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Comparative evaluation of three ^{64}Cu -labeled *E. coli* heat-stable enterotoxin analogs for PET imaging of colorectal cancer

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Abstract

Analogues of the *E. coli* heat-stable enterotoxin (STh) are currently under study as both imaging and therapeutic agents for colorectal cancer. Studies have shown the guanylate cyclase C (GC-C) receptor is commonly expressed in colorectal cancers. It has also been shown that STh peptides inhibit the growth of tumor cells expressing GC-C. The ability to determine GC-C status of tumor tissue using in vivo molecular imaging techniques would provide a useful tool for the optimization of GC-C-targeted therapeutics. In this work we have compared receptor binding affinities, internalization/efflux rates, and in vivo biodistribution patterns of an STh analog linked to N-terminal DOTA, TETA, and NOTA chelating moieties and radiolabeled with Cu-64. The peptide F¹⁹-STh(2–19) was N-terminally labeled with three different chelating groups via NHS ester activation and characterized by RP-HPLC, ESI-MS, and GC-C receptor binding assays. The purified conjugates were radiolabeled with Cu-64 and used for in vitro internalization/efflux, in vivo biodistribution, and in vivo PET imaging studies. In vivo experiments were carried out using SCID mice bearing T84 human colorectal cancer tumor xenografts. Incorporation of DOTA-, TETA-, and NOTA-chelators at the N-terminus of the peptide F¹⁹-STh(2–19) resulted in IC₅₀'s between 1.2–3.2 nM. In vivo, tumor localization was similar for all three compounds, with 1.2–1.3 %ID/g at 1 hr pi and 0.58–0.83 %ID/g at 4 hr pi. The principal difference between the three compounds related to uptake in nontarget tissues, principally kidney and liver. At 1 hr pi, ^{64}Cu -NOTA-F¹⁹-STh(2–19) demonstrated significantly ($p < 0.05$) lower uptake in liver than ^{64}Cu -DOTA-F¹⁹-STh(2–19) (0.36 ± 0.13 vs. 1.21 ± 0.65 %ID/g), and significantly ($p < 0.05$) lower uptake in kidney than ^{64}Cu -TETA-F¹⁹-STh(2–19) (3.67 ± 1.60 vs. 11.36 ± 2.85 %ID/g). Use of the NOTA chelator for coordination of Cu-64 in the context of *E. coli* heat-stable enterotoxin analogs results in higher tumor:nontarget tissue ratios at 1 hr pi than either DOTA or TETA macrocycles. Heat-stable enterotoxin-based radiopharmaceuticals such as these provide a means of noninvasively determining GC-C receptor status in colorectal cancers by PET.

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INTRODUCTION

Colorectal cancer is an enormous problem within the United States, with an estimated 148,000 new cases diagnosed and almost 50,000 deaths in 2008 alone (1). Of patients diagnosed with colorectal cancer, the majority will at some time during the course of the disease develop distant metastases (2). The liver is the most common site of colorectal cancer metastasis, and patients with liver metastases in general have a poor prognosis, with 5-year survival rates of 25–40% following resection with curative intent (2). Recent research has resulted in the approval of several new agents for the treatment of metastatic colorectal cancer. While fluorouracil/leucovorin therapy was formerly the standard treatment regimen, advances made within the last decade have added the topoisomerase I inhibitor irinotecan (Camptosar, Pfizer) and the alkylating agent oxaliplatin (Eloxatin, Sanofi-Aventis) to combination therapy protocols (3,4). More recently, monoclonal antibodies targeted against the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF) have received FDA approval for treatment of metastatic colorectal cancer (3,4). Both EGFR-targeted Cetuximab (Erbix[®], ImClone Systems/Bristol-Myers Squibb) and the α -VEGF mAb Bevacizumab (Avastin[®], Genentech/Roche) have been shown to synergize with existing chemotherapy protocols and lengthen duration of response (5,6). Additionally, EGFR-targeted Panitumumab (Vectibix[®], Amgen) has been recently approved for treatment of chemotherapy-refractory metastatic CRC expressing wild-type KRAS (7). Despite these advances however, the median life expectancy for people with advanced colorectal cancer is approximately 20 months, suggesting that new forms of treatment are still required (4).

