Supplemental Information

Utilizing the multi-radionuclide resolving power of SPECT and dual radiolabeled single molecules to assess treatment response of tumors

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Materials and Methods

Due to the admixture of short and long lived isotopes in some of the products, proper disposal methods should follow Environment Health and Safety guidelines.

Radiochemistry

^{125}I-Labeling-LS370 or LS734 (10 µg) was dissolved in 100 µL of 0.1 M Tris (hydroxymethyl) aminomethane-hydrochloride (Tris-HCl) buffer, pH 7.6, and iodinated with 1.0 mCi $Na^{125}I$, using 2 µg Iodogen (Pierce, Rockford, IL, USA). After 30 min, the reaction was stopped by removing the supernatant to another vessel. Purification was performed by reverse phase-high-performance liquid chromatography RP-HPLC) on the C18 column (Supelcosil ABZ $^{+}$ PLUS, HPLC Column, 150 x 4.0mm, 5µm) with a gradient of H_2O containing 0.1% TFA and ACN containing 0.1% TFA for 30 min at a flow rate of 1 mL/min. The quality control (QC) testing of the radiochemical purity was done by RP-HPLC on the C18 column (Alltima HP, HPLC Column C18, 3µ, 53 x 7mm) with the gradient elution with H_2O containing 0.1% TFA and ACN containing 0.1% TFA for 8 minutes at a flow rate of 2.5 mL/min. Radioactivity of each fraction was determined by γ-counting. ^{125}I-LS734 eluted at 12.5 min and ^{125}I-LS370 eluted at 12 min. The radiochemical purities of peptides used in this study was >95%.

^{111}Indium Labeling of LS734

LS734 (20 µg) in 0.1 M acetic acid (300 µL) was added to ^{111}In (2 mCi) in 0.02 M HCl (100 µL), and the mixture was incubated for 30 min at room temperature. The reaction was applied to RP-HPLC in 0.1% TFA and purified by chromatography on a C18 reverse phase column (Waters, Milford, MA, USA). ^{111}In-LS734 was obtained by linear gradient elution consisting of solvent A (0.1% TFA in water) and B (0.1% TFA in ACN)

(1 mL/min flow rate, 30 min). Radioactivity of each fraction was determined by γ-counting. . The radiochemical purity by HPLC was >95%.

Dual ^{125}I/^{99m}Tc-Labeling

To synthesize the dual-labeled ^{125}I/^{99m}Tc-LS370, the HPLC purified ^{125}I-LS370 was incubated with ^{99m}Tc-glucoheptonate in ethanol/saline (9:1) at room temperature. The progress of the reaction was monitored *via* a radio-TLC scanner using water as eluent. While the dual labeled ^{125}I/^{99m}Tc-LS370 probe was found to be retained at the origin, the excess ^{99m}Tc-glucoheptonate eluted with the solvent front. A mobile eluent mixture of methanol/saline/TFA (90:8:2) and radio-TLC scanner was used to confirm the absence of any ^{99m}Tc oxides in the dual labeled peptide. The peptide eluted with the solvent front under these conditions without significant retention at the origin, indicating completion of ligand exchange reaction and lack of any ^{99m}Tc oxides in the preparation. Following the completion of the ligand–exchange reaction, the dual-labeled ^{125}I/^{99m}Tc-LS370 probe was further purified on HPLC system equipped with the radio-detector and C-18 column. While the excess ^{99m}Tc-glucoheptonate eluted with the injection front, the dual-labeled ^{125}I/^{99m}Tc-LS370 peptide showed retention times that are about 2 min faster than the single labeled ^{125}I-LS370 peptide alone. Additionally, the formation of the dual-labeled probe was also confirmed using the nanoSPECT. Finally, appropriate fractions of the dual labeled ^{125}I-^{99m}Tc-LS370 were collected and reconfirmed *via* radio-TLC. Radiochemical purity was >98%.

