[CANCER RESEARCH 63, 6864–6869, October 15, 2003]

Subcellular Localization of Radiolabeled Somatostatin Analogues: Implications for Targeted Radiotherapy of Cancer[1]

Mu Wang, Amy L. Caruano, Michael R. Lewis,[2] Laura A. Meyer, Robert P. VanderWaal, and Carolyn J. Anderson[3]

Mallinckrodt Institute of Radiology [M. W., A. L. C., M. R. L., L. A. M., C. J. A.], and Department of Radiation Oncology [R. P. V.], Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT

Copper-64 ($T_{1/2}$ = 12.7 h; β^+, 17.4%; β^-, 39%) has been used both in positron emission tomography imaging and in radiotherapy. Copper-64 radiopharmaceuticals have shown tumor growth inhibition with a relatively low radiation dose in animal models; however, the mechanism of cytotoxicity has not been fully elucidated. These studies incorporate the use of somatostatin receptor-positive AR42J rat pancreatic tumor cells *in vitro* to understand the cell killing mechanism of ^{64}Cu by focusing on subcellular distribution of the somatostatin analogues ^{64}Cu-labeled 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid-octreotide (^{64}Cu-TETA-OC) and ^{111}In-labeled diethylenetriaminepentaacetic acid-octreotide (^{111}In-DTPA-OC). Cell uptake and organelle isolation studies were conducted on ^{64}Cu-TETA-OC and ^{111}In-DTPA-OC. Nuclear localization of ^{64}Cu and ^{111}In from ^{64}Cu-TETA-OC and ^{111}In-DTPA-OC, respectively, increased over time, with 19.5 ± 1.4% and 6.0 ± 1.0% in the cell nucleus at 24 h, respectively. In pulse-chase experiments, in which ^{64}Cu-TETA-OC was incubated with AR42J cells for 4 h, it was found that the nuclear localization of ^{64}Cu increased significantly over the next 20 h (from 9.8 ± 1.0% to 26.3 ± 5.4%). In a control pulse-chase experiment, levels of ^{64}Cu from [^{64}Cu]cupric acetate decreased from 4 to 24 h postadministration (20.6 ± 8.7 to 5.4 ± 1.9), suggesting that the redistribution mechanism, or the kinetics of ^{64}Cu from ^{64}Cu-TETA-OC is different from that for ^{64}Cu from [^{64}Cu]cupric acetate. The amount of ^{64}Cu from ^{64}Cu-TETA-OC also increased in the mitochondria over time, with 21.1 ± 3.6% in the mitochondria at 24 h postadministration. These results suggest that localization of substantial quantities of ^{64}Cu to the cell nucleus and mitochondria may contribute to cell killing with ^{64}Cu radiopharmaceuticals.

INTRODUCTION

Over the last several years, considerable progress has been made in the investigation of radiolabeled somatostatin analogues as radiotherapeutic agents for somatostatin-receptor-positive tumors. Because of the use of radiolabeled somatostatin analogues for targeted radiotherapy of cancer (1, 2), the intracellular fate of the radiolabeled somatostatin analogues after binding to cell surface receptors has been a topic of considerable interest (3, 4). For example, in experiments in which ^{111}In-DTPA-OC,[4] a clinically approved imaging agent for somatostatin-receptor-positive tumors in the United States and Europe, was incubated with cells grown in culture, uptake of ^{111}In in the cell nuclei was observed (3). This suggests a possible mechanism for the therapeutic efficacy of this Auger electron-emitting radiopharmaceutical. We are interested in ^{64}Cu [$T_{1/2}$ = 12.7 h; β^+, 0.655 MeV (17.4%); β^-, 0.573 MeV (39%)] because of its decay by β^+ emission for diagnostic imaging by PET, along with decay by β^- emission for cancer therapy applications. The decay characteristics of ^{111}In and ^{64}Cu are summarized in Table 1. ^{64}Cu-TETA-OC, in which TETA = 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid, is a somatostatin analogue that has been shown to have applications for PET imaging and targeted radiotherapy of cancer (5–7). The structures of DTPA-OC and TETA-OC are shown in Fig. 1.

In a previous study, we observed enhanced therapeutic efficacy of an internalizing ^{64}Cu-labeled mAb, ^{64}Cu-BAT-2IT-1A3, in a tumor-bearing rodent model compared with published studies of ^{131}I- or ^{90}Y-labeled mAbs in the same animal model (8). The mechanism for this improved tumor cell killing is unclear. One hypothesis that has been set forth is that, after cellular internalization, ^{64}Cu may localize to the nuclei of tumor cells because of the dissociation of the radiometal from macrocyclic chelators *in vivo* (9). Copper-64 decays by β^- (and β^+) emission, and it would seem that the range of β particles is too large for nuclear uptake to affect tumor cell killing. There is considerable evidence for the binding of Cu(II) to DNA and/or other structures in the nucleus. Copper ions have been suggested to play an important role in the maintenance of nuclear matrix organization and DNA folding (10, 11). Chiu *et al.* have found that treatment of isolated nuclei with levels as low as 1 μM Cu(II) causes nuclear matrix-associated DNA to bind to nuclear matrix proteins and further, Cu(II) causes DNA protein cross-linking, as well as DNA double-strand breaks on subsequent irradiation (12, 13). The fact that copper has been shown to bind to radiation-sensitive areas in the nucleus, such as the nuclear matrix proteins and DNA, suggests that in comparison with other radionuclides, radiopharmaceuticals labeled with copper radioisotopes may have enhanced effectiveness for targeted radiotherapy.

