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Title: ^{225}Ac-Macropatate: A Novel Alpha Particle Peptide Receptor Radionuclide Therapy for Neuroendocrine Tumors

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Disclosures

JJW and NAT are authors of a patent for the use of macropa as a chelator for ^{225}Ac chelation. No other potential conflicts of interest relevant to this article exist.

Short running title: ^{225}Ac-Macropatate TAT for NETs

ABSTRACT

Neuroendocrine tumors (NETs) express Somatostatin Receptor-2 (SSTR2) and Somatostatin Receptor-5 (SSTR5). Modified variants of somatostatin, the cognate ligand for SSTR2/5, are used in treatment for metastatic and locoregional disease. Peptide receptor radionuclide therapy (PRRT) with ^{177}Lu-Dotatate (DOTA-Octreotate), a beta particle-emitting somatostatin derivative, has demonstrated survival benefit in patients with SSTR^{+} NETs. Despite excellent results, a subset of patients has tumors that are resistant to treatment, and alternative agents are needed. Targeted alpha particle therapy (TAT) has been shown to kill tumors that are resistant to targeted beta-particle therapy, suggesting that TAT may offer a promising treatment option for patients with ^{177}Lu -Dotatate resistant disease. While Dotatate can chelate the clinically relevant alpha particle-emitting radionuclide ^{225}Ac, the labeling reaction requires high temperatures, and the resulting radioconjugate has suboptimal stability. **Methods**: Here, we design and synthesize Macropatate (Macropa-octreotate), a novel radioconjugate capable of chelating ^{225}Ac at room temperature, and assess its *in vitro* and *in vivo* performance. **Results:** Macropatate demonstrated comparable affinity to Dotatate (K_D = 21 nM) in U2-OS-SSTR2, a SSTR2^{+} transfected cell line. ^{225}Ac-Macropatate demonstrated superior serum stability at 37 °C over time compared to ^{225}Ac-Dotatate. Biodistribution studies demonstrated higher tumor uptake of ^{225}Ac-Macropatate relative to ^{225}Ac-Dotatate in mice engrafted with subcutaneous H69 neuroendocrine tumors. Therapy studies showed that ^{225}Ac-Macropatate exhibits significant antitumor and survival benefit compared to saline control in mice engrafted with SSTR^{+} tumors. However, the increased accumulation of ^{225}Ac-Macropatate in liver and kidneys and subsequent toxicity to these organs decreased its therapeutic index compared to ^{225}Ac-Dotatate. **Conclusions**: ^{225}Ac-Macropatate and ^{225}Ac-Dotatate exhibit favorable therapeutic efficacy in animal models. Because of elevated

liver and kidney accumulation and lower administered activity for dose limiting toxicity of ^{225}Ac-Macropatate, ^{225}Ac-Dotatate was deemed the superior agent for TAT PRRT.

Keywords: Oncology, Actinium, Targeted Alpha Therapy, Neuroendocrine Tumors, Octreotate, Somatostatin, Peptide Receptor Radionuclide Therapy

INTRODUCTION

Neuroendocrine tumors (NETs) are a heterogenous family of neoplasms originating in cells within the endocrine and nervous systems, including the gastrointestinal tract, lungs, pancreas, thyroid, and gonads (*1*,*2*). Many NETs overexpress somatostatin receptors (SSTRs) (*3*). This high receptor expression offers a targetable vulnerability in NETs, which has long been exploited for therapy.

Somatostatin-like derivatives have been used as drugs themselves or as scaffolds to deliver radioisotopes for peptide receptor radionuclide therapy (PRRT). One of the most successful of these is the pairing of [Tyr 3] octreotate coupled to the chelator DOTA, yielding Dotatate (*4*,*5*). Radiolabeled Dotatate has been successfully used for both PET imaging (*6*) (^{68}Ga, ^{64}Cu) and therapeutic (^{177}Lu) purposes. The Phase 3 randomized controlled clinical trial, NETTER-1, showed that patients with treatment refractory NETs who received ^{177}Lu-Dotatate had significantly higher progression free survival versus patients receiving SSA (*7*). The results of this trial led to ^{177}Lu-Dotatate (Lutathera®) being approved by the FDA in January 2018 for the treatment of SSTR^{+} gastroenteropancreatic-NETs (*8*).

While these results made PRRT a first-in-class treatment option for patients with NETs, many are *ab initio* resistant to, or develop resistance following treatment with, beta-particle emitting ^{177}Lu-Dotatate. Alpha particle-emitting radionuclides are an attractive alternative to beta particle-emitting radionuclides due to their short range, which can mitigate off target effects, and

the high energy deposited by these particles over that short range (a.k.a. high linear energy transfer or LET) (*9,10*). The alpha particle-emitting nuclide ^{225}Ac has been coupled to prostate specific membrane antigen (PSMA)-targeting ligands to successfully treat prostate cancers refractory to treatments with androgen deprivation, taxanes, and ^{177}Lu-PSMA-617 (Pluvicto^{TM}), which is approved for treatment of patients with PSMA^{+} castration resistant prostate cancer in the U.S. (*11-14*). Recently, a Phase I clinical trial of patients with gastroenteropancreatic NETs previously treated with ^{177}Lu-Dotatate receiving ^{225}Ac-Dotatate therapy showed stable disease or partial response in 82% of patients (*15*). Similarly, another study with ^{225}Ac-Dotatate found to have efficacy in patients with SSTR^{+} paraganglioma (*16*). Alpha-emitting PRRT with ^{213}Bi ($t_{1/2}$ = 45 min) and ^{212}Pb ($t_{1/2}$ = 10.6 h) have shown promising clinical results as well (*17,18*).

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and its derivatives are used to chelate ^{225}Ac and many of the previously mentioned radionuclides. However, in order to chelate ^{225}Ac with DOTA to yield high specific activity radioconjugates, temperatures >70 °C are typically required. Even if these temperatures are used, the resulting complex's thermodynamic stability (*19*) and labeling kinetics are sub-optimal (*20*). Recently, Thiele *et al*. showed that Macropa, an 18-membered macrocycle, is capable of chelating ^{225}Ac at room temperature, faster, and at lower concentrations than DOTA (*21*). The ^{225}Ac-Macropa complex showed comparable stability (8 d) to ^{225}Ac-DOTA in human serum and in C57BL6 mice. Further preclinical studies have demonstrated the suitability of ^{225}Ac-labeled macropa-containing radioconjugates for targeted alpha therapy (TAT) with both small-molecule and antibody conjugates (*22,23*).

Here, we synthesize and characterize Macropatate, consisting of Macropa-coupled to [Tyr 3] octreotate, and compare its performance to Dotatate with respect to ^{225}Ac labeling

efficiency, serum stability, target engagement, and therapeutic efficacy. We show that Macropatate exhibits improved stability over Dotatate when complexed to ^{225}Ac, maintains high SSTR binding affinity, demonstrates favorable *in vivo* target localization, and has significant antitumor activity.

METHODS

Synthesis and Radiolabeling of Macropatate and Dotatate

Macropatate and Dotatate were prepared by conjugating isothiocyanate-activated Macropa and DO3A-tri-t-butyl ester, respectively, to immobilized octreotate (*21*,*24*,*25*). After synthesis and deprotection, the products were characterized for purity and identity by HPLC and LC-MS, respectively. Full synthetic details for both conjugates are reported in the Supplemental Information (Supplemental Figure 1). Radiolabeling of Macropatate and Dotatate with ^{225}Ac was performed at room temperature or 70°C, respectively, in NH_4OAc (pH 5.5), and the products were characterized using instant-thin layer chromatography (ITLC). Full radiolabeling and characterization details are provided in the Supplemental Information.

Cell Culture and In Vitro Assays

A panel of SSTR2/5 expressing cell lines were cultured and their SSTR2 and SSTR5 expression levels were evaluated using flow cytometry. The highly positive U2OS-SSTR2 cell line was used to confirm the binding affinity of radiolabeled ^{225}Ac-Macropatate in a saturation binding assay. Full cell culture details and experimental procedures for flow cytometry and saturation assays are reported in the Supplemental Information.

Serum Stability Studies

^{225}Ac-Macropatate and ^{225}Ac-Dotatate were evaluated for stability in human serum (EMDMillipore, Temecula, CA) at 37°C and pH 7.4. Radiochelate was diluted to 370 kBq in 1

mL of human serum and placed on an Eppendorf Thermomixer set to 37°C. At fixed intervals, aliquots were removed from the reactions and analyzed by ITLC as described in the Supplemental Information.

Murine Subcutaneous Xenograft Models

All procedures used and animal studies followed a protocol approved by the National Institutes of Health Institutional Animal Care and Use Committee (protocol ROB104). Female athymic homozygous nude mice (NCI Athymic NCr-nu/nu strain 553, Charles River Laboratories, Wilmington, MA), 8–10 weeks old, were subcutaneously engrafted with 8×10^6 H69 cells in 200 μL ice-cold PBS. Treatment of tumors with radiotracers for biodistribution or therapy studies was performed once palpable tumors developed, approximately 1 month post-inoculation. The biodistributions of both ^{225}Ac-Macropatate and ^{225}Ac-Dotatate were evaluated in H69 subcutaneous tumor models, both with and without D-lysine pre-treatment (*26*). Full experimental details for biodistribution experiments are reported in the Supplemental Information.

Dose Finding Study for ^{225}Ac-Macropatate

To evaluate the therapeutic potential of ^{225}Ac-Macropatate, we performed a dose finding study in mice. Mice ($n \geq 3$) bearing H69 tumor xenografts were first injected with D-lysine hydrochloride (35 mg/mouse), then treated with 148, 93.3, 46.3, or 23.1 kBq of ^{225}Ac-Macropatate, and their body weights and tumor growth were monitored over several weeks. The highest tested dose of ^{225}Ac-Macropatate was chosen based on a recent report of therapy using ^{225}Ac-Dotatate, which found that 148 kBq was well-tolerated in mice (*27*).