Dual ^{125}I/^{111}In-Labeling

LS734 (20 µg) was dissolved in Tris-HCl buffer (0.1 M, 100 µL, pH 7.6) and iodinated with $Na^{125}I$ (2.0 mCi) using 2 µg Iodogen (Pierce, Rockford, IL, USA). After 30 min, the reaction was stopped by transferring the solution into another tube. QC was performed on ^{125}I-LS734 (5 µL) by radio-HPLC. For the subsequent ^{111}In labeling, acetic acid (400 µL, 0.1 M) and ^{111}In (10 µL, 2 mCi) in HCl (0.02 M) was added to the iodinated fraction and the mixture was incubated for 30 min at room temperature. After reaction, the mixture was purified by RP-HPLC on a C18 column (Waters, Milford, MA, USA). ^{125}I-^{111}In-LS734 was obtained and purified by linear gradient elution consisting of solvent A (0.1% TFA in water) and B (0.1% TFA in ACN) (1 mL/min flow rate, 30 min). Eluted fractions (500 µL) were collected into tubes. Radioactivity of each fraction was determined by γ-counting. The final product was >95% pure.

Cell Culture

All cell handling was aseptically performed in a laminar flow hood. 4T1/*luc* murine breast cancer cells were purchased from Sibtech (Brookfield, CT) and MDA-MB-231 human mammary gland/breast cancer cells were purchased from the American Tissue Culture Collection (ATCC) and were grown until 60-75% confluence in T75 tissue culture flasks. The cells were grown in Dulbeccos's Modified Eagles Medium (GIBCO-BRL) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO_2 in a Revco Elite II incubator. To determine cell density, equal amounts of cell suspension and trypan blue exclusion were added to a hemocytometer to calculate a cells/mL concentration and ensure cell viability.

Dual isotope SPECT/CT imaging in spontaneous breast cancer model:

The MMTV-PyMT transgenic mice carrying the polyoma middle T oncogene driver by the MMTV promoter in the FVB background were bred in house*(15)*. The animal model recapitulates the human condition of early breast tumor-genesis including complex interactions of immune cells within the tumor, and multifocal lesions throughout the mammary tissue[19]. Multifocal tumors enabled internal controls as treated and untreated tumors within the same animal. For the proof-of-principle small animal nanoSPECT imaging study, a 12 week old MMTV-PyMT female mouse was used when the bilateral tumors were palpable and greater than 3 mm diameter.

The left pectoral mammary tumor was injected intratumorally with bromopyruvate (150 µL, 1.75 µM), an inhibitor of GDPH[16]. The contralateral tumor was injected with saline. At 24 h after bromopyruvate injection, the mouse was injected intravenously with dual radiolabeled ^{125}I-^{111}In-LS734 (500 µCi) and imaged with nanoSPECT 4 h post-injection. SPECT scans were performed using 16 projections, 180 s per projection. Reconstruction of SPECT and CT scans was performed using *In vivo* scope software (Bioscan).

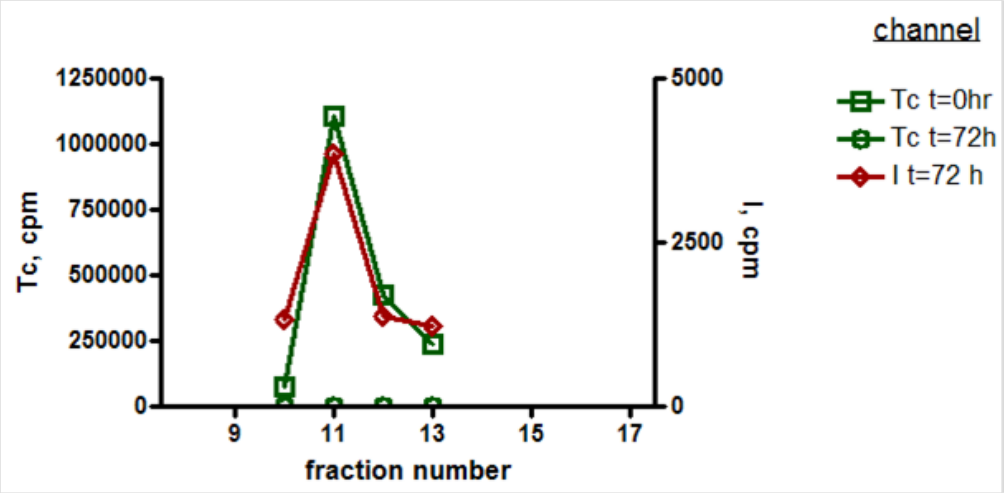
Data Analysis and Statistics: All data are presented as mean ± SD. For statistical classification, a Student's t test (two-tailed, unpaired) was used to compare individual datasets. All statistical analyses were performed using Prism software. P values less than 0.05 were considered significant.

