Molecular Cancer Therapeutics

Preclinical Investigation of ^{212}Pb-DOTAMTATE for Peptide Receptor Radionuclide Therapy in a Neuroendocrine Tumor Model

Tania A. Rozgaja Stallons[1], Amal Saidi[1], Izabela Tworowska[2], Ebrahim S. Delpassand[2], and Julien J. Torgue[1]

Abstract

Somatostatin analogues have been examined as a treatment for somatostatin receptor overexpressing tumors for years; specifically, octreotate (TATE) and octreotide (TOC). Several versions of these analogues coupled to beta or gamma nuclides are currently used as imaging agents, as treatments with peptide receptor radionuclide therapy (PRRT) for patients with neuroendocrine tumors or are being explored in preclinical and clinical settings. Our study describes the use of ^{212}Pb-DOTAMTATE, the octreotate analogue, in combination with ^{212}Pb, the parent of an alpha emitter. Preclinical studies demonstrated tumor targeting of ^{212}Pb-DOTAMTATE of >20% ID/g up to 24 hours post drug injection. The addition of kidney protection agents, including L-lysine and L-arginine decreases drug accumulation in the kidneys and the addition of ascorbic acid to the chelation mixture reduces oxidation of the drug product. ^{212}Pb-DOTAMTATE displays a favorable toxicity profile with single-dose injections of 20 μCi showing 100% survival and with nontoxic cumulative doses up to 45 μCi, when fractionated into three smaller doses of 15 μCi. In an initial efficacy study, a single 10 μCi injection of ^{212}Pb-DOTAMTATE extended the mean survival 2.4-fold. Efficacy was enhanced by giving three treatment cycles of ^{212}Pb-DOTAMTATE and reducing the time between injections to two weeks. Efficacy was optimized further by the addition of a chemo-sensitizing agent, 5-fluorouracil, given in combination with three cycles of 10 μCi ^{212}Pb-DOTAMTATE. These conditions led to 79% of the animals being tumor free at the end of the 31-week study suggesting that ^{212}Pb-DOTAMTATE alone or in combination with a chemotherapeutic may have positive clinical implications.

Introduction

Although great strides have been taken to increase the success of cancer treatments, new and more specific strategies are urgently needed to increase cancer cytotoxicity while minimizing damage to healthy tissue. One such strategy is to link peptides targeting tumor-associated receptors to radioisotopes, to direct the killing power of these isotopes to tumor cells. For successful targeted radiation, crucial considerations must be addressed regarding emission type, energy/range of emission, and half-life. ^{212}Pb provides a radiotherapeutic agent with short-range cancer cell destruction (α-particles) and potential imaging (γ-ray) capabilities. The ^{212}Pb half-life of 10.6 hours provides clinical feasibility and allows for its production and world-wide distribution.

Peptide receptor radiotherapy (PRRT), specifically with somatostatin analogues, has been examined as a treatment for somatostatin-overexpressing tumors for years. The SSTR binding Tyr3-octreotate (TATE) peptide used in this study has been extensively evaluated in clinical studies in the United States and worldwide. Octreotate-based compounds are routinely used in clinical studies for diagnosis of patients with SSTR-positive neuroendocrine tumors (NET) using gamma-emitting isotopes such as ^{68}Ga (U.S. commercial name Netspot, Novartis) and ^{64}Cu as well as other radiolabeled analogues, ^{111}In-octreoscan and 99mTc-/EDDA/HYNIC-octreotide (1–4). They have shown favorable results in therapy of patients with neuroendocrine tumors using beta-emitting isotopes (^{177}Lu and ^{90}Y; refs. 5, 6) and more recently with alpha particle–emitting isotopes such as ^{225}Ac and ^{213}Bi (7). The ^{177}Lu-DOTATATE phase III study NETTER-1 trial demonstrated a statistically significant and clinically meaningful risk reduction of 79% in disease progression or death versus a treatment with a double dose of Octreotide LAR versus standard of care in patients with progressed midgut carcinoid tumors (8). This study also demonstrated a favorable safety profile of ^{177}Lu-DOTATATE. The median progression-free-survival in the ^{177}Lu-DOTATATE arm (NETTER-1) at 30 months has not yet been reached, while the median progression-free survival in the Octreotide LAR 60 mg arm was only 8.4 months. While beta-emitter PRRT showed very promising results and ^{177}Lu-DOTATATE (Lutathera) has recently been approved in United States and Europe, it is known to be limited in some populations. Patients previously resistant to beta PRRT have responded favorably to alpha therapy (9). Previous studies have demonstrated the low toxicity profile of alpha-emitter–labeled SSTR-targeting agents (7), but limited preclinical data are available. Further studies showed that PRRT could be combined with chemotherapeutics to enhance efficacy (10–14). The studies presented here

[1]Orano Med LLC, Plano, Texas. [2]RadioMedix Inc, Houston, Texas.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Tania A. Rozgaja Stallons, Orano Med LLC, 700 Klein Rd., Plano, TX 75074. Phone: 469-786-6069; Fax: 469-814-9116; E-mail: tania.stallons@oranomed.com

doi: 10.1158/1535-7163.MCT-18-1103

further support the use of alpha SSTR agents as treatment for neuroendocrine tumors.

Extensive preclinical work, including relevant xenograft models of SSTR-overexpressing tumors, has been accomplished showing tumor uptake > 20% ID/g at 1 hour postinjection and remaining for up to 24 hours, a reduction in kidney accumulation by the addition of positively charged amino acids and the reduction of drug oxidation by ascorbic acid added during the chelation step. Furthermore, ^{212}Pb-DOTAMTATE showed a favorable toxicity profile with a Highest Non-Severely Toxic Dose (HNSTD) of 20 μCi and efficacy that can be improved by decreasing the timing between drug injections from three weeks to two weeks. Efficacy data showed a 2.4-fold increase in median survival in mice treated with a single 10 μCi dose of ^{212}Pb-DOTAMTATE. This could be further enhanced by the addition of a chemo-sensitizing agent, 5-fluorouracil (5-FU), which when given in combination of ^{212}Pb-DOTAMTATE (at 2-week intervals) yielded 79% tumor-free mice at the end of the 31-week study. These data suggest that there is therapeutic potential for ^{212}Pb-DOTAMTATE alone or in combination with a chemotherapy as a treatment of SSTR-positive neuroendocrine tumors and have supported the initiation of a phase I clinical study with ^{212}Pb-DOTAMTATE (NCT03466216).

Materials and Methods

Cell line and mice

AR42J rat pancreatic cell line was purchased from ATCC. The cells were tested for *Mycoplasma* by Hoechst DNA stain, Agar culture, and PCR-based assay by ATCC and were not detected as per certificate of analysis. The cells were maintained in F12K media (Gibco) containing 20% FBS (Gibco). Athymic nude mice were purchased from Charles River (Crl:NU(NCr)-*Foxn1*^{nu}) or Envigo (Hsd: Athymic Nude-*Foxn1*^{nu}) and CD-1 mice were purchased from Envigo (HSD:ICR (CD-1)). All studies were conducted using female mice unless otherwise mentioned. All studies were conducted under the approval of the Institutional Animal Care and Use committee.

Manufacturing and radiolabeling

GMP DOTAMTATE ($C_{65}H_{93}N_{17}O_{16}S_2$; Fig. 1) was manufactured by Macrocyclics using Fmoc solid-phase peptide synthesis. DOTAMTATE was added to purified ^{212}Pb at a ratio of 2.4 μCi/ng and incubated at 50°C for 10 minutes with shaking at 300 rpm. For studies using the ascorbic acid–enriched formulation, metal-free L-ascorbic acid (Honeywell) was diluted in Optima water (Thermo Fisher Scientific) and added prior to the drug chelation to a final injection concentration of 10 mmol/L.

iTLC was used to confirm chelation was greater than 95%. Samples were diluted to appropriate activity in PBS or saline prior to injection.

