1 The pre-clinical characterization of an alpha-emitting sigma-2 receptor targeted
2 radiotherapeutic

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4 Authors: ¹Mehran Makvandi, ¹Brian P. Lieberman, ¹Ben LeGeyt, ¹Catherine Hou, ¹David
5 A. Mankoff, ¹Robert H. Mach, and ¹Daniel A. Pryma

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7 Abbreviated Title: ²¹¹At-MM3, an alpha-emitting therapeutic

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9 Affiliations: 1. University of Pennsylvania, Department of Radiology, Division of Nuclear
10 Medicine and Clinical Molecular Imaging, Radiological Chemistry and Biology
11 Laboratories

12

13 Corresponding Author: Daniel A. Pryma, MD

14 Address: 231 S. 34 Street Philadelphia, PA 19104

15 Telephone: (215)349-5272

16 Fax: 215-349-5843

17 Email: Daniel.Pryma@uphs.upenn.edu

18

19 First Author: Mehran Makvandi, PharmD, Postdoctoral Research Fellow

20 Address: 231 S. 34 Street Philadelphia, PA 19104

21 Telephone: (215)746-8755

22 Fax: 215-349-5843

23 Email: makvandi@mail.med.upenn.edu

24

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31 **Key Words:** sigma-2, astatine-211, radiotherapeutic, alpha-emitter, targeted alpha
32 therapy

33 **Abstract**

34 Rationale: The sigma-2 receptor is a protein with a Heme binding region and is capable
35 of receptor-mediated endocytosis. It is overexpressed in many cancers making it a
36 potential vector for therapeutic drug delivery. Our objective was to introduce an alpha-
37 emitting radionuclide, astatine-211, into a selective sigma-2 ligand moiety to provide
38 cytotoxic capabilities without adversely altering the pharmacological characteristics. In
39 this study we investigated the in vitro/in vivo tumor targeting and estimated dosimetry of
40 alpha-emitting sigma-2 ligand, 5-(astato-²¹¹At)-N-(4-(6,7-dimethoxy-3,4-
41 dihydroisoquinolin-2(1H)-yl)butyl)-2,3-dimethoxybenzamide (²¹¹At-MM3), in a pre-clinical
42 human breast cancer model.

43 Methods: Astatine-211 was produced in a cyclotron and isolated by dry distillation.
44 Radiosynthesis of ²¹¹At-MM3 was performed using a tin precursor through
45 radioastatodestannylation. In vitro sigma-2 binding experiments using ²¹¹At-MM3 were
46 carried out in live EMT6 and MDA-MB-231 breast cancer cells and liver homogenate
47 tissue. In vivo biodistribution experiments were performed using EMT-6 mouse breast
48 cancer cells in BALB/c female mice. Approximately 370 kBq of ²¹¹At-MM3 was
49 administered intravenously and at time points of 5 min, 1, 2, 4, 8, and 24 hrs
50 organs/tissue were harvested. Estimated human dosimetry was extrapolated from
51 biodistribution data using OLINDA/EXM (VU e-Innovations).

52 Results: Astatine-211 was successfully produced and isolated in quantities suitable for in
53 vitro and small animal in vivo experiments. Radiosynthesis of ²¹¹At-MM3 was
54 reproducible with high radiochemical purity. Astatine-211-MM3 exhibited picomolar
55 affinity to the sigma-2 receptor in contrast to the iodinated analog that had nanomolar
56 affinity. Prolonged tumor targeting was measured through biodistribution studies with a
57 maximal tumor to muscle ratio of 9.02 at 4 hrs. Estimated human dosimetry revealed

58 doses of up to 370 MBq in an adult female patient were below organ radiation limits with
59 the potential to provide a high therapeutic dose to tumors.

60 Conclusion: The sigma-2 receptor could serve as a suitable targeting platform for
61 designing radiotherapeutics. ²¹¹At-MM3 showed tumor targeting properties in vitro/in vivo
62 and favorable estimated human dosimetry establishing the proof of concept for future
63 development as a radiotherapeutic for the treatment of breast cancer.

