1. Synthesis

1.1 General Information: Reagents for *in vitro* assays were purchased from the following sources: DMEM media (Lonza); Fetal Bovine Serum (FBS) (Atlanta Biologicals); NKH477 (Cayman Chemical Company); the GloSensor 22F plasmid (Promega); D-Luciferin (GoldBio); Anti-HA antibody (mouse monoclonal antibody HA.11 (from ascites)) (Covance); Goat anti-mouse secondary antibody (KPL); G418 (Sigma); 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) (Roche); CO2-independent media (GIBCO); BSA, Fraction V (Sigma); FuGENE 6 (Promega); Hygromycin B (Invitrogen).

1.2 Synthesis of (di-*tert*-butyl 2, 2'-(4-(2-oxo-2-phenoxyethyl)-1, 4, 7, 10-tetraazacyclododecane-1, 7-diyl) diacetate (2): Benzyl bromoacetate (367 mg, 1.6 mmol) was dissolved in 30 mL CH_2Cl_2 (anhydrous) and added dropwise into a mixture of **1** (800 mg, 2 mmol) and anhydrous K_2CO_3 (417 mg, 1.5 equiv. of **1**) in CH_2Cl_2 (25 mL). The reaction mixture was stirred for 4 h at r.t., then filtered, evaporated, and purified by silica gel chromatography (2% methanol in CH_2Cl_2, v/v) to give compound **2** (820 mg) with 75% yield. The compound was identified by NMR and mass spectrometry. ^{1}H NMR ($CDCl_3$, 300 MHz), δ (ppm): 9.96(1H, broad-NH), 7.39-7.28(m, 5H), 5.15(s, 2H) 3.50(s, 2H), 3.46(s, 4H), 3.07-2.85(m, 16H), 1.45(s, 18H). ^{13}C NMR ($CDCl_3$, 300 MHz), δ (ppm): 170.53,128.70,128.63,128.51,127.59,127.02,81.81 ,58.39,51.69,49.35,31.21,28.18. ESI-MS (*m/z*): $[M+H]^+$ calculated for $C_{29}H_{48}N_4O_6$, 548.71; found, 549.3.

1.3 Synthesis of (2-(4, 10-bis (2-(*tert*-butoxy)-2-oxoethyl)-1, 4,7,10 tetraazacyclododecan-1-yl) acetic acid (3): To a stirred solution of **2** (800 mg, 1.45 mmol) in absolute ethanol (20 mL),

10% Pd/C was added followed by 1, 4-cyclohexadiene (4 mL) using published methods (*1*). The suspension was stirred at 50°C for 5 h. The catalyst was removed by filtration through celite and the filtrate was evaporated to dryness under vacuum. The solid mass was washed several times with ether to remove impurities and give the product **3** with 89% yield (594 mg) as a pale yellow solid. The compound was identified by NMR and mass spectrometry. ^{1}H NMR (MeOD, 300 MHz), δ (ppm): 3.63(s, 2H), 3.48(s, 4H), 3.31-3.02(m, 16H), 1.47(s, 18 H). ^{13}C NMR ($CDCl_3$, 300 MHz), δ (ppm): 170.44, 83.40, 57.90, 55.44, 53.37, 50.45, 44.16, 28.54. ESI-MS (*m/z*): $[M+H]^+$ calculated for $C_{22}H_{42}N_4O_6$, 458.59; found, 459.1.

1.4 Synthesis of (2-(7-(5-azidopentanoyl)-4, 10-bis (2-(*tert*-butoxy)-2-oxoethyl)-1, 4, 7, 10-tertaazacyclododecan-1-yl) acetic acid (4): 5-azido pentanoic acid (300 mg, 2.09 mmol), 400 mg (2.09 mmol) of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 280 mg (2.09 mmol) of 1-Hydroxybenzotriazole (HOBt) were combined in 30 mL CH_2Cl_2 solution and stirred for 4 h at room temperature. The solution of compound **3** (600 mg, 1.3 mmol) in CH_2Cl_2 (10 mL) was then added to the reaction mixture followed by addition of 600 µL of *N, N*-diisopropylethyl amine. The reaction mixture was stirred overnight at r.t. and the solvent was removed under vacuum. The crude product was purified by silica gel chromatography (2% methanol in CH_2Cl_2, v/v) to give **4** (500 mg, 65%) as a pale yellow oil. The compound was identified by NMR and mass spectrometry. ^{1}H NMR ($CDCl_3$, 300 MHz), δ (ppm): 3.58(s, 2H), 3.53(s, 4H), 3.40-3.05(m, 16H), 2.36-2.328(m, 2H), 1.71-1.65(m, 2H), 1.61-1.57(m, 2H), 1.46-1.41(m, 20H). ^{13}C NMR ($CDCl_3$, 300 MHz), δ (ppm): 170.35, 83.33, 57.21, 55.41, 54.08, 51.24, 46.85, 42.30, 2.69, 28.40, 28.02, 22.30. ESI-MS (*m/z*): $[M+H]^+$ calculated for $C_{27}H_{49}N_7O_7$, 583.72; found, 584.1.