Guanylate cyclase C (GC-C) is a type I transmembrane glycoprotein overexpressed on colorectal cancer cells. Recent clinical trials have shown a correlation between expression of the GUY2C mRNA for the GC-C protein in lymph nodes of CRC patients and disease recurrence (8). GC-C agonists such as the *E. coli* heat-stable enterotoxin (STh) and uroguanylin have been shown to have antiproliferative effects on human colorectal cancer cells (9,10), and have demonstrated efficacy in the treatment of colorectal cancers in animal models (11). Mechanistically, agonist binding to GC-C stimulates production of intracellular cyclic guanosine 3'-5'-monophosphate (cGMP), which can lead either to cell cycle arrest (9) or apoptosis (11). A substantial body of experimental evidence supports the concept that the intracellular signaling pathway regulated by the second messenger molecule cGMP may be a therapeutic target for the treatment of colon cancer as well as other malignant tumors (12–15). STh-based imaging agents could therefore be useful for assessment of GC-C receptor status in colorectal cancer metastases, thus defining subsets of patients who may benefit from GC-C agonist-based adjuvant therapy.

Although ¹¹¹In- and ^{99m}Tc-labeled GC-C agonists have shown promise as SPECT imaging agents (16–18), peptides in this class have not as yet been exploited for PET imaging. Existing previously characterized constructs have employed an N-terminal DOTA macrocycle for coordination of ¹¹¹In (17,18), and could easily be employed for M²⁺ or M³⁺ PET radionuclides such as ⁶⁴Cu, ⁸⁶Y, and ⁶⁸Ga. In the case of ⁶⁴Cu, extensive research using diverse targeting vectors has documented some degree of in vivo instability of ⁶⁴Cu-DOTA complexes, giving rise to observations of increased uptake in nontarget tissues such as liver (19,20). The azomacrocyclic chelator TETA has often been used in ⁶⁴Cu radiopharmaceuticals in preference to DOTA, since the larger TETA macrocycle forms a more stable Cu(II) complex (19,21). However, ⁶⁴Cu-TETA complexes have also demonstrated in vivo instability, resulting in uptake of free ⁶⁴Cu by cytosolic proteins such as superoxide dismutase (22–24). These findings have catalyzed continuing research into the development of ⁶⁴Cu chelators with increased stability in vivo (20,22–25). In this study, we have compared three different macrocyclic chelating systems for the coordination of ⁶⁴Cu combined with an analog of the *E. coli* heat-stable enterotoxin, to address the hypothesis

that ^{64}Cu -labeled GCC agonists could be effective PET agents for in vivo imaging of GC-C receptor status.

MATERIALS AND METHODS

Reagents

The peptide F¹⁹-STh(2–19) was obtained from Bachem (King of Prussia, PA) and used as received. 1,4,8,11-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (TETA) and the NHS ester of 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) were obtained from Macrocyclics, Inc. 1,4,7-triazacyclononane-N,N',N''-triacetic acid (NOTA) was synthesized as previously described (25). All solvents were either ACS certified or HPLC grade solvents obtained from Fischer Scientific and used as received. MALDI-TOF mass spectral analyses were performed by the Proteomics Center at the University of Missouri-Columbia. ^{64}Cu ($T_{1/2}$ 12.7 h, β^+ , 17.4%, $E_{\beta^+\text{max}}$, 656 keV; β^- , 39%, $E_{\beta^-\text{max}}$, 573 keV) was obtained from Trace Life Sciences, or from the cyclotron facility at the University of Wisconsin-Madison. T-84 human colon cancer cells were obtained from American Type Culture Collection (ATCC) and maintained and grown for use in these studies in the University of Missouri Cell and Immunology Core facilities.

