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The Influence of Linker Length on the Properties of Cathepsin S Cleavable ¹⁷⁷Lu-labeled HPMA Copolymers for Pancreatic Cancer Imaging

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Abstract

N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers have shown promise for application in the detection and staging of cancer. However, non-target accumulation, particularly in the liver and spleen, hinders the detection of resident or nearby metastatic lesions thereby decreasing diagnostic effectiveness. Our laboratory has pursued the development of cathepsin S susceptible linkers (CSLs) to reduce the non-target accumulation of diagnostic/radiotherapeutic HPMA copolymers. In this study, we ascertain if the length of the linking group impacts the cleavage and clearance kinetics, relative to each other and a non-cleavable control, due to a reduction in steric inhibition. Three different CSLs with linking groups of various lengths (0, 6 and 13 atoms) were conjugated to HPMA copolymers. *In vitro* cleavage studies revealed that the longest linking group (13 atoms) led to more rapid cleavage when challenged with cathepsin S. The CSL incorporated HPMA copolymers demonstrated significantly higher levels of excretion and a significant decrease in long-term hepatic and splenic retention relative to the non-cleavable control. Contrary to *in vitro* observations, the length of the linking group did not substantially impact the non-target

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in vivo clearance. In the case of HPAC tumor retention, the CSL with the null (0 atom) linker demonstrated significantly higher levels of retention relative to the other CSLs. Given these results, we find that the length of the linking group of the CSLs did not substantially impact non-target clearance, but did influence tumor retention. Overall, these results demonstrate that the CSLs can substantially improve the non-target clearance of HPMA copolymers thereby enhancing clinical potential.

Keywords

HPMA; Cathepsin S; Cleavable linker; Mononuclear phagocyte system; Pancreatic cancer; SPECT/CT imaging

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in the United States with a 5-year survival rate of only 6% and a median survival of approximately 6 months.[1] The poor prognosis for pancreatic cancer patients is mainly due to the asymptomatic nature of the early disease state with symptoms typically presenting only in advanced stages, where effective treatment options are severely limited.[2, 3] For the small portion of the pancreatic cancer population (< 10%) diagnosed with localized disease, surgical resection has been shown to be an effective treatment and is the current standard of care. Pre-operative staging is crucial in determining whether a patient is a viable candidate for surgical resection.[4] Inaccurate identification of patients with unresectable tumors leads to unnecessary surgeries that can result in significant increases in patient morbidity and mortality.[4] To date, accurate staging of pancreatic cancer represents a major challenge in patient treatment.

Several noninvasive imaging techniques are currently used for the diagnosis and staging of pancreatic cancer. These modalities include contrast-enhanced computed tomography (CT), abdominal ultrasound (US), magnetic resonance imaging (MRI) and MR cholangiopancreatography (MRCP).[4] While these techniques have superb anatomical resolution, these imaging modalities do not have the sensitivity and specificity associated with nuclear imaging instrumentation, namely Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET).[5, 6] Recently, the fusion of nuclear imaging with the CT modality has resulted in hybrid imaging systems, SPECT/CT and PET/CT, which have been demonstrated to more accurately detect and stage a variety of cancers.[6] Unfortunately, the potential of these hybrid imaging systems for improving pancreatic cancer staging has not been fully realized due to the lack of an effective radiotracer to target the disease.[4]

The development of polymer-based diagnostics and therapeutics for human disease has been an area of intense research and has yielded several drugs that have progressed to the clinic. [7] N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer is a polymeric platform that has been extensively investigated for a variety of biomedical applications including the development of SPECT and PET tracers.[8–18] HPMA copolymers are capable of targeting solid tumors either passively, through the enhanced permeability and retention (EPR) effect,

and/or actively by inclusion of targeting vectors into the polymeric construct.[13, 14, 19, 20] However, one major challenge for the development of diagnostic and/or therapeutic HPMA based drugs, as well as other polymer and nanomedicine systems, is opsonization and sequestration by the mononuclear phagocyte system (MPS).[21, 22] In many cases, the sequestration of these polymeric drugs leads to substantial accumulation in the liver and spleen. From a diagnostic perspective, this MPS accumulation in non-target organs can hinder identification of resident or nearby metastatic lesions thereby decreasing the diagnostic effectiveness. For therapeutic applications, the non-target accumulation of these polymeric drugs can lead to significant toxicities which may be dose-limiting.

Our laboratory is interested in developing synthetic approaches in which HPMA copolymers can be modified to significantly reduce the MPS accumulation thereby enhancing the diagnostic and/or radiotherapeutic efficacy of the agent. Recently, we have described the development of cathepsin S susceptible linkers (CSLs),[23] which degrade in the presence of cathepsin S, a lysosomal protease that is selectively and highly expressed in MPS tissues. [24–26] From our initial investigation, we found that CSLs significantly reduced long-term retention of ¹⁷⁷Lu-labeled HPMA copolymers in tissues associated with the MPS (i.e., liver and spleen). However, the high molecular weight HPMA copolymer (343 kDa) utilized in the study gave blood circulation times that were not ideal for diagnostic or therapeutic use. Herein, we report our continued investigation of the CSL design by evaluating the structure-activity impact of linking groups of varying size, Figure 1, on the *in vitro* and *in vivo* efficacy of HPMA copolymer based radiopharmaceuticals. In conjunction with these studies, we utilize a lower molecular weight HPMA (109 kDa) copolymer with a blood circulation time that is more suitable for diagnostic and/or radiotherapeutic applications.

MATERIALS AND METHODS

Materials

All chemicals were used without further purification unless otherwise noted. Fluorescein isothiocyanate (FITC), N-hydroxysuccinimide (NHS), N, N'-dicyclohexylcarbodiimide (DCC), 4,4'-Azobis(4-cyanovaleric acid) (V-501), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTP), thioanisole, 3,6-dioxo-1,8-octanedithiol (DOTD), triisopropylsilane (TIS), Tween-20, Tris-buffered saline, RIPA buffer, protease inhibitor cocktail, ethylenediaminetetraacetic acid (EDTA), ninhydrin, glycine, sodium acetate, insulin, transferrin, hydrocortisone and cysteine cathepsin S isolated from human spleens were obtained from Sigma-Aldrich (U.S.). Acetonitrile (HPLC Grade), formic acid (HPLC Grade), N,N-dimethylformamide (DMF, Peptide Synthesis Grade), dichloromethane (DCM, Peptide Synthesis Grade), methanol (MeOH), N-methylpyrrolidone (NMP, Peptide Synthesis Grade), N,N-Diisopropylethylamine(DIEA), trifluoroacetic acid (TFA, Peptide Synthesis Grade), L-ascorbic acid, ethylene glycol, tin(II) chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin (BSA), sodium hydroxide, sodium dodecyl sulfate (SDS), sodium bicarbonate, glutathione, epidermal growth factor (EGF), 2-mercaptoethanol, 0.9% sodium chloride (NaCl) solution (irrigation USP), Dulbecco's Modified Eagle Medium (DMEM), and phosphate buffered saline (PBS) were obtained from Fisher Scientific (U.S.). N-(2-Hydroxypropyl) methacrylamide (HPMA) and

