 1.6–2.3 nM), respectively, whereas it was slightly lower for Fab fragments (8.9 nM; CI 7.1–11.0 nM Fig. 2), which could be due to the monovalency of Fab fragments.

3.2. Biodistribution studies

In the dose escalation study an inverse correlation between the D2B antibody protein dose and the uptake in the LNCaP

tumor was observed: LNCaP tumor uptake of ^{111}In -D2B IgG decreased from $52 \pm 5\%$ ID/g at $0.1 \mu\text{g}/\text{mouse}$ to $26 \pm 1.5\%$ ID/g at $100 \mu\text{g}/\text{mouse}$. Highest tumor uptake ($54 \pm 4\%$ ID/g)

Figure 4. Ex vivo biodistribution of nude mice bearing subcutaneous PSMA-negative PC3 and PSMA-positive LNCaP xenografts at several time points after intravenous injection of (a) ^{111}In -D2B IgG, (b) ^{111}In -F(ab')₂, (c) ^{111}In -Fab fragments and (d) ^{111}In -capromab pendetide. Data are presented as mean $\pm$ SD.

was observed at D2B doses $\leq 3 \mu\text{g}/\text{mouse}$ at 3 days p.i. (Fig. 3). At that time point, tumor-to-blood ratios ranged from 4.3 (at $0.1 \mu\text{g}/\text{mouse}$) to 1.8 (at $100 \mu\text{g}/\text{mouse}$). Tumor weight was $0.04 \text{ g} \pm 0.02 \text{ g}$ (D2B IgG group), $0.08 \text{ g} \pm 0.03 \text{ g}$ (F(ab')₂ group), $0.07 \text{ g} \pm 0.02 \text{ g}$ (Fab group) and $0.10 \text{ g} \pm 0.02 \text{ g}$ (capromab group).

For biodistribution studies, ¹¹¹In-D2B IgG (0.4 MBq , $3.0 \mu\text{g}/\text{mouse}$), ¹¹¹In-F(ab')₂ (0.4 MBq , $2.2 \mu\text{g}/\text{mouse}$), ¹¹¹In-Fab fragments (0.4 MBq , $1 \mu\text{g}/\text{mouse}$) or ¹¹¹In-capromab pendetide (0.4 MBq , $5 \mu\text{g}/\text{mouse}$) was injected intravenously. All three ¹¹¹In-labeled D2B antibody formats specifically accumulated in the s.c. PSMA⁺ LNCaP tumors (right flank), while uptake in PSMA⁻ PC3 xenografts (left flank) remained low. LNCaP tumor uptake increased for ¹¹¹In-D2B IgG from $2.2 \pm 2.0\%$ ID/g at 1 h p.i. to $94.8 \pm 19.2\%$ ID/g at 168 h after injection, the latter representing the highest tumor uptake observed in these studies (Fig. 4). For ¹¹¹In-F(ab')₂ and ¹¹¹In-Fab, LNCaP tumor uptake ranged from 1.6 ± 0.6 and $4.1 \pm 0.9\%$ ID/g at 1 h p.i. to 12.1 ± 3.0 and $15.1 \pm 2.9\%$ ID/g at 24 h after injection, respectively. For ¹¹¹In-labeled capromab pendetide, LNCaP tumor uptake ranged from $0.7 \pm 0.4\%$ ID/g at 1 h p.i. to $16.7 \pm 2.2\%$ ID/g at 168 h after injection, reaching a maximum of $17.4 \pm 2.8\%$ ID/g 3 days after injection.

Tumor uptake of the three D2B formats could be blocked by coinjection of an excess of unlabeled mAb. The uptake of ¹¹¹In-D2B IgG decreased from 60.9 ± 11.0 to $12.3 \pm 7.1\%$ ID/g at 72 h p.i.. The LNCaP uptake of ¹¹¹In-F(ab')₂, decreased from $4.8 \pm 2.1\%$ ID/g to $3.5 \pm 1.8\%$ ID/g (4 h p.i.), while the LNCaP uptake of ¹¹¹In-Fab decreased from $8.5 \pm 2.4\%$ ID/g to $3.9 \pm 0.4\%$ ID/g, respectively at 4 h p.i.. Maximum uptake in the PSMA-negative PC3 tumor was $9.1 \pm 1.3\%$ (24 h p.i.), $6.8 \pm 1.3\%$ (4 h p.i.) and $6.3 \pm 1.1\%$ ID/g (2 h p.i.) for ¹¹¹In-labeled IgG, F(ab')₂ and Fab, further confirming the specific uptake of the D2B antibody formats in the PSMA⁺ tumor.

