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Evaluation of copper-64-labeled somatostatin agonists and antagonist in *sstr2*-transfected cell lines that are positive and negative for p53: implications for cancer therapy

Kim Nguyen^{1,3}, Jesse J. Parry², Buck E. Rogers^{1,2}, and Carolyn J. Anderson^{1,3,4,*}

¹Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO, USA.

²Department of Radiation Oncology, Washington University School of Medicine, St. Louis, MO, USA.

³Department of Chemistry, Washington University, St. Louis, MO, USA.

Abstract

Objectives—Radiolabeled somatostatin analogs have become important agents for molecular imaging and targeted radiotherapy of somatostatin receptor-positive tumors. Here we determine the effect of the tumor suppressor protein, p53, on trafficking ⁶⁴Cu to tumor cell nuclei from DOTA vs. CB-TE2A-conjugated agonist Y3-TATE and the antagonist ⁶⁴Cu-CB-TE2A-sst2-ANT in cell lines that are positive or negative for p53.

Methods—Receptor binding, internalization, cAMP and nuclear localization studies were performed with the SSTR2 agonists, ⁶⁴Cu-CB-TE2A-Y3-TATE and ⁶⁴Cu-DOTA-Y3-TATE vs. antagonist, ⁶⁴Cu-CB-TE2A-sst2-ANT, in SSTR2-transfected p53 +/+ and -/- HCT116 colorectal carcinoma cells.

Results—The antagonist, ⁶⁴Cu-CB-TE2A-sst2-ANT, bound 8-9-fold more SSTR2 binding sites than did the ⁶⁴Cu-labeled agonists. ⁶⁴Cu-CB-TE2A-Y3-TATE was more efficiently internalized than ⁶⁴Cu-DOTA-Y3-TATE, while ⁶⁴Cu-CB-TE2A-sst2-ANT showed lower, yet significant levels of internalization. CB-TE2A-Y3-TATE acted as a full agonist, inhibiting cAMP production, whereas CB-TE2A-sst2-ANT showed no inhibition of cAMP production. The ⁶⁴Cu from agonists ⁶⁴Cu-DOTA-Y3-TATE and ⁶⁴Cu-CB-TE2A-Y3-TATE showed greater nuclear localization at 24 h in p53 +/+ vs. -/- cells; however, there was no difference in the levels of ⁶⁴Cu from the antagonist based on p53 status. Surprisingly, the DOTA and CB-TE2A-conjugated agonists showed similar nuclear localization in the p53 +/+ and -/- cells, suggesting no difference in ⁶⁴Cu release from these chelators in the HCT116 cell lines.

Conclusion—Based on these *in vitro* data, the agonist ⁶⁴Cu-CB-TE2A-Y3-TATE demonstrated the most promise as an agent for targeted radiotherapy in p53 positive, SSTR2-positive tumors.

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*Corresponding Author: Mallinckrodt Institute of Radiology Washington University School of Medicine 510 S. Kingshighway Blvd., Campus Box 8225 St. Louis, MO 63110 Phone: 314-362-8427 Fax: 314-362-9940 andersoncj@upmc.edu .

⁴Current address: Department of Radiology University of Pittsburgh 100 Technology Drive, Suite 450K Pittsburgh, PA 15219 Phone: 412-624-6887 andersoncj@upmc.edu

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Keywords

Copper-64; somatostatin; agonist; antagonist; p53

INTRODUCTION

Radiolabeled somatostatin analogs have become important agents for molecular imaging and targeted radiotherapy of somatostatin receptor-positive tumors. While considerable research has been conducted to develop targeted ligands for all 5 receptor subtypes, the somatostatin receptor subtype 2 (SSTR2) has received the most attention since it is expressed in variety of malignancies [1-10].

Considerable research has focused on the development of somatostatin agonists and antagonists [11, 12]. The somatostatin agonist, Y3-TATE, has higher affinity for the somatostatin receptor than other agonists, such as Y3-OC or OC, both *in vitro* and *in vivo* [13-15]. Y3-TATE conjugated to CB-TE2A and labeled with ^{64}Cu demonstrated higher tumor uptake for somatostatin-positive tissues, lower accumulation in blood and liver at all time points, and less accumulation in the kidney at earlier time points compared to ^{64}Cu -TETA-Y3-TATE [15]. While agonists readily internalize into tumor cells, antagonists do not, and therefore until recently were not considered for tumor targeting. The SSTR2 antagonist, ^{64}Cu -CB-TE2A-sst2-ANT, demonstrated excellent tumor-to-background contrast and better tumor-to-blood and tumor-to-muscle ratios, but slower liver and kidney clearance compared to the agonist ^{64}Cu -CB-TE2A-Y3-TATE in AR42J tumor-bearing rats [16]. This suggests that even though somatostatin antagonists do not internalize, they have high binding affinity and high, prolonged tumor uptake that may result in an improved PET imaging agent.

In addition, it has been shown that the degree of ^{64}Cu localization in tumor cell nuclei depends on the stability of the Cu(II) chelator [17, 18]. Therefore, in this study the less stable chelator DOTA was compared to more stable CB-TE2A to determine whether ^{64}Cu -chelate stability affects ^{64}Cu localization. The more metabolically stable ^{64}Cu -CB-TE2A-Y3-TATE has shown higher tumor and lower non-specific organ uptake compared to less stable ^{64}Cu -TETA-Y3-TATE; therefore, a more stable chelating agent may be a better choice for imaging [15]. Conversely, ^{64}Cu is more likely to dissociate from a weaker chelating agent resulting in higher nuclear localization, which may be beneficial for cancer therapy [18, 19].