To investigate this hypothesis further, we chose to evaluate the subcellular distribution of ^{64}Cu-TETA-OC compared with ^{111}In-DTPA-OC, because radiolabeled somatostatin analogues are known to be internalized (14). Other investigators have observed the localization of ^{111}In to the nuclei of tumor cells after incubation with ^{111}In-DTPA-OC (3, 4), and a comparison of ^{64}Cu-TETA-OC with this agent is warranted. Most importantly, determining the extent of the nuclear localization of ^{64}Cu from ^{64}Cu-TETA-OC will be a first step in determining whether the delivery of ^{64}Cu radiopharmaceuticals to the nuclei of tumor cells has implications for cancer therapy.

MATERIALS AND METHODS

Materials. High specific activity $^{64}CuCl_2$ was produced from enriched ^{64}Ni targets on a CS-15 cyclotron at Washington University (St. Louis, MO) as described previously (15). Indium-111 chloride ($^{111}InCl_3$) was obtained from Mallinckrodt, Inc. (St. Louis, MO). Ammonium acetate and ammonium citrate were purchased from Fluka (Buchs, Switzerland). AR42J rat pancreatic tumor cells were obtained from Mallinckrodt, Inc. AR42J cell media [1× DMEM (Cellgro) and Hams F12K], 1× trypsin-EDTA [0.05% trypsin/0.02% EDTA (Cellgro)], and EDTA were obtained from Fisher Scientific (Pittsburgh, PA). The protease inhibitor, Pefabloc-SC, was purchased from Roche Diagnostics

Received 4/25/03; revised 7/21/03; accepted 8/5/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by National Cancer Institute Grant R01 CA64475 and in part by Department of Energy Training Grant DE F0101 NE23051 (to A. L. C.). The production of copper radionuclides at Washington University is supported by National Cancer Institute Grant R24 CA86307 (to Dr. Michael J. Welch).

[2] Present address: Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri-Columbia, Columbia, MO 65211.

[3] To whom requests for reprints should be addressed, at Mallinckrodt Institute of Radiology, Washington University School of Medicine, 510 South Kingshighway Boulevard, Campus Box 8225, St. Louis, MO 63110. Phone: (314) 362-8427; Fax: (314) 362-9940; E-mail: andersoncj@mir.wustl.edu.

[4] The abbreviations used are: DTPA, diethylenetriaminepentaacetic acid; TETA, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; OC, octreotide; mAb, monoclonal antibody; BAT, bromoacetamidobenzyl-TETA; 2IT, 2-iminothiolane; CSK, cytoskeleton; LDH, lactate dehydrogenase; SSTR2, somatostatin receptor subtype 2; PET, positron emission tomography.

Table 1 *Decay characteristics of ^{111}In and ^{64}Cu*

Isotope	$T_{1/2}$	β^- MeV (%)	β^+ MeV (%)	EC[a] MeV (%)	γ MeV (%)	No. of Auger and/or conversion e^- per decay
^{64}Cu	12.7 h	0.573 (39.6%)	0.655 (17.4%)	0.33 (0.6%) 1.68 (40.5%)	0.51 (34.8%) 1.35 (0.6%)	2
^{111}In	67 h			1.1 (99+%)	0.173 (89%) 0.247 (94%)	8

[a] EC, electron capture; e^-, electrons.

(Indianapolis, IN). All of the other reagents were from Sigma-Aldrich (St. Louis, MO). An IEC Centra MR 4R centrifuge (Fisher Scientific, Pittsburgh, PA) was used for cell pelleting (280 × *g*, 5 min, 4°C) and nuclear isolation (560 × *g*, 5 min, 4°C). A Sorvall Superspeed RL2-B centrifuge was used for the isolation of mitochondria (3,000 × *g*, 20 min, 4°C), and a Beckman Ultracentrifuge (Spinco Division, Palo Alto, CA) was used for the Percoll gradients (60,000 × *g*, 30 min, 4°C). A nitrogen cavitation device (Parr Instrument Co., Moline, IL) was used for cell disruption to isolate mitochondria and lysosomes. Whole cell and cell nuclei counts were determined with a Coulter Counter (Miami, FL). Fluorescence microscopy was accomplished with an Olympus BX40 microscope equipped with a Diagnostic Equipment SPOT CCD digital camera interfaced to PC-based Optimas software for data acquisition and analysis. Radioactive samples were counted using a Beckman 8000 automated well-type counter (Fullerton, CA).

TETA-OC was obtained from Mallinckrodt, Inc. and labeled with [^{64}Cu]cupric acetate as described previously (5). Briefly, 1–11 mCi (37–407 MBq) of [^{64}Cu]cupric acetate in 100–250 μl of 0.1 M ammonium acetate (pH 5.5) were added to 1 μg of TETA-OC in 100–250 μl of 0.1 M ammonium acetate (pH 5.5). The reaction mixture was incubated at room temperature for 30–60 min, and then ^{64}Cu-TETA-OC was purified with a C18 SepPak cartridge. DTPA-OC (1 μg; Mallinckrodt, Inc.) was labeled with $^{111}InCl_3$ [1–9 mCi (37–333 MBq)] as described previously (5). Radiochemical purity was determined by radio-thin layer chromatography as described previously (5).

Internalization Assay. The amount of cell membrane-bound ^{64}Cu-TETA-OC was determined using modifications of the procedure of Zinn *et al.* (16). AR42J cells were seeded in three 12-well plates containing Hams F12K with 20% fetal bovine serum and were incubated at 37°C until ~80% confluency. Cells were washed with HBSS (pH 7.2), and then internalization medium (30 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% BSA in DMEM) was added to each well, and the mixture was incubated for 10 min at room temperature. For determination of nonspecific internalization, one set of wells was incubated with unlabeled TETA-OC (2 μg/10 μl) on a shaker at 37°C for 10 min to block somatostatin receptors. ^{64}Cu-TETA-OC (300,000 cpm/10 μl; specific activity = 440 μCi/μg; 4 pmol peptide) was added to all of the wells and was incubated with rocking at 37°C for 15 min or 1, 4, or 24 h, respectively. At each time point, radioactive media were aspirated, and the plate was washed twice with HBSS (pH 7.2). To collect the surface-bound fraction, each well was treated with 20 mM sodium acetate-HBSS (pH 4.0) and was incubated with rocking at 37°C for 10 min followed by a second 20-mM sodium acetate-HBSS (pH 4.0) wash without incubation and pooled with the first rinse. After removal of the surface-bound fraction, trypsin was used to dislodge the pellet. All of the fractions were counted for radioactivity on the gamma counter. The percentage internalized was the amount of activity in the final cell pellet, corrected for activity in the blocked fractions and background activity.