Cell binding assay

Peptide binding to somatostatin receptors 2 (SSTR2) and K_d was evaluated in SSTR2-expressing AR42J cells by growing 2.5×10^5 cells into the wells of a 24-well plate for 48 hours. Concentrations from 0.5 nmol/L to 64 nmol/L of ^{212}Pb-DOTAMTATE were incubated in the AR42J-containing wells for 10 minutes at 37°C. Four replicates were performed for each concentration. Cells were then washed with PBS and cells from each well were counted for the presence of radioactivity. Binding curves were then created and K_d calculated using GraphPad Prism software.

Cell killing assay

A total of 3×10^4 AR42J cells were grown in a 96-well plate for 48 hours. Cells were then incubated for 4 hours with increasing ^{212}Pb-DOTAMTATE ranging from 0 nCi/mL to 800 nCi/mL. Eight wells per group were treated. Cells were washed with PBS to remove the unbound peptide fraction and then fresh media was introduced. Cells were allowed to incubate for 4 days at 37°C. Cells were then rinsed and incubated with fluorescein diacetate for 30 minutes and read with a fluorimeter at 485/535 nm. Percentage of viable cells was calculated on the basis of untreated cells as a control.

Tumor models

For all tumor studies, 2×10^6 AR42J cells were implanted subcutaneously, in an equal volume mixture of GFR-Matrigel (Corning) and RPMI media (Gibco), into the right flank of each mouse and grown to a volume of approximately 200–300 mm^3.

Preparation of kidney protection agents

Two-hundred microliters of L-lysine-L-arginine (35 mg/mL of each) diluted in saline or 10% dextrose, 200 μL of L-lysine (35 mg/mL or 70 mg/mL) in saline, or 200 μL of L-arginine (70 mg/mL) in saline were given via intravenous injection 5 minutes prior to drug injection.

Biodistribution studies

Tumors were grown in female mice until an approximate volume of 300 mm^3 was reached. Two hundred microliters of ^{212}Pb-DOTAMTATE (5 μCi) was administered to the mice via the tail vein and mice were euthanized at predetermined timepoints. The background was automatically subtracted from the counts. A standard is also used for decay correction. %ID/g was calculated for each organ collected.

Alpha imaging

Ex *vivo* assessment of ^{212}Pb-DOTAMTATE localization and microdosimetry was performed on frozen sections (10–12 μm) of AR42J xenograft tumors placed on a phosphor sheet (Eljen Technology) and imaged using a high-sensitivity QHYCCD camera (Andor). Images were analyzed with Micromanager software (ImageJ)

Radio HPLC studies

^{212}Pb-DOTAMTATE was analyzed on an Agilent 1220 HPLC using a C18 reverse phase column (Restek) with an acetonitrile gradient. Fractions were collected off the column every 10 seconds for a total of 10 minutes and then analyzed for radiometric detection by auto gamma counter (Perkin Elmer).

Toxicity studies

Female athymic nude mice received an injection of either 10 μCi, 20 μCi, 40 μCi, or 60 μCi of ^{212}Pb-DOTAMTATE or control PBS intravenously. Animals were weighed three times per week and monitored daily for signs of termination criteria over a 4-week period. For fractionated toxicity, study animals ($n = 10$ per group) received a single injection of 40 μCi ^{212}Pb-DOTAMTATE, 2× 20 μCi of ^{212}Pb-DOTAMTATE, or 3× 15 μCi of ^{212}Pb-DOTAMTATE. Repeat injections were given at 3-week intervals. Control mice received PBS only. Blood was sampled via the retro-orbital plexus using potassium-EDTA capillaries and tubes

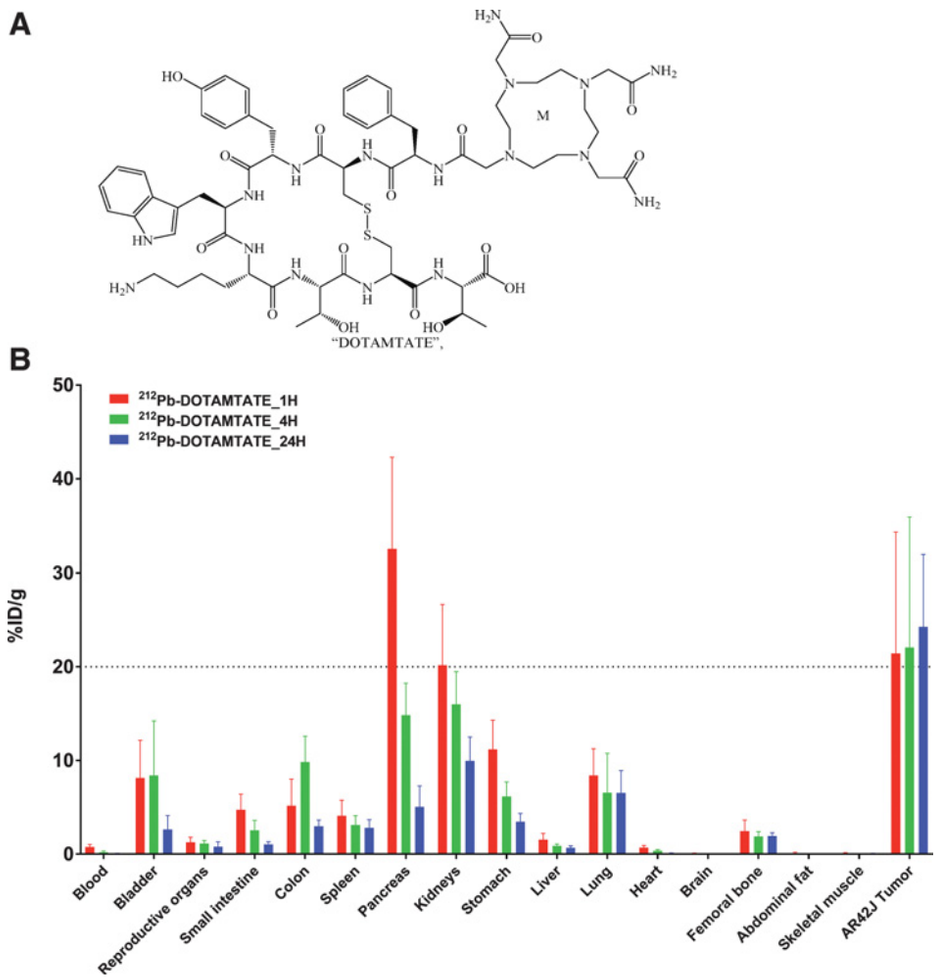

Figure 1.
Drug structure and initial biodistribution. This figure shows the chemical structure of DOTAMTATE and biodistribution of ^{212}Pb-DOTAMTATE in athymic nude tumor-bearing mice. **A,** Chemical structure of DOTAMTATE (Formula: C65H93N17O16S2). **B,** Drug was administered and organs were collected from 5 mice per timepoint: 1 hour post (red), 4 hours (green), and 24 hours (blue) post injection.

(Greiner Bio-one) and complete cell blood count (CBC) was obtained using VETSCAN HM5 Hematology Analyzer (Abaxis). Animals were euthanized when termination criteria were met.

Efficacy study

Tumor-bearing animals were injected with 100 μL of 5 μCi or 10 μCi ^{212}Pb-DOTAMTATE or control (PBS or cold peptide). After 3 weeks, mice who received the 5 μCi dose, received a second dose of ^{212}Pb-DOTAMTATE. Animals were monitored daily and calipered three times per week to monitor tumor volume. Mice were sacrificed when termination criteria were met.