The p53 protein, which induces cell cycle arrest and/or apoptosis in response to various stress conditions, has been studied extensively because of its role as a tumor suppressor. The loss of p53 tumor-suppressor activity, either by mutation/deletion of the TP53 gene or by inhibition of the p53 protein, favors the development of cancer. p53 is involved in effective cancer treatment in which radiotherapy induces various proteins that recognize damage and transmit this information to p53, thus inducing cell death [20]. However, p53 is essentially kept inactive in non-stressed cells through the actions of the ubiquitin ligase MDM2 and is activated by various stress mechanisms (i.e. chemotherapy agents, chemical carcinogens, activated oncogenes, hypoxia etc.) [21, 22].

The tumor suppressor protein, p53, may directly or indirectly be involved in ^{64}Cu nuclear localization [19, 23]. Wang and co-workers showed that ^{64}Cu from ^{64}Cu -TETA-octreotide localized in the nucleus of tumor cells, and hypothesized it occurred by ^{64}Cu dissociation from the chelate, although the exact mechanism remains unknown [18]. Eiblmaier and colleagues observed higher nuclear accumulation of ^{64}Cu in the p53 positive human

HCT116+/+ colorectal cancer cell line when compared to the same cell line that does not express p53 (HCT116-/-) [23], suggesting p53 involvement in ^{64}Cu trafficking into the nucleus of tumor cells. In HCT116-/- cells, both wild-type alleles of p53 present in the HCT116+/+ clone were knocked out [24]) providing a well-controlled system for evaluating the effects of p53 on tumor imaging and therapy. These findings may have important implications for cancer therapy since the transport of ^{64}Cu , which emits β^- particles and Auger electrons, may deliver a higher radiation dose to nuclear DNA resulting in enhanced tumor cell killing.

A series of *in vitro* experiments were designed to elucidate the role of p53 in trafficking ^{64}Cu into tumor cell nuclei by comparing nuclear localization studies of three ^{64}Cu -labeled somatostatin analogs in p53 positive (+/+) and p53 negative (-/-) HCT116 cells transfected with SSTR2. As it was previously reported that the degree of ^{64}Cu localization depends on the stability of the Cu(II) chelator [17], nuclear uptake of ^{64}Cu was evaluated for two somatostatin agonists (Y3-TATE containing a weaker (DOTA) and stronger (CB-TE2A) Cu(II) chelator). Furthermore, it has been reported that ligand composition is a critical factor in the intracellular trafficking and retention of somatostatin analogs [25]. To assess this, nuclear localization studies of ^{64}Cu -CB-TE2A conjugated to somatostatin agonist (Y3-TATE) vs. antagonist (sst2-ANT) were performed. Understanding the molecular mechanisms of p53 in ^{64}Cu nuclear localization may aid in the design of improved imaging and therapeutic agents.

MATERIALS AND METHODS

General

Copper-64 was produced as previously described on a biomedical CS-15 cyclotron at Washington University School of Medicine [26]. Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint Louis, Missouri). 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono(N-hydroxysuccinimide ester) (DOTA-NHS) was obtained from Macrocyclics (Dallas, Texas), and 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (CB-TE2A) was synthesized as previously described [27]. Y3-TATE was synthesized and conjugated to DOTA and CB-TE2A by CSBio (San Diego, California) using previously published methods [16]. The somatostatin antagonist, sst2-ANT, was also synthesized and conjugated to CB-TE2A (CSBio) using previously published methods [28]. SSTR2-transfected HCT116 cell medium (Iscove's) was obtained from Gibco (Carlsbad, California) and supplemented with 10% fetal bovine serum (FBS, Gibco) and 1 mM Zeocin (Gibco).

Preparation of SSTR2-positive HCT116+/+ and -/- clones

The stable transfection of human colorectal cancer cells HCT116+/+ and HCT116-/- (provided by Dr. Bert Vogelstein, Johns Hopkins University) with SSTR2 was performed as previously described [29]. Briefly, the full length human SSTR2 containing an N-terminal hemagglutinin(HA) epitope was cloned into pcDNA3.1/Zeo (Invitrogen; Carlsbad, California) to produce pcSSTR2. The resulting plasmid was then stably transfected into both HCT116+/+ and HCT116-/- using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. The following day, the transfected cells were collected, diluted, and plated with the addition of Zeocin for the formation of colonies. The cells were monitored daily, and upon colony formation, isolated colonies for each cell line were picked for expansion and subsequent analysis.

Radiochemistry

Preparation of ^{64}Cu -labeled somatostatin analogs—Radiolabeling of DOTA-Y3-TATE with ^{64}Cu was achieved by incubating DOTA-Y3-TATE (1 μg) with 30–37 MBq (0.8–1 mCi) of [^{64}Cu]cupric acetate in 100 μl of 0.1 M ammonium acetate (pH 5.5) for 1 h at 95°C. CB-TE2A-Y3-TATE and CB-TE2A-sst2-ANT (1 μg) were each labeled with 30–37 MBq (0.8–1 mCi) of [^{64}Cu]cupric acetate by incubation in 100 μl of 0.1 M ammonium acetate (pH 8.1) for 1.5 h at 95°C. For all ^{64}Cu -labeled conjugates, radiochemical purity was confirmed by radio-TLC or radio-HPLC.