64

65 **Introduction:**

66 The sigma-2 receptor is a promising target for cancer therapy due to its
67 overexpression in multiple malignancies including: breast, pancreatic, ovarian, lung,
68 renal, melanoma, neuroendocrine and colon.^{1,2} The molecular function of the sigma-2
69 receptor has been defined through pharmacological studies that have validated it as a
70 biomarker that can be utilized to non-invasively measure the proliferative status of
71 tumors.³⁻⁵ During cell proliferation the sigma-2 receptor is overexpressed by a factor of 10
72 compared with quiescent cells.³ Confocal microscopy studies have shown the subcellular
73 localization of sigma-2 receptors on cell membrane, lysosomes, endoplasmic reticulum,
74 and mitochondria.⁶ Sigma-2 ligands, classified as azabicyclononane derivatives, have
75 been shown to undergo rapid internalization and are capable of inducing caspase
76 dependent and independent cell death pathways.⁶⁻⁸ More recently, the molecular identity
77 of the sigma-2 receptor was identified as the progesterone receptor membrane
78 component 1 (PGRMC1), providing new insight and potential for further defining its
79 biological, pharmacological and molecular properties.⁹ Three classes of sigma-2 ligands
80 have been characterized pharmacologically through caspase-3/7 activation as agonist,
81 partial agonist or antagonist.¹⁰ Caspases are a group of proteolytic enzymes that cleave
82 proteins during apoptosis and other cell death pathways. Azabicyclononane and tropane
83 analogs induce or partially induce downstream caspase 3/7 activation, and therefore are
84 considered agonist or partial agonist to the sigma-2 receptor. The benzamide class of
85 ligands act as antagonists, unable to induce caspase activity, and are believed to interact
86 with binding sites on the surface membranes, although passive diffusion across the cell
87 membrane has not been evaluated. Sigma-2 ligands are suitable as diagnostic imaging
88 agents and therapeutics due to selective tumor targeting observed in vivo.¹¹⁻¹³

89 Numerous selective sigma-2 ligands have been reported;¹⁴⁻¹⁶ however, ISO-1 is
90 the only diagnostic imaging agent used clinically in a research setting.¹⁷ Other sigma-2
91 ligands have been applied in chemosensitization and the targeted delivery of cancer
92 therapeutics showing a beneficial therapeutic response in pre-clinical models.¹⁸⁻²²

93 Currently, only sigma-2 agonist conjugates have been used as non-radiolabeled
94 therapeutics, although non-selective sigma ligands containing therapeutic radionuclides
95 have been reported.^{23,24} This is most likely due to the lack of cytotoxicity observed with
96 sigma-2 antagonists and a lack of evidence to support that antagonists are internalized,
97 which is an important characteristic of a therapeutic to reach the site of action. By using
98 an iodinated sigma-2 antagonist RHM-4, a ligand similar to ISO-1 (see **Figure 1**), we
99 intended to afford cytotoxicity through the introduction of astatine-211 (²¹¹At) to produce
100 5-(astato-²¹¹At)-N-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-2,3-
101 dimethoxybenzamide (²¹¹At-MM3).

102 Astatine-211 is a radionuclide that decays through the emission of a high-energy
103 alpha particle and has a half-life of 7.21 hr.²⁵ Alpha-particles travel approximately 2-3 cell
104 diameters (50-100 µm) and cause dense ionizations along the track resulting in clustered
105 DNA damage capable of inducing cell death.²⁶ The short path-length of an alpha particle
106 also translates to a highly specific cell killing capability. Cells only within immediate
107 proximity to the radioactive decay event are affected. It has been proposed that as few as
108 10 alpha particles traversing a cell have a high probability of inducing cell death.²⁷
109 Currently, radium-223 (²²³Ra), an alpha emitter and calcium mimetic, has been shown to
110 improve overall survival in metastatic castrate resistant prostate cancer validating the
111 clinical utility of targeted alpha therapy.²⁸ Although there has been a renewed interest in
112 applying alpha-emitting radionuclides in therapy, production and supply of isotopes make
113 developmental research efforts difficult.²⁵ The current global production of astatine-211 is
114 low; however, attributes like the method of production, half-life and chemistry, make it a
115 feasible radionuclide with high potential for global application in therapy.²⁵ Alpha-emitting
116 radionuclides have the potential for new therapeutic drug development and can result in
117 new treatments to combat cancer. One of the potential advantages of ²¹¹At compared to
118 other alpha-emitters is a relatively straightforward decay scheme and half-life well suited
119 to fast targeting characteristics of small molecules whereas many other alpha emitters