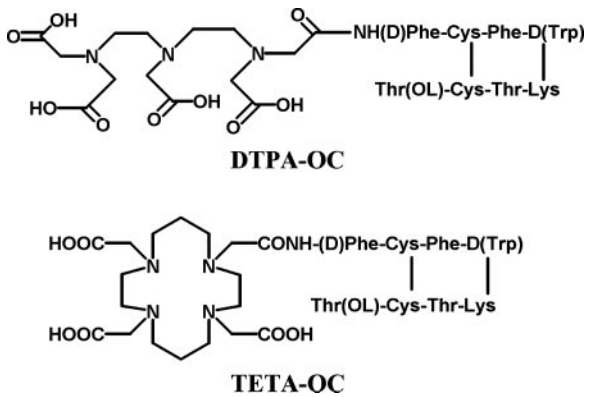

Fig. 1. Structures of DTPA-OC and TETA-OC.

Isolation of AR42J Nuclei after Administration of ^{64}Cu-TETA-OC or ^{111}In-DTPA-OC. ^{111}In-DTPA-OC or ^{64}Cu-TETA-OC was added to AR42J cells (1×10^7 cells/flask). To maintain a molar ratio of somatostatin receptors: SSTR2 ligand that was at least 10:1, we added 0.15–0.20 pmol of cold DTPA-OC or TETA-OC. This was based on a B_{max} (number of receptors as determined by Scatchard analysis of 244.4 fmol/mg of membrane protein (17). After incubation times of 1, 4, and 24 h, the cells were pelleted and resuspended in CSK buffer [0.5% Triton X-100, 300 mM sucrose, 100 mM NaCl, 1 mM EGTA, 2 mM $MgCl_2$, and 10 mM PIPES (pH 6.8)] and were incubated on ice for 2 min. Cell lysates were centrifuged at 560 × *g* for 5 min at 4°C, and the supernatant was discarded. The nuclear pellet was resuspended in 1 ml of CSK buffer without Triton X-100, and centrifuged at 560 × *g* at 4°C for 5 min. The supernatant was discarded and the postwash nuclear pellet was counted in the gamma counter. Aliquots of nuclei were assayed qualitatively for purity by fluorescence microscopy after staining with a 1:10 dilution of FITC (30 μg/ml) and propidium iodide (70 μg/ml). Micrographs were obtained at ×100, ×200, and ×1000 magnification. The yield of nuclei was determined by counting the initial cell number and the collected nuclei using a Coulter Counter. The percentage in the cell nucleus was determined by the gamma counts in the pure nucleus divided by gamma counts associated with whole cells, and was corrected for the yield of nuclei.

Subcellular Fractionation. ^{111}In-DTPA-OC or ^{64}Cu-TETA-OC (0.15–0.20 pmol) was added to AR42J cells (1×10^7 cells/flask) as described above. Cell disruption and subcellular fractionation were performed using a modification of the procedure described by Morton *et al.* (18). Briefly, the cell pellets were resuspended in homogenization buffer [0.25 M sucrose, 10 mM HEPES-KOH (pH 7.3), containing 1 mM EDTA and 1 mM Pefabloc-SC] and then were disrupted by nitrogen cavitation (200 psi, 10 min). Nuclei and nonlysed cells were pelleted by centrifugation (400 × *g* for 20 min, 4°C). The supernatant was then centrifuged at 3,000 × *g* for 20 min at 4°C to pellet mitochondria. The postmitochondrial supernatants were collected and layered over a 37.5% (v/v) Percoll gradient. The gradient was centrifuged at 60,000 × *g* for 30 min at 4°C and fractions of 1.0 ml were collected from the bottom of the gradient. Enzymatic assays for LDH (cytosol) and β-hexosaminidase (lysosomes) were performed on each fraction and on the mitochondrial pellets. Enzyme assays were also done on nuclei isolated as described above. Mitochondrial pellets and Percoll gradient fractions were counted for radioactivity in the gamma counter.

RESULTS

Radiochemistry. Radiochemical purity for both ^{64}Cu-TETA-OC and ^{111}In-DTPA-OC were >95% as determined by radio-thin-layer chromatography. The specific activity for ^{64}Cu-TETA-OC ranged from 1497 to 16,467 mCi/μmol (55,389–498,501 MBq/μmol) and for ^{111}In-DTPA-OC was 1507–13,563 mCi/μmol (55,759–501,831 MBq/μmol).

