Synthesis and evaluation of a ^{99m}Tc tricarbonyl-labeled somatostatin receptor-targeting antagonist peptide for imaging of neuroendocrine tumors

Abbreviated title: ^{99m}Tc antagonist peptide for imaging NETs

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Abstract

Introduction: A somatostatin receptor (SSTR)-targeting antagonist peptide (sst₂-ANT) was radiolabeled with ^{99m}Tc tricarbonyl via a tridentate [N,S,N]-type ligand (L) to develop a radiodiagnostic agent, ^{99m}TcL-sst₂-ANT, for imaging of SSTR-expressing neuroendocrine tumors.

Methods: Receptor affinity was assessed *in vitro* with the nonradioactive analogue, ReL-sst₂-ANT, via a challenge experiment in AR42J cells with ¹²⁵I-SS-14 as the competing radioligand. Preparation of ^{99m}TcL-sst₂-ANT was achieved via reaction of [^{99m}Tc(CO)₃(H₂O)₃]⁺ with L-sst₂-ANT. To test the stability of the radiolabeled complex, challenge experiments were performed in phosphate-buffered saline solutions containing cysteine or histidine and also in mouse serum. Biodistribution and micro-SPECT/CT imaging studies were performed in AR42J tumor-bearing female ICR SCID mice.

Results: The half maximal inhibitory concentration (IC₅₀ value) of ReL-sst₂-ANT in AR42J cells was 15 nM. Preparation of ^{99m}TcL-sst₂-ANT was achieved with ≥97% radiochemical yield (RCY) and was verified by HPLC co-elution with the ReL-sst₂-ANT analogue. The radiolabeled complex remained intact for up to 24 h in high concentration solutions of cysteine and histidine at 37 °C. Furthermore, the radiotracer was 90% stable for 1 h at 37 °C in mouse serum. Micro-SPECT/CT images showed clear uptake in tumors and were supported by the biodistribution data, in which the 3.2% ID/g tumor uptake at 4 h was significantly blocked by co-administration of nonradioactive SS-14.

Conclusions: A [^{99m}Tc(CO)₃(N,S,N)]⁺ chelate was employed for radiolabeling of a SSTR-targeting antagonist peptide. Synthesis of ^{99m}TcL-sst₂-ANT was achieved in high RCY,

and the resulting complex displayed high *in vitro* stability. Somatostatin receptor affinity was retained in both cells and in tumor-bearing mice, where the complex successfully targeted SSTR-positive tumors via a receptor-mediated process.

Advances in Knowledge and Implications for Patient Care: This first ^{99m}Tc -tricarbonyl-labeled SSTR antagonist peptide showed promising *in vivo* tumor targeting in mice.

Future studies may lead to translation of a similar design into the clinic.

Introduction

Imaging and treatment of neuroendocrine tumors (NETs) through targeting of somatostatin receptors (SSTRs) is well established; the most important SSTR target being subtype two (SSTR2) due to its prevalence in the majority of NETs.[1–3] Physiological imaging of NETs is crucial, not only for profiling metastases, but also in providing valuable insight on treatment plan selection and efficacy determinations.[1,4] Currently used diagnostics for NETs include the FDA-approved agents ^{111}In -DTPA-octreotide (OctreoScan™) and ^{68}Ga -DOTA-Tyr³-octreotate (NETSPOT™) and the European agent $^{99\text{m}}\text{Tc}$ -EDDA/HYNIC-Tyr³-octreotide ($^{99\text{m}}\text{Tc}$ -Tektrotyd), all of which utilize cyclic octapeptide agonists for SSTR2 targeting. The use of agonist peptides to cause internalization (and thus accumulation) of radioactivity in tumor cells was historically deemed necessary for high target to non-target ratios. Recently, however, the development and study of antagonist peptides as practical targeting vectors has increased. In general, they have shown potential to bind to more receptor conformations and have longer tumor retention than some of their agonist counterparts, while retaining nanomolar receptor affinities,[5–7] making them worthy of further study as targeting vectors. For example, the antagonist peptide In-DOTA-[4-NO₂-Phe-c(DCys-Tyr-DTrp-Lys-Thr-Cys)-DTyr-NH₂] (In-DOTA-sst₂-ANT) exhibited high *in vitro* binding affinity for SSTR2, and its radiotracer analogue ^{111}In -DOTA-sst₂-ANT demonstrated increased tumor retention with better tumor to non-tumor ratios than the agonist ^{111}In -DTPA-TATE in HEK-sst₂ tumor-bearing mice.[8] Additionally, ^{111}In -DOTA-sst₂-ANT detected a higher percentage of lesions and demonstrated higher tumor-to-kidney ratios in NET patients with

progressive disease in a small clinical imaging trial compared directly against OctreoScan™.[9]