Combination efficacy with 5-FU

All animals were grown with tumors as described above. Control groups were injected with saline alone or 15 mg/kg 5-FU (Acros) once per week for 9 weeks (5-FU alone). Radiotherapy only groups received 10 μCi of ^{212}Pb-DOTAMTATE at 2-week or 3-week intervals. Combination therapy groups received a treatment of 5-FU (15 mg/kg) followed 24 hours later by 10 μCi of ^{212}Pb-DOTAMTATE. The 5-FU was continued weekly for a total of 9 weeks for both treatment groups. 10 μCi of ^{212}Pb-DOTAMTATE was given 24 hours after the first 5-FU injection and then at 2- or 3-week intervals for a total of three injections. Animals were monitored daily for signs of termination criteria and calipered three times per week to monitor tumor volume. Animals were euthanized when termination criteria were met.

Termination criteria

Mice were sacrificed when tumor volumes reached 3,000 mm^3 or other predetermined termination criteria were met (weight loss over 15% for two consecutive days or 20% weight loss from initial weight, serious bleeding, necrosis or ulceration of the tumor, scruffiness or lack of grooming over 5 days, lethargy over 3 days, weakness/balance issues over 5 days, hunchback appearance, diarrhea, or hypothermia).

Statistical analysis

Animals were randomly assigned to each group. An unpaired *t* test was used for statistical analysis.

Patient studies

The study was conducted in accordance with the Declaration of Helsinki ethical guidelines and upon signature of the Institutional review board–approved informed consent form. Studies were performed under FDA IND 130960.

Results

In vitro data

An *in vitro* binding study of ^{212}Pb-DOTAMTATE to SSTR2-expressing AR42J cells yielded a K_d of 12.9 nmol/L (Supplementary Fig. S1A), which is in line with other studies that have examined the binding of octreotate peptides to somatostatin-expressing cell lines (15). In addition, a cytotoxicity assay showed a dose-dependent cytotoxic effect of ^{212}Pb-DOTAMTATE for AR42J cells with complete death observed at 800 nCi/mL and 50% viability observed between 12.5 nCi/mL to 25 nCi/mL (Supplementary Fig. S1B). A ^{212}Pb-chelate only negative control did not show a dose-dependent cytotoxic effect with viability ranging from 47% to 156%.

Biodistribution studies

All studies were conducted in female mice, as a biodistribution study showed that there was no significant difference in organ uptake between male and female mice (Supplementary Fig. S2A) and the literature suggests that female mice may be more susceptible to toxicity and may provide a worst case scenario between the two sexes (16). When animals were injected with a single dose of 5 μCi ^{212}Pb-DOTAMTATE, the average tumor uptake exceeded 20% ID/g 1 hour after drug administration and remained constant through 4 and 24 hours post drug administration (Fig. 1). The pancreas and kidneys were the two organs with the highest nontarget uptake, but these organs also showed significantly less accumulation by 24 hours postinjection. In further examining AR42J tumors for ^{212}Pb-DOTAMTATE distribution, no correlation between tumor volume and tumor uptake is visible in tumors up to 1,500 mm^3 (Supplementary Fig. S3) and alpha imaging of tumors treated with ^{212}Pb-DOTAMTATE showed homogenous distribution of the drug at all tumor sizes up to 1,500 mm^3 (Supplementary Fig. S4A). Three specific activities of 4.1 ng, 22 ng, or 110 ng per 10 μCi were also examined via biodistribution study (Supplementary Fig. S2B). 10 μCi per 4.1 ng (2.4 μCi/ng) has been primarily used in ^{212}Pb-DOTAMTATE studies to date; however, a decrease in the specific activity does not appear to have a significant effect on tumor uptake. This suggests that receptor saturation is not occurring even at over 25-fold lower specific activity then what has been primarily used in these studies.

Reduction of renal retention of ^{212}Pb-DOTAMTATE

As kidney protection agents are often given with targeted radiotherapies to minimize nephrotoxicity, several kidney protection agents and diuretics were tested in combination with ^{212}Pb-DOTAMTATE through biodistribution studies for their ability to minimize drug accumulation in the kidneys (Fig. 2). Of the five versions of amino acid combinations/concentrations given, all were able to significantly reduce ^{212}Pb-DOTAMTATE uptake in the kidneys ($P \leq 0.0001$) at 1 hour post drug injection. Additional studies conducted with higher levels of L-lysine in tumor bearing mice at three timepoints showed that while kidney uptake is reduced no effect on drug accumulation in the tumor was observed (Supplementary Fig. S4B).

Enhancing stability of ^{212}Pb-DOTAMTATE binding with ascorbic acid

Oxidation of DOTATATE peptides, specifically on the indol ring of the tryptophan residue has been shown to occur when the peptide is labeled with radioisotopes and can be minimized by the addition of ascorbic acid (17). The presence of an oxidized form of DOTAMTATE was also witnessed in our studies when the drug was prepared and not used immediately; however, it was not known whether this oxidation influenced the drug binding to its SSTR targets. To test whether the presence of oxidized DOTAMTATE influenced overall drug binding, a biodistribution study was conducted in AR42J tumor-bearing mice. ^{212}Pb-DOTAMTATE prepared with and without ascorbic acid present during chelation was left overnight (to obtain a worst-case scenario) and oxidation confirmed the following day by Radio-HPLC before the biodistribution was conducted (Fig. 3). Drug binding to the tumor was significantly enhanced ($P < 0.01$) in the presence of ascorbic acid during the chelation reaction at 1, 4, and 24 hours (33% ID/g 24 hours post drug injection) compared with the ascorbic acid–free formulation (10% ID/g 24 hours post drug injection) suggesting

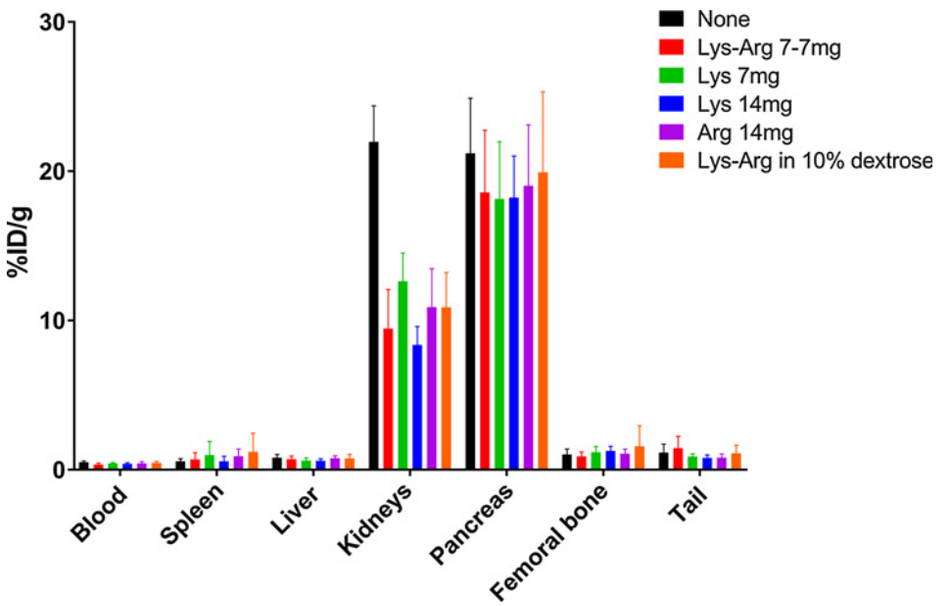

Figure 2.
^{212}Pb-DOTAMTATE in the presence of kidney protection agents. This figure shows a biodistribution of ^{212}Pb-DOTAMTATE in CD-1 mice at 1-hour post drug injection with kidney protection agents. Two-hundred microliters of kidney protections agents 7 mg Arg–7 mg Lys in saline (red), 7 mg Lys in saline (green), 14 mg Lys in saline (blue), 14 mg Arg in saline (purple), or 7 mg Arg–7 mg Lys in 10% dextrose (orange) were given intravenously 5 minutes before drug injection. No kidney protection agent control shown in black. Five mice per group. Average of two studies displayed.