N-(3-aminopropyl) methacrylamide (APMA) hydrochloride were purchased from Polysciences (U.S.). 5-(3-(methacryloylaminopropyl) thioureidyl) fluorescein (APMA-FITC) was synthesized in our lab as previously described[23]. 1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminoxy) dimethylaminomorpholino)] uronium hexafluorophosphate (COMU), fluorenylmethyloxycarbonyl (Fmoc)-protected natural amino acids, Fmoc-NH-(PEG)₂-COOH(13 atoms), H-Pro-2-Trt resin and the 2-chlorotritylchloride (2-ClTrt-Cl) resin were purchased from NovaBiochem (U.S.). Fmoc-5-aminovaleic acid (Fmoc-5-Ava-OH) was obtained from Advanced ChemTech (U.S.). 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid (DOTA) was purchased from Macrocyclics (U.S.). Lutetium-177 trichloride was obtained from PerkinElmer (U.S.). The human pancreatic adenocarcinoma (HPAC) CRL-2119 cell line was purchased from American Type Culture Collection (ATCC) (U.S.). Matrigel™ was obtained from BD Biosciences (U.S.). Human AB serum, DMEM/Ham's F12 medium, l-glutamine and sodium pyruvate were obtained from Mediatech (U.S.). Fetal bovine serum (FBS), LysoTracker Red DND-99 and NucBlue Live ReadyProbe reagent were purchased from Invitrogen/GIBCO (U.S.). Goat anti-cathepsin S antibody (ab18822) was purchased from Abcam (U.S.). Mouse anti-β actin antibody (A5441) was from Sigma-Aldrich (U.S.). Rabbit anti-goat (sc-2768) and goat anti-mouse IgG-HRP (sc-2302) were from Santa Cruz Biotechnology (U.S.). Female Carworth Farms No. 1 (CF-1) mice were purchased from Charles River Laboratories (U.S.). Five-to-six week old, female Institute of Cancer Research severe combined immunodeficient (ICR SCID) mice were purchased from Taconic Farms (U.S.). All procedures utilizing animals conform to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center.

Equipment

The peptides were synthesized on a Liberty microwave peptide synthesizer from CEM (U.S.). A Waters (U.S.) e2695 system equipped with a Waters 2489 absorption detector and a Waters Qtof Micro electrospray ionization mass spectrometer was used to perform reversed-phase high performance liquid chromatography/mass spectrometry (RP-HPLC/MS) analyses. A Phenomenex (U.S.) Jupiter 10μ Proteo 250 × 10 mm semiprep column was used for the purification of bulk amounts of peptides. A GE HealthCare (U.S.) ÄKTA Fast Protein Liquid Chromatography (FPLC) system equipped with a Superose 6 column (HR 10/30) was used for HPMa copolymer molecular weight estimates. A Sephadex™ LH-20 size exclusion resin obtained from GE HealthCare was used for chromatographic separations. Amino acid analysis was performed by the UNMC Protein Structure Core Facility using a Hitachi L8800 amino acid analyzer. Evaluation and purification of radiolabeled conjugates were performed on a Waters 1515 binary pump equipped with a Waters 2489 absorption detector and a Bioscan (U.S.) Flow Count radiometric detector system using an Agilent PL aquagel-OH MIXED-H 300 × 7.5 mm (U.S.) size exclusion chromatography (SEC) column. Purification and concentration of radioconjugates was performed using Amicon Ultra-4 Centrifugal Filter (10 kDa) Devices from Millipore (U.S.). Gamma decay detection of ¹⁷⁷Lu-radioconjugates for biodistribution studies were accomplished using a NaI (Tl) well detector constructed by AlphaSpectra, Inc. (U.S.). UV spectroscopy was performed on a Biochrom WPA Biowave II+ (U.K.). Confocal

microscopy images were taken on a Leica LSM 510 META Microscope equipped with an argon laser (405 nm blue excitation, 488 nm green excitation and 568 nm red excitation). Small animal SPECT/CT image acquisition was achieved with the GE Healthcare dual Flex Triumph CT/SPECT instrument (U.S.).

Synthesis and Characterization of the HPMA Copolymer

The HPMA copolymerization was accomplished using the reversible addition-fragmentation chain transfer (RAFT) polymerization reaction with V-501 as the initiator and CTP as the chain transfer agent. The feed ratio of the polymerization consisted of 98.75:0.85:0.40 mole % HPMA, APMA and APMA-FITC, respectively. The polymerization was run at a 1 M total monomer concentration in 1 M acetate buffer (pH = 5.2) with a molar ratio of total monomer: chain transfer agent: initiator of 1200:3:1. Prior to proceeding with the polymerization reaction, the glass ampoule and contents were purged with argon for 20 min. Subsequently, the ampoule was flame sealed and allowed to heat at 70 °C in an oil bath for 14 h. After cooling the HPMA copolymer was evaporated to dryness, dissolved in methanol and purified by size exclusion chromatography using an LH-20 packing material with methanol as the eluent. The conversion rate was 42.3 % as determined by mass measurement after purification. The weight average molecular weight and polydispersity of the copolymers were determined by FPLC equipped with UV and RI detectors. The average molecular weights of the copolymers were estimated using HPMA copolymer standards with narrow polydispersity indices. Quantification of the primary amine content of the HPMA copolymer was assessed using a ninhydrin assay.[23]

Solid Phase Peptide Synthesis

For the synthesis of the CSLs with linking groups (CSL2 and CSL3), the resin was manually pre-loaded. Briefly, to a fritted reaction vessel, 2-ClTrt-Cl resin (3 g, 3.60 mmol), 1 equiv of the linker group (Fmoc-5-Ava-OH or Fmoc-NH-(PEG)₂-COOH) and 4 equiv of DIEA in dry DMF (30 mL) was added. The reaction mixture was stirred for 2 h, filtered and washed sequentially with: (1) 3 X 100 mL of a solution consisting DCM/MeOH/DIEA (17:2:1); (2) 3 X 100 mL of DCM (3) 2 X 100 mL of DMF and (4) 2 X 100 mL of DCM. The resin was dried in vacuum over KOH. For estimating residue loading, the Fmoc group from a small sample of the resin was cleaved with 2% DBU in DMF and the concentration of liberated dibenzofulvene was measured by UV spectroscopy (304 nm). Using known standards of Fmoc-Gly-OH as a reference, the residue loading was estimated from the equation: mmole/g = (Abs_{sample}-Abs_{ref}) *16.4/mg of resin.[27] Estimation of the level of residue attachment indicated substitution of 0.60 and 0.65 mmol/g for the Fmoc-5-Ava-trityl and Fmoc-NH-(PEG)₂-trityl resin, respectively.

The CSLs were synthesized using automated solid phase peptide synthesis. Briefly, the resin (containing 0.25 mmole of peptide anchors) was deprotected using piperidine resulting in the formation of the primary amine. The carboxylic acids of the Fmoc protected amino acids (1 mmol) were activated using COMU and conjugated to the primary amines of the growing peptide on the resin. The process of deprotection, activation and conjugation was repeated until the desired peptide was synthesized. The selective cleavage of the peptide from the 2-chlorotrityl resin anchor was achieved by reaction with 1% TFA in dry DCM and

subsequent filtration. The combined crude filtrates were dried by a rotary concentrator. Purification of the peptides was performed using a semi-preparative Proteo C₁₂ column with a flow rate of 5.0 mL/min. HPLC solvents consisted of H₂O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). For all peptides, the initial gradient of 70% A: 30% B linearly decreased to 60% A: 40% B over a 15 min time period. At the end of the run time for all HPLC experiments, the column was flushed with the gradient 5% A: 95% B and re-equilibrated to the starting gradient. The yields after purification were 0.1326 g (37.5%) for CSL1; 0.166 g (44%) for CSL2 and 0.109 g (27.1%) for CSL3. The peptides were confirmed to be 95% purity as characterized by LC/MS prior to *in vitro* or *in vivo* use.