Internalization of ^{64}Cu-TETA-OC in AR42J Cells. We previously showed that ^{64}Cu-TETA-OC and other ^{64}Cu-labeled somatostatin analogues are taken up by AR42J cells (19). Here, experiments were performed to determine the amount of AR42J cell-associated activity that was membrane-bound *versus* internalized from 15 min to 24 h postadministration of ^{64}Cu-TETA-OC (Fig. 2). Internalization of ^{64}Cu-TETA-OC (4 pmol) by AR42J cells (1×10^6) increased over time. Internalization was significantly higher at 24 h (6.89 ± 1.42%

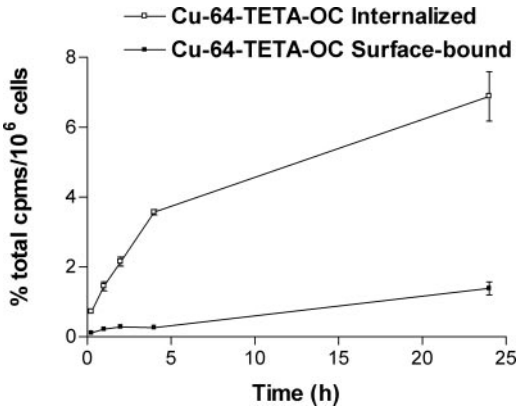

Fig. 2. Internalization of ^{64}Cu-TETA-OC (4 pmol) by AR42J cells (1×10^6) increases from 15 min to 24 h postadministration. Internalization was significantly higher at 24 h than at 4 and 1 h. The percentage of cell-associated activity that was surface bound ranged from 6.86 ± 0.33% to 16.55 ± 0.85%, with the amount of surface-bound activity increasing at 24 h, indicating that the majority of ^{64}Cu-TETA-OC is internalized.

of total activity added per 1×10^6 cells) than at 4 h (3.57 ± 0.16%; $P < 0.005$) and 1 h (1.45 ± 0.26%; $P < 0.001$). The percentage of cell-associated activity that was surface bound ranged from 6.86 ± 0.33% to 16.55 ± 0.85%, with the amount of surface-bound activity increasing at 24 h, indicating that the majority of ^{64}Cu-TETA-OC is internalized. Blocking studies were performed at all time points, and in all cases less than 1% of the ^{64}Cu-TETA-OC was cell associated when 2 μg of unlabeled TETA-OC was added to the wells, demonstrating that internalization was receptor mediated.

The data reported here indicate that ^{64}Cu-TETA-OC is internalized in AR42J cells by receptor-mediated endocytosis. The amount internalized increases steadily over the 24-h time course, with low amounts of cell-surface-associated activity, suggesting a rapid turnover and recycling of SSTR2 receptors. These data are consistent with other SSTR2 analogues in AR42J cells. The internalization of ^{111}In-DTPA-OC in AR42J cells over time was reported by de Jong *et al.* (20). In their assay, the internalized activity increased 2-fold from 1 to 4 h postadministration. The reported percentage increase of ^{111}In-DTPA-OC is consistent with the increased internalized activity observed with ^{64}Cu-TETA-OC from 1 to 4 h postadministration.

Uptake of ^{111}In-DTPA-OC, ^{64}Cu-TETA-OC and [^{64}Cu]Cupric Acetate in AR42J Nuclei. The isolation of AR42J nuclei by centrifugation techniques yielded high purity nuclei as demonstrated by fluorescence staining with FITC and propidium iodide, which stain for CSK and nuclei, respectively. As seen in Fig. 3, the nuclei isolated by our protocol showed no detectable cytoskeletal debris. In addition, enzyme assays were performed on purified nuclei to determine the contamination from lysosomes, cytosol, mitochondria, or plasma membrane. The overall contamination from the other organelles was ~12%, with the majority of the contamination being lysosomal (data not shown).

Experiments to determine the extent of nuclear localization of ^{64}Cu-TETA-OC and ^{111}In-DTPA-OC were performed with the radiotracer continuously incubated with cells for various times. In a control experiment, the nuclear localization of [^{64}Cu]cupric acetate was also determined. Under these conditions, the nuclear uptake of ^{64}Cu from ^{64}Cu-TETA-OC increased significantly over the time of incubation [1 h, 1.5 ± 0.19 ($n = 6$); 4 h: 9.28 ± 2.152 ($n = 5$); 24 h: 19.5 ± 1.40 ($n = 4$); $P < 0.0001$]. The amount of ^{64}Cu from [^{64}Cu]cupric acetate also increased from 1 to 24 h (1 h, 13.5 ± 0.37 to 21.0 ± 3.4; $n = 3$ for each time point), although in this case the ^{64}Cu had much higher initial uptake in the nucleus (Fig. 4). The uptake of ^{111}In in the nucleus increased significantly over the time of incubation (1 h, 1.0 ± 0.35 ($n = 3$); 4 h, 2.4 ± 0.4 ($n = 4$); 24 h, 6.0 ± 1.1 ($n = 5$); $P < 0.002$). ^{64}Cu from ^{64}Cu-TETA-OC in the cell nuclei showed a 13-fold increase from 1 to 24 h, whereas the amount of ^{111}In from ^{111}In-DTPA-OC showed a 6-fold increase over the same time period. The nuclear uptake of ^{111}In from ^{111}In-DTPA-OC was significantly less than ^{64}Cu from ^{64}Cu-TETA-OC at all time points ($P < 0.05$).

Another experiment was performed to determine whether the nuclear localization of ^{64}Cu and ^{111}In from their respective OC analogues varied with time under pulse-chase conditions. In these experiments, the radiolabeled somatostatin analogues were incubated with cells for 4 h, and then the media containing the tracers were removed and replaced with fresh media. The cells were then incubated for another 4 or 20 h before harvesting for the determination of ^{111}In or ^{64}Cu in the cell nucleus. After internalization into AR42J cells and replacement with fresh media, the amount of ^{64}Cu from ^{64}Cu-TETA-OC and ^{111}In from ^{111}In-DTPA-OC in the nuclei increased, ^{64}Cu to a greater extent than ^{111}In (Fig. 5). Interestingly, in the control pulse-chase experiment, levels of ^{64}Cu from [^{64}Cu]cupric acetate decreased significantly from 4 to 24 h postadministration (20.6 ± 8.7 to

