Selective Targeting of *E. coli* Heat-stable Enterotoxin Analogs to Human Colon Cancer Cells

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Abstract. *Background: Radiolabeled analogs of the E. coli heat-stable enterotoxin (ST_h) are currently under study as imaging and therapeutic agents for colorectal cancer. The aim of these studies is to compare in vitro and in vivo characteristics of two novel ST_h analogs with appended DOTA chelating moieties. Materials and Methods: ST_h analogs were synthesized with pendant N-terminal DOTA moieties and radiolabeled with indium-111. In vitro cell binding was studied using cultured T-84 human colorectal cancer cells, and in vivo biodistribution studies were carried out using T-84 human colorectal tumor xenografts in SCID mice. Results: Competitive radioligand binding assays employing T-84 human colon cancer cells demonstrated similar IC_{50} values for the F^{19}-ST_h(2-19) and F^9-ST_h(6-19) analogs. Addition of DOTA to the N-terminus of these peptides elicited distinctly different effects on binding affinities in vitro, effects that were largely unchanged by metallation with nonradioactive ^{nat}In. In vivo pharmacokinetic studies in SCID mice bearing T-84 human colon cancer-derived tumor xenografts demonstrated tumor uptake of 0.74±0.1 %ID/g at 4 h post-injection (p.i.) for the ^{111}In-DOTA-F^{19}-ST_h(2-19) analog, and significantly reduced tumor localization (0.27 + 0.08 %ID/g) for the ^{111}In-DOTA-F^9-ST_h(6-19) analog. Conclusion: These results demonstrate that placement of a DOTA moiety immediately adjacent to Cys 6 in ST_h significantly inhibits receptor binding in vitro and in vivo, highlighting the need for intervening spacer residues between the pharmacophore and the DOTA chelating moiety in effective ST_h-based radiopharmaceutical constructs.*

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Colorectal cancer is the third most common neoplasm and the second most common cause of cancer mortality in the United States (1, 2). The current gold standard for initial diagnosis of colorectal cancer is colonoscopy, and CT and MRI both have roles in the staging of local disease, assessment of regional lymph node involvement and identification of hepatic and extrahepatic metastases (1). Recent results suggest that ^{18}F-FDG PET may play a significant role in the management of colorectal cancer (1, 2, 4). Colorectal cancer metastases most commonly localize to the pelvis, liver and lungs, and the majority of patients who die from colon cancer have liver metastases (3). Development of a colorectal cancer-specific radio-pharmaceutical agent could significantly impact the ability to treat individuals with metastatic colorectal cancer.

Interest in using radiolabeled peptides as diagnostic and therapeutic agents has rapidly increased over the last decade (5, 6). Radiolabeled variants of somatostatin (SST) receptor-targeting peptides are the archetypical radio-pharmaceutical peptides, and are currently used for imaging of neuroendocrine tumors and pulmonary masses (5). OctreoScan® (Mallinkrodt, Inc.) is a commercially available SST analog (^{111}In-DTPA-D-Phe^1-Octreotide, ^{111}In-pentetreotide) that is routinely used for imaging of neuroendocrine tumors (7). ^{99m}Tc depreotide (NeoSpect®, NeoTect®, 99mTc-p829) and ^{99m}Tc-tricine-HYNIC-Tyr^3-octreotide are two ^{99m}Tc-labeled SST analogs that have been tested in the clinic (8). Analogs of bombesin (9-12), α-MSH (13-16), and numerous other receptor-avid peptides are also under investigation for oncologic imaging of a variety of tumor types. Vasoactive intestinal peptide (VIP) and the *E. coli* heat-stable enterotoxin (ST_h) are both under investigation as imaging agents for colorectal cancer (17-20).

Guanylate cyclase C (GC-C) is a type I transmembrane glycoprotein expressed on brush borders of intestinal epithelial cells, as well as on transformed human colon cancer cell lines, such as the T-84 cell line (21, 22).

Importantly, GC-C is also expressed at much lower levels in normal human extraintestinal tissues (23-25). Circulation accessible when expressed on primary and metastatic colon cancer tissues, GC-C provides a uniquely specific target for receptor avid peptide radiopharmaceuticals (18-20, 23, 26). As normally expressed within the lumen of the gut, GC-C binds the endogenous peptides guanylin and uroguanylin, which act to regulate ion and fluid homeostasis and also may regulate epithelial cell turnover (27, 28). Perhaps *via* gene transfer from vertebrate hosts, enteropathogenic strains of *E. coli* have evolved a homologous peptide, the human *E. coli* ST peptide (ST_h), which has the highest affinity for GC-C of any known ligand (27). Several studies have established the applicability of radiolabeled ST_h analogs to the diagnosis and treatment of human colorectal cancers *in vivo* (18-20, 35).