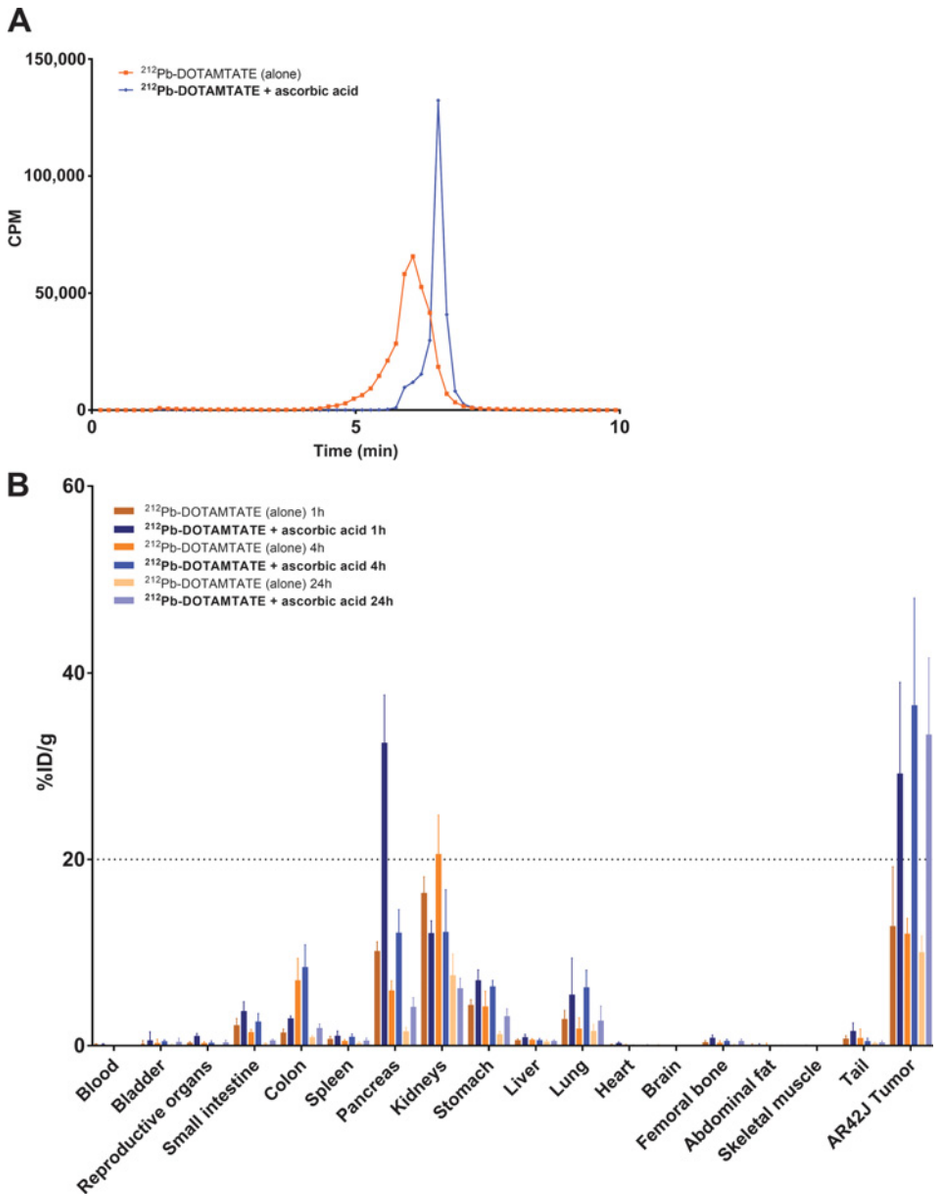

Figure 3.
Addition of ascorbic acid to drug preparation. This figure shows radio HPLC and biodistribution studies of ^{212}Pb-DOTAMTATE with ascorbic acid. **A,** Radio-HPLC of ^{212}Pb-DOTAMTATE without ascorbic acid present during chelation (orange) or with 10 mmol/L final concentration of ascorbic acid (blue) prior to biodistribution. **B,** Biodistribution of ^{212}Pb-DOTAMTATE in AR42J tumor-bearing mice ($n = 5$ per group) at 1, 4, and 24 hours post drug injection in the presence of 10 mmol/L ascorbic acid (blue) or none (orange).

that oxidation was having a negative effect on the drug but could be minimized with the addition of the antioxidant.

^{212}Pb-DOTAMTATE toxicity studies

To assess the toxicity profile of ^{212}Pb-DOTAMTATE, a dose range–finding study in female, athymic nude mice was first conducted as a basis for dose selection for subsequent efficacy studies and for a GLP toxicity study. An MTD was determined to be between 20 μCi and 40 μCi (Fig. 4A).

A single dose GLP toxicity study was conducted in female CD-1 nontumor-bearing mice at doses of ^{212}Pb-DOTAMTATE ranging from 0 μCi to 40 μCi per mouse. Body weights, clinical chemistry, and hematology parameters were examined throughout the 9-month duration of the study. The 2 and 10 μCi doses appeared to be reasonably well tolerated, whereas administration of a single 40 μCi intravenous dose of ^{212}Pb-DOTAMTATE was associated with adverse findings including mortality, decreased body weight gain, leukocyte, erythrocyte, serum albumin, and organ weights as well as histopathologic findings of bone marrow depletion and gastrointestinal lesions. At 20 μCi, there were relatively mild and reversible effects on weight gain and leukocyte counts along with chronic glomerular nephritis, which appears late in the study due to a combination of aging and dosing. On the basis of the study findings, the dose of 10 μCi was considered a no-observable effect limit (NOEL) dose and an HNSTD of 20 μCi was determined (Supplementary Fig. S5).

To further examine whether the HNSTD determined in the single-dose toxicity study could be overcome through fractionation of the ^{212}Pb-DOTAMTATE, a repeat dose toxicity study was conducted in nontumor-bearing CD-1 mice (Fig. 4B). As