Synthesis of Cathepsin S Cleavable Copolymers (CSCs)

The CSCs were prepared by condensation of the HPMA copolymer and the CSLs as previously described. Briefly, in a 10 mL round bottom flask, 16.5 (28.9 μ mol, 5.0 equiv), 16.5 (11.6 μ mol, 2.0 equiv), 17.4 (11.6 μ mol, 2.0 equiv) or 18.6 mg (11.6 μ mol, 2.0 equiv) of CSL0, CSL1, CSL2 or CSL3 was dissolved in 600 μ L of DMF. The solution was cooled to 0 °C and 2.4 equivalents of NHS and DCC, relative to the CSL, were added to the flask. The solution was stirred at 0 °C for 3 h. At the end of that time, the HPMA copolymer was added and stirred for another 2 h at 0 °C. Subsequently, the reaction was allowed to warm to room temperature and continued overnight. The precipitate generated from the reaction was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in methanol, isolated by size exclusion chromatography using an LH-20 packing material and evaporated to dryness. The attached protected peptides of the resulting CSCs were deprotected using standard peptide cleavage conditions. In the case of CSC0, the copolymer was added to 4 mL of a cleavage cocktail consisting of a 1:1:1:37 volumetric ratio of DODT, water, TIS and TFA. This solution was kept at 0 °C and stirred for 2 h. For CSC1-3, the cleavage cocktail contained a 1:1:1:0.75:46.25 volumetric ratio of DODT, water, TIS, thioanisole, and TFA. After deprotection, the mixture was evaporated to dryness and the polymers were finally purified by size exclusion chromatography using an LH-20 packing material and methanol as the eluent. The conjugation yield for CSC0 was determined by the ninhydrin assay. For CSC1-3, the conjugation yield was determined using amino acid analysis conducted by the UNMC Protein Structure Core Facility.

Radiolabeling of the CSCs

The radiolabeling of the CSCs was accomplished by heating 200 μ g of the CSC at 90 °C for 1 h in the presence of 37 MBq (~1mCi) of ¹⁷⁷LuCl₃. After cooling, the resulting ¹⁷⁷Lu-radiolabeled CSC was purified by radio-SEC-HPLC. Purification of the ¹⁷⁷Lu-CSCs was accomplished using a Biosep-SEC-S2000 column with an isocratic mobile phase consisting of PBS with 0.02 M EDTA at pH 7.2. The linear flow rate was 0.80 mL/min. UV analysis was performed at a wavelength of 494 nm. After collection of the peak-purified ¹⁷⁷Lu-CSC, approximately 10 mg of L-ascorbic acid was added to inhibit radiolysis. The purified ¹⁷⁷Lu-CSC was concentrated using an Amicon Ultra-4 centrifugal filter (10 kDa) and washed with 2 X 4 mL of deionized water. The radiochemical purity of all radioconjugates was re-evaluated prior to *in vitro/in vivo* use and found to be 95 % pure as determined by the radio-HPLC.

Cathepsin Cleavage Assay of Radiolabeled Copolymers

Human cathepsin S (20 ng/ μ L) in 100 μ L of buffer solution (50 mM sodium acetate, 1 mM EDTA and 10 mM DTT, pH 6.0) was incubated with 100 μ g of each purified ^{177}Lu -CSC0-3 at 25 $^{\circ}\text{C}$. After 12 h, a 50 μ L aliquot of each copolymer solution was analyzed by radio-SEC-HPLC to determine the relative amount of cathepsin S cleavage product. The analysis utilized an isocratic gradient at a linear flow rate of 0.80 mL/min with a mobile phase consisting of PBS with 0.02 M EDTA at pH 7.2. The UV analysis of the HPLC eluent was carried out at a wavelength of 494 nm.

Human Serum Stability of Radiolabeled Copolymers

Briefly, 10 μ g of purified ^{177}Lu -CSC was incubated with 1 mL of human AB serum at 37 $^{\circ}\text{C}$. After 144 hours, the stability was determined by centrifuge filtration using an Amicon Ultra-4 10 kDa filter. A 100 μ L aliquot was loaded in the filter and diluted to 4 mL using DI water. The loaded sample was centrifuged and washed with 4 mL of DI water. The percent of intact ^{177}Lu -CSC was calculated by dividing the remaining radioactivity in the 10 kDa filter to the total radioactivity added.

Cell Culture

The HPAC cells were cultured in our laboratory, as per ATCC protocols, in DMEM/Ham's F12 medium containing 5% FBS, 1.562 nM EGF, 14.3 mM sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate supplemented with 0.350 μ M insulin, 0.0625 μ M transferrin and 0.110 μ M hydrocortisone. Cells were incubated at 37 $^{\circ}\text{C}$ at 5% CO_2 in air.

Human monocytes were provided by the UNMC Elutriation Core Facility. Monocytes were plated into T75 tissue culture flasks at a concentration of 20×10^6 cells in 20 mL of macrophage differentiation medium: DMEM containing 10% FBS, 1% NEAA, 500 U rhM-CSF, 1% Penicillin/Streptomycin, 2 mM l-glutamine and 1% sodium-pyruvate. Media was half-exchanged every 2 to 3 days for 7–10 days, at which time, visual confirmation of differentiation led to media replacement with the macrophage maintenance medium (differentiation media without the rhM-CSF). Cells were maintained in a 37 $^{\circ}\text{C}$ humidified atmosphere with 5% CO_2 .

Confocal Microscopy

Macrophage cells and HPAC cells were seeded onto 4-well chambered coverglass units with 1×10^4 and 5×10^4 cells, respectively. FITC-labeled polymer conjugates were diluted in blank media to 100 μ g/mL concentration. The cells were then incubated with the polymer for 24 h in a 37 $^{\circ}\text{C}$ humidified atmosphere with 5% CO_2 . Half an hour before the end of this incubation, LysoTracker Red DND-99 and Hoechst 33342 were added in the media to stain the lysosomal compartment and the nuclei. Following incubation, the media was aspirated, and the cells were washed with PBS to remove free polymers that were not internalized prior to confocal imaging.

Immunoblot Analysis

Whole cell HPAC and differentiated macrophage lysate as well as mice HPAC tumor homogenate were prepared using RIPA buffer and a cocktail of proteases inhibitors. The lysates and homogenate were centrifuged at 13,000 RPM at 4 °C for 20 minutes. The supernatants containing 50 µg total proteins were mixed with sample buffer and then separated by 10% SDS-PAGE, followed by transfer to PVDF membrane. After blocking in 5% nonfat milk, 0.1% Tween-20, and Tris-buffered saline at room temperature for 1 h, membranes were probed with primary antibodies in blocking solution for 1 h at room temperature, followed by extensive washing in TBS. Rabbit anti-goat and goat anti-mouse secondary antibodies were used for the detection of immunoreactive proteins by chemiluminescence.

Biodistribution Studies

Biodistribution studies were carried out using age-matched normal CF-1 and HPAC tumor bearing SCID mice. For biodistribution studies involving HPAC xenograft mice, the mice were inoculated in each flank with 100 µL of a 1:1 ratio of suspended HPAC cells and Matrigel™ to obtain a final concentration of 5×10^6 HPAC cells per 100 µL. Upon achieving sufficient tumor size, (10–15 mm in diameter) the mice were considered viable for biodistribution studies. The CF-1 mice and HPAC xenograft mice were injected with a 0.37 MBq (10 µCi) intravenous bolus of the purified ^{177}Lu -CSCs. The mice were sacrificed and their tissues excised at 4, 24, 72 and 144 h time points post-injection (p.i.). The blood and excised tissues were weighed, the ^{177}Lu radioactivity measured using a gamma counter, and the percentage injected dose (%ID) and the percentage injected dose per gram (%ID/g) calculated for each tissue.