Results such as these are promising, however it remains that none of the radiolabeled antagonist peptides that target NETs use ^{99m}Tc , a much more widely available radionuclide. The FDA-approved $^{99m}\text{Tc}/^{99}\text{Mo}$ generator is delivered worldwide, and ^{99m}Tc scans account for approximately 70% of all nuclear imaging diagnostics.[10] In addition to its availability, the favorable decay properties of ^{99m}Tc (6 h $t_{1/2}$, 140 keV γ) continue to make it a popular choice for SPECT imaging. These combined facts support the need for additional ^{99m}Tc radiopharmaceuticals, in order to make diagnostic imaging capabilities such as disease staging and treatment efficacy monitoring available to a larger percentage of the population. The goal of this study, therefore, was to develop the first ^{99m}Tc -labeled antagonist peptide for imaging NETs.

We recently reported an [N,S,N]-type tridentate ligand, 3-(2-aminoethylthio)-3-(1*H*-imidazol-4-yl)propanoic acid (L), which coordinates *fac*- $[\text{}^{99m}\text{Tc}/\text{Re}(\text{CO})_3]^+$ via an imidazole nitrogen, a thioether sulfur, and an amine nitrogen to form stable complexes in high yield (Figure 1A).[11] In that study, a small bioconjugate model of the ligand (L-pyr) and its $^{99m}\text{Tc}/\text{Re}(\text{CO})_3$ complexes were also reported (Figure 1B). The model ligand used the same bifunctional chelator, L, but mimicked conjugation of a peptide through amide bond formation with pyrrolidine. Upon reaction with $[\text{Re}(\text{CO})_3(\text{H}_2\text{O})_3]^+$, L-pyr chelated via the predicted [N,S,N] mode and formed isomeric products. Radiotracer level stability challenge experiments with the analogous ^{99m}Tc L-pyr complexes demonstrated the effectiveness of the [N,S,N] chelator; the complexes were stable for up to 24 h in

competing ligand solutions. These studies prompted the production of L-sst₂-ANT, which was successfully used to synthesize ReL-sst₂-ANT (Figure 2).

In this work, the Re-labeled antagonist peptide was assessed for SSTR affinity in an *in vitro* cell binding assay. Synthetic conditions were translated to the ^{99m}Tc radiotracer scale, and the resulting ^{99m}TcL-sst₂-ANT products were evaluated *in vitro* for stability and *in vivo* for potential use as a SPECT radiopharmaceutical for NET imaging.

Methods and Materials

General

All chemicals were reagent grade from Sigma-Aldrich, unless otherwise specified, and used as received. Somatostatin-14 [SS-14; H-Ala-Gly-c(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys)-OH] was purchased from Bachem (Torrance, CA), and [¹²⁵I]Tyr¹¹-SS-14 was purchased from Perkin Elmer (Waltham, MA).

Peptide synthesis was performed on an Apex 396 solid-phase peptide synthesizer from AAPPTEC (Louisville, KY). Semi-preparative high-performance liquid chromatography (HPLC) purification was performed with a Waters Nova-Pak C18 preparative column (300 mm × 19 mm × 6 μm) attached to a Beckmann Coulter Gold HPLC system equipped with a 168 diode array detector, a 507e auto-injector, and the 32 KARAT software package (Beckmann Coulter, Fullerton, CA). Solvent A was 0.1% trifluoroacetic acid (TFA) in water, and solvent B was 0.1% TFA in acetonitrile (ACN). The semi-preparative purification method was a linear gradient of 10% to 35% B in A over 50 min at a 10 mL/min flow rate. This HPLC system was coupled to an ion trap mass analyzer (LCQ FLEET instrument with positive ion ionization, Thermo Fisher

Scientific, Waltham, MA) in order to perform liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analyses. LC-ESI-MS data were analyzed using Thermo Fisher Scientific XCalibur software.