Head-to-Head Therapy Study with ^{225}Ac-Macropatate or ^{225}Ac-Dotatate

For our therapy study, we wished to identify the highest administered activities that exhibited

acceptable toxicity as measured by mouse weight loss and survival, and found 46.3 kBq of ^{225}Ac-Macropatate and 148 kBq of ^{225}Ac-Dotatate to be suitable. Animals engrafted with H69 cells were treated with either of the two radioconjugates or saline (n = 8–10, each). Local control and survival were primary outcomes. All groups were tracked for humane endpoints including, but not limited to > 2000 mm^3 tumors, > 20 % weight loss.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (v 9.0, GraphPad Software, San Diego, CA, USA). Statistical analysis of survival curves was performed using the log-rank test. Comparisons of organ uptake, tumor volume, and stability were performed using the Student's t-test.

RESULTS

Macropatate Forms Stable Complex with ^{225}Ac at Room Temperature

We successfully synthesized Dotatate and Macropatate with good yields and high purity, characterizing the identity and purity of both molecules via HPLC and mass spectrometry (Figure 1, Supplemental Figure 1). After synthesis, we labeled both Dotatate and Macropatate with ^{225}Ac. Radiolabeling was conducted in mildly acidic (pH 5.5) NH_4OAc buffer (0.1 M). We found that Macropatate could be radiolabeled quantitatively after 1 h incubation at room temperature (18–20 °C), whereas Dotatate required heating at 70 °C for 1 h to achieve comparable purity and yield. Typical specific activities for both radioconjugates were approximately 185 GBq/mmol. A representative ITLC of ^{225}Ac-Macropatate is shown in Figure 2A and Supplemental Figure 2, and an ITLC of ^{225}Ac-Dotatate is shown in Supplemental Figure 3.

We evaluated the stability of both molecules in human serum at 37°C using ITLC (Figure

2B and 2C, Supplemental Figures 4–7). ^{225}Ac-Macropatate and ^{225}Ac-Dotatate were stable to 10 days. Stability experiments conducted in human serum indicate that ^{225}Ac-Macropatate has significantly greater stability than ^{225}Ac-Dotatate (98% vs 95%, P = 0.0097), and our results compare well to a recent stability investigation of ^{225}Ac-Dotatate reported by others, which found 90% intact ^{225}Ac-Dotatate after 10 days (*27*). Thus, ^{225}Ac-Macropatate exhibited a modest, yet significant stability advantage over the ^{225}Ac-Dotatate.

^{225}Ac-Macropatate Retains Affinity for SSTR

After evaluating the purity and stability of the ^{225}Ac-Macropatate conjugate, we sought to confirm its binding affinity using SSTR2-expressing cells *in vitro* (*28-30*). Saturation binding assays showed a binding affinity of 21 nM (Figure 2D), comparable to that reported for Eu-Dotatate (22 nM), which has been used as a surrogate for ^{225}Ac-labeled Dotatate (*27*). This value is also in a similar range to other studies examining radiopeptide somatostatin derivatives (*31*,*32*). These results confirm that Macropa conjugation to octreotate does not adversely affect SSTR binding.

Selection of Cell Lines for *in Vivo* Studies

To find a suitable model for our murine subcutaneous xenograft models, we assessed SSTR2 and SSTR5 expression by flow cytometry in several cell lines U2-OS, U2-OS-SSTR2, AR42J, H69, Bon-1, and U937 (Figure 3). US-OS exhibited low to negligible levels of both SSTR2 and SSTR5. All other cell lines were SSTR2^{+}, with expression decreasing in the order U2OS-SSTR2 > H69 > AR42J > U937 > Bon-1. Cell lines H69, AR42J, and U937 also displayed moderate expression of SSTR5. The H69 cell line was chosen for *in vivo* experiments due to its high expression of SSTR2/5 and established history as a model system for investigating SSTR-targeting radioconjugates (*33*,*34*).

^{225}Ac-Macropatate Demonstrates Target Engagement *in Vivo*

After confirming the stability and SSTR binding of ^{225}Ac-Macropatate *in vitro*, we evaluated its biodistribution and compared it to that of ^{225}Ac-Dotatate in mice bearing SSTR^{+} H69 tumor xenografts. Both tracers showed favorable tumor uptake, with %IA/g of 9% and 5% at 2 h, and 4% and 2% at 24 h for ^{225}Ac-Macropatate and ^{225}Ac-Dotatate, respectively (Figure 4A and 4B, Supplemental Figures 8–11). Although both tracers display excellent tumor:muscle ratios > 50:1 at 4 h (Figure 4C), they also have high renal uptake, as is typical of SSTR-targeting peptides (*35*,*36*). ^{225}Ac-Macropatate displayed significantly higher tumor accumulation at 2 and 24 h than ^{225}Ac-Dotatate ($P < 0.05$). However, the liver and kidney uptake of ^{225}Ac-Macropatate were also higher, possibly because the higher hydrophobicity of the conjugate could slow clearance leading to increased liver uptake. The liver accumulation of ^{225}Ac-Macropatate was 2–3x higher than ^{225}Ac-Dotatate at all time points investigated.

The high kidney accumulation of peptide radioconjugates is routinely lowered by pre-administration of D-lysine (*26*). Accordingly, we observed lower kidney %IA/g of both ^{225}Ac-Macropatate and ^{225}Ac-Dotatate after D-lysine administration compared to kidneys of animals not receiving D-lysine. This lowered kidney accumulation of the radiotracers was most pronounced at 4 h post-injection, with the kidney signal for ^{225}Ac-Macropatate changing from 11.5% to 5.2% ($P = 0.0057$) and ^{225}Ac-Dotatate decreasing from 6.0% to 3.1% at this time point ($P = 0.0039$).

^{225}Ac-Macropatate and ^{225}Ac-Dotatate Delay Tumor Growth and Improve Survival of Mice Bearing NET Xenografts

The promising biodistribution profile of ^{225}Ac-Macropatate led us to investigate its therapeutic efficacy. As a preliminary investigation, we evaluated a series of treatment activities of ^{225}Ac-

Macropatate ranging from 23.1 to 148 kBq in mice bearing H69 tumor xenografts. All mice in the 148 kBq treatment group (10/10) and 1/3 mice in the 92.3 kBq group were sacrificed within 10 days of treatment due to substantial weight loss (>20%). All other mice displayed minimal weight loss, and a clear dose-dependent reduction in tumor volume was evident (Supplemental Figures 12–14). Based on these results, 46.3 kBq of ^{225}Ac-Macropatate was selected as the appropriate dose for further investigation.

Animals treated with ^{225}Ac-Macropatate and ^{225}Ac-Dotatate demonstrated significant tumor growth delay and improvements in survival compared to saline-treated controls (Figure 5A, B, and C; Supplemental Figures 15–17). Mice treated with ^{225}Ac-Macropatate exhibited an initial reduction in tumor volume lasting approximately 3 weeks post-treatment. However, the tumors subsequently relapsed in most mice (7 of 8). Conversely, ^{225}Ac-Dotatate treatment resulted in complete, durable tumor remission for all mice. However, two mice in the ^{225}Ac-Dotatate treatment group were euthanized due to weight loss. Although mice in the Macropatate treatment group also displayed some weight loss immediately following treatment, their weights stabilized within two weeks (Figure 5D). ^{225}Ac-Macropatate significantly improved median survival relative to the vehicle control (55 days v. 26 days, log rank, $P = 0.0006$), while 8/10 mice (80%) treated with ^{225}Ac-Dotatate survived the full 100-day duration of the study. Overall, ^{225}Ac-Macropatate exhibited favorable local control and survival benefit over saline treated animals. However, mice treated with ^{225}Ac-Dotatate showed significantly better local control and overall survival ($P<0.02$).

DISCUSSION

PRRT with ^{177}Lu-Dotatate represents a significant advance for patients with SSTR-expressing NETs. Nevertheless, treatment options for tumors refractory to ^{177}Lu-based peptide

receptor radionuclide therapy are needed. By exploiting the unique properties of alpha particles, namely high energy deposition over a short path, we may be able to overcome resistance to ^{177}Lu-based PRRT (*16*,*27*,*37*,*38*). However, the DOTA chelator used in these and other studies is suboptimal for chelation of the large Ac^{3+} ion (*19*). With the goal of achieving a more stable SSTR-targeting radioconjugate for ^{225}Ac TAT, we designed, synthesized, and characterized the conjugate Macropatate, wherein we replace the DOTA of Dotatate with the expanded macrocyclic chelator macropa, which has been shown to more stably chelate $^{225}Ac^{3+}$ compared to DOTA (*21*).

We confirmed that ^{225}Ac-Macropatate displayed high tumor accumulation, tumor growth delay, and survival benefit in xenograft models of NETs. However, our radioconjugate also exhibited a narrow therapeutic index as evinced by toxicity at lower injected activities compared to ^{225}Ac-Dotatate. As such, the head-to-head therapy study was performed with 3-fold higher injected activity in animals receiving ^{225}Ac-Dotatate compared to those receiving ^{225}Ac-Macropatate. Biodistribution studies indicate relatively high liver accumulation for ^{225}Ac-Macropatate. Notably, unlike for most other organs, this liver signal does not appear to diminish over time. This persistent accumulation of ^{225}Ac may arise from metabolism of the radioconjugate and could be responsible for the observed higher toxicity of ^{225}Ac-Macropatate. Previous investigations of ^{177}Lu-Dotatate have indicated significant degradation of the targeting octreotate portion of the tracer, likely due to metabolism (*39*). Such metabolism could significantly impact the biodistribution of the ^{225}Ac radioconjugates explored in this work. Thus, the disparate off-target uptake of ^{225}Ac-Macropatate and ^{225}Ac-Dotatate despite their similar tumor accumulation may also reflect accumulation of fragmented species in non-target organs. While ^{225}Ac-Macropatate demonstrates significant anti-tumor activity in SSTR-expressing

models of NET, it remains inferior to ^{225}Ac-Dotatate *in vivo*. Therefore, Macropatate requires significant optimization to decrease off-target accumulation and associated toxicity. For instance, variation of the specific activity or molar amount of ^{225}Ac-Macropatate injected might provide decreased background accumulation while preserving tumor uptake, for such optimization has been shown to greatly improve the pharmacokinetic profile of ^{177}Lu-Dotatate (*40*).