Maximum LNCaP tumor-to-blood ratio for ¹¹¹In-D2B IgG was 13.0 ± 2.3 (168 h p.i.). For D2B Fab and F(ab')₂ fragments, high tumor-to-blood ratios were reached faster compared with tumor-to-blood ratios of D2B IgG, with a maximum of 23.0 ± 4.0 and 6.2 ± 0.7 , respectively, 24 h after injection (Table 1). The highest

LNCaP tumor-to-blood ratio of ¹¹¹In-labeled capromab pendetide was 4.5 ± 0.6 , reached 7 days after injection. LNCaP tumor-to-muscle ratio for ¹¹¹In-D2B IgG (168 h p.i.), ¹¹¹In-F(ab')₂ (24 h p.i.), ¹¹¹In-Fab (24 h p.i.) and ¹¹¹In-capromab pendetide (168 h p.i.) were 94.8 ± 19.2 , 12.1 ± 3.0 , 15.1 ± 2.9 , and 16.7 ± 2.2 , respectively.

3.3. MicroSPECT/CT Imaging

Coronal sections of SPECT/CT images of all ¹¹¹In-labeled antibody formats through the center of subcutaneous PSMA⁺ LNCaP and PSMA⁻ PC3 tumors are displayed in Fig. 5. At 24 h p.i., LNCaP tumors were clearly visualized with all formats. The optimal time point for imaging with ¹¹¹In-D2B IgG and ¹¹¹In-capromab pendetide was 168 h p.i. and for ¹¹¹In-F(ab')₂ and ¹¹¹In-Fab this was 48 h p.i. Tumor uptake was specific for ¹¹¹In-D2B IgG, ¹¹¹In-F(ab')₂ and ¹¹¹In-Fab since tumor uptake was markedly lower in mice coinjected with an excess of unlabeled D2B IgG (Fig. 5). Uptake in the PSMA⁻ PC3 tumors remained low for all antibody formats at all time points.

For D2B F(ab')₂ and Fab fragments, high accumulation of ¹¹¹In in the kidneys, especially the renal cortex, was observed, indicating that these antibody preparations were cleared via the kidneys apparently with tubular reabsorption of the ¹¹¹In-labeled metabolites.

4. DISCUSSION

In the present study, the *in vivo* tumor targeting characteristics of ¹¹¹In-D2B IgG, ¹¹¹In-F(ab')₂, and ¹¹¹In-Fab fragments for targeting of prostate cancer were examined in mice with human prostate cancer xenografts using ¹¹¹In-labeled capromab pendetide as a reference.

Since intact antibodies clear slowly from the circulation and maximum tumor uptake is reached at 3–7 days after injection, we included F(ab')₂ and Fab fragments of D2B in our studies to investigate whether further improvements in prostate cancer targeting would be possible by using D2B antibody fragments. The slow accumulation of intact antibodies has been attributed

Table 1. *Ex vivo* biodistribution (tissue uptake % ID/g) and LNCaP tumor-to-organ ratios of ¹¹¹In-capromab pendetide, ¹¹¹In-D2B IgG, ¹¹¹In-F(ab')₂ and ¹¹¹In-Fab fragments in nude mice with subcutaneous prostate-specific membrane antigen (PSMA)-negative PC3 xenografts and PSMA-positive LNCaP xenografts

