Development of Alpha-emitting radioembolization for Hepatocellular Carcinoma: Longitudinal Monitoring of Actinium-225's Daughters Through SPECT Imaging

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Abstract

Hepatocellular carcinoma is the most common primary liver cancer and the fifth most frequently diagnosed cancer worldwide. Most patients with advanced disease are offered non-surgical palliative treatment options. This work explores the first α -emitting radioembolization for the treatment and monitoring of hepatic tumors. Furthermore, this work demonstrates the first *in vivo* simultaneous multiple-radionuclide SPECT images of the complex decay chain of an [^{225}Ac]Ac-labeled agent using a clinical SPECT system to monitor the temporal distribution.

Methods: A DOTA chelator was modified with a lipophilic moiety and radiolabeled with Actinium-225. The resulting agent, [^{225}Ac]Ac-DOTA-TDA, was emulsified in Lipiodol® and evaluated *in vivo* in mouse model and the VX2 rabbit technical model of liver cancer. SPECT imaging was performed to monitor distribution of the TAT agent and the free daughters.

Results: [^{225}Ac]Ac-DOTA-TDA was shown to retain within the HEP2G tumors and VX2 tumor, with minimal uptake within normal tissue. In the mouse model, significant improvements in overall survival were observed. SPECT imaging was able to distinguish between the Actinium-225 agent (Francium-221) and the loss of the longer lived daughter, Bismuth-213.

Conclusion: A TAT agent emulsified in Lipiodol® is capable of targeting liver tumors with minimal accumulation in normal tissue, providing a potential therapeutic agent for the treatment of HCC as well as a variety of hepatic tumors. In addition, SPECT imaging presented here provides a foundation for imaging methodology and protocols that can be rapidly translated into the clinic to monitor Actinium-225-labeled agents.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the fifth most frequently diagnosed cancer worldwide, as well as the second most frequent cause of cancer death[1-5]. Most patients with advanced disease are offered only non-surgical palliative treatment options, including systemic chemotherapy, radiofrequency ablation, or intra-arterial therapies[6]. Hepatic tumors derive their blood supply primarily from the hepatic artery, whereas normal liver is mainly fed by the portal vein. Image-guided intra-arterial therapies, such as transarterial embolization (TAE), chemoembolization (TACE) and radioembolization (TARE), exploit the arterial route to selectively deliver therapy to tumors. However, these therapies have several limitations, urging the development of new treatment strategies[7, 8].

Ethiodized oil or Lipiodol® is an FDA-approved radio-opaque agent composed of a variety of oils including the ethyl esters of fatty acids of poppy seed oil. Lipiodol® accumulates and remains in liver cancer while clearing out of normal liver tissue when injected via the hepatic artery, providing an excellent vehicle for the selective delivery of therapeutic radionuclides. β -emitting radiopharmaceutical therapies, such as the commercially available Yttrium-90 glass or resin microspheres have been developed over the

past 20 years[7, 9] but have shown limited survival benefits in patients with HCC as compared to TACE, the standard of care treatment option. Compared to Yttrium-90 microspheres that accumulate into arterioles, Lipiodol®-based β -emitting agents, such as [^{131}I]I-Lipiodol®, seem to be directly up taken by tumor cells, resulting in a higher tumor radiation dose[10]. However, HCC patients treated with [^{131}I]I-Lipiodol® vs. TACE had similar survival up to three years post-treatment; yet patients that had a portal vein thrombosis or more advanced disease demonstrated a significantly higher mean survival as compared to patients treated with TACE[10]. The incorporation of Iodine-131 into Lipiodol® is costly and the exploration of alternate β -emitting radionuclides has been explored, such as chelation of Rhenium-188 into Lipiodol®[11-13]. Despite initial efficacy of [^{188}Re]Re-Lipiodol®, the increased hepatic and hematologic toxicities have been a concern[13]. In addition, the low energy transfer of β -particles introduces the ability of the remaining tumors to develop resistance mechanisms further limiting the treatment options for patients[14, 15].