120 have multi-day half-lives and long lived daughters that could complicate targeting and
121 dose distribution.

122 The dosimetry of alpha-emitting therapeutics plays an important role in
123 evaluating whether the radiotherapeutic will be safe and effective in a clinical setting.
124 Microdosimetric calculations are required for accurate dose estimates if non-uniform
125 tissue distribution is expected due to alpha particles short range in tissue and stochastic
126 interactions with biological matter.²⁹ When homogenous organ/tissue distribution is
127 expected standard dosimetry can be used. OLINDA/EXM 1.1 is an FDA approved
128 software package that is utilized in this study to estimate human dosimetry from animal
129 data.³⁰

130 In this study we investigated the in vitro/in vivo properties and estimated human
131 dosimetry of an astatinated sigma-2 ligand, ²¹¹At-MM3, in a breast cancer model as a
132 proof of concept to show retained selective sigma-2 binding and in vivo tumor targeting.
133 We hypothesize the substitution of At-211 at the 5 position on the benzamide parent
134 compound will not affect the in vitro/in vivo selectivity for sigma-2 receptors in breast
135 cancer models and an injected activity of up to 370 MBq for an adult female will be below
136 organ radiation limits while having the potential to deliver an effective radiation dose to
137 tumors.

138 **Materials and Methods:**

139 Production and Distillation

140 Astatine-211 was produced via the nuclear reaction ²⁰⁹Bi(α,2n)²¹¹At on a Japan
141 Steel Works (JSW) BC3015 cyclotron using an external solid target positioned
142 perpendicular to the beam line. The solid target diameter was 1.6 cm and consisted of a
143 75 μm thick ²⁰⁹Bi layer backed by 1100 aluminum alloy. The beam energy was degraded
144 from 30 MeV to 28.4 MeV by using a 75 μm thick aluminum degrader foil attached
145 directly to the ²⁰⁹Bi surface. A defocused beam profile was used to spread the beam
146 across a larger surface area to mitigate pinpoint heating and subsequent melting due to
147 the poor thermal properties of ²⁰⁹Bi and the perpendicular incident beam angle.

Radionuclidic purity was analyzed by gamma and alpha spectroscopy on an Ortec GEM10P4-70 (Oak Ridge, TN) High-Purity Germanium Detector (HPGe) and Ortec ULTRA alpha-spectrometer equipped with Maestro Version 7 software operated on Windows 10. Metallic bismuth or other non-radioactive metal species that could be present in the distillate mixture were not evaluated.

Dry distillation was performed to isolate ^{211}At from the solid target using a method that has been previously reported.³¹ The foil was removed from the solid target; the $^{209}\text{Bi}/^{211}\text{At}$ layer was cleaned to remove any thermal paste using alcohol wipes, hexane and acetone. The clean and dry target was placed in a quartz boat and was positioned inside the quartz furnace tube. 1 mL of 0.1 M NaOH was added to the collection vessel and served as the medium in which ^{211}At was collected. The entire distillation apparatus was assembled and vacuum initiated. Once the entire system was evacuated, a slow argon gas stream was turned on along with the furnace. The furnace maximum temperature was set to 700 °C and was held for 10 minutes. Radioactivity in the collection vessel was monitored in real time with a dose calibrator (Capintec, Inc, Ramsey NJ) using the xenon-133 setting, and a 2.3 conversion factor to calculate ^{211}At radioactivity.³² At the end of the distillation the furnace was turned off, the system was depressurized and the 1 mL of $^{211}\text{At}/\text{NaOH}$ solution was collected.