	¹¹¹ In-capromab (168 h p.i.) (% ID/g tissue uptake)	LNCaP tumor- to-organ ratio (¹¹¹ In-capromab)	¹¹¹ In-D2B IgG (168 h p.i.) (% ID/g tissue uptake)	LNCaP tumor- to-organ ratio (¹¹¹ In-D2B IgG)	¹¹¹ In-F(ab') ₂ (24 h p.i.) (% ID/g tissue uptake)	LNCaP tumor- to-organ ratio (¹¹¹ In-F(ab') ₂)	¹¹¹ In-Fab (24 h p.i.) (% ID/g tissue uptake)	LNCaP tumor-to- organ ratio (¹¹¹ In-Fab)
Blood	3.7 ± 0.3	4.5 ± 0.6	7.3 ± 0.7	13.0 ± 2.3	2.0 ± 0.3	6.2 ± 0.7	0.7 ± 0.0	23.1 ± 4.0
Muscle	0.5 ± 0.1	31.9 ± 6.5	0.9 ± 0.2	110.4 ± 41.1	0.7 ± 0.2	18.4 ± 3.4	0.5 ± 0.4	38.2 ± 19.8
Lung	3.0 ± 0.4	5.5 ± 0.7	4.3 ± 0.8	22.8 ± 6.3	2.1 ± 0.1	5.7 ± 1.2	0.9 ± 0.1	20.0 ± 4.2
Spleen	5.3 ± 0.4	3.5 ± 0.6	3.1 ± 0.13	30.9 ± 6.4	5.5 ± 0.7	2.8 ± 0.4	1.1 ± 0.1	13.2 ± 2.4
Pancreas	1.1 ± 0.1	14.9 ± 1.0	1.0 ± 0.2	98.9 ± 18.6	0.9 ± 0.0	14.1 ± 3.4	0.3 ± 0.0	45.8 ± 12.1
Kidney	9.0 ± 0.6	1.9 ± 0.3	2.7 ± 0.4	35.5 ± 7.5	57.8 ± 6.4	0.2 ± 0.0	102.8 ± 8.5	0.2 ± 0.0
Stomach	1.00 ± 0.1	17.6 ± 2.5	0.9 ± 0.2	104.0 ± 31.0	1.1 ± 0.1	11.4 ± 1.5	0.4 ± 0.0	35.5 ± 8.8
Duodenum	1.4 ± 0.2	11.9 ± 2.4	1.5 ± 0.1	64.3 ± 14.3	1.4 ± 0.2	8.5 ± 2.5	0.4 ± 0.0	37.3 ± 9.7
Liver	5.5 ± 0.7	3.1 ± 0.7	3.4 ± 0.3	28.2 ± 4.6	7.3 ± 0.4	1.7 ± 0.3	1.4 ± 0.1	10.8 ± 2.3
Prostate	1.8 ± 0.2	9.5 ± 1.8	1.7 ± 0.5	59.2 ± 14.4	1.6 ± 0.3	8.0 ± 3.2	0.6 ± 0.1	25.7 ± 5.8
PC3 tumor	7.1 ± 1.2	2.4 ± 0.4	6.7 ± 0.9	14.0 ± 1.2	6.5 ± 1.0	1.9 ± 0.2	2.2 ± 0.4	7.1 ± 2.0
LNCaP tumor	16.7 ± 2.2		94.8 ± 19.2		12.1 ± 3.0		15.1 ± 2.9

Figure 5. Representative SPECT/CT images of mice bearing subcutaneous PSMA-negative PC3 (left flank, green arrows) and PSMA-positive LNCaP (right flank, blue arrows) prostate cancer xenografts at several time points after intravenous injection of ^{111}In -D2B IgG, ^{111}In - $\text{F}(\text{ab}')_2$, ^{111}In -Fab fragments and ^{111}In -capromab pendetide.

to the slow blood clearance of intact IgG in combination with the presence of various physiological barriers between the circulation and the tumor cell surface (18). It has been pointed out that the vascular endothelium, the relatively large transport distances in the tissue and the enhanced interstitial fluid pressure in the tumor tissue hamper the penetration of antibodies into the tumor tissue to bind to their target antigen. Antibody fragments such as F(ab')₂ and Fab fragments are cleared more rapidly from the blood, mainly because they lack the CH₂ domain, which causes IgG molecules to recycle into the bloodstream via the FcRn receptor (19). On the other hand, the sustained blood levels of intact IgG molecules are the driving force for their accumulation in tumors. Therefore, the uptake of antibody fragments in tumors is lower than that of intact IgGs, as was also observed in the present study. Because the diffusion rate of a molecule is linearly correlated to its molecular size (20), antibody fragments diffuse more rapidly in the interstitial fluid. Owing to the more rapid blood clearance, higher tumor-to-blood ratios are obtained. With the antibody fragments, tumors can be visualized more rapidly: optimal images can be acquired within 24 h p.i., whereas intact IgG can be used when high concentrations of the antibody in the tumor are required (e.g. in therapeutic applications when high payloads of toxic agents should be targeted to the tumor).

Here, we observed that the tumor uptake of ¹¹¹In-D2B IgG was higher than that of ¹¹¹In-F(ab')₂ and ¹¹¹In-Fab at all time points, even as early as 24 h after injection. However, at early time points after injection, tumor–nontumor ratios of ¹¹¹In-F(ab')₂ and ¹¹¹In-Fab were higher than those of ¹¹¹In-D2B IgG, indicating that the antibody fragments are preferred for imaging purposes. For the evaluation of response to therapy, when imaging early after tracer injection is required, and to determine tracer uptake before and shortly after intervention, antibody fragments should be used.