Targeted alpha therapy (TAT) has emerged as a highly potent treatment for cancer[16-18]. The high potency of TAT impacts normal tissue as well, highlighting a need to accurately determine absorbed doses to provide well-defined dosing strategies and effective treatment while minimizing toxicities. Currently, surrogate imaging agents are utilized to evaluate TAT agents; however, complex decay schemes of α -emitting radionuclides are not accurately portrayed using surrogate imaging strategies, urging the development of methodologies and instrumentation to image α -emitting radionuclides. Actinium-225 is one of the α -emitting radionuclides currently being explored in the clinic. Actinium-225 has a complex decay scheme and energy spectrum (**Figure 1**). Please note, even though there are several low abundant X-rays from Actinium-225 and its daughters at the 70 keV to 90 keV energy range, the peak around 80 keV shown in Figure 1 is dominated by the lead X-rays generated from interactions of high-energy photons with the collimator. This is a well known phenomena in SPECT physics [19]. In addition, this region is also heavily contaminated by downscatter, and thus should not be used for imaging on current clinical SPECT systems. The decay of Actinium-225 and its daughters primarily releases energy as α -particles; however, γ -emissions suitable for SPECT imaging occur, but are in low abundance. The activity administered for TAT is extremely small due to their high potency, compounding the low abundance of rays suitable for imaging and resulting in a small number of γ photons for SPECT imaging. Furthermore, the initial alpha decay with its high recoil energy breaks the bonds within the chelate, resulting in the release of the free decay daughters. These daughters may migrate to other organs, complicating dosimetry calculations. Surrogate imaging radionuclides do not replicate the complex decay schemes that are commonly found for α -emitting radionuclides. Therefore, it is urgent and essential to develop quantitative SPECT imaging methodologies specifically for these TAT radionuclides to improve the accuracy and precision of the imaging and the resulting knowledge of the distributions and dose deposition of the agent and its daughters.

In this study we investigate the potential of an [^{225}Ac]Ac-labeled TAT Lipiodol®-based agent to selectively target liver tumors for TAT. Furthermore, we evaluated the prospect of using a clinical

SPECT/CT system to image and monitor the distribution of the [^{225}Ac]Ac-labeled TAT Lipiodol®-based agent as well as its daughters in the VX2-tumor bearing rabbit model over a 6-day window.

Materials And Methods

Reagents

All chemicals were purchased from Sigma-Aldrich Chemical Co. (ST. Louis, MO, USA) or Thermo Fisher Scientific (Pittsburgh, PA, USA), unless otherwise specified. DOTA-tris(tert-butyl ester) was purchased from Macroyclics, Inc. (Dallas, TX, USA). Indium-111 ($[^{111}\text{In}]\text{InCl}_3$) was purchased from BWZXT ITG Canada, Inc. (Ottawa, ON, Canada). Actinium-225 nitrate was purchased from Oak Ridge National Laboratory (Oak Ridge, TN, USA).

Chemical Syntheses. *DOTA-tris(t-Bu ester)-TDA* was synthesized as previously described with modifications [20]. Briefly, the DOTA-tris(t-Bu) ester (50 mg, 0.09 mmol) and HBTU (0.168 mg, 0.44 mmol) were dissolved in 2 mL of DMF. The resulting solution was stirred for 30 minutes at room temperature (RT) then 1-tetradecylamine (TDA) (22 mg, 0.11 mmol) and DIPEA (76 μL , 0.44 mmol) were added to the solution, which was stirred overnight at RT. The solution was extracted with ethyl acetate washed with water (2x) then brine (1x). The ethyl acetate solution was dried over MgSO_4 , filtered and the ethyl acetate was evaporated under vacuum to yield a yellow powder. The DOTA-tris(t-Bu ester)-TDA was purified by silica gel chromatography (eluent: hexane/ethyl acetate, 3/1 v/v), purity confirmed by TLC and the product was used without further confirmation.

DOTA-TDA. DOTA-tris(t-Bu ester)-TDA (30 mg, 0.04 mmol) was dissolved in trifluoroacetic acid (125 μL) and dichloromethane (400 μL) and stirred overnight at RT. The solvent was evaporated under vacuum yield a pure white solid, 51.3% was recovered. DOTA-TDA was successfully synthesized (overall yield 53.1%) and confirmed by mass spectrometry. DOTA-TDA was confirmed by using a Thermo Scientific Q Exactive Plus Orbitrap Mass Spectrometer coupled with a Vanquish UPLC system at the Metabolomics Facility at Johns Hopkins School of Medicine (Director – Dr. Anne Le) (M+H): calculated 600.4336, found 600.4316; (M-H) calculated 598.4185 found 598.4203.