TAT agents directed at NETs, such as ^{225}Ac-Dotatate, have demonstrated promising results in small clinical studies and warrant further investigation. Other alpha particle-emitting radionuclides being investigated include ^{213}Bi and ^{212}Pb. Recently, a Phase I study with the alpha particle emitting PRRT agent ^{212}Pb-Dotamtate, which has a Pb-optimized chelator, has shown good tolerability and overall response rates of 80% in patients naïve to PRRT (*18*). The Phase II study (NCT05153772) is open and recruiting. These studies indicate that the intrinsic properties of alpha emitters can elicit responses in tumors otherwise refractory to beta-emitting PRRT (*41*,*42*).

More broadly, several strategies toward aiming to improve the efficacy of ^{177}Lu-PRRT in NETs are being investigated, which could apply to TAT PRRT as well. For instance, deploying epigenetic modulators have shown to increase the membrane expression of SSTR and subsequent accumulation of PRRT agent (*43-46*). Combinatorial approaches exploiting inhibitors of DNA damage repair are also being explored (NCT04086485, NCT04375267, NCT03958045).

CONCLUSION

We have successfully synthesized Macropatate, a novel SSTR2/5 targeting PRRT agent tailored to deliver ^{225}Ac to NETs. Importantly, we show that Macropatate is able to chelate ^{225}Ac at room temperature, and that this complex has four-fold lower susceptibility to degradation than ^{225}Ac-Dotatate in human serum. Both ^{225}Ac-Macropatate and ^{225}Ac-Dotatate demonstrated excellent *in*

vivo target engagement in NET xenografts, and exhibited superior local control and survival compared to saline. However, while ^{225}Ac-Macropatate had *in vitro* stability superior to ^{225}Ac-Dotatate, because it underperformed ^{225}Ac-Dotatate *in vivo*, optimization is needed prior to further translation.

Disclosure

JJW and NAT are authors of a patent for the use of macropa as a chelator for ^{225}Ac chelation.

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KEY POINTS

Question: Can chemical conjugation of the Macropa chelator to SSTR-targeting octreotate result in a superior SSTR-targeting radioconjugate for targeted-alpha therapy compared to Dotatate?

Pertinent Findings: ^{225}Ac-Macropatate demonstrates comparable *in vitro* SSTR2 affinity and higher *in vivo* uptake in SSTR^{+} xenografts versus the standard ^{225}Ac-Dotatate. However, its efficacy is limited by a poor therapeutic index, highlighting a need for further optimization prior to translation.

Implication for Patient Care: We confirm that targeted alpha therapies for neuroendocrine tumors demonstrate high efficacy in preclinical studies. Such agents warrant further clinical investigation to offer a therapeutic option for patients with disease refractory to beta particle peptide receptor radionuclide therapy.

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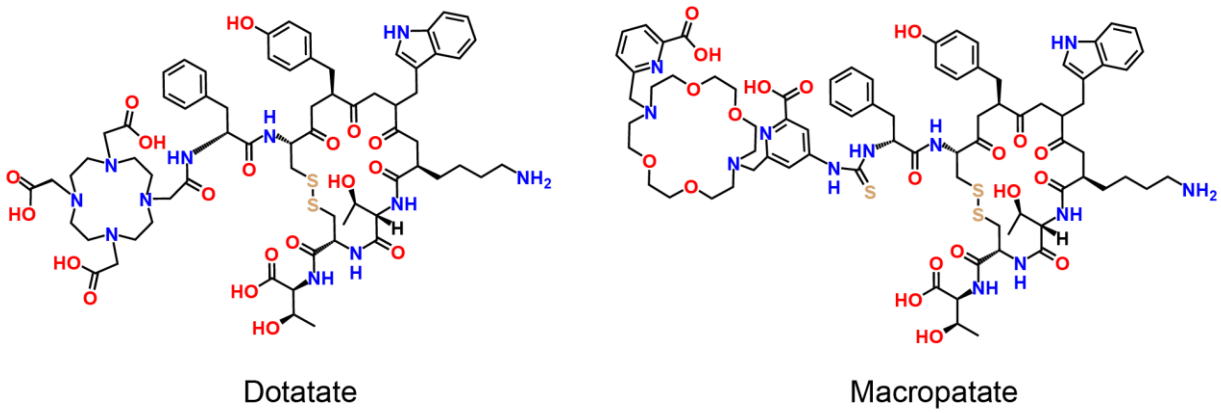


Figure 1. Structures of Dotatate (Left) and Macropatate (Right).

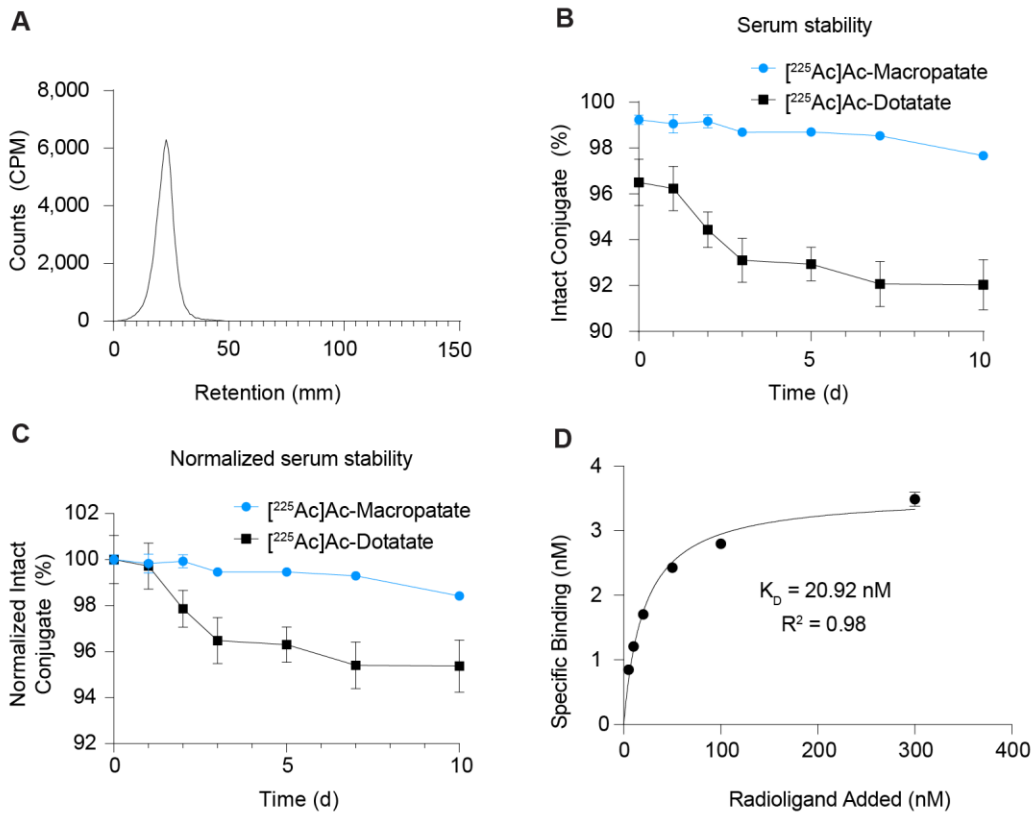


Figure 2. Macropatate stably chelates Ac-225 and binds to SSTR. (A) Representative ITLC chromatogram of ^{225}Ac-Macropatate. (B) Intact conjugate remaining over time of ^{225}Ac-Macropatate and ^{225}Ac-Dotatate in human serum incubated at 37°C, as measured by ITLC. (C) Percent of initial intact conjugate remaining over time, normalized to starting amount, after incubation in human serum at 37°C. (D) Assessment of SSTR2 binding affinity of ^{225}Ac-Macropatate in U2-OS SSTR2 cells using saturation binding assay.

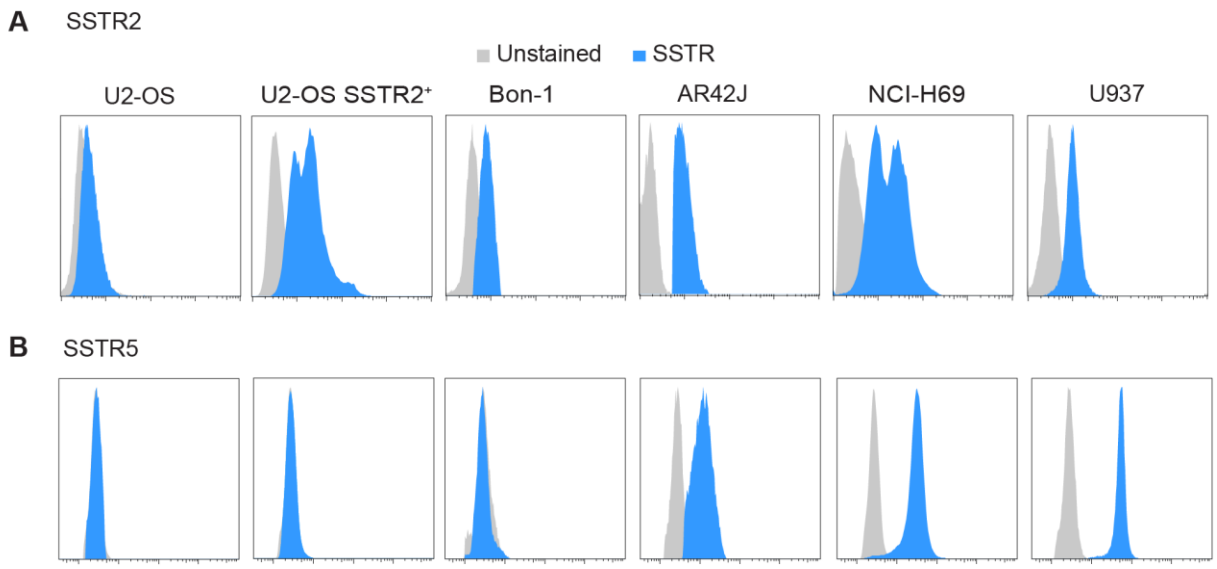


Figure 3. Flow cytometry assessment of (A) SSTR2 and (B) SSTR5 in a panel of SSTR-expressing cell lines.