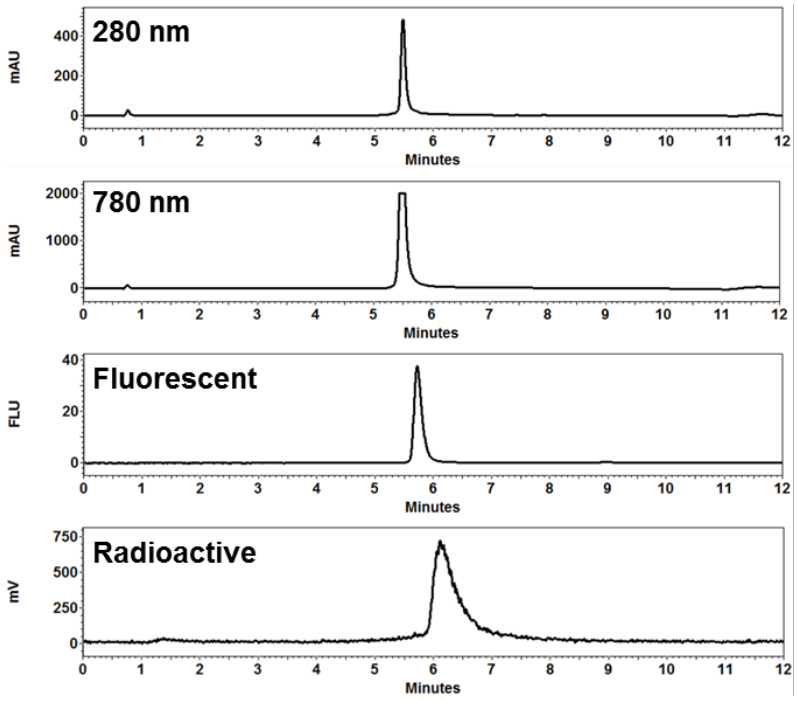
1.5 Synthesis of (2-(7-(5-azidopentanoyl)-4, 10-bis (2-(*tert*-butoxy)-2-oxoethyl)-1, 4, 7, 10-tertaazacyclododecan-1-yl) (5) (MMC-TOC): TOC was synthesized by Bachem (Torrance, CA) using standard Fmoc/tBu chemistry. Briefly, **4** was conjugated to the N-terminus of resin-bound TOC *via in-situ* aminium-based activation. Protecting groups were removed from the MMC and the side chains of the peptide in a single step with TFA, and the thiol groups were oxidized to form **5** (referred to as MMC-TOC). MMC-TOC was subsequently purified by reversed-phase high-performance liquid chromatography (RP-HPLC) and identified by mass spectrometry. ESI-MS (*m/z*): $[M-H]^-$ calculated for $C_{68}H_{97}N_{17}O_{17}S_2$, 1488.73; found, 1487.50.

1.6 Synthesis of MMC(IR800)-TOC: The fluorescently labeled dual conjugate**, 6** (Scheme 1, herein referred to as MMC(IR800)-TOC), was synthesized using DO2A (Fig. 1) and obtained by conjugating IRDye 800-DBCO to MMC-TOC (synthesis methods described in Supplemental Data) *via* a copper-free strain-promoted alkyne azide cycloaddition reaction. In brief, an aqueous solution of MMC-TOC (1.8 mg, 1.2 mmol) was added to IRDye 800-DBCO (1.6 mg, 1.2 mmol) in a mixture of water and DMSO (3:1). After stirring at 37°C for 3 h, and then overnight at room temperature in the dark, the dye-conjugated product was purified with a Vivaspin ultrafiltration spin column (2 kDa molecular weight cutoff, Sartorius) and yielded 2.7 mg of MMC(IR800)-TOC after SpeedVac. The compound was identified by RP-HPLC using a Kinetex C18 column (2.6 μm) (Phenomenex) with a mobile phase of A = 0.1% TFA in H_2O, B = 0.1% TFA in CH_3CN; gradient, 0 min = 10% B, 12 min = 90% B; flow rate, 1 mL/min. Electrospray ionization (ESI) mass spectra were acquired on a LCQ FLEET instrument (Thermo Scientific).