Radiolabeling of DOTA-TDA. For radiolabeling the following were added to acid wash Eppendorf tube 1 μL of Indium-111, 40 μL of 0.4M NaOAc buffer pH=4.5, and 1 μL of DOTA-TDA (1 $\mu\text{g}/\mu\text{L}$). The solution was heated at 95°C for 15 minutes and purified using a Waters Sep-Pak C18 (Milford, MA, USA). $[^{111}\text{In}]\text{In-DOTA-TDA}$ was eluted with ethanol, dried, and resuspended in sterile PBS with greater than 95% radiopurity as determined by thin layer chromatography (TLC).

For Actinium-225, 5 μL of Actinium-225 solution, 5 μL of 2M Tris buffer pH=7, and 2.5 μL of DOTA-TDA were added to an acid washed tube, heated at 95°C for 15 minutes. Radiolabeling yields were $\geq 95\%$; [^{225}Ac]Ac-DOTA-TDA was used without further purification.

Partition Coefficient (Log P). Log P were determined for the radiolabeled conjugates, [^{111}In]In- or [^{225}Ac]Ac-DOTA-TDA, were dissolved in 1 mL of octanol in a centrifuge tube. The centrifuge tube was vigorously vortexed for a minute with 1 mL of 0.9% saline. The tube was centrifuged at 1000 rpm for 5 minutes and allowed to sit for 30 minutes until the phases were clearly separated. The organic phase (Octanol) and the aqueous phase (Saline) were collected separately and counted at equilibrium of Actinium-225 in an automatic γ -well counter (Perkin-Elmer 2470 WIZARD²® Automatic Gamma Counter, Waltham, MA, USA). The Log P values were calculated using the following formula: $\text{Log P} = \text{Log} ([\text{Counts}]_{\text{octanol}}/[\text{Counts}]_{\text{saline}})$.

Animal Studies

Animal studies were performed using 7-8-week-old healthy male NCG mice obtained from Charles River Laboratories (Wilmington, MA, USA). Adult New Zealand rabbits were used weighing approximately 4 kg (Myrtle's Rabbitry, Thompson Station, TN, USA). All animal studies were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. For tumor retention and survival studies the mice were injected subcutaneously in the right flank with approximately 1×10^6 HEP2G cells in 100 μL of sterile PBS and Matrigel (1:1).

Mouse Model

Tumor Retention. Biodistribution studies were carried out as previously described in healthy NCG male mice ($n=3/\text{group}$) bearing HEP2G subcutaneous tumors [21, 22]. To assess tumor retention the mice were divided into two groups that were injected intratumorally with ^{111}In -DOTA-TDA (37kBq, 60 μL) alone or ^{111}In -DOTA-TDA (37kBq, 60 μL) emulsified in Lipiodol. At 48-hours post-injection (p.i.), the mice were euthanized. The blood, heart, lungs, liver, spleen, kidneys, stomach (with content), intestines (with content), bone, muscle, and tumors were harvested, weighed, and measured in an automatic γ -well counter. The percentage of injected dose per gram (%ID/g) was calculated by comparison with a weighed, diluted standard.

Therapeutic Efficacy: Survival Studies. Subcutaneous liver cancer models were used to evaluate the therapeutic efficacy of [^{225}Ac]Ac-DOTA-TDA/Lipiodol emulsions. The following treatments were administered in subcutaneously HEP2G tumors ($266.5 \pm 23.2 \text{ mm}^3$): 1. Saline (60 μL) 2. Lipiodol alone (60 μL) or 3. [^{225}Ac]Ac-DOTA-TDA/Lipiodol (37 kBq, 60 μL). Mice were observed for signs of pain and distress (lethargy, hunched back, paralysis, etc.), weight loss, and tumor size [volume= $0.5(\text{length} \times \text{width}^2)$]; mice were euthanized if one of the following conditions were met: 1. weight loss ($>20\%$); 2. primary tumor reaching $1,500 \text{ mm}^3$; 3. signs of pain or distress; or 4. 180 days post-treatment. Survival fractions were plotted as a Kaplan-Meier survival curve using Prism 8 (GraphPad; La Jolla, CA, USA).

VX2 Rabbit Model

Anesthesia. Rabbits were administered a mixture of ketamine (30-50 mg/kg) and xylazine (3-5 mg/kg) intramuscularly prior to tumor harvesting, implantation, and imaging. Anesthesia was maintained with 3% isoflurane in oxygen as well as additional injections of the ketamine/xylazine solution.

