**Establishing $^{177}$Lu-PSMA-617 radioligand therapy in a syngeneic model of murine prostate cancer**

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ABSTRACT

Clinical $^{177}$Lu-PSMA-617 radioligand therapy (RLT) is applied in advanced stage prostate cancer. However, to the best of our knowledge murine models to study the biological effects of various activity levels have not been established. **Aim:** To optimize specific and total activity for $^{177}$Lu-PSMA-617 RLT in a syngeneic model of murine prostate cancer. **Methods:** 0.1 x $10^6$ murine reconstituted, oncogene-driven prostate cancer cells (RM1), transduced to express human prostate-specific membrane antigen (PSMA), were injected into the left flank of C57Bl6 immunocompetent mice. RLT was performed by administering a single tail vein injection of $^{177}$Lu-PSMA-617 at different formulations for specific (60 MBq at high, 62 MBq/nmol; intermediate, 31 MBq/nmol; or low 15 MBq/nmol specific activity) or total activity (30, 60, or 120 MBq). Organ distribution was determined by ex-vivo gamma counter measurement. DNA double-strand breaks were measured using anti-gamma-H2A.X (phospho S139) immunohistochemistry. Efficacy was assessed by serial computed tomography (CT) tumor volumetry and 2-deoxy-2-($^{18}$F)fluoro-D-glucose positron emission tomography ($^{18}$F-FDG PET) metabolic volume. Toxicity was evaluated four weeks after start of RLT. **Results:** Mean±standard error (SEM) tumor-to-kidney ratio was 19±5, 10±5, and 2±0 for high, intermediate, and low (each n=3) specific activity, respectively. Four of six (67%) mice treated with intermediate or high specific activity and none of six (0%) mice treated with low specific activity or formulation demonstrated significant DNA double-strand breaks (≥5% gamma H2A.X positive cells). High when compared to intermediate or low specific activity resulted in lower mean±SEM tumor load by histopathology (vital tissue 4±2 versus 8±3 mm$^2$, n=3 versus 6), day 4 $^{18}$F-FDG PET (metabolic volume 87±23 mm$^3$ versus 118±14 mm$^3$, n=6 versus 12), and day 7 CT (volume 323±122 versus 590±46 mm$^3$, n=3 versus 6, *p=0.039). 120 MBq $^{177}$Lu-PSMA-617 with high specific activity induced superior tumor growth inhibition (*p=0.021, n=5 per group) without subacute hematologic toxicity (n=3 per group). **Conclusion:** 120 MBq $^{177}$Lu-PSMA-617 and high specific activity resulted in highest efficacy in a syngeneic model of murine prostate cancer. The
model will be useful for studying the effects of PSMA-directed RLT combined with potentially synergistic pharmacologic approaches.
INTRODUCTION

PSMA targeted radioligands have been introduced for treating patients with metastatic castrate resistant prostate cancer (mCRPC) in several countries (1-3). The safety and effectiveness of $^{177}$Lu-PSMA-617 RLT is described in a recently published German multi-center study (2). Rahbar et al. reported a 50% decrease in serum PSA levels in about half and hematologic adverse events in only 12% of 145 patients after up to four cycles of $^{177}$Lu-PSMA-617 (2). The high demand for this therapy led to the publication of first expert recommendations for clinical practice (4).

The clinical translation of PSMA-targeted RLT occurred before compound properties were published (5,6). Since then $^{177}$Lu-PSMA-617 RLT has been successfully performed in hundreds of prostate cancer patients worldwide. Given such rapid clinical translation few attempts have been made to optimize radiation dose and specific activity for increased efficacy. Moreover, as PSMA-targeted RLT is not curative synergistic pharmacologic approaches need to be identified.