Precursor and Standard Synthesis

The precursor, N-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-2,3-dimethoxy-5-(tributylstannyl)benzamide, and standard, 5-(iodo)-N-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-2,3-dimethoxybenzamide (RHM-4), were synthesized as previously reported.^{12,33} Since no stable isotopes of ^{211}At exist, RHM-4 was the standard of choice due to the chemical similarity between astatine and iodine.

Radiosynthesis

At-211-MM3 was synthesized by electrophilic radioastatodestannylation. Briefly, to 100 µg of precursor 1 mL of 0.1 M NaOH containing ^{211}At (37-148 MBq) was added followed by 100 µL of 3:1, glacial acetic acid: hydrogen peroxide. Radioactivity used in each

176 reaction was determined by the needs of the planned experiments from that reaction.
177 Animal studies required starting with a higher concentration of ^{211}At to account for a more
178 concentrated product to avoid ethanol percentages > 10% in the injectate preparation
179 and cell studies required much less radioactivity. The reaction was vortexed periodically
180 over 20 minutes, then quenched with 3.5 mL ammonium formate (AMF) pH 4.5 and
181 purified by High Performance Liquid Chromatography (HPLC) on a reversed phase C-18
182 Phenomenex column (250x10mm, 5 μM). Isocratic mobile phase of 30:70 acetonitrile: 0.1
183 M AMF pH 4.5 was used with UV wavelength of 288 nm and flow rate of 4 mL/min.
184 Radioactive peak was collected at 23 minutes and was diluted to 50 mL with water, then
185 concentrated on a C-18 light Sep-Pak cartridge (Waters, Milford MA). Product was eluted
186 from the cartridge using 200 proof ethanol in a volume of 100 μL . Quality control was
187 performed using the same mobile phase as above on a reversed phase C-18 Agilent
188 Zorbax (4.6x150 mm, 5 μM) analytical column. There are no stable isotopes of ^{211}At so a
189 non-radioactive standard capable of UV absorption is not feasible. To account for this we
190 used the non-radioactive iodinated sigma ligand RHM4 due to the chemical similarities of
191 iodine and astatine. Co-injection of RHM-4 standard was used to identify ^{211}At -MM3.
192 Since the theoretical mass of ^{211}At -MM3 is below the limit of detection of the analytical
193 HPLC system used the specific activity of the product was not measured.

194 Cell Culture

195 MDA-MB-231 were cultured in Minimum Essential Media (MEM) with added nutrients,
196 and EMT-6 were cultured in Dulbecco's Modified Eagle's Media (DMEM), with both
197 containing 10% FBS and 1% Penicillin/Streptomycin. Cells were incubated at 5% CO_2
198 and 37 $^\circ\text{C}$.

199 Live Cell Binding Experiments

200 ^{211}At -MM3 was evaluated in MDA-MB-231 breast cancer cells known to have high sigma-
201 2 receptor density.³⁴ 24 hours before assay MDA-MB-231 cells were plated at 50,000
202 cell/well in 96-well format. Two solutions of freshly synthesized radioligand were diluted
203 to a concentration of approximately 2,000 cpm/ μL in phosphate buffered saline with

204 calcium and magnesium (PBS w/ Ca^{2+} and Mg^{2+}), with and without non-radioactive 100
205 μM RHM-4 to determine non-specific binding. At the time of assay, culture media was
206 removed and $^{211}\text{At-MM3}$ or $^{211}\text{At-MM3}$ with 100 μM RHM-4 was added to the wells. Total
207 volume of treatment in each well was 50 μL . PBS w/ Ca^{2+} and Mg^{2+} without substrate was
208 added to the last row of wells and was used for protein quantification at the end of the
209 experiment. Treatment was removed at time points over the course of 2 hrs ($n=4/\text{time}$
210 point). At each time of treatment removal, wells were washed with PBS x 3. At the end of
211 the experiment wells were counted on an automatic gamma counter using a custom
212 protocol with an energy window of 70-110 keV (Wizard 2470, Perkin Elmer, Waltham
213 MA). Protein quantification was performed using a modified Lowry method. Independent
214 experiments were repeated in triplicate.