Ultrasound Guided Tumor Implantation. The implantation of the VX2 tumor was done as previously described with modifications[23]. Briefly, the solid VX2 tumor for implantation was obtained from a carrier rabbit that had been injected intramuscularly in the thigh approximately 2 weeks prior. The VX2 tumor was harvested, placed in 0.9% sodium chloride, and minced into small pieces. The minced tumor was prepared in a 16-gauge Angiocath (2 inches long), using ultrasound guidance, the needle punctured the liver and the tumor pieces were pushed into the liver with a guide wire. Manual compression was administered after the needle was removed.

Intrahepatic Arterial Injection including Agents and Dosing. Hepatic angiography was performed as previously described[23]. Briefly, rabbits with VX2 hepatic tumors, confirmed by ultrasound were anesthetized and surgical cutdowns were performed to expose the femoral artery. A 4-F sheath (Cook) was inserted to allow a 2-F catheter tip (JB1 catheter; Cook) to access the common hepatic artery. Fluoroscopy was utilized to select the arterial branch and radioembolization was performed.

SPECT Imaging in VX2-bearing Rabbit. SPECT/CT imaging was performed on a Siemens Symbia T16 SPECT/CT system. The anesthetized rabbit (n=1) was placed supine on the imaging bed with the whole body in the field-of-view. The images were acquired at 24 hours, 72 hours and 6 days p.i.. Projection data were acquired in 120 views over 360° with a 128 x 128 matrix and 4.795 mm pixel size. The imaging time were 75 seconds per view for the 24-hour imaging, and 90 seconds per view for the 72-hour and 6-day imaging. The high-energy collimators were used. Data were binned into two energy windows - 20% centered at 440.45 keV peak of Bismuth-213 and 20% centered at 218 keV peak of Francium-221, respectively. Both isotopes are daughters of Actinium-225 decay and emit gamma photons that can be imaged by SPECT. Francium-221 has a short half-life of 4.9 minutes, therefore its distribution represents those of Actinium-225's. Bismuth-213 has a half-life of approximately 45.6 minutes and could have redistributed from the parent isotope. After SPECT acquisition, a CT scan was performed to provide anatomical images and an attenuation map. The SPECT images were reconstructed with Siemens Flash3D OSEM algorithm with compensation for attenuation and resolution[24]. A total of 6 iterations with 15 subsets per iteration were used.

Ex Vivo Biodistribution VX2-bearing and non-tumor bearing Rabbits. Biodistribution studies were carried out following the SPECT imaging approximately 6-days post-injection of [²²⁵Ac]Ac-DOTA-TDA (0.49 MBq) emulsified in 1 mL of Lipiodol in the VX2-bearing rabbit (n=1) and at approximately 24 hours for non-tumor bearing rabbits (n=2). At the end timepoint the rabbits were euthanized (Euthasol®, 1 mL minimum then 1mL/4.5 kg). The blood, heart, lungs, liver, spleen, kidneys, stomach, intestines, marrow, femur+marrow, muscle, gall bladder, bile, and if applicable the tumor were harvested, weighted, and measure in an automatic γ-well counter and/or a Capintec CRC-7 dose calibrator. The percentage of

injected dose per gram (%ID/g) was calculated based on the dose injected and converted to Bq/g using an efficiency coefficient.

Statistical Analysis was performed using the software Graphpad Prism 8. All data are presented as mean $\pm$ SD. Tumor retentions were compared using 2-way ANOVA. Survival studies used Kaplan-Meier curves that were analyzed using the Log Rank (Mantel-Cox) test. Values were considered significant when $P < 0.05$.

Results

Radiochemistry. [^{111}In]In-DOTA-TDA was radiolabeled at a specific activity of 2.13 MBq/nmol with greater than 95% radiopurity following purification. The [^{225}Ac]Ac-DOTA-TDA was radiolabeled at a specific activity of 0.36 MBq/nmol with greater than 95% radiolabeled yield.

Determining Partition Coefficients (LogP). The LogP for [^{111}In]In-DOTA-TDA was 1.57 ± 0.01 and the LogP for [^{225}Ac]Ac-DOTA-TDA was 1.65 ± 0.02 . For comparison, doxorubicin, a chemotherapeutic used for TACE, has a reported LogP of 1.27[25].