GC-C agonists, such as ST_h and uroguanylin have been shown to have antiproliferative effects on human colorectal cancer cells (28, 30), and have shown efficacy in treatment of colorectal cancers in animal models (29). Mechanistically, agonist binding to GC-C stimulates production of intracellular cGMP, which can lead either to cell cycle arrest (28) or apoptosis (29). 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 (31-34). Therefore, in addition to the potential role of radiolabeled ST_h analogs in diagnosis, staging and treatment of colorectal cancer, ST_h-based imaging agents could also 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. The work presented here defines structural requirements for optimal *in vivo* imaging using ^{111}In-labeled analogs of ST_h with N-terminal pendant DOTA chelating moieties.

Materials and Methods

Reagents. The peptides F^{19}-ST_h(2-19) and F^{9}-ST_h(6-19) were obtained from Bachem (King of Prussia, PA, USA) and Microbia (Cambridge, Mass), respectively. The NHS ester of 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) was obtained from Macrocyclics, Inc. 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, USA. $^{111}InCl_3$ was obtained from Mallinckrodt Medical, Inc. (St. Louis, MO, USA) as a 0.05 N HCl solution. 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 DOTA-peptides. 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. Under these conditions, >90% yield of DOTA-peptides was achieved. DOTA-conjugated peptides were purified by high performance liquid chromatography (HPLC). HPLC was performed on a Waters 600E system equipped with a Varian 2550 variable absorption detector, sodium iodide crystal radiometric detector, Eppendorf TC-50 column temperature controller and Hewlett Packard HP3395 integrators. HPLC solvents consisted of H_2O 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, 4.6x250 mm) column was used with a flow rate of 1.5 ml/min. Gradient purification of all compounds was achieved during a linear 30 min ramp from 20% B to 30% B, followed by column rinse and re-equilibration.

Radiolabeling. F^{19}-ST_h(1-19) was iodinated by a modified lactoperoxidase method (20). Briefly, 2 μg peptide was suspended in 50 μl 100 mM sodium phosphate buffer, pH 7.5, containing 2 μg lactoperoxidase and 300 μCi $Na^{125}I$. The reaction was initiated by addition of 2 μl of a 1:10,000 dilution 30% H_2O_2. The reaction was incubated for 30 min at room temperature with occasional mixing, then diluted with dH_2O and purified to homogeneity by RP-HPLC.

For the synthesis of ^{111}In-labeled compounds, aliquots of $^{111}InCl_3$ (0.2-2.5 mCi, 7.4-92.5 MBq, 4-50 μl) were added to solutions of DOTA-F^{19}-ST_h(2-19) (50 μg), or DOTA-F^{9}-ST_h(6-19) (50 μg) in 0.4 M ammonium acetate (200 μl). The pH of the reaction mixture was adjusted to 6.0. The reaction mixture was incubated for 1 h at 80˚C. Then, an aliquot of 0.002 M EDTA (50 μl) was added to the reaction mixture to complex unreacted $^{111}In^{+3}$. The resulting conjugates were purified to homogeneity by RP-HPLC. The ^{111}In-metallated conjugates eluted between 0.7-2.1 min before the associated non-metallated species, enabling collection of high-specific activity, no carrier-added ^{111}In-ST_h conjugates. All purified ^{111}In-ST_h conjugates were then concentrated by passing through a 3 M Empore C-18 HD high performance extraction disk (7 mm/3 ml) 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 $N_{2(g)}$, and finally diluted with 0.1 M NaH_2PO_4 buffer, pH 7.0, to a final activity of approximately 2 μCi / 100 μl.

Macroscopic quantities of indium complexes were synthesized by an analogous synthetic protocol. 0.2 mg DOTA-F^{19}-ST_h(2-19) or DOTA-F^{9}-ST_h(6-19), each in 100 μl 0.4 M ammonium acetate, pH 6.0, were added to 100 μg indium chloride. Reactions were incubated at 80˚C for 1 h. The resulting indium complexes were purified by RP-HPLC and analyzed by MALDI-TOF MS.

