

PSMA-reactive NB7 single domain antibody fragment: A potential scaffold for developing prostate cancer theranostics

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ARTICLE INFO

Keywords:

Prostate specific membrane antigen
Prostate cancer
Single domain antibody fragment
Nanobody
²¹¹Astatine

ABSTRACT

Introduction: Single domain antibody fragments (sdAbs) are an appealing scaffold for radiopharmaceutical development due to their small size (~15 kDa), high solubility, high stability, and excellent tumor penetration. Previously, we developed NB7 sdAb, which has very high affinity for an epitope on PSMA that is different from those targeted by small molecule PSMA inhibitors. Herein, we evaluated NB7 after radioiodination using [¹²⁵I]SGMIB (1,3,4-isomer) and iso-[¹²⁵I]SGMIB (1,3,5-isomer), as well as their ²¹¹At-labeled analogues.

Methods: [¹²⁵I]SGMIB, iso-[¹²⁵I]SGMIB, [²¹¹At]SAGMB, and iso-[²¹¹At]SAGMB conjugates of NB7 sdAb were synthesized and their binding affinity, cell uptake and internalization were assessed in PSMA⁺ PC3 PIP and PSMA⁻ PC3 flu cells. Biodistribution studies were performed in mice bearing PSMA⁺ PC3 PIP xenografts. First, a single-label experiment evaluated the tissue distribution of a NB7 bearing a His₆-tag (NB7H6) and labeled with iso-[¹²⁵I]SGMIB. Three paired-label experiments then were performed to compare: a) NB7 labeled using [¹²⁵I]SGMIB and iso-[¹²⁵I]SGMIB, b) ¹³¹I- vs ²¹¹At-labeled NB7 conjugates and c) [¹²⁵I]SGMIB-NB7H6 to the small molecule PSMA inhibitor [¹³¹I]YF2.

Results: All NB7 radioconjugates bound specifically to PSMA with dissociation constants, K_d, in the low nM range (1.4–6.4 nM). An initial biodistribution study demonstrated good tumor uptake for iso-[¹²⁵I]SGMIB-NB7H6 (7.2 ± 1.5 % ID/g at 1 h) and no deleterious effect of the His₆-tag on renal activity levels, which declined to 3.1 ± 1.1 % ID/g by 4 h. Paired-label biodistribution found no distinction between the two SGMIB isomer NB7 conjugates with the [¹³¹I]SGMIB-NB7-to-iso-[¹²⁵I]SGMIB-NB7 tumor uptake ratios not significantly different from unity: 1.06 ± 0.08 at 1 h, 1.04 ± 0.12 at 4 h, and 1.07 ± 0.09 at 24 h. Both isomer conjugates cleared rapidly from normal tissues and exhibited very low uptake in thyroid, lacrimal and salivary glands. Paired-label biodistribution of [¹³¹I]SGMIB-NB7H6 and [²¹¹At]SAGMB-NB7H6 demonstrated similar tumor uptake and kidney clearance for the two radioconjugates. However, levels of ²¹¹At in thyroid, stomach, salivary and lacrimal glands were significantly higher (*P* < 0.05) than those for ¹³¹I suggesting greater dehalogenation for [²¹¹At]SAGMB-NB7H6. Finally, co-administration of [¹²⁵I]SGMIB-NB7H6 and [¹³¹I]YF2 demonstrated good tumor uptake for both with considerably more rapid renal clearance for the NB7 radioconjugate.

Conclusion: NB7 radioconjugates exhibited good accumulation in PSMA-positive xenografts with rapid clearance from kidney and other normal tissues. We conclude that NB7 is a potentially useful scaffold for developing PSMA-targeted theranostics with different characteristics than current small molecule and antibody-based approaches.

1. Introduction

Prostate-specific membrane antigen (PSMA) is an attractive target for the imaging and therapy of prostate cancers with its levels linked to disease progression and transition to metastatic castration-resistant prostate cancer (mCRPC) [1–3]. For decades, researchers have

explored various strategies to target PSMA ranging from small molecule inhibitors to antibodies [4–6]. Regarding small molecule PSMA inhibitors, [¹⁷⁷Lu]PSMA-617 (Pluvicto) was approved by FDA in 2022 while the proteins targeting PSMA developed to date have been intact monoclonal antibodies (mAbs) [4,5]. These mAbs have prolonged serum half-lives, leading to increased risk of undesired side effects. The large

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<https://doi.org/10.1016/j.nucmedbio.2024.108913>

Received 31 January 2024; Received in revised form 29 March 2024; Accepted 22 April 2024

Available online 25 April 2024

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size of mAbs also hinders tumor penetration reducing drug delivery efficiency [7,8]. On the contrary, rapid blood clearance of small molecule inhibitors usually necessitates multi-dose regimens that can potentially lead to increased toxicity in patients [9].

With the objective of reducing side effects while still delivering effective treatment, there is a growing interest in single domain antibody fragments (sdAbs), also known as nanobodies or VHH. *Camelidae*-derived sdAbs are fragments of heavy chain-only antibodies with notable characteristics including high affinity, moderate size (15 kDa), and better tumor penetration [10] making them an excellent platform for cancer imaging and therapy. There are a few reports describing radiolabeled PSMA-targeted sdAbs providing proof of concept [11] but not with properties likely to merit clinical translation. Believing that a PSMA-targeted sdAb with more favorable characteristics is needed, we recently developed four high affinity PSMA-targeting sdAbs that bind to an epitope on PSMA different from that recognized by small molecule inhibitors [12]. Of these, NB7 exhibited the best combination of high PSMA affinity and tumor cell internalization.

Regarding the choice of radionuclide for PSMA targeted radiotherapy, our approach has been to utilize ^{211}At , which emits a single alpha particle per decay. This allows simplification of dosimetry calculations and minimizing the risks of off-target side effects from daughter radionuclides that confound alternate alpha-emitters, notably ^{225}Ac . Moreover, astatine belongs to the same chemical group as iodine, making it possible to adapt radioiodination chemistry and use radioiodinated analogues to facilitate the development of ^{211}At -labeled therapeutics. Previously, we reported promising results for several ^{211}At -labeled small molecule PSMA inhibitors including tumor growth suppression [13–16]. Likewise, three ^{211}At -labeled analogues - [^{211}At]PSMA1, [^{211}At]PSMA5 and [^{211}At]PSMA6 based on the structure of PSMA-1007 have recently been developed [17]. Although results have been encouraging in some cases, none of these compounds have advanced to clinical trial.

In this study, we examined two versions of NB7 sdAb - without or with His₆-tag (aka NB7H6) - conjugated to four guanidino-substituted prosthetic agents: *N*-succinimidyl 4-guanidinomethyl 3-[*I]iodobenzoate ([$^{125,131}\text{I}$]SGMIB; 1,3,4 isomer), *N*-succinimidyl 3-guanidinomethyl 5-[*I]iodobenzoate (*iso*-[$^{125,131}\text{I}$]SGMIB; 1,3,5 isomer), *N*-succinimidyl 3-[* ^{211}At]astato-4-guanidinomethyl benzoate ([^{211}At]SAGMB) and *N*-succinimidyl 3-[* ^{211}At]astato-5-guanidinomethyl benzoate (*iso*-[^{211}At]SAGMB) [18]. The in vitro and in vivo behavior of these NB7 sdAb radioconjugates was assessed in PSMA⁺ PC3 PIP and PSMA⁻ PC3 flu cells and PSMA⁺ PC3 PIP xenograft models.

2. Materials and methods

2.1. General

Chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) or ThermoFisher (Waltham, MA) unless stated otherwise. Sodium [^{125}I]iodide in 0.1 N NaOH (629 GBq/mg, 12.95 GBq/mL) was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). Sodium [^{131}I]iodide (936 GBq/mg, 236.097 GBq/mL) in 0.04 M sodium thiosulfate, 0.2 M NaOH and 0.2 M sodium carbonate was obtained from International Isotopes Inc. (Idaho Falls, ID). Astatine-211 was produced on the Duke University CS-30 Cyclotron via the $^{209}\text{Bi}(\alpha, 2n)^{211}\text{At}$ reaction by bombarding natural bismuth metal targets with 28 MeV α -particles as reported [19]. The Boc-protected tin precursors 1,3,4-SGMTB and its 1,3,5-SGMTB were synthesized as previously reported [20,21]. The NB7 sdAb without His₆-tag was produced by BioIntron Biological Inc. (Shanghai, China). SDS-PAGE performed upon arrival indicated two bands – one corresponding to an sdAb monomer (~75 %) and the other ~25 % with a molecular weight consistent with an sdAb dimer – and was used without further purification. The NB7 sdAb with His₆-tag at the C-terminus (NB7H6) was produced and characterized in Ben-Gurion University of the Negev, Beer-Sheva, Israel [12]. Details of the

synthesis and radiolabeling of the small molecule PSMA inhibitor YF2 [16], a DOTAGA analogue of compound 3 reported in Mease et al. [14] will be published elsewhere.