215 On and Off Rates for the Sigma-2 Receptor and Dissociation Constant Determination

216 On and off rates of $^{211}\text{At-MM3}$ were measured in live EMT6 cells and liver homogenates
217 in 96 well format. Live EMT6 cells were plated as previously described in the live cell
218 binding experiment. Liver homogenate tissue was prepared as previously reported.¹⁵ The
219 on rate (K_{on}) was determined by adding picomolar quantities of $^{211}\text{At-MM3}$ to cells or liver
220 homogenate tissue at different time points over 2 hrs ($n=4/\text{time point}$). The off rate (K_{off})
221 was determined by first incubating picomolar quantities of $^{211}\text{At-MM3}$ for 1 hr to establish
222 equilibrium then at time points over 2 hrs known sigma-2 ligand haloperidol was added at
223 a concentration of 10 μM to competitively inhibit sigma-2 binding and force dissociation of
224 $^{211}\text{At-MM3}$ ($n=4/\text{time point}$). The use of haloperidol provides a structurally different sigma-
225 2 compound for competitive inhibition and is considered a gold standard for sigma
226 pharmacology assays.¹⁵ At the end of 2 hrs, all tissues were washed or filtered and
227 radioactivity was measured as previously described. Independent experiments were
228 repeated in triplicate. One-phase association or dissociation kinetics were modeled using
229 Prism version 6.0 (La Jolla, CA) and the dissociation constant was calculated from
230 $K_{\text{off}}/K_{\text{on}}$ with units of $\text{min}^{-1}/\text{min}^{-1}\text{pMol}^{-1}$.

231 In Vivo Biodistribution

232 Tumors were grown by subcutaneously injecting 1×10^6 EMT-6 mouse breast cancer
233 cells into the flanks of female BALB/c mice. Tumors were an appropriate size of
234 approximately 200 mm^3 after 14 days of growth. On the day of experiment 370 kBq of
235 newly synthesized ^{211}At -MM3 in saline (ethanol <5%) was administered intravenously to
236 mice under isoflurane anesthesia. Mice were sacrificed by cervical dislocation followed by
237 immediate cardiac puncture post administration at time points of 5 min, 1, 2, 4, 8, and 24
238 hrs (n=4 mice/time point). Tissue harvested included: blood, heart, muscle, lung, kidney,
239 pancreas, spleen, liver, skin, brain, thyroid, stomach and EMT-6 tumor. Tissues were
240 weighed and counted on an automatic gamma counter as previously described. All
241 animal experiments were conducted under IACUC-approved protocols compliant with
242 University of Pennsylvania guidelines for the care and use of research animals.

243 Estimated Human Dosimetry

244 Biodistribution data was utilized to estimate human dosimetry in an adult female model
245 that was a predefined model available in OLINDA/EXM 1.1 software (VU e-Innovations,
246 Nashville TN). Kinetic data was fitted as percent-injected dose/organ (%ID/organ) over
247 time using OLINDA/EXM 1.1.³⁰ By fitting the kinetic data using %ID/organ we assume
248 ^{211}At -MM3 biodistribution studied in mouse would be relative to human and therefore did
249 not need to apply a scaling factor accounting for organ weight to subject total body weight
250 ratios between mouse and human.³⁵ Also, OLINDA/EXM 1.1 provides an adult female
251 model that includes adjustable organ weights in dose calculations. An OLINDA/EXM 1.1
252 sphere model was used to calculate absorbed tumor dose.