Tumor Retention of ^{111}In -DOTA-TDA-Lipiodol®. The retention of [^{111}In]In-DOTA-TDA alone or as a Lipiodol® emulsion (**Figure 2**) were compared 48-hours p.i. using a 2-way ANOVA test in HEP2G tumor-bearing mice. The [^{111}In]In-DOTA-TDA-Lipiodol® emulsion had significantly higher uptake within the tumor as compared to [^{111}In]In-DOTA-TDA alone after 48 hours (216 ± 145 %ID/g vs. 20.0 ± 15.9 %ID/g; $p \leq 0.0001$), supporting the statement that Lipiodol® is able to retain the proposed agents in a model of liver cancer.

Therapeutic Efficacy: Survival Studies. Kaplan-Meier survival curves (**Figure 3**) showed significant improvement in survival of HEP2G tumor-bearing mice when treated with [^{225}Ac]Ac-DOTA-TDA-Lipiodol® as compared to both the untreated control ($p < 0.005$) and Lipiodol® alone ($p < 0.001$). The median survival for [^{225}Ac]Ac-DOTA-TDA-Lipiodol® treated mice was 45 days, ranging from 26-87 days, as compared to 24-days (untreated, [15-29 days]) and 26-days (Lipiodol® alone, [15-40 days]). In addition, the rate of tumor growth in the [^{225}Ac]Ac-DOTA-TDA-Lipiodol® treated group was slowed as demonstrated by the individual tumor growth curves (**Figure 4**).

SPECT Imaging in VX2-bearing Rabbit. The fused SPECT/CT images from 24, 72, and 144-hours p.i. shown in **Figure 5** demonstrated that the activity of [^{225}Ac]Ac-DOTA-TDA-Lipiodol® agent accumulated mainly in the VX2 tumor with gradual clearance out of normal liver tissue. At 72 hours and 6-day p.i., the [^{225}Ac]Ac-DOTA-TDA-Lipiodol® agent is concentrated within the tumor; however, the figures clearly show that there were differences in the distributions between the Francium-221 energy window and Bismuth-213 energy window - there was increased signal in the normal liver tissue above the tumor in the Bismuth-213 window, most likely associated with the escape of the free Bismuth-213 daughter from TAT agent.

Ex-Vivo Biodistribution in VX2-bearing Rabbit. Following the SPECT imaging, biodistribution studies were carried out in the VX2-tumor bearing rabbit at 6-days p.i. of the [^{225}Ac]Ac-DOTA-TDA-Lipiodol® emulsion (**Table 1**). Accumulation of the TAT agent was predominately in the VX2 tumor (40.6 %ID/g). The next highest uptake was seen in the normal liver tissue (0.23 %ID/g), resulting in having approximately 177:1 tumor to liver ratio. The TAT agent accumulation in normal tissues were minimal, only slightly above background as determined by uptake in the muscle (0.001 %ID/g). This low uptake in normal tissue is consistent with the *ex-vivo* biodistributions in non-tumor bearing rabbits (n=2) at approximately 24-hours p.i. via the hepatic artery as shown in **Table 1**.

Discussion

Due to its indolent course, most patients with HCC have advanced and unresectable disease at the time of diagnosis and are being offered only non-surgical palliative treatment options[6, 7]. In addition, the liver is also the most common site for metastatic disease, including colorectal liver metastases. Intra-arterial therapies are widely used for treatment of patients with HCC or metastatic liver cancer[6]. Nevertheless, these therapies have shown limited survival benefit for patients with HCC, urging the need for developing novel improved therapeutic strategies [6, 26-30].

β -emitting radiopharmaceutical agents have shown promising results but the survival benefits in patients with HCC are comparable to the standard of care, TACE[31]. TAT provides highly potent agents[32-45] that are highly damaging to tumor cells, effectively causing irreparable DNA damage to tumor cells, needing only a few α -tracks as compared to 10^3 - 10^4 tracks for a β -emitting agents. Consequently, there is growing interest in TAT agents for cancer therapy[46-48]. Recently, the first in human experience with [^{213}Bi]Bi-DOTATOC, a TAT agent for neuroendocrine cancer, was selectively targeted via the hepatic artery to treat metastatic disease in the liver of neuroendocrine cancer patients not responding to standard therapies as well as [^{177}Lu]Lu-DOTATOC, a β -emitting targeted therapy. Kratochwil, et. al. demonstrated in a small subset of patients that [^{213}Bi]Bi-DOTATOC was able to overcome refractory disease and provided positive responses in patients[49]. Biomarkers for targeted radiopharmaceutical therapies are limited for HCC; however, the selective deliver of therapies via the hepatic artery for primary liver cancers are common. In this study, we investigated the potential to exploit intra-arterial delivery of the TAT agent, [^{225}Ac]Ac-DOTA-TDA-Lipiodol®, for its ability to target primary liver tumors as well as its therapeutic efficacy in a mouse model of HCC.