In vitro cell binding studies. IC_{50}'s of F^{19}-ST_h(2-19) and F^{9}-ST_h(6-19) and the corresponding DOTA- and ^{nat}In-DOTA-complexes were determined by a competitive displacement cell binding assay using ^{125}I-F^{19}-ST_h(1-19). Briefly $3x10^6$ 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 h in presence of approximately 20,000 cpm ^{125}I-tracer and increasing concentrations of ST_h 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 Packard Riastar gamma counting system. The %

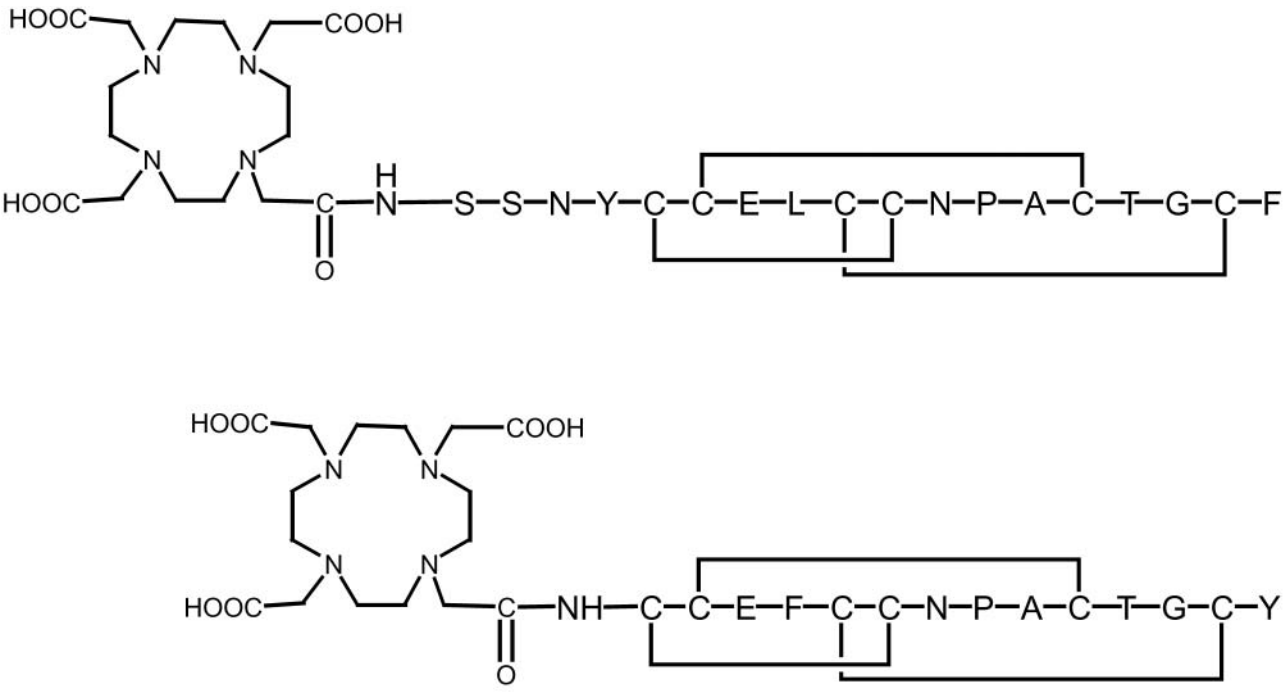

Figure 1. *Structures of ST_h analogs. Top: DOTA-F^{19}-ST_h(2-19). Bottom: DOTA-F^{9}-ST_h(6-19).*

^{125}I-F^{19}-ST_h(1-19) bound to cells was plotted *vs.* increasing concentrations of peptides to determine the respective IC_{50} values. An ST_h analog lacking tertiary structure by virtue of alanine substitutions for the six cysteine residues present in the wild-type molecule evaluated using this same system does not exhibit measurable binding to GC-C receptors (*i.e.*, $IC_{50}>10^{-6}$ M) (data not shown). 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 the program grafit (Erithacus Software, Ltd.).

In vitro studies to measure rates of internalization of labeled compounds in T-84 human colon carcinoma cells were carried out, as described previously (20). Briefly, $3x10^6$ 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 30-60 KCPM ^{111}In-DOTA-F^{19}-ST_h(2-19) and ^{111}In-DOTA-F^{9}-ST_h(6-19). At designated times, binding media was aspirated and cells were washed 3x with 4˚C binding media, then washed 2x 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.

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

Human colon cancer T-84 cells were injected on the bilateral subcutaneous (*s.c.*) flank with ~$5x10^6$ cells in a suspension of 100 μl normal sterile saline per injection site. T-84 cells were allowed to grow *in vivo* four to six weeks post inoculation, developing tumors ranging in sizes from 0.01-0.77 grams. The biodistribution and uptake of ^{111}In-DOTA-F^{19}-ST_h(2-19) and ^{111}In-DOTA-F^{9}-ST_h(6-19) in tumor bearing SCID mice was studied. The mice (average weight, 25 g) were injected with aliquots (50-100 μl) of the radiolabeled peptide solution (55-90 kBq) *via* the tail vein. Tissues, organs and tumors were excised from animals sacrificed at 1, 4 and 24 h *p.i.*, weighed and counted. Radioactivity was measured in a Wallac 1480 automated gamma counter and the percent-injected dose per organ and the percent-injected dose per gram tissue were calculated. Differences in organ uptake between the two ^{111}In-labeled peptides were analyzed by Student *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.

