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A Third Generation PSMA-targeted Agent [²¹¹At]YF2: Synthesis and In Vivo Evaluation

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Abstract

Introduction: Targeted α -particle therapy agents have shown promising responses in patients who have developed resistance to β -particle emitting radionuclides, albeit off-target toxicity remains a concern. Astatine-211 emits only one α -particle per decay and may alleviate the toxicity from α -emitting daughter radionuclides. Previously, we developed the low-molecular-weight PSMA-targeted agent [²¹¹At]L3-Lu that showed suitable therapeutic efficacy and was well tolerated in mice. Although [²¹¹At]L3-Lu had good characteristics, we now have evaluated a closely related analogue, [²¹¹At]YF2, to determine the better molecule for clinical translation.

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Declaration of competing interest

Ganesan Vaidyanathan, Martin Pomper and Michael Zalutsky are co-inventors on a patent related to the labeled PSMA inhibitors described in this study and discussed in this publication. They are entitled to royalty distributions related to this technology, which was originally licensed by Precision Molecular, Inc. and now transferred to z-alpha, inc. Martin Pomper and Michael Zalutsky are co-founders and paid consultants of z-alpha and are minority shareholders via stock options. No other potential competing interests related to this work are reported.

Editor conflict of interest statement

Given his role as Editorial Board Member, Michael Zalutsky was not involved in the peer-review of this article and had no access to information regarding its peer-review.

CRediT authorship contribution statement

Yutian Feng: Methodology, Formal analysis, Investigation, Data Curation, Writing - original draft, Visualization. **Rebecca L. Meshaw:** Investigation. **Sean W. Finch:** Methodology, Formal analysis, Investigation, Data Curation. **Yongxiang Zheng:** Investigation. **Il Minn:** Investigation. **Ganesan Vaidyanathan:** Methodology, Validation, Writing - Review & Editing. **Martin G. Pomper:** Conceptualization, Resources, Writing - Review & Editing. **Michael R. Zalutsky:** Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Appendix A. Supplementary data

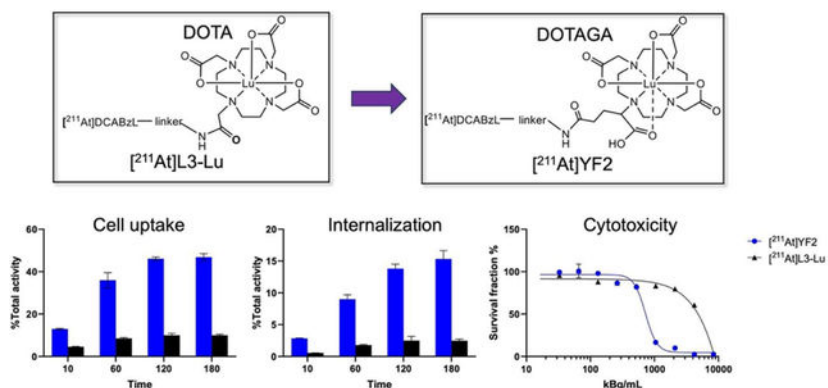
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nucmedbio.2024.xxxxxx>.

Methods: The tin precursors and unlabeled iodo standards for [^{211}At]YF2 and [^{211}At]L3-Lu each were synthesized and a new one-step labeling method was developed to produce [^{211}At]YF2 and [^{211}At]L3-Lu from the respective tin precursor. RCY and RCP were determined using RP-HPLC. Cell uptake, internalization and *in vitro* cell-killing (MTT) assays were performed on PSMA+ PC-3 PIP cells in parallel experiments to compare [^{211}At]YF2 and [^{211}At]L3-Lu directly. A paired-label biodistribution study was performed in athymic mice with subcutaneous PSMA-positive PC-3 PIP xenografts as a head-to-head comparison of [^{131}I]YF2 and [^{125}I]L3-Lu. The tissue distribution of [^{211}At]YF2 and [^{211}At]L3-Lu were determined individually in the same animal model. Finally, the effect of co-injecting unlabeled iodinated YF2 on the biodistribution of [^{211}At]YF2 was evaluated in the same animal model.

Results: The syntheses of tin precursors and unlabeled iodo standards were accomplished in reasonable yields. A streamlined and scalable radiolabeling method (1 h total synthesis time) was developed for the radiosynthesis of both [^{211}At]YF2 and [^{211}At]L3-Lu with $86 \pm 7\%$ ($n=10$) and $87 \pm 5\%$ ($n=7$) RCY, respectively, and $>95\%$ RCP for both. The maximum activity of [^{211}At]YF2 produced to date was 666 MBq. An alternative method that did not involve HPLC purification was developed that provided similar RCY and RCP. Significantly higher cell uptake, internalization and cytotoxicity was seen for [^{211}At]YF2 compared with [^{211}At]L3-Lu. Significantly higher uptake and longer retention in tumor was seen for [^{131}I]YF2 than for co-administered [^{125}I]L3-Lu, while considerably higher renal uptake was seen for [^{131}I]YF2. The biodistribution of [^{211}At]YF2 was consistent with that of [^{131}I]YF2. Co-injection of 2 nmole iodinated YF2 reduced renal uptake of [^{211}At]YF2 by 6-fold without significantly changing tumor uptake.

Conclusion: [^{211}At]YF2 exhibited higher cellular uptake, internalization and cytotoxicity than [^{211}At]L3-Lu on PSMA-positive PC3 PIP cells. Likewise, higher uptake and longer retention in tumor was seen for [^{211}At]YF2. Experiments to evaluate the dosimetry and therapeutic efficacy of [^{211}At]YF2 are under way.

Graphical Abstract



Keywords

Prostate-specific membrane antigen; Prostate cancer; ^{211}At Astatine; Targeted alpha-particle therapy; Radionuclide therapy

1. Introduction

Prostate-specific membrane antigen (PSMA)-targeted radiopharmaceutical therapy (RPT) has rejuvenated the field of nuclear medicine, marked by the FDA approval of [^{177}Lu]Lu-PSMA-617, namely Pluvicto[®] (1). While earlier work with low-molecular-weight (LMW) PSMA-inhibitors was dominated by β^- -particle emitting radionuclides, there is accumulating evidence that α -particle emitting radionuclides may outperform β -emitters such as ^{177}Lu (2–5). In the registration trial for [^{177}Lu]Lu-PSMA-617, a 4 to 6 cycle treatment regimen increased overall survival compared with standard of care from 11.3 months to 15.3 months (6). Targeted α -particle therapy (TAT) has shown even more dramatic results in some studies. For instance, a study using the ^{225}Ac -labeled analogue of PSMA-617 demonstrated significant responses, even in individuals nonresponsive to prior β -emitter therapy (7). PSMA-targeted radiotherapy has exhibited positive results with a reasonable therapeutic index. However, the challenge ahead is to determine the optimal application of this therapy within the spectrum of protocols for managing prostate cancer. Questions persist regarding the ideal TAT agent, given existing toxicities that can compromise quality of life. That has motivated many groups including our own to evaluate different chemical entities and radionuclides that may be provide a more favorable balance between maximizing efficacy and minimizing normal tissue toxicity.

Our research in this area has focused on the α -particle emitter ^{211}At in part because its half-life (7.2-h) is a good match to the biological half-life of LMW PSMA-targeted agents (8). In addition, ^{211}At offers practical advantages; notably, ^{211}At can be readily measured using its 79–92 keV polonium x-rays, permitting the quality control of ^{211}At -labeled radiopharmaceuticals to be performed in real time using HPLC or TLC equipped with a standard radioactive detector under GMP guidelines (9, 10). Also, unlike other α -emitters such as ^{225}Ac and ^{212}Pb that require sophisticated methodology of measurement (11–13), ^{211}At can be measured using dose calibrators that are in wide use throughout the world. The first ^{211}At -labeled LMW PSMA-targeted agent, [^{211}At]DCABzL, reported by Kiess et al. (14), showed significant therapeutic efficacy in both subcutaneous and micrometastatic models but was compromised by dose-limiting renal toxicity (14). In search for an agent with a more favorable tumor-to-kidney dose ratio, a small-scale medicinal chemistry campaign was conducted (15) that focused on strategies used successfully for labeling internalizing proteins. That led to the development of a second-generation agent, [^{211}At]L3-Lu, bearing a Lu-DOTA moiety, which demonstrated robust tumor growth inhibition with little off-target toxicity (16).

Although clinical evaluation of [^{211}At]L3-Lu is an attractive option, we decided it would be prudent first to evaluate a close analogue, [^{211}At]YF2, in which the Lu-DOTA was replaced with Lu-DOTAGA. That was motivated in part by recent studies demonstrating that the renal uptake in patients of [^{177}Lu]Lu-PSMA I&T (17), which also bears a Lu-DOTAGA moiety, was considerably less than previously thought based on mouse data (18). Herein we report the synthesis of [^{211}At]YF2 and its evaluation in head-to-head comparison *in vitro* and *in vivo* with the previous lead [^{211}At]L3-Lu. We also report on the optimization and scale-up production of both [^{211}At]YF2 and [^{211}At]L3-Lu using a novel radiolabeling chemistry to lay the foundation for clinical translation in the near future. Finally, we have provided the

details of our HPGe detector-based procedure, similar to a method reported by others (9), to identify ^{211}At and possible radionuclidic impurities, and to calibrate dose calibrators for routine use.