Thus, there is a need to establish murine models of prostate cancer to permit optimization of RLT alone or in combination with drug treatments. Models should be based on immunocompetent mice to account for any effects of RLT on anti-tumor immune response. Here we report on an immunocompetent model of prostate cancer and investigate the impact of specific and total $^{177}$Lu-PSMA-617 activity on organ distribution and RLT efficacy. The findings will help optimize $^{177}$Lu-PSMA-617 RLT both for murine models, and potentially for use in clinical routine or clinical trials to ultimately achieve better outcomes for mCRPC patients. Finally, the model will provide a platform to develop rational RLT protocols in combination with other therapeutic approaches.

MATERIALS AND METHODS

Cell Culture
RM1 carcinoma was previously induced by transfection of ras and myc oncogenes in reconstituted cells of C57BL/6 prostate origin (7). RM1 parental cells and RM1 cells stably transduced with human PSMA and SFG-Egfp/Luc (RM1-PGLS) were provided by Dr. Michel Sadelain (Memorial Sloan-Kettering Cancer Center, New York, USA). A pMIY II-hPSMA plasmid was generated by the insertion of the human PSMA coding sequence into the multiple cloning site of the pMIY II (pMSCV-IRES-YFP II, Addgene 52108) plasmid. Amphotropic retroviruses were generated as previously described (8). To generate RM1-YFP and RM1-hPSMA lines, RM1 parental cells underwent transduction with the respective amphotropic retroviruses and were then sorted by flow cytometry to isolate pure populations of transduced cells. C4-2 cells were provided by Dr. George Thalmann (Department of Urology, Inselspital Bern, Switzerland). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine (GIBCO) and 5% fetal bovine serum (Omega Scientific) and were grown at 37°C, 20% O₂, and 5% CO₂.

**Flow Cytometry and PSMA Expression Level**

Absolute numbers of PSMA proteins on the cell surface were quantified by flow cytometry based on mean fluorescence intensity (MFI) values using the Quantum Simply Cellular (Bangs Laboratory) anti-human IgG quantification beads according to the manufacturer's instructions. PSMA was detected using APC-labeled anti-human PSMA (clone REA408, Miltenyi Biotec). Briefly, 0.5x10⁶ cells were incubated with the anti-PSMA antibody at a 1:5 dilution for 30 min at 4°C. Data were acquired on a five laser LSR II cytometer (BD). Data were analyzed using FlowJo (Three Star) software.

**Mice**

Male C57BL/6 or SCID mice were bred and housed under pathogen-free conditions. Studies were approved by the UCLA Animal Research Committee. The mice were 5 to 12 weeks
of age at the onset of experiments. Four days before commencement of RLT 0.1×10⁶ tumor cells (RM1-hPSMA or RM1-PGLS) were injected subcutaneously into the shoulder region of C57BL/6 mice. C4-2 tumors were induced after s.c. injection of 5×10⁶ cells in SCID mice.

**Radiosynthesis**

PSMA-617 precursor was obtained from ABX GmbH (Radeberg, Germany) and no-carrier-added ¹⁷⁷LuCl₃ was obtained from ITG GmbH (Garching, Germany). The precursor was stored in 1 mg/mL aliquots in 0.1% aqueous trifluoroacetic acid until use. For radiolabeling, 0.1M sodium acetate buffer pH 4.8 containing 10 mg/mL dihydroxybenzoic acid was added to 1, 2, or 3 µg precursor and mixed with 60 MBq ¹⁷⁷LuCl₃ to obtain high (62 MBq/nmol), intermediate (31 MBq/nmol), or low (15 MBq/nmol) specific activity. The mixture was heated to 100 °C for 30 min, diluted with phosphate-buffered saline and sterilized by filtration. 30 and 120 MBq ¹⁷⁷Lu-PSMA-617 were labelled at high (62 MBq/nmol) specific activity following the same protocol. Radiochemical purity was always >99% without further purification.