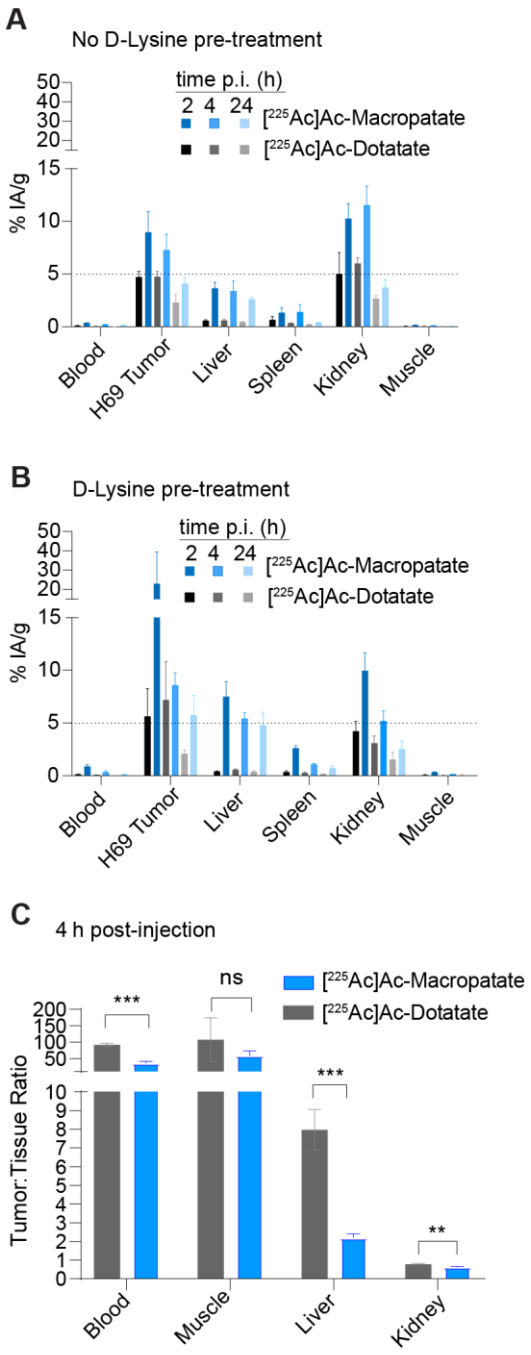


Figure 4. ^{225}Ac-labeled Macropatate and Dotatate bind to SSTR^{+} tumors. Selected-organ biodistribution (n = 3) of ^{225}Ac-Macropatate and ^{225}Ac-Dotatate (37 kBq) without (A) or with (B) pre-administration of D-Lysine (35 mg/mouse). Corresponding Tumor:Tissue ratios for selected organs with lysine pre-treatment are shown in panel C. Full 12-organ biodistribution data are reported in Supplemental Figures 8–11. Error bars represent standard deviation. %IA/g: percent injected activity per gram tissue. *** $P < 0.005$, * $P < 0.01$.

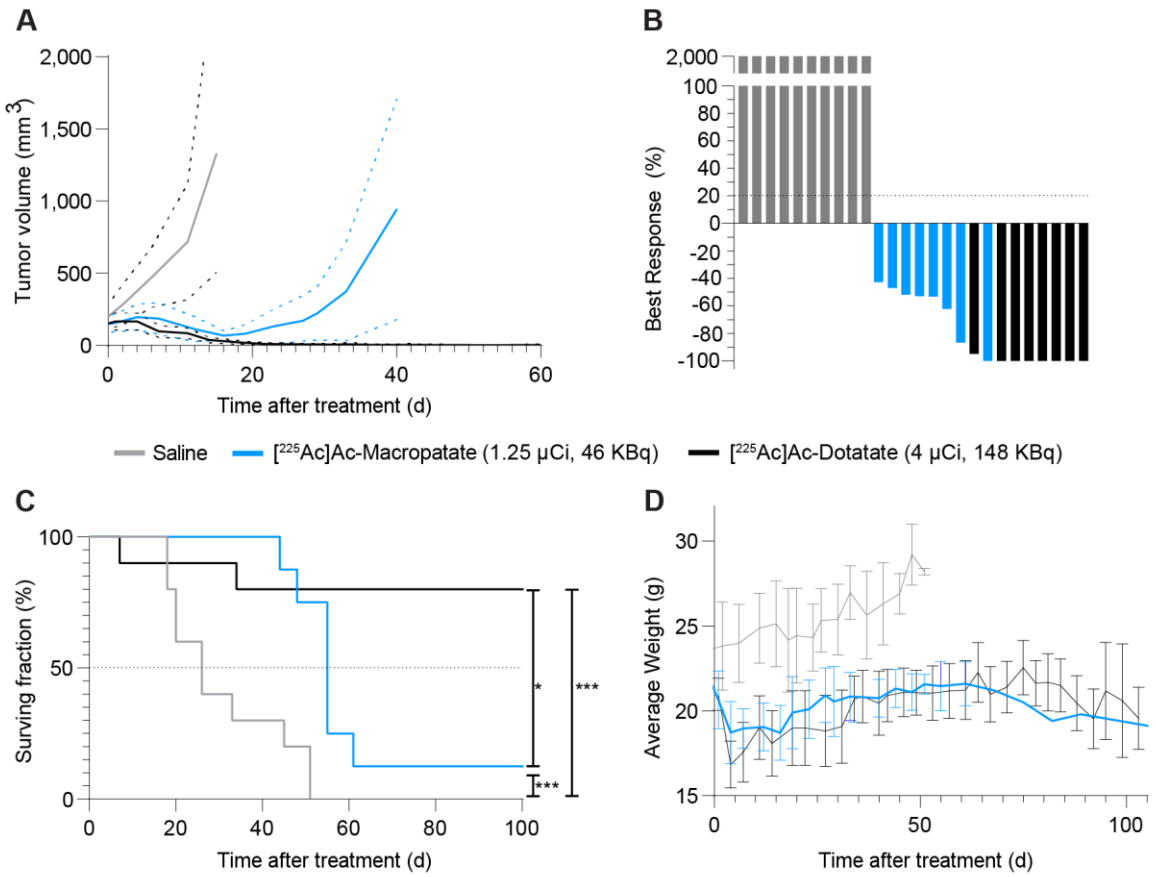
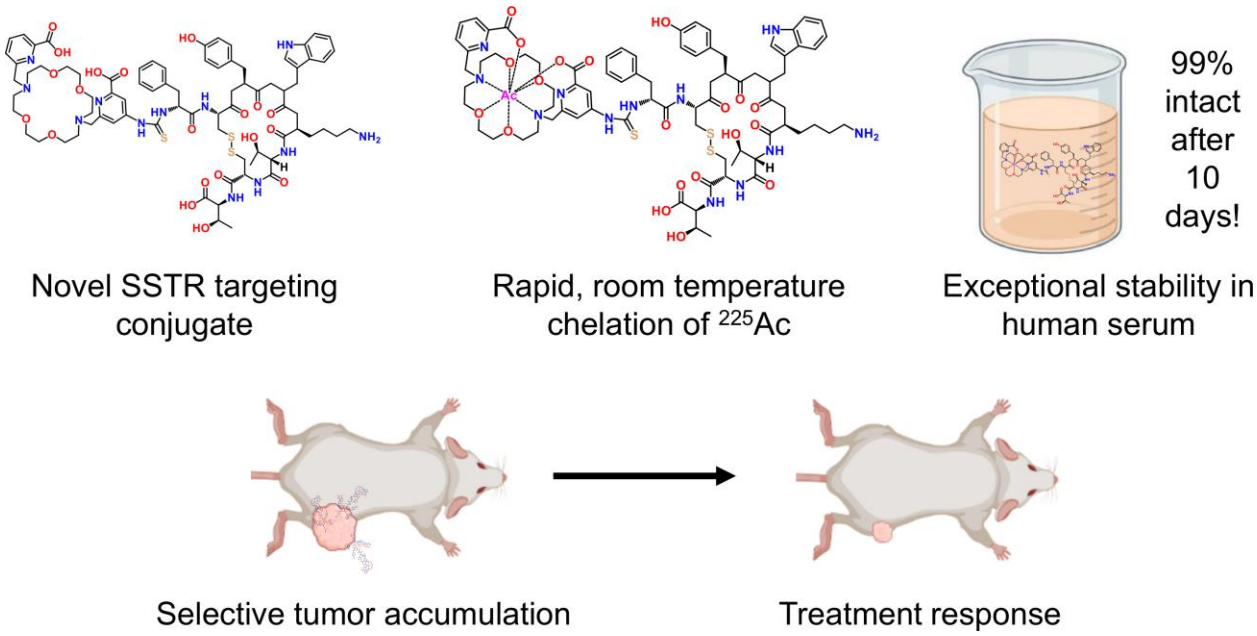


Figure 5. Targeted alpha therapy with ^{225}Ac-Macropatate and Ac-Dotatate is effective. Therapeutic response of mice bearing H69 lung neuroendocrine tumor xenografts treated with ^{225}Ac-Macropatate (46 kBq), ^{225}Ac-Dotatate (148 kBq), or vehicle control. (A) Tumor volume measurements over time. Solid lines represent the average volume and dashes lines represent the 95% confidence interval. Plots for each data set are discontinued after the first mouse death due to excessive tumor volume. Full tumor volume measurements for the study duration for each mouse are reported in Supplemental Figures 15–17. (B) Maximal response to treatment (tumor volume growth %) of individual mice. Non-responding mice are represented as full tumor growth (2000% increase). (C) Mouse survival over time. Endpoint was defined as tumor volume >2000 mm^3 or weight loss over 20% of starting weight. (D) Average mouse body weights over time for each treatment group. Error bars represent the standard deviation. *** $P < 0.005$, *$P < 0.05$.