2. Materials and methods

2.1 General

The chemicals and reagents used in this work were purchased from Sigma-Aldrich except as noted below. Sodium [^{125}I]iodide (629 GBq/mg) in 0.1 M NaOH was obtained from Perkin Elmer Life and Analytical Sciences (Boston, MA). Sodium [^{131}I]iodide in a solution of 0.04 M sodium thiosulfate, 0.2 M NaOH and 0.2 M sodium carbonate (936 GBq/mg, 236.097 GBq/mL) was purchased from International Isotopes Inc. (Idaho Falls, ID). Normal phase column chromatography was performed with the Biotage Isolera chromatography system (Charlotte, NC) using their prepacked columns. High performance liquid chromatography (HPLC) was conducted using two Agilent 1260 Infinity systems equipped with a 1260 Infinity Multiple Wavelength Detector (Santa Clara, CA). For monitoring radioactivity, one system was connected to a Dual Scan-RAM flow radioactivity detector/TLC scanner and the other to a Flow-RAM detector (LabLogic, Tampa, Florida); the HPLC and the gamma detectors were controlled by LabLogic Laura software. Reversed phase HPLC (RP-HPLC) was performed with an Agilent Poroshell 120 EC-C18 column (2.7 μm , 4.6 $\times$ 100 mm, with guard column). Mobile phase A was deionized water (DI) and mobile phase B was acetonitrile, both containing 0.1% formic acid; the flow rate was 2 mL/min. Preparative RP-HPLC (Prep-HPLC) was performed with an Agilent ZORBAX SB-C18 preparative column (5 μm , 9.4 $\times$ 150 mm, with guard column) with the same mobile phases above. Radioactivity levels were measured using a CRC-7 dose calibrator (Capintec, Pittsburgh, PA) for higher activities, and for lower activities, either an LKB 1282 (Turku, Finland) or a Perkin Elmer Wizard II (Shelton, CT) automated gamma counter were used. Gamma-ray spectroscopy was performed using an Ortec high-purity germanium (HPGe) detector connected to a Canberra Multiport-II multichannel analyzer (MCA) running the associated Genie 2000 software. Proton NMR spectra were obtained on a 400 MHz spectrometer (Varian/Agilent; Inova), and chemical shifts are reported in δ units using the residual solvent peak as a reference. Mass spectra were obtained using an Advion (Ithaca, NY) ExpressionL CMS LC-MS System attached to an Agilent 1260 infinity HPLC. This mass spectrometer has the capability of determining molecular weights of compounds directly from TLC plates (Plate Express), and by ESI, atmospheric pressure chemical ionization (APCI), and atmospheric solids analysis probe (ASAP). High resolution mass spectra were obtained by flow injection into an Agilent 6224 TOF LC/MS system under positive ion mode. Reagents for cell culture were obtained from Thermo Fisher Scientific (Waltham, MA), except where noted.

2.2 Chemistry

(S)-2-[3((S)-(5-Benzyloxycarbonylamino)-1-*tert* butoxycarbonyl)pentyl-ureido]pentanedioic acid di-*tert*-butyl ester (1).—Compound 1 was synthesized following reported procedures (19). The crude mixture was purified by normal phase chromatography using a 100 g BIOTAGE SNAP ULTRA column and 9:1 hexanes:EtOAc as the mobile phase to afford the product as a pale-yellow oil (11.3 g, 68% yield). ^1H NMR ($\text{DMSO}-d_6$): 7.21 –

7.38 (m, 5H), 6.24 – 6.3 (m, 3H), 4.99 (s, 2H), 3.93 – 4.06 (m, 2H), 2.95 – 3.00 (m, 2H), 2.19 – 2.25 (m, 2H), 1.99 – 1.49 (m, 8H), 1.44 – 1.39 (m, 27H, *t*-Bu's). HRMS Calcd for C₃₂H₅₁N₃O₉ (M + H)⁺: 622.36. Found: 622.60.

2-[3-(5-Amino-1-*tert*-butoxycarbonylpentyl)ureido]pentanedioic acid di-*tert*-butyl ester (2).—Compound 2 was synthesized following reported procedures (19).

Briefly, Compound 1 (1 g, 1.59 mmol) was dissolved in ethanol (30 mL) under an inert atmosphere (using nitrogen), to which solution ammonium formate (1 g, 10 eq.) was added, followed by the addition of 10% Pd-C (100 mg, 10%w/w) under an inert atmosphere. The mixture was stirred at room temperature (RT) overnight. The solvent was removed to afford the product (white solid, 0.75 g, 95% yield), which was used for the next step without further purification. ¹H NMR (DMSO-*d*₆): 6.24 – 6.3 (m, 2H), 3.93 – 4.06 (m, 2H), 2.19 – 2.25 (m, 2H), 1.99 – 1.49 (m, 10H), 1.44 – 1.39 (m, 27H, *t*-Bu's), 0.86 (m, 2H). HRMS Calcd for C₂₄H₄₅N₃O₇ (M + H)⁺: 488.33. Found: 488.61.

Di-*tert*-butyl (((S)-1-(*tert*-butoxy)-6-((4-iodobenzyl)amino)-1-oxohexan-2-yl)carbamoyl)-L-glutamate (3).—Compound 2 (100 mg, 205 μmol, 1 eq.) was dissolved in methanol (1 mL) at 0 – 5°C under an inert atmosphere (N₂), to which 4-iodobenzaldehyde (47.6 mg, 205 μmol, 1 eq.) dissolved in 1 mL methanol was added dropwise. The

reaction mixture was stirred at room temperature for 1 h and then treated with sodium cyanoborohydride (51 mg, 820 μmol, 4 eq.). The resultant mixture was stirred overnight at room temperature (RT) and concentrated under vacuum. The crude product was purified by flash column chromatography using a 20 g BIOTAGE SNAP ULTRA column eluted with 40% ethyl acetate in hexanes, affording Compound 3 in 49% yield (71 mg) as a pale-yellow oil. ¹H NMR (CDCl₃): 7.63 – 7.70 (m, 2H), 7.06 – 7.13 (m, 2H), 4.97 – 5.03 (m, 2H), 4.29 – 4.34 (m, 2H), 3.73 (s, 2H), 2.60 (t, 2H), 2.27 – 2.36 (m, 2H), 2.04 – 2.09 (m, 1H), 1.91 – 1.53 (m, 3H), 1.45 – 1.49 (m, 32H). HRMS Calcd for C₃₁H₅₀IN₃O₇ (M + H)⁺: 704.27. Found: 704.38.

Di-*tert*-butyl (((S)-6-(5-amino-N-(4-iodobenzyl)pentanamido)-1-(*tert*-butoxy)-1-oxohexan-2-yl)carbamoyl)-L-glutamate (4).—A mixture of 5-(Fmoc-amino)valeric acid (48.2 mg, 142 μmol, 1 eq.), TSTU (42.8 mg, 142 μmol, 1 eq.) and DIPEA (50 μL,

260 μmol, 1.83 eq.) in DMF (1 mL) were stirred at RT for 1 h. Compound 3 (100 mg, 142 μmol, 1 eq.) dissolved in DMF (1 mL) was added dropwise to the above. The resultant mixture was stirred at RT for 4 h and concentrated in vacuum. The crude product from this reaction, Fmoc-4, was purified by flash column chromatography by elution with 40% ethyl acetate in hexanes using a 20 g BIOTAGE SNAP ULTRA column. The purified Fmoc-4 intermediate (100 mg, 97.6 μmol, 1 eq.) was treated with 20% piperidine/DMF (2.5 mL) and the mixture stirred at RT for 3 h. The reaction mixture was concentrated under vacuum and purified by flash chromatography (20 g BIOTAGE SNAP ULTRA column) using a mobile phase of 10% MeOH in dichloromethane (DCM) with 1% NH₄OH. Fractions containing Compound 4 were pooled and concentrated under vacuum to afford a pale-yellow oil (27.4 mg, 35% yield). ¹H NMR (DMSO-*d*₆): 7.63 – 7.75 (m, 2H), 7.03 – 7.15 (m, 2H), 6.25 – 6.32 (m, 2H), 4.44 – 4.51 (d, 2H), 3.93 – 4.03 (m, 2H), 3.14 – 3.36 (m, 2H), 2.16 – 2.39

(m, 6H), 1.38 – 1.81 (m, 6H), 1.31 – 1.37 (s, 27H), 1.16 – 1.30 (m, 6H), 0.84 – 0.87 (t, 2H). HRMS Calcd for $C_{36}H_{59}IN_4O_8$ (M + H)⁺: 803.34. Found: 803.92.