High performance liquid chromatography (HPLC) was performed using an Agilent Technologies 1260 Affinity HPLC system equipped with two-solvent modules. Normal phase HPLC was employed for the purification of radiohalogenated products. For this, a 4.6×250 mm Zorbax Rx-SIL 5 μm column (Agilent Technologies, Santa Clara, CA) was eluted with a gradient consisting of hexanes and ethyl acetate, each containing 0.2 % acetic acid. Radiolabeled sdAbs were purified using PD-10 desalting columns obtained from GE Healthcare (Piscataway, NJ).

2.2. Cells and cell culture conditions

The PC3 PIP and PC3 flu cell lines were generously gifted by Dr. Pomper at Johns Hopkins University and were originally developed by Dr. W. Heston (Cleveland, OH). These cell lines, namely PSMA-expressing (PC3 PIP) and non-expressing (PC3 flu) prostate cancer cell lines, were screened for mycoplasma contamination using the MycoAlert Mycoplasma detection kit (Lonza). The passage numbers typically ranged from P20 to P40. PC3 PIP cells were cultured in RPMI 1640 medium containing 10 % fetal bovine serum, Pen-Strep (10 mL/L), plasmocin (5 mg/L) and puromycin (20 mg/L) (InvivoGen, San Diego CA). Adding puromycin to PC3 PIP cells serves the purpose of maintaining PSMA expression. PC3 flu cells were cultured in RPMI 1640 medium containing 10 % fetal bovine serum and Pen-Strep (5 mL/L). Cells were cultured at 37 °C in a humidified incubator under 5 % CO₂.

2.3. Radiolabeling of NB7 sdAbs

The prosthetic agents were synthesized by the radiohalogenation of their respective tin precursors as reported previously [18]. Briefly, a solution of NCS in MeOH (0.2 mg/mL) containing 0.6–1 % (v/v) acetic acid was added to a vial containing 50 μg tin precursor and 37–74 MBq (1–2 mCi) of ^{125}I or ^{131}I . For ^{211}At labeling, a solution of 74–148 (2–4 mCi) ^{211}At in NCS/MeOH was added to a half-dram vial containing 50 μg tin precursor, followed by the addition of acetic acid (1 % v/v). The mixture was stirred at room temperature for 30 min and injected onto a normal phase HPLC column that was eluted with a linear gradient of 30 to 70 % ethyl acetate/hexanes containing 0.2 % AcOH at a flow rate of 1.5 mL/min. HPLC fractions containing the radiohalogenated product were collected and solvents evaporated to dryness under a stream of argon gas.

The Boc groups from the protected intermediates were removed by treatment with trifluoroacetic acid (TFA; 100 μL) at room temperature for 20 min. TFA was evaporated using an argon stream followed by the addition and evaporation of 3×100 μL of ethyl acetate to ensure complete removal of TFA. The NB7 or NB7H6 sdAbs were conjugated with the radiolabeled prosthetic agents as previously reported [18,21]. Briefly, ~100 μg of sdAb in 100 μL 0.1 M borate buffer pH 8.5 was added to about 1–2 mCi of the dry prosthetic agents in a half-dram vial, and the mixture was kept at room temperature for 20 min. The radiolabeled sdAb was isolated by gel filtration over a PD10 column. The sdAb concentration was determined using a NanoDrop microvolume spectrophotometer (Thermo Fisher). The radiochemical purity of radiohalogenated sdAb was determined by SDS-PAGE/phosphor imaging.

2.4. Saturation binding assays

The binding affinity of NB7 radioconjugates and specificity for PSMA was determined by saturation binding assays using PSMA⁺ PC3 PIP and PSMA⁻ PC3 flu cell lines. Briefly, cells were plated in 24-well plates at a density of 1×10^4 cells/well/mL and incubated overnight at 37 °C. On the next day, cells were acclimatized at 4 °C for 30 min and then 0.1–100 nM of NB7 radioconjugates were added to PC3 PIP cells,

followed by incubation at 4 °C for 2 h. The medium containing unbound activity was then removed, the cells were washed twice with ice-cold RPMI 1640 media and solubilized by 1 % SDS in deionized H₂O. The supernatants were collected and counted on an automated gamma counter. Nonspecific binding was determined in parallel assays as above but using PC3 flu cells. The data were fitted using GraphPad Prism software to determine K_d values. Experiments were repeated three times.

2.5. In vitro cell uptake and internalization

Uptake and internalization of radiolabeled NB7 sdAbs were in a paired-label format. Briefly, PC3 PIP and PC3 flu cells (5 × 10⁵ cells/well/2 mL medium) were plated in 6-well plates the day prior to the study. For cellular binding studies, cells were incubated with 11.1–22.2 kBq (0.3–0.6 µCi) of NB7 radioconjugates at 37 °C for 1, 2, 4 and either 24 h (radioiodine) or 21 h (²¹¹At). The medium containing unbound radioactivity was removed, and the cells were washed twice with cold RPMI 1640 media. The cells were then treated with 2 × 0.05 M glycine buffer, pH 2.8, and the supernatants were collected as surface-bound radioactivity. The cells were solubilized with 1 mL 0.1 % SDS and counted to determine internalized fraction.

To determine retention of radioactivity after binding, cells were acclimatized at 4 °C for 30 min, after which 0.3–0.5 µg NB7 radioconjugate was added and then incubated at 4 °C for 1 h. The unbound fraction was removed, cells were supplemented with fresh medium, and then incubated at 37 °C for different times. At each time point, the supernatant (effluxed fraction) was removed and counted for radioactivity. Then the cells were treated with 2 × 0.05 M glycine buffer, pH 2.8, and the supernatants were collected as the membrane-bound fraction. Subsequently, cells were solubilized with 1 mL of 0.1 % SDS and counted to determine the internalized fraction.

Finally, we evaluated whether NB7 sdAb and the small molecule PSMA inhibitor YF2 competed for binding to PSMA. The uptake and internalization of [¹²⁵I]SGMIB-NB7 and [¹³¹I]YF2 both in paired-label and single-label formats was determined using protocols as above but doing the assay just for one time point - 2 h.

In all experiments, nonspecific uptake was assessed using PC3 flu cells and all studies were performed in triplicate.

2.6. Biodistribution studies

All experiments involving animals were carried out in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committee (IACUC) of Duke University and described in approved protocol number A204-21-10. For biodistribution studies, 4–6-week old male athymic nude mice (25 g; Jackson Labs) were inoculated subcutaneously in the flank with 3 × 10⁶ PC3 PIP cells. When the tumors reached 100–500 mm³, groups of five mice were injected with the radioconjugate(s) via the tail vein. At 1, 4, and at 24 h (radioiodine) or 21 h (²¹¹At) post injection, blood and urine samples were collected and necropsied. Tissues were weighed and counted for radioactivity along with injection standards to permit calculation percent injected dose (ID) per organ, ID/g and tumor-to-tissue ratios. All radioactivity measurements were decay corrected.

In a preliminary study, the biodistribution of *iso*-[¹²⁵I]SGMIB-NB7H6 (1 µCi; 1 µg) was evaluated at 1, 4 and 24 h in single-label format primarily to determine whether the presence of a His₆-tag resulted in excessive retention of activity in the kidney as has been reported for sdAbs labeled with radiometals [22]. In the first paired-label study, the tissue distribution of [¹³¹I]SGMIB-NB7 (5 µCi; 4 µg) and *iso*-[¹²⁵I]SGMIB-NB7 (2.5 µCi; 4 µg) were compared. In the second paired-label experiment, mice received both [¹³¹I]SGMIB-NB7H6 (122 kBq/3.3 µCi; 4 µg) and [²¹¹At]SAGMB-NB7H6 (122 kBq/3.3 µCi; 4 µg). The third paired-label experiment was performed to directly compare the biodistribution of the small molecule PSMA inhibitor YF2 and the PSMA-

targeted sdAb. Mice were injected intravenously with 148 kBq (5 µCi; 4 µg) of [¹²⁵I]SGMIB-NB7H6 and 148 kBq (5 µCi; 0.02 µg) of [¹³¹I]YF2. Biodistribution was performed as above at 1 and 4 h.

2.7. Statistical analyses

Results are presented as mean ± SD. For experiments done in paired-label format, a two-tail, paired Student's t-test was used to evaluate significance of differences. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Radiolabeling

The yield for prosthetic agent conjugation to NB7 was 20–31 % for *iso*-[¹²⁵I]SGMIB, 23–33 % for [¹²⁵I]SGMIB, and 15–50 % for [²¹¹At]SAGMB. The specific activity of the labeled sdAbs were similar for ¹²⁵I and ¹³¹I and were: [¹²⁵I]SGMIB-NB7 (70–90 MBq/mg), *iso*-[¹²⁵I]SGMIB-NB7 (70–110 MBq/mg), [¹²⁵I]SGMIB-NB7H6 (70–110 MBq/mg) and *iso*-[¹²⁵I]SGMIB-NB7H6 (60–100 MBq/mg). The specific activity of [²¹¹At]SAGMB-NB7H6 was about 60–100 MBq/mg.