**Binding and Internalization Assay**

10⁵ cells (triplicates in 24-well plates) were seeded and allowed to attach to the plate overnight. Cells were incubated with 4 nM ⁶⁸Ga-PSMA-11 in the absence or presence of 10 µM 2-(Phosphonomethyl)pentane-1,5-dioic acid (PMPA) for 60 min at 37°C. Supernatant was collected and cells were washed with glycine HCl pH 2.8 to remove membrane-bound ligand. For collection of the internalized fraction, cells were lysed with 0.3N NaOH. Samples were measured in a γ counter (Cobra II Auto-Gamma, Packard Instrument Co, Meriden, USA). For analysis of retention cells were incubated with 4 nM ⁶⁸Ga-PSMA-11 for 60 min at 37°C and supernatant was collected. Cell were incubated in fresh medium for another 60 min in the absence or presence of 10 µM PMPA.
Decay-corrected data were expressed as percentage of bound, internalized or retained radioligand, respectively, normalized to the total cell number.

**Radioligand Therapy**

Mice bearing subcutaneous RM1-hPSMA tumors were used to study specific activity in RLT (experiment 1; n=6 per group). RLT was started by administering a tail vein injection of formulation (no treatment, NT) or 60 MBq $^{177}$Lu-PSMA-617 at high (62 MBq/nmol), intermediate (31 MBq/nmol), or low (15 MBq/nmol) specific activity. Animals were sacrificed at day 4 after start of RLT for $^{177}$Lu-PSMA-617 biodistribution and tissue analysis (n=3 per group, Fig. 2A) or were kept alive for tumor growth monitoring and survival (n=3 per group, Fig. 4A).

Mice bearing subcutaneous RM1-PGLS tumors were used to study total activity in RLT (experiment 2, n=5 per group). Animals were administered formulation (NT group) or 120, 60, or 30 MBq at high (62 MBq/nmol) specific activity and were kept alive for tumor growth monitoring and survival (Fig. 5A).

In experiment 3, RM1-hPSMA tumor bearing mice were sacrificed at week 4 after administration of formulation (NT group) or 120, 60, or 30 MBq $^{177}$Lu-PSMA-617 at high (62 MBq/nmol) specific activity to assess subacute toxicity (n=3 per group).

**PET/CT Image Acquisition**

Baseline $^{68}$Ga-PSMA-11 PET/CT was performed two days while baseline $^{18}$F-FDG PET/CT was done one day prior to start of RLT to confirm PSMA expression and formation of viable tumor, respectively. Follow-up $^{18}$F-FDG PET/CT was acquired on day 4. Tumor growth was monitored by serial CT acquisitions, three times per week until day 14; two times per week thereafter.
Acquisition of static PET images commenced 60 min after application of 0.7 to 1.5 MBq of tracer using a preclinical PET/CT scanner (Genisys 8 PET/CT, Sofie Biosciences; successor of Genisys 4 (9)). Maximum-likelihood expectation maximization with 60 iterations was used for PET image reconstruction. All images were corrected for photon attenuation. The CT acquisition parameters were 40 kVp, 190 mA, and 720 projections with an exposure time of 55 ms at each projection.

**PET/CT Image Analysis**

PET/CT images were analyzed using OsiriX Imaging Software (Version 3.9.3; Pixmeo SARL, Bernex, Switzerland). The single reader was blinded for specific and total $^{177}$Lu-PSMA-617 activity.

At baseline, PSMA expression and glucose metabolic activity were confirmed visually by focal uptake above background at the tumor cell injection site. To assess metabolic response, the $^{18}$F-FDG PET volume was measured at baseline and follow-up using the Osirix region grow function to include all tumor voxels exceeding mean liver SUV plus two standard deviations, a definition proposed in the PET Response Criteria in Solid Tumors (10).

For CT size measurements entire tumors were delineated visually on more than seven axial slices for each timepoint and volume was calculated using the Osirix compute volume function.