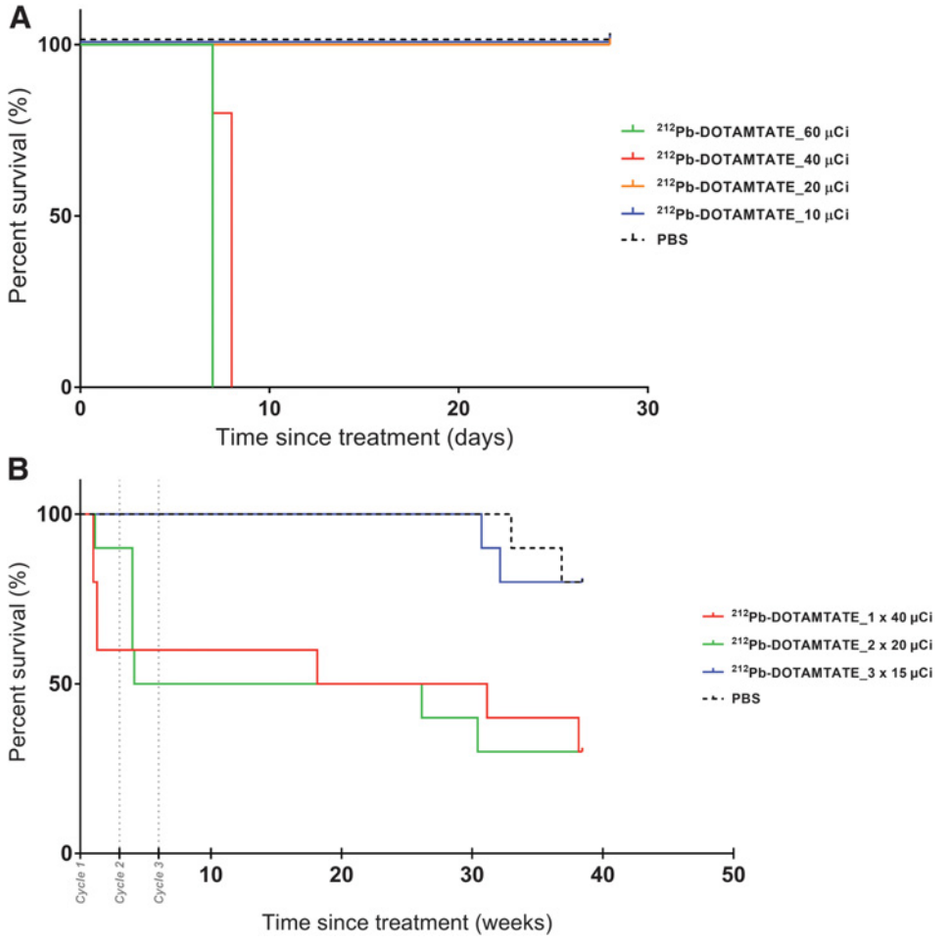

Figure 4.
Dose range finding and toxicity studies. This figure shows initial dose range–finding studies and fractionated dose toxicity studies with ^{212}Pb-DOTAMTATE in athymic nude mice. **A,** Kaplan–Meier survival curve of ^{212}Pb-DOTAMTATE–treated athymic nude mice. Animals received a single dose of 10 μCi (blue), 20 μCi (orange), 40 μCi (red), or 60 μCi (green) of ^{212}Pb-DOTAMTATE or PBS control (black). $n = 5$ mice per group. Survival of the animals are shown in days post injection during the 4-week study. **B,** Kaplan–Meier curve of ^{212}Pb-DOTAMTATE in CD-1 mice in a fractionated dose toxicity study. PBS alone, $n = 10$ (black), 1 × 40 μCi, $n = 10$ (red), 2 × 20 μCi, $n = 10$ (green), and 3 × 15 μCi, $n = 10$ (blue) treatment groups. Drug cycles 1, 2, and 3 are shown with gray dots.

hematologic toxicity is routinely dose limiting for radiotherapeutics and is usually reversible with time at lower doses, fractionation was expected to overcome the lower HNSTD determined in the single dose study. Animals were given a single dose of 40 μCi of ^{212}Pb-DOTAMTATE, two cycles of 20 μCi, or three cycles of 15 μCi ^{212}Pb-DOTAMTATE every 3 weeks. Almost 40% of animals in the 1 × 40 μCi group died 9 days after injection, but those that survived were able to survive through the remainder of the study. Fifty percent of the animals in the 2 × 20 μCi group died within 4 weeks of the study and 1 week after receiving the second dose. The animal group that received 3 × 15 μCi of ^{212}Pb-DOTAMTATE were consistent with the control group. Hematologic toxicity appeared to be the reason for death in the first two groups. This was evident by the significantly low white blood cell counts (WBC) and platelets (PLT) in the 1 × 40 μCi and 2 × 20 μCi groups after drug injections (Supplementary S6). Animals who received 3 × 15 μCi doses of ^{212}Pb-DOTAMTATE also had a decrease in their WBC and PLT counts but were able to recover after each dose. This study suggests that a fractioned dose of drug is optimal as it allows the same cumulative dose but with recoverable hematologic effects.

Efficacy studies with ^{212}Pb-DOTAMTATE

An initial low-dose efficacy study of ^{212}Pb-DOTAMTATE was performed to examine the effectiveness of the drug in tumor bearing mice at 25% of the HNSTD. Animals were given one or two cycles of 5 μCi ^{212}Pb-DOTAMTATE or 10 μCi ^{212}Pb-DOTAMTATE. Control animals received cold-DOTAMTATE or PBS. Animals that were injected with cold-DOTAMTATE or PBS had similar median survival of 3.4 weeks and 3.5 weeks, respectively, post injection. Mice that received one injection of 5 μCi ^{212}Pb-DOTAMTATE had a median survival of 6.3 weeks while mice who received one injection of 10 μCi ^{212}Pb-DOTAMTATE had a median survival of 8.5 weeks showing a dose-dependent effect. Two injections of 5μCi ^{212}Pb-DOTAMTATE led to a median survival of 7.1 weeks (Fig. 5). The median survival time was similar between animals that received 1 × 10 μCi versus 2 × 5 μCi of drug suggesting that at low doses a fractionated dose does not appear to be beneficial. Overall, however, the ^{212}Pb-DOTAMTATE does show efficacy at these low doses, but efficacy could likely be improved with higher treatment doses.

With this data, efficacy studies with ^{212}Pb-DOTAMTATE were further optimized with a combination therapy and treatment cycle study. The aim of this study was to optimize the timing of treatment cycles and to combine the radiotherapeutic with a subtherapeutic (~40 mg/m^2 versus at least 400 mg/m^2 in human) chemotherapy dose of 5-FU to maximize tumor devastation. Animals received saline only, 5-FU only, 3 × 10 μCi of ^{212}Pb-DOTAMTATE at 2-week or 3-week intervals, or a combination of 5-FU and 3 ×10 μCi ^{212}Pb-DOTAMTATE at 2-week or 3-week