Synthesis and purification of chelator-peptide conjugates

Folded *E. coli* heat-stable enterotoxin analogs were N-terminally labeled with the DOTA chelating moiety via incubation with the NHS ester of DOTA at 100-fold molar excess at 4 °C overnight in 0.3 M HEPES, pH 8.5. TETA and NOTA conjugations were performed by in situ formation of the activated esters using a 100-fold molar excess of EDC and sulfo-NHS in 0.2 M sodium phosphate buffer, pH 8.0. Polyaminocarboxylate-conjugated peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC). HPLC was performed using a Shimadzu system with SCL-10A controller, LC-20AT pumps, and in-line SPD-20A UV-Vis/NaI solid crystal scintillation detectors. Column temperature was maintained at 37 °C using an Eppendorf TC-50 column heater, and data acquisition took place with EZstart software version 7.4 (Shimadzu). HPLC solvents consisted of H₂O containing 0.1% trifluoroacetic acid (Solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (Solvent B). Conditions: A Phenomenex Jupiter C-18 (5 μm , 300 Å, 4.6 $\times$ 250 mm) column was used with a flow rate of 1.5 ml/min. Gradient purification of compounds is achieved during a linear 30 minute ramp from 23% B to 33 % B, followed by column rinse and reequilibration. Masses of each peptide conjugate were confirmed post-purification by MALDI-TOF MS analysis (DOTA-F¹⁹-STh(2–19) (M+H)⁺ Calc. = 2297.6, Obs. = 2297.8; TETA-F¹⁹-STh(2–19) (M+H)⁺ Calc. = 2325.6, Obs. = 2325.9; NOTA-F¹⁹-STh(2–19) (M+H)⁺ Calc. = 2196.6, Obs. = 2196.8.).

Radiolabeling

F¹⁹-STh(1–19) was iodinated by a modified lactoperoxidase method. Briefly, 2 μg peptide was suspended in 50 μl 100 mM sodium phosphate buffer, pH 7.5, containing 2 μg lactoperoxidase and 10–20 MBq Na¹²⁵I. The reaction was initiated by addition of 2 μl of a 1:10,000 dilution 30% H₂O₂. The reaction was incubated 30 min. at room temp. with occasional mixing, then diluted with dH₂O and purified to homogeneity by RP-HPLC.

For the synthesis of ^{64}Cu -labeled peptides, aliquots of $^{64}\text{CuCl}_2$ (7.4 – 74 MBq, 4 – 40 μl) were added to solutions of DOTA-, TETA-, and NOTA-F¹⁹-STh(2–19) (2–5 μg) in 0.4M ammonium acetate (200 μl). The pH of the reaction mixture was adjusted to 6.0. The reaction mixture was incubated for 1 hour at 80 °C. After 1 hour, an aliquot of 2 mM EDTA (50 μl) was added to the reaction mixture to complex unreacted $^{64}\text{Cu}^{+2}$. Radiochemical yields were typically >70%, with higher yields obtainable with increasing peptide

concentration in the reaction mix. The resulting radiolabeled conjugates were purified to homogeneity by RP-HPLC. All purified ^{64}Cu -STh conjugates were then concentrated by passing through a 3M Empore C-18 HD high performance extraction disk (7mm/3ml) cartridge and eluting with 50% ethanol in 0.1 M NaH_2PO_4 buffer (500 μl). The concentrated fractions were then reduced in volume under a stream of $\text{N}_{2(g)}$, and finally diluted with 0.1 M NaH_2PO_4 buffer, pH 7.0, to a final activity of approximately 74 kBq/100 μl . Calculated specific activities of ^{64}Cu - radiotracers ranged between 240–1200 Ci/mmol.

In vitro cell binding studies

IC_{50} 's of DOTA-, TETA-, and NOTA- F^{19} -STh(2–19) were determined by a competitive displacement cell binding assay using ^{125}I - F^{19} -STh(1–19). Briefly, 3×10^6 cells suspended in DMEM/F-12 media containing 15 mM MES and 0.2% BSA, pH 5.5, were incubated at 37 °C for 1 hr in presence of approximately 20,000 cpm ^{125}I -tracer and increasing concentrations of STh peptides. After the incubation, the reaction medium was aspirated and cells were washed three times with media. The radioactivity bound to the cells was counted in a Wallac Wizard 1480 gamma counting system. The % ^{125}I - F^{19} -STh(1–19) bound to cells was plotted vs. increasing concentrations of peptides to determine the respective IC_{50} values. For statistical considerations, three separate *in vitro* cell-binding experiments with each analog were performed in duplicate. IC_{50} calculations were performed using the 4 parameter logistic model within GraphPad Prism.