**Termination Criteria**

Mice were sacrificed if tumor volume exceeded 1000 mm$^3$ by caliper measurement or with the occurrence of skin ulcerations or other symptoms of deteriorating mouse condition. The UCLA Animal Research Committee protocol guidelines were applied. Caliper measurements were obtained by one blinded investigator three times per week throughout the experiment and daily when tumors approached critical size.
Immunohistochemistry

Tumor sections were stained for gamma H2A.X (phospho S139), a marker of DNA double-strand breaks. Tissues were fixed overnight in 10% buffered formalin and transferred to 70% ethanol for storage. Paraffin embedded samples were sectioned in the center of the tumor to obtain 4 μm slices. Paraffin was removed with xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Heat-induced antigen retrieval was carried out for all sections in 0.01M citrate buffer, pH 6.0 using a Biocare decloaker (Biocare Medical, Concord, USA) at 95°C for 25 min. Primary rabbit polyclonal anti-gamma H2A.X (phospho S139) IgG (ab2893; Abcam, Cambridge, MA, USA) was added at 1:500 dilution in BSA and incubated overnight at 4°C. Secondary staining was performed using the Dakocytomation Envision System labeled polymer HRP anti-rabbit (K4003; DakoCytomation, Carpinteria, USA) and visualized with the diaminobenzidine reaction (#BDB2004L, Biocare Medical) according to the manufacturer’s instructions. The sections were counterstained with hematoxylin. All slides were mounted with Cytoseal (Fisher Scientific, Pittsburgh, USA). Entire slides were scanned digitally at 40x using ScanScope AT (Leica Biosystems, Vista, USA).

The area around the edges was disregarded to minimize influence of the edge effect. For visual analysis, a blinded reader with prior immunohistochemistry experience delineated vital tumor tissue and categorized samples by percentage of gamma H2A.X positive cells into negative, (+) 5-10%, (++) 10-20%, or (+++) >20%. For software analysis, high resolution images of the entire section were diverted into brown nuclear staining for gamma H2A.X positive cells and blue nuclear staining for gamma H2A.X negative cells. ImageJ (Version 1.51g, National Institutes of Health, Bethesda, USA) was used to count positive/negative cells >15 μm in diameter.
Biodistribution and Toxicity

Biodistribution and toxicity were assessed in two separate experiments with n=3 mice each. On day 4 after the start of RLT, the distributed $^{177}$Lu-PSMA-617 activity was measured ex vivo in selected organs, tumor and blood using a γ counter. Uptake was expressed as percentage injected activity per gram tissue (%IA/g).

Toxicity was assessed 4 weeks after the start of RLT. Immediately after sacrifice ≥100 μl blood was drawn via cardiac puncture and organs were inspected for any signs of deposits or damage. Blood analyses, including hemoglobin concentration, red and white blood cells and platelets count, were performed using GENESIS Hematology Analyzer (Oxford Science, Oxford, USA).

Statistics

Data are presented as single values or mean±SEM. Normal distribution of baseline variables was rejected by Shapiro-Wilk test and Mann-Whitney test was used for unpaired comparison between two subgroups. Kaplan-Meier survival analysis was performed based on the interval between the start of RLT and the date of sacrifice or the end of the experiment. Correlation was assessed using Spearman’s rho. Significance was set at *p<0.05. The SPSS software package (version 15.0, SPSS, Inc.) was used for all statistical analysis.

RESULTS

Mouse (RM1) and Human (C4-2) Prostate Cancer Cells Demonstrate similar PSMA Expression and Internalization in Vitro

PSMA expression was determined in vitro by flow cytometry for RM1-hPSMA, RM1-PGLS, and C4-2, respectively (Fig. 1A). RM1-PGLS cells demonstrate heterogeneous expression with a smaller PSMA-low (MFI 44) and a larger PSMA-high sub-population (MFI 466). PSMA expression was quantified based on MFI using anti-human IgG quantification beads. Median absolute PSMA numbers range from 19x10^3 (RM1-hPSMA) to 238x10^3 (C4-2,
Fig. 1B) per cell. RM1-PGLS (56x10^3) and its PSMA-high sub-population (99x10^3) demonstrated mean absolute PSMA numbers within one order of magnitude of C4-2. In vitro, 68Ga-PSMA-11 cell surface binding and overall retention was highest for C4-2, internalization was highest for RM1-PGLS with less than four-fold difference between the cell lines (Table 1). High uptake of 68Ga-PSMA-11 was confirmed for all cell lines in vivo by PET/CT. Representative maximum intensity projection images of mice bearing equally sized s.c. RM1 allograft or C4-2 xenograft are shown in Fig. 1C.