HPLC analyses and analytical purifications were performed on a Shimadzu Prominence HPLC system with a SPD-20AV multiple wavelength detector connected in-line to a NaI(Tl) detector. A Thermo Scientific BetaBasic reversed-phase C18 column (150 mm $\times$ 4.6 mm $\times$ 5 μ m) was used. The flow rate was 1 mL/min. Linear gradient method 1 was 25% to 45% solvent B in A over 30 min, and linear gradient method 2 was 28% to 35% solvent B in A over 20 min.

Baker-flex plastic-backed silica 60 thin layer chromatography (TLC) strips were developed with 0.5% HCl (conc.) in MeOH. The strips were analyzed with a BioScan 200 Imaging Scanner (Poway, California). Inductively coupled plasma mass spectrometry (ICP-MS) analyses were performed on a VG Axiom SC high-resolution ICP-MS (Thermo Fisher, Waltham, MA).

Synthesis of ReL-sst₂-ANT

The ReL-sst₂-ANT complex was synthesized by refluxing the *fac*-[Re(CO)₃(H₂O)₃]⁺ precursor with the L-sst₂-ANT bioconjugate as previously described, [11] and the three isomeric products were collectively isolated by HPLC (method 1) and used as such for the studies reported herein. Purity and identity were confirmed at the time of study by LC-ESI-MS characterization: (*m/z*) 1622.4 calc. for [M⁺], 1622.1 found and 811.7 calc. for [M+H]²⁺, 811.9 found.

Concentration determination of L-sst₂-ANT and ReL-sst₂-ANT

Purified, lyophilized ReL-sst₂-ANT was dissolved in 10% methanol in water at an approximate concentration of 1 mg/mL. A solution of L-sst₂-ANT in 20% methanol in water was also made at a similar target concentration. ICP-MS analyses were employed to determine actual solution concentrations. After dispensing duplicate aliquots (13-15 μ L) into pre-cleaned/pre-weighed tubes, the tubes were reweighed and the solutions were digested with 150 μ L of concentrated HNO₃ for 1 h at 100°C. The digestate volume was brought to ~5 mL with ultrapure water and then weighed again, after which aliquots were removed for further dilution and fortification with Be/Sc/Y/Tl internal standards of known concentration (undiluted digestate was also analyzed). Internal standards were likewise added to a series of linearity standards prepared from High Purity Standards MS-D multi-element stock solutions, which contained Re and S at ppb levels. Mean results are reported, as measured by signals from ³²S for S and both ¹⁸⁵Re and ¹⁸⁷Re for Re. Solvent and digestion blanks were analyzed in similar fashion.

In Vitro Receptor Binding (IC₅₀) Studies

The half maximal inhibitory concentration (IC₅₀ value) of ReL-sst₂-ANT for SSTRs on AR42J rat pancreatic cancer cell line was determined by competition with the radioligand standard [¹²⁵I]Tyr¹¹-SS-14, following a modified procedure.[12] The AR42J cells were cultured by the Cell and Immunobiology Core at the University of Missouri (Columbia, MO) in RPMI 1640 medium (GIBCO-Invitrogen, Carlsbad, CA) modified with 4.5 g/L glucose supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μ g/mL gentamycin and were incubated at 37 °C in a 5% CO₂ atmosphere. The cells were harvested prior to use with TrypLE Express (GIBCO-Invitrogen, Carlsbad, CA),

and the trypsin was quenched with 1 mg of soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO).