In vitro studies to measure rates of internalization and efflux of labeled compounds in T-84 human colon carcinoma cells were carried out as described previously (17). For internalization measurements, 3×10^6 cells suspended in DMEM/F-12 media containing 15 mM MES and 0.2% BSA, pH 5.5 were incubated for varying times at 37 °C with 30,000 – 60,000 cpm ^{64}Cu -labeled peptides. At designated times, binding media was aspirated and cells were washed 3 $\times$ with 4 °C binding media, then washed 2 $\times$ with 4 °C acid/saline solution (0.5 M NaCl, 1.2% CH_3COOH). The activity of the acid/saline rinse and the cell pellet were determined, and represent surface and internalized activity, respectively. For efflux experiments, all samples were initially incubated for 1 hr under the above conditions, then washed 3 $\times$ with 37 °C binding media to remove free label. The initial ($t = 0$) sample was then washed 3 $\times$ with 4 °C binding media, then 2 $\times$ with 4 °C acid/saline wash. Subsequent timepoints were treated identically, with surface and internalized activity determined as above.

Biodistribution studies

Four- to 5-week old female ICR SCID (severe combined immunodeficient) outbred mice were obtained from Taconic (Germantown, NY). The mice were housed four animals per cage in sterile micro isolator cages in a temperature- and humidity-controlled room with a 12-hour light/12-hour dark schedule. The animals were fed sterile rodent chow (Ralston Purina Company, St. Louis, MO) and water *ad libitum*. Animals were housed one week prior to inoculation of tumor cells and anesthetized for injections with isoflurane (Minrad, Inc.) at a rate of 2.5% with 0.4L/min oxygen through a non-rebreathing anesthesia vaporizer.

Human T-84 colorectal carcinoma cells were injected on the subcutaneous (s.c.) flank with $5\text{--}10 \times 10^6$ cells in a suspension of 100 μl 3:1 phosphate buffered saline : matrigel (BD Biosciences) per injection site. T-84 cells were allowed to grow *in vivo* four to six weeks post inoculation, developing tumors ranging in sizes from 0.06 – 0.30 grams. Mice (average weight, 25 g) were injected with aliquots (50–100 μl) of radiolabeled peptide solutions (55–90 kBq) *via* the tail vein. To demonstrate specificity of tumor localization, blocking studies were carried out in which ^{64}Cu -NOTA- F^{19} -STh(2–19) was co-injected with 2 mg/kg unlabeled F^{19} -STh(2–19). Tissues, organs and tumors were excised from animals sacrificed

at 1 and 4 hr p.i., weighed, and counted. Radioactivity was measured in a Wallac 1480 automated gamma counter and the percent-injected dose per gram tissue was calculated. Differences in organ uptake between labeled constructs were analyzed by Student's t-test. Differences at the 95% confidence level ($P < 0.05$) were considered significant. Animal studies were conducted in accordance with the highest standards of care as outlined in the NIH guide for Care and Use of Laboratory Animals and the Policy and Procedures for Animal Research at the Harry S. Truman Memorial Veterans' Hospital and according to approved protocols.

microPET/microCT imaging

MicroPET imaging was performed using a MOSAIC small-animal PET unit (Philips, USA). The unit has a gantry port diameter of 21 cm, a transverse field of view (FOV) of 12.8 cm and an axial length of 11.6 cm. The microPET scanner operates in a 3D volume imaging acquisition mode facilitating measurements of relative changes in radiotracer pharmacokinetics in vivo either as in frame, sequential or dynamic scanning capabilities. Small animals are frequently laser aligned at the center of the scanner FOV for subsequent imaging. SCID mice bearing T84 human colorectal cancer tumor xenografts were injected intravenously with 10.1 MBq, 36.4 MBq, and 13.9 MBq ^{64}Cu -DOTA-, ^{64}Cu -TETA-, and ^{64}Cu -NOTA- F^{19} -Sth(2–19), respectively. 0.8, 2.1, and 1.0 MBq of activity remained in the each animal, respectively, at time of image acquisition (1 hr pi). Tumor bearing mice were imaged and data was collected using an emission scan protocol and image reconstruction was performed with the use of a 3D row action maximum likelihood algorithm (RAMLA) without tissue attenuation correction. The MicroPET data was filtered with a 1.1 mm Gaussian FWHM filter. The MicroCT unit (MicroCAT IITM CT/SPECT, Siemens Pre-Clinical Solutions, Knoxville, TN) consists of a CCD x-ray detector and an 80 kVp micro-focus x-ray source (40 μm focal spot). MicroCT imaging was performed preceding microPET imaging for the purpose of anatomic/molecular data fusion and concurrent microCT image reconstruction was achieved with the use of a Fanbeam (Feldkamp) Filtered back projection algorithm. Co-registration, visualization and image analysis of PET/CT data was achieved with the use of the Amira 3.1 software package (TGS, Germany).