Thin-layer chromatography of ^{64}Cu -labeled somatostatin analogs was performed using thin-layer chromatography plates (MKC18F silica gel 60 Å, 2.5×7.5 cm, layer thickness 200 μm ; Whatman, Florham Park, New Jersey) with a mobile phase of 10% ammonium acetate:methanol(3:7). High-performance liquid chromatography of ^{64}Cu -labeled somatostatin analogs was performed using an Alltima C18 3 μ Rocket column with a mobile phase of A (water/0.1% trifluoroacetic acid) and B (acetonitrile/0.1% trifluoroacetic acid) = 0–40% eluted at a flow rate of 3 ml/min.

Flow cytometry

Flow cytometry was used to sort out high SSTR2 expressing HCT116 clones against the HA epitope inserted on the N-terminus of SSTR2. FACS analysis was performed using a FACScan flow cytometer (BD Biosciences, San Jose, California), and data were analyzed using CellQuest Pro (BD Biosciences). Cells were harvested with a cell scraper, washed twice with PBS, counted, and resuspended (1×10^7 cells per 50 μl) in a 96-well V bottom plate (Midwest Scientific, Saint Louis, Missouri) containing the respective growth medium. Anti-hemagglutinin monoclonal antibody conjugated with Alexa Fluor 488 (1 ml of 1:10 dilution; 1 mg/ml) (Covance, Emeryville, California) was added to the cell suspension and incubated on ice for 1 h in the dark. Wild type HCT116+/+ and HCT116–/– cells incubated with this antibody were used as a negative control. After the incubation, the antibody medium was removed, and the plate was washed twice with 1 ml PBS. The cells were resuspended in 1 ml PBS and transferred to FACS tubes (VWR, West Chester, Pennsylvania).

Receptor binding studies

SSTR2-transfected HCT116 cell membrane preparations were used for receptor binding assays using previously described methods [17]. Specific binding was obtained by subtraction of non-specific binding from total binding. Maximum binding capacities (B_{max}) and binding affinities (K_d) were determined from non-linear curve regression of specific binding vs. the concentration of the ^{64}Cu radiopharmaceutical using Prism v. 5.0 (GraphPad; San Diego, CA).

Internalization studies

Before each assay, SSTR2-transfected HCT116+/+ and HCT116–/– cells (1×10^6 cells per 500 μl) were harvested with a cell scraper, washed twice with PBS, counted, and resuspended in 1.7-ml microcentrifuge tubes containing growth medium as previously described [16]. The percentage of radioligand internalized was the amount of activity in the final cell pellet counted against a standard, and normalized to protein content.

Intracellular cAMP assay

The intracellular levels of induced cAMP were measured using the cAMP Biotrak EIA System non-acetylation procedure (GE Healthcare Bio-Sciences, Piscataway, New Jersey). Briefly, HCT116+/+ SSTR2 cells were seeded in a 96-well plate at a concentration of 5×10^4

cells/well. On the following day, the media from each well was aspirated and replaced with 180 μ l of DMEM containing 10 μ M forskolin plus either somatostatin-14, CB-TE2A-Y3-TATE, or CB-TE2A-sst2-ANT at concentrations ranging from 0–100 nM, in triplicate. Isobutylmethylxanthine was added to the DMEM at a final concentration of 1 mM to act as non-specific inhibitor of cAMP phosphodiesterase. The cells were incubated at 37°C for 30 min, after which 20 μ l of lysis reagent was added to each well with mechanical manipulation and a 20 min room temperature incubation to facilitate cell lysis. An aliquot of each lysate was diluted 50-fold in sample buffer, and 100 μ l of each diluted lysate was analyzed for cAMP content following the manufacturer's instructions. The data are plotted as the percent of intracellular cAMP produced upon treatment with various concentrations of somatostatin analogs, normalized to the amount produced in the absence of these ligands.

Nuclear localization studies

Isolation of HCT116 tumor cell nuclei was performed according to previously described methods with some modifications [18]. The experiments were performed in T-25 flasks containing approximately 3×10^7 cells at the beginning of the time course. Cisplatin suspended in 1X PBS was added to T-25 flasks to a final concentration of 40 μ M per flask to stress the cells and induce p53 expression, and this was then incubated for 24 h at 37°C, 5% CO₂, before the addition of ⁶⁴Cu-labeled somatostatin analogs. After 24 h, media containing cisplatin was replaced with fresh media before adding ⁶⁴Cu-labeled somatostatin analogs. Copper-64-labeled somatostatin analogs were added in a ratio of receptor:peptide of 3:1, as determined by the respective B_{max}, and after various incubation times, the cells were pelleted, resuspended in CSK buffer (0.5% Triton X-100, 300 mM sucrose, 100 mM NaCl, 1 mM EGTA, 2 mM MgCl₂, and 10 mM PIPES, pH 6.8) and incubated on ice for 2 min. Cell lysates were centrifuged at 560 x g for 5 min at 4°C, and the supernatant was discarded. The nuclear pellet was resuspended in 1 ml CSK buffer without Triton X-100, incubated on ice for 2 min, and centrifuged at 560 x g at 4°C for 5 min. The supernatant was discarded, and the nuclear pellet was counted on a gamma counter. To confirm purity, aliquots of nuclei were assayed qualitatively by fluorescence microscopy after staining with a 1:10 dilution of FITC (3 μ g/ml) and propidium iodide (PI) (7 μ g/ml). The identification of nuclei by microscopy was evaluated using a double staining technique with FITC (cytoplasm and membrane) and PI (nuclei). Micrographs were obtained at 400-fold magnification. The percentage of cell associated activity was determined by the gamma counts of the pure nuclei divided by gamma counts internalized in whole cells.

Statistical analysis

A Student T-test (two-tailed, unpaired) was performed using Prism software. A p-value less than 0.05 was considered significant.