Graphical Abstract



Supplementary Information For: ^{225}Ac-Macropatate: A Novel Alpha Particle Peptide Receptor Radionuclide Therapy for Neuroendocrine Tumors

Table of Contents

Synthesis of peptide segments:

Resins, reagents and solvents used for the synthesis of peptide segment were procured from Chem-Impex Int'l Inc. The sequences were assembled starting from preloaded amino acid/alcohol on 2-chlorotrityl resins. Standard Fmoc chemistry was used to construct the required sequences. For coupling, 8.0 eq. of suitably protected amino acids (0.4 M in DMF) were activated with HBTU (0.4 M in DMF, 8.0 eq.) and NMM (0.8 M, in DMF, 16.0 eq.) and transferred to the peptide vessels containing the resin. The peptide vessels were flushed with argon and shaken. After a coupling time of 60 min, the activated amino acid solution was drained off the resin under a positive pressure of nitrogen and the resin was washed with DMF (3 x 15.0 ml/g of resin). Fmoc- protecting groups were removed with 20% piperidine in DMF (15.0 ml/g, 15.0 min), drained, washed with DMF and taken to the next coupling with the respective amino acid. The deprotection step was repeated two times before the next step. The coupling and deblocking cycles were repeated until the last amino acid was added to the growing peptide chain on the resin.

The following three sequences were assembled on the resin using manual synthesis.

1) Fmoc-Dphe-Cys(Trt)-Tyr(tBu)-DTrp(Boc)-Lys(Boc)-Thr(tBu)Cys(Trt)-Thr(tBu)-2ClTrt Resin
2) Fmoc-Dphe-Cys(Trt)-Tyr(tBu)-DTrp(Boc)-Lys(ivDde)-Thr(tBu)Cys(Trt)-Thr(tBu)-ol-2ClTrt Resin

Dotatate (*1*):

Completed sequence 1 (starting with 0.25 mmol of preloaded amino acid) was deprotected with 20% piperidine in DMF using standard protocol and washed with DMF. Bromoacetic acid was activated with HOBt and EDC (8.0 equiv. each) in DMF and transferred to the resin with free N-terminus amine in the peptide vessel and shaken for 30 min. The resin was drained, washed with DMF and the alkyl bromide on the resin displaced with DO3A-tri-t-butyl ester (*2*) using DIPEA as base in DMF for 20h (20.0 equiv. of macrocyclic ester as its hydrobromide salt and 40.0 equiv. of base). The resin was drained, washed with DMF (3 x 15 ml), DCM (3 x 15.0 ml) and cleaved with 95:2.5:2.5 - TFA: water: triisopropylsilane cocktail for 1h. The resin was filtered, the filtrate was freeze-dried and the residue was dissolved at a peptide concentration of 0.025 M in 10% DMSO in water. The pH was adjusted to about 8.5 with sodium bicarbonate and stirred for 48h at RT. The solution was then freeze-dried, and the residue was purified by preparative HPLC.

Conditions: Column – Waters Corp. X-Bridge C18; 5.0 microns, 30 x 150 mm; Solvent A – water with 0.1% TFA and solvent B – acetonitrile with 0.1% TFA; Elution rate: 30.0 ml/min; Detection @ 220 nm. Fractions with the required mass and purity of >95% were pooled and freeze dried to provide the required material as colorless fluffy solid.

Analytical HPLC: Column; Agilent Corp. Zorbax 300SB C18; 3.5 microns; 4.6 x 50 mm; Solvent A – water with 0.1% TFA and solvent B – acetonitrile with 0.1% TFA ; Elution rate – 1.0 ml/min. Detection @ 220 nm; Gradient – 5%B to 95% B over 7min; t_R: 3.15 min

Yield: 0.065g (18%). MS: [M-H] 1433.6

Macropatate:

Sequence 2 on the resin (0.25 mmol scale) was cleaved with the same cocktail used for sequence 1, cyclized, and purified by prep. HPLC using the conditions described for Dotatate, resulting in the intermediate Dphe-Cys*-Tyr-DTrp-Lys(ivDde)-Thr-Cys*-Thr-ol . Yield: 0.11g (20%). t_R: 4.95 min; MS – [M-H] 1253.5

The above sequence (35.0 mg, 0.028 mmol) and MACROPA-NCS (*3*) (35.0 mg, 0.033 mmol) were dissolved in DMF (0.2 ml) and DIPEA (85.0 mg, 0.66 mol) was added and stirred for 2h at RT. To the crude conjugate, hydrazine hydrate (42.0 mg, 0.84 mmol) was added and stirred for 6h at RT. The crude peptide was purified by prep. HPLC.

Conditions: Waters X-Bridge C18 column; 50 x 250 mm; 5.0 microns; Solvent A – water and solvent B – acetonitrile; Elution rate – 50.0 ml/min; Detection @ 220 nm; Gradient – 10%B to 95% B over 85.0 min. Fractions with the required mass and purity of >95% were pooled and freeze dried to yield the product as colorless fluffy solid. Yield: 44.5 mg (90%). Analytical HPLC conditions were the same as for DOTA-TATE. t_R: 3.8 min; MS: [M-H] 1637.4

Radiolabeling Macropatate and Dotatate with ^{225}Ac (Representative Procedure)

Solid [^{225}Ac]Ac(NO_3)$_3$, produced by bombardment of a ^{232}Th target, was obtained from Oak Ridge National Laboratory. The actinium salt was dissolved in nitric acid (0.1 M) to make a ^{225}Ac stock solution. The radioactive stock (20 µL, 125 µCi, 4.63 MBq) was mixed with gentisic acid (10 µL, 10 mg/mL in H_2O). The pH was adjusted to 5.5 with NH_4OAc (2 µL, 5 M) before Macropatate in DMSO (20 µL, 20 µg, 12.2 nmol) or Dotatate in H_2O (20 µL, 20 µg, 14 nmol) were added to the reaction vial. Water was added to bring the total volume of the reaction to 100 µL. The Macropatate reaction mixture was incubated at RT for 1 h, while the Dotatate reaction mixture was incubated at 70ºC for 1 h. Purity of the product was assessed using ITLC. Approximately 1 µL of the product was spotted on ITLC-SA TLC strips and the TLCs were developed using a mobile phase consisting of 50 mM EDTA in 100 mM NH_4OAc (pH 5). After allowing at least 12 h for daughter equilibration, TLC strips were scanned on a Bioscan AR-2000 (Eckert and Ziegler, Hopkinton, MA). Successfully radiolabeled product remains at the origin under these conditions, while unchelated material migrates at the solvent front. Typical purities for both radioconjugates were >95% and conjugates were used without purification.

Cell culture

We obtained the U2-OS, AR42J, H69, and U-937 cell lines from ATCC (ATCC, Manassas, VA), U2-OS SSTR2, a transfected SSTR2$^+$ cell line derived from U2-OS and engineered to stably express human SST2, was graciously provided by Dr. Julie Nonnekens, (Erasmus MC, Netherlands). The Bon-1 cell line, obtained from a lymph node metastasis of a neuroendocrine tumor, was provided by Dr. Mark Hellmich (University of Texas Medical Branch at Galveston). Both U2-OS lines were cultured in McCoy's 5A media (ATCC). The H69 and U-937 cell lines were cultured in RPMI-1640 (ATCC), while AR42J cells were cultured in Kaighn's Modification of Ham's F-12 (ATCC). All media was supplemented with 10% Fetaplex (GeminiBio, Sacramento, CA). All cell lines tested negative for mycoplasma in monthly tests and were used for experiments within 15 passages.

Flow cytometry

U2-OS, U2-OS-SSTR2, AR42J, H69, Bon-1 and U937 cell lines were harvested, washed once and resuspended in 60 µL of ice-cold 1% BSA in PBS. To minimize nonspecific staining, cell suspensions were incubated for 15 minutes on ice with human FcR block (Miltenyi Biotec, Bergisch Gladbach, Germany). 1 ×10^6 cells were then distributed into control and experimental groups. Unstained samples were used as controls, and experimental samples were stained with a 50 nM concentration of either commercially available anti-hSSTR2 specific mouse IgG2a conjugated to AlexaFluor 488 (R&D Systems, Minneapolis, USA) or commercially available anti-hSSTR5 specific mouse IgG1 conjugated to AlexaFluor 647 (R&D Systems, Minneapolis, USA). The suspensions were incubated for 1 hour on ice in

the dark. Data were collected using a BD FACSCalibur cytometer running BD CellQuest Pro software (v6.0), and results were analyzed with FlowJo (v10.6.1).

Cell Saturation Assays

Saturation studies were performed to determine the K_D of ^{225}Ac-Macropatate using the SSTR2$^+$ U2-OS SSTR2 transfected small cell lung carcinoma line. Cells were plated (12 well plates with 100,000 cells/well) and varying concentrations of ^{225}Ac-Macropatate were introduced to corresponding wells; non-specific binding was determined by adding unlabeled Dotatate (1000-fold mass excess of each concentration used) to another set of triplicates. After incubation (1.5 h, 37 °C), the bound ^{225}Ac-Macropatate was separated from the free as plated cells were washed with phosphate buffered saline (PBS), treated with trypsin, and collected in vials. The bound radioactivity for these samples was determined by measuring gamma radiation (2480 Wizard, PerkinElmer, Shelton, CT). From the saturation studies, the K_D was determined from six concentrations of ^{225}Ac-Macropatate. Specific binding was calculated by subtracting non-specific binding from total binding and analyzed using non-linear regression curve fitting (one-site specific binding), GraphPad Prism (v 7.0, GraphPad Software, San Diego, CA, USA).

Biodistribution Experiments

The biodistributions of ^{225}Ac-Macropatate and ^{225}Ac-Dotatate at 2, 4, and 24 h post-injection were determined using H69 subcutaneous models. Mice were injected with 37 kBq of radiotracer, and at 2, 4, and 24 h post-injection, mice (n = 3, per time point) were euthanized. Twelve tissues including the tumor were collected. Each sample was weighed and then measured in a gamma counter (2480 Wizard^3, Perkin Elmer Inc, Shelton, CT) calibrated for an open window (0–2000 keV). The counts from each sample were converted into percent injected activity per gram (% IA/g) To probe the effect of amino-acid saturation on radiotracer kidney uptake, mice were treated with D-Lysine hydrochloride, using a previously reported method(*4*). A solution of D-Lysine (Thermo Fisher Scientific, Waltham, MA) in PBS (175 mg/mL, 200 µL) was injected intraperitoneally, and then after 5 minutes mice were injected intravenously with either ^{225}Ac-Macropatate or ^{225}Ac-Dotatate (37 kBq). Biodistribution of the radiotracers was then analyzed as described for mice without lysine pretreatment.