RESULTS

The polyaminocarboxylate chelating moieties DOTA, TETA, and NOTA were appended to the N-terminus of F^{19} -Sth(2–19) via NHS ester activation. The addition of DOTA was carried out using a commercially available NHS ester of DOTA, while TETA and NOTA were appended via in situ NHS ester activation using a 100-fold excess of sulfo NHS and EDC in aqueous solution. Addition of TETA- and DOTA- to the N-terminus of F^{19} -Sth(2–19) resulted in 0.5 – 0.6 minute shifts to earlier retention times, respectively, while addition of NOTA-resulted in a retention time 0.4 minutes later, as assessed by C18 RP-HPLC. The identity of each conjugate was confirmed post-purification by MALDI-TOF mass spectral analyses.

Each peptide conjugate was subsequently radiolabeled with ^{64}Cu and purified by C18 RP-HPLC (Figure 2). Radiolabeling efficiencies observed for each peptide were typically in the range of 70–80%, with quantitative radiolabeling achievable with the use of higher concentrations of peptide substrate. Coordination of ^{64}Cu by DOTA and TETA macrocycles resulted in radiolabeled STh analogs which coeluted with unlabeled peptides, while ^{64}Cu -labeling of the NOTA peptide resulted in a retention time 1.3 minutes earlier than the unlabeled NOTA-peptide.

Affinities of each conjugate for GC-C were measured in vitro in a competitive radioligand binding assay using ^{125}I -F 19 -STh(1–19) and intact T84 human colorectal cancer cells. Incorporation of each chelating macrocycle at the N-terminus of the targeting peptide had a minimal impact on measured receptor binding affinities (Figure 3). Binding affinities for the three peptide conjugates were 3.2 ± 1.9 , 2.0 ± 0.5 , and 1.2 ± 0.4 nM for DOTA-, TETA-, and NOTA-F 19 -STh(2–19), respectively, similar to the previously reported value of 2.2 ± 0.4 nM for the unmodified F 19 -STh(2–19) molecule (18).

Internalization and efflux studies were also performed with each ^{64}Cu -labeled peptide. The fraction of internalized radioactivity for each ^{64}Cu -labeled peptide was similar over time, with 45.1 ± 7.2 , 43.6 ± 3.6 , and 49.1 ± 2.7 % of total cell-associated radioactivity internalized at 100 min. for DOTA, NOTA, and TETA constructs, respectively. The degree of residualization of ^{64}Cu within cells was also not significantly different between DOTA- and TETA-labeled peptides. However, ^{64}Cu -NOTA-F 19 -STh(2–19) demonstrated an increased rate of efflux from T84 cells in vitro (Figure 4).

Biodistribution studies of ^{64}Cu -labeled peptides were conducted in SCID mice bearing T84 human colorectal cancer tumor xenografts. At 1 hr pi, all three bioconjugates showed rapid elimination via the renal/urinary route, with 88.6 ± 4.3 , 85.1 ± 5.8 , and 91.7 ± 2.6 % ID in urine for DOTA, TETA, and NOTA peptides, respectively. Tumor uptake of all three peptides was similar, with 1.18–1.37 %ID/g in tumor tissue. At 1 hr pi, uptake in tumor for each construct was higher than for all other normal tissues with the exception of kidney (Table 1). Blocking studies, carried out by co-administration of unlabeled F 19 -STh(2–19), reduced accumulation of the NOTA conjugate in tumor tissue by $\approx 42\%$ ($p < 0.05$), comparable to previous results obtained for this class of peptides (17,18). Kidney uptake values at 1 hr pi for the NOTA conjugate (3.7 ± 1.6 %ID/g) were lower than for either the DOTA (6.5 ± 2.7 %ID/g) or TETA (11.4 ± 2.9 %ID/g) constructs (Table 1, Figure 5). Uptake in liver was also lower for the NOTA conjugate (0.36 ± 0.13 %ID/g) than for either the DOTA (1.21 ± 0.67 %ID/g) or TETA (0.49 ± 0.15 %ID/g) constructs (Table 1, Figure 5). Resulting tumor:normal tissue ratios for the three conjugates ranged between 1.8–2.5 for blood, 11.4–13.1 for muscle, 1.1–3.3 for liver, and 0.12–0.32 for kidney, with the NOTA conjugate yielding the highest ratio in each instance.