Tetra-*tert*-butyl (3*S*,7*S*)-12-(4-iodobenzyl)-5,13,19-trioxo-22-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)-4,6,12,18-tetraazadocosane-1,3,7,22-tetracarboxylate (5).—A solution/mixture of DOTAGA tetra *t*-Bu ester (87.3 mg, 125 μ mol, 1 *eq.*, Macrocyclics, Plano, TX), TSTU (37.5 mg, 125 μ mol, 1 *eq.*) and DIPEA (40 μ L, 228 μ mol, 1.83 *eq.*) in DMF (1 mL) was stirred at RT for 1 h. Compound **4** (100 mg, 125 μ mol, 1 *eq.*) dissolved in DMF (1 mL) was added dropwise to the above. The resultant mixture was stirred at RT for 4 h and concentrated under vacuum. The crude product was purified by flash column chromatography using a 20 g BIOTAGE SNAP ULTRA column eluted with 50% ethyl acetate in hexanes to afford Compound **5** (clear oil, 121 mg, 65% yield). ¹H NMR (DMSO-*d*₆): 7.65 – 7.80 (m, 2H), 6.99 – 7.02 (m, 2H), 6.23 – 7.02 (m, 2H), 4.43 – 4.51 (d, 2H), 3.93 – 4.06 (m, 2H), 3.39 – 3.44 (m, 2H), 3.31 (s, 7H), 2.67 – 3.20 (m, 8H), 2.50 – 2.54 (m, 16H), 1.84 – 2.36 (m, 14H), 1.16 – 1.69 (m, 63H), 0.94 – 0.96 (m, 3H). HRMS Calcd for $C_{71}H_{121}IN_8O_{17}$ (M + H)⁺: 1485.79. Found: 1486.11.

Iodinated YF2 standard (6).—Compound **5** (87.3 mg, 74 μ mol) was treated with TFA (1% v/v triethylsilane, 1 mL) at RT for 1 h. The crude product was concentrated under vacuum, and the residue was re-dissolved in 0.2 M NH_4OAc buffer solution (50 mL). A solution of $Lu(NO_3)_3 \cdot xH_2O$ (26 mg, 71 μ mol) in 0.1 M HCl (25 mL) was added to the above. The reaction mixture was stirred at RT for 1 h and then passed through a C18 Sep-Pak cartridge, which was subsequently eluted with 1 mL of methanol. The methanolic solution was concentrated under vacuum and the crude product re-dissolved in 20% acetonitrile in water (1 mL) and purified using Prep-HPLC (described above). The gradient was 15% B to 75% B in 28 min at a 4 mL/min flow rate, and the product was eluted at 8 min, the fractions pooled and lyophilized to yield a white solid with 76% yield (71 mg). ¹H NMR (D_2O): 7.78 – 7.85 (m, 2H), 7.06 – 7.10 (m, 2H), 4.80 (s, 2H), 4.68 (t, 1H), 4.58 (t, 1H), 4.29 – 4.32 (m, 1H), 4.19 – 4.23 (m, 1H), 3.65 (single broad peak, 9H), 3.00 – 3.45 (m, 13H), 2.82 – 2.67 (m, 9H), 2.53 – 2.59 (m, 4H), 2.44 – 2.48 (t, 3H), 2.19 – 2.24 (m, 1H), 1.81 – 2.06 (m, 4H), 1.58 – 1.79 (m, 6H), 1.48 – 1.58 (m, 1H), 1.36 – 1.42 (m, 2H). HRMS Calcd for $C_{43}H_{62}ILuN_8O_{17}$ (M - H)⁻: 1263.27. Found: 1263.68.

YF2 tin precursor (7).—Compound **5** (100 mg, 68 μ mol) was treated with TFA (1% v/v triethylsilane, 1 mL) at RT for 1 h. The crude product (deprotected **5**) was concentrated under vacuum and used without further purification for the next step. DIPEA (129 μ L, 670 μ mol, 10 *eq.*), bis(triphenylphosphine)palladium(II) dichloride (9.4 mg, 13 μ mol, 0.2 *eq.*) and 1,1,1,2,2,2-hexamethyldistannane (220 mg, 670 μ mol, 10 *eq.*) were added to a solution of deprotected **5** (73 mg, 67 μ mol, 1 *eq.*) in DMF (1 mL). The solution was stirred at 100°C for 1 h and concentrated under vacuum. The crude product in methanol (5 mL) was filtered through a bed of Celite. The methanol from the filtrate was removed under vacuum and the residue was re-dissolved in 50 mL of 0.2 M NH_4OAc buffer solution. A solution of $Lu(NO_3)_3 \cdot xH_2O$ (26 mg, 71 μ mol) in 0.1 M HCl (25 mL) was added to the above solution. The reaction mixture was stirred at RT for 1 h, and then passed through a C18 Sep-Pak

cartridge and the cartridge eluted with 1 mL of methanol. The methanol was evaporated under vacuum, the crude product re-dissolved in 20% acetonitrile in water (1 mL), and purified using Prep-HPLC. The gradient was 15% B to 75% B in 28 min at a 4 mL/min flow rate, and the product was eluted at 12 min, the fractions pooled and lyophilized to yield the product as a white solid in 17% yield (15 mg). ¹H NMR (D₂O): 7.79 – 7.86 (m, 2H), 7.04 – 7.08 (m, 2H), 4.80 (s, 2H), 4.68 (t, 1H), 4.58 (t, 1H), 4.29 – 4.32 (m, 1H), 4.19 – 4.23 (m, 1H), 3.65 (single broad peak, 9H), 3.00 – 3.45 (m, 13H), 2.82 – 2.67 (m, 9H), 2.53 – 2.59 (m, 4H), 2.44 – 2.48 (t, 3H), 2.19 – 2.24 (m, 1H), 1.81 – 2.06 (m, 4H), 1.58 – 1.79 (m, 6H), 1.48 – 1.58 (m, 1H), 1.36 – 1.42 (m, 2H), 0.25 (s, 9H). HRMS Calcd for C₄₆H₇₁LuN₈O₁₇Sn (M + H)⁺: 1303.34. Found: 1303.41.

Tri-*tert*-butyl (14*S*,18*S*)-9-(4-iodobenzyl)-2,8,16-trioxo-1-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)-3,9,15,17-tetrazaicosane-14,18,20-tricarboxylate (8).—The synthesis of Compound **8** was accomplished under conditions similar to those for Compound **5** using DOTA-*tert*-Bu ester (71.6 mg, 125 μmol, 1 *eq.*, Macrocyclics, Plano, TX) in lieu of the DOTAGA reagent. The crude product was purified by flash column chromatography (20 g BIOTAGE SNAP ULTRA column) using 50% ethyl acetate in hexanes to afford Compound **8** (clear oil, 113 mg, 67% yield). ¹H NMR (DMSO-*d*₆): 7.65 – 7.80 (m, 2H), 6.99 – 7.02 (m, 2H), 6.23 – 7.02 (m, 2H), 4.43 – 4.51 (d, 2H), 3.93 – 4.06 (m, 2H), 3.39 – 3.44 (m, 2H), 3.32 (s, 6H), 3.29 (s, 2H), 2.67 – 3.20 (m, 8H), 2.50 – 2.54 (m, 10H), 1.84 – 2.36 (m, 14H), 1.16 – 1.69 (m, 54H), 0.94 – 0.96 (m, 3H). HRMS Calcd for C₆₄H₁₀₉IN₈O₁₅ (M + H)⁺: 1356.71. Found: 1357.11.

L3-Lu iodinated standard (9).—This synthesis was accomplished under conditions similar to those for the iodinated YF2 standard. The product was purified using the Prep-HPLC conditions identical to those for Compound **7**, and the product was eluted at 7 min. The fractions were pooled and lyophilized as a white solid in 76% yield (67 mg). ¹H NMR (D₂O): 7.78 – 7.85 (m, 2H), 7.06 – 7.10 (m, 2H), 4.80 (s, 2H), 4.68 (t, 1H), 4.58 (t, 1H), 4.29 – 4.32 (m, 1H), 4.19 – 4.23 (m, 1H), 3.65 (single broad peak, 9H), 3.00 – 3.45 (m, 13H), 2.82 – 2.67 (m, 8H), 2.53 – 2.59 (m, 4H), 2.44 – 2.48 (t, 3H), 1.58 – 1.79 (m, 8H), 1.48 – 1.58 (m, 1H), 1.36 – 1.42 (m, 2H). HRMS Calcd for C₄₀H₅₈ILuN₈O₁₅ (M - H)⁻: 1191.25. Found: 1191.64.