The harvested cells were washed twice with, and then resuspended in, RPMI 1640 with 0.25% BSA as binding buffer, which was used for the remainder of the experiments. Serial dilutions (10× dilution factor) of ReL-sst₂-ANT in buffer were performed, with three 100-μL replicates prepared for each concentration. An additional three 100-μL replicates containing buffer only (in lieu of ReL-sst₂-ANT) were prepared as zero concentration standards. To all replicates, 100 μL of [¹²⁵I]Tyr¹¹-SS-14 in buffer (100,000 cpm per tube) and 100 μL of AR42J cells (2×10⁶ cells per tube) were added. Final ReL-sst₂-ANT concentrations ranged from 1×10⁻⁵ M to 1×10⁻¹² M, plus 0 M. The tubes were incubated for 1 h in a laminar flow hood at room temperature, centrifuged at 6000 rpm for 30 s, and the supernatant from each was then removed. The cells were subsequently handled at approximately 4 °C using cold tube racks and washed three times with refrigerated buffer to remove unbound [¹²⁵I]Tyr¹¹-SS-14. As the final step, the supernatant was removed and the pellets were counted on a Wallac 1480 Wizard 3” automated gamma counter (PerkinElmer Life Sciences, Gaithersburg, MD, USA). Data analysis was performed using Graph Pad Prism® 6 software (La Jolla, CA).

Preparation of ^{99m}TcL-sst₂-ANT

The ^{99m}Tc(I) tricarbonyl precursor, [^{99m}Tc(CO)₃(H₂O)₃]⁺, was prepared according to the literature[13] by adding 0.8 mL of ^{99m}TcO₄⁻ (370-740 MBq, 10-20 mCi) in saline, from a ⁹⁹Mo/^{99m}Tc generator, to an IsoLink kit (Mallinckrodt, St. Louis, MO; currently available through the Paul Scherrer Institute). Subsequent heating at 100°C for

approximately 25 min resulted a precursor solution with >98% radiochemical yield (RCY), which was then pH adjusted to 5-6 using 1 M HCl. The formation of $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ was verified by HPLC ($t_R = 5.1$ min; method 2). In this method, $^{99m}\text{TcO}_4^-$ has a $t_R = 2.9$ min.

To 50 μL of HPLC-purified L-sst₂-ANT (0.5 mM in an aqueous solution of 20% MeOH), 450 μL of freshly prepared $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ (259-444 MBq, 7-12 mCi) was added. The solution was heated at 75 °C for 30 min to give $^{99m}\text{TcL-sst}_2\text{-ANT}$ with a RCY of 90-100%. The isomer products were collectively isolated via analytical HPLC (method 2), and their identities were subsequently verified via HPLC co-injection with ReL-sst₂-ANT. Prior to further use, the purified $^{99m}\text{TcL-sst}_2\text{-ANT}$ solution was concentrated. The product-containing HPLC fraction was diluted to approximately 20 mL with ultrapure water (18.2 M Ω ·cm, Thermo Barnstead) and then loaded onto a Waters Sep-Pak C18 Light cartridge (preconditioned with 10 mL of absolute EtOH followed by 10 mL of water). Following a 10 mL H₂O wash, the cartridge was eluted with 400 μL of absolute EtOH (Decon Laboratories, Inc., King of Prussia, PA). An alternative to the Sep-Pak process was pH neutralization of the HPLC fraction by addition of 100 μL of a 1 M NaHCO₃ solution. In either case, 90 μL of 1% Tween 80 in 10 mM phosphate-buffered saline (PBS) was added to the $^{99m}\text{TcL-sst}_2\text{-ANT}$ solution, and the total volume was then reduced to 90 μL using argon gas flow and gentle heat from a water bath (~55 °C).

In vitro $^{99m}\text{TcL-sst}_2\text{-ANT}$ stability studies

Purified, concentrated $^{99m}\text{TcL-sst}_2\text{-ANT}$ was diluted 10-fold with cysteine, histidine, or mouse serum (Sigma Life Science, St. Louis, MO). The final concentrations

of histidine and cysteine were 1 mM in 10 mM PBS, and the final concentration of Tween 80 was 0.1%. The resulting solutions were incubated at 37 °C, and aliquots were removed at various time points (1 h, 4 h, and either 12 or 24 h) to be assessed for stability via HPLC. Histidine and cysteine solutions were directly injected into the HPLC for analysis. In the case of mouse serum, an aliquot of the incubation solution was removed, diluted three-fold with ACN to precipitate serum proteins, vortexed thoroughly and then centrifuged at 2500 rpm for 5 min. The supernatant was removed and the pellet was washed once more with ACN in the same manner. The activities of the combined supernatants and the pellet were each counted on a dose calibrator (Capintec; Ramsey, NJ). A portion of the supernatant was then injected for HPLC monitoring using method 2.