At 4 hr pi, $> 94\%$ of all three ^{64}Cu -labeled peptides had been excreted into urine. Kidney uptake for all three compounds was significantly ($p < 0.05$) reduced, with 57–62% lower uptake relative to 1 hr pi. Tumor and blood %ID/g values for both the TETA- and NOTA-peptides were significantly lower than at 1 hr pi, while these values for the DOTA-peptide were not ($p < 0.05$). Tumor uptake of the three labeled peptides ranged between 0.58–0.83 %ID/g. Tumor:blood, tumor:muscle, and tumor:liver ratios for the three conjugates ranged between 3.5–11.8, 15–29, and 0.8–1.5, respectively.

The results of small-animal microPET/microCT imaging experiments performed on SCID mice bearing hind flank T84 human colorectal cancer tumor xenografts are shown in Figure 6. Images obtained using DOTA, TETA, and NOTA conjugates at 1 hr pi demonstrate their potential as site directed PET agents for in vivo characterization of GC-C-expressing colorectal cancers. These images visually recapitulate the in vivo biodistribution results at the same timepoint, with tumor uptake clearly visualized in each case. Uptake in non-target tissues is also evident to varying extents, with uptake in liver most evident for the DOTA conjugate, as predicted from biodistribution data (Table 1).

DISCUSSION

Stable in vivo complexation of ^{64}Cu in the context of receptor-targeted radiopharmaceuticals is essential for minimization of off-target uptake in normal tissues such as liver and kidneys with concomitant maximization of receptor-mediated tumor uptake. While the cyclam- and cyclen-based chelating backbones TETA and DOTA have been most commonly used for ^{64}Cu -coordination in targeted radiopharmaceuticals, other polyaminocarboxylate macrocycles have been extensively studied for their increased kinetic inertness to both H^+ -mediated $\text{Cu}(\text{II})$ dissociation and protein transchelation (24). Among these alternative chelators, both cross-bridged DOTA- and TETA-based chelators and the 9-membered NOTA macrocycle have been found to offer increased resistance to in vitro and in vivo ^{64}Cu demetallation (22,25,26).

Characterization of the three ^{64}Cu -labeled heat-stable enterotoxin peptides demonstrated significantly earlier elution of the NOTA compound from a C18 RP-HPLC column relative to the DOTA and TETA peptides. In the ^{64}Cu -NOTA peptide, each of two carboxylate arms is thought to be involved in metal coordination, resulting in a neutral complex (25). DOTA and TETA peptides, by contrast, each possess a free carboxylate arm, resulting in metal centers with an overall net -1 charge (21,27).