Lipiodol® has been demonstrated to have high selectivity and retention in the liver [50]; Lipiodol® has shown to be retained in hepatic tumors for months [51]. [^{188}Re]Re-labeled chelators, modified to introduce lipophilicity, have been successfully incorporated into Lipiodol® for β -emitting radiopharmaceutical therapy[52]. Here, we used a similar approach and altered the DOTA chelator, which is suitable for [^{225}Ac]Ac-labeling, by incorporating an alkyl side chain to introduce lipophilicity. The resulting agent, DOTA-TDA, was radiolabeled with both Indium-111 and Actinium-225 in high radiolabeling yields and purities. Both the [^{111}In]In- and [^{225}Ac]Ac-DOTA-TDA were shown to be lipophilic as determined by the

partition coefficients, helping them to be emulsified into Lipiodol®. The emulsification of [^{111}In]In-DOTA-TDA with Lipiodol® resulted in an approximate 11-fold increase in retention of the radiolabel agent within the mouse model of primary liver cancer. The potency of the TAT agent, [^{225}Ac]Ac-DOTA-TDA-Lipiodol®, was evaluated and demonstrated significant increases in survival in TAT treated mice as compared to mice receiving no treatment or Lipiodol® alone. [^{225}Ac]Ac-DOTA-TDA-Lipiodol® treated mice almost doubled (1.8x) their median survival as compared to the control groups, with the first TAT-treated mouse not reaching its endpoint until the median survival of the control. The preliminary results in the mouse model of primary liver cancer are promising, and we further evaluated the TAT agent in a technical model to confirm selective delivery and retention via intra-arterial delivery.

The rabbit VX2 model provides a technical animal model for intra-arterial injections, allowing us to explore the selective delivery of [^{225}Ac]Ac-DOTA-TDA-Lipiodol® to a hepatic tumor. We have confirmed delivery, accumulation, and retention of the TAT-Lipiodol® agents within the VX2 tumor by *ex-vivo* biodistribution, and *in-vivo* SPECT imaging of Actinium-225's daughters (Francium-221 and Bismuth-213) using a clinical SPECT/CT system. Even though feasibility of SPECT imaging of Actinium-225 decay chain has been demonstrated [53, 54] – to our knowledge, this work is the first to demonstrate it can be done longitudinally *in-vivo* on a clinical SPECT/CT system with low activities, allowing for dosimetry without the use of a surrogate imaging agent. Furthermore, the fact that the two energy windows showed different distributions indicates the importance of using SPECT to monitor both parent and daughter isotopes. Clearance from normal tissues including the liver was seen in early biodistribution studies in non-tumor-bearing rabbits at approximately 24 hours p.i. of [^{225}Ac]Ac-DOTA-TDA-Lipiodol® with the highest uptake, less than 0.2 %ID/g, in the normal liver. The VX2-bearing rabbit's biodistribution showed that the normal liver had slightly higher uptake (0.23 %ID/g) of [^{225}Ac]Ac-DOTA-TDA-Lipiodol® at 6-days p.i., most likely associated with clearance of the TAT agent from the VX2 tumor, which had 40.6 %ID/g uptake at 6-days p.i.. This is further confirmed by the SPECT images that demonstrated the clearance of the TAT agent from the normal liver tissue over time. More importantly, the SPECT images also demonstrated the differences in the distribution of Actinium-225/Francium-221 from that of free Bismuth-213, indicating the importance of obtaining the daughter isotopes' distribution for accurate dosimetry for TAT.