RESULTS

Radiochemistry

The radiolabeling of ⁶⁴Cu-DOTA-Y3-TATE, ⁶⁴Cu-CB-TE2A-Y3-TATE, and ⁶⁴Cu-CB-TE2A-sst2-ANT reactions [30] proceeded to 70-99% completion after heating. The R_f's for ⁶⁴Cu-DOTA-Y3-TATE, ⁶⁴Cu-CB-TE2A-Y3-TATE, and ⁶⁴Cu-CB-TE2A-sst2-ANT were 0.59, 0.63, and 0.53, respectively, by radio-TLC, whereas free ⁶⁴Cu remained at the origin. These results were confirmed by radio-HPLC, where the retention times were 5.4, 5.5, and 6.2 min for ⁶⁴Cu-DOTA-Y3-TATE, ⁶⁴Cu-CB-TE2A-Y3-TATE, and ⁶⁴Cu-CB-TE2A-sst2-ANT, respectively. Non-complexed ⁶⁴Cu eluted at 0.5-1 min. For reactions with less than 95% radiochemical purity, free ⁶⁴Cu was chelated using ethylenediaminetetraacetic acid (EDTA) and purified with a C-18 SepPak Light cartridge (Waters, Milford, Massachusetts). Approximately 75% of the radiolabeled complex was eluted from the cartridge using

absolute ethanol containing 0.5% acetic acid. A speed vacuum was used to evaporate most of the solvent, and the product was re-dissolved in an appropriate solvent for *in vitro* or *in vivo* studies. Final radiochemical purity of >95% was confirmed by radio-TLC and/or radio-HPLC, and a specific activity range of 18-37 MBq/μg was obtained.

Flow cytometry

HCT116+/+ clones 4, 17, and 39, and HCT116-/- clones 3, 12, 17, 26, 29, and 31 showed the highest levels of expression (Figure 2A and 2B). SSTR2 expression in HCT116+/+ cells was higher than in HCT116-/- cells. Wild-type HCT116+/+ and HCT116-/- were used as negative controls. HCT116+/+ clone 4 and HCT116-/- clone 3 were utilized for all subsequent experiments.

Receptor binding studies

⁶⁴Cu-DOTA-Y3-TATE vs. ⁶⁴Cu-CB-TE2A-Y3-TATE (Agonists)—The B_{max} values for ⁶⁴Cu-DOTA-Y3-TATE and ⁶⁴Cu-CB-TE2A-Y3-TATE in SSTR2-transfected HCT116+/+ clone 4 cells were 4700 ± 270 and 4200 ± 200 fmol/mg, respectively (Table 1). The dissociation constants (K_d) for ⁶⁴Cu-DOTA-Y3-TATE and ⁶⁴Cu-CB-TE2A-Y3-TATE were 1.6±0.4 and 0.5±0.2 nM, respectively. These values are in the range of those previously reported for ⁶⁴Cu-CB-TE2A-Y3-TATE in AR42J rat pancreatic tumor cells [15].

The B_{max} values for ⁶⁴Cu-DOTA-Y3-TATE and ⁶⁴Cu-CB-TE2A-Y3-TATE in HCT116-/- clone 3 cells were 3400 ± 150 and 2900 ± 160 fmol/mg, respectively (Table 1). The SSTR2 expression was higher in HCT116+/+ clone 4 compared to HCT116-/- clone 3, which is consistent with FACS analysis. The two agonists occupy a similar number of receptor sites on the cell surface, similar to what was observed for HCT116+/+ cells. The dissociation constant K_d for these two agonists (⁶⁴Cu-DOTA-Y3-TATE: K_d = 1.5 ± 0.3 nM and ⁶⁴Cu-CB-TE2A-Y3-TATE: K_d = 0.5 ± 0.1 nM) are consistent with previously reported values and are similar compared to HCT116+/+ cells from above [15].

⁶⁴Cu-CB-TE2A-sst2-ANT (Antagonist)—The number of binding sites (B_{max}) for the antagonist, ⁶⁴Cu-CB-TE2A-sst2-ANT, in SSTR2-transfected HCT116+/+ clone 4 was found to be 38,000 ± 2100 fmol/mg, which is 8-fold higher than agonist ⁶⁴Cu-CB-TE2A-Y3-TATE (B_{max} = 4200 ± 200 fmol/mg). This phenomenon was observed previously with AR42J cells where the antagonist/agonist ratio was a > 14-fold [16]. The binding affinity for ⁶⁴Cu-CB-TE2A-sst2-ANT was significantly lower than ⁶⁴Cu-CB-TE2A-Y3-TATE (10.5 ± 1.7 nM vs. 0.5 ± 0.2 nM) (p = 0.04), verifying that the agonist has higher binding affinity than the antagonist. The K_d value from the Scatchard plot (17.9 nM) (13.12-28.28 nM) in HCT116+/+ clone 4 cells is in the range of the confidence interval as the published value for ⁶⁴Cu-CB-TE2A-sst2-ANT in AR42J cells (26 ± 2.4 nM)(21.72-31.17 nM) [16].

In the HCT116-/- clone 3 cells, the number of binding sites for the antagonist, ⁶⁴Cu-CB-TE2A-sst2-ANT, (B_{max} = 25000 ± 1700 fmol/mg) is 8-fold higher than the agonist, ⁶⁴Cu-CB-TE2A-Y3-TATE (B_{max} = 2900 ± 160 fmol/mg) (p = 0.008); however, the antagonist has a lower SSTR2 binding affinity (K_d = 12 ± 2.4 nM) than the agonist. Table 1 summarizes the binding parameters for these three somatostatin analogs in SSTR2-transfected HCT116+/+ cell lines.