L3-Lu tin precursor (10).—This synthesis was accomplished under conditions similar to those for the YF2 tin precursor. The product was purified using the Prep-HPLC conditions identical to those for Compound **7**, and the product was eluted at 12 min. The fractions were pooled and lyophilized as a white solid in 15% yield (14 mg). ¹H NMR (D₂O): 7.78 – 7.85 (m, 2H), 7.06 – 7.10 (m, 2H), 4.80 (s, 2H), 4.68 (t, 1H), 4.58 (t, 1H), 4.29 – 4.32 (m, 1H), 4.19 – 4.23 (m, 1H), 3.65 (single broad peak, 9H), 3.00 – 3.45 (m, 13H), 2.82 – 2.67 (m, 8H), 2.53 – 2.59 (m, 4H), 2.44 – 2.48 (t, 3H), 1.58 – 1.79 (m, 8H), 1.48 – 1.58 (m, 1H), 1.36 – 1.42 (m, 2H), 0.25 (s, 9H). HRMS Calcd for C₄₃H₆₇LuN₈O₁₅Sn (M + H)⁺: 1230.32. Found: 1230.41.

2.3 Astatine-211 production and measurement

Astatine-211 was produced on the Duke University CS-30 cyclotron via the $^{209}\text{Bi}(\alpha, 2n)^{211}\text{At}$ nuclear reaction, by irradiating natural bismuth metal target with 28 MeV α -particles (20–22). Astatine-211 was isolated from the target by dry distillation and trapped into a solution of *N*-chlorosuccinimide in methanol (NCS, 0.2 mg/mL) as previously described (20–22). The nature of the ^{211}At species present was evaluated using a modification of a previously reported method (23). The NCS/methanol ^{211}At solution was injected onto the RP-HPLC described above; the mobile phase was 5% B from 0 to 15 min at 1 mL/min flow rate. Based on previous experiments (23), At(1), was speculated to be the At^+ species and eluted at 2 min, while At(2) was speculated to be At^- and eluted at 14.5 min.

An aliquot of the solution containing ^{211}At was measured using the dose calibrator (Capintec®) with the ^{133}Xe setting, and then analyzed using a HPGe detector located at the Triangle University Nuclear Laboratory (TUNL) at Duke University. The HPGe detector was calibrated prior to the measurements using a fixed counting geometry. A cadmium plate was placed between the source and the detector to attenuate the X-rays facilitating analysis of higher energy, less abundant γ -rays. Standard reference sources (Eckert & Ziegler), specifically ^{137}Cs , ^{54}Mn , ^{65}Zn , ^{134}Cs , and ^{88}Y , were used to determine counting efficiencies as a function of γ -ray energy. Eckert and Ziegler maintain traceability of these reference sources to the National Institute of Standards and Technology (NIST). The ^{211}At solution was measured using the same geometry with the cadmium plate in place, and counts were accumulated every hour for 24 h. The ^{211}At radioactivity was determined based on the γ -ray emissions at 687.0 keV ($I_g = 0.261\%$) from the decay of ^{211}At and 897.8 keV ($I_g = 0.321\%$) from the decay of the daughter ^{211}Po , and then used as the true value to cross-calibrate the dose calibrators. The half-life of the ^{211}At sample was calculated from HPGe counting data and compared with the value reported in the literature. The ^{210}At radioactivity was determined by measuring the 1181.39 keV γ -ray emission ($I_g = 99\%$).

2.4 Radiochemistry

Two methods were used for the synthesis of ^{211}At]YF2, its radioiodinated analogues $^{125/131}\text{I}$]YF2, as well as ^{211}At]L3-Lu and $^{125/131}\text{I}$]L3-Lu.

Method 1: A solution of ^{211}At in methanol containing NCS (37–740 MBq, 200–500 μL , 0.2 mg/mL NCS) was added to a half-dram glass vial containing 100 μg (equivalent to 77 nmole for the YF2 precursor, and 81 nmole for the L3-Lu precursor) of the YF2 tin precursor, followed by the addition of acetic acid (1% v/v of total reaction volume). The reaction mixture was vortexed for 30 s and left at RT for 30 min. The methanol was removed with a gentle stream of nitrogen, and the residual activity was reconstituted in 20% ACN in water (100 μL). This solution was injected onto the RP-HPLC using the conditions described above, and the gradient was run from 20% B to 40% B over 8 min at 2 mL/min flow rate. The fractions containing ^{211}At]YF2 ($t_R = 2$ min) were collected, diluted with 10 mL of DI water, and passed through a C18 Sep-Pak (C18 Sep-Pak Plus Light®, 130 mg, Waters) cartridge. The ^{211}At]YF2 activity was then eluted from the cartridge using ethanol (500 μL), diluted to 10.5 mL with 10 mL of sterile PBS and passed through a 0.22 μm sterile

syringe filter. For the radioiodinated analogues, Na¹²⁵I or Na¹³¹I (1–10 μ L, 37–370 MBq) was added to a half-dram glass vial containing the YF2 tin precursor (100 μ g, 77 nmole), followed by the addition of 100 μ L of methanol containing NCS (20 μ g) and acetic acid (0.6 μ L). Subsequent steps were as described for [²¹¹At]YF2.

Method 2: The astatodestannylation reaction was run as described for Method 1. Then, *N*-iodosuccinimide (NIS, 1 mg/mL) in methanol was added to the reaction vial (10% of total volume). The reaction mixture was vortexed for 10 s, left at RT for 10 min, diluted with 10 mL of DI water, passed through a C18 Sep-Pak, and the cartridge was eluted with ethanol (500 μ L). A silver-filled cartridge was prepared using an empty SPE column (Agilent empty SPE cartridge with frits, 1 mL) filled with 100 mg of silver powder (99.99%, <250 micron, Sigma-Aldrich) between two 0.2 μ m frits. The [²¹¹At]YF2 in ethanol obtained above was diluted with 2 mL of sterile PBS, and passed through the silver-filled cartridge. The resultant solution was further diluted to 10.5 mL with sterile PBS, and passed through a 0.22 μ m sterile syringe filter. To measure the iodinated YF2 content in the final product, a standard curve was developed by plotting the UV peak area vs. known quantities of iodinated YF2 injected onto the RP-HPLC. For the syntheses of radioiodinated analogues, Na¹²⁵I or Na¹³¹I (1–10 μ L, 37–370 MBq) was added to a half-dram glass vial containing the YF2 tin precursor (100 μ g, 77 nmole), followed by the addition of 100 μ L of methanol containing NCS (20 μ g) and acetic acid (0.6 μ L). Subsequent steps were as described for [²¹¹At]YF2.

Quality control of the radiolabeled compounds was conducted by analyzing an aliquot of the product using the same RP-HPLC system, with the retention times verified by comparison to authentic non-radioactive iodo standards.

2.5 In vitro stability of [²¹¹At]YF2

The [²¹¹At]YF2 produced using Method 2 was formulated in PBS containing sodium ascorbate (25 mg/mL) and 5% (v/v) ethanol at an activity concentration of 74 MBq/mL (2 mCi/mL). This solution was stored at room temperature, and an aliquot was taken and analyzed by RP-HPLC at 6 h and 21 h after [²¹¹At]YF2 formulation using the same gradient as described for quality control. The area under the curve was integrated for all peaks to determine the percentage of ²¹¹At activity present as intact [²¹¹At]YF2 (RCP) at these time points.

2.6 Cell uptake and internalization assay

All cell uptake and internalization assays were performed using ²¹¹At-labeled PSMA inhibitors produced via Method 1. PSMA-positive PC-3 PIP and PSMA-negative PC-3 flu cells were maintained as previously described (24). PC-3 PIP cells were plated in 12-well plates at a density of 1×10^5 per well and incubated overnight. After adding [²¹¹At]YF2 (3.7 kBq/100 μ L) cells were incubated at 37°C for 10 min, with 60 min, 120 min, and 180 min. A parallel experiment was performed at each time point with the addition of 2-PMPA (33 μ M final concentration) to determine specific and non-specific uptake. At each time point, the supernatant was removed followed by two sequential washings using 1 mL PBS. The supernatants were pooled as efflux fractions. The surface-bound activity fractions

were determined by incubating the cells with 1 mL of 50 mM glycine-HCl buffer (pH 2.8) at 37°C for 5 min, removing the supernatant, washing the cells with 1 mL of PBS twice, and pooling the solutions. Finally, the internalized activity fractions were measured by solubilizing cells with 1% SDS cell lysis buffer, followed by washing the wells with PBS. The total uptake, surface-bound fraction and internalized fraction were calculated as percentages of total input activity. The cellular uptake and internalization for [²¹¹At]L3-Lu also was determined as above

2.7 In vitro cytotoxicity studies

All cytotoxicity assays were performed using ²¹¹At-labeled PSMA inhibitors produced via Method 1. The studies were performed using a Cell Proliferation Kit I (MTT, Roche Diagnostics GmbH, Germany). PSMA-positive PC-3 PIP cells were plated in 96-well plates at 5×10^3 per well and incubated overnight. Media were removed from each well and cells were washed three times with 0.1 mL PBS. Cells were then incubated with various concentrations (0–8409.1 kBq/mL; n=5) of [²¹¹At]YF2 in cell culture media at 37°C for 4 h. Media were removed and cells were washed with PBS. Fresh media (100 μ L) and 10 μ L of MTT reagent were added to each well. The plate was incubated at 37°C for 3 h when a purple precipitate was visible. Then, 100 μ L of detergent reagent was added to each well and the plate was left at RT for 2 h in the dark. Absorbance was measured at 570 nm on a microplate reader and results were plotted using GraphPad Prism V9.0.2. The *in vitro* cytotoxicity of [²¹¹At]NaAt and [²¹¹At]L3-Lu were determined in an identical fashion. Nonlinear regression using the least squares fit method was used to process the data to determine IC₅₀ values. The goodness of the fit was expressed as an R-squared value.