SPECT/CT imaging. A combined micro-SPECT/CT unit (microCAT II, Siemens Medical Systems) was employed for imaging studies. One SCID mouse bearing T-84 human colorectal cancer tumor xenografts was injected intravenously with 1.6 mCi ^{111}In-DOTA-F^{19}-ST_h(2-19) and sacrificed at 1 h p.i. 71 μCi of In-111 remained in the animal at the time of SPECT data acquisition. Micro-SPECT scans of 60 projections were performed using a symmetrical 20%

Table I. *Calculated and observed molecular weights and IC_{50} values for DOTA-ST_h analogs.*

Peptide	IC_{50} (nM)	$(M+H)^+$ Calc	$(M+H)^+$ Obs
F^{19}-ST_h(2-19)	2.2±0.4	1911.6	1911.7
F^9-ST_h(6-19)	2.6±0.8	1510.4	1510.4
DOTA-F^{19}-ST_h(2-19)	3.9±1.4	2297.6	2297.8
DOTA-F^9-ST_h(6-19)	45.5±5.4	1896.4	1896.6
In-DOTA-F^{19}-ST_h(2-19)	5.6±1.4	2409.4	2409.8
In-DOTA-F^9-ST_h(6-19)	60.2±28.3	2008.2	2008.6

photopeak discriminating window. A total acquisition of 4,974,144 counts was obtained in this experiment. Volumetric data from SPECT and CT was visualized and image fused using Amira 3.1 (TGS, San Diego, CA, USA).

Results

Synthesis and radiolabeling. In the studies described here, two analogs of the *E. coli* heat-stable enterotoxin were derivatized with the DOTA chelating moiety and radiolabeled with ^{111}In. These analogs differ from one another in three respects (Figure 1). First, F^9-ST_h(6-19) lacks 4 amino terminal residues, Ser-Ser-Asn-Tyr-, which are present both in the F^{19}-ST_h(2-19) analog and in the wild-type peptide. Second, F^9-ST_h(6-19) possesses a L9F substitution, while F^{19}-ST_h(2-19) retains the wild-type leucine residue in position 9. Lastly, F^9-ST_h(6-19) retains a tyrosine residue in position 19, while in F^{19}-ST_h(2-19) this residue has been altered to phenylalanine.

Each peptide was coupled to the DOTA chelating moiety through the use of the NHS ester of DOTA. Each of these peptides possesses a single amino group at its N-terminus, which allows targeted synthesis of the N-terminally modified peptide. All synthesized peptide analogs were characterized by analytical HPLC and MALDI-TOF MS (Table I). Addition of the DOTA moiety in each case resulted in a more hydrophilic molecule, with DOTA-peptides eluting 0.8 and 2.1 min prior to the unmodified peptide for F^{19}-ST_h(2-19) and F^9-ST_h(6-19), respectively.

DOTA-peptides were subsequently metallated with ^{111}In and ^{nat}In. ^{111}In conjugates were prepared in high yield (>90%) and purified by C18 RP-HPLC to attain high specific activity radioconjugates (Figure 2). ^{111}In-DOTA-F^9-ST_h(6-19) had a retention time of 8.8 min, eluting 0.7 min earlier than the unlabeled DOTA-peptide. The more hydrophobic ^{111}In-DOTA-F^{19}-ST_h(2-19) had a retention time of 16.8 min on the same gradient, eluting 2.1 min earlier than the unlabeled DOTA-peptide.

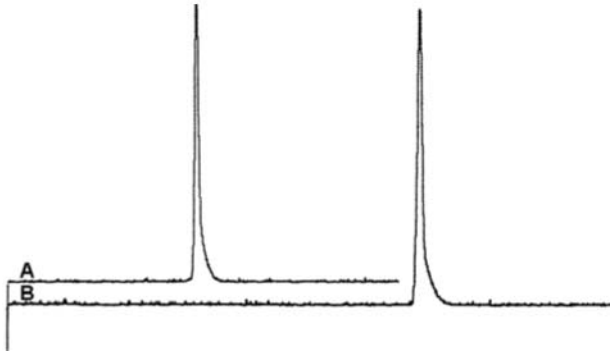

Figure 2. *C18 RP-HPLC chromatograms of purified ^{111}In-labeled peptides. A. ^{111}In-DOTA-F^9-FST_h(6-19), retention time = 8.8 min. B. ^{111}In-DOTA-F^{19}-ST_h(2-19), retention time = 16.8 min. Chromatographic conditions were as described in Materials and Methods.*