Internalization studies

⁶⁴Cu-DOTA-Y3-TATE vs. ⁶⁴Cu-CB-TE2A-Y3-TATE (Agonists)—The nonzero y-intercept suggested a rapid early internalization phase at 10 min, followed by a steady rise at 60 min, and there is no significant change in the radiotracer internalization at 120 min (Figures 3A and 3B). Addition of excess Y3-TATE with the respective radiotracer, as well as

incubation at 4°C, inhibited the internalization for both agonists. However, the ^{64}Cu conjugate with the more stable chelator, CB-TE2A, is observed to be internalized more efficiently than the conjugate containing the less stable chelator, DOTA. This phenomenon was observed in both HCT116 clones, but internalization was greater in the positive clone due to the higher number of binding sites.

^{64}Cu -CB-TE2A-sst2-ANT (antagonist)—A rapid internalization was observed during the first 10 min followed by a plateau at 120 min, which signifies no significant change in the radiotracer internalization (Figures 3C and 3D). Addition of excess cold peptides along with the respective radiotracer, as well as incubation at 4°C, inhibited the internalization for both agonist and antagonist. However, although ^{64}Cu -CB-TE2A-sst2-ANT internalized to a lesser extent (~2-fold less) compared to the agonist ^{64}Cu -CB-TE2A-Y3-TATE, the antagonist internalized at a significant level. This phenomenon was observed in both SSTR2-transfected HCT116 cell lines. ^{64}Cu -CB-TE2A-sst2-ANT internalized to a greater extent in SSTR2-transfected HCT116+/+ clone 4 compared to HCT116-/- clone 3 due to the higher number of binding sites.

Intracellular cAMP

HCT116+/+ SSTR2 clone 4 cells were treated with increasing concentrations of either somatostatin-14, CB-TE2A-Y3-TATE, or CB-TE2A-sst2-ANT in order to determine the effects on intracellular cAMP production (Figure 4). The cells were treated with forskolin to induce cAMP production, and the IC_{50} values for the compounds effect on cAMP inhibition were determined using a sigmoidal dose response binding analysis. Somatostatin-14 produced an IC_{50} of 1.0 ± 0.1 nM, which was not significantly different from the IC_{50} value of 0.4 ± 0.2 nM for CB-TE2A-Y3-TATE. However, when the cells were treated with CB-TE2A-sst2-ANT there was no inhibition of cAMP production even at the highest concentration, suggesting that CB-TE2A-sst2-ANT is an antagonist.

Nuclear localization studies

Agonists ^{64}Cu -DOTA-Y3-TATE vs. ^{64}Cu -CB-TE2A-Y3-TATE—In order to evaluate the role of p53 in trafficking ^{64}Cu to tumor cell nuclei, nuclear localization studies were performed with the ^{64}Cu -labeled agonists in the SSTR2-transfected HCT116 cell lines. Without the addition of the stress factor, cisplatin, no difference in nuclear localization was observed for p53 positive vs negative cells for both agonists (Figure 5A). However, significantly more nuclear localization of ^{64}Cu was observed for p53 positive vs. negative cells with the addition of cisplatin (Figure 5B) since cisplatin promoted p53 expression as shown by Western plot analysis (Figure 5D). The nuclear uptake was low initially, but increased substantially at 24 h, suggesting that p53 plays a role in ^{64}Cu trafficking into the tumor cell nuclei. In addition, there was no difference in nuclear localization for the ^{64}Cu -DOTA compared to the ^{64}Cu -TE2A conjugate. The 24 h data (^{64}Cu -DOTA-Y3-TATE (p53 positive = 17.5 ± 2.59 % CAA vs. p53 negative = 7.72 ± 2.04 % CAA) ($p=0.006$) and ^{64}Cu -CB-TE2A-Y3-TATE (p53 positive = 18.5 ± 3.89 % CAA vs. p53 negative = 5.56 ± 0.13 % CAA) ($p=0.03$)) suggest that the degree of ^{64}Cu nuclear localization did not depend on the stability of Cu(II) chelator in these cell lines ($p > 0.05$ for both cell lines).

Agonist ^{64}Cu -CB-TE2A-Y3-TATE vs. antagonist ^{64}Cu -CB-TE2A-sst2-ANT—Wadas and colleagues demonstrated that the somatostatin antagonist, ^{64}Cu -CB-TE2A-sst2-ANT, internalized 10-fold less than the agonist, ^{64}Cu -CB-TE2A-Y3-TATE in rat pancreatic AR42J cell line [16]. Here we conducted experiments to compare the nuclear localization of the ^{64}Cu -labeled somatostatin agonist and antagonist and whether p53 mediated nuclear localization. In contrast to the the agonist ^{64}Cu -CB-TE2A-Y3-TATE (p53 positive = 18.48 ± 3.89 vs. p53 negative = 5.56 ± 0.13 as % CAA at 24 h) ($p=0.03$), similar nuclear localization

of ^{64}Cu -CB-TE2A-sst2-ANT was observed in both cell lines (p53 positive = 7.98 ± 1.44 vs. p53 negative = 8.81 ± 1.02 as %CAA at 24 h) ($p=0.82$))(Figure 5C). To correct for the increased internalization of ^{64}Cu -CB-TE2A-Y3-TATE, the data were plotted as a percentage of cell-associated activity (%CAA) rather than %ID/mg. The higher nuclear uptake of ^{64}Cu from the agonist vs. antagonist suggests that structural modifications may effect nuclear localization of ^{64}Cu .