2.8 Biodistribution studies

All biodistribution studies were performed using radiohalogenated PSMA inhibitors produced via Method 1. 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. Male athymic mice (4–6 weeks old) were implanted subcutaneously with PSMA⁺ PC-3 PIP cells (3×10^6) in 100 μ L of 1:1 (v/v) Matrigel (Corning, Inc., NY) in cell culture media. Biodistribution experiments commenced when tumor volumes reached ~ 200 mm³. First, a paired-label biodistribution of [¹³¹I]YF2 and [¹²⁵I]L3-Lu was performed. For this, each mouse received ~ 111 kBq each of [¹³¹I]YF2 and [¹²⁵I]L3-Lu via the tail vein. At 1, 2, 4 and 24 h, mice (n=5) were killed by an overdose of isoflurane. Tissues were harvested, blot-dried, weighted, and counted along with injection standards using a dual-channel automated γ -counter with crossover correction capability, allowing simultaneous measurement of ¹²⁵I and ¹³¹I activity levels. The percentage injected dose per gram of tissue (%ID/g) was calculated and presented as mean $\pm$ SD. The biodistribution of [²¹¹At]YF2 was evaluated in the same animal model. Groups of five mice were injected with [²¹¹At]YF2 (~ 185 kBq/mouse) via the tail vein. At 30 min, 1, 4, 8, 16, and 21 h, mice (n=5) were killed and subjected to the same procedures as above. Mice for the 1, 4 and 21 h groups were from one cohort while those for 30 min, 8 and 16 h groups were from a different cohort.

Statistics

Results are presented as mean $\pm$ SD. For experiments done in paired-label format ($[^{131}\text{I}]\text{YF2}$ and $[^{125}\text{I}]\text{L3-Lu}$), a two-tail, paired Student t-test was used to evaluate significance of differences. A P value of <0.05 was considered statistically significant.

3. Results

3.1 Chemistry

Synthetic details are outlined in Schemes 1 and 2. Compound **1** was synthesized using the “Route B” reported in the literature, via the isocyanate intermediate (19). Removal of the benzyl chloroformate protecting group (Cbz) took place via hydrogenolysis using a palladium-carbon catalyst in quantitative yield. The reductive amination of Compound **2** afforded Compound **3** in a 49% yield, with the primary impurity being the product from subsequent reductive amination of **3** with 4-iodobenzaldehydes (speculated based on LC-MS results). Compound **3** was conjugated with 5-(Fmoc-amino)valeric acid using TSTU as a coupling reagent, and the Fmoc-4 intermediate was purified and the Fmoc protective group was removed under basic conditions to give Compound **4** in reasonable yield. Compound **4** served as a common intermediate for synthesizing both YF2 and L3-Lu analogues, with Compound **5** (intermediate for YF2 analogues) produced using DOTAGA conjugation while Compound **8** (intermediate for L3-Lu analogues) used DOTA. The reaction conditions for both coupling reactions were identical and both **5** and **8** were obtained in $>65\%$ yields. Deprotection of Compound **5** using TFA with triethylsilane as a scavenger and the complexation of the crude product with Lu^{3+} in situ afforded the iodinated YF2 standard (**6**) after HPLC purification. For the synthesis of the YF2 tin precursor, the crude deprotected **5** was treated with 1,1,1,2,2,2-hexamethyldistannane using a palladium(II) catalyst under basic conditions via the addition of DIPEA. The crude product, isolated after the removal of DMF, subjected to complexation with lutetium in situ. After Prep-HPLC purification, YF2 tin precursor was obtained in 17% yield and $>95\%$ purity. Alternatively, the YF2 tin precursor could be synthesized by conducting stannylation after the Lu-DOTA complexation. The syntheses of the iodinated L3-Lu standard (**9**) and L3-Lu tin precursor (**10**) were carried out similarly.

3.2 Astatine-211 production and measurement

A procedure to measure ^{211}At radioactivity using an HPGe detector was implemented and dose calibrators were calibrated based on the HPGe data. Using the ^{133}Xe setting on the dose calibrators, the measured radioactivity of the ^{211}At sample was multiplied by the calibration factor 2.3 to obtain the ^{211}At activity level, which was within $\pm 5\%$ of the ^{211}At radioactivity value acquired using the HPGe detector. The half-life of the ^{211}At was determined to be 7.22 ± 0.08 h, in good agreement with the literature value. The ^{210}At content was 3.0 ± 0.7 parts per million (ppm) of ^{211}At . The primary species of ^{211}At present in the NCS methanolic solution was determined to be At(1) in a RCP $>90\%$ ($n>3$).

3.3 Radiochemistry

The synthesis of [^{211}At]YF2 is described in Scheme 3. Radiolabeling of the YF2 tin precursor with ^{211}At was conducted in one step in $86 \pm 7\%$ RCY ($n=10$, decay-corrected) using Method 1. The RCY using this approach for [^{211}At]L3-Lu was essentially identical ($87 \pm 5\%$; $n=7$, decay-corrected) and labeling both tin precursors with ^{125}I and ^{131}I generally gave similar RCYs (80–85%, decay-corrected). To date, a maximum of 666 MBq (18 mCi) of [^{211}At]YF2 has been produced using Method 1 starting from 851 MBq (23 mCi) of ^{211}At with an RCP >95%. With Method 1, any remaining YF2 tin precursor was separated from the [^{211}At]YF2 product via RP-HPLC purification. Because no iodinated YF2 was detected by HPLC, the preparation was assumed to be at a no-carrier-added (or detected) level (Fig. S1 Supplementary Data). The retention time of [^{211}At]YF2 was 2.3 min, the same as that observed for the iodinated YF2 standard, while the retention time of YF2 tin precursor was 4.6 min. In the case of L3-Lu analogues, the iodinated standard and radiolabeled analogue had a retention time of 3.2 min while the L3-Lu tin precursor eluted at 5.6 min.

Method 2 was developed to avoid the need for RP-HPLC, which could be logistically advantageous under some circumstances. Compared with Method 1, a slightly lower RCY ($84 \pm 6\%$; $n=10$, decay-corrected) was obtained but RCP was still >95%. In Method 2, an excess of NIS was added after the radiolabeling to convert any unreacted YF2 tin precursor to iodinated YF2. Although the unreacted NCS and NIS were removed during the Sep-Pak purification, the iodinated YF2 was eluted along with the [^{211}At]YF2. Hence, the [^{211}At]YF2 obtained via Method 2 had iodinated YF2 as a pseudo carrier. Based on the standard curve, the iodinated YF2 content in the [^{211}At]YF2 product was determined to be $9 \pm 1 \mu\text{g}$ ($n=4$) in 10.5 mL of final formulation. Residual NCS and NIS were not detected in the quality control HPLC profiles. The silver-filled column was included to remove residual unreacted ^{211}At , which eluted with the [^{211}At]YF2 from the Sep-Pak cartridge. However, 5–10% of the [^{211}At]YF2 also was absorbed onto the silver, accounting for the lower RCY compared with Method 1. The total radiolabeling time for both methods was an hour.

For both of these methods, a similar RCY was obtained when using freshly distilled ^{211}At or when using ^{211}At in methanol/NCS that was allowed to remain at RT for more than 8 h. Excellent *in vitro* stability was seen for [^{211}At]YF2 at an activity concentration of 74 MBq/mL (2 mCi/mL), with a RCP >95% retained at 6 and 21 h. The RP-HPLC chromatograms from the stability study are shown in Fig. S2.

3.4 Cell uptake and internalization assay

Incubation of [^{211}At]YF2 with PSMA⁺ PC-3 PIP cells at 37°C resulted in 13.0% of input radioactivity cell bound after 10 min, which increased to 36.0%, 46.1%, and 46.8% at 1, 2, and 3 h, respectively (Fig. 1A). The cell-bound fraction of [^{211}At]YF2 was more than 2-fold higher than that of [^{211}At]L3-Lu at 10 min (4.6%), increasing to 4-fold higher at later times (Fig. 1B). The internalized fractions of [^{211}At]YF2 were 2.8%, 9.0%, 13.8% and 15.3% at 10 min, 1, 2, and 3 h, respectively (Fig. 1A), levels that were 4- to 6-fold higher than those for [^{211}At]L3-Lu (Fig. 1B).