ESI-MS (*m/z*): calculated for $C_{132}H_{165}N_{21}O_{32}S_6$, 2750.23; found, 1375.51 $[M+2H]^{2+}$ and 917.56 $[M+3H]^{3+}$.

1.7 Discussion-Synthesis of 6

Synthesis of MMC(IR800)-TOC is shown in Scheme 1. Selective functionalization of **1** was achieved and optimized to favor formation of the monosubstituted product **2**. This was accomplished by comparing different solvents (CH_3CN, DMF, and CH_2Cl_2) and reaction temperatures (r.t. vs. 50°C), and adding benzyl bromoacetate dropwise in a high reaction volume (55 mL final volume). As a result, **2** was obtained in 75% yields which allowed scale-up of subsequent compounds for SPPS. DA-TOC was formed on solid-phase using commercially available reagents (DOTA-NHS and ε-azido-norleucine). Each peptide conjugate was efficiently labeled with IRDye 800 in solution-phase with >95% purity as determined by RP-HPLC. Chromatograms for each agent showed excellent correlation between UV (280 and 780 nm), fluorescent, and radioactive peaks (Supplemental Fig. 1).



Supplemental Fig. 1. Representative RP-HPLC analysis of ^{64}Cu-MMC(IR800)-TOC showing the corresponding UV (280 and 780 nm), fluorescent, and radioactive traces.

1.8 Synthesis of DA(IR800)-TOC: A more conventionally synthesized, hybrid somatostatin (SST) analog (referred to as DA-TOC) was prepared by sequential N-terminal functionalization of resin-bound TOC with ε-azido-norleucine and DOTA-NHS. Protecting groups were removed as described above to afford DA-TOC. The peptide conjugate (1.8 mg, 1.1 mmol) was then reacted with IRDye 800-DBCO (1.6 mg. 1.2 mmol) as described above to give 1.8 mg of DA(IR800)-TOC. The compound was identified by RP-HPLC and mass spectrometry. ESI-MS (*m/z*): calculated for $C_{135}H_{170}N_{23}O_{34}S_6$, 2837.31; found, 1417.3 $[M-2H]^{2-}$ and 944.6 $[M-3H]^{3-}$.

1.9 Serum Stability: Stability of the radiotracers was examined in serum as previously described (*2*). Briefly, an aliquot of ^{64}Cu-MMC(IR800)-TOC and ^{64}Cu-DOTA-TOC was diluted

in PBS, added to mouse serum, and incubated at 37ºC. Samples were taken immediately, 1, 2, and 4 h after incubation and analyzed by radio-HPLC to determine the percent of intact agent. The data were normalized to 100% at t=0 and represent experiments performed in triplicate.

1.10 Cold Cu Labeling: Labeling with non-radioactive copper was performed according to methods established for the radiolabeled compounds. Briefly, the peptide conjugates (150 µg, 60 nmol) were mixed with a 4-fold molar excess of $CuCl_2$ and heated at 50°C for 1 h. The crude mixtures were purified by ultrafiltration and the pure products were characterized by RP-HPLC and mass spectrometry. ESI-MS (*m/z*): calculated for $C_{132}H_{165}N_{21}O_{32}S_6$ Cu (Cu-MMC(IR800)-TOC), 2813.77; found, 1405.5 $[M-2H]^{2-}$ and 936.2 $[M-3H]^{3-}$. ESI-MS (*m/z*): calculated for $C_{135}H_{170}N_{23}O_{34}S_6$ Cu (Cu-DA(IR800)-TOC), 2900.85; found, 964.2 $[M-3H]^{3-}$.