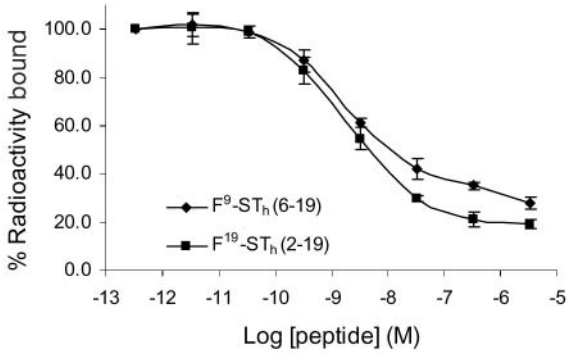

Figure 3. *Measurement of ST_h peptide IC_{50} values in a competitive receptor-binding assay with ^{125}I-F^{19}-ST_h(1-19) using cultured T84 human colorectal cancer cells.*

Receptor-binding studies. The effect of DOTA addition on peptide binding was assessed using T-84 human colorectal cancer cells. The abilities of both unmodified peptides to competitively displace ^{125}I-F^{19}-ST_h(1-19) were similar, with IC_{50}'s of 2.2±0.4 and 2.6±0.8 nM for F^{19}-ST_h(2-19) and F^9-ST_h(6-19), respectively (Table I, Figure 3). This indicates that together, the combined structural alterations between these peptides result in a minimal impact on binding affinities for the GC-C receptor. However, the addition of the DOTA moiety to the N-terminus of each peptide resulted in dramatic alterations in binding affinities (Table I). For the F^{19}-ST_h(2-19) analog, DOTA addition resulted in a less than 2-fold increase in IC_{50} (3.9±1.4 nM *vs.* 2.2±0.4 nM with and without the DOTA moiety, respectively). By contrast, addition of DOTA to the F^9-ST_h(6-19) analog resulted in a greater than 10-fold loss of binding affinity relative to the unmodified peptide (45.4±5.4

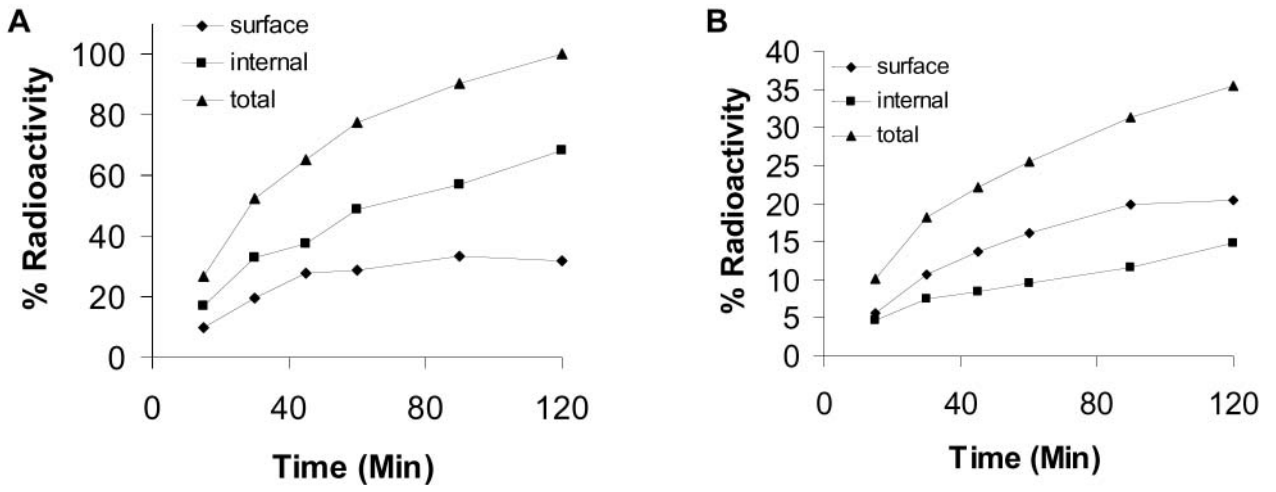

Figure 4. *Comparison of surface binding and internalization for (A) ^{111}In-DOTA-F^{19}-ST$_h$(2-19) and (B) ^{111}In-DOTA-F^{9}-ST$_h$(6-19). T-84 human colon carcinoma cells were incubated with 30,000-60,000 CPM ^{111}In-labeled peptides and processed as described in Materials and Methods.*

nM *vs.* 2.6±0.8 nM with and without the N-terminal DOTA group, respectively). Measured IC_{50}'s of ^{nat}In-DOTA-F^{19}-ST$_h$(2-19) and ^{nat}In-DOTA-F^{9}-ST$_h$(6-19) were similar to those of the uncomplexed peptides (Table I). *In vitro* receptor binding studies of each ^{111}In-labeled peptide also showed decreased binding of the ^{111}In-DOTA-F^{9}-ST$_h$(6-19) analog relative to ^{111}In-DOTA-F^{19}-ST$_h$(2-19). T-84 whole cell binding of ^{111}In-DOTA-F^{9}-ST$_h$(6-19) was only 35% that of the ^{111}In-DOTA-F^{19}-ST$_h$(2-19) after 2 h at 37˚C (Figure 4). Further, the majority of cell-associated ^{111}In-DOTA-F^{9}-ST$_h$(6-19) at this time point remained surface bound, while the bulk of cell associated ^{111}In-DOTA-F^{19}-ST$_h$(2-19) was internalized.