3.5 In vitro cytotoxicity studies

PSMA-specific cell killing efficacy was observed when PSMA⁺ PC-3 PIP cells were incubated with [²¹¹At]YF2 (Fig. 2). An IC₅₀ of 725.5 kBq/mL was calculated for [²¹¹At]YF2 via a non-linear regression ($R^2=0.9915$). The IC₅₀ for [²¹¹At]L3-Lu was 4670.0 kBq/mL ($R^2=0.9630$). Thus, [²¹¹At]YF2 showed significantly higher *in vitro* cytotoxicity compared to [²¹¹At]L3-Lu ($P<0.0001$). No significant decrease in cell viability was seen for the nonspecific control, [²¹¹At]NaAt, even at its highest activity concentration.

3.6 Biodistribution studies

A paired-label study was performed to directly compare the *in vivo* behavior of [¹³¹I]YF2 and [¹²⁵I]L3-Lu. Both agents showed fast tumor uptake with $9.9 \pm 5.0\%$ ID/g for [¹²⁵I]L3-Lu and $10.4 \pm 5.3\%$ ID/g for [¹³¹I]YF2 at 1 h (Fig. 3). Maximum tumor uptake occurred at 2 h for both agents – $12.4 \pm 3.8\%$ ID/g for [¹²⁵I]L3-Lu and $16.8 \pm 5.2\%$ ID/g for [¹³¹I]YF2. Differences in tumor uptake were not statistically significant, except at 24 h ($P=0.0275$). The paired-label format allowed calculation of tumor uptake ratios of [¹³¹I]YF2 to [¹²⁵I]L3-in the same animal. The [¹³¹I]YF2:[¹²⁵I]L3-Lu tumor uptake ratios were $1.4 \pm 0.0\%$, $1.4 \pm 0.1\%$, and $2.3 \pm 0.4\%$ at 2, 4, and 24 h, respectively (Fig. 3). Both agents were excreted primarily through the kidneys, but [¹²⁵I]L3-Lu displayed faster clearance and lower renal retention. The kidney uptake of [¹²⁵I]L3-Lu declined from $84.6 \pm 38.1\%$ at 1 h to $1.2 \pm 0.7\%$ ID/g at 24 h compared with values of $167.8 \pm 32.0\%$ ID/g and $53.3 \pm 46.4\%$ ID/g, respectively, for [¹³¹I]YF2 ($P<0.05$) (Fig. 3). Low activity levels were observed for both agents in other normal tissues including salivary and lacrimal glands as well as stomach and thyroid, indicating high *in vivo* stability (Table S1).

The biodistribution of [²¹¹At]YF2 was in mice with subcutaneous PSMA⁺ PC3 PIP xenografts and the results are summarized in Fig. 4 and Table S2. Tumor uptake was maintained at a level of ~15–20% ID/g from 1–21 h, a period covering 3 half-lives of ²¹¹At. Kidney uptake was highest at 1 h ($163.9 \pm 34.9\%$ ID/g) and declined to $10.8 \pm 6.5\%$ ID/g at 16 h. No significant uptake was observed in other normal tissues. Notably, activity levels of ²¹¹At in thyroid and stomach were quite low, suggesting only minimal dehalogenation had occurred *in vivo*. Because the biodistribution of [²¹¹At]L3-Lu had previously had only been evaluated in NOD SCID mice (16), its biodistribution was repeated in the current study in athymic mice to facilitate comparison of the two agents. As shown in Fig. S4, the *in vivo* behavior of [²¹¹At]L3-Lu was comparable to that of its radioiodinated analogue (Table S1). Both the tumor and kidney uptake levels observed in athymic mice for [²¹¹At]L3-Lu were consistently lower than those reported previously when the study was performed in NOD SCID mice (Fig. S4).

4. Discussion

In this work, we introduced [²¹¹At]YF2, a closely related analogue to [²¹¹At]L3-Lu, and compared the two PSMA inhibitors head-to-head. In addition to comparing their properties, our goal was to improve the radiolabeling chemistry of both agents to lay the foundation for evaluating one or both of these agents clinically.

Robust production methodologies for RPT agents are necessary not only for supporting clinical studies, but also to enable preclinical dose-escalation efficacy and toxicity assessment, which are an important part of IND-enabling studies. This can be problematic for TAT agents because of the radiolysis resultant from the high radiation dose induced by α -particles, which complicates their clinical translation. For example, the total production time for [^{225}Ac]Ac-PSMA I&T was about 1.5 days, and the final product formulation consisted of 1 M ascorbate and 100 μg of PSMA I&T mass to stabilize the active ingredient, [^{225}Ac]Ac-PSMA I&T (11). That required dilution of the product with saline just before administration to avoid excessive osmolality and the shelf life of the final formulation was only about 3 h (11). Likewise, limitations in radiolabeling chemistry of [^{211}At]L3-Lu in terms of duration of process and yield hindered its preclinical evaluation when prepared more than a half-life after ^{211}At production.

Previously, [^{211}At]L3-Lu was produced with $49.6 \pm 6.8\%$ ($n=3$) RCY using freshly produced ^{211}At , while the RCY decreased to $\sim 20.8\%$ when labeling was done 9 h after ^{211}At production (16). Total production time was long, about 3 h, and the highest amount of [^{211}At]L3-Lu produced was ~ 185 MBq (5 mCi) (16). A problem with the radiolabeling chemistry was that all the carboxyl groups in the precursor were protected as their *t*-butyl esters, which necessitated a deprotection step before complexing with the Lu^{3+} ion. The two additional steps and HPLC purification compromised the overall RCY motivating the development of a new radiolabeling process in which the precursor had the Lu^{3+} metal ion incorporated, permitting radiolabeling to be done in one step.

Because of their structural similarity, the YF2 and L3-Lu tin precursors were synthesized in the same way except using a different chelator (DOTA for L3-Lu, DOTAGA for YF2). Converting compounds **5** and **8** to the respective tin precursors **7** and **10** was a critical step due to the instability of the C-Sn bond to acidic conditions. The presence of multiple acidic carboxylic acid functions in these compounds thus may not be conducive for stannylation. To circumvent this, after removal of *t*-Bu ester groups by treatment with TFA, it was necessary to remove the TFA residue completely and perform the stannylation in the presence of a base. Care must be taken when performing LC purification of the YF2 tin precursor as it could deteriorate in aqueous solution at RT, especially at lower pH (<4). Radiolabeling of [^{211}At]YF2 and [^{211}At]L3-Lu from their respective tin precursor already complexed with Lu^{3+} was performed in a single step with significantly improved RCY allowing the total synthesis time to be reduced from 3 h to 1 h. A maximum of 666 MBq (18 mCi) of [^{211}At]YF2 was produced starting from 740 MBq (20 mCi) of ^{211}At using Method 1, which was more than 3-fold higher than previously reported for [^{211}At]L3-Lu starting with higher ^{211}At activity levels (16). Similar scaled up yields with this labeling strategy would be expected for [^{211}At]L3-Lu.

Although Method 1 permitted production of clinically relevant levels of [^{211}At]YF2 in less than 1 h, we also wanted to develop a method that did not require HPLC purification at all. Our objective was to make the procedure more amenable to automation and make [^{211}At]YF2 production possible at locations without HPLC equipment, facilitating its clinical translation. To accomplish this, Method 2 was developed, which used NIS as a quencher to convert any remaining tin precursor to its non-radioactive iodo-analogue. With

this procedure, the need for HPLC purification to remove unreacted tin precursor was avoided. The unreacted ^{211}At along with small-molecule impurities such as NCS, NIS, and acetic acid, were removed using a C18 Sep-Pak cartridge. However, about 5% of unreacted ^{211}At co-eluted with ^{211}At YF2 its iodo-analogue. Inspired by the natural affinity between silver and halides and the use of tellurium in ^{211}At purification (25), a column of silver was used to remove the remaining free ^{211}At . Although some ^{211}At YF2 also bound to the silver column, this method still resulted in RCYs that were similar to those obtained using Method 1. The maximum amount of ^{211}At YF2 produced using Method 2 was 370 MBq (10 mCi) starting from 518 MBq (14 mCi) ^{211}At .

A potentially important difference between ^{211}At YF2 prepared via the two methods is that the generation of iodo YF2 in Method 2 results in a lower effective molar activity. In Method 2, the amount of iodo YF2 in the product was determined to be $9 \pm 1 \mu\text{g}$ (7 nmol) with the level not dependent on the ^{211}At activity level for 4 batches ranging from 74 to 298 MBq (2 to 8 mCi) of ^{211}At YF2. That gave a molar activity range of 11 to 44 GBq/ μmol . On the other hand, Method 1 produced ^{211}At YF2 without any iodo analogue generated and no signs of YF2 tin precursor or other impurities detected in the final product. Therefore, its molar activity is considered to be the theoretical value of 16,100 GBq/ μmol . For comparison, molar activities of 48 GBq/ μmol and 88 GBq/ μmol were reported for ^{177}Lu Lu-PSMA-617 (Pluvicto[®]) for its synthesis starting with carrier-added ^{177}Lu and no-carrier-added ^{177}Lu , respectively (26–29). We note that each of the six 7.4 GBq clinical doses of Pluvicto[®] thus contain 84 nmole or 154 nmole of carrier, which is more than 10- to 20-fold higher obtained for ^{211}At YF2 produced using Method 2.