2. *In Vitro* Experiments

2.1 Generation and Culture of Stable Cell Lines: Human embryonic kidney (HEK)-293 cells stably expressing the HA3-tagged rsstr2 receptor were generated as described previously (*3*) and used for internalization experiments. Briefly, HEK293 cells were transfected with the HA3-rat-sst2A pcDNA3.0 plasmid and selected with G418 (750 µg/ml). Individual clones were isolated by limited dilution and grown in DMEM + 10% FBS + G418 (250 µg/ml). The clone used for experiments was named HEK293-HA3-rsstr2-c1D.

For real time cyclic adenosine monophosphate (cAMP) assays, the HEK293-HA3-rsstr2 cell line was further transfected with the cAMP biosensor 22F using FuGENE 6 (Promega). Stably transfected cells were selected with Hygromycin B (750 µg/ml) and individual clones were

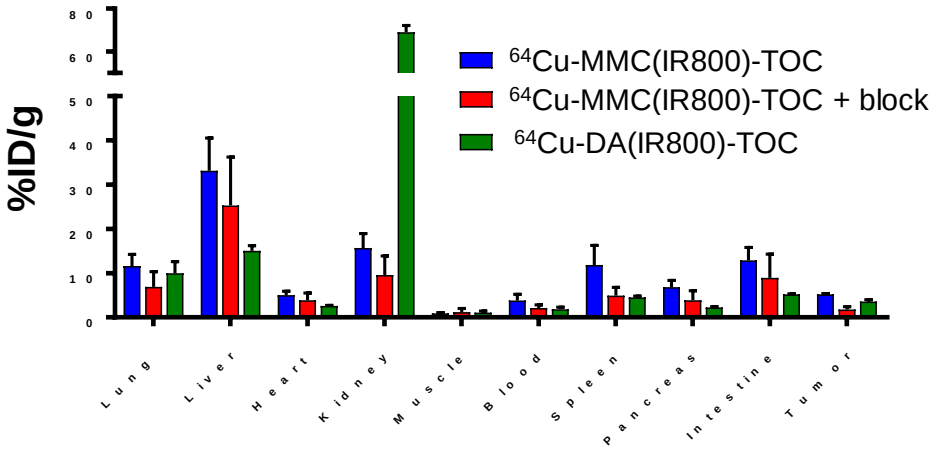
isolated by limiting dilution. Clonal cell lines were grown in GloSensor growth media (90% DMEM + 10% FBS + 200 µg/ml Hygromycin B). The clone used for experiments was named HEK293-HA3-rsstr2-Glo.

2.2 Measurement of Intracellular cAMP with the GloSensor Assay: HEK293-HA3-rsstr2-Glo cells, expressing both the GloSensor 22F plasmid (Promega) and HA-tagged sst2 receptor, were seeded at 100,000 cells/well in 96 well plates. After 24 h, the medium was aspirated and replaced with 90 µl of warm (28°C) equilibration medium (DMEM + 10% FBS + 10 mM HEPES + 2% D-Luciferin). Plates were pre-incubated in a dark humidified chamber at 28°C for 2 h and then placed in a PolarStar Optima multiplate reader (BMG Labtech). Basal bioluminescence was measured and then 10 µl of NKH477 (final concentration = 10 µM) was added either with or without the appropriate concentration of SST analog. Readings were taken every 2.5 min for 1 h. Data shown were obtained 20 min after agonist addition and are expressed as a percentage of the luminescence measured in the presence of NKH477 alone.

2.3 Measurement of Receptor Internalization: Changes in cell surface expression of rsstr2 were measured in HEK293-HA3-rsstr2 cells using ELISA, as previously described (*20*). Briefly, HEK293-HA3-rsstr2 cells were incubated with labeled peptides for 30 min at 37°C. After washing, cells were fixed, blocked with 1% BSA for 30 min, and incubated overnight at 4°C with mouse anti-HA antibody (1:10,000). Cells were then washed with PBS and incubated at room temperature for 1 h with goat anti-mouse HRP-labeled secondary antibody (1:10,000). Cell surface receptor level was determined by incubating for 45-60 min with ABTS and then measuring optical density at 405 nm.