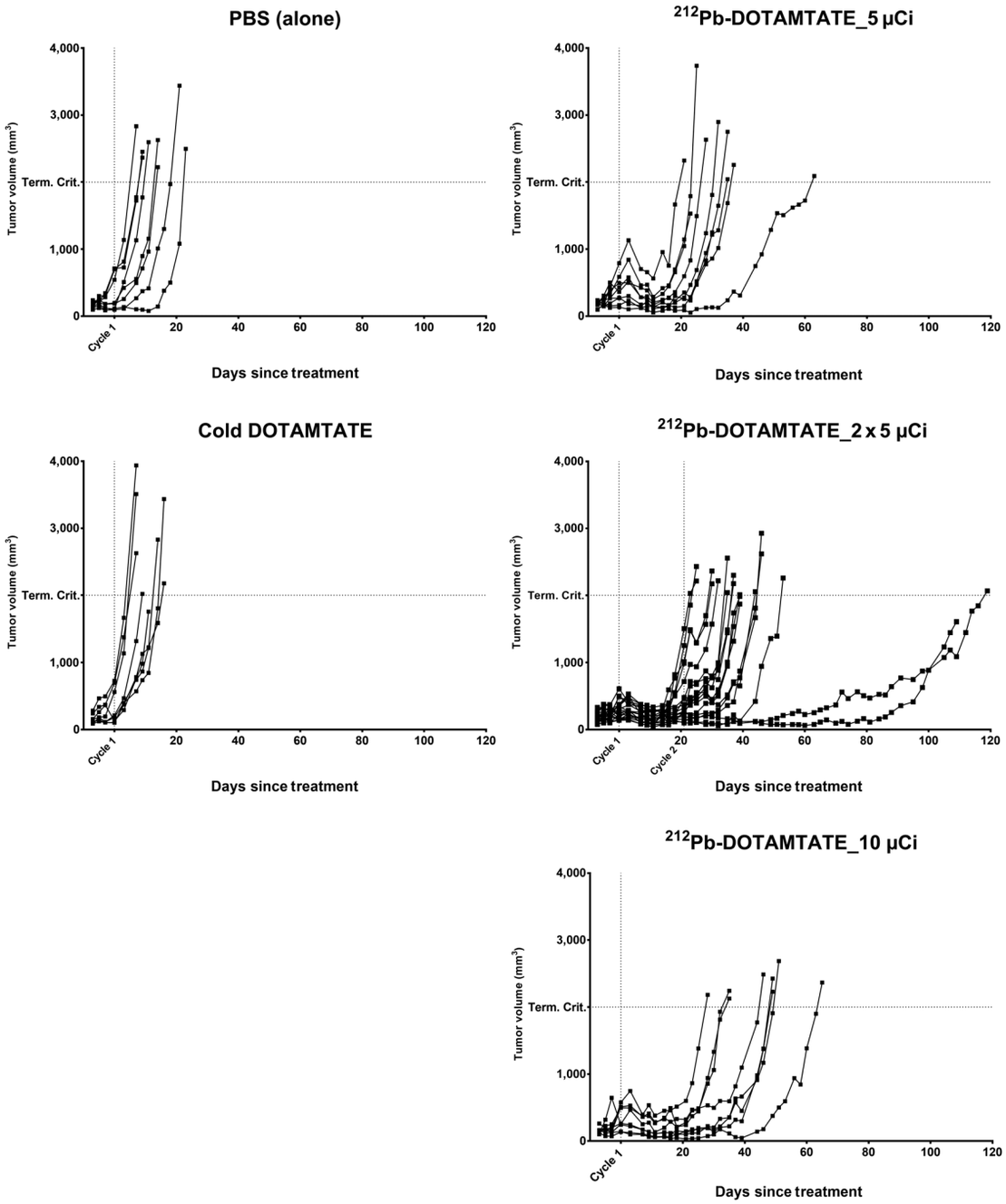

Figure 5.

Single versus multiple dose efficacy study. This figure shows the efficacy of mice treated with ^{212}Pb-DOTAMTATE in a single and multi-injection dose setting. Groups of animals were injected with cold DOTAMTATE ($n = 8$), PBS ($n = 8$), 5 µCi ^{212}Pb-DOTAMTATE ($n = 9$), 10 µCi ^{212}Pb-DOTAMTATE ($n = 8$), 2 × 5 µCi ^{212}Pb-DOTAMTATE ($n = 18$). Tumor volumes for individual mice per group are shown in mm^3 over time.

intervals (Fig. 6). Animals that were injected with 5-FU alone had a median survival of 2.4 weeks while the saline alone group had a median survival of 3.1 weeks post cell injection. Mice that received three injections of ^{212}Pb-DOTAMTATE only at three-week intervals had a median survival rate of 9.4 weeks while combination therapy with 5-FU led to a longer median survival of 11.1 weeks with 20% of the mice alive and tumor free at the termination of the 31-week study. When ^{212}Pb-DOTAMTATE was given at 2-week intervals the median survival was 11.9 weeks and this was further improved by the addition of 5-FU where 79% of the animals survived to the end of the 31-week study. This suggests that the timing of the drug treatment is critical in maximizing its effectiveness. Furthermore, optimal timing of the radiotherapeutic combined with a radiosensitizer can significantly enhance

efficacy versus the drug alone and lead to a significant group of tumor-free animals.

Discussion

Extensive preclinical work and optimization has been accomplished to demonstrate the feasibility, safety, and therapeutic potential of ^{212}Pb-DOTAMTATE alone or in combination as a treatment for SSTR-positive neuroendocrine tumors. Specifically, *in vitro* assays have shown that the peptide binds to its SSTR receptor with an appropriate affinity for therapeutic use and has cytotoxic effects. Furthermore, *in vivo* tissue distribution studies in tumor-bearing animals showed ^{212}Pb-DOTAMTATE has a high uptake in the tumor relative to other organs. Although some drug

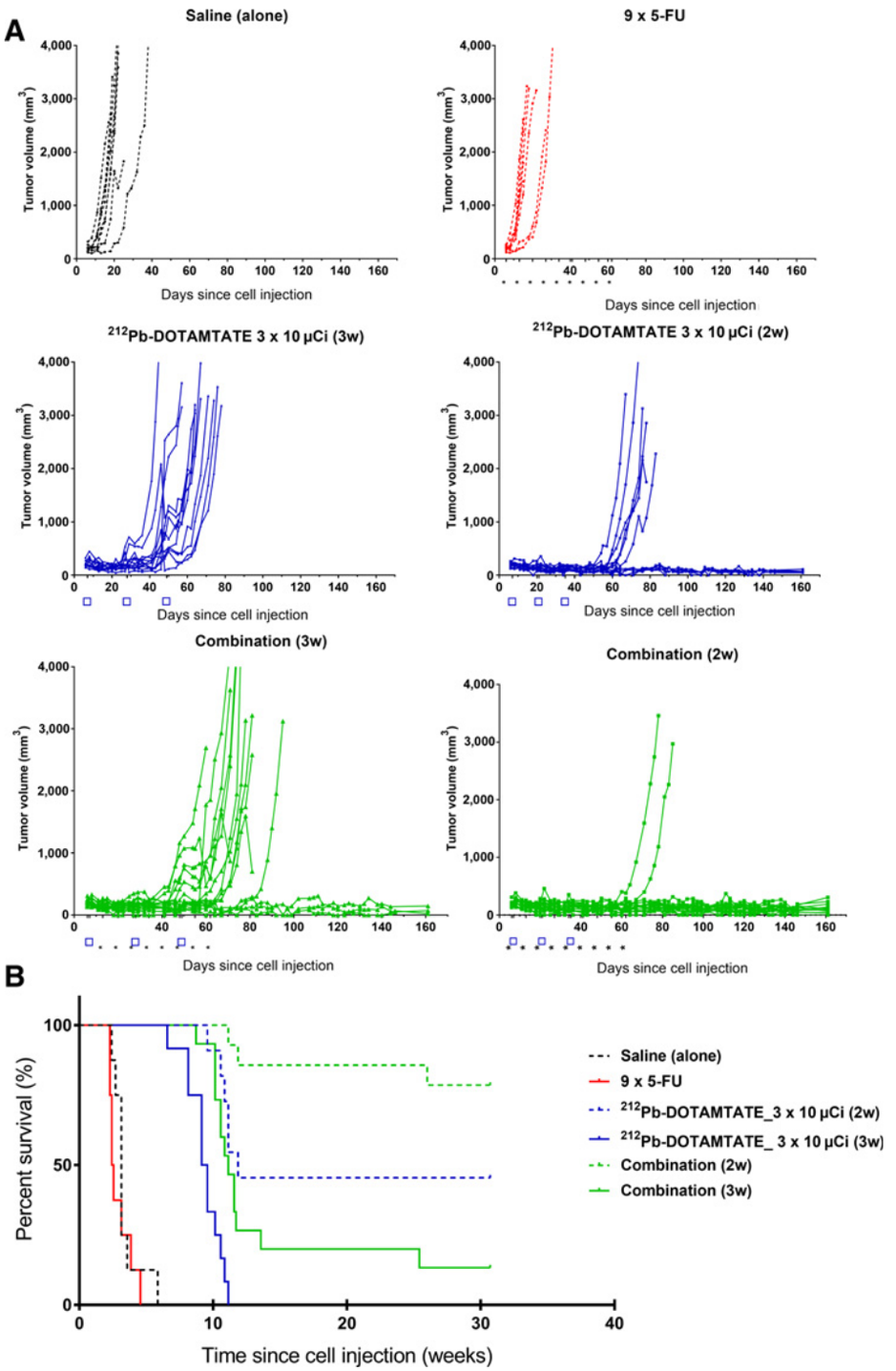

Figure 6.
Combination efficacy study. This figure shows an efficacy study of AR42J tumor-bearing mice treated with ^{212}Pb-DOTAMTATE in combination with 5-FU. **A,** Individual mouse efficacy data showing tumor volumes of mice injected with saline (black), 5-FU only (red), ^{212}Pb-DOTAMTATE only (blue) and in combination with 5-FU (green). **B,** Kaplan–Meier of animals treated with saline (black), 5-FU only (red), ^{212}Pb-DOTAMTATE only at 2-week intervals (blue dashed), 3-week intervals (blue solid), in combination with 5-FU at 2-week intervals (green dashed) or 3-week intervals (green solid).