Many different scaffolds have been evaluated for PSMA-targeted RLT with PSMA-617 and PSMA I&T labeled with radiometals receiving the widest attention in the clinical domain (30) and thus useful benchmarks for further research. However, it is difficult and perhaps misleading to compare biodistribution data reported for these and other PSMA-targeted agents because mouse strain, xenograft characteristics and molar activity of the labeled compound can influence biodistribution (16, 31). For example, as illustrated in Fig. S4, the tissue distribution of ^{211}At L3-Lu in athymic and SCID mice was significantly different even though the same xenograft model was used in both cases. Fortuitously, Banerjee et al. conducted head-to-head comparisons of different PSMA-targeted scaffolds labeled with ^{177}Lu , including PSMA-617 and PSMA I&T, as well as ^{177}Lu -L9 (an ^{211}At YF2 analogue in which ^{177}Lu was used instead of unlabeled lutetium and bromine was used instead of ^{211}At), and ^{177}Lu -L1 (an ^{211}At L3-Lu analogue in which unlabeled lutetium was replaced with ^{177}Lu and ^{211}At with bromine) (32). Tumor uptake of ^{177}Lu Lu-PSMA-617, ^{177}Lu Lu-PSMA I&T and ^{177}Lu -L1 was similar, while ^{177}Lu -L9 showed significantly higher tumor uptake and retention (32). However, ^{177}Lu -L9 exhibited significantly higher kidney levels than ^{177}Lu Lu-PSMA-617 and ^{177}Lu -L1, but cleared faster than ^{177}Lu Lu-PSMA I&T (32).

Those results provided motivation to synthesize and evaluate ^{211}At YF2 as an ^{211}At -labeled analogue of ^{177}Lu -L9 with the expectation that ^{211}At YF2 should have improved tumor targeting over that of ^{177}Lu Lu-PSMA-617 and ^{211}At L3-Lu, and lower renal absorbed dose than would be likely with a PSMA I&T analogue. As expected, high *in vitro* cell

uptake and internalization were seen for [^{211}At]YF2, with as much as a 4-fold higher internalized fraction than that observed with [^{211}At]L3-Lu. That was consistent with *in vitro* results showing higher cell uptake and internalization for ^{177}Lu -L9 (^{177}Lu -labeled analogue of [^{211}At]YF2) compared to ^{177}Lu -L1 (32). Consequently, the IC_{50} for [^{211}At]YF2 in the *in vitro* cytotoxicity assay was more than 6-fold lower than that observed for [^{211}At]L3-Lu, indicating that [^{211}At]YF2 is a much more potent cytotoxic agent. Because [^{211}At]YF2 has an extra $-\text{COOH}$ function group in its structure compared with [^{211}At]L3-Lu, we speculate that the additional negative charge likely resulted in its enhanced cellular internalization and retention. No decrease in cell viability was seen for [^{211}At]NaAt at the highest radioactivity concentration evaluated, confirming that the cytotoxicity of [^{211}At]YF2 and [^{211}At]L3-Lu was PSMA-specific. Microdosimetry calculations predict that α -particle emitters are more cytotoxic when they are internalized and closer to the cell nucleus, and our results with [^{211}At]YF2 and [^{211}At]L3-Lu provide further evidence that that is the case (33–35).

We plan to focus future development on [^{211}At]YF2 in order to exploit its significantly higher tumor uptake and retention compared with [^{211}At]L3-Lu. It is also noteworthy that in a paired-label biodistribution study (not possible with astatine), [^{131}I]YF2 had 1.4-fold and >2-fold higher tumor uptake than [^{125}I]L3-Lu at 2 and 24 h post-injection, respectively. On the other hand, both [^{211}At]YF2 and [^{131}I]YF2 had considerably higher kidney uptake and retention than the corresponding L3-Lu agents. This was likely due to the difference in the total charge between the DOTA and DOTAGA moiety, where the one additional negative charge of the DOTAGA moiety likely contributed to the higher kidney uptake and retention. This is supported by similar differences in the renal activity levels observed for ^{177}Lu -labeled PSMA-617 and PSMA I&T (18) bearing DOTA and DOTAGA moieties, respectively. Spleen, salivary glands and lacrimal glands are other PSMA-expressing normal tissues (32). The slightly elevated accumulation of YF2 analogues in these tissues were consistent with the distributions observed for ^{177}Lu -PSMA-I&T and ^{68}Ga -PSMA-I&T which also contain a DOTAGA chelate (32, 36–37). In addition, free iodide and astatide accumulate in the salivary and lacrimal glands (38–39), so if any was present as an impurity in the preparation or generated *in vivo*, this could elevate activity levels in salivary and lacrimal glands. This problem can be circumvented by administering blocking agents such as Cytomel and potassium iodide which is done routinely in patients receiving ^{211}At -labeled TAT agents (40) but was not done in these preclinical studies.

Because of differences in PSMA expression in humans and mice, it is not certain that the high renal levels observed in mice for [^{211}At]YF2 is a valid harbinger of a high potential for renal toxicity in patients. That is supported by two head-to-head comparisons of [^{177}Lu]Lu-PSMA-617 and [^{177}Lu]Lu-PSMA I&T – one in mice and the other, in patients – with different conclusions about the renal uptake and retention of the two agents. Ruigrok et al. reported that in mice [^{177}Lu]Lu-PSMA I&T showed a ~40-fold higher peak kidney uptake than that of [^{177}Lu]Lu-PSMA-617, resulting in a ~30-fold higher cumulative kidney activity for [^{177}Lu]Lu-PSMA I&T (18). In contrast, a clinical dosimetry study comparing the two agents determined that the median kidney absorbed dose for [^{177}Lu]Lu-PSMA I&T was 0.73 Gy/GBq compared with 0.49 Gy/GBq for [^{177}Lu]Lu-PSMA-617 (17). Thus, the factor of 30 higher kidney radiation dose for [^{177}Lu]Lu-PSMA I&T predicted in mice was actually only a factor of about 1.5 in patients.

Potential limitations of murine data aside, efforts to better understand and address the potential for renal toxicity will be critical to the success of RPT in prostate cancer treatment. Signs of potential long-term renal toxicity have been reported especially in patients who received more than 4 cycles of [^{177}Lu]Lu-PSMA RLT (41). Due to the lack of comprehensive clinical data, current dosimetry guidelines for PSMA-targeted RPT are derived from external beam radiation treatment, which set 23 Gy as the critical renal radiation dose limit (42). That benchmark is probably of limited relevance to RPT because of intrinsic differences between the two modalities in critical parameters such as dose rate and homogeneity (35). The European guidelines suggest a 40 Gy biological effective dose as the threshold for renal toxicity for [^{177}Lu]Lu-PSMA based on clinical experience in Lutathera[®] (43). As more extensive clinical experience with PSMA-targeted radiotherapeutics becomes available, it is expected that more realistic guidelines for renal toxicity thresholds will continue to emerge.

5. Conclusion

In summary, a low-molecular-weight PSMA-inhibitor, [^{211}At]YF2, was synthesized and studies on PSMA⁺ PC-3 PIP cells demonstrated that this agent had superior cellular uptake, internalization and cytotoxicity, as well as higher in vivo accumulation in xenografts compared with our previous lead, [^{211}At]L3-Lu. The radiolabeling chemistry for both ^{211}At -labeled PSMA inhibitors was optimized and streamlined such that current production capabilities are sufficient for clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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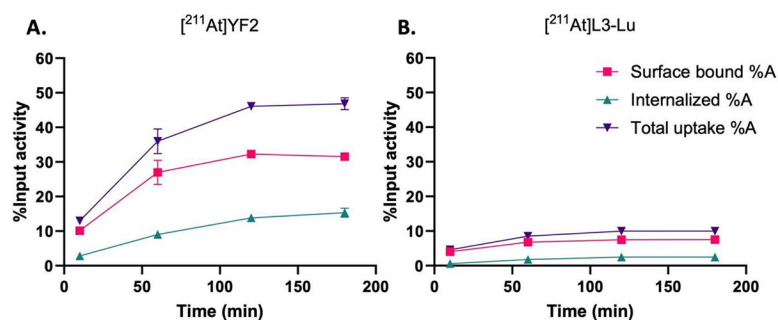


Figure 1. Cell uptake and internalization of $[^{211}\text{At}]\text{YF2}$ (A) and $[^{211}\text{At}]\text{L3-Lu}$ (B) on PSMA+ PC-3 PIP cells. The activity associated with each fraction is reported as percentage of input activity (%A).