DISCUSSION

Radiolabeled somatostatin analogs have been evaluated for both diagnostic and therapeutic applications. A significant number of studies involving radiolabeled somatostatin analogs have been performed with rat pancreatic cell lines AR42J and CA20948 [31-33]. Here, the human colorectal cancer cell line HCT116 was stably transfected with hemagglutinin(HA)-tagged human SSTR2. HCT116+/+ and -/- cells were utilized because they provide a model system for evaluating the role of p53 in ^{64}Cu intracellular trafficking.

Small structural modifications of the radioligand molecule, such as the introduction of a metal, or the alteration of a metal or a chelator, may considerably affect the binding properties of the analogs for selected SSTR subtypes and may have an important impact on the quality of *in vivo* imaging and therapy [11]. As a result, two BFCs (DOTA vs. CB-TE2A) with different chelating stabilities were compared in the SSTR2-transfected HCT116 cell lines. Similar receptor levels ($B_{\max}$) and binding affinities (K_d) were observed for the two agonists, ^{64}Cu -DOTA-Y3-TATE and ^{64}Cu -CB-TE2A-Y3-TATE. These results differed from what was observed previously with AR42J cells for ^{64}Cu -TETA-Y3-TATE ($B_{\max} = 192$ fmol/mg) and ^{64}Cu -CB-TE2A-Y3-TATE ($B_{\max} = 1596$ fmol/mg) [15], and this may be attributed to a broader specificity to SSTR binding for ^{64}Cu -CB-TE2A-Y3-TATE than for ^{64}Cu -TETA-Y3-TATE, since AR42J tumors express SSTR2 as well as SSTR1, SSTR3, and SSTR5 [15]. In addition, it may reflect the difference in binding affinity for ^{64}Cu -CB-TE2A-Y3-TATE in AR42J ($K_d = 1.7$ nM), since the assay cannot distinguish binding to various somatostatin receptor subtypes [15]. Furthermore, the data presented here suggest that the change from ^{64}Cu -DOTA to ^{64}Cu -CB-TE2A does not alter the SSTR2 binding profile of the somatostatin analog.

There were two important findings in the saturation binding assay for the somatostatin antagonist ^{64}Cu -CB-TE2A-sst2-ANT. Firstly, although the dissociation constant is an order of magnitude higher (weaker binding affinity) than its agonist ^{64}Cu -CB-TE2A-Y3-TATE, it still had an affinity in the nanomolar range. Secondly, the receptor concentration ($B_{\max}$) is 8-fold greater compared to the agonist ^{64}Cu -CB-TE2A-Y3-TATE. This increase in $B_{\max}$ was also observed for ^{111}In -DOTA-sst2-ANT in sst2-HEK cells and ^{64}Cu -CB-TE2A-sst2-ANT in AR42J cells [16, 34]. The elevated $B_{\max}$ may be attributed to stabilization of sst2-ANT in the lipid-rich environment of the receptor membrane, and its (sst2-ANT) ability to interact and bind to a larger number of SSTR2 conformations, resulting in high tumor uptake [34].

Between the two agonists, faster internalization kinetics were observed for the ^{64}Cu -labeled Y3-TATE analogs containing the more stable chelator CB-TE2A compared to the less stable DOTA. This may be attributed to the stabilization of the more lipophilic CB-TE2A conjugate in the lipid-rich environment of the receptor membrane and/or due to the fact that ^{64}Cu is more likely to dissociate from the weaker chelating agent DOTA before internalization. In addition, after SSTR2/somatostatin complexes are internalized by receptor-mediated endocytosis, both receptors and analogs are recycled to the cell surface, and the analogs can be re-internalized, in which the recycling rate of the ^{64}Cu -CB-TE2A conjugate may be more rapid compared to ^{64}Cu -DOTA [35]. Thus, ^{64}Cu -CB-TE2A-Y3-TATE was

predicted to have improved specific target tissues uptake compared to ^{64}Cu -DOTA-Y3-TATE.

The antagonist internalized at a significant level, in contrast to that observed with ^{111}In -DOTA-sst2-ANT in SSTR2 transfected HEK229 cells [34]. There was no reported internalization for the antagonist ^{111}In -DOTA-sst2-ANT in an immunofluorescence internalization assay using sst2-HEK cells. Reubi et al. have demonstrated that relatively small changes in the radioligand molecule, such as changing the metal (^{67}Ga , ^{90}Y , and ^{111}In) or chelator (DOTA and DTPA) can markedly change the SSTR binding profile of the somatostatin analogs (OC, Y3-OC, and Y3-TATE) [11]. As a result, these small changes (from ^{111}In to ^{64}Cu , and from the chelators DOTA to CB-TE2A) may be sufficient enough to affect the binding of the somatostatin analog, and may explain the significant internalization level of ^{64}Cu -CB-TE2A-sst2-ANT that was observed in this study. Moreover, the results reported here differ from what was observed for ^{64}Cu -CB-TE2A-sst2-ANT in AR42J cells [16]. Wadas et al. reported that ^{64}Cu -CB-TE2A-sst2-ANT internalized weakly, 10-fold less instead of 2-fold less as observed here, than its agonist ^{64}Cu -CB-TE2A-Y3-TATE, in rat AR42J pancreatic tumor cells [16]. It was reported that in human SSTRs-transfected CHO-K1 cells, agonist-induced internalization was significantly different than in rat SSTRs-transfected HEK-293 cells [25, 36, 37]. The differences observed with the two transfected cell lines suggest that the cell type as well as the origin of the SSTR (human vs. rat) might play a role in SSTR internalization. The differences between rat and human cell lines may explain the significant internalization level of ^{64}Cu -CB-TE2A-sst2-ANT in SSTR2-transfected human HCT116 cells compared to AR42J rat pancreatic cancer cells.