uptake and retention was observed in the kidneys and pancreas of animals, it decreased significantly by 24 hours post drug injection. This uptake is not unexpected as these organs have also shown high uptake in other nonclinical rodent studies involving alpha emitters, which have not transliterated into adverse effects in human studies. (7, 18–21). However, given the particularly high tumor uptake, the DOTAMTATE peptide has potential not only for therapeutic applications with ^{212}Pb but also for imaging applications using longer-lived and gamma-emitting lead isotopes such as ^{203}Pb. Biodistribution studies conducted in our laboratory have shown that CD-1 mice given ^{203}Pb-DOTAMTATE did not show significantly different tissue uptake compared with mice treated with ^{212}Pb-DOTAMTATE in all critical organs (Supplementary Fig. S7). This was confirmed by an exploratory IND (IND 102,590) conducted to examine the dosimetry and biodistribution of ^{203}Pb-DOTAMTATE in patients with SSTR-expressing neuroendocrine tumors as a surrogate for ^{212}Pb-DOTAMTATE. ^{203}Pb-DOTAMTATE showed similar pharmacokinetic properties to other commercially available octreotate drugs but with the advantage that the same metal could be used for imaging and therapeutic applications. This further confirms that the two isotopes have a similar physical property and pharmacokinetic profile and could therefore be used for theranostic purposes.

As with many PRRT treatments, the presence of radiolabeled somatostatin analogues in the kidneys is common due to their renal clearance and retention by megalin/cubulin receptors (20–22). Kidney protection agents including L-lysine-L-arginine, mixtures of positively charged amino acids and amifostine are often given in combination with radiolabeled drugs (23–25). With ^{212}Pb-DOTAMTATE specifically, multiple kidney protection agents were tested, and all were found to significantly reduce drug uptake in the kidneys 1 hour post drug injection. It should be noted, however, that these were given as a bolus injection 5 minutes prior to drug injection rather than an intravenously over the course of 4 hours, which is done with patients, due to animal model constraints; therefore, the data may not directly translate into a clinical setting.

In addition to kidney uptake, another factor that must be considered with PRRT is the oxidation of peptides in the proximity of radionuclides. The presence of an oxidized form of DOTAMTATE was detected in our studies by radio-HPLC and was shown to have a negative impact on tumor binding through biodistribution studies. Free radicals have been shown to form in solutions containing high energy β-particles and tryptophan residues, specifically, can become oxidized (26–28). The addition of the antioxidant, ascorbic acid, during the chelation reaction significantly enhanced (3× vs. a mostly oxidized peptide) tumor binding presumably by minimizing this tryptophan oxidation within the ^{212}Pb-DOTAMTATE peptide as confirmed by Radio-HPLC analysis.

To better characterize the safety profile of ^{212}Pb-DOTAMTATE a dose range–finding study in female, athymic nude mice was conducted as a basis for dose selection for subsequent efficacy studies and a single-dose GLP toxicity study with ^{212}Pb-DOTAMTATE. The dose range–finding study led to a MTD between 20 μCi and 40 μCi and provided the preliminary information for a single-dose GLP toxicity study, which included a 9-month follow-up to determine potential delayed toxicities in radiation-sensitive organs. On the basis of histopathology, body weights, hematology, and clinical chemistry from this GLP toxicity study, an HNSTD of 20 μCi was determined. An additional toxicity study showed that fractionating the dose was optimal and allowed for a cumulative dose that would be toxic if given as a single injection.

Efficacy studies showed that ^{212}Pb-DOTAMTATE has therapeutic potential as it was able to extend median life span 2.4-fold with a single treatment at low doses. Furthermore, it was able to cure approximately 50% of the animals when the timing of the drug was optimized. The time between cycles must be sufficient to allow for acute hematologic toxicity recovery without being too long of a duration that the tumor growth rate renders the drug less effective. Furthermore, combination therapy with PRRT can be used to enhance the efficacy of the drugs beyond the additive efficacy of each. By targeting multiple mechanisms involved in tumor cell proliferation and resistance, combination therapies using two or more drugs achieve efficacy with lower doses or toxicity than individual treatments. Several radiosensitizers have shown additive or synergistic effects when combined with PRRT (10–14). Fluorouracil acts as an inhibitor of thymidylate synthase (TS), which is a nucleoside required for DNA replication and DNA repair (29–31). Fluorouracil's mechanism of action makes it an ideal candidate for combination therapy with PRRT as the main goal is to maximize irreversible DNA damage. ^{212}Pb-DOTAMTATE and fluorouracil combination therapy showed a significant improvement in tumor regression. A three-cycle ^{212}Pb-DOTAMTATE treatment combined with weekly subtherapeutic fluorouracil dosing was able to durably cure approximately 80% of the animals.

Overall, the nonclinical studies provide appropriate justification on the safety and efficacy of ^{212}Pb-DOTAMTATE in animals and have provided sufficient data to warrant a clinical trial study. The rodent models showed a promising safety index with a 3.2-fold increase in median survival and one third of the animals being tumor free. Somatostatin analogues have long been studied and used in preclinical and clinical settings for the treatment of SSTR-expressing neuroendocrine tumors, but a successful TAT treatment remains elusive. The preclinical data presented support the further progression of ^{212}Pb-DOTAMTATE into a clinical setting and was used to support the initiation of a phase I study (NCT03466216, https://clinicaltrials.gov/ct2/show/NCT03466216?term = radiomedix&rank=2).

Disclosure of Potential Conflicts of Interest

T. Stallons, A. Saidi, and J. Torgue are all full-time employees of Orano Med. E.S. Delpassand has ownership interest (including stock, patents, etc.) in RadioMedix. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: T.A.R. Stallons, A. Saidi, I. Tworowska, E.S. Delpassand, J.J. Torgue

Development of methodology: T.A.R. Stallons, I. Tworowska, J.J. Torgue

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.A.R. Stallons, A. Saidi, J.J. Torgue

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.A.R. Stallons, A. Saidi, I. Tworowska, E.S. Delpassand, J.J. Torgue

Writing, review, and/or revision of the manuscript: T.A.R. Stallons, A. Saidi, I. Tworowska, J.J. Torgue

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Saidi

Study supervision: T.A.R. Stallons, A. Saidi, E.S. Delpassand, J.J. Torgue

Acknowledgments

The authors are grateful to the working group of Comparative Bioscience, Inc for their contribution to the GLP-Toxicity analyses and reporting. RAPID is

particularly acknowledged for their technical assistance in the dosimetry study of ^{203}Pb-DOTAMTATE in patients. This work was supported, in part, by The National Cancer Institute (NCI SBIR phase I Contract HHSN261201600015C, awarded to I. Tworowska, RadioMedix Inc.). I. Tworowska (RadioMedix) has been awarded NCI SBIR Phase I Contract HHSN261201600015C (2016) "Targeted radionuclide therapy of neuroendocrine tumors using Pb212-octreotate analogues."

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Received September 25, 2018; revised November 29, 2018; accepted March 15, 2019; published first March 29, 2019.