SDS-PAGE/phosphor imaging was performed to evaluate the radiochemical purity of radiohalogenated sdAbs immediately after PD10 purification (Fig. S2A). A single radioactive band at approximately 16 kDa was observed for [¹²⁵I]SGMIB-NB7H6, *iso*-[¹²⁵I]SGMIB-NB7H6 and [²¹¹At]SAGMB NB7H6, consistent with the molecular weight of NB7 sdAb. Two radioactive bands were observed for [¹²⁵I]SGMIB-NB7 and *iso*-[¹²⁵I]SGMIB-NB7, corresponding to both the expected sdAb monomer (75 %) as well as the second species (25 %) of unknown origin that was likely a dimer.

3.2. Saturation binding assays

Saturation binding assays on PC3 PIP and PC3 flu cells (for nonspecific uptake) revealed K_d values for PSMA-specific binding of 6.4 ± 2.0 nM and 1.4 ± 0.4 nM for [¹³¹I]SGMIB-NB7 and [¹³¹I]SGMIB-NB7H6, respectively (Fig. S2). The K_d value was 4.0 ± 0.5 nM for [²¹¹At]SAGMB NB7H6 (Fig. S2B).

3.3. In vitro cell uptake and internalization

Paired-label assays were performed to evaluate the cellular uptake and internalization of NB7 and NB7H6. No significant differences in either parameter were observed between [¹²⁵I]SGMIB and *iso*-[¹²⁵I]SGMIB. For example, total cellular binding was about 24 % of input activity with ~7 % internalized into PC3 PIP cells for both [¹³¹I]SGMIB-NB7 and *iso*-[¹²⁵I]SGMIB-NB7 after 2 h incubation at 37 °C (Fig. S3). Similarly, no significant differences in cell uptake and internalization were observed between [¹³¹I]SGMIB-NB7H6 and *iso*-[¹²⁵I]SGMIB-NB7H6 (Fig. S4). It should be noted that cell uptake (about 32 % of input activity at 2 h) was higher than observed with the NB7 radioconjugates lacking a His₆-tag (Fig. S3A). In all cases, uptake by PSMA[−] PC3 flu cells at 2 h was <0.3 %, confirming the PSMA specificity of radiolabeled NB7 uptake.

In the retention assays (Fig. S3B), about 12–14 % of the input activity was taken up by the cells after 1 h incubation at 4 °C for both [¹³¹I]SGMIB-NB7 and *iso*-[¹²⁵I]SGMIB-NB7. The percentage of initially bound activity that was internalized was about 26–31 % with no significant differences detected between the two prosthetic agent isomers. Similar to the NB7 constructs without His₆-tags, [¹³¹I]SGMIB-NB7H6 and *iso*-[¹²⁵I]SGMIB-NB7H6 showed no isomer-related differences in cell uptake or internalization; however initial uptakes were 20–24 % of input counts (Fig. S4B), about twice those observed for the corresponding NB7 radioconjugates.

Given the differences in cell uptake noted above, paired-label assays were performed to directly compare the in vitro behavior of NB7 with

and without a His₆-tag at the C-terminus (Fig. 1). After 2 h incubation at 37 °C, uptake by PC3 PIP cells for *iso*-[¹³¹I]SGMIB-NB7H6 was 51.6 ± 1.1 % compared with 42.8 ± 2.2 % for *iso*-[¹²⁵I]SGMIB-NB7 in PC3 PIP cells ($P = 0.003$). However, at 24 h, the cell uptake of the two isomer conjugates was not significantly different (*iso*-[¹³¹I]SGMIB-NB7H6, 54.0 ± 0.9 %; *iso*-[¹²⁵I]SGMIB-NB7, 53.2 ± 0.9 %; $P > 0.05$). In the retention assay, *iso*-[¹³¹I]SGMIB-NB7H6 also showed a slightly higher uptake (23.7 ± 1.5 %) compared with that for *iso*-[¹²⁵I]SGMIB-NB7 (20.4 ± 1.6 %) after 1 h incubation at 4 °C, which was significant by a paired t-test ($P < 0.001$). In contrast, the percentage of initially bound activity that remained internalized at 4 h and 24 h was slightly higher for *iso*-[¹²⁵I]SGMIB-NB7 compared to *iso*-[¹³¹I]SGMIB-NB7H6 ($P < 0.05$).

In order to evaluate potential effects of substituting radioiodine with ²¹¹At, paired-label studies were performed to compare [¹³¹I]SGMIB-NB7H6 and [²¹¹At]SAGMB-NB7H6. When the radioconjugates were incubated with PC3 PIP cells at 37 °C, no significant differences in cellular uptake or internalization were observed between ¹³¹I and ²¹¹At (Fig. 2A). For example, after 2 h incubation, cell uptake of [¹³¹I]SGMIB-NB7H6 was 31.5 ± 0.7 % of input activity compared with 31.2 ± 1.0 % for [²¹¹At]SAGMB-NB7H6. Likewise, no significant differences were observed between [¹³¹I]SGMIB-NB7H6 and [²¹¹At]SAGMB-NB7H6 in the internalization and retention assay (Fig. 2B).

To investigate whether NB7 and the PSMA small molecule inhibitor YF2 compete for binding to PSMA, [¹²⁵I]SGMIB-NB7H6 and [¹³¹I]YF2 were incubated with PC3 PIP cells in single- and paired-label format at 37 °C for 2 h (Fig. S5). No significant differences in cell uptake or internalization levels between [¹²⁵I]SGMIB-NB7H6 and [¹³¹I]YF2 when the radiotracers were incubated individually or together ($P > 0.05$). In addition, in the retention assay (1 h initial incubation at 4 °C) $24.8 \pm$

0.3 % of initially cell-bound [¹²⁵I]SGMIB-NB7H6 remained internalized at 2 h in the presence of [¹³¹I]YF2 compared with 25.8 ± 0.5 % in the single-label assay. Overall, co-incubation with [¹³¹I]YF2 had no significant effect on the cell uptake and internalization of [¹²⁵I]SGMIB-NB7H6 ($P > 0.05$).

3.4. Biodistribution studies

Results from the single-label biodistribution study of *iso*-[¹²⁵I]SGMIB-NB7H6 in athymic mice with subcutaneous PC3 PIP xenografts are summarized in Fig. 3 and Table S1. Tumor uptake was highest at 1 h post injection (7.2 ± 1.5 % ID/g), and only declined slightly to 5.2 ± 1.2 % ID/g at 24 h. Radioactivity levels in kidneys were high at 1 h (72.5 ± 21.2 % ID/g), but rapidly cleared to $<0.2 \pm 0.0$ % ID/g at 24 h. Dehalogenation was minimal, as evidenced by thyroid and stomach accumulation of <0.25 % ID/organ at all-time points. The uptake in salivary and lacrimal glands was <1.5 % ID/g at 1 h, which declined to <0.5 % ID/g by 4 h.

The results from a paired-label study comparing the biodistribution of [¹³¹I]SGMIB-NB7 and *iso*-[¹²⁵I]SGMIB-NB7 are summarized in Fig. 4 and Table S2. At all-time points, tumor uptake of the two NB7 radioconjugates was not significantly different. Kidney activity levels of both tracers cleared rapidly and were similar at 1 h. However, at later time points, [¹³¹I]SGMIB-NB7 exhibited better kidney clearance with 0.3 ± 0.0 % ID/g seen at 24 h compared with 1.3 ± 0.3 % ID/g for *iso*-[¹²⁵I]SGMIB-NB7 ($P < 0.05$). Stomach, thyroid, salivary gland, and lacrimal gland activity levels were low for both isomer conjugates; however, they were consistently lower for [¹³¹I]SGMIB-NB7 with the differences significant ($P < 0.05$) in salivary gland at 1 and 4 h.