SPECT/CT Imaging Studies

Xenograft mice were intravenously injected with 400–600 µCi of CSCs in 100 µL saline and then anesthetized with 5% isoflurane delivered in 0.5 mL/min oxygen. Image acquisition with a 5-pinhole (1.0 mm/pinhole) collimator was acquired at 4, 24, 72 and 144 h time points p.i. Five hundred and twelve CT projections for each image were acquired and reconstructed using Triumph X-O 4.1 at a matrix size of $512 \times 512 \times 512$ with a 0.15-mm voxel dimension. Sixty four SPECT projections for each image were acquired using a Triumph SPECT with a 20% window at 208 keV for ^{177}Lu and reconstructed using Triumph SPECT Reconstruction Application 1.0.8.0 at a matrix size of $64 \times 64 \times 64$ with a 1.14 mm voxel dimension. Co-registration of anatomical CT images and functional SPECT was performed using 3D image visualization and analysis software VIVID based on Amira 4.1. A median filter was applied to all SPECT images.

Statistics

Statistical analyses were done using a two-tailed unpaired Student's t-test (2 groups) or one way ANOVA (> 2 groups) with Bonferroni post-tests, a $p < 0.05$ indicating statistically significant differences.

RESULTS AND DISCUSSION

Design and Synthesis of Cathepsin S Cleavable Linkers (CSLs)

Herein, we report the evaluation of a series of CSLs incorporated into a ^{177}Lu -labeled HMPA copolymer construct. The objective of our approach is to utilize the CSLs to enhance the diagnostic/therapeutic potential of HPMA copolymer based drugs by improving the clearance properties of these agents from MPS associated tissues, in particular the liver and spleen. The CSLs, depicted in Figure 1, are composed of four components: 1) the DOTA moiety that is responsible for chelation of the diagnostic/therapeutic radiometal; 2) the P-M-G-L-P substrate (**Y**) which cleaves in the presence of cathepsin S; [23, 28] 3) a clearance sequence (**X**), in this case G-(D)S-D(S), that can be modified to influence the clearance rate of the resulting radiometabolite; and finally 4) the linker group (**Z**) that is used to optimize the distance of the cathepsin S substrate from the polymer backbone. The distance of the cathepsin S substrate from the HPMA copolymer backbone is expected to be a factor, due to steric inhibition, that influences the cleavage rate of the cathepsin S substrate. In this study we investigate the impact of linking groups of various lengths (0, 6 and 13 atoms) on the *in vitro* cleavage rate and *in vivo* performance of CSLs incorporated into ^{177}Lu -labeled HPMA copolymers.

As shown in Figure 1, we chose to investigate CSL1-3 containing a null, a six atom hydrocarbon (5-Ava) and a 13 atom polyethylene glycol (PEG) linking group, respectively. In the case of CSL3, a PEG linker was chosen in place of a 13 atom hydrocarbon chain in order to reduce the potential for hydrophobic, intramolecular associations that would have likely limited separation of the cathepsin S substrate and the HPMA copolymer backbone. [29, 30] The control for our study, **0**, is simply the DOTA chelation system absent of any peptide components. The CSL1-3 were synthesized with intact orthogonal protection using automated solid-phase peptide synthesis. The synthesis of the CSLs with orthogonal protection results in the generation of a single carboxylic acid functionality at the C-terminus which will later be used for conjugation to the HPMA copolymer. Purification of the crude synthetic peptides by HPLC gave yields of 37.5, 44.0 and 27.1 for CSL1-3, respectively. The HPLC and mass spectrometric data of the CSLs are given in Table 1.

Synthesis and Characterization of Cathepsin S Cleavable Copolymers (CSCs)

The scheme for synthesis of the CSCs is depicted in Figure 2. The HPMA copolymer consisting of HPMA, APMA and APMA-FITC monomers were prepared by RAFT polymerization. The estimated molecular weight of the synthesized HPMA copolymer was 109 kDa with a polydispersity of 1.3 as determined by FPLC analysis. Ninhydrin assay revealed a total amine concentration of 57.2 $\mu\text{mol/g}$ ($n=1$), which corresponds, based on the feed content, to 97% incorporation of the APMA monomer. On average, this yields approximately six APMA moieties per polymer backbone. Conjugation of CSLs to HPMA copolymers was accomplished using EDC/NHS amidation chemistry. The orthogonal protection of the CSLs allowed for the chemoselective conjugation of the C-terminal carboxylic acid to the APMA moiety on the HPMA copolymer. Conjugation of the DOTA chelation system to the HPMA copolymer (CSC0) gave a DOTA content of 31.3 $\mu\text{mol/g}$ polymer ($n=1$), corresponding to a 54% conjugation yield, as evaluated by ninhydrin assay.

Amino acid analysis of the conjugation of CSL1-3 to the HPMA copolymer gave a peptide content of 15.0, 13.4 and 12.7 $\mu\text{mol/g}$ polymer ($n=3$), which corresponds to 26.0, 23.4 and 22.2 % for CSC1-3, respectively. The orthogonal protecting groups were removed with a cleavage cocktail (92.5% TFA) to give the deprotected CSCs. Size exclusion chromatography was used to purify the CSCs prior to radiolabeling.

Synthesis of ^{177}Lu -labeled CSCs

The ^{177}Lu -labeled CSCs were prepared by heating $^{177}\text{LuCl}_3$ in the presence of CSCs at 90°C for 1 h. The radiochemical yields for CSC0-4, as determined by radio-SEC-HPLC, were on average ($n=3$) 77%, 90%, 43% and 81%, respectively. Correspondingly, the achievable specific activities were 142.45, 166.50, 79.55 and 149.85 MBq/mg of polymer.

Serum Stability and Cleavage Studies of ^{177}Lu Labeled CSCs

The stability of the ^{177}Lu -CSCs in human serum was investigated over the course of 144 h at 37°C . At the end of this incubation period, 98% of the ^{177}Lu -CSCs remained intact thus suggesting that the ^{177}Lu -CSCs would be stable to serum proteases *in vivo* while in circulation. It is important to note that this stability study does not reflect potential uptake and metabolism by cellular components of the blood (e.g., circulating monocytes), which likely impacts the stability of the ^{177}Lu -CSCs in circulation.

The *in vitro* susceptibility of the ^{177}Lu -CSCs to cathepsin S cleavage was investigated by incubating the radiolabeled copolymers at 37°C for 12 h in the presence of cathepsin S. Analysis of aliquots of the reaction medium by radio-SEC-HPLC, Figure 3, revealed that ^{177}Lu -CSC1-3 underwent proteolytic cleavage when challenged with cathepsin S. The non-cleavable control CSC0, not shown, was found to be stable in the presence of cathepsin S over 72 h. All of the intact ^{177}Lu -CSCs demonstrated a retention time of approximately 10.5 min on the SEC column. Upon cleavage, the generated radiometabolite resulted in a substantially higher retention time of 12.4 min, which corresponds to the expected reduction in molecular weight. We have previously demonstrated that the cleavage site for the CSLs are between the glycine and leucine residues (P-M-G-L-P) of the substrate sequence.[23] Over the 12 h incubation period, the ^{177}Lu -CSC1-3 demonstrated an average ($n=2-3$) of 8.4, 10.1 and 25.9 % cleavage, correspondingly.