To determine whether CB-TE2A-Y3-TATE (agonist) and CB-TE2A-sst2-ANT (antagonist) regulate the same signaling pathway as native SS-14, we measured their ability to inhibit cAMP production in SSTR2-transfected HCT116 cells (Figure 4). CB-TE2A-Y3-TATE inhibited forskolin-stimulated cAMP production at lower concentrations than SS-14, indicating that CB-TE2A-Y3-TATE behaved as a full agonist, whereas there was no inhibition of cAMP production for CB-TE2A-sst2-ANT, even at the highest concentration. Cescato and colleagues observed Tyr³-octreotide inhibiting forskolin-stimulated cAMP accumulation more than SS-14 in SSTR2a-expressing HEK293 and AR42J cells [38]. In addition, Liu et al. demonstrated that the effect of somatostatin analogs to induce receptor internalization did not closely correlate with their effect on receptor signaling as well as with binding affinities in CHO-sst2 cells [39].

Copper-64 emits 2 Auger electrons (840 eV (57.7%) and 6.5 keV (22.5%)), and therefore nuclear localization of this radionuclide may enhance therapeutic efficacy. Nuclear localization is particularly important, as the range for the 840 eV electron is 0.05 μm , and the range for the 6.5 keV electron is less than 1.5 μm , while the diameter of HCT116 tumor cell nuclei was determined to be 12-13 μm [25]. Therefore, optimal cancer radiotherapy using this Auger electron emitter requires nuclear localization of radionuclide in close proximity to tumor DNA to obtain maximum cytotoxicity [40]. In addition, the recoil energy associated with the transmutation of ^{64}Cu to stable ^{64}Ni (β^+ decay) or ^{64}Zn (β^- decay) occurring in the cell nuclei, within the DNA molecule itself, may enhance tumor cell killing, since DNA is very sensitive to changes in its structure [41]. Rapid internalization of SSTR2 ligands was observed in SSTR2-positive tumors via a receptor mediated process, but it is still unknown how ^{64}Cu is transported into the tumor cell nucleus. As a result, it is important to understand the mechanisms of action.

Cisplatin upregulated p53 expression in p53 positive cells (Figure 5D) and higher nuclear localization of ^{64}Cu from the agonists was observed for p53 positive compared to p53 negative cell lines at 24 h (Figure 5B), suggesting that p53 plays a role in ^{64}Cu trafficking

into the tumor cell nuclei. Hainaut et al. demonstrated that copper is capable of displacing zinc from metal binding sites and binds to p53 *in vitro* [42]. p53 may directly bind to ^{64}Cu and transport it into the tumor cell nuclei, or ^{64}Cu may bind to a protein binding partner of p53 that is localized into the tumor cell nuclei. Alternatively, p53 may indirectly affect copper transport to the cell nucleus by acting as a transcriptional activator or inhibitor for one or several unknown proteins involved in the nuclear transport of copper whose genes may be upregulated by p53 after exposure to copper. Genes involved in cell cycle arrest (e.g., *p21*, *Tp53INP1*), apoptotic processes (e.g., *IGFBP-6*, *PUMA*), and heat-shock proteins (e.g., Hsp70, HSP27) were reported to be regulated by p53 after exposure to copper, but their involvement in copper transport has not been reported [43].

Surprisingly, there was no significant difference in nuclear localization between the ^{64}Cu -DOTA and ^{64}Cu -CB-TE2A conjugates, suggesting that Cu(II) chelate stability may not affect the degree of ^{64}Cu nuclear localization in the SSTR2-transfected HCT116 cell lines. However, Eiblmaier et al. reported that higher ^{64}Cu nuclear localization was observed for the weaker chelated ^{64}Cu -TETA-Y3-TATE compared to more stably chelated ^{64}Cu -CB-TE2A-Y3-TATE in the SSTR2-transfected non-small cell lung carcinoma cell line A427 [17]. It is possible that different cell types (A427 vs. HCT116) metabolize somatostatin analogs differently, or may even deliver them to different cellular compartments. Another possibility is that the pH in the lysosome of HCT116 cells is lower than that of A427 cells, and the lower pH may be causing more release of ^{64}Cu from CB-TE2A in HCT116 cells. Other researchers have proposed the transport of intact somatostatin radiopharmaceuticals into the cell nuclei [40]. The data presented here suggest that both ^{64}Cu -DOTA-Y3-TATE and ^{64}Cu -CB-TE2A-Y3-TATE are equally effective at localizing ^{64}Cu to the nucleus in HCT116 cells; however, the greater internalization of ^{64}Cu -CB-TE2A-Y3-TATE would favor this tracer over the DOTA analog. Future studies to address this discrepancy will include determining the stability of the ^{64}Cu -chelator-somatostatin analogs in nuclei of different somatostatin receptor-positive cells. It is important to understand whether the results obtained in the SSTR2-transfected cells will have relevance to *in vivo* or future clinical endeavors.

Similar nuclear localization was observed in the p53 positive and negative cell lines when incubated with the antagonist, ^{64}Cu -CB-TE2A-sst2-ANT (Figure 5B). This may be attributed to structural differences between the agonist Y3-TATE and antagonist sst2-ANT. Roth et al. demonstrated that ligand composition is a critical factor in the intracellular trafficking and retention of somatostatin analogs, and it may also be true for nuclear trafficking [25]. Therefore, the structure of the antagonist sst2-ANT may affect its binding to p53 or to a protein binding partner of p53, thus preventing it from being trafficked into the tumor cell nuclei. As a result, ^{64}Cu -CB-TE2A-sst2-ANT may not be as effective for therapy.