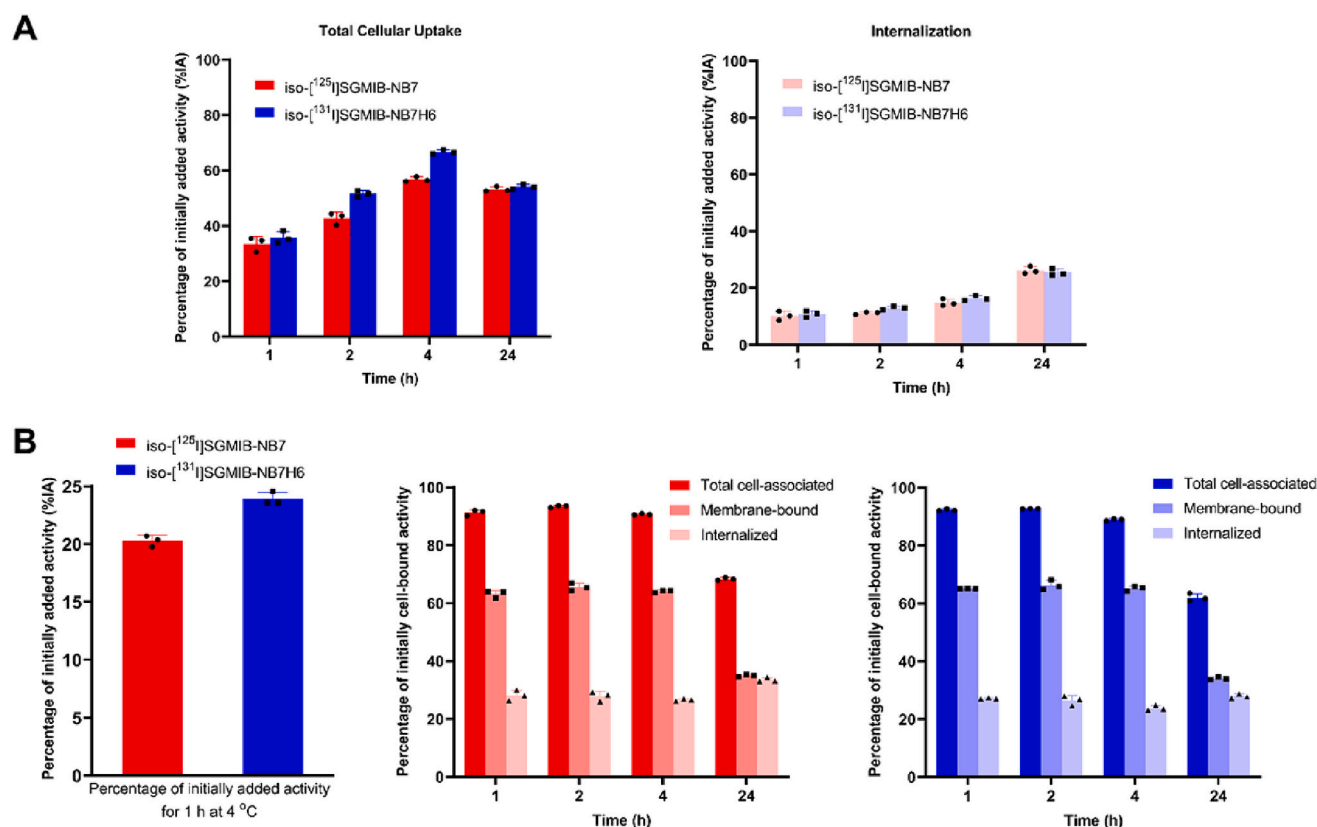


Fig. 1. In vitro binding and specificity of paired-label *iso*-[¹²⁵I]SGMIB-NB7 (red) and *iso*-[¹³¹I]SGMIB-NB7H6 in PC3 PIP cells ($n = 3$). (A) Total cellular uptake and internalization levels at 1, 2, 4 and 24 h after incubation at 37 °C. Values are expressed as percentages of applied radioactivity (B) Retention of ¹²⁵I,¹³¹I activity after incubation at 37 °C following 1 h incubation at 4 °C at various time points. Membrane-bound and internalized % activity are expressed as percentages of initially cell-bound radioactivity after an initial incubation at 4 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

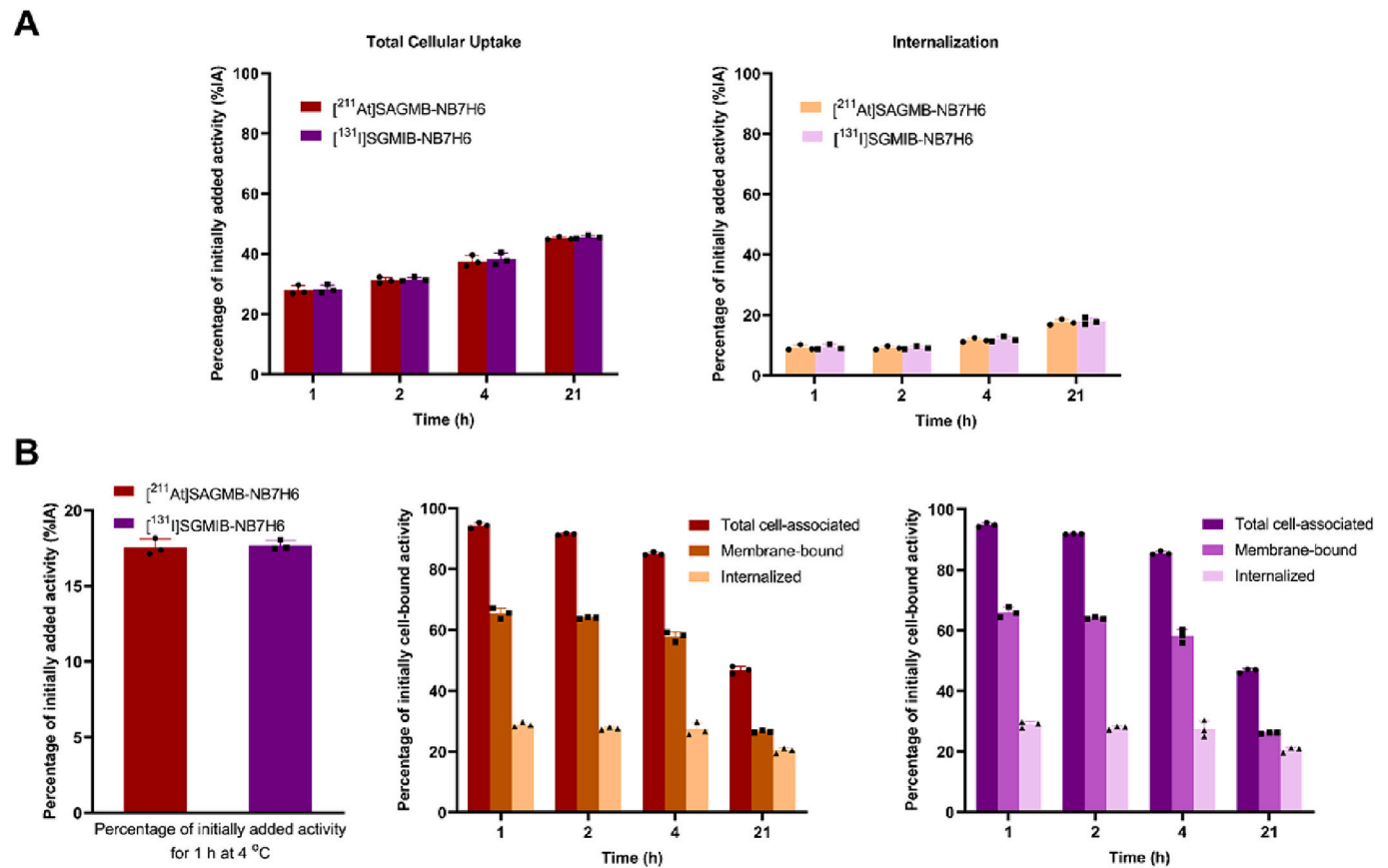


Fig. 2. In vitro binding and specificity of paired-label [^{211}At]SAGMB-NB7H6 (brown) and [^{131}I]SGMIB-NB7H6 (purple) in PC3 PIP cells ($n = 3$). (A) Total cellular uptake and internalization levels at 1, 2, 4 and 21 h after incubation at 37 °C. Values are expressed as percentages of applied radioactivity (B) Retention of ^{125}I , ^{131}I activity after incubation at 37 °C following 1 h incubation at 4 °C at various time points. Membrane-bound and internalized % activity are expressed as percentages of initially cell-bound radioactivity after an initial incubation at 4 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

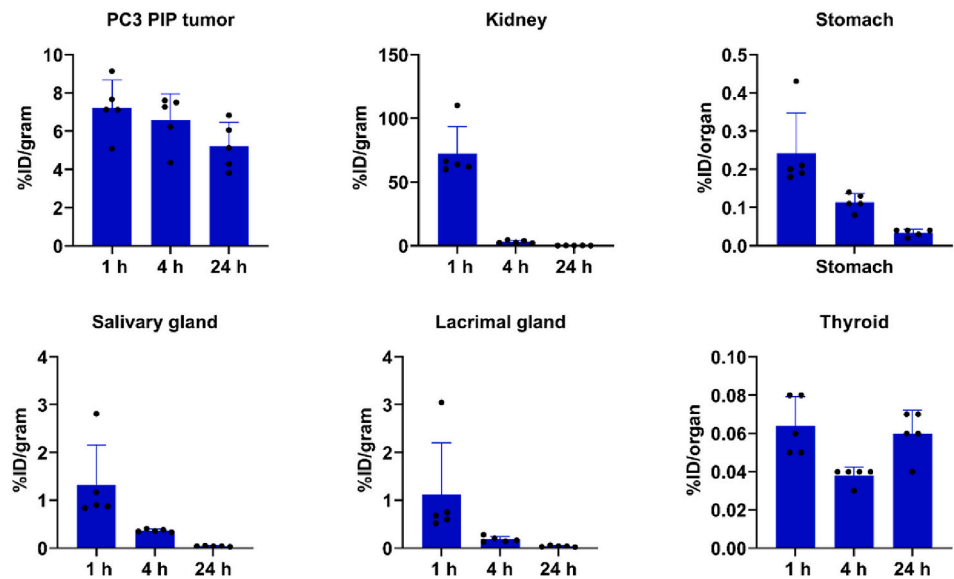


Fig. 3. Uptake in selected organs from the single-label biodistribution of iso- ^{125}I]SGMIB-NB7H6 in athymic mice with subcutaneous PC3 PIP xenografts ($n = 5$).