References

1. Pfeifer A, Knigge U, Binderup T, Mortensen J, Oturai P, Loft A, et al. 64Cu-DOTATATE PET for neuroendocrine tumors: a prospective head-to-head comparison with 111In-DTPA-Octreotide in 112 patients. J Nucl Med 2015;56:847–54.
2. Maxwell JE, Howe JR. Imaging in neuroendocrine tumors: an update for the clinician. Int J Endoc Oncol 2015;2:159–68.
3. Olsen JO, Pozderac RV, Hinkle G, Hill T, O'Dorisio TM, Schirmer WJ, et al. Somatostatin receptor imaging of neuroendocrine tumors with indium-111 pentetreotide (Octreoscan). Semin Nucl Med 1995;25:251–61.
4. Storch D, Behe M, Walter MA, Chen J, Powell P, Mikolajczak R, et al. Evaluation of [99mTc/EDDA/HYNIC0]octreotide derivatives compared with [111In-DOTA0,Tyr3, Thr8]octreotide and [111In-DTPA0]octreotide: does tumor or pancreas uptake correlate with the rate of internalization? J Nucl Med 2005;46:1561–9.
5. Bushnell D, Menda Y, O'Dorisio T, Madsen M, Miller S, Carlisle T, et al. Effects of intravenous amino acid administration with Y-90 DOTA-Phe1-Tyr3-Octreotide (SMT487[OctreoTher) treatment. Cancer Biother Radiopharm 2004;19:35–41.
6. Kwekkeboom DJ, de Herder WW, Kam BL, van Eijck CH, van Essen M, Kooij PP, et al. Treatment with the radiolabeled somatostatin analog [177 Lu-DOTA 0,Tyr3]octreotate: toxicity, efficacy, and survival. J Clin Oncol 2008;26:2124–30.
7. Kratochwil C, Giesel FL, Bruchertseifer F, Mier W, Apostolidis C, Boll R, et al. (213)Bi-DOTATOC receptor-targeted alpha-radionuclide therapy induces remission in neuroendocrine tumours refractory to beta radiation: a first-in-human experience. Eur J Nucl Med Mol Imaging 2014;41: 2106–19.
8. Strosberg J, El-Haddad G, Wolin E, Hendifar A, Yao J, Chasen B, et al. Phase 3 Trial of 177Lu-dotatate for midgut neuroendocrine tumors. N Engl J Med 2017;376:125–35.
9. Nayak TK, Norenberg JP, Anderson TL, Prossnitz ER, Stabin MG, Atcher RW. Somatostatin-receptor-targeted alpha-emitting 213Bi is therapeutically more effective than beta(-)-emitting 177Lu in human pancreatic adenocarcinoma cells. Nucl Med Biol 2007;34:185–93.
10. Barber TW, Hofman MS, Thomson BNJ, Hicks RJ. The potential for induction peptide receptor chemoradionuclide therapy to render inoperable pancreatic and duodenal neuroendocrine tumours resectable. Eur J Surg Oncol 2012;38:64–71.
11. Claringbold PG, Brayshaw PA, Price RA, Turner JH. Phase II study of radiopeptide 177Lu-octreotate and capecitabine therapy of progressive disseminated neuroendocrine tumours. Eur J Nucl Med Mol Imaging 2011; 38:302–11.
12. van Essen M, Krenning EP, Kam BL, de Herder WW, van Aken MO, Kwekkeboom DJ. Report on short-term side effects of treatments with (177)Lu-octreotate in combination with capecitabine in seven patients with gastroenteropancreatic neuroendocrine tumours. Eur J Nucl Med Mol Imaging 2008;35:743–8.
13. Kong G, Johnston V, Ramdave S, Lau E, Rischin D, Hicks RJ. High-administered activity In-111 octreotide therapy with concomitant radiosensitizing 5FU chemotherapy for treatment of neuroendocrine tumors: preliminary experience. Cancer Biother Radiopharm 2009;24:527–33.
14. Ballal S, Yadav MP, Damle NA, Sahoo RK, Bal C. Concomitant 177Lu-DOTATATE and capecitabine therapy in patients with advanced neuroendocrine tumors: a long-term-outcome, toxicity, survival, and quality-of-life study. Clin Nucl Med 2017;42:e457–66.
15. Ullrich M, Bergmann R, Peitzsch M, Zenker EF, Cartellieri M, Bachmann M, et al. Multimodal somatostatin receptor theranostics using [(64)Cu]Cu-/[(177)Lu]Lu-DOTA-(Tyr(3))octreotate and AN-238 in a mouse pheochromocytoma model. Theranostics 2016;6:650–65.
16. Lipnick RL, Cotruvo JA, Hill RN, Bruce RD, Stitzel KA, Walker AP, et al. Comparison of the up-and-down, conventional LD50, and fixed-dose acute toxicity procedures. Food Chem Toxicol 1995;33:223–31.
17. Mu L, Hesselmann R, Oezdemir U, Bertschi L, Blanc A, Dragic M, et al. Identification, characterization and suppression of side-products formed during the synthesis of high dose (6)(8)Ga-DOTA-TATE. Appl Radiat Isot 2013;76:63–9.
18. Norenberg JP, Krenning BJ, Konings IR, Kusewitt DF, Nayak TK, Anderson TL, et al. 213Bi-[DOTA0, Tyr3]octreotide peptide receptor radionuclide therapy of pancreatic tumors in a preclinical animal model. Clin Cancer Res 2006;12:897–903.
19. Kulaksiz H, Eissele R, Rossler D, Schulz S, Hollt V, Cetin Y, et al. Identification of somatostatin receptor subtypes 1, 2A, 3, and 5 in neuroendocrine tumours with subtype specific antibodies. Gut 2002;50:52–60.
20. Rolleman EJ, Valkema R, Melis M, Krenning EP, Visser TJ, de Jong M. Cubilin and megalin in radiation-induced renal injury with labelled somatostatin analogues: are we just dealing with the kidney? Eur J Nucl Med Mol Imaging 2006;33:749–50.
21. Vegt E, Melis M, Eek A, de Visser M.Brom M, Oyen WJG, et al. Renal uptake of different radiolabelled peptides is mediated by megalin: SPECT and biodistribution studies in megalin-deficient mice. Eur J Nucl Med Mol Imaging 2011;38:623–32.
22. Vegt E, de Jong M, Wetzels JF, Masereeuw R, Melis M, Oyen WJ, et al. Renal toxicity of radiolabeled peptides and antibody fragments: mechanisms, impact on radionuclide therapy, and strategies for prevention. J Nucl Med 2010;51:1049–58.
23. Melis M, Valkema R, Krenning EP, de Jong M. Reduction of renal uptake of radiolabeled octreotate by amifostine coadministration. J Nucl Med 2012; 53:749–53.
24. Rolleman EJ, Valkema R, de Jong M, Kooij PP, Krenning EP. Safe and effective inhibition of renal uptake of radiolabelled octreotide by a combination of lysine and arginine. Eur J Nucl Med Mol Imaging 2003;30: 9–15.
25. Chan HS, Konijnenberg MW, de Blois E, Koelewijn S, Baum RP, Morgenstern A, et al. Influence of tumour size on the efficacy of targeted alpha therapy with (213)Bi-[DOTA(0),Tyr(3)]-octreotate. EJNMMI Res 2016;6:6.
26. Liu S, Edwards DS. Stabilization of 90Y-Labeled DOTA-Biomolecule conjugates using gentisic acid and ascorbic acid. Bioconjug Chem 2001;12: 554–8.
27. Garrison WM. Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins. Chem Rev 1987;87:381–98.
28. Simat TJ, Steinhart H. Oxidation of free tryptophan and tryptophan residues in peptides and proteins. J Agric Food Chem 1998;46: 490–8.
29. Kjellström J, Kjellén E, Johnsson A. In vitro radiosensitization by oxaliplatin and 5-fluorouracil in a human colon cancer cell line. Acta Oncol 2005;44:687–93.
30. Ojima E, Inoue Y, Watanabe H, Hiro J, Toiyama Y, Miki C, et al. The optimal schedule for 5-fluorouracil radiosensitization in colon cancer cell lines. Oncol Rep 2006;16:1085–91.
31. Valdes G, Iwamoto KS. Re-evaluation of cellular radiosensitization by 5-fluorouracil: High-dose, pulsed administration is effective and preferable to conventional low-dose, chronic administration. Int J Radiat Biol 2013; 89:851–62.