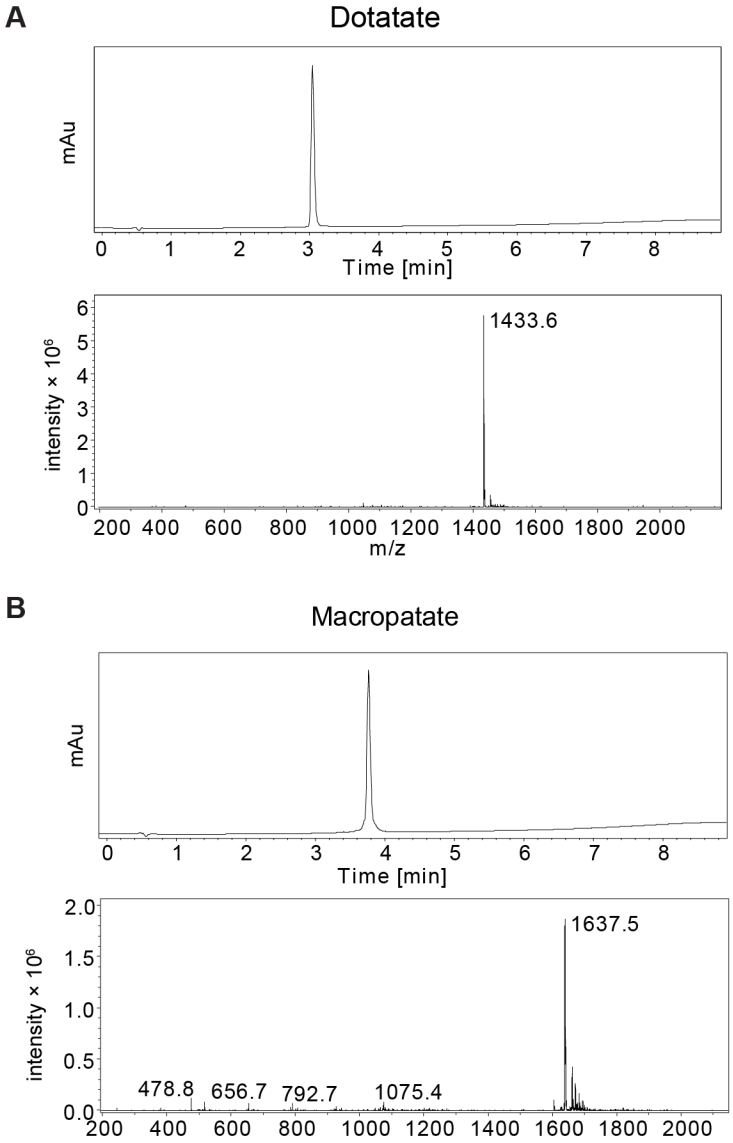


FIGURE S1. Characterization data of Dotatate and Macropatate (A) RP-HPLC with 280 nm detection (top panel) and mass spectrum in positive ion mode (bottom panel) of Dotatate. (B) RP-HPLC with 280 nm detection (top panel) and mass spectrum in positive ion mode (bottom panel) of Dotatate.

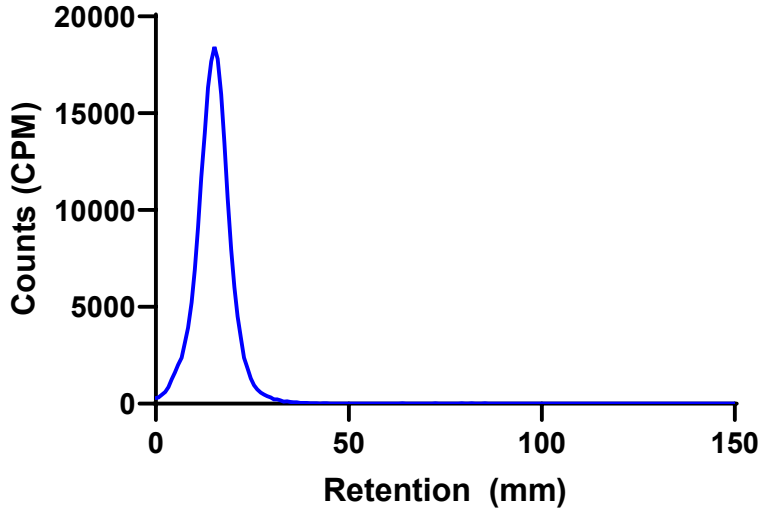


FIGURE S2. ITLC chromatogram of ^{225}Ac-Macropatate after radiolabeling.

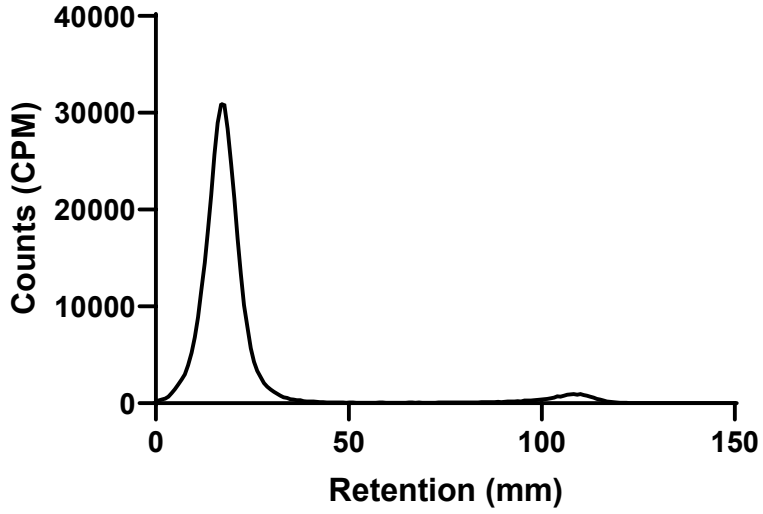


FIGURE S3. ITLC chromatogram of ^{225}Ac-Dotatate after radiolabeling.

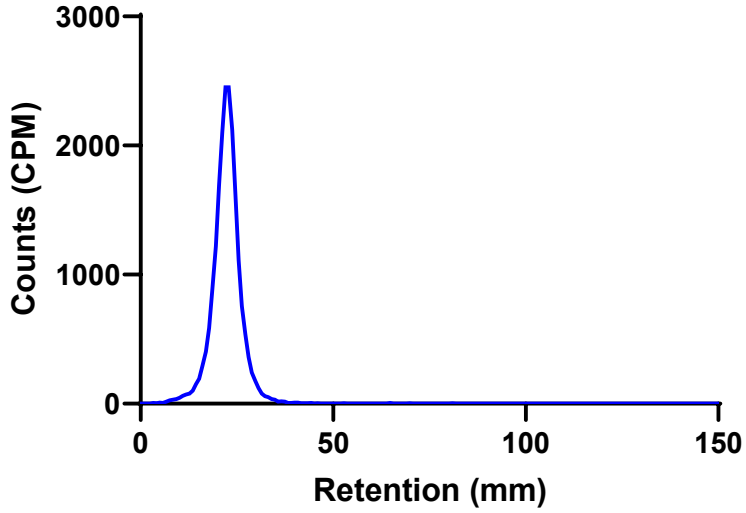


FIGURE S4. ITLC chromatogram of ^{225}Ac-Macropatate in human serum (0 h incubation).

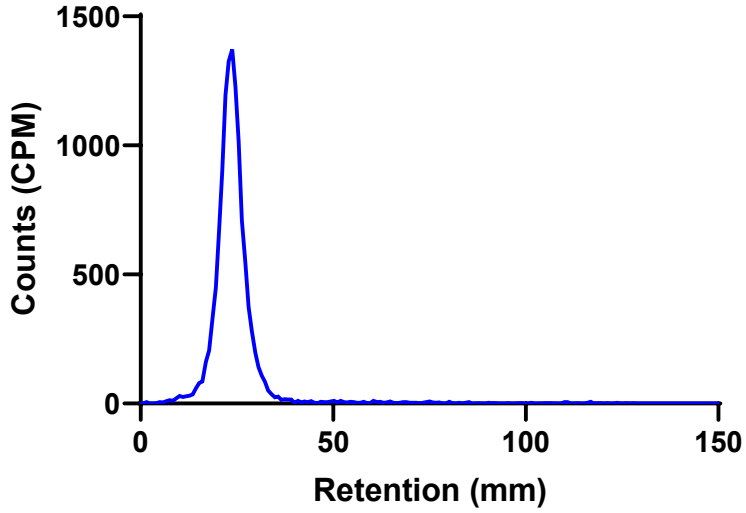


FIGURE S5. ITLC chromatogram of ^{225}Ac-Macropatate in human serum (10 day incubation).

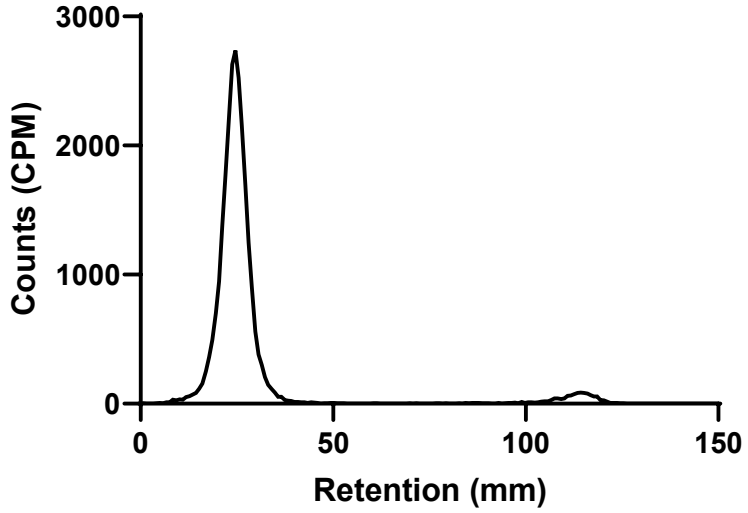


FIGURE S6. ITLC chromatogram of ^{225}Ac-Dotatate in human serum (0 h incubation).