Another paired-label study was performed to evaluate the tissue distribution of [^{211}At]SAGMB-NB7H6 in direct comparison to that for [^{131}I]SGMIB-NB7H6. No significant differences in tumor and kidney activity levels up to 21 h, the time when injected ^{211}At had decayed to

~10 % of injected activity (Fig. 5 and Table S3). However, notable differences were observed in stomach, thyroid, and lacrimal/salivary glands, where ^{211}At activity levels were more than twice those for co-administered [^{131}I]SGMIB-NB7H6. Higher ^{211}At uptake levels were

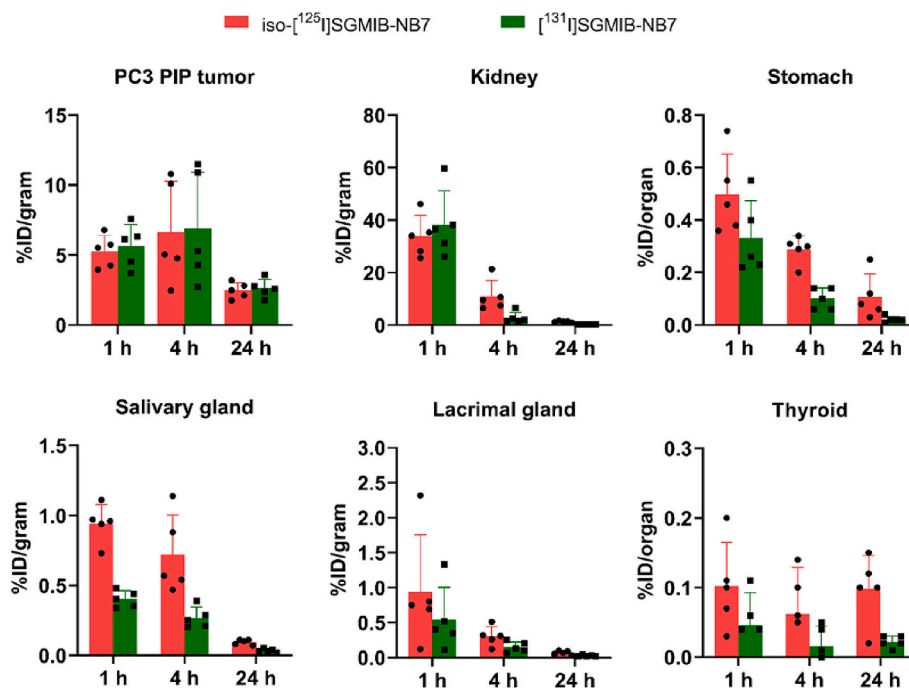


Fig. 4. Uptake in selected organs from a paired-label biodistribution of $iso-[^{125}\text{I}]SGMIB-NB7$ and $[^{131}\text{I}]SGMIB-NB7$ in athymic mice with subcutaneous PC3 PIP xenografts ($n = 5$).

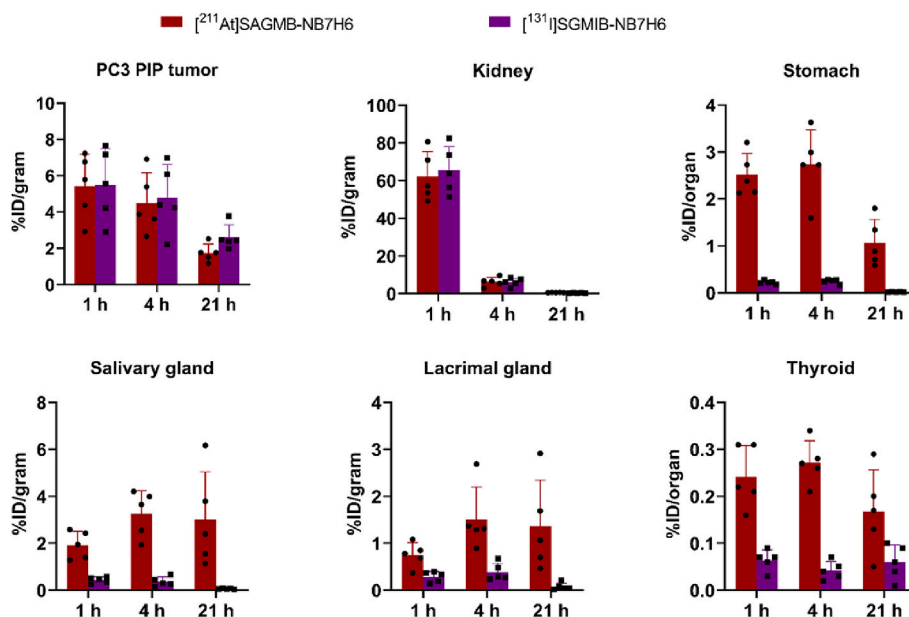


Fig. 5. Uptake in selected organs from a paired-label biodistribution of $[^{211}\text{At}]SAGMB-NB7H6$ (brown) and $[^{131}\text{I}]SGMIB-NB7H6$ (purple) in athymic mice bearing subcutaneous PC3 PIP xenografts ($n = 5$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

also seen in lungs, liver, and spleen (Table S3).

In order to compare the PSMA-targeting ability of NB7 sdAb with a small molecule PSMA inhibitor, a paired-label biodistribution of $[^{125}\text{I}]SGMIB-NB7H6$ in tandem with $[^{131}\text{I}]YF2$ was performed at 1 and 4 h. Tumor uptake of $[^{125}\text{I}]SGMIB-NB7H6$ was 2–3-fold lower ($P < 0.05$) than that for co-administered $[^{131}\text{I}]YF2$ at both time points (Fig. 6 and Table S4). However, renal activity levels of $[^{125}\text{I}]SGMIB-NB7H6$ were 4- and 84-fold lower than those observed for $[^{131}\text{I}]YF2$ at 1 and 4 h, respectively; ($P < 0.05$). Additionally, uptake of $[^{125}\text{I}]SGMIB-NB7H6$ was lower than $[^{131}\text{I}]YF2$ in thyroid, stomach, salivary glands and lacrimal glands ($P < 0.05$ except for thyroid at 1 h and stomach at 4 h)

(Table S4). Tumor-to-kidney ratios at 1 and 4 h for $[^{125}\text{I}]SGMIB-NB7H6$ rapidly increased from 0.2 ± 0.0 at 1 h to 5.8 ± 4.0 at 4 h, values that were higher than those observed for $[^{131}\text{I}]YF2$ (1 h: 0.1 ± 0.0 ; $P < 0.02$; 4 h: 0.2 ± 0.1 ; $P < 0.02$) (Fig. 7).