High Specific Activity is Critical for High 177Lu-PSMA-617 Tumor Uptake in Vivo (experiment 1)

PSMA expression was confirmed by baseline 68Ga-PSMA-11 PET/CT for all mice (Supplemental Fig. 1A). RM1-hPSMA tumors were chosen for this experiment due to their homogeneous PSMA expression (Fig. 1A). To investigate the role of specific activity, RM1-hPSMA tumor bearing mice (n=6 per group) received RLT as outlined in Fig. 2A. Day 4 biodistribution and 177Lu-PSMA-617 tumor-to-organ uptake ratios are shown in Fig. 2B and C (n=3 per group). The kidneys had the highest organ uptake. Tumor uptake was higher (mean±SEM, 0.57±0.15 versus 0.23±0.10 %IA/g, p=0.071) and kidney uptake was lower (0.03±0.00 versus 0.04±0.00 %IA/g, *p=0.020) for high versus pooled low or intermediate specific activity (Fig. 2B). High specific activity resulted in highest tumor-to-kidney (mean±SEM, 19±5, *p=0.039), -liver (42±13), -lung (230±97) and -salivary gland (214±50) ratios (Fig. 2C).

Higher 177Lu-PSMA-617 Uptake Translates into more severe DNA Damage and Tumor Growth Inhibition (experiment 1)
Tumor tissue taken at day 4 was stained for gamma H2A.X by immunohistochemistry (n=3 per group). Representative images are shown in Fig. 3A. RLT using intermediate or high specific activity induced significant DNA damage (≥5% positive cells) in 4/6 (67%) tumors both by visual and software analysis (Fig. 3A and B). One out of 3 (33%) tumors demonstrated significant DNA damage by visual analysis in mice that received low specific activity. In the no treatment (NT) group all samples were negative (<5% positive cells).

On day 4 the mean±SEM 18F-FDG PET metabolic volume was 87±23 (high), 91±14 (intermediate), 145±19 (low), and 134±13 mm$^3$ (NT; n=6 per group, Fig. 4D). Area of vital tumor tissue was 4±2 (high), 4±1 (intermediate), 12±6 (low), and 7±1 mm$^2$ (NT, n=3 per group, Supplemental Fig. 3A). Metabolic volume correlated significantly with the area of vital tumor tissue (*p<0.01, Spearman’s rho=0.73; Supplemental Fig. 3B), despite one outlier in the low group.

High specific activity resulted in significantly lower mean±SEM CT tumor volume at day 7 (323±122 mm$^3$) when compared to intermediate or low specific activity (590±46 mm$^3$, *p=0.039, n=3 per group; Fig. 4B). Kaplan-Meier curves demonstrate survival of >50 days for each one mouse in the high and intermediate groups (n=3 per group; Fig. 4C).

120 MBq $^{177}$Lu-PSMA-617 Induced Most Efficient Tumor Growth Inhibition (experiment 2)

High specific activity was associated with high efficacy. Based on a more consistent tumor growth (Supplemental Fig. 2), RM1-PGLS-bearing mice were chosen for experiment 2. To investigate the impact of total activity, RM1-PGLS bearing mice received formulation (NT group) or RLT at three different activity levels (30, 60 or 120 MBq; n=5 per group), each at high specific activity (Fig. 5A). Mice that received 120 and 60 MBq $^{177}$Lu-PSMA-617 survived longer than animals that received 30 MBq or no treatment (Fig. 5C). 120 MBq induced most efficient tumor growth inhibition as determined by serial CT (*p=0.021). The mean±SEM tumor volume
on day 12 was 113±50 (high), 393±214 (intermediate), 412±127 (low) and 774±291 mm³ (NT, Fig. 5B).