253 **Results:**

254 Production and Distillation

255 Astatine-211 production yields up to 340.4 MBq were achieved and are presented in
256 **Table 1**. Dry distillation of ^{211}At resulted in decay corrected recovery yields from 50-89%,
257 median=60% (**Table 1**). Monitoring the collection vessel radioactivity in real time assisted
258 method development by providing instant feedback of the distillation process and helped
259 adequately fine tune the parameters of temperature and argon flow. The entire distillation

process lasted approximately 45 min. Radionuclidic purity was confirmed by gamma and alpha spectroscopy, spectra are presented in **Figure 2**.

Radiosynthesis

²¹¹At-MM3 was synthesized with average recovery yield of 70% and is highly comparable to similar iodination chemistry. Radiochemical purity was >95% with no observable UV mass. Activity of the final product ranged from 18.5 - 74 MBq, depending on the in vitro or in vivo experiments for which the final product was intended. Co-injection of ²¹¹At-MM3 and non-radioactive standard RHM-4 resulted in UV and radioactive peak elution at similar retention times.

In Vitro Binding Studies

Live cellular binding studies with ²¹¹At-MM3 in MDA-MB-231 cells resulted in sigma-2 binding and demonstrated retained affinity to tumor cells. Fast kinetics were observed with 50% maximal binding occurring at 4.3 min. Selective sigma-2 competitive inhibition was shown with an 80% reduction in total binding of ²¹¹At-MM3 through the addition of RHM-4. **Figure 3-a** represents data from 3 independent experiments. Average protein quantification for experiments was 7.4 +/- 0.75 µg. By measuring on and off rates we were able to calculate the K_d of ²¹¹At-MM3 for the sigma-2 receptor and the constants are presented in **Figure 3** and **Figures 3-b and 3-c** represent the association and dissociation curves. There was no significant difference in K_d determination when using live EMT6 cells or liver homogenates.

In Vivo Biodistribution

Biodistribution data is presented in and tumor: tissue ratios are presented in **Figure 4**. Biodistribution was evaluated over 24 hrs showing high initial kidney uptake at 5 min (47.7% ID/g) with rapid clearance over 1 hr (5.83% ID/g). Similar results were also seen, although to a lesser extent, in the liver. Tumor accumulation of ²¹¹At-MM3 remained above 4% ID/g from 1 hr to at least 8 hrs. At 24 hrs, the majority of decay corrected radioactivity was cleared from all tissues. Considerable uptake was noted in the stomach, thyroid and pancreas. High uptake of radioactivity in the stomach and thyroid might be due to in vivo deastatination. Future metabolite studies are needed to verify this and

289 define what percentage of deastatination occurs. The high uptake in the pancreas is
290 expected due to the high expression of the sigma-2 receptor.

291 Estimated Human Dosimetry

292 Using OLINDA/EXM software estimated human dosimetry was calculated for an adult
293 female. The organs that received the highest doses of radiation were pancreas, thyroid,
294 spleen and kidney. However, all organs received were below 16 Gy. See **Table 2** for
295 results. In contrast, using the sphere model to calculate tumor dosimetry for a patient
296 dose of 370 MBq revealed a high radiation dose to tumor of 88 Gy. In this model it was
297 noted that the tumor dose remained constant irrespective to tumor size due to the short
298 path-length of alpha particles.

299 **Discussion:**

300 Historically, astatinated small molecules have been reported as non-stable in vivo and
301 readily deastatinate. Astatine-211-*meta*astatobenzylguanidine(²¹¹At-MABG) is the only
302 small molecule reported that has paradoxical in vivo stability.³⁶ Theories proposed involve
303 the guanidinium group stabilizing the astatine-carbon bond at the meta position. Attempts
304 to functionalize antibodies with astato-benzylguanidine have also been reported (SNMMI
305 annual meeting 2015). The atomic radius of astatine leads to a very weak bond formation
306 between aromatic carbons that can readily dissociate in aqueous environments.³⁷ Newer
307 methods of functionalizing antibodies with carborane cages have resulted in a dramatic
308 increase in the stabilization of astatine in vivo.³⁷ This is believed to be a property of
309 thermodynamically favorable halogen-boron bond strength. Although previous attempts
310 at directly astatinating small molecules have failed to produce stable compounds suitable
311 for biological purposes we have shown that ²¹¹At-MM3 possess characteristics that
312 attribute to favorable in vivo stability. This finding establishes a foundation to investigate a
313 large class of small molecules and their respective properties to astatination and in vivo
314 stability.