As stated previously, the proximity of the metabolic sequence to the HPMA copolymer backbone is expected to influence the cleavage rate of the CSCs. Our basis for the introduction of linking groups of various lengths (**Z**) into the CSLs was to investigate the structure-activity relationship, with respect to cathepsin S activity, of the CSLs when integrated into the HPMA copolymers. From the above data, some insight can be gleaned regarding the structure-activity relationship of the HPMA copolymer incorporated CSLs. The inclusion of the smaller linking group, 5-Ava, into the CSL (^{177}Lu -CSC2) gave no statistically significant increase in cleavage relative to the null analog (^{177}Lu -CSC1). In the case of ^{177}Lu -CSC3, the introduction of the longer PEG linking group gave much higher rates of cathepsin S cleavage. These data suggest that the length of the linking group is capable of influencing the cathepsin S cleavage activity likely through a reduction of steric hindrance.

Cathepsin S Expression in HPACs and Macrophages

It is well known that the MPS is primarily responsible for the non-target accumulation of nanomedicine platforms, particularly in the liver and spleen.[31] The MPS is composed of phagocytic cells (e.g., macrophages and monocytes) that sequester drug delivery systems which typically results in loss of diagnostic/therapeutic effectiveness and potential toxicity. These phagocytic cells are also known as antigen presenting cells (APCs) due to their function in adaptive immunity where these cells process pathogenic material and present antigen fragments on the cellular surface. Cathepsin S is a predominantly lysosomal proteolytic enzyme that has been shown to be highly regulated, relative to other cathepsins (e.g., cathepsin B), and predominately expressed in APCs.[32] The substantial expression of cathepsin S in APCs is thought to be due to its unique role in the catabolism of pathogenic proteins.[33–35] Our design seeks to take advantage of the cathepsin S expression/activity in APCs to preferentially enhance clearance from non-target tissues.[3, 23]

In this report, we utilize the HPAC human pancreatic cancer cell line as our *in vitro* and *in vivo* model of pancreatic cancer. Human monocytes have been differentiated to macrophages to serve as our model of tissue-resident macrophages. In order to determine cathepsin S expression, immunoblotting was performed on both cell types. As shown in Figure 4, cathepsin S is highly expressed in differentiated macrophages with negligible detectable expression in the HPAC cells. Overall, this corresponds well with our previous observations that the cathepsin S protease activity is much higher (tenfold) in differentiated macrophages versus the HPAC cell line.[23] Evaluation of the cathepsin S expression in HPAC tumor homogenates revealed increased levels of cathepsin S relative to cultured cells. This relative increase in expression may be a consequence of the dissimilarity of the *in vivo* tumor microenvironment versus the *in vitro* cultured milieu. However, it seems likely that the escalation in cathepsin S signal may, at least partially, be attributable to tumor-associated macrophages (TAM), which exist in almost all solid tumors.[36]

The intracellular trafficking of FITC-labeled copolymers in both the differentiated macrophages and the HPAC cell line was investigated using confocal microscopy, Figure 5. In these studies, the CSC0 was utilized as our model HPMA copolymer. LysoTracker® Red and Hoechst fluorescent markers were used to delineate the lysosome and nucleus compartments, respectively. After 24 h incubation with CSC0, the differentiated macrophages (Figure 4A) demonstrated substantially higher levels of cellular accumulation relative to the HPAC cell line (Figure 4B). This is in accordance with our previously reported flow cytometric studies working with a higher molecular weight HPMA copolymer.[23] As can be seen in the overlay, the CSC0 accumulation in the macrophages is co-localized with the LysoTracker® Red marker. The accumulation of HPMA copolymers in lysosomal compartments is in agreement with previous literature reports.[37, 38] By accumulating in the macrophage lysosomes, the CSCs are co-localized with the highest sub-cellular concentrations of cathepsin S. The selective uptake of the CSCs in macrophages and the accumulation of the copolymer in the lysosomal compartments are expected to substantially and selectively increase the CSC cleavage kinetics in macrophages and other APC cell types. The proteolytic degradation of the CSCs will result in the formation of low

molecular weight radiometabolite which are anticipated to clear more rapidly from the APC cells residing in non-target tissues and ultimately result in higher clearance.

Biodistribution Studies of ^{177}Lu -CSCs

The biodistribution properties of the ^{177}Lu -CSCs were investigated at 4, 24, 72 and 144 h post-administration in both a normal CF-1 (supporting information) and HPAC xenograft mouse models. With exception of the blood clearance rate (elaborated on below), the ^{177}Lu -CSCs demonstrated similar biodistribution trends in both animal models. The data obtained from the biodistribution studies of the ^{177}Lu -CSCs in a HPAC xenograft mouse model are given in Table 2. The calculated tumor-to-non-target ratios for selective tissues are given in Table 3. At the 4 h p.i. time point, the ^{177}Lu -CSCs revealed excretion values that ranged from $23.82 \pm 3.23 - 34.57 \pm 2.41$ %ID. Those HPMA copolymers with the cathepsin S cleavable linkers (^{177}Lu -CSC1-3) demonstrated significantly higher levels of radioactivity excretion relative to the non-cleavable control (^{177}Lu -CSC0) by the 24 h time point ($p < 0.05$). At the 144 h time point, $83.29 \pm 1.24 - 87.93 \pm 1.49$ %ID of the ^{177}Lu -CSC1-3 had been eliminated from the body in comparison to only 55.65 ± 2.26 %ID of ^{177}Lu -CSC0 ($p < 0.0001$). The overall elimination of radioactivity from the mouse corresponded well to the blood retention profiles of the ^{177}Lu -CSCs. At 4 h p.i., all four ^{177}Lu -CSCs demonstrated blood retention profiles that were statistically identical. By the 24 h time point, ^{177}Lu -CSC1 and ^{177}Lu -CSC2 demonstrated significantly lower blood retention ($p < 0.05$) relative to the control. The blood retention of ^{177}Lu -CSC1-3 at 72 and 144 h post-administration were all substantially lower ($p < 0.001$), 2.2 – 2.9 and 3.0 – 4.4 fold lower, respectively, in comparison to ^{177}Lu -CSC0. Comparison of the blood clearance profile of the ^{177}Lu -CSCs in both the CF-1 and HPAC xenograft model revealed that the CF-1 model demonstrated a relative two-fold lower retention for all ^{177}Lu -CSCs at all time points investigated. This suggests that the alterations made to the SCID model (i.e., absence of functional T and B lymphocytes) may be impacting the blood clearance of the ^{177}Lu -CSCs. However, when the blood clearance profiles are taken into account, the overall trends of the ^{177}Lu -CSCs, with respect to uptake, retention and clearance, in solid tissues remain consistent among the two models.

As with most nanomedicine platforms, the ^{177}Lu -CSCs exhibited liver and spleen retention as a result of MPS driven sequestration.[39] The liver and spleen uptake of the non-cleavable control, ^{177}Lu -CSC0, plateaued by 24 h post-injection with retention values of 4.74 ± 0.65 and 7.79 ± 1.47 %ID/g, respectively. The retention of ^{177}Lu -CSC0 associated radioactivity in the liver and spleen remained essentially constant throughout the remaining time points investigated. As a result of the incorporation of the CSLs, the ^{177}Lu -CSC1-3 demonstrated substantially lower levels of long-term retention in comparison to the control. Significant differences ($p < 0.05$) in the spleen retention of the ^{177}Lu -CSC1-3 versus the ^{177}Lu -CSC0 were observed by 24 h post-administration. By 72 h p.i., both spleen and liver retentions of the ^{177}Lu -CSC1-3 were considerably lower ($p < 0.01$) than control. At our terminal time point, the liver and spleen retentions of the ^{177}Lu -CSC1-3 were 3.5 – 4.4 and 2.2 – 2.5 fold lower, correspondingly, than the ^{177}Lu -CSC0.