Biodistribution and Imaging Studies

All animal studies were conducted in compliance with a protocol approved by the Subcommittee for Animal Studies of the Harry S. Truman Memorial Veterans' Hospital and the Animal Care and Use Committee of the University of Missouri-Columbia Animal Care Quality Assurance Office. Female ICR SCID mice (Taconic; Hudson, NY), age 6 weeks, were inoculated with 5×10^6 AR42J cells via subcutaneous injection in the right hind flank, and studies were conducted 3.5 weeks after inoculation. Injections were prepared by purifying the $^{99m}\text{TcL-sst}_2\text{-ANT}$ via HPLC (method 2) and concentrating by Sep-Pak, as described above, with the exception that both 1% Tween 80 in saline and 1-2 mg of the radioprotectant gentisic acid (GA) were added to the EtOH elution vial. Following removal of the EtOH as above, the solution was further diluted with 0.1% Tween 80 in sterile saline and 1 M NaOH was used to adjust its pH into a biocompatible

range (here pH 5-6). The tumor-bearing mice were injected via the tail vein, with final radioactivity concentrations as follows: (1) biodistribution, regular dose, 0.37 MBq (10 μ Ci) in 100 μ L; (2) biodistribution, blocking dose, 0.37 MBq (10 μ Ci) in 150 μ L containing 150 μ g SS-14; (3) imaging, regular dose, 5.6 MBq (150 μ Ci) in 100 μ L; (4) imaging, blocking dose, 5.6 MBq (150 μ Ci) in 150 μ L containing 150 μ g SS-14. For biodistribution studies, mice were anesthetized and humanely euthanized by cervical dislocation at selected time points (1 h, 4 h, 4 h block, and 24 h), and their organs and tissues were collected and counted on a Wallac 1480 Wizard 3" gamma counter from Perkin Elmer.

Micro-SPECT/CT imaging studies were performed on live mice at 1 and 4 h for both the regular and blocking doses. The mice were anesthetized using 3% isoflurane at induction and 2.5% isoflurane during imaging and were kept warm throughout the study with a heated water pad located on the imaging platform. Imaging data were collected using a SIEMENS Inveon Micro-SPECT/CT scanner (Siemens; Knoxville, TN). Spiral SPECT imaging was performed for 40 min using MWB 1.0 mm collimators with a 35 mm radius of rotation and 120 projections. The images were reconstructed using OSEM3D. The CT images were captured over 6 min using an 80 kVp x-ray source and 360° data acquisition for 3D volume rendered data. The images were reconstructed in real-time using Fan beam (Feldkamp) filtered back projection algorithms and were processed using Inveon Research Workplace processing software. Upon completion of the study, the anesthetized mice were humanely euthanized by cervical dislocation.

Results and Discussion

The sst₂-ANT antagonist peptide was selected due to its high affinity for the subtype 2 SSTR,[8] which is an excellent target in neuroendocrine cancer research due to its expression in the majority of NETs.[14,15] This targeting peptide has also been shown to maintain low nanomolar affinity for SSTR2 despite *N*-terminal modifications, such as the conjugation of bifunctional chelating agents.[6,8] We previously reported the [N,S,N]-type tridentate bifunctional chelator, L and its pyrrolidine-derivitized model conjugate, L-pyr, each of which produced exceptionally stable complexes in high yield with Re and ^{99m}Tc tricarbonyl cores.[11] The ligand-peptide bioconjugate, L-sst₂-ANT, was also prepared and labeled with nonradioactive Re(CO)₃⁺, as a labeling proof-of-principal study.

Here, we successfully translated these synthetic conditions to the labeling of L-sst₂-ANT at the radiotracer level with [^{99m}Tc(CO)₃]⁺. Using a 10⁻⁴ M ligand concentration and a 75 °C reaction temperature yielded the ^{99m}TcL-sst₂-ANT complex with ≥97% RCY. Two isomer products of ^{99m}TcL-sst₂-ANT were observed by HPLC (Figure 3; method 1, *t_R* = 18.2 min, 18.9 min). As expected, chelation of the small organometallic core by L-sst₂-ANT resulted in an increased lipophilicity of the complex, as noted by the large increase in retention time from L-sst₂-ANT to ^{99m}TcL-sst₂-ANT (method 2, *t_R* = 7 min to ~12 min, respectively). Such retention time shifts for peptides after being labeled with [^{99m}Tc(CO)₃]⁺ are common.[16,17]