Conclusion

In summary, these studies demonstrate that the developed TAT agent, [^{225}Ac]Ac-DOTA-TDA-Lipiodol®, has promise as a therapeutic agent for hepatic tumors, including primary liver tumors. This work highlights that the combination of a TAT agent labeled with a long-lived α -emitting radionuclide emulsified with Lipiodol® is capable of being selectively delivered to hepatic tumors, delivering a highly potent therapeutic dose to hepatic tumors over an extended period of time. Furthermore, we have shown that an [^{225}Ac]Ac-labeled TAT agent can be imaged and longitudinally monitored through clinical SPECT imaging using its daughters Francium-221 and Bismuth-213 even with very low injected activity. The development of SPECT imaging methodologies to monitor the complex decay scheme of α -emitting

radionuclides, and their daughters, such as Actinium-225, are important for accurately monitoring the distribution of TAT agents *in-vivo* over time through molecular imaging, providing essential pharmacokinetic data to optimize the therapeutic efficacy of [²²⁵Ac]Ac-DOTA-TDA-Lipiodol®. More importantly, these SPECT imaging methodologies can be implemented for [²²⁵Ac]Ac-labeled TAT agents currently in clinical trials helping to develop well-defined dosing strategies and optimal patient-specific treatment planning.

Declarations

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Conflicts of interest: Drs. Nedrow and Liapi currently hold a patent application on the presented work (PCT/US2019/029051).

Ethics Approval: All animal studies were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Consent to participate: N/A

Consent to publication: N/A

Code Availability: N/A

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Tables

Table 1
Ex vivo biodistribution of [225Ac]Ac-DOTA-TDA in a technical rabbit model

	Tumor-bearing 6 days p.i.		Non-tumor bearing 23.5 hours p.i.		Non-Tumor bearing 28 hours p.i.	
	%ID/g	Bq/g	%ID/g	Bq/g	%ID/g	Bq/g
Blood	0.001	4.291	0.006	161.4	0.036	592.2
Heart	0.002	5.641	0.003	94.95	0.004	59.36
Lungs	0.014	46.23	0.028	781.0	0.023	382.0
Liver	0.233	753.2	0.153	4352	0.172	2796
Spleen	0.092	296.4	0.078	2214	0.092	1495
Kidneys	0.020	64.59	0.039	1114	0.045	731.0
Stomach	0.010	30.65	0.047	1346	0.044	708.8
Intestines	0.020	826.3	0.031	880.3	0.028	449.9
Marrow	0.018	66.02	0.013	372.1	0.013	210.1
Whole Bone	Indistinguishable from background		0.054	1521	0.011	182.7
Muscle	0.001	2.637	0.001	18.54	0.002	26.93
Gall Bladder	0.081	260.134	0.098	1600.62	0.105	2986.55
Bile	0.023	74.533	0.091	1483.13	0.064	1812.35
Tumor	40.62	148370	—		—	

Figures

Figure 1

(A) Actinium-225 decay scheme [simplified] (B) Energy spectrum of Actinium-225 from a Siemens' SPECT system). Dashed lines indicate gamma and x-ray emissions and their abundance, including 218 keV representing Francium-221 and 440 keV representing Bismuth-213 for SPECT imaging.

Figure 2

Biodistribution of ^{111}In -DOTA-TDA (37 kBq) with and without Lipiodol® emulsification in Hep2G tumor-bearing NCG mice at 48 hours post-injection ($n=3$, $p \leq 0.0001$).

Figure 3

Kaplan-Meier survival plots in Hep2G-tumor bearing mice receiving the following treatments: 1. Saline (60 μL) 2. Lipiodol® alone (60 μL) or 3. ^{225}Ac -DOTA-TDA/Lipiodol® (37 kBq, 60 μL).

Figure 4

Individual tumor growth curves in a subcutaneous model of liver cancer. NCG mice were injected in the right flank with HEP2G cells when the tumors reached an average volume of $266.5 \pm 23.2 \text{ mm}^3$ the following treatments were administered intratumorally: 1. Saline (60 μL) 2. Lipiodol alone (60 μL) or 3. [^{225}Ac]Ac-DOTA-TDA/Lipiodol (37 kBq, 60 μL).

Figure 5

Fused SPECT/CT images of rabbit with a VX2 hepatic tumor at 24, 72 and 144-hours post-injection of $[^{225}\text{Ac}]\text{Ac-DOTA-TDA/Lipiodol}^{\circledR}$ emulsion. Left Panels: Francium-221 energy window. Right panels: Bismuth-213 energy window.