Several differences exist between in vivo biodistribution patterns of ^{64}Cu -DOTA- F^{19} -STh(2–19) and the previously described ^{111}In -complex of this DOTA-peptide (18). At 1 hr pi, tumor uptake of ^{64}Cu -DOTA- F^{19} -STh(2–19) was significantly lower than that of ^{111}In -DOTA- F^{19} -STh(2–19) (1.33 ± 0.52 vs. 2.35 ± 0.43 %ID/g, respectively). Simultaneously, the liver uptake of the ^{64}Cu -labeled peptide was higher than for the ^{111}In -complex (1.21 ± 0.65 vs. 0.2 ± 0.09 , respectively). At 4 hr pi, both ^{111}In - and ^{64}Cu -DOTA- F^{19} -STh(2–19) were retained in tumor to a similar degree (0.74 ± 0.1 vs. 0.83 ± 0.3 %ID/g, respectively). This was due to slower washout of ^{64}Cu -labeled peptides from tumor tissue- 42–62% of 1 hr ^{64}Cu activity remained in tumor at 4 hr pi, contrasted with only 31% of ^{111}In activity. Uptake in liver of the ^{111}In -labeled peptide at 4 hr pi was only 0.07 ± 0.01 %ID/g, while 88% of ^{64}Cu activity in liver tissue at 1 hr pi remained at 4 hr pi (1.07 ± 0.8 %ID/g, Table 2). The 6–15-fold higher accumulation of the ^{64}Cu -DOTA-labeled peptide in liver is likely due to instability of the ^{64}Cu -DOTA complex, with transchelation of ^{64}Cu by proteins such as superoxide dismutase, ceruloplasmin, serum albumin, and metallothionein (22). Similar results have previously been observed in the context of other peptide radiopharmaceuticals (20,28), and great effort has been put toward the development of alternate Cu-chelating frameworks (19–25).

The major distinguishing characteristic between the ^{64}Cu -DOTA-, ^{64}Cu -TETA-, and ^{64}Cu -NOTA-peptides in vivo relates not to tumor uptake, but to uptake in nontarget tissues such as liver and kidney (Figure 5, Figure 6). The ^{64}Cu -NOTA conjugate displays significantly lower liver uptake at 1 hr pi than the ^{64}Cu -DOTA conjugate. Although experiments to characterize products of in vivo metabolism were not carried out in this work, one possible explanation for this could be increased in vivo kinetic stability of the ^{64}Cu -NOTA complex (25,26). Previously published studies have demonstrated similar effects in the context of other receptor-targeted radiopharmaceuticals containing cross-bridged macrocyclic chelators for the coordination of Cu-64 (20,23,24). The ^{64}Cu -NOTA conjugate also displays lower kidney uptake at 1 and 4 hr pi than either the ^{64}Cu -TETA or the ^{64}Cu -DOTA conjugates. This difference is most probably due to the differences in formal charge between these species. ^{64}Cu -TETA and ^{64}Cu -DOTA conjugates possess an additional negatively charged carboxylate group at physiological pH relative to ^{64}Cu -NOTA conjugates, which can increase tubular reabsorption of radiolabeled peptides following glomerular filtration (29). Previous studies have also shown higher kidney uptake for ^{64}Cu -DOTA/ ^{64}Cu -TETA

peptides in comparison with ^{64}Cu -TE2A peptides, which again is correlated with the formal charge of the Cu complexes (20,23).

MicroPET/CT images obtained with each radioconjugate at 1 hr pi (Fig.6) essentially recapitulate the in vivo biodistribution results. T84 tumor xenografts are clearly visualized in the normalized images, with only kidney uptake more clearly evident. Both ^{64}Cu -TETA and ^{64}Cu -NOTA conjugates display decreased liver uptake with respect to the ^{64}Cu -DOTA conjugate. Similarly, the markedly reduced kidney uptake of the ^{64}Cu -NOTA conjugate relative to the ^{64}Cu -TETA conjugate in the normalized images also reflects the in vivo biodistribution results.

CONCLUSION

This study demonstrates the utility of ^{64}Cu -labeled heat-stable enterotoxin analogs as PET imaging agents for the in vivo detection of colorectal cancers expressing GC-C. Employment of the NOTA chelator in this context gave superior tumor: liver and tumor: kidney ratios relative to DOTA- and TETA-functionalized peptides, respectively. This class of PET imaging agents could prove useful in stratification of subpopulations of individuals who may benefit from treatment with GC-C-targeted therapeutics.

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FIGURE 1.
Structures of STh analogs. Top: DOTA-F¹⁹-STh(2–19). Middle: TETA-F¹⁹-STh(2–19).
Bottom: NOTA-F¹⁹-STh(2–19).

FIGURE 2.

C18 RP-HPLC chromatograms of purified ^{64}Cu -labeled peptides. Upper: ^{64}Cu -NOTA- F^{19} -S Th (2–19), Tr =10.0 min. Middle: ^{64}Cu -DOTA- F^{19} -S Th (2–19), Tr =10.5 min. Lower: ^{64}Cu -TETA- F^{19} -S Th (2–19), Tr =10.6 min.