4. Discussion

Herein, we have evaluated NB7 as a PSMA-targeted vector in part because sdAbs offer an intermediately sized molecule between PSMA inhibitors like PSMA-I&T and intact antibodies like J591. Combinations of these two targeting vectors are now being investigated clinically with

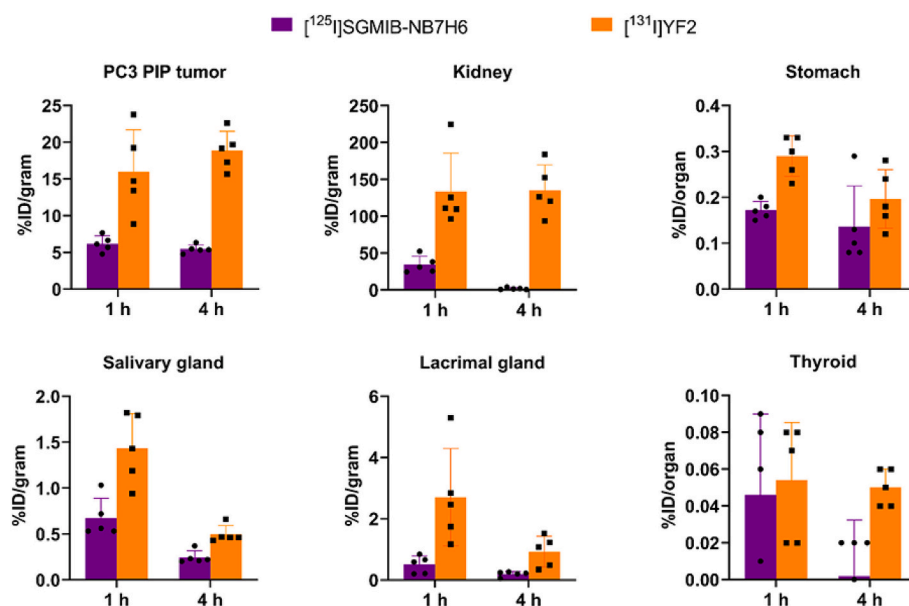


Fig. 6. Paired-label uptake of [^{125}I]SGMIB-NB7H6 (purple) and [^{131}I]YF2 (orange) in selected organs in athymic mice with subcutaneous PC3 PIP xenografts ($n = 5$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

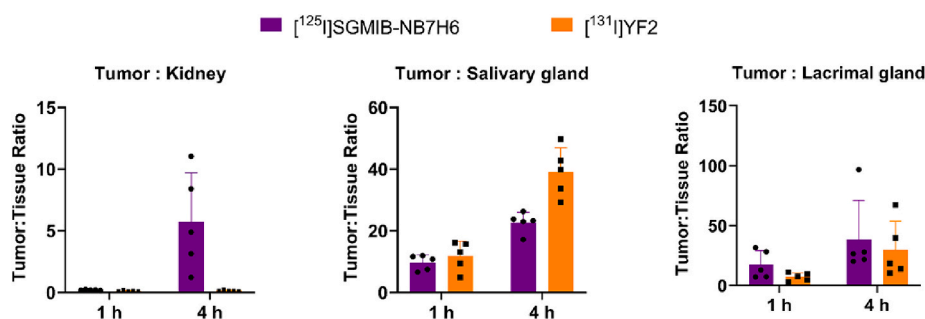


Fig. 7. Tumor-to-normal-tissue ratios calculated from the biodistribution of [^{125}I]SGMIB-NB7H6 (purple) and [^{131}I]YF2 (orange) in athymic mice with subcutaneous PC3 PIP xenografts ($n = 5$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the goal of providing synergistic effects in tumor while offering lower toxicity to normal tissue [23]. Perhaps a PSMA-targeted sdAb alone, with its intermediate properties, might provide a simpler way of achieving this goal. Alternatively, an sdAb would provide a more rapidly diffusing alternative to an intact mAb in therapeutic cocktail with a small molecule PSMA inhibitor. Because NB7 binds to a different site on PSMA than PSMA inhibitors [12], this should be feasible. Moreover, NB7 exhibits an avidity effect due to its simultaneous interaction with two PSMA monomers [12] so that if one site is detached from PSMA, the other site may remain bound.

Regarding selection of a radiolabeling strategy for NB7, previous studies have documented that residualizing radiohalogenated prosthetic agents offer significant advantages compared with radiometals for labeling sdAbs [24,25]. For example, head-to-head studies have demonstrated comparable tumor uptake with both labeling approaches but considerably higher kidney retention of radioactivity with radiometals [24]. Specifically, we have shown that SGMIB and iso-SGMIB, as well as their ^{211}At -labeled analogues, were excellent residualizing prosthetic agents for labeling HER2-targeted sdAbs [18,21,26]. These radiohalogenated sdAbs demonstrated excellent tumor uptake, minimal in vivo dehalogenation and high tumor-to-normal-tissue ratios.

In addition to or central objective of evaluating the potential utility of NB7 as a scaffold for developing PSMA-targeted theranostics, we also wished to evaluate variables of potentially broader impact on radio-pharmaceutical design: the effects of a His₆-tag on kidney activity levels

of an sdAb after SGMIB labeling and whether the choice of SGMIB isomer used for radiohalogenation had a significant effect on its in vivo behavior.

With radiometals, the presence of His₆-tag can lead to high and prolonged kidney levels when used to label sdAbs, which for therapy, could result in dose limiting renal toxicity. For example, in a study conducted by D'Huyvetter et al., evaluating 2Rs15d, an anti-HER2 sdAb, labeled with ^{177}Lu , kidney radioactivity levels decreased 88 % for untagged [^{177}Lu]2Rs15d compared with that for Myc-His₆-tagged [^{177}Lu]2Rs15d [22]. Similarly, Chatalic et al. reported that another anti-PSMA sdAb, JVZ-007 radiolabeled with ^{111}In displayed substantially higher renal radioactivity levels when Myc-His₆-Tag was present at its C-terminus [27]. This has been attributed to the high polarity of the C-terminal of the sdAb with a His₆-tag [27].

In contrast, radioactivity levels in the kidneys were similar from 1 to 24 h for *iso*-[^{125}I]SGMIB-NB7 and *iso*-[^{125}I]SGMIB-NB7H6 despite the presence of a His₆-tag in NB7H6. While the reason for this observation needs to be investigated, we speculate that differences in the renal retention of labeled catabolites generated from proteins labeled with SGMIB-like prosthetic agents and radiometals likely plays a role. When small proteins are labeled with radiometals via either DOTA [28] or DTPA [29] bifunctional chelates, radiometal-chelate- ϵ -lysine catabolites are generated, and these species are retained at high levels in the kidney. On the other hand, the main catabolites generated from proteins labeled using SGMIB are 4-guanidinomethyl-3-iodobenzoic acid (GMIBA) and

its glycine conjugate, both of which rapidly pass through the kidney into the urine [30,31]. We speculate that the increased polarity induced by the His₆-tag leads to increased uptake of sdAbs in the kidney within a few minutes [27], leading to generation of more radiolabeled catabolites which are retained in the kidney for radiometals and rapidly excreted for SGMIB. This was one of the factors guiding our selection of ²¹¹At the preferred α -emitter for labeling sdAbs instead of a radiometal like ²²⁵Ac that when used for sdAb labeling results in high and prolonged kidney activity levels [32].

We note that there is a second factor that could account for the increased kidney retention of radiometals when a His₆-tag is present. Although divalent cations such as Ni²⁺ are used most frequently in immobilized metal affinity chromatography, trivalent cations including Ga(III) and Fe(III) as well as Zr(IV) also have been used successfully [33,34]. Thus, it seems plausible that when sdAb-chelate conjugates are reacted with radiometals like ¹⁷⁷Lu and ²²⁵Ac, unanticipated labeling at the His₆-tag could occur. This could lead to release of the metal *in vivo* as well as the generation of different catabolites, which might contribute to enhanced kidney retention of the radiometal. Experiments are planned to investigate these possibilities.

The 1,3,5-*iso*-SGMIB isomer originally was developed to improve precursor and radiolabeling synthesis efficiencies by providing less steric hindrance than the 1,3,4- SGMIB isomer, and this was achieved [21]. When the biodistribution of an intact mAb, trastuzumab, labeled using [¹³¹I]SGMIB and *iso*-[¹²⁵I]SGMIB were compared in paired-label format, no significant differences in tissue distribution were observed. In a subsequent study using HER2-targeted 5F7 sdAb done in single-label format, the 1,3,5-isomer conjugates for both ¹³¹I and ²¹¹At exhibited higher tumor uptake and lower activity levels in thyroid and stomach than their 1,3,4-isomer counterparts [18]. However, the sdAb comparison was not done in paired-label format so the well-known differences that can occur among groups of experimental animals could have played a role. For this reason, we performed paired-label experiments to assess whether changing the position of the guanidino-methyl group in the SGMIB template would affect the residualizing ability of NB7 radioconjugates. Paired-label studies in PC3 PIP cells demonstrated that total cell uptake and internalization of two isomer conjugates were nearly identical; consistent with this, no significant isomer dependent differences in PC3 PIP xenograft uptake were observed *in vivo*. Radioactivity cleared rapidly from normal tissues with both SGMIB conjugates; however, when small differences were significant by paired t-test, activity levels were higher for the *iso*-[¹²⁵I]SGMIB-NB7 conjugate. Thus, while both isomers performed well, *iso*-SGMIB appeared to offer an advantage with anti-HER2 sdAb 5F7 [18]. It is plausible that the disparity maybe due to differential catabolism of two isomeric conjugates and the differing susceptibility of labeled catabolites towards dehalogenation. Several factors could play a role including microenvironment of the labeling site(s) on NB7 and 5F7, different intracellular routing/processing of HER2- and PSMA-targeted sdAbs, and strain of the mice (athymic for NB7 and SCID for 5F7). Finally, the 5F7 experiments were done in single-label format so differences in variables such as tumor size and HER2 expression level could have played a role.