In contrast to ¹¹¹In-D2B IgG, the renal clearance of ¹¹¹In-F(ab')₂ and ¹¹¹In-Fab resulted in high accumulation in the kidneys, most likely owing to tubular reabsorption after glomerular filtration of these antibody preparations. Usually, the threshold for glomerular filtration is approximately 60 kDa, suggesting that the antibody fragments are catabolized into smaller molecules in the kidneys (21). Renal clearance and accumulation of F(ab')₂ and Fab fragments might be a disadvantage, since it might interfere with visualization of malignant tissue located in the vicinity of the kidneys and the urinary bladder.

For the characterization of the tumor targeting capacities of ¹¹¹In-D2B IgG, ¹¹¹In-F(ab')₂ and ¹¹¹In-Fab fragments, ¹¹¹In-labeled capromab pendetide was used as a reference in this study. Since capromab pendetide targets an intracellular epitope of PSMA, internalization or uptake into the cell by another process is required to bind to PSMA. It has been postulated that capromab pendetide may only bind dying or necrotic cells with cellular membranes that are permeable for IgG (22). Therefore, ¹¹¹In-capromab pendetide has low sensitivity for viable tumor lesions, which makes it a suboptimal agent for diagnostic imaging of prostate cancer. Still, specific tumor targeting of the PSMA⁺ LNCaP tumors was observed with ¹¹¹In-labeled capromab pendetide, although at a much lower level than ¹¹¹In-D2B IgG.

Overall, the new mAb D2B IgG has excellent *in vivo* tumor targeting characteristics. In addition, ¹¹¹In-F(ab')₂ and ¹¹¹In-Fab fragments of D2B IgG are feasible for SPECT or PET imaging of PSMA-expressing prostate cancer. The preparations based on antibody fragments allow imaging at early time points after

injection, while the preparation based on intact D2B IgG has a higher absolute uptake in the tumor.

5. CONCLUSION

¹¹¹In-labeled D2B IgG can be used for imaging of PSMA-expressing cancer, as shown in mice with s.c. PSMA-expressing tumors. The xenografts were clearly visualized in SPECT/CT with specific differentiation between PSMA[−] and PSMA⁺ tumors. Compared with ¹¹¹In-capromab pendetide, higher tumor-to-blood ratios were obtained. Owing to renal accumulation of F(ab')₂ and Fab fragments, visualization of malignant tissue located in the vicinity of the kidneys might be hampered. However, especially in settings where multiple images within a short time interval are required, ¹¹¹In-D2B F(ab')₂ and Fab fragments can be used.

Acknowledgments

We thank Bianca Lemmers-van de Weem, Henk Arnts, Iris Lamers-Elmians and Kitty Lemmens-Hermans for their excellent technical assistance in the animal experiments. We greatly acknowledge Fondazione Cariverona (Verona Nanomedicine Initiative and Interceptin project), AIRC 5 × 1000 and Fondazione Cariverona/AIRC Progetto Regionale. This study was performed within the framework of CTMM, the Center for Translational Molecular Medicine, PCMM project (grant 030-203) and the Dutch Cancer Society (grant KUN 2010-4820).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Heidenreich A, Bastian PJ, Bellmunt J, Bolla M, Joniau S, van der Kwast T, Mason M, Matveev V, Wiegel T, Zattoni F, Mottet N. EAU Guidelines on prostate cancer. Part 1: screening, diagnosis, and local treatment with curative intent – update 2013. *Eur Urol* 2014; 65(1): 124–137.
- Fletcher JW, Djulbegovic B, Soares HP, Siegel BA, Lowe VJ, Lyman GH, Coleman RE, Wahl R, Paschold JC, Avril N, Einhorn LH, Suh WW, Samson D, Delbeke D, Gorman M, Shields AF. Recommendations on the use of 18F-FDG PET in oncology. *J Nucl Med* 2008; 49(3): 480–508.
- Jana S, Blafox MD. Nuclear medicine studies of the prostate, testes, and bladder. *Semin Nucl Med* 2006; 36(1): 51–72.
- Israeli RS, Powell CT, Fair WR, Heston WDW. Molecular cloning of a complementary DNA encoding a prostate specific membrane antigen. *Cancer Res* 1993; 53(2): 227–230.
- Ross JS, Sheehan CE, Fisher HA, Kaufman RP Jr, Kaur P, Gray K, Webb I, Gray GS, Mosher R, Kallakury BV. Correlation of primary tumor prostate-specific membrane antigen expression with disease recurrence in prostate cancer. *Clin Cancer Res* 2003; 9(17): 6357–6362.
- Horoszewicz JS, Kawinski E, Murphy GP. Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. *Anticancer Res* 1987; 7(5B): 927–935.
- FDA. Capromab pendetide product approval information. Available from: <http://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/ucm080734.htm>
- Barren RJ 3rd, Holmes EH, Boynton AL, Misrock SL, Murphy GP. Monoclonal antibody 7E11.C5 staining of viable LNCaP cells. *Prostate* 1997; 30(1): 65–68.
- Smith-Jones PM, Vallabhaajosula S, Goldsmith SJ, Navarro V, Hunter CJ, Bastidas D, Bander NH. In vitro characterization of radiolabeled monoclonal antibodies specific for the extracellular domain of prostate-specific membrane antigen. *Cancer Res* 2000; 60(18): 5237–5243.