FIGURE 3. Measurement of F^{19} -STh(2–19) peptide conjugate IC_{50} values in a competitive receptor-binding assay with ^{125}I - F^{19} -STh(1–19) using cultured T84 human colorectal cancer cells.

FIGURE 4.

Determination of % efflux of radioactivity from T-84 human colon carcinoma cells for ^{64}Cu -DOTA- F^{19} -Sth(2-19), ^{64}Cu -TETA- F^{19} -Sth(2-19), and ^{64}Cu -NOTA- F^{19} -Sth(2-19).

FIGURE 5. Differences in A. liver and B. kidney uptake between ^{64}Cu -DOTA-, ^{64}Cu -TETA-, and ^{64}Cu -NOTA- $\text{F}^{19}\text{-STh}(2-19)$ at 1 hr pi (* $p < 0.05$ vs. NOTA).

FIGURE 6.

PET/CT images of SCID mice bearing hind flank T84 human colorectal cancer tumor xenografts 1 hr post-injection of 10 – 36 MBq labeled peptides in three-dimensional views (A–C) and as transaxial sections (D–F). ^{64}Cu -DOTA- F^{19} -S Th (2–19) (A,D), ^{64}Cu -TETA- F^{19} -S Th (2–19) (B,E), and ^{64}Cu -NOTA- F^{19} -S Th (2–19) (C,F). Pictured data has been normalized to the highest pixel intensity within each image. Axial cross sections through tumor xenografts are shown as red horizontal lines in A–C.

TABLE 1

Biodistribution of ^{64}Cu -DOTA-, ^{64}Cu -TETA-, and ^{64}Cu -NOTA- $\text{F}^{19}\text{-S}^{\text{Th}}(2-19)$ (%ID/g ($\pm$ SD), N=4) at 1 hr pi in SCID mice bearing T-84 human colorectal cancer tumor xenografts.

Organ	DOTA	TETA	NOTA
Blood	0.57 (0.21)	0.76 (0.25)	0.48 (0.22)
Heart	0.28 (0.14)	0.25 (0.08)	0.20 (0.10)
Lung	0.79 (0.33)	0.82 (0.18)	1.00 (0.73)
Liver	1.21 (0.65)	0.49 (0.15)	0.36 (0.13)
Spleen	0.29 (0.30)	0.20 (0.11)	0.10 (0.09)
Intestine	0.98 (0.49)	0.64 (0.13)	0.59 (0.20)
Kidney	6.52 (2.74)	11.36 (2.85)	3.67 (1.60)
Muscle	0.11 (0.05)	0.12 (0.06)	0.09 (0.03)
Bone	0.08 (0.17)	0.05 (0.05)	0.06 (0.09)
Tumor	1.33 (0.52)	1.37 (0.27)	1.18 (0.40)

TABLE 2

Biodistribution of ^{64}Cu -DOTA-, ^{64}Cu -TETA-, and ^{64}Cu -NOTA- F^{19} -S Th (2–19) (%ID/g ($\pm$ SD), N=3) at 4 hr pi in SCID mice bearing T-84 human colorectal cancer tumor xenografts.

Organ	DOTA	TETA	NOTA
Blood	0.24 (0.24)	0.07 (0.03)	0.05 (0.01)
Heart	0.21 (0.17)	0.06 (0.01)	0.04 (0.06)
Lung	0.54 (0.35)	0.28 (0.13)	0.17 (0.04)
Liver	1.07 (0.80)	0.47 (0.13)	0.40 (0.06)
Spleen	0.26 (0.29)	0.08 (0.11)	0.15 (0.12)
Intestine	0.69 (0.37)	0.48 (0.12)	0.57 (0.04)
Kidney	2.81 (1.37)	4.34 (0.10)	1.47 (0.37)
Muscle	0.03 (0.03)	0.02 (0.01)	0.04 (0.04)
Bone	0.15 (0.25)	0.04 (0.03)	0.002 (0.003)
Tumor	0.83 (0.30)	0.58 (0.12)	0.59 (0.11)