In vivo biodistribution. In vivo biodistribution studies of each ^{111}In-labeled ST$_h$ analog were carried out using SCID mice bearing T-84 human colorectal cancer tumor xenografts (Figure 5, Table II). ^{111}In-DOTA-F^{19}-ST$_h$(2-19) displayed rapid clearance from the bloodstream, with >90% of injected activity excreted into the urine at 1 h p.i. Tumor uptake at 1 h p.i. for this analog was 2.35±0.43 %ID/g, a value higher than for any other tissue with the exception of kidneys, which showed retention of 3.43±0.97 %ID/g at this time point. At 4 h p.i., tumor uptake was reduced to 0.74±0.1 %ID/g, and 0.37±0.04 %ID/g remained in tumor tissue at 24 h p.i. Kidney-associated activity fell over this same period to 2.22±0.62 and 0.93±0.12 %ID/g at 4 and 24 h p.i., respectively. Urinary clearance of the ^{111}In-DOTA-F^{9}-ST$_h$(6-19) analog was similarly high, and at 4 h p.i. the only significant difference ($p<0.05$) between these two compounds was in the level of retention in tumor. Injected activity remaining in tumor at 4 h p.i. was 0.27±0.08 %ID/g,

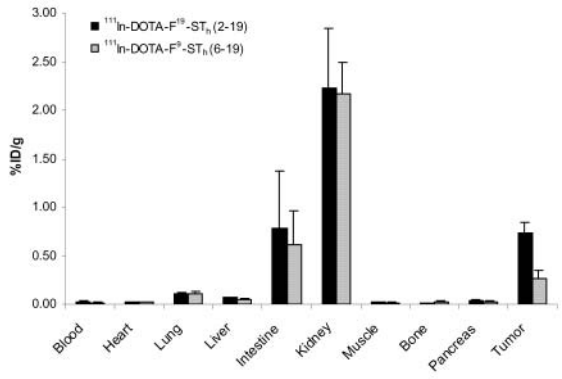

Figure 5. *Graphical comparison of biodistribution of ^{111}In-DOTA-F^{19}-ST$_h$(2-19) and ^{111}In-DOTA-F^{9}-ST$_h$(6-19) in SCID mice bearing T84 human colonic tumor xenografts at 4 h p.i.*

Table II. *^{111}In-DOTA-F^{19}-ST$_h$(2-19) biodistribution (%ID/g (+SD), N=3) in T-84 human colorectal cancer tumor xenografts in SCID mice.*

Tissue	1 h p.i.	4 h p.i.	24 h p.i.
Blood	0.77±0.7	0.02±0.01	0.02±0.01
Heart	0.26±0.15	0.02±0.01	0.01±0.01
Lung	0.76±0.26	0.1±0.02	0.04±0.01
Stomach	0.2±0.1	0.16±0.12	0.05±0.00
Liver	0.2±0.09	0.07±0.01	0.02±0.02
Intestines	0.89±0.13	0.79±0.58	0.93±1.25
Kidney	3.43±0.97	2.22±0.62	0.93±0.12
Muscle	0.1±0.05	0.02±0.01	0.01±0.01
Tumor	2.35±0.43	0.74±0.1	0.37±0.04
Urine (%ID)	91.2±1.85	96.7±1.5	92.8±6.27

a value 2-3-fold lower than that of the ^{111}In-DOTA-F^{19}-ST$_h$(2-19) analog. For ^{111}In-DOTA-F^{19}-ST$_h$(2-19) at 4 h p.i., tumor-to-blood, -muscle and -kidney radioactivity ratios were 37.0, 37.0 and 0.3, respectively, while for ^{111}In-DOTA-F^{9}-ST$_h$(6-19) these values were significantly lower (27.0, 13.5, and 0.1, respectively).

Imaging. In vivo imaging of SCID mice bearing T-84 human tumor xenografts was carried out using a combined micro-SPECT/CT unit (microCAT II, Siemens Medical Systems). Scintigraphic images obtained using the ^{111}In-DOTA-F^{19}-ST$_h$(2-19) analog overlaid upon anatomical CT data are shown in Figure 6. These data, acquired 1 h p.i. of 1.6 mCi of labeled peptide, visually recapitulate the biodistribution data given in Table II. High uptake of labeled peptide is evident in the bilateral hind flank T-84 human tumor xenografts. Only kidney uptake and diffuse retention in intestinal tissue is otherwise evident.