120 MBq $^{177}$Lu-PSMA-617 Was Safe (experiment 3)

To evaluate toxicity RM1-hPSMA bearing mice received formulation (NT group) or RLT with 30, 60 or 120 MBq $^{177}$Lu-PSMA-617 (n=3 each) at high specific activity. Four weeks after the start of RLT none of the mice demonstrated pathologic findings on gross examination with reference to organ size, shape, color and texture. The mean hemoglobin concentration, red and white blood cell counts and platelet numbers were within normal range for each group (Fig. 6).

DISCUSSION

Here we established a syngeneic mouse model of prostate cancer aimed at optimizing specific and total activity levels for $^{177}$Lu-PSMA-617 RLT. We selected three specific activity levels based on clinical $^{177}$Lu-PSMA-617 RLT protocols (2) and three total activity levels established in mouse models of somatostatin receptor-targeted RLT (11). High specific activity was associated with the highest tumor uptake and more prominent DNA damage. Mice that received 120 MBq $^{177}$Lu-PSMA-617 RLT with high specific activity displayed no adverse health effects throughout the study and had hemoglobin and blood count levels within a safe range at four weeks post injection. 120 MBq $^{177}$Lu-PSMA-617 RLT also resulted in most effective tumor growth inhibition. RM1-based cells used in our studies demonstrate similar PSMA expression, internalization and retention when compared to human prostate cancer cells in vitro. This mouse model can now be used to test rationally-designed combination therapies.

Our findings have implications for clinical and preclinical $^{177}$Lu-PSMA-617 RLT. First, the critical role of high specific activity for $^{177}$Lu-PSMA-617 was established for tumor-to-organ uptake ratios of the radioligand. More important, high specific activity was associated with high
levels of DNA double strand break, determined by gamma H2A.X staining, a prerequisite for effective tumor growth inhibition. The range of specific activity tested in our mouse model was representative of clinical protocols. Twelve centers reported specific activity for a total of 123 \(^{177}\text{Lu-PSMA-617}\) RLT applications in mCRPC patients (2). Specific activity within the German dataset ranged from 23 to 78 MBq/nmol (mean 48 MBq/nmol; personal correspondence with Bernd Krause from the German Society of Nuclear Medicine) matching our experiment parameters (range 15 to 62 MBq/nmol). Improved tumor uptake by high specific activity is in line with previous findings for a similar compound (PSMA-I&T) labeled with \(^{111}\text{In}\) (12).

Our findings suggest that the use of intermediate or low specific activity \(^{177}\text{Lu-PSMA-617}\), as reported previously (13) may be suboptimal. A comparison of different specific activity levels is important, given prior reports of improved tumor-to-organ uptake for other radiolabeled compounds, including \(^{68}\text{Ga-OPS202}/^{177}\text{Lu-OPS201}\) (14) and \(^{90}\text{Y-Ibritumomab}\) (15).

Furthermore, mouse data on safety and efficacy of different activity levels are now available for future evaluation. Of the three total activity levels assessed in this study (30, 60, 120 MBq), 120 MBq \(^{177}\text{Lu-PSMA-617}\) resulted in the most effective tumor growth inhibition. 120 MBq \(^{177}\text{Lu-PSMA-617}\) did not result in subacute or life-threatening bone marrow toxicity. Radiation nephropathy, a late event previously reported after application of \(^{90}\text{Y-labeled radioligands}\) (16), was not analyzed in these experiments given the short median survival of 25 to 32 days after start of therapy and the lack of accurate early biomarkers.