Results

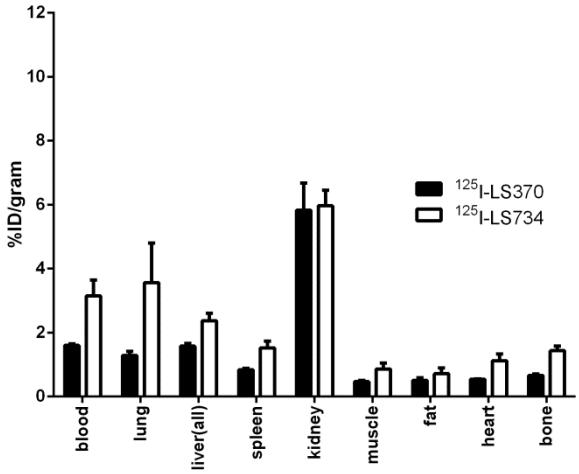
Radiochemistry

The achieved specific activities were: ^{125}I-LS370: 40-50 × 10^6 MBq/mmol, ^{125}I-LS734: 6-10 × 10^6 MBq/mmol, ^{111}In-LS734: 12 × 10^6 MBq/mmol. The specific activity of ^{125}I-^{111}In-LS734 was 3× 10^6 MBq/mmol. We also determined the specific activity of ^{99m}Tc-LS370 from the purified ^{125}I-^{99m}Tc-LS370. Based on a specific activity of 50 x 10^6 MBq/mmol of ^{125}I-LS370, the specific activity of ^{99m}Tc in ^{125}I-^{99m}Tc -LS370 was calculated. After 72 h of decay, 0.02% of ^{99m}Tc remained but this fraction did not have a significant impact on the ^{125}I counting window (15-75 keV). Based on the sum of the counts per minute (CPM) from fractions 10-12 and the counting efficiency (0.679 CPM/DPM; see **Supplemental Fig. 1**), the calculated specific activity of ^{99m}Tc in ^{125}I-^{99m}Tc-LS370 was 17 × 10^{10} MBq/mmol. This value is in good agreement with the literature reported maximum specific activity of ^{99m}Tc, which is ~20 × 10^{10} MBq/mmol[17].

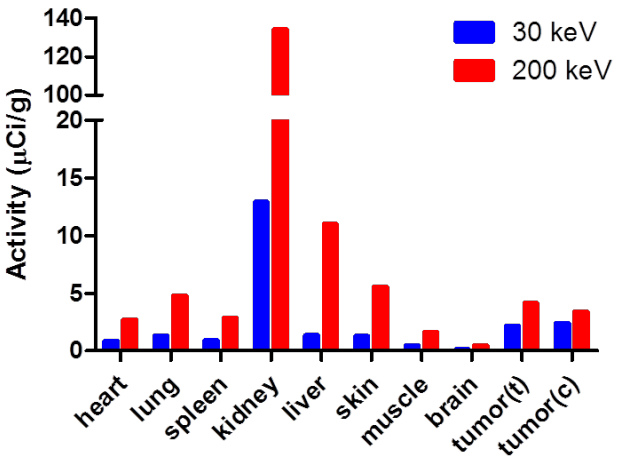
Supplemental Figures



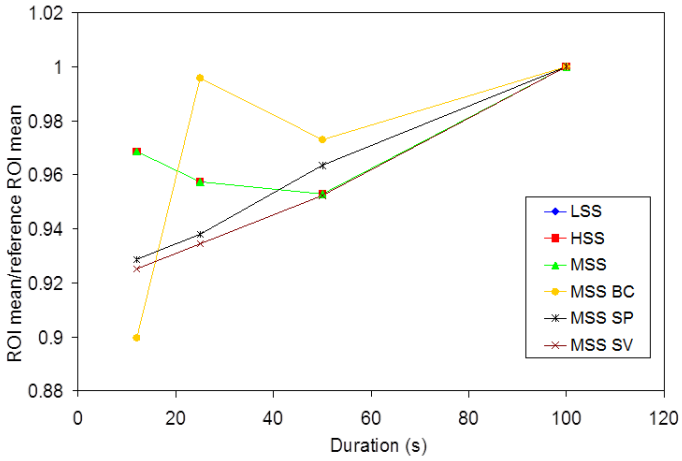
Supplemental Fig. 1: Decay counts of ^{125}I and ^{99m}Tc in LS370.