The increased excretion and lower blood, liver and spleen retention profiles of ^{177}Lu -CSC1-3 are undoubtedly linked to the incorporation of the cathepsin S cleavable linker into the HPMA copolymer. The lower liver and spleen retention is likely due primarily to the processing of ^{177}Lu -CSC1-3 by tissue-resident macrophages (e.g., Kupffer cells of the liver) thus resulting in low-molecular weight radiometabolites that are more readily cleared.[22, 39] With respect to blood retention, the ^{177}Lu -CSC1-3 are being cleared from circulation significantly faster than the non-cleavable control. The inclusion of the CSLs into the HPMA copolymers is possibly leading to a markedly higher opsonization and sequestration by the MPS. Such responses have been observed with functionalization of other drug delivery systems, but this seems unlikely given the limited functionalization (< 1 mole%) of the HPMA copolymers used in these experiments.[39] Alternatively, and to us more likely, is the possibility that the ^{177}Lu -CSCs are being internalized and processed by cellular components of the blood. The human serum studies that were performed suggest that the ^{177}Lu -CSCs are stable in the presence of extracellular serum proteases. However, many drug delivery platforms are known to be taken up by circulating blood monocytes, an APC known to express cathepsin S.[40–42] If this is the case, the ^{177}Lu -CSCs would be expected to be taken up and processed by phagocytic cells in the blood, presumably circulating monocytes. Metabolism of the CSLs by these APCs would lead to the generation of radiometabolites which would likely favor clearance from the processing cell as well as the circulation relative to the non-cleavable ^{177}Lu -CSC0.

The ^{177}Lu -CSCs exhibited substantial accumulation in the HPAC xenograft tumors through the EPR effect.[43, 44] ^{177}Lu -CSC0 demonstrated the highest tumor retention with a peak accumulation of 5.00 ± 1.40 %ID/g at 72 h post-administration. For the ^{177}Lu -CSC1-3, maximal tumor retention for each copolymer was observed by the 24 h time point with values of 3.70 ± 0.52 , 2.71 ± 0.34 and 3.98 ± 2.16 %ID/g, respectively. By the 144 h time point, the tumor retention of ^{177}Lu -CSC1 (2.61 ± 0.93 %ID/g) was statistically identical to the non-cleavable control (3.21 ± 0.68 %ID/g), but was higher than ^{177}Lu -CSC2 ($p < 0.05$) and ^{177}Lu -CSC3 ($p < 0.05$) (1.49 ± 0.31 and 1.68 ± 0.43 , correspondingly).

The tumor to non-target ratios for selected tissues at 4, 24, 72 and 144 h p.i. are given in Table 3. For ^{177}Lu -CSC1, the HPAC tumor retention and non-target clearance properties yielded superior tumor-to-non-target ratios by 24 h p.i. relative to the other ^{177}Lu -CSCs, including the control. The superiority of ^{177}Lu -CSC1, relative to the other copolymers, improved longitudinally with maximal divergence in tumor to non-target ratios being observed at the 144 h time point. At this time point, the tumor-to-liver, spleen and kidney ratios of ^{177}Lu -CSC1 are on average 3.0 ($p < 0.01$), 2.1 ($p < 0.05$) and 2.0 ($p = 0.067$) higher, respectively, than the control. Evaluation of the biodistribution data for the CSL containing HPMA copolymers reveals some interesting trends with regard to the structure-activity profiles of the ^{177}Lu -CSC1-3 in HPAC tumor and non-target tissues. As described earlier, *in vitro* assays revealed that the addition of the PEG linker (^{177}Lu -CSC3), the longest (13 atoms) linker, significantly enhanced the cathepsin S cleavage rate relative to ^{177}Lu -CSC1 or ^{177}Lu -CSC2. Given this, one would anticipate that ^{177}Lu -CSC3 would have exhibited the fastest clearance rates from MPS associated non-target tissues. However, *in vivo* biodistribution studies revealed that the ^{177}Lu -CSC1, null (0 atom) linker, exhibited overall

the fastest clearance of radioactivity from the blood, liver and spleen among the CSL containing HPMA copolymers. In fact, at 24 h p.i., the clearance of ^{177}Lu -CSC1 from MPS associated non-target tissues is statistically better ($p < 0.05$) than ^{177}Lu -CSC3. Though, by 72 h and beyond, the clearance of radioactivity in the blood, liver and spleen is statistically identical for all three copolymers. Interestingly, this trend is somewhat reversed with regard to HPAC tumor retention. At 144 h p.i., ^{177}Lu -CSC1 exhibits substantially higher tumor retention relative to both ^{177}Lu -CSC2 and ^{177}Lu -CSC3. At this point, the factors driving these observations remain unclear. Likely, the clearance kinetics of the ^{177}Lu -CSCs relies primarily on a combination of the cleavage kinetics and the vascular and lymphatic efficiencies of the tissue. The inequalities between the non-target, normal tissues and the HPAC tumors with regard to the abundance of cathepsin S expressing APCs and the vasculature and lymphatic systems are likely the driving influences behind these observations. Currently, we are pursuing studies that will hopefully yield a better understanding of the *in vivo* cathepsin S cleavage kinetics and clearance mechanisms of the ^{177}Lu -CSCs.

Small Animal SPECT/CT Imaging Studies

For the development of nuclear imaging agents based on nanomedicine platforms, the clinical success of antibody based agents (e.g., ProstaScint[®] and OncoScint[®]) can serve as an example, due to the similar pharmacokinetic profiles of the two systems.[45, 46] Similar to many nanomedicine platforms, monoclonal antibodies are large macromolecules that typically don't achieve optimal tumor-to-non-target ratios until days after injection. As a consequence, image acquisitions for clinical imaging agents based on monoclonal antibodies are typically obtained between 2 and 5 days post-injection.[45, 47] For the investigated ^{177}Lu -CSCs, the biodistribution studies suggest that this same time frame would be suitable, if not optimal, for diagnostic imaging.

In order to better evaluate the imaging potential of the ^{177}Lu -CSCs, micro-SPECT/CT studies were performed for ^{177}Lu -CSC0, ^{177}Lu -CSC1 and ^{177}Lu -CSC3 in mice bearing bilateral flank HPAC xenografts. ^{177}Lu -CSC2 was not chosen for microSPECT/CT analysis due to the similarity in its biodistribution profile with ^{177}Lu -CSC3. Whole body images, depicted in Figure 6, were collected at 4, 24 and 72 h post-administration. At 4 h p.i., visual inspection of the normalized images revealed substantial accumulation in the abdominal cavity as well as the bladder for all three copolymers. The bladder activity at such an early time point suggests that the clearance of activity is likely from the copolymer fraction that is below the renal excretion threshold.[48] Visualization of the flank HPAC tumors was limited at 4 h p.i., but became clearly evident by the 24 h time point. At 24 h p.i., decreases in the abdominal activity, which includes the liver, were noted for the three copolymers. By the 72 h time point, all of the investigated ^{177}Lu -CSCs demonstrated considerable uptake/retention in the HPAC tumors. In addition, ^{177}Lu -CSC1 and ^{177}Lu -CSC3 demonstrated superior abdominal clearance relative to the non-cleavable control (^{177}Lu -CSC0). Overall, the SPECT/CT images agree well with the data obtained from the biodistribution study. The substantial retention of ^{177}Lu -CSC1 in the HPAC tumors coupled with the enhanced clearance of activity from the abdominal region suggest that the CSL1 is

the most promising linker to move forward with in the development of diagnostic and radiotherapeutic HPMA copolymers.