\(^{177}\text{Lu-PSMA-617}\) RLT is safe and effective in patients with mCRPC. Biochemical response was documented in about half of patients undergoing \(^{177}\text{Lu-PSMA-617}\) RLT (2). RLT is well tolerated with low rates (<15%) of grade 3 to 4 hematologic adverse events (2) and no relevant kidney toxicity has been documented thus far (1,2,17-20). However, \(^{177}\text{Lu-PSMA-617}\) RLT alone is not curative. Therefore, rational combination of \(^{177}\text{Lu-PSMA-617}\) RLT with other
synergistic therapies, including immunotherapy, will help to improve outcomes for mCRPC patients. Our model can be used for this purpose.

Limitations

We observed shrinkage of RM1-hPSMA tumors starting from day 14 after inoculation in about one third of untreated C57Bl6 mice (Supplemental Fig. 2). Adaptive immune response was indicated by tumor re-challenge experiment using PSMA-positive (RM1-hPSMA, RM1-PGLS) and PSMA-negative (RM1-YFP) cell lines (Supplemental Fig. 4). Adaptive immune response directed at human PSMA poses a limitation of the RM1-hPSMA cells with high expression level for PSMA. Tumor rejection was not seen for RM1-PGLS cells with heterogeneous PSMA expression (Fig. 1A).

Conclusion

In summary, we established $^{177}$Lu-PSMA-617 RLT in a syngeneic model of murine prostate cancer. *In vitro*, RM1 murine cell lines were similar to human prostate cancer cells in terms of PSMA expression, internalization and retention. *In vivo*, high specific activity was critical for optimal tumor growth inhibition. Up to 120 MBq $^{177}$Lu-PSMA-617 was applied without relevant subacute toxicity. Long term toxicity was not evaluated. This model will serve future evaluation of combination therapy with potentially synergistic pharmacologic approaches.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

C.R. and J.C. are co-founders of Sofie Biosciences.

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REFERENCES


FIGURE LEGENDS

Figure 1: PSMA expression of mouse RM1 versus human C4-2 prostate cancer cell lines.
(A) PSMA expression was determined in vitro by flow cytometry for RM1-hPSMA (green), RM1-PGLS (blue) and C4-2 (red). RM1-PGLS cells exhibit heterogeneous PSMA expression, with PSMA-low (light blue) and PSMA-high (dark blue) sub-populations. (B) Mean absolute numbers of PSMA on the cell surface were quantified based on mean fluorescence intensity values using anti-human IgG quantification beads. (C) Representative $^{68}$Ga-PSMA-11 PET/CT maximum intensity projection of mice bearing equally sized s.c. mouse allograft or human xenograft. Mean %IA/g was 22.7, 17.4, and 15.5 for RM1-hPSMA, RM1-PGLS, and C4-2, respectively (n=2 each).
Figure 2: Biodistribution of $^{177}$Lu-PSMA-617 at different levels of specific activity (experiment 1). (A) Mice received 60 MBq $^{177}$Lu-PSMA-617 at high (62 MBq/nmol), intermediate (31 MBq/nmol), or low (15 MBq/nmol) specific activity. Mice were sacrificed on day 4 to determine $^{177}$Lu-PSMA-617 biodistribution (B) and tumor-to-organ uptake ratios (C). Data are mean %IA/g tissue+SEM (n=3 per group). The difference between high versus intermediate or low specific activity groups was assessed by Mann Whitney test. *, $P<0.05$; GI=gastrointestinal tract.
Figure 3: DNA damage induced by $^{177}$Lu-PSMA-617 radioligand therapy using high, intermediate, or low specific activity (experiment 1). (A) Representative areas of tumor tissue stained for gamma H2A.X are shown at 10-fold magnification (conditions in separate rows). Proportion of gamma H2A.X positive cells was evaluated by a blinded reader visually (A) and by ImageJ software (B). + indicates 5-10%, ++ 10-20%, +++ >20% gamma H2A.X positive cells. Dots represent single values, bars are mean values (n=3 per group). NT=no treatment.
Figure 4: Response after $^{177}$Lu-PSMA-617 radioligand therapy using high, intermediate, or low specific activity (experiment 1). (A) Mice received formulation (NT group) or 60 MBq $^{177}$Lu-PSMA-617 at high, intermediate, or low specific activity. CT tumor volume is shown until the first animal per group was required to be sacrificed. Higher tumor uptake translates into more effective tumor growth inhibition (B, $p=0.039$), prolonged survival (C), and metabolic response (D). One mouse in the high group had a complete response and remained tumor free for >60 days (C). The difference between high versus intermediate to low specific activity groups was assessed by Mann Whitney test. Data are presented as mean+SEM of n=3 (B, C) or n=6 (D) per group. *, $P<0.05$; NT=no treatment.
Figure 5: Tumor growth inhibition (A) and survival (B) after RLT using 30, 60, or 120 MBq $^{177}$Lu-PSMA-617 (experiment 2). (A) Mice received formulation (NT group) or 120, 60, or 30 MBq $^{177}$Lu-PSMA-617 at high (62 MBq/nmol) specific activity. 120 MBq $^{177}$Lu-PSMA-617 induced most effective tumor growth inhibition (B, *p=0.021) with improved survival (C). ‡, one mouse each died during PET/CT. The difference between the 120 MBq versus other groups was assessed by Mann Whitney test on day 12. Data are mean+SEM of n=5 per group. *, P<0.05; NT=no treatment.
Figure 6: Hemoglobin and blood count level four weeks after radioligand therapy using 30, 60, or 120 MBq $^{177}$Lu-PSMA-617 (experiment 3). $^{177}$Lu-PSMA-617 RLT was not associated with relevant hematotoxicity. Data are presented as mean±SEM of n=3 per group. LLN=lower limit of the normal range, NT=no treatment.
TABLES