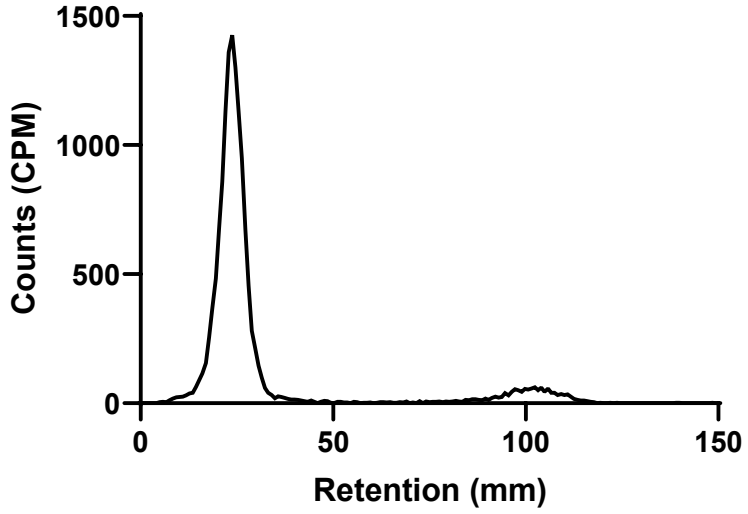


FIGURE S7. ITLC chromatogram of ^{225}Ac-Dotatate in human serum (10 day incubation).

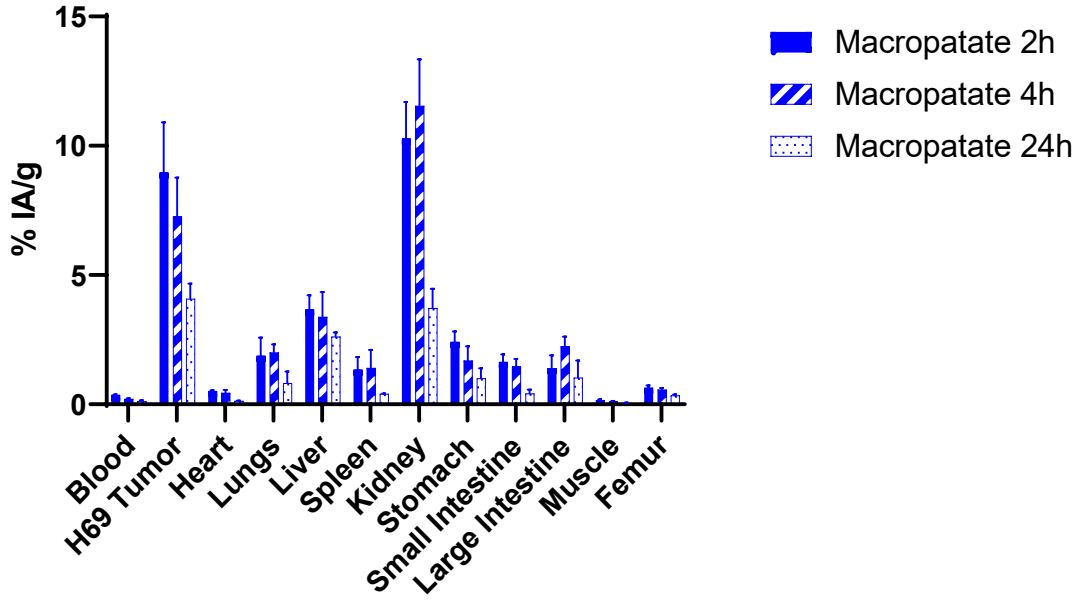


FIGURE S8. Biodistribution at 2, 4, and 24 h post-injection of ^{225}Ac-Macropatate (37 kBq) in mice (n=3) bearing H69 tumor xenografts. Error bars represent the standard deviation.

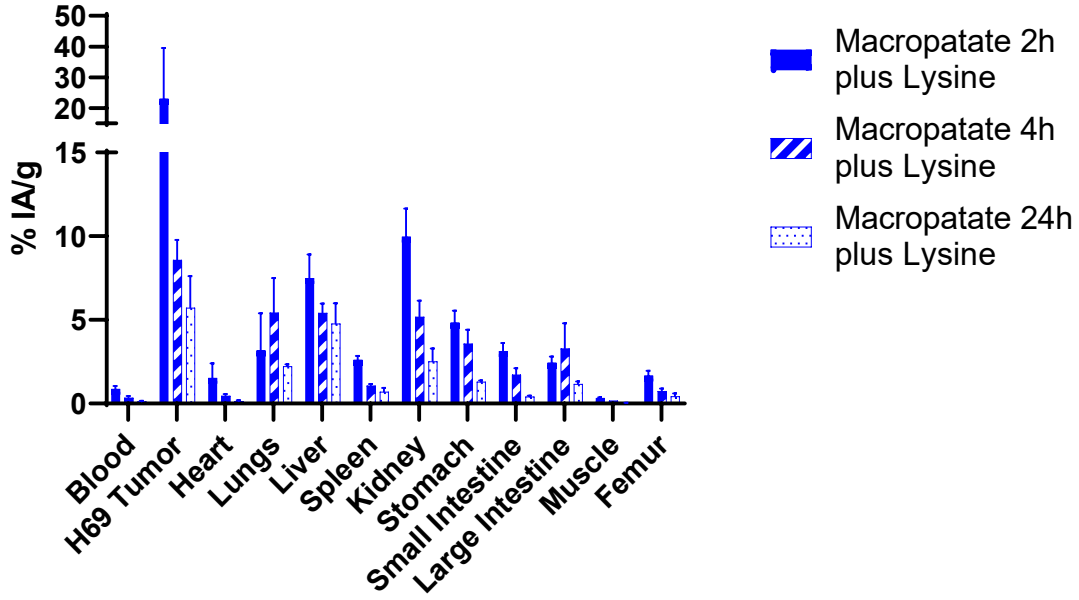


FIGURE S9. Biodistribution at 2, 4, and 24 h post-injection of ^{225}Ac-Macropatate (37 kBq) in mice (n=3) bearing H69 tumor xenografts. Mice were injected with 35 mg of D-lysine hydrochloride immediately prior to radioconjugate administration. Error bars represent the standard deviation.

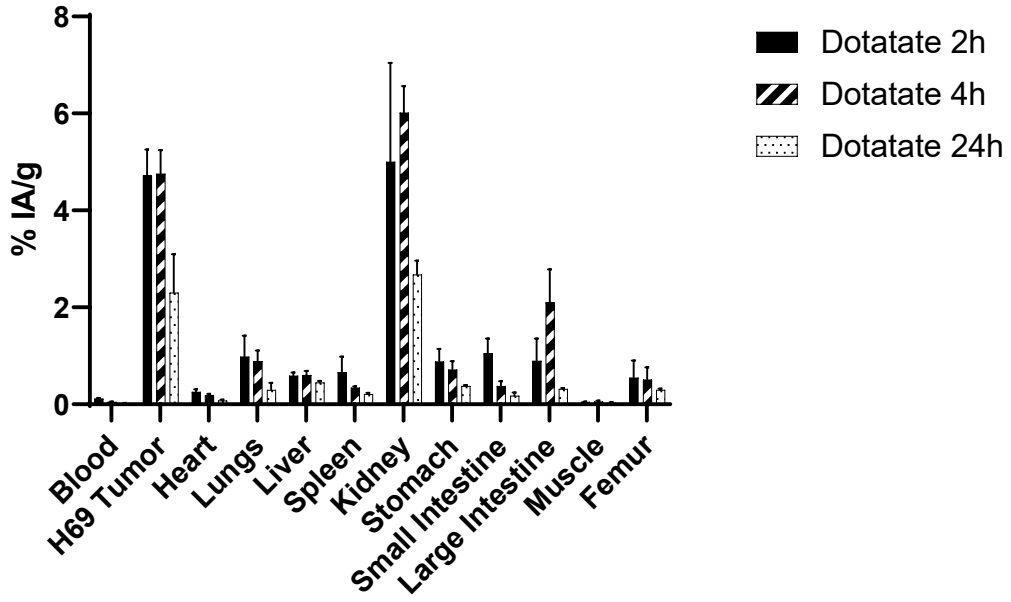


FIGURE S10. Biodistribution at 2, 4, and 24 h post-injection of ^{225}Ac-Dotatate (37 kBq) in mice (n=3) bearing H69 tumor xenografts. Error bars represent the standard deviation.

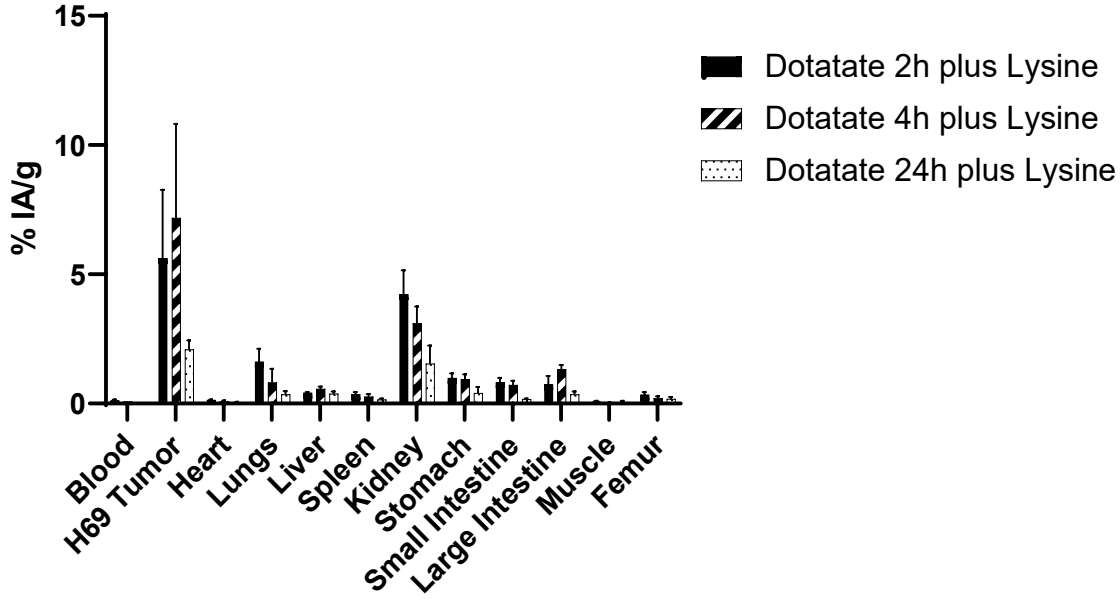


FIGURE S11. Biodistribution at 2, 4, and 24 h post-injection of ^{225}Ac-Dotatate (37 kBq) in mice (n=3) bearing H69 tumor xenografts. Mice were injected with 35 mg of D-lysine hydrochloride immediately prior to radioconjugate administration. Error bars represent the standard deviation.