CONCLUSION

In summary, our laboratory is pursuing the development of CSLs that cleave in the presences of cathepsin S, to reduce the non-target accumulation of diagnostic/radiotherapeutic HPMA copolymers in MPS tissues. To this end, we have evaluated a series of CSLs incorporated into a ^{177}Lu -labeled HMPA copolymer construct with the purpose of understanding the structure activity relationship with regard to the length of the linking groups. *In vitro* cleavage assays revealed that the longest linking group (13 atoms) demonstrated the highest cathepsin S cleavage kinetics. Additionally, confocal microscopy confirmed the selective uptake of HPMA copolymers in differentiated macrophages and trafficking to the lysosomal cellular compartments. Contrary to *in vitro* observations, *in vivo* biodistribution studies revealed that the linking group length in the CSL design did not substantially impact the non-target clearance. However, in the case of HPAC tumor retention, the CSC1 demonstrated significantly higher levels of retention relative to the other CSCs. This gave CSC1 superior tumor-to-non-target ratios as confirmed by longitudinal SPECT/CT imaging studies. Taken together, our results suggest that the dissimilarities in the metabolism and clearance kinetics of the CSL incorporated HPMA copolymers are tissue specific. Our future work will focus on gaining an understanding of the tissue-specific underpinnings influencing CSL metabolism and clearance. Lastly, these results demonstrate that the CSLs can substantially improve the non-target clearance of HPMA copolymers, and likely other nanomedicine platforms, leading to an enhancement in the tumor-to-non-target ratios and ultimately clinical potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic design of CSLs with intact orthogonal protection.

Figure 2.

Schemes for the synthesis of the ¹⁷⁷Lu-CSC conjugates. For CSC0, CSC1, CSC2 and CSC3, **X** = **Y** = **Z** = null; **X** = GSS, **Y** = PMGLP; **X** = GSS, **Y** = PMGLP **Z** = 5-Ava and **X** = GSS, **Y** = PMGLP **Z** = PEG3, respectively.

Figure 3.
Chromatograms of CSC1-3 after a 12 h incubation with cathepsin S.

Figure 4. Western Blot analysis of cathepsin S expressions in cultured HPACs (HPAC1), differentiated macrophages, and HPACs from corresponding *in vivo* tumor-derived cells (HPAC2). 50 μ g was loaded in each sample.

Figure 5.
After 24 hours incubation, FITC-labeled HPMA copolymer showed intense accumulation in lysosomes of macrophages (A) (B).

Figure 6.
Micro-SPECT/CT imaging studies of CSC0, CSC1 and CSC3 in HPAC xenograft mice.

Table 1

Characterization of CSLs.

CSL Peptides	Sequences	Formula	Mass Expected[M+H] ⁺	Mass Observed[M+H] ⁺
CSL0	DOTA(tBu) ₃	C ₂₈ H ₅₂ N ₄ O ₈	573.4	573.2
CSL1	DO3A(tBu) ₃ -G- (D)S(tBu)-(D)S(tBu)-P- M-G-L-P	C ₆₇ H ₁₁₈ N ₁₂ O ₁₈ S	1411.8	1411.5
CSL2	DO3A(tBu) ₃ -G- (D)S(tBu)-(D)S(tBu)-P- M-G-L-P-5-Ava	C ₇₂ H ₁₂₇ N ₁₃ O ₁₉ S	1510.9	1510.6
CSL3	DO3A(tBu) ₃ -G- (D)S(tBu)-(D)S(tBu)-P- M-G-L-P-PEG3	C ₇₆ H ₁₃₅ N ₁₃ O ₂₂ S	1615.0	1614.7

Table 2

Biodistribution of ^{177}Lu -labeled CSCs in HPAC tumor bearing mice. N=5 at each time point. Data are represented as mean $\pm$ SD %ID/g.

Tissue	$^{177}\text{Lu-0}$	4 h p.i.			24 h p.i.		
		$^{177}\text{Lu-1}$	$^{177}\text{Lu-2}$	$^{177}\text{Lu-3}$	$^{177}\text{Lu-0}$	$^{177}\text{Lu-1}$	$^{177}\text{Lu-2}$
Blood	21.02 $\pm$ 6.09	17.62 $\pm$ 2.87	16.42 $\pm$ 2.87	20.47 $\pm$ 2.19	11.34 $\pm$ 2.24	5.80 $\pm$ 0.62	6.87 $\pm$ 1.15
Heart	6.33 $\pm$ 2.04	5.05 $\pm$ 0.97	4.64 $\pm$ 1.30	5.12 $\pm$ 1.81	3.77 $\pm$ 0.35	2.44 $\pm$ 0.19	2.65 $\pm$ 1.10
Lung	6.68 $\pm$ 1.09	5.32 $\pm$ 1.26	5.73 $\pm$ 1.48	6.44 $\pm$ 2.22	3.87 $\pm$ 1.18	2.83 $\pm$ 0.40	2.78 $\pm$ 0.58
Liver	4.97 $\pm$ 0.74	4.20 $\pm$ 0.64	4.27 $\pm$ 0.38	5.13 $\pm$ 0.64	4.74 $\pm$ 0.65	3.33 $\pm$ 0.05	4.21 $\pm$ 0.32
Pancreas	2.92 $\pm$ 0.57	2.48 $\pm$ 0.12	2.50 $\pm$ 0.66	2.98 $\pm$ 0.76	3.09 $\pm$ 1.07	2.05 $\pm$ 0.76	1.96 $\pm$ 0.47
Stomach	1.04 $\pm$ 0.29	1.15 $\pm$ 0.27	1.00 $\pm$ 0.27	1.15 $\pm$ 0.28	0.93 $\pm$ 0.35	0.76 $\pm$ 0.18	0.72 $\pm$ 0.29
Spleen	3.35 $\pm$ 0.56	2.88 $\pm$ 0.83	4.04 $\pm$ 1.56	3.55 $\pm$ 0.61	7.79 $\pm$ 1.47	3.24 $\pm$ 1.29	4.53 $\pm$ 2.21
Small Int.	1.96 $\pm$ 0.34	2.06 $\pm$ 0.74	1.56 $\pm$ 0.29	1.66 $\pm$ 0.52	1.03 $\pm$ 0.13	0.68 $\pm$ 0.11	0.69 $\pm$ 0.13
Large Int.	1.88 $\pm$ 0.20	3.84 $\pm$ 3.42	2.33 $\pm$ 0.59	1.93 $\pm$ 0.43	1.25 $\pm$ 0.37	1.24 $\pm$ 0.16	1.50 $\pm$ 0.26
Kidney	5.28 $\pm$ 1.25	5.08 $\pm$ 1.06	3.81 $\pm$ 0.76	4.63 $\pm$ 0.88	3.01 $\pm$ 1.01	2.38 $\pm$ 0.44	2.58 $\pm$ 0.20
Muscle	1.21 $\pm$ 0.24	1.22 $\pm$ 0.24	1.58 $\pm$ 0.95	1.22 $\pm$ 0.31	1.89 $\pm$ 1.14	1.26 $\pm$ 0.70	1.07 $\pm$ 0.31
Bone	1.52 $\pm$ 0.21	1.68 $\pm$ 0.21	1.65 $\pm$ 0.37	1.59 $\pm$ 0.21	1.42 $\pm$ 0.34	1.39 $\pm$ 0.19	1.14 $\pm$ 0.20
Brain	0.68 $\pm$ 0.09	0.70 $\pm$ 0.10	0.48 $\pm$ 0.08	0.56 $\pm$ 0.10	0.46 $\pm$ 0.08	0.29 $\pm$ 0.10	0.28 $\pm$ 0.04
HPAC Tumors	3.67 $\pm$ 0.72	3.29 $\pm$ 0.64	2.57 $\pm$ 0.39	3.04 $\pm$ 1.01	3.66 $\pm$ 0.62	3.70 $\pm$ 0.52	2.71 $\pm$ 0.34
Excretion (%ID)	23.82 $\pm$ 3.23	25.40 $\pm$ 3.08	34.57 $\pm$ 2.41	25.85 $\pm$ 4.68	40.22 $\pm$ 4.70	56.54 $\pm$ 1.75	56.44 $\pm$ 4.30
144 h p.i.							
Blood	5.15 $\pm$ 0.86	1.85 $\pm$ 0.35	1.75 $\pm$ 0.47	2.36 $\pm$ 0.47	2.35 $\pm$ 0.43	0.53 $\pm$ 0.19	0.68 $\pm$ 0.25
Heart	3.60 $\pm$ 1.06	2.05 $\pm$ 0.43	1.22 $\pm$ 0.16	1.49 $\pm$ 0.39	2.79 $\pm$ 1.38	1.24 $\pm$ 0.36	0.88 $\pm$ 0.20
Lung	3.24 $\pm$ 0.43	1.33 $\pm$ 0.20	1.21 $\pm$ 0.36	1.44 $\pm$ 0.22	1.89 $\pm$ 0.33	0.74 $\pm$ 0.22	0.50 $\pm$ 0.26
Liver	5.29 $\pm$ 1.17	2.46 $\pm$ 0.30	2.83 $\pm$ 0.56	2.96 $\pm$ 0.47	5.13 $\pm$ 0.64	1.40 $\pm$ 0.33	1.16 $\pm$ 0.19
Pancreas	3.10 $\pm$ 1.70	1.02 $\pm$ 0.43	0.92 $\pm$ 0.13	0.84 $\pm$ 0.30	1.71 $\pm$ 0.56	0.75 $\pm$ 0.62	0.41 $\pm$ 0.21
Stomach	0.82 $\pm$ 0.33	0.52 $\pm$ 0.23	0.32 $\pm$ 0.07	0.29 $\pm$ 0.10	0.48 $\pm$ 0.24	0.17 $\pm$ 0.06	0.14 $\pm$ 0.07
Spleen	8.22 $\pm$ 2.63	2.72 $\pm$ 0.72	3.00 $\pm$ 1.78	2.70 $\pm$ 0.69	8.27 $\pm$ 1.91	3.25 $\pm$ 0.86	3.43 $\pm$ 1.46
Small Int.	0.59 $\pm$ 0.15	0.57 $\pm$ 0.12	0.45 $\pm$ 0.05	0.62 $\pm$ 0.15	0.55 $\pm$ 0.19	0.12 $\pm$ 0.04	0.18 $\pm$ 0.07
Large Int.	1.15 $\pm$ 0.12	0.58 $\pm$ 0.09	0.63 $\pm$ 0.11	0.90 $\pm$ 0.53	0.92 $\pm$ 0.30	0.29 $\pm$ 0.04	0.31 $\pm$ 0.10