Co-elution with the spectroscopically-characterized ReL-sst₂-ANT (method 1, *t_R* = 17.7 min, 18.3 min, 18.7 min) upon HPLC co-injection verified formation of the analogous monocationic ^{99m}TcL-sst₂-ANT isomers, though only two of the expected three

were observed. As described in our previous work,[11] the Re complexation yielded three isomers of the same molecular mass, that formed due to: 1) the chiral carbon and prochiral sulfur of the chelate and 2) the peptide's additional chirality. The same isomeric products with ^{99m}Tc are therefore possible, and were expected based on the L-pyr labeling results, in which the Re/ ^{99m}Tc -labeling reactions each yielded two co-eluting peaks. The elution profiles of the ^{99m}Tc - and Re-labeled L-sst₂-ANT products were very similar, with a ratio of 0.15:1 for the first isomer to the second for the ^{99m}Tc products and 0.22:1 for the Re products (when combining the second and third peaks). Thus, it is likely that the third ^{99m}Tc isomer was indeed formed, perhaps at a lower relative percentage, and was simply unresolved by HPLC. Alternatively, it is possible that formation of the third isomer was not favored at the ^{99m}Tc tracer level. No interconversion between the product isomers was observed in our studies.

In addition to using ReL-sst₂-ANT to identify the ^{99m}Tc L-sst₂-ANT products, it was used as the nonradioactive analogue to assess receptor affinity of the radiotracer complex. All studies evaluating the Re/ ^{99m}Tc L-sst₂-ANT complexes were performed on the collectively purified isomers, due to the close proximity of their retention times and therefore incomplete resolution by HPLC. The IC₅₀ value (15 ± 4 nM, Figure 4) was determined through a competitive *in vitro* binding assay using SSTR2-expressing AR42J cells and ^{125}I -SS-14 as the competing radioligand. This low nanomolar value demonstrated that receptor affinity for SSTR2 was retained after addition of the ReL complex to sst₂-ANT, and it compared favorably with the measured affinity for SS-14,

the native SSTR ligand, which was found to have an IC_{50} value of 8 ± 4 nM by the same experimental methods.

For *in vitro* stability studies, the HPLC fractions containing purified ^{99m}TcL -sst₂-ANT were initially neutralized using $NaHCO_3$ and concentrated with a gentle stream of argon prior to 10-fold dilution in solutions of cysteine, histidine or in serum. Due to the relatively short half-life of ^{99m}Tc , a rapid method for concentration and acid removal was developed to facilitate the *in vivo* evaluations of the new radiotracer complex. The Sep-Pak cartridge method simultaneously removed the TFA and concentrated the complex into ethanol, which was then quickly evaporated under argon with gentle heating. Comparison stability replicates confirmed that the method of concentration neither affected product purity nor its stability determinations.

Excellent stability of the ^{99m}TcL -sst₂-ANT isomers was observed in cysteine (Table 1), with no decomposition found through 12 h of incubation at 37 °C in this challenge solution. In histidine, high stability was also observed at both 1 and 4 h time points; however, only 30% of the complex remained intact at 24 h. Hypothesizing that this decomposition was caused by radiolysis at the relatively high radioactivity concentration of 37 kBq/ μ L (versus 2.6-11 kBq/ μ L used in the ^{99m}TcL -pyr studies[11]), the experiment was repeated with the addition of the radioprotectant gentisic acid (GA). Though ^{99m}Tc has relatively low decay energetics, we have observed radiolysis in other peptide-based complexes labeled with ^{99m}Tc . [18] The described stability procedure was modified by the addition of GA to the incubation vial and pH readjustment to 7.4 using a 1 M $NaHCO_3$ solution. The histidine/GA results demonstrated a marked improvement in

stability, with $^{99m}\text{TcL-sst}_2\text{-ANT}$ remaining highly intact ($\geq 99\%$) through 24 hours, supporting the radiolysis hypothesis. Lending further support, cysteine is a known radioprotectant[19], which is consistent with the excellent stability observed in the cysteine studies despite the absence of GA.