Discussion

Guanylate cyclase C is highly expressed on cells originating from the intestinal epithelium (22, 23). In advanced colorectal cancer, GC-C is expressed on metastatic cells within the bloodstream and is therefore accessible to iv-injected imaging agents (18-20, 23, 26). This contrasts with GC-C expression in normal intestinal epithelia, which is primarily on the luminal face of the epithelium and isolated from the bloodstream by cell-cell tight junctions. This allows imaging agents targeting GC-C to achieve high specificity for colorectal cancer with low uptake in non-target tissues (18-20).

In the studies described here, the effects of L9F and Y19F substitutions and deletion of the N-terminal amino acid tail of the ST$_h$ peptide were studied. Wild-type ST$_h$ displays a 5 amino acid sequence at its N-terminus (NSSNY-), which lies outside of the disulfide-stabilized 6-18 core of the molecule. Deletion of the N-terminal asparagine residue allows for the preparation of a more stable peptide analog due to inhibition of possible formation of an N-terminal pyroglutamate residue in the full-length molecule. Further elimination of the N-terminal 4 amino acids (SSNY-) in an ST$_h$ analog bearing an L9F substitution and a wild-type Y19 residue slightly decreased receptor binding affinity, as assessed by IC$_{50}$ analysis using ^{125}I-F^{19}-ST$_h$(1-19) and whole T-84 human colorectal cancer cells (Table I). Addition of the DOTA chelating moiety to this analog did however significantly decrease receptor-binding affinity, suggesting that the close proximity of the DOTA moiety to the receptor-binding region of STh interferes with binding through steric and/or charge effects. DOTA addition to the analog bearing the 4 amino acid spacer present in native STh did not have such a dramatic effect on receptor binding affinity. Coordination of In^{3+} by the peptide-linked DOTA moiety results in formation of a neutral complex. The fact that ^{nat}In coordination did not significantly alter IC$_{50}$ values of either peptide indicates that the disruptive effect on receptor binding of DOTA incorporation into F^{9}-ST$_h$(6-19) is primarily due to steric hindrance, rather than electrostatic effects.

Despite demonstrable differences in hydrophobicity between these two labeled peptides (Figure 2), *in vivo* biodistribution of the two compounds was similar. Each compound cleared rapidly from the bloodstream into the urine, with >95% of the injected dose excreted into urine at 4 h p.i. Only one significant difference was observed in tissue distribution between these two compounds at 4 h p.i., that is, the extent to which each compound was retained within T-84 human tumor xenografts (Figure 5). The 2- to 3-fold decrease in tumor localization for the ^{111}In-DOTA-F^{9}-ST$_h$(6-19) analog mirrors the decrease in receptor binding affinity of this species *in vitro*, and serves to emphasize the importance of incorporation of the appropriate linker moiety between the chelator and the core 6-19 ST pharmacophore.

Previous results obtained using full-length ST$_h$(1-19) analogs, either with or without an additional 11 carbon aliphatic spacer group demonstrated, neither increased receptor binding affinity nor increased tumor localization at 4 h p.i. *in vivo,* as a result of including this spacer group (20). This suggests that the negative impact of the chelating moiety on receptor binding of ST$_h$ peptides can be entirely attenuated by inclusion of the intervening 5 amino acid N-terminal "tail" present in wild-type ST$_h$. However, tumor uptake at 4 h p.i. of the ^{111}In-DOTA-F^{19}-ST$_h$(2-19) analog described here is significantly lower than that of the previously described ^{111}In-DOTA-F^{19}-ST$_h$(1-19) analog. Taken together, results for these two species, which only differ by a single amino acid deletion in the N-terminal linker, suggest that the 5 residue linker sequence may represent the minimal distance required between the 6-18 core and the DOTA moiety for full activity of ST$_h$ conjugates. *In vivo* SPECT images obtained with ^{111}In-DOTA-F^{19}-ST$_h$(2-19) demonstrate the specific tumor localization coupled with low background in nontarget tissues obtainable using radiolabeled ST$_h$ analogs.