315 The sigma-2 receptor possesses several attributes that make it a valid target in the
316 treatment of cancer. The overexpression of the sigma-2 receptor on proliferating cells

allows for therapeutic drug delivery at sites of rapid tumor growth.⁵ This concept could be beneficial for DNA damaging agents, such as alpha-emitting radiotherapeutics. Secondly, while sigma-2 expression is highest on proliferating cells, it is expressed on quiescent cells³, which could allow for targeting dormant tumors and residual disease. Lastly, the sigma-2 receptor is overexpressed in many forms of human cancer allowing for a therapeutic platform with potential for impacting multiple patient populations.^{38,39}

Currently, there is high interest in sigma-2 ligands as targeting vectors for therapeutic drug delivery. Sigma-2 antagonists have been historically developed as imaging agents due to their safe toxicity profile and high affinity and selectivity for the sigma-2 receptor, as well as fast tumor targeting and clearance in vivo.⁴⁰ While internalization is required for ligand-drug conjugates, a radiotherapeutic does not have this limitation so the kinetic and affinity advantages of sigma-2 antagonists can be utilized for therapeutic purposes. Through the introduction of astatine-211, we afford a sigma-2 targeted alpha-emitting radiotherapeutic capable of in vitro and in vivo tumor localization.

²¹¹At-MM3 exhibited selective sigma-2 binding in vitro and tumor targeting in vivo. Interestingly, through association and dissociation experiments we revealed that ²¹¹At-MM3 exhibited an affinity for the sigma-2 receptor that was orders of magnitude higher than iodinated and parent compound RHM4 with K_d value 0.6 nM.¹² The introduction of astatine-211 into the molecular structure actually improved the pharmacological properties for the sigma-2 receptor. In support of these findings, results from the biodistribution study show retention of radioactivity in the tumor from 1-8 hrs post administration. The pharmacological nature of sigma-2 antagonist predicts the interaction between the ligand and receptor is reversible and this class of sigma-2 ligands has previously shown quick tumor clearance kinetics in vivo.³³ The iodinated sigma-2 ligand RHM-4 was shown to have tumor clearance by 4 hrs with a lower tumor uptake of 2% ID/g.¹² Tumor retention of ²¹¹At-MM3 is highly desirable as a therapeutic and provides a tumor residence time approximately equal to the half-life of ²¹¹At (t_{1/2}=7.21 hr) providing at least 50% of total possible radiation dose to the tumor before washout.

345 In vivo deastatination is the suspected cause of partial off-target localization in the
346 thyroid and stomach, which is consistent with literature reports of in vivo biodistribution of
347 free astatine-211.⁴¹ Similar in vivo effects have been reported with iodinated sigma-2
348 ligands.^{12,13} In this study, thyroid blockade with stable iodide was not utilized. Other
349 possible reasons for off-target localization might be due to sigma-2 receptor expression in
350 the thyroid and stomach.⁴² The pancreas and spleen also had an appreciable amount of
351 radioactivity localized and can be explained by the known high expression of sigma-2
352 receptors on endocrine glands.⁴³

353 Estimated human dosimetry revealed a high therapeutic ratio for small tumors at an
354 expected relatively safe dose of 370 MBq in an adult female. All organ doses remained
355 under 16 Gy, which are within acceptable normal organ radiation limits.⁴⁴ Future pre-
356 clinical studies will be performed to further explore the therapeutic effectiveness
357 associated with dosimetry calculations in mouse models using OLINDA/EXM 1.1, as well
358 as microdosimetric calculations in comparison.