Stability studies in mouse serum showed that 29% of the radioactivity in the 1 h aliquot remained with the serum proteins when precipitated by acetonitrile. The radioactivity left in the supernatant revealed minimal decomposition of $^{99m}\text{TcL-sst}_2\text{-ANT}$ at 1 h (Table 1). By 4 h, the pellet-associated radioactivity had increased to 55%, a behavior also exhibited by other ^{99m}Tc -labeled somatostatin peptide analogues[20], and just 6% of the complex remained intact in the supernatant; the balance of the supernatant radioactivity was found as hydrophilic metabolites and/or the oxidized metal form of $^{99m}\text{TcO}_4^-$. Given that peptides have fast pharmacokinetics and can reach tumors early after administration, we concluded that the $^{99m}\text{TcL-sst}_2\text{-ANT}$ radiotracer was stable enough for further *in vivo* investigations.

Biodistribution studies were performed in female ICR SCID mice at time points of 1, 4 and 24 h following intravenous injection of $^{99m}\text{TcL-sst}_2\text{-ANT}$ (Table 2). A blocking study was also carried out at 4 h, in which SS-14 was co-administered to saturate tumor receptors. Tumor targeting by $^{99m}\text{TcL-sst}_2\text{-ANT}$ was successful, with uptake increasing from 2.3% ID/g at 1 h to 3.2% ID/g at 4 h, and over a third of the maximal uptake retained through 24 h. Tumor uptake was reduced by nearly 50% in the 4 h blocking group, supporting receptor-mediated targeting. The formation of $^{99m}\text{TcO}_4^-$, which is readily taken up by the stomach, was not supported by the data, as this organ

had low uptake at 1 h that did not increase at 4 h. Elimination of $^{99m}\text{TcL-sst}_2\text{-ANT}$ was slow and occurred primarily through the hepatobiliary route, presumably due to the lipophilic character of the complex. High kidney and liver uptake occurred early and persisted for the duration of the experiment. This is a common finding in kidneys for SSTR-targeting peptides,[7,21] likely augmented for $^{99m}\text{TcL-sst}_2\text{-ANT}$ by the positive charge of the metal-ligand complex. While consistent with the high protein binding observed in the *in vitro* stability studies, the blood and liver profile is markedly different from other radiolabeled antagonist peptides, where rapid clearance was observed in female nude mice bearing either rat (HEK-rsst2) or human (HEK-hsst2) pancreatic tumors[6–8]. Differences in the studies themselves as well as the chelate, lipophilicities and overall charges of the complexes make direct comparisons difficult, but these factors can certainly lead to large pharmacokinetic differences.

Micro-SPECT/CT imaging was performed on live female ICR SCID mice at 1 and 4 h post-injection of $^{99m}\text{TcL-sst}_2\text{-ANT}$, with and without the use of SS-14 as a SSTR blocking agent. The SPECT/CT imaging data supported the biodistribution study findings. The unblocked tumor was visible at both 1 and 4 h, with increased intensity at 4 h (Figure 5A), while the blocked tumor (Figure 5B) showed a dramatic decrease in intensity at both time points. An appreciable amount of radioactivity was apparent in the gastrointestinal tract, consistent with the biodistribution data. This high abdominal uptake could impede selective imaging of gastroenteropancreatic NETs, which often form in the gut and metastasize to the liver[22]. Although $^{99m}\text{TcL-sst}_2\text{-ANT}$ was successful at detecting the tumors in the images, structural modifications to reduce the overall

lipophilic character are needed to enhance the imaging capabilities of this radiotracer. Such alterations would be expected to reduce liver uptake and speed clearance from non-targeted tissues, thus allowing for higher target to non-target ratios with improved imaging sensitivity.