NB7 radioconjugates exhibited excellent binding affinity to the PSMA⁺ PC3 PIP cell lines with K_d values in the low nanomolar range compared with <0.1 nM affinity to the PSMA molecule as measured previously by surface plasmon resonance [12]. Such differences between affinity to a molecule when in pure form and presented on a cell are not uncommon [16,26]. The binding affinity for the [¹³¹I]SGMIB conjugate of NB7H6 was better than that for NB7 (1.4 ± 0.4 nM versus 6.4 ± 2.0 nM), which might reflect differences in their purification method. Nonetheless, cell binding and internalization *in vitro*, and tumor accumulation *in vivo* were similar for NB7 constructs with and without a His₆-tag.

There are two previous reports on the development of radiolabeled sdAbs for targeting PSMA. Evazalipour et al. reported a K_d of 4.6 nM for

PSMA30, their best sdAb, in unlabeled form but the affinity for ^{99m}Tc-labeled PSMA30 was not given [11]. Chatalic et al. obtained a K_d value of 27.4 nM for the ¹¹¹In-labeled PSMA targeted sdAb JVZ-007-*c-myc*-his [27]. With the caveat that different cell lines and xenografts were used in these studies, the affinity values we obtained for our radiolabeled NB7 sdAbs were comparable or considerably better. Moreover, the NB7 radioconjugates described herein also exhibited substantially higher cell uptake and internalization than those reported for the ^{99m}Tc- and ¹¹¹In-labeled anti-PSMA sdAb [11,27]. Importantly, tumor targeting for the NB7 radioconjugates was considerably higher than those reported for ^{99m}Tc-labeled PSMA30 (1.57 ± 0.38 % ID/g at 90 min) and ¹¹¹In-labeled JVZ-007-cys (3.70 ± 0.29 % ID/g at 4 h). In addition, renal activity levels for the radiohalogenated NB7 conjugates were considerably lower except in the case of ¹¹¹In-labeled JVZ-007-cys when co-administered with lysine and gelofusine [27].

Although ¹³¹I-labeled sdAbs are currently being evaluated as therapeutics in patients [35], the long-term translational objective of this work is to develop a ²¹¹At-labeled NB7 conjugate for targeted alpha particle therapy. Our *in vitro* studies demonstrated that the levels of cellular uptake and intracellular trapping for [²¹¹At]SAGMB-NB7H6 were virtually identical to those for [¹³¹I]SGMIB-NB7H6. Moreover, *in vivo* studies revealed no notable differences in tumor uptake and kidney clearance between [²¹¹At]SAGMB-NB7H6 and its ¹³¹I-labeled analogue at any time point. However, ²¹¹At activity levels were significantly higher than those for ¹³¹I in thyroid, stomach, and lacrimal and salivary gland, tissues known to sequester iodide and also [²¹¹At]astatide [36]. On the other hand, the uptake of ²¹¹At in the thyroid after injection of [²¹¹At]SAGMB-NB7H6 was <0.3 % ID at all-time points suggesting that the absolute level of deastatination *in vivo* was low. It is worth noting that the thyroid and stomach accumulation of ²¹¹At after injection of [²¹¹At]SAGMB-NB7H6 comparable to that reported previously for [²¹¹At]SAGMB-5F7 conjugates, which have been shown to be very effective at a single dose in preclinical models [37]. Finally, it should be noted that blocking agents such as saturated solution of potassium iodide are available and are effective at preventing tissue uptake of [²¹¹At]astatide [38] and have been used clinically for this purpose [39].

Molecular docking simulation analysis indicated that NB7 binds to human PSMA far from its catalytic active site and at a different site than PSMA small molecule inhibitors [12] suggesting that the two PSMA targeting strategies should not compete. Head-to-head comparisons were performed to confirm this and to directly compare [¹²⁵I]SGMIB-NB7 and the recently developed small molecule PSMA inhibitor, YF2. We note that the cross reactivity of NB7 and YF2 with murine PSMA has not been determined. No discernible differences in PSMA⁺ PC3 PIP cell uptake and internalization were observed when the compounds were incubated with the cells together or individually. On the other hand, notable differences were observed in mice with PSMA⁺ PC3 PIP xenografts where [¹³¹I]YF2 exhibited 2.5-fold higher uptake than co-administered [¹²⁵I]SGMIB-NB7. However, the radioactivity levels in kidney for [¹²⁵I]SGMIB-NB7 were substantially lower than [¹³¹I]YF2 with about 2-fold, 4-fold and 84-fold differences observed at 1, 4 and 24 h post-injection, respectively. Renal radiation toxicity after treatment with radiolabeled small molecule PSMA inhibitors can be problematic [40]. Thus, NB7 sdAbs labeled with these guanidino-substituted residualizing prosthetic agents could provide an attractive treatment option, either alone or in tandem with a small molecule PSMA, for achieving tumor control while delivering less radiation dose to the kidneys. Pre-clinical therapeutic efficacy experiments are being planned to evaluate these possibilities.

5. Conclusion

In summary, we have shown that PSMA-targeted sdAb NB7 could be labeled with radioiodine and ²¹¹At using the guanidino-bearing residualizing prosthetic agents SGMIB and SAGMB with excellent retention of affinity. Biodistribution studies in athymic mice with PSMA⁺ PC3 PIP

xenografts demonstrated rapid tumor accumulation and clearance from kidneys, accompanied by minimal uptake in other non-targeted organs. A paired-label biodistribution study demonstrated that with SGMIB labeling, that presence of a C-terminal His₆-tag on NB7 did not result in significant increase of radioiodine levels in kidneys. In a second head-to-head comparison of NB7 labeled using SGMIB and *iso*-SGMIB prosthetic agents, no isomer-dependent differences in tumor uptake or meaningful normal tissue levels were observed. In a final paired-label experiment, [¹²⁵I]SGMIB-NB7 exhibited 2.5-fold lower tumor uptake than [¹³¹I]YF2 but considerably more favorable tumor-to-kidney uptake ratios. Given that its biological properties are compatible with the physical characteristics of ²¹¹At, evaluation of the therapeutic potential of ²¹¹At-labeled NB7 appears warranted.

CRediT authorship contribution statement

Truc T. Huynh: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Yutian Feng:** Methodology, Investigation, Formal analysis, Data curation. **Rebecca Meshaw:** Investigation. **Xiao-Guang Zhao:** Investigation. **Lior Rosenfeld:** Investigation. **Ganesan Vaidyanathan:** Writing – review & editing, Validation. **Niv Papo:** Writing – review & editing, Resources, Conceptualization. **Michael R. Zalutsky:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

LR and NP have filed a patent application that includes the NB7 single domain antibody fragment. The authors declare that they have no other known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nucmedbio.2024.108913>.