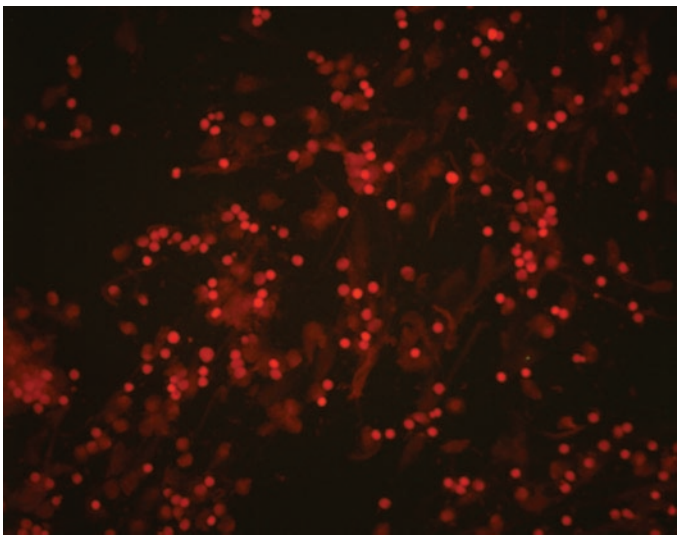

Fig. 3. Fluorescence microscopy of isolated nuclei from AR42J cells (×100) demonstrates the absence of green FITC stain, which suggests that the nuclei are free of cell debris and other contamination.

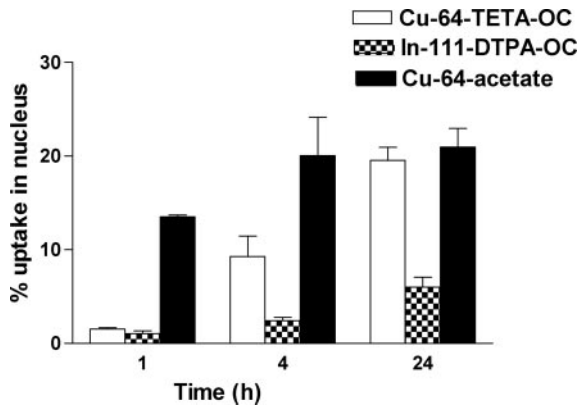

Fig. 4. Percentage of nuclear uptake of radioactivity in AR42J tumor cells incubated with ^{64}Cu-TETA-OC, ^{111}In-DTPA-OC, or [^{64}Cu]cupric acetate (*Cu-64-acetate*) for 1, 4, or 24 h. The nuclear uptake of ^{64}Cu from ^{64}Cu-TETA-OC increases significantly from 1 to 24 h. The nuclear uptake after incubation of cells with ^{111}In-DTPA-OC is significantly less than that with ^{64}Cu-TETA-OC at all time points. The amount of ^{111}In from ^{111}In-DTPA-OC increases significantly over the time of incubation. The uptake of [^{64}Cu]cupric acetate increases significantly from 1 to 24 h.

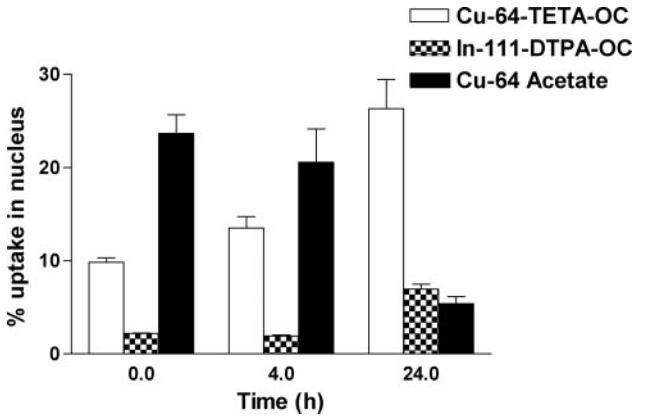

Fig. 5. Localization of ^{64}Cu from ^{64}Cu-TETA-OC compared with ^{111}In from ^{111}In-DTPA-OC and ^{64}Cu from [^{64}Cu]cupric acetate after a pulse with AR42J cells for 4 h or 1 h (for [^{64}Cu]cupric acetate; on graph, time = 0). After the 4-h incubation (or 1 h with [^{64}Cu]cupric acetate), the medium with the radiotracer was replaced with fresh medium, and the cells were incubated for another 4 and 24 h, and nuclear localization was determined. For ^{64}Cu-TETA-OC, there was a significant increase in ^{64}Cu activity in AR42J nuclei from 0 to 24 h postincubation in the fresh medium, and the amount of ^{111}In from ^{111}In-DTPA-OC in the cell nuclei also increased significantly from 0 to 24 h. In the case of ^{64}Cu-acetate, there was a significant decrease in ^{64}Cu activity in AR42J nuclei from 4 to 24 h post-incubation in the fresh medium. These data demonstrate that there is further migration of radionuclides from the somatostatin analogues after the tracer is removed from the medium; however, in cells pulsed with [^{64}Cu]cupric acetate, the ^{64}Cu is released from nuclei.

5.4 ± 1.9; $P < 0.05$) rather than increased as in the case of ^{64}Cu-TETA-OC.

Localization of ^{64}Cu-TETA-OC in Mitochondria, Lysosomes, and Cytosol. The mitochondrial, lysosome, and cytosolic fractions were found to have low levels of cross-contamination from each of the other fractions as determined by their marker enzyme assays, succinate cytochrome *c*-reductase, β-hexosaminidase and LDH. Fig. 6, *A* and *B* shows the typical profile of cytosolic and lysosomal enzyme markers, respectively, and ^{64}Cu activity of the Percoll gradient for AR42J cells incubated with ^{64}Cu-TETA-OC for 4 h. After a 4-h incubation, there was minimal ^{64}Cu associated with the fractions containing higher concentrations of cytosolic enzymes; however, there was a significant amount of ^{64}Cu activity associated with the lysosomal fractions of the Percoll gradient. Because of the difficulties in isolating high purity lysosomal fractions without significant loss of the organelle, we were unable to conduct reliable quantitative experiments. Fig. 7 demonstrates that the ^{64}Cu from ^{64}Cu-TETA-OC associated with the mitochondrial fraction increased over time, similar to uptake in the cell nucleus.