CONCLUSIONS

Experiments with two somatostatin agonists (^{64}Cu -DOTA-Y3-TATE and ^{64}Cu -CB-TE2A-Y3-TATE) in SSTR2-transfected HCT116 cell lines (p53 positive and negative) indicated that the stability of the Cu(II) chelates attached to Y3-TATE did not significantly alter binding affinity and nuclear localization, although the chelator did affect internalization kinetics. The somatostatin antagonist, ^{64}Cu -CB-TE2A-sst2-ANT, showed significant levels of internalization in the HCT116 cell lines; however, the cAMP assay confirmed there was no agonist activity of the agent. The tumor suppressor protein p53 plays a role in ^{64}Cu trafficking into tumor cell nuclei for the somatostatin agonists but not the antagonist. Of the agents studied, ^{64}Cu -CB-TE2A-Y3-TATE shows the most promise as a therapeutic radiopharmaceutical of somatostatin receptor-positive tumors.

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Figure 1.
Structures of somatostatin analogs A) DOTA-Y3-TATE, B) CB-TE2A-Y3-TATE, and C) CB-TE2A-sst2-ANT, that were utilized in this study.

Figure 2.

A) FACS analysis for 42 clones of the SSTR2-transfected HCT116+/+ (p53 positive) cell line. Clones 4, 17, and 39 demonstrated high expression of SSTR2. B) FACS analysis for 35 clones of the SSTR2-transfected HCT116-/- (p53 negative) cell line. Clones 3, 12, 17, 26, 29 and 31 showed high expression of SSTR2. HCT116+/+ clone 4 and HCT116-/- clone 3 were chosen for subsequent studies.

Figure 3. Internalization of two somatostatin agonists, ^{64}Cu -DOTA-Y3-TATE (■) and ^{64}Cu -CB-TE2A-Y3-TATE (●), in SSTR2-transfected HCT116+/+ clone 4 (A) and HCT116-/- clone 3 (B) at 37°C. Internalization results for somatostatin agonist ^{64}Cu -CB-TE2A-Y3-TATE (●) vs. ^{64}Cu -CB-TE2A-sst2-ANT (▲) in SSTR2-transfected HCT116+/+ clone 4 (C), (E), and HCT116-/- clone 3 (D), (F) at 37°C and 4°C, respectively. Blocking studies were performed with the addition of excess cold peptide Y3-TATE or sst2-ANT (^{64}Cu -DOTA-Y3-TATE with block: □, ^{64}Cu -CB-TE2A-Y3-TATE with block: ○, ^{64}Cu -CB-TE2A-sst2-ANT with block: ▲).

Figure 4. Intracellular cAMP measurement of somatostatin-14 (■), CB-TE2A-Y3-TATE (agonist) (▲), and CB-TE2A-sst2-ANT (antagonist) (●) in SSTR2-transfected HCT116/+ clone 4. The data represent the percent of forskolin-induced cAMP production in the presence of increasing concentrations of either somatostatin-14, CB-TE2A-Y3-TATE, or CB-TE2A-sst2-ANT, normalized to the amount produced in the absence of stimulus. Each curve represents the mean $\pm$ SD of 2 experiments, with each experiment consisting of triplicate points.

Figure 5. Nuclear uptake shown as percentage of cell associated activity (%CAA) in SSTR2 transfected HCT116 at 1, 4, and 24 h time points comparing ^{64}Cu -DOTA-Y3-TATE [p53 positive (+/+) (solid line, ■); p53 negative (-/-) (dash line, □)], and ^{64}Cu -CB-TE2A-Y3-TATE [p53 positive (+/+) (solid line, ●); p53 negative (-/-) (dash line, ○)] without (A) and with cisplatin (B); ^{64}Cu -CB-TE2A-Y3-TATE [p53 positive (+/+) (solid line, ●); p53 negative (-/-) (dash line, ○)] and ^{64}Cu -CB-TE2A-ss2-ANT [p53 positive (+/+) (solid line, ▲); p53 negative (-/-) (dash line, △)] with cisplatin (C). Western blot in HCT116 cell lines with and without the addition of 40μM cisplatin at different incubation times (D).

Table 1

Summary of binding affinity data for ^{64}Cu -DOTA-Y3-TATE, ^{64}Cu -CB-TE2A-Y3-TATE, and ^{64}Cu -CB-TE2A-sst2-ANT in SSTR2-transfected HCT116+/+ clone 4 and HCT116-/- clone 3. The units for B_{max} and K_d are fmol/mg and nM, respectively.

	^{64}Cu -DOTA-Y3-TATE	^{64}Cu -CB-TE2A-Y3-TATE	^{64}Cu -CB-TE2A-sst2-ANT
HCT116+/+ clone 4	$B_{\text{max}} = 4700 \pm 270$ $K_d = 1.6 \pm 0.4$	$B_{\text{max}} = 4200 \pm 200$ $K_d = 0.5 \pm 0.2$	$B_{\text{max}} = 38,000 \pm 2100$ $K_d = 10.5 \pm 1.7$
HCT116-/- clone 3	$B_{\text{max}} = 3400 \pm 150$ $K_d = 1.5 \pm 0.3$	$B_{\text{max}} = 2900 \pm 160$ $K_d = 0.5 \pm 0.1$	$B_{\text{max}} = 25,000 \pm 1700$ $K_d = 12 \pm 2.4$