Conclusions

A new ^{99m}Tc -labeled SSSTR-targeting peptide was prepared and evaluated for potential use as a radiodiagnostic agent for NETs. The monocationic ^{99m}Tc tricarbonyl core was coordinated by a tridentate [N,S,N]-type ligand that was conjugated to a SSSTR2-targeting antagonist peptide. The nonradioactive ReL-sst₂-ANT complex was used to demonstrate both analogous labeling in the ^{99m}Tc product and low nanomolar receptor affinity. Exceptional labeling yields were achieved for ^{99m}TcL -sst₂-ANT, and *in vitro* stability studies indicated effective chelation of the ^{99m}Tc tricarbonyl core by the L-sst₂-ANT bioconjugate. Additionally, ^{99m}TcL -sst₂-ANT successfully targeted SSSTR2-positive tumors *in vivo* by a receptor-mediated process. The slow and predominantly hepatobiliary clearance of the complex, attributed to its lipophilic nature, diminished the imaging potential of ^{99m}TcL -sst₂-ANT. However, enhancements in imaging capabilities may be realized through ongoing structural modification efforts designed to increase overall hydrophilic character and improve the pharmacokinetic profile.

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Figure Captions

Figure 1: Previously synthesized complexes using the [N,S,N] tridentate chelator L (A) and its small bioconjugate model L-pyr (B); M = ^{99m}Tc , Re.[11]

Figure 2: Proposed structure of labeled SSTR antagonist peptide ML-sst₂-ANT; M = ^{99m}Tc , Re.

Figure 3: HPLC chromatograms of ReL-sst₂-ANT (black, UV at 280 nm) and ^{99m}Tc L-sst₂-ANT (dashed gray, NaI(Tl) detector) using linear gradient method 1.

Figure 4: IC₅₀ curve for ReL-sst₂-ANT obtained from *in vitro* SSTR binding competition against the ^{125}I -Tyr¹¹-SS-14 radioligand in AR42J cells.

Figure 5: Micro-SPECT/CT images of live female ICR SCID mice bearing AR42J tumors at 4 h post injection of 5.6 MBq (150 μCi) ^{99m}Tc L-sst₂-ANT alone (A) or co-administered with a 150 μg blocking dose of SS-14 (B). Mice were anaesthetized using isoflurane and imaged for a total of 46 min.

Table 1. Stability of $^{99m}\text{TcL-sst}_2\text{-ANT}$ in various challenge solutions.^a

	1 h	4 h	24 h
Cysteine	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0 [*]
Histidine	99 $\pm$ 1	95 $\pm$ 2	30 $\pm$ 2
Histidine/GA ^b	100	99	99
Mouse Serum ^c	90 $\pm$ 13	6 $\pm$ 4	NP ^{**}

^a Mean percentage values $\pm$ SD are listed relative to intact complex at 0 h; n=3

^b n=1

^c n=2

^{*} Carried out to 12 h

^{**} Not performed

Table 2. Biodistribution of ^{99m}Tc L-sst₂-ANT in AR42J tumor-bearing mice.^a

Organ	1 h	4 h	4 h blocking ^b	24 h
Blood	3.5 ± 0.6	2 ± 1	2 ± 1	0.5 ± 0.1
Lung	10 ± 7	5 ± 2	3.8 ± 0.5	2 ± 1
Liver	60 ± 13	79 ± 8	63 ± 5	27 ± 7
Spleen	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	0.6 ± 0.1
Stomach	1.1 ± 0.4	1.3 ± 0.3	1.3 ± 0.8	3 ± 1
L Int	1.0 ± 0.1	9 ± 9	0.6 ± 0.1 ^c	8 ± 3
Sm Int	8 ± 3	12 ± 4	11 ± 3	2.6 ± 0.4
Kidney	55 ± 16	56 ± 10	53 ± 9	10 ± 2
Muscle	0.22 ± 0.02	0.15 ± 0.05	0.30 ± 0.08	0.07 ± 0.02
Adrenals	1.5 ± 0.5	1.8 ± 0.7	2 ± 1	0.8 ± 0.1
Fat	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.10 ± 0.03
Brain	0.12 ± 0.02	0.10 ± 0.04	0.08 ± 0.01	0.03 ± 0.01
Pancreas	1.9 ± 0.4	1.5 ± 0.7	1.2 ± 0.3	0.26 ± 0.02
Tumor	2.3 ± 0.7	3.2 ± 0.6	1.7 ± 0.8	1.2 ± 0.2

^a Mean %ID/g values are reported ± SD; n=5

^b Co-administration of a 150 µg SS-14 blocking dose

^c n=4, Q-test was used to remove outlier with 90% probability

A

B