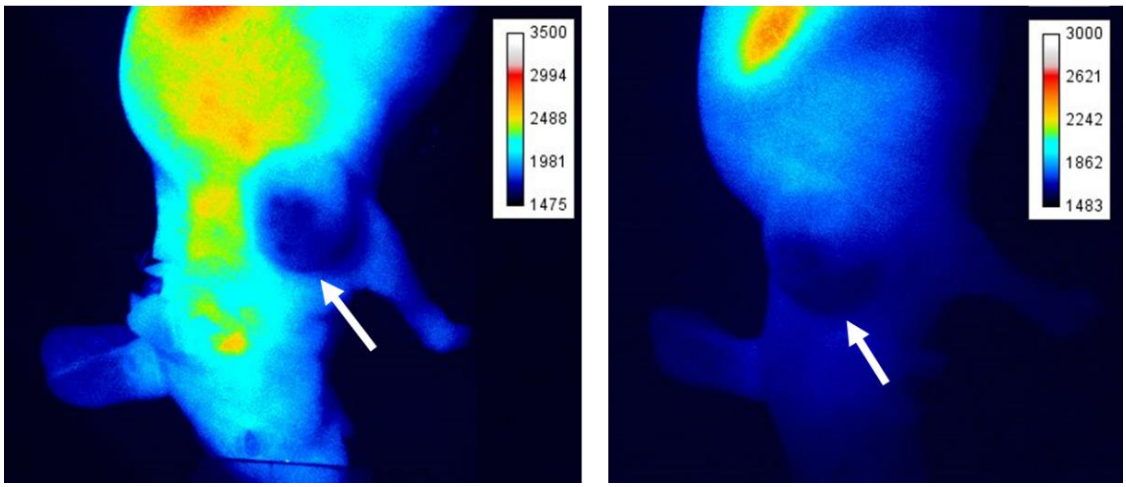
3. *In Vivo* Experiments

3.1 Biodistribution: Biodistribution studies were performed following multimodality imaging. AR42J tumor-bearing mice were injected intravenously with 200 μCi (2 nmol) of ^{64}Cu-MMC(IR800)-TOC (with and without octreotide block) or ^{64}Cu-DA(IR800)-TOC. At 4 h post-injection, the mice were euthanized and selected tissues were excised, weighed, and counted for radioactivity using a γ counter. Supplemental Fig. 2 summarizes the biodistribution of the peptide conjugates. Tumor uptake was highest for ^{64}Cu-MMC(IR800)-TOC and reduced by 66% when co-injected with a 10-fold excess of octreotide. Prominent uptake of ^{64}Cu-MMC(IR800)-TOC in the liver and spleen suggest clearance through the reticuloendothelial system, whereas ^{64}Cu-DA(IR800)-TOC exhibited high renal clearance. Intestinal accumulation was higher for ^{64}Cu-MMC(IR800)-TOC and may be attributable to increased transchelation of ^{64}Cu from the MMC (contains 2 acid groups compared to the 3 acid groups in DA) and subsequent formation of radiometabolites.



Supplemental Fig. 2. *Ex vivo* analysis of tracer biodistribution in AR42J xenografts 4 h post-injection.

3.2 ***In Vivo*** **NIRF Imaging:** NIRF imaging of AR42J tumor-bearing mice was performed with an exposure time of 200 msec. Images were acquired at 4 and 24 h post-injection with ^{64}Cu-MMC(IR800)-TOC and analyzed with the ImageJ software package. Liver uptake was prominent but tumors were not efficiently detected due to hyperpigmentation which reduced the emitted fluorescence signal (Supplemental Fig. 3).



Supplemental Fig. 3. *In vivo* NIRF images of AR42J xenografts following injection of ^{64}Cu-MMC(IR800)-TOC. Images were taken 4 (left) and 24 h (right) post-injection. Arrows indicate cold spots caused by tumor pigmentation.

4. References

1. Bajwa JS. Chemoselective deprotection of benzyl esters in the presence of benzyl ethers, benzyloxymethyl ethers and n-benzyl groups by catalytic transfer hydrogenation. *Tetrahedron Letters.* 1992;33:2299-2302.

2. Ghosh SC, Pinkston KL, Robinson H, et al. Comparison of DOTA and NODAGA as chelators for (64)Cu-labeled immunoconjugates. *Nucl Med Biol.* 2015;42:177-183.

3. Liu Q, Cescato R, Dewi DA, Rivier J, Reubi JC, Schonbrunn A. Receptor signaling and endocytosis are differentially regulated by somatostatin analogs. *Mol Pharmacol.* 2005;68:90-101.