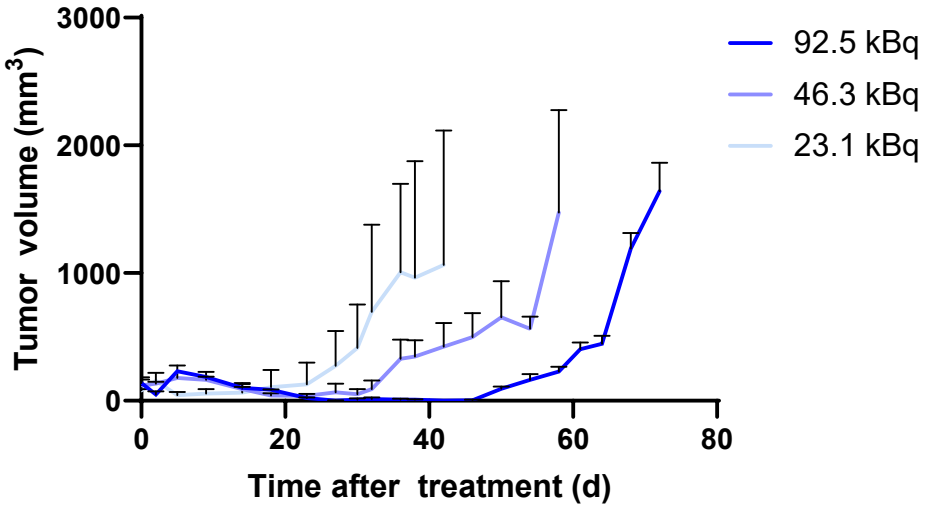


FIGURE S12. Average tumor volumes over time of H69 tumor-bearing mice treated with varying doses of ^{225}Ac-Macropatate (n=3, per group). Error bars represent the standard deviation. Tumor volume tracking ceases after the first mouse in each group was sacrificed due to excessive tumor growth.

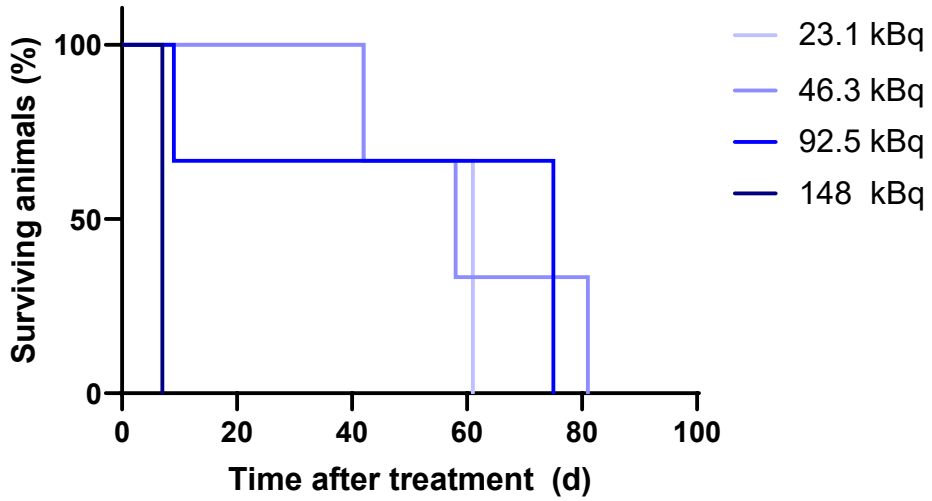


FIGURE S13. Survival over time of H69 tumor-bearing mice treated with varying doses of ^{225}Ac-Macropatate.

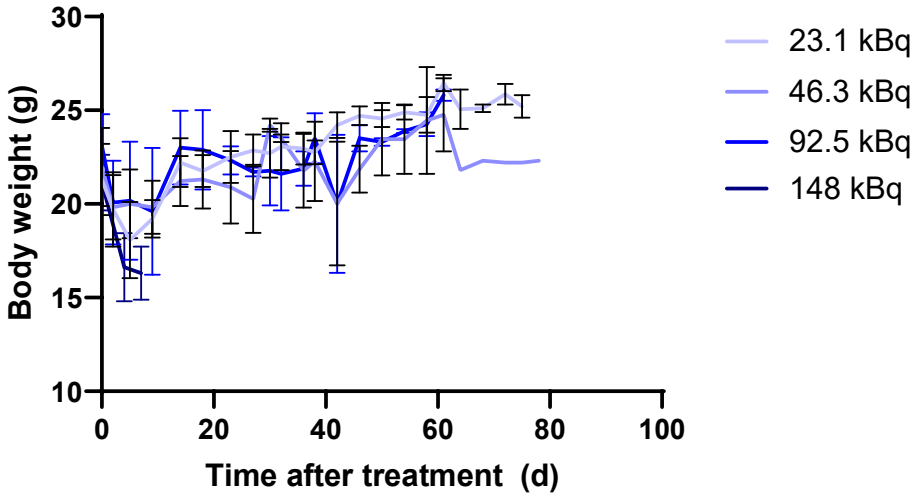


FIGURE S14. Body weights over time of H69 tumor-bearing mice treated with varying doses of ^{225}Ac-Macropatate.

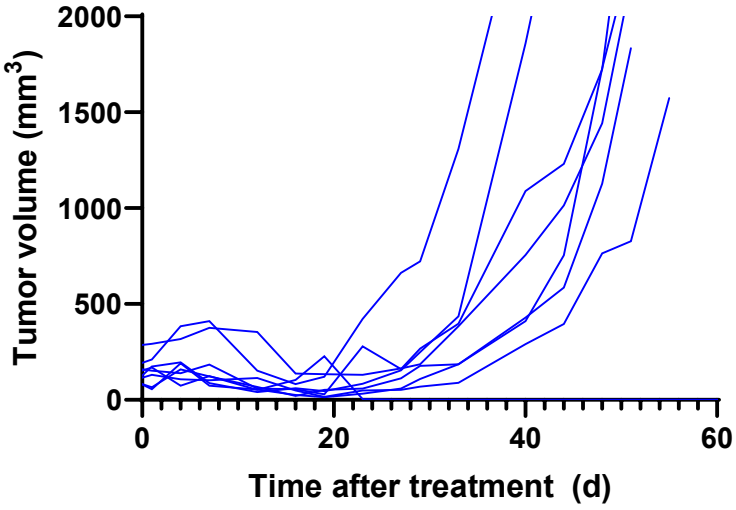


FIGURE S15. Tumor growth over time of H69-xenograft bearing mice (n=8) treated with ^{225}Ac-Macropatate (46.3 kBq). Each line represents an individual mouse.

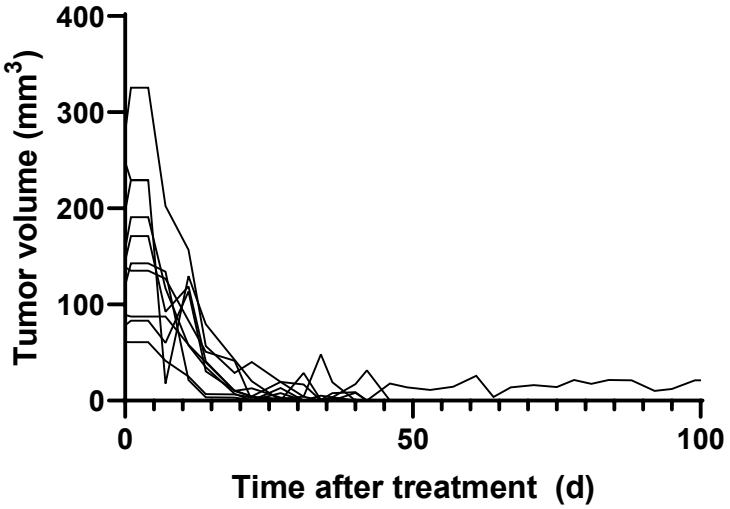


FIGURE S16. Tumor growth over time of H69-xenograft bearing mice (n=10) treated with ^{225}Ac-Dotatate (148 kBq). Each line represents an individual mouse.

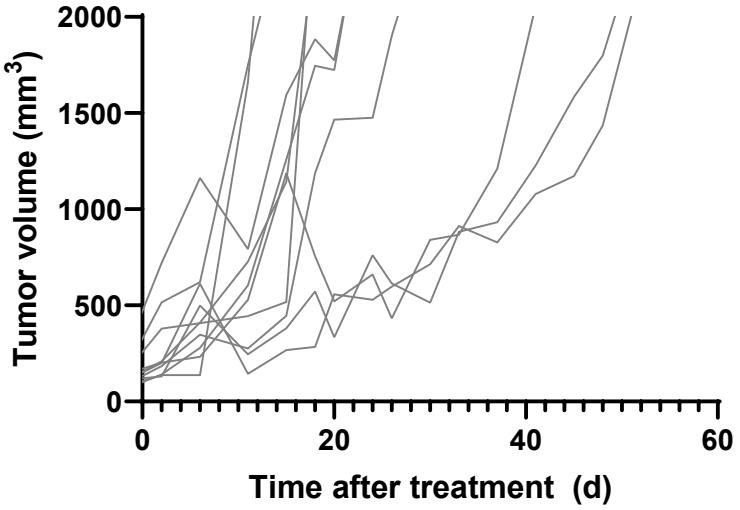


FIGURE S17. Tumor growth over time of H69-xenograft bearing mice (n=10) treated with PBS. Each line represents an individual mouse.

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