Conclusion

Two novel *E. coli* heat-stable enterotoxin analogs were radiolabeled with N-terminal ^{111}In-DOTA moieties and compared *in vitro* and *in vivo*. Each indium-labeled peptide bound specifically to the GC-C receptor expressed on T-84 human colorectal cancer cells. However, placement of the DOTA chelating group immediately proximal to the Cys6 residue in ST$_h$ was found to inhibit

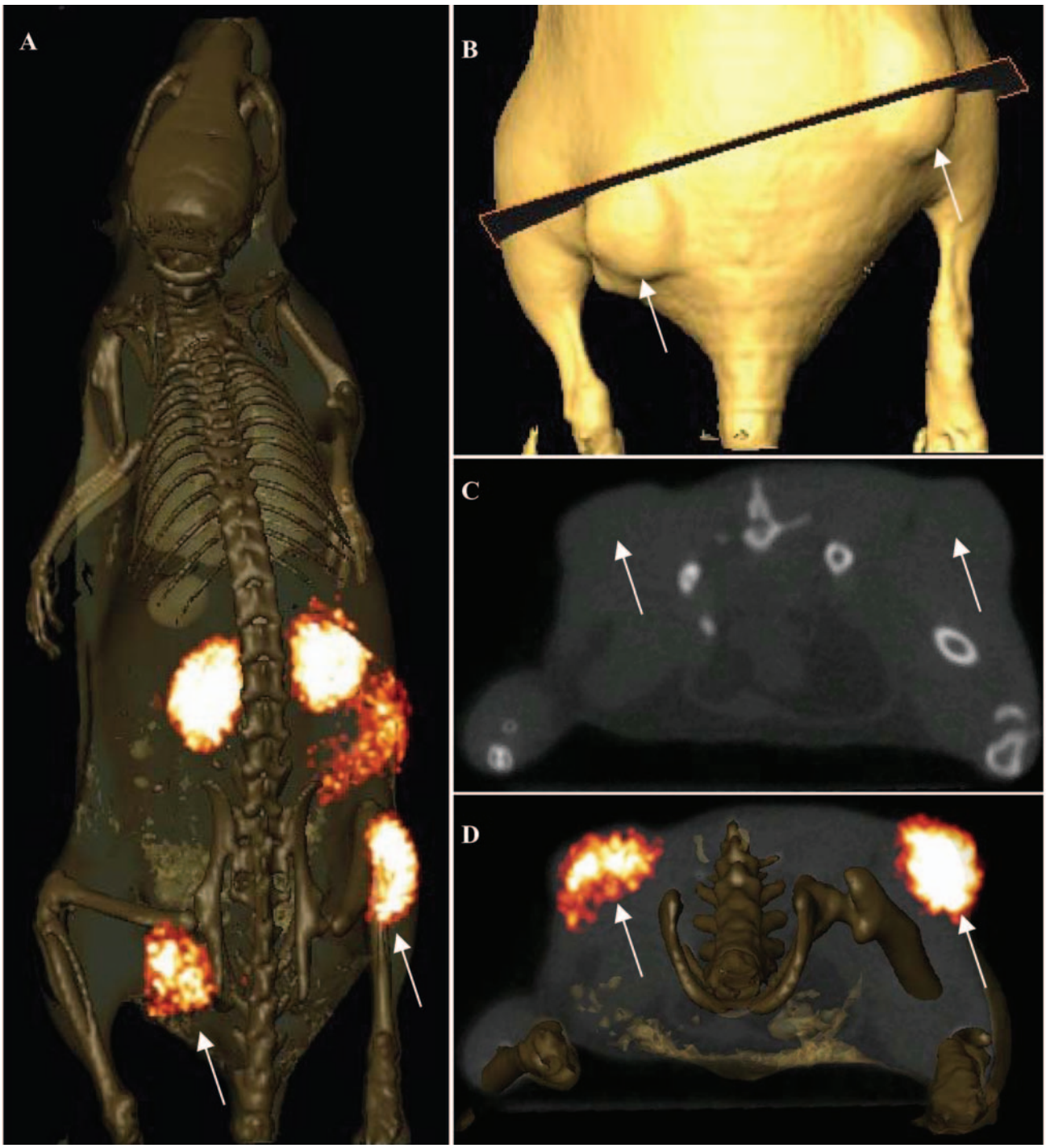

Figure 6. *In vivo SPECT/CT image of ^{111}In-DOTA-F^{19}-ST$_h$(2-19) in a SCID mouse bearing two hind flank T84 human colorectal cancer tumor xenografts. Data was acquired on a Siemens Medical Systems MicroCAT II Micro-SPECT/CT System 1 h post-injection of 1.6 mCi labeled peptide. A. Whole body fused SPECT/CT image. B. Skin surface reconstruction from micro X-ray CT data, showing cross section detailed in C, D. C. Transaxial X-ray CT image. D. Transaxial fused SPECT/CT image. Arrows mark locations of tumors.*

receptor binding both *in vitro* and *in vivo*. Inclusion of a four amino acid spacer group between the DOTA moiety and the ST$_h$(6-18) pharmacophore resulted in an ST$_h$ conjugate with increased receptor binding affinity and increased ability to successfully localize T-84 human tumor xenografts *in vivo*.

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