10. Milowsky MI, Nanus DM, Kostakoglu L, Vallabhajosula S, Goldsmith SJ, Bander NH. Phase I trial of yttrium-90-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for androgen-independent prostate cancer. *J Clin Oncol* 2004; 22(13): 2522–2531.
11. Bander NH, Trabulsi EJ, Kostakoglu L, Yao D, Vallabhajosula S, Smith-Jones P, Joyce MA, Milowsky M, Nanus DM, Goldsmith SJ. Targeting metastatic prostate cancer with radiolabeled monoclonal antibody J591 to the extracellular domain of prostate specific membrane antigen. *J Urol* 2003; 170(5): 1717–1721.
12. Bander NH, Milowsky MI, Nanus DM, Kostakoglu L, Vallabhajosula S, Goldsmith SJ. Phase I trial of 177lutetium-labeled J591, a monoclonal antibody to prostate-specific membrane antigen, in patients with androgen-independent prostate cancer. *J Clin Oncol* 2005; 23(21): 4591–4601.
13. Colombatti M. Isolated monoclonal antibody or fragment thereof binding prostate specific membrane antigen, conjugates and uses thereof. WO 2009/130575 A2 International Patent Application (PCT/IB2009/005326).
14. Steiner M, Neri D. Antibody-radionuclide conjugates for cancer therapy: historical considerations and new trends. *Clin Cancer Res* 2011; 17(20): 6406–6416.
15. Parham P, Androlewicz MJ, Brodsky FM, Holmes NJ, Ways JP. Monoclonal antibodies: purification, fragmentation and application to structural and functional studies of class I MHC antigens. *J Immunol Meth* 1982; 53(2): 133–173.
16. Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA Jr. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J Immunol Meth* 1984; 72(1): 77–89.
17. Heskamp S, van Laarhoven HW, Molkenboer-Kueneen JD, Franssen GM, Versleijen-Jonkers YM, Oyen WJ, van der Graaf WT, Boerman OC. ImmunoSPECT and immunoPET of IGF-1R expression with the radiolabeled antibody R1507 in a triple-negative breast cancer model. *J Nucl Med* 2010; 51(10): 1565–1572.
18. Jain RK. Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. *Cancer Res* 1990; 50(3 suppl): 814s–819s.
19. Gehlsen K, Gong R, Bramhill D, Wiersma D, Kirkpatrick S, Wang Y, Feng Y, Dimitrov DS. Pharmacokinetics of engineered human monomeric and dimeric CH2 domains. *MAbs* 2012; 4(4): 466–474.
20. Pluen A, Boucher Y, Ramanujan S, McKee TD, Gohongi T, di Tomaso E, Brown EB, Izumi Y, Campbell RB, Berk DA, Jain RK. Role of tumor-host interactions in interstitial diffusion of macromolecules: cranial vs. subcutaneous tumors. *Proc Natl Acad Sci U S A* 2001; 98(8): 4628–4633.
21. Heskamp S, van Laarhoven HW, Molkenboer-Kueneen JD, Bouwman WH, van der Graaf WT, Oyen WJ, Boerman OC. Optimization of IGF-1R SPECT/CT Imaging Using ¹¹¹In-Labeled F(ab')₂ and Fab Fragments of the Monoclonal Antibody R1507. *Mol Pharm* 2012; 9(8): 2314–2321.
22. Troyer JK, Feng Q, Beckett ML, Wright GL Jr. Biochemical characterization and mapping of the 7E11-C5.3 epitope of the prostate-specific membrane antigen. *Urol Oncol* 1995; 1(1): 29–37.