DISCUSSION

Somatostatin is a tetradecapeptide that acts to inhibit secretory and proliferative effects in a variety of tissue types. The binding of SSTR2 ligands to SSTR2-positive tumor cells results in rapid internalization of the ligand. However, uncertainty still remains in regard to the fate of the SSTR2 ligand after internalization. Hornick *et al.* (3) suggested that ^{111}In-DTPA-OC localized intact in the nuclei of human neuroblastoma cells that were grown in cell culture; however, no characterization of the chemical form ^{111}In was reported to confirm this. In the Hornick study, the nuclear uptake was determined by rupturing cells, separating cell components on a Percoll gradient and analyzing fractions for DNA content (3). It was not clear that the investigators were able to distinguish between fractions containing DNA and those containing lysosomal enzymes, which also coeluted with the ^{111}In activity (3).

Our interest in ^{64}Cu-TETA-OC was initially to design a PET imaging agent for SSTR2-positive tumors (5, 7), because of the superior imaging capabilities of PET *versus* γ scintigraphy. Concurrent studies evaluating ^{64}Cu-TETA-OC for therapy showed that the agent inhibited tumor growth with low toxicity in a CA20948 tumor-bearing rat model (6), which was consistent with our finding using ^{64}Cu-labeled mAb 1A3 in tumor-bearing hamsters (8). Like ^{64}Cu-BAT-2IT-1A3, ^{64}Cu-TETA-OC is internalized in tumor cells, and elucidating the subcellular distribution might help provide an understanding of whether therapeutic efficacy might be linked with localization in specific tumor cell organelles.

Our hypothesis for both ^{111}In-DTPA-OC and ^{64}Cu-TETA-OC is that dissociation of the radiometal occurs inside cells, which is followed by trafficking of the radiometal to the cell nucleus. This occurs to a much greater extent for ^{64}Cu than for ^{111}In, most likely because

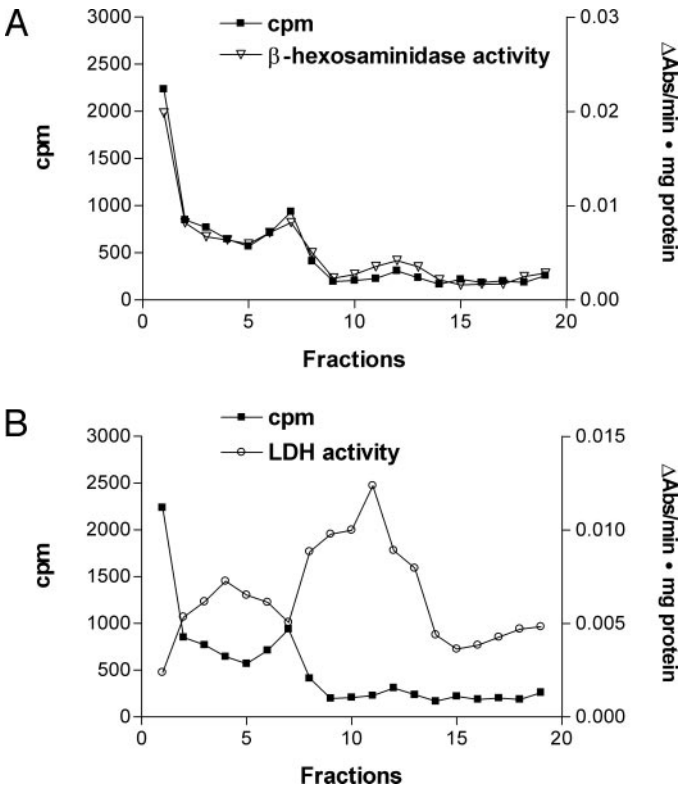

Fig. 6. Subcellular fractionation of AR42J tumor cells incubated with ^{64}Cu-TETA-OC for 4 h. Cells were disrupted by nitrogen cavitation and fractionated on 37.5% Percoll gradient as described in "Materials and Methods." Fractions were collected from the bottom of the gradient and assayed for radioactivity (cpm) in *A* and *B*; a lysosome enzyme, β-hexosaminidase (*A*); and a cytosol enzyme, LDH (*B*). Δ*Abs*, change in absorbance.

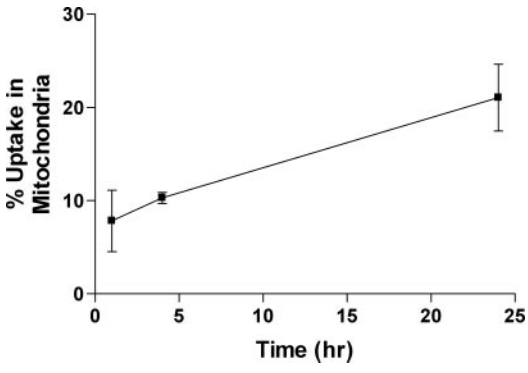

Fig. 7. Mitochondrial uptake of ^{64}Cu from ^{64}Cu-TETA-OC in AR42J tumor cells. ^{64}Cu-TETA-OC uptake in mitochondria increased over time. The increase from 4 to 24 h was statistically significant ($P < 0.05$).

^{111}In is more stably chelated to DTPA *in vivo* than ^{64}Cu is to TETA as demonstrated by *in vivo* metabolism studies with ^{111}In-DTPA-OC and ^{64}Cu-TETA-OC (9, 21). As a metal ion, In(III) may possibly be mimicking the behavior of Fe(II), which binds nonspecifically to exposed regions of DNA (22).