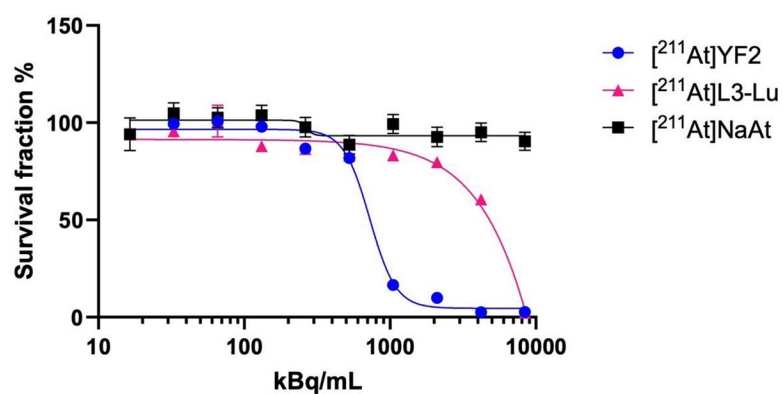


Figure 2.

Cytotoxicity of [²¹¹At]YF2, [²¹¹At]L3-Lu and [²¹¹At]NaAt on PSMA+ PC-3 PIP cells determined by MTT assay. Cell viability was measured and expressed as fractions of the total cell number. IC₅₀ values were calculated using nonlinear regression fit. Data were processed in GraphPad Prism 9.0.2.

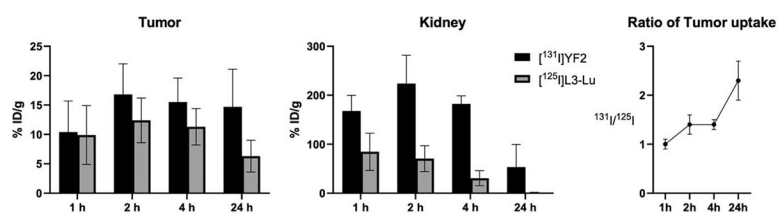


Figure 3. Paired-label biodistribution of [¹³¹I]YF2 and [¹²⁵I]L3-Lu in male athymic mice bearing subcutaneous PSMA+ PC-3 PIP xenografts. Data are presented as percent injected dose per gram of tissue (%ID/g) (n=5). The ratio of tumor uptake was calculated by averaging the [¹³¹I]YF2 -to-[¹²⁵I]L3-Lu tumor uptake in each mouse.

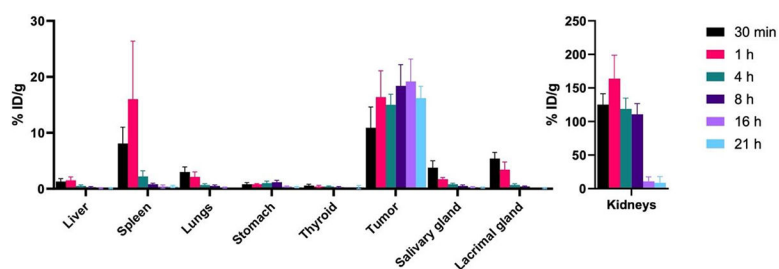


Figure 4. Biodistribution of $[^{211}\text{At}]\text{YF2}$ in male athymic mice bearing subcutaneous PSMA+ PC-3 PIP xenografts. Data were presented as percent injected dose per gram of tissue (%ID/g). (n=5).

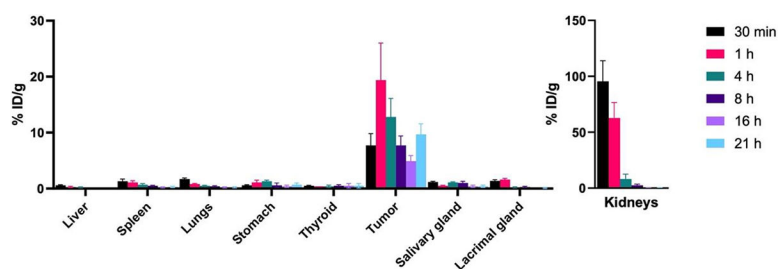
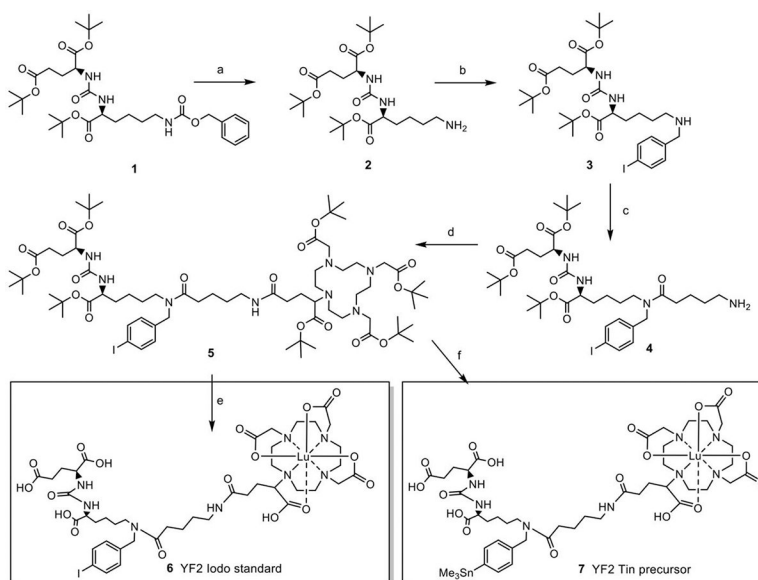


Figure 5. Biodistribution of $[^{211}\text{At}]\text{L3-Lu}$ in male athymic mice bearing subcutaneous PSMA+ PC-3 PIP xenografts. Data are presented as percent injected dose per gram of tissue (%ID/g). (n=5).

**Scheme 1.**

Synthesis of the iodinated YF2 standard and YF2 tin precursor.

a: Ammonium formate, 10% Pd-C, ethanol

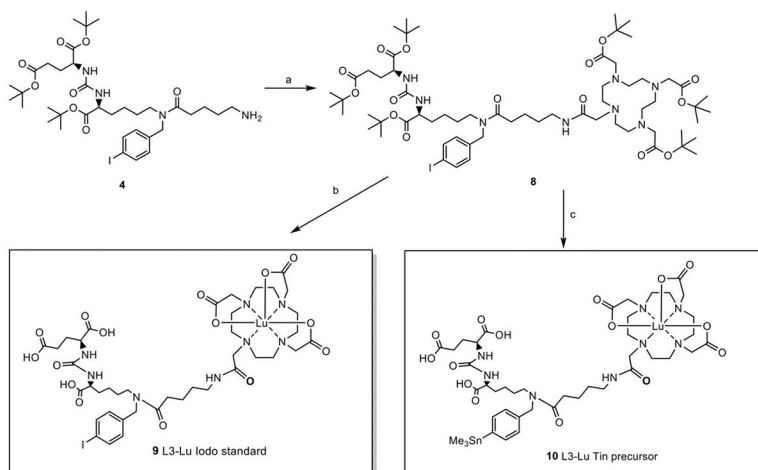
b: 4-Iodobenzaldehyde, sodium cyanoborohydride, methanol

c: i, 5-(Fmoc-amino)valeric acid, TSTU, DIPEA, DMF; ii, 20% piperidine/DMF

d: DOTAGA tetra(t-Bu ester), TSTU, DIPEA, DMF

e: i, TFA, TES; ii, $\text{Lu}(\text{NO}_3)_3 \cdot x\text{H}_2\text{O}$, 0.2 M NH_4OAc

f: i, TFA, TES; ii, bis(triphenylphosphine)palladium(II) dichloride, 1,1,1,2,2,2-hexamethyldistannane, DIPEA, DMF, 100 °C, 1 h

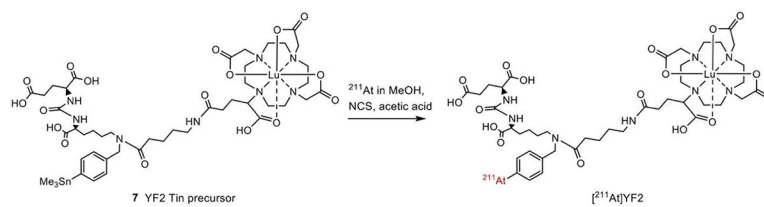
**Scheme 2.**

Synthesis of the iodinated L3-Lu standard and L3-Lu tin precursor.

a: DOTA tri(t-Bu ester), TSTU, DIPEA, DMF

b: i, TFA, TES; ii, $\text{Lu}(\text{NO}_3)_3 \cdot x\text{H}_2\text{O}$, 0.2 M NH_4OAc

c: i, TFA, TES; ii, bis(triphenylphosphine)palladium(II) dichloride, 1,1,1,2,2,2-hexamethyldistannane, DIPEA, DMF, 100 °C, 1 h



Scheme 3.
Synthesis of [^{211}At]YF2 from its tin precursor.