359 Potential limitations of this study include not harvesting intestines or bladder during
360 biodistribution. The intestine is another organ which is reported in the literature as a site
361 of free astatine-211 accumulation in vivo. However, it is difficult to propose the bladder
362 tissue uptake.³⁷ Despite the high amount of renal clearance observed we could only
363 assume the bladder wall has a high level of radiation exposure. Other limitations such as
364 metabolism and in vivo stability studies will be addressed in future work.

365 **Conclusion:**

366 ²¹¹At-MM3 was evaluated in vitro and in vivo for pharmacological properties to
367 the sigma-2 receptor. The results from the in vitro work presented show that the
368 substitution of iodine for astatine-211 increased the affinity of the compound for the
369 sigma-2 receptor. These results are consistent with other sigma-2 compounds that have
370 bromine or iodine substitution at the 5 position on the benzamide backbone.
371 Pharmacological studies are needed to define how atomic size and hydrophobicity effect
372 sigma-2 receptor interactions.

373 Additional biodistribution studies are needed using a non-iodinated/astatinated
374 sigma-2 ligand to assess thyroid and stomach uptake. There are multiple reports of
375 astatinated small molecules that lack in vivo stability, however; our in vivo results showed
376 no significant evidence of complete deastatination. There is most likely a partial in vivo
377 deastatination that occurs and will be addressed in future work.

378 The data presented serves as a foundation for further exploring astatinated
379 sigma-2 targeted small molecules as potential candidates for radiotherapeutic drug
380 development.

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387 **Table 1:** Astatine-211 production and distillation. Distillation yield presented as recovered
388 radioactivity from measured solid target. This is a representative sample of over 30
389 production runs.

Avg Current	$\mu\text{A}\cdot\text{Hr}$	Production Yield (MBq/ $\mu\text{A}\cdot\text{Hr}$)	²¹¹ At Measured on Solid Target	Distillation Recovery (MBq)	Decay Corrected Distillation % Yield
10	30.1	10.4	313.04	238	89
10	20	9.8	196.00	107	61
6.8	20.1	9.2	184.92	104	64
9.9	13.3	8.6	114.38	70	66
7.5	16.95	7.5	127.13	85	76
9	45.4	8.7	394.98	196	58

390

391 **Table 2:** Estimated human dosimetry for 370 MBq of ^{211}At -MM3 using OLINDA/EXM 1.1
392 with an adult female model.

Target Organ	Total
Brain	0.027
Stomach Wall	0.045
Heart Wall	0.285
Kidneys	2.827
Liver	1.083
Lungs	0.808
Muscle	0.073
Pancreas	15.206
Red Marrow	0.843
Osteogenic Cells	6.089
Spleen	3.985
Thyroid	7.980
Total Body	0.140

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395 **Figure 1:** Chemical structures of a) ISO-1, b) RHM4 and c) MM3.

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398 **Figure 2: A)** Gamma photopeaks of [^{211}At] are represented by **1)** **2)** and **4)**,
 399 corresponding to 76.8/79.2, 89.8 and 685/687 KeV. Gamma photopeaks of the daughter
 400 [^{211}Po] are represented by **3)** and **5)** corresponding to 569.6 and 897.8 KeV. The main
 401 suspected isotopic impurity [^{210}At] was not detected at 1181 and 1483 KeV. **B)** Alpha
 402 spectra of [^{211}At] is presented with alpha-peaks represented by **6)** and **7)** which
 403 correspond with [^{211}At] and [^{211}Po] at 5.87 and 7.45 MeV.

406 **Figure 3**

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408

409 **Figure 4-a)** Bar graph representing the tumor:muscle and tumor:blood ratios at time
 410 points over the course of 24 hrs. Error bars are represented as standard error of
 411 measurement (S.E.M). **Figures 4-b/4-c/4-d** represent biodistribution data for different
 412 organs grouped based off **b)** low background organs **c)** high blood flow organs and **d)** off-
 413 target organs.

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