References

- Caromile LA, Dortche K, Rahman MM, Grant CL, Stoddard C, Ferrer FA, et al. PSMA redirects cell survival signaling from the MAPK to the PI3K-AKT pathways to promote the progression of prostate cancer. *Sci Signal* 2017;10:470.
- Wright Jr GL, Grob BM, Haley C, Grossman K, Newhall K, Petrylak D, et al. Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. *Urology* 1996;48:326–34.
- Ross JS, Sheehan CE, Fisher HA, Kaufman Jr RP, Kaur P, Gray K, et al. Correlation of primary tumor prostate-specific membrane antigen expression with disease recurrence in prostate cancer. *Clin Cancer Res* 2003;9:6357–62.
- Chang SS, Reuter VE, Heston WD, Bander NH, Grauer LS, Gaudin PB. Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. *Cancer Res* 1999;59:3192–8.
- Smith-Jones PM, Vallabhaajosula S, Goldsmith SJ, Navarro V, Hunter CJ, Bastidas D, et al. In vitro characterization of radiolabeled monoclonal antibodies specific for the extracellular domain of prostate-specific membrane antigen. *Cancer Res* 2000;60:5237–43.
- Eder M, Eisenhut M, Babich J, Haberkorn U. PSMA as a target for radiolabelled small molecules. *Eur J Nucl Med Mol Imaging* 2013;40:819–23.
- Vaneycken I, D'Huyvetter M, Hernot S, De Vos J, Xavier C, Devoogdt N, et al. Immuno-imaging using nanobodies. *Curr Opin Biotechnol* 2011;22:877–81.
- Polakis P. Antibody drug conjugates for cancer therapy. *Pharmacol Rev* 2016;68:3–19.
- Gerber DE. Targeted therapies: a new generation of cancer treatments. *Am Fam Physician* 2008;77:311–9.
- Sun S, Ding Z, Yang X, Zhao X, Zhao M, Gao L, et al. Nanobody: a small antibody with big implications for tumor therapeutic strategy. *Int J Nanomedicine* 2021;16:2337–56.
- Evazalipour M, D'Huyvetter M, Tehrani BS, Abolhassani M, Omidfar K, Abdoli S, et al. Generation and characterization of nanobodies targeting PSMA for molecular imaging of prostate cancer. *Contrast Media Mol Imaging* 2014;9:211–20.
- Rosenfeld L, Sananes A, Zur Y, Cohen S, Dhara K, Gelkop S, et al. Nanobodies targeting prostate-specific membrane antigen for the imaging and therapy of prostate cancer. *J Med Chem* 2020;63:7601–15.
- Kiess AP, Minn I, Vaidyanathan G, Hobbs RF, Josefsson A, Shen C, et al. (2S)-2-(3-(1-carboxy-5-(4-²¹¹At-astatobenzamido)pentyl)ureido)-pentanedioic acid for PSMA-targeted α -particle radiopharmaceutical therapy. *J Nucl Med* 2016;57:1569–75.
- Mease RC, Kang CM, Kumar V, Banerjee SR, Minn I, Brummet M, et al. An improved (211)At-labeled agent for PSMA-targeted α -therapy. *J Nucl Med* 2022;63:259–67.
- Vaidyanathan G, Mease RC, Minn I, Choi J, Chen Y, Shallal H, et al. Synthesis and preliminary evaluation of 211At-labeled inhibitors of prostate-specific membrane antigen for targeted alpha particle therapy of prostate cancer. *Nucl Med Biol* 2021;94:95:67–80.
- Feng Y, Meshaw R, Finch S, Zheng Y, Minn I, Vaidyanathan G, Pomper M, Zalutsky M. A third generation PSMA-targeted agent [²¹¹At]YF2: Synthesis and in vivo evaluation. *Nucl Med Biol* 2024. <https://doi.org/10.1016/j.nucmedbio.2024.108916>.
- Watabe T, Kaneda-Nakashima K, Shirakami Y, Kadonaga Y, Ooe K, Wang Y, et al. Targeted α -therapy using astatine ((211)At)-labeled PSMA1, 5, and 6: a preclinical evaluation as a novel compound. *Eur J Nucl Med Mol Imaging* 2023;50:849–58.
- Choi J, Vaidyanathan G, Koumariannou E, Kang CM, Zalutsky MR. Astatine-211 labeled anti-HER2 5F7 single domain antibody fragment conjugates: radiolabeling and preliminary evaluation. *Nucl Med Biol* 2018;56:10–20.
- Vaidyanathan G, Pozzi OR, Choi J, Zhao XG, Murphy S, Zalutsky MR. Labeling monoclonal antibody with α -emitting (211)At at high activity levels via a tin precursor. *Cancer Biother Radiopharm* 2020;35:511–9.
- Vaidyanathan G, Zalutsky MR. Synthesis of N-succinimidyl 4-guanidinomethyl-3-[¹²⁵I]iodobenzoate: a radio-iodination agent for labeling internalizing proteins and peptides. *Nat Protoc* 2007;2:282–6.
- Choi J, Vaidyanathan G, Koumariannou E, McDougald D, Pruszyński M, Osada T, et al. N-Succinimidyl guanidinomethyl iodobenzoate protein radiohalogenation agents: influence of isomeric substitution on radiolabeling and target cell residualization. *Nucl Med Biol* 2014;41:802–12.
- D'Huyvetter M, Vincke C, Xavier C, Aerts A, Impens N, Baatout S, et al. Targeted radionuclide therapy with a 177Lu-labeled anti-HER2 nanobody. *Theranostics* 2014;4:708–20.
- Nauseef JT, Osborne J, Gregos P, Thomas C, Bissassar M, Singh S, et al. Phase I/II study of 225Ac-J591 plus 177Lu-PSMA-I&T for progressive metastatic castration-resistant prostate cancer. *J Clin Oncol* 2022;40 [TPS5100-TPS].
- Feng Y, Sarrett SM, Meshaw RL, Vaidyanathan G, Cornejo MA, Zeglis BM, et al. Site-specific radiohalogenation of a HER2-targeted single-domain antibody fragment using a novel residualizing prosthetic agent. *J Med Chem* 2022;65:15358–73.
- D'Huyvetter M, De Vos J, Xavier C, Pruszyński M, Sterckx YGJ, Massa S, et al. (131)I-labeled anti-HER2 camelid sdAb as a theranostic tool in cancer treatment. *Clin Cancer Res* 2017;23:6616–28.
- Feng Y, Meshaw R, McDougald D, Zhou Z, Zhao X-G, Jannetti SA, et al. Evaluation of an 131I-labeled HER2-specific single domain antibody fragment for the radiopharmaceutical therapy of HER2-expressing cancers. *Sci Rep* 2022;12:3020.
- Chatalic KL, Veldhoven-Zweistra J, Bolkestein M, Hoebe S, Koning GA, Boerman OC, et al. A novel ¹¹¹In-labeled anti-prostate-specific membrane antigen nanobody for targeted SPECT/CT imaging of prostate cancer. *J Nucl Med* 2015;56:1094–9.
- Tsai SW, Li L, Williams LE, Anderson AL, Raubitschek AA, Shively JE. Metabolism and renal clearance of 111In-labeled DOTA-conjugated antibody fragments. *Bioconjug Chem* 2001;12:264–70.
- Rogers BE, Franano FN, Duncan JR, Edwards WB, Anderson CJ, Connett JM, et al. Identification of metabolites of 111In-diethylenetriaminepentaacetic acid-monoclonal antibodies and antibody fragments in vivo. *Cancer Res* 1995;55:5714s–20s.
- Vaidyanathan G, Affleck DJ, Li J, Welsh P, Zalutsky MR. A polar substituent-containing acylation agent for the radioiodination of internalizing monoclonal antibodies: N-succinimidyl 4-guanidinomethyl-3-[131I]iodobenzoate ([131I]SGMIB). *Bioconjug Chem* 2001;12:428–38.
- Shankar S, Vaidyanathan G, Affleck DJ, Peixoto K, Bigner DD, Zalutsky MR. Evaluation of an internalizing monoclonal antibody labeled using N-succinimidyl 3-[131I]iodo-4-phosphonomethylbenzoate ([131I]SIPMB), a negatively charged substituent bearing acylation agent. *Nucl Med Biol* 2004;31:909–19.
- Rodak M, Dekempeneer Y, Wojewódzka M, Caveliers V, Covens P, Miller BW, et al. Preclinical evaluation of 225Ac-labeled single-domain antibody for the treatment of HER2pos cancer. *Mol Cancer Ther* 2022;21:1835–45.
- Posewitz MC, Tempst P. Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal Chem* 1999;71:2883–92.
- Block H, Maertens B, Spriestersbach A, Brinker N, Kubicek J, Fabis R, et al. Immobilized-metal affinity chromatography (IMAC): a review. *Methods Enzymol* 2009;463:439–73.
- D'Huyvetter M, Vos J, Caveliers V, Vaneycken I, Heemskerk J, Duhoux FP, et al. Phase I trial of (131)I-GMIB-anti-HER2-VHH1, a new promising candidate for HER2-targeted radionuclide therapy in breast cancer patients. *J Nucl Med* 2021;62:1097–105.
- Watabe T, Liu Y, Kaneda-Nakashima K, Sato T, Shirakami Y, Ooe K, et al. Comparison of the therapeutic effects of [(211)At]NaAt and [(131)I]NaI in an NIS-expressing thyroid cancer mouse model. *Int J Mol Sci* 2022;23.
- Feng Y, Meshaw R, Zhao XG, Jannetti S, Vaidyanathan G, Zalutsky MR. Effective treatment of human breast carcinoma xenografts with single-dose (211)At-labeled anti-HER2 single-domain antibody fragment. *J Nucl Med* 2023;64:124–30.

- [38] Larsen RH, Slade S, Zalutsky MR. Blocking [211At]astatide accumulation in normal tissues: preliminary evaluation of seven potential compounds. *Nucl Med Biol* 1998;25:351–7.
- [39] Zalutsky MR, Reardon DA, Akabani G, Coleman RE, Friedman AH, Friedman HS, et al. Clinical experience with α -particle-emitting 211At: treatment of recurrent brain tumor patients with 211At-labeled chimeric antitenascin monoclonal antibody 81C6. *J Nucl Med* 2008;49(1):30–8.
- [40] Steinhelfer L, Lunger L, Cala L, Pfof CH, Lapa C, Hartrampf PE, et al. Long-term nephrotoxicity of 177Lu-PSMA radioligand therapy. *J Nucl Med* 2024;65(1):79–84.