The role of Cu(II) binding to the DNA and/or other structures in the nucleus has been widely reported. Copper ion has been suggested to play an important role in the maintenance of nuclear matrix organization and DNA folding (10, 11). Chiu *et al.* have found that treatment of isolated nuclei with micromolar levels of Cu(II) not only binds nuclear matrix-associated DNA to matrix proteins but also enhances the production of additional DNA-protein cross-linking, as well as DNA double-strand breaks on subsequent irradiation (12, 13). Studies by George *et al.* (10) suggest that cell killing or malfunction at the nuclear level that is induced by ionizing radiation is caused by the reduction of Cu(II) to Cu(I) and also by specific hydroxyl radical attack on proteins or DNA at a copper site. More recently, Trumbore *et al.* (23) reported that, when concentrations of copper that are an order of magnitude lower than DNA base concentrations are present in different DNAs, ranging from small, double-stranded oligonucleotides to high-molecular-weight DNA from natural sources, γ irradiation induces changes in conformation from a right- to a left-handed DNA helix. Taken together, interactions of radiolytic intermediates in the region of the nuclear matrix could have significant biological consequences if changes in DNA conformation adversely affect transcription or translation processes.

Although the above mentioned studies suggest that copper binding to structures in the nucleus may enhance radiation damage to DNA, these studies do not directly compare with the studies presented here. The majority of the above-mentioned studies were performed using isolated nuclei or isolated DNA, not whole cells. The amount of copper added to the isolated nuclei in the study by Chiu *et al.* was 1–1000 μM (13). In the studies with isolated DNA, the amount of copper used was also in the μM range. In the studies performed here, the concentration of ^{64}Cu-TETA-OC in the wells containing intact AR42J cells was approximately 4 nM. Additional studies are warranted to determine whether the localization of copper radionuclides to tumor cell nuclei enhances cell killing, as are microdosimetry experiments to determine absorbed doses to the cell nuclei.

Our studies demonstrating the translocation of ^{64}Cu from ^{64}Cu-TETA-OC to the nuclei of tumor cells are consistent with the hypothesis that there are binding sites for copper in the cell nucleus as described above. ^{64}Cu-TETA-OC was not designed to target the cell nucleus, although it was assumed that the somatostatin receptor ligand would be internalized because of previous studies with ^{111}In-DTPA-OC (14). The instability of the ^{64}Cu-TETA moiety under biological conditions (9) is likely the cause of ^{64}Cu translocating to the nucleus. This hypothesis is supported by the similarity in ^{64}Cu uptake in the nucleus when ^{64}Cu-TETA-OC is delivered, either continuously over 24 h (Fig. 4) or in a 4-h pulse followed by removal of the ligand from the media (Fig. 5). It was previously shown that ^{111}In-DTPA-OC is internalized via receptor-mediated endocytosis and that the ligands then accumulate in lysosomes (14). There is no known pathway for somatostatin receptor ligands to accumulate in the cell nucleus. The fact that ^{64}Cu continues to localize to the cell nucleus after the removal of ^{64}Cu-TETA-OC from the media suggests the ^{64}Cu may be translocating to another protein in the lysosomes and then migrating to the nucleus and/or the mitochondria. In another study, the amount of ^{111}In from ^{111}In-DTPA-OC increased in the nucleus over time, and it was suggested that for therapeutic purposes, a continuous infusion of ^{111}In-DTPA-OC over a long period of time might improve therapy (3). Our data suggest that ^{111}In also translocates to the nucleus after a 4-h pulse, although not to the extent that occurs with ^{64}Cu-TETA-OC. However, a slow continuous infusion of ^{111}In-DTPA-OC may not substantially improve therapy, because our experiments demonstrated that a 24-h *versus* a 4-h exposure to ^{111}In-DTPA-OC did not increase nuclear uptake of the radiometal to a large extent.

As a control, the nuclear localization of a non-receptor-mediated tracer, [^{64}Cu]cupric acetate, was also performed in AR42J cells. ^{64}Cu from [^{64}Cu]cupric acetate was taken up more rapidly in the nucleus, with ~14% taken up at 1 h postadministration. Under conditions in which the cells were continuously incubated with [^{64}Cu]cupric acetate, the nuclear localization leveled out at ~20% by 4 h. One of the goals of this study was to better understand mechanisms of uptake of ^{64}Cu tracers into cell nuclei. Mechanisms may depend on how the ^{64}Cu is taken up into cells, whether by a general mechanism most likely involving the Ctr1 copper transporter (most likely in the case of [^{64}Cu]cupric acetate), or whether the initial uptake of the ^{64}Cu tracer is receptor-mediated, as in the case of ^{64}Cu-TETA-OC. It is noteworthy that, in the pulse-chase experiment, levels of ^{64}Cu from [^{64}Cu]cupric acetate decreased significantly from 4 to 24 h postadministration (20.6 ± 8.7 to 5.4 ± 1.9, respectively; $P < 0.05$) rather than increased as in the case of ^{64}Cu-TETA-OC. The data suggest that the redistribution mechanism or the kinetics of ^{64}Cu from ^{64}Cu-TETA-OC, is different from those for ^{64}Cu from [^{64}Cu]cupric acetate. Additional studies to elucidate the mechanisms of nuclear uptake of ^{64}Cu from receptor- *versus* transporter-mediated uptake are warranted.

Data presented here also show that an accumulation of ^{64}Cu activity occurs in mitochondria in a fashion similar to that in nuclei, although to a lesser extent (Fig. 7). Mitochondria are the energy-producing centers of the cell, and many cell functions are bioenergy-requiring processes. Mechanisms for delivery of copper to the mitochondria have been elucidated in yeast (24) and mammalian tissues and cells (25) via the Cox17p chaperone protein, which delivers copper to cyctochrome *c* oxidase, the terminal complex of mitochondrial and bacterial respiratory chains. To the best of our knowledge, the presence of this mitochondrial copper chaperone has not been verified in mammalian tumor cell lines. Damage to the mitochondria in cells has also been implicated in cell death (26). In the present studies, the administration of ^{64}Cu-TETA-OC resulted in substantial accumulation of ^{64}Cu in the mitochondria of AR42J cells. This uptake may have implications for cytotoxicity resulting from mitochondrial DNA damage or disruption of critical structures or functions in that organelle.