Table 1: Mouse and human prostate cancer cell surface binding, internalization and retention of $^{68}$Ga-PSMA-11.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Surface binding (%IA per $10^5$ cells)</th>
<th>Internalization (%IA per $10^5$ cells)</th>
<th>Retention (%IA per $10^5$ cells)</th>
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<td>Mouse</td>
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<td>RM1-hPSMA</td>
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<td>6.7±0.2</td>
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</table>

Data are mean %IA±SEM of n=3 per group; representative of two independent experiments.
Supplemental Figure 1: Baseline and follow-up PET/CT of RM1-hPSMA tumor-bearing mice. Representative maximum intensity projection images of (A) baseline 68Ga-PSMA-11.
PET/CT, (B) baseline $^{18}$F-FDG PET/CT, and (C) day four $^{18}$F-FDG PET/CT are shown (one mouse per group).

Supplemental Figure 2: Tumor growth of the RM1 cell lines in vivo. $0.1 \times 10^6$ tumor cells were injected subcutaneously into the shoulder region of C57BL/6 mice. Tumor growth curves are shown for untreated RM1-hPSMA (green) or RM1-PGLS (blue). Data are mean±SEM of n=5 per group.
Supplemental Figure 3: Vital tumor tissue after $^{177}$Lu-PSMA-617 radioligand therapy using high, intermediate, or low specific activity (experiment 1). (A) Sections collected from the center of the tumor, stained for gamma H2A.X and counterstained with hematoxylin are shown at 2-fold magnification (n=3 per group). Vital tumor tissue was delineated visually by a blinded reader (green). Scale is given for all samples in right lower corner; larger scale was chosen for one outlier (sample 1 in the low specific activity group). (B) $^{18}$F-FDG PET volume correlates with area of vital tumor tissue after sacrifice on the same day ($^*P<0.01$, rho=0.73). NT=no treatment.
Supplemental Figure 4: Tumor re-challenge in mice with prior rejection of RM1-hPSMA. In a separate experiment, subcutaneous re-challenge with equal and 10-fold amounts of PSMA-expressing tumor cells (RM1-hPSMA or RM1-PGLS) did not result in engraftment. Re-challenge with PSMA-negative RM1-YFP led to sustainable tumor growth. Findings indicate adaptive immune response directed at human PSMA. Individual mice are shown.