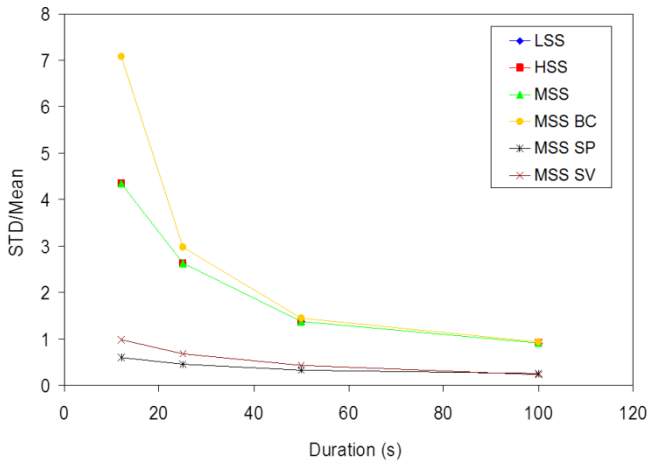
Supplemental Fig. 2: Tissue bio-distribution of ^{125}I-LS370 and ^{125}I-LS734 in tumor naïve Balb/c mice after 1h of administration of 4-7 µCi of the respective radiopharmaceutical.



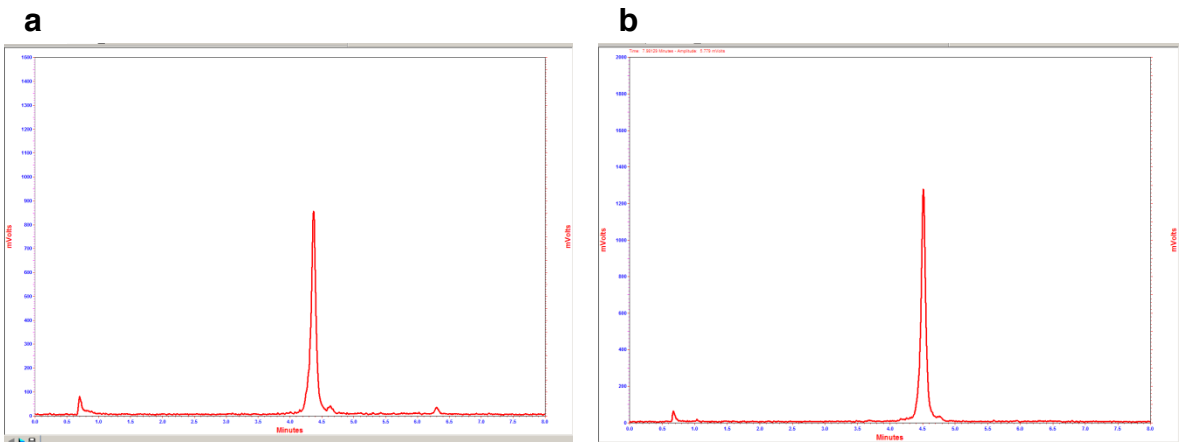
Supplemental Fig. 3: Biodistribution analysis for ^{125}I and ^{111}In fragments of LS734 in mouse tissues 4 h after intravenous injection. The 30 keV signal is mixture of ^{125}I and ^{111}In decay and demonstrates significant difference in biodistribution from the ^{111}In individual signal about 200 keV.



Supplemental Fig. 4: Ratio of the mean ROI value and the reference mean ROI value *vs* the scan duration (counting statistics) with various reconstruction options. The standard number of iterations (9) and standard pixel size (0.3 mm) were used in the reconstruction for all low, medium, and high background corrections (i.e. LSS, MSS, HSS corresponding to Low, Medium and High background correction for Standard number of iteration (9) and Standard pixel size 0.3 mm), and the BC, SP, and SV options were separately applied to the MSS reconstruction.



Supplemental Fig. 5: Coefficient of variation (STD/mean) for the ROI values *vs* the scan duration (counting statistics) with various reconstruction options; the standard number of iterations (9) and standard pixel size (0.3 mm) were used in the reconstruction for all low, medium, and high background corrections (i.e. LSS, MSS, HSS), and the BC, SP, and SV options were separately applied to the MSS reconstruction.



Supplemental Fig. 6: Radio-HPLC chromatogram of ^{125}I-LS734 at two different time points. **Panel a** is the radio-HPLC-QC trace obtained right after the purification of the iodinated product (LS734). The sample was stored in PBS for up to 48 h. **Panel b** represents the radio-HPLC-QC trace obtained at 48 h.