Tissue	$^{177}\text{Lu-0}$	$^{177}\text{Lu-1}$	$^{177}\text{Lu-2}$	$^{177}\text{Lu-3}$	$^{177}\text{Lu-0}$	$^{177}\text{Lu-1}$	$^{177}\text{Lu-2}$	$^{177}\text{Lu-3}$
Kidney	2.40 ± 0.70	1.45 ± 0.30	1.45 ± 0.36	1.44 ± 0.18	1.95 ± 0.36	0.83 ± 0.20	0.91 ± 0.18	1.03 ± 0.07
Muscle	1.57 ± 0.59	0.85 ± 0.48	0.81 ± 0.32	1.10 ± 0.29	1.21 ± 0.39	0.41 ± 0.21	0.46 ± 0.24	0.64 ± 0.16
Bone	1.88 ± 0.82	1.45 ± 0.68	0.49 ± 0.21	0.78 ± 0.14	1.30 ± 0.42	0.48 ± 0.27	0.48 ± 0.33	0.83 ± 0.24
Brain	0.30 ± 0.04	0.32 ± 0.35	0.07 ± 0.05	0.08 ± 0.02	0.20 ± 0.07	0.06 ± 0.04	0.07 ± 0.09	0.09 ± 0.09
HPAC Tumors	5.00 ± 1.40	3.28 ± 0.74	2.38 ± 0.59	2.81 ± 0.52	3.21 ± 0.68	2.61 ± 0.93	1.49 ± 0.31	1.68 ± 0.43
Excretion (%ID)	51.84 ± 2.65	72.12 ± 1.28	75.70 ± 1.35	68.96 ± 3.13	55.65 ± 2.26	83.29 ± 1.24	87.93 ± 1.49	84.10 ± 0.82

Tumor to non-target organ ratios of ^{177}Lu -labeled CSCs in HPAC tumor bearing mice. N=5 at each time point. Data are represented as mean $\pm$ SD%ID/g

Table 3

T/NT Ratios	$^{177}\text{Lu-0}$	$^{177}\text{Lu-1}$	$^{177}\text{Lu-2}$	$^{177}\text{Lu-3}$	$^{177}\text{Lu-0}$	$^{177}\text{Lu-1}$	$^{177}\text{Lu-2}$	$^{177}\text{Lu-3}$
4 h p.i.				24 h p.i.				
Blood	0.19 $\pm$ 0.10	0.19 $\pm$ 0.02	0.16 $\pm$ 0.03	0.15 $\pm$ 0.06	0.33 $\pm$ 0.05	0.65 $\pm$ 0.12	0.41 $\pm$ 0.09	0.44 $\pm$ 0.17
Liver	0.74 $\pm$ 0.12	0.79 $\pm$ 0.08	0.60 $\pm$ 0.06	0.61 $\pm$ 0.22	0.79 $\pm$ 0.18	1.11 $\pm$ 0.16	0.65 $\pm$ 0.08	0.81 $\pm$ 0.31
Spleen	1.13 $\pm$ 0.35	1.23 $\pm$ 0.42	0.73 $\pm$ 0.32	0.86 $\pm$ 0.22	0.49 $\pm$ 0.12	1.26 $\pm$ 0.44	0.70 $\pm$ 0.32	0.80 $\pm$ 0.32
Kidney	0.72 $\pm$ 0.19	0.68 $\pm$ 0.19	0.69 $\pm$ 0.14	0.66 $\pm$ 0.17	1.32 $\pm$ 0.43	1.59 $\pm$ 0.28	1.06 $\pm$ 0.16	1.39 $\pm$ 0.84
72 h p.i.				144 h p.i.				
Blood	0.98 $\pm$ 0.17	1.83 $\pm$ 0.39	1.46 $\pm$ 0.53	1.25 $\pm$ 0.41	1.41 $\pm$ 0.45	5.49 $\pm$ 2.32	2.47 $\pm$ 1.09	2.36 $\pm$ 0.87
Liver	0.96 $\pm$ 0.18	1.35 $\pm$ 0.26	0.84 $\pm$ 0.15	0.96 $\pm$ 0.12	0.63 $\pm$ 0.13	1.92 $\pm$ 0.68	1.31 $\pm$ 0.28	1.14 $\pm$ 0.17
Spleen	0.66 $\pm$ 0.22	1.28 $\pm$ 0.38	0.90 $\pm$ 0.28	1.08 $\pm$ 0.26	0.40 $\pm$ 0.08	0.82 $\pm$ 0.27	0.47 $\pm$ 0.11	0.48 $\pm$ 0.15
Kidney	2.14 $\pm$ 0.39	2.34 $\pm$ 0.55	1.67 $\pm$ 0.32	1.97 $\pm$ 0.36	1.69 $\pm$ 0.45	3.31 $\pm$ 1.65	1.67 $\pm$ 0.32	1.63 $\pm$ 0.35