The cited examples of copper binding to structures in the cell nucleus suggest that copper plays a role in the radiation chemistry and biology of DNA. Data presented here show an increase in ^{64}Cu localization to the cell nucleus and mitochondria after the addition of ^{64}Cu-TETA-OC to intact AR42J cells over time. These processes may induce cytotoxic responses resulting from radiochemical and radiobiological effects on nuclear structure and function, as well as on cellular respiration. Specifically designing copper radiopharmaceuticals to target the cell nucleus or mitochondria could have a significant impact on the field of targeted radiotherapy of cancer. Gaining a better understanding of the subcellular and subnuclear localization of copper and mechanisms of cell killing by copper radionuclides will also benefit the development of therapeutic radiopharmaceuticals. Future studies are planned to determine the intranuclear structure of ^{64}Cu localization, as well as the chemical form of the ^{64}Cu in the cell nucleus.

ACKNOWLEDGMENTS

We acknowledge Dr. Wenping Li for radiochemistry assistance, Virginia Richey and Susan Adams for cell culture assistance, Dr. David Piwnica-

Worms for the use of the ultracentrifuge, and Drs. Buck E. Rogers and James R. Duncan for helpful discussions.We are grateful to Mallinckrodt Inc. (St. Louis, MO) for the gift of AR42J cells and to Drs. A. Srinivasan and Michelle Schmidt (Mallinckrodt Inc.) for providing TETA-OC.

REFERENCES

1. Krenning, E. P., Kooij, P. P. M., Bakker, W. H., Breeman, W. A. P., Postema, P. T. E., Kwekkeboom, D. J., Oei, H. Y., de Jong, M., Visser, T. J., Reijs, A. E. M., and Lamberts, S. W. J. Radiotherapy with a radiolabeled somatostatin analogue, ^{111}In-DTPA-Phe1-octreotide: a case history. Ann. N. Y. Acad. Sci., *733:* 496–506, 1994.
2. Krenning, E. P., de Jong, M., Kooij, P. P. M., Breeman, W. A. P., Bakker, W. H., de Herder, W. W., van Eijck, C. H. J., Kwekkeboom, D. J., Jamar, F., Pauwels, S., and Valkema, R. Radiolabelled somatostatin analogue(s) for peptide receptor scintigraphy and radionuclide therapy. Ann. Oncol., *10* (Suppl. 2)*:* S23–S29, 1999
3. Hornick, C. A., Anthony, C. T., Hughey, S., Gebhardt, B. M., Espenan, G. D., and Woltering, E. A. Progressive nuclear translocation of somatostatin analogs. J. Nucl. Med., *41:* 1256–1263, 2000.
4. Janson, E. T., Westlin, J-E., Ohrvall, U., and Lukinius, A. Nuclear localization of ^{111}In after intravenous injection of [^{111}In-DTPA-D-Phe^{1}]-octreotide in patients with neuroendocrine tumors. J. Nucl. Med., *41:* 1514–1518, 2000.
5. Anderson, C. J., Pajeau, T. S., Edwards, W. B., Sherman, E. L. C., Rogers, B. E., and Welch, M. J. *In vitro* and *in vivo* evaluation of copper-64-labeled octreotide conjugates. J. Nucl. Med., *36:* 2315–2325, 1995.
6. Anderson, C. J., Jones, L. A., Bass, L. A., Sherman, E. L. C., McCarthy, D. W., Cutler, P. D., Lanahan, M. V., Cristel, M. E., Lewis, J. S., and Schwarz, S. W. Radiotherapy, toxicity and dosimetry of copper-64-TETA-octreotide in tumor-bearing rats. J. Nucl. Med., *39:* 1944–1951, 1998.
7. Anderson, C. J., Dehdashti, F., Cutler, P. D., Schwarz, S. W., Laforest, R., Bass, L. A., Lewis, J. S., and McCarthy, D. W. Copper-64-TETA-octreotide as a PET imaging agent for patients with neuroendocrine tumors. J. Nucl. Med., *42:* 213–221, 2001.
8. Connett, J. M., Anderson, C. J., Guo, L. W., Schwarz, S. W., Zinn, K. R., Rogers, B. E., Siegel, B. A., Philpott, G. W., and Welch, M. J. Radioimmunotherapy with a Cu-64-labeled monoclonal antibody: a comparision with Cu-67. Proc. Nat. Acad. Sci. USA, *93:* 6814–6818, 1996.
9. Bass, L. A., Wang, M., Welch, M. J., and Anderson, C. J. *In vivo* transchelation of copper-64 from TETA-octreotide to superoxide dismutase in rat liver. Bioconj. Chem., *11:* 527–532, 2000.
10. George, A. M., Sabovljev, S. A., Hart, L. E., Cramp, W. A., Harris, G., and Hornsey, S. DNA quaternary structure in the radiation sensitivity of human lymphocytes—a proposed role of copper. Br. J. Cancer, *55* (Suppl. VIII)*:* 141–144, 1987.
11. Lewis, D. C., and Laemmli, U. K. Higher order chromatin structure: evidence for metallo protein interactions. Cell, *29:* 171–181, 1982.
12. Chiu, S. M., Xue, L. Y., Friedman, L. R., and Oleinick, N. L. Chromatin compaction and the efficiency of formation of DNA-protein crosslinks in γ-irradiated mammalian cells. Radiat. Res., *129:* 184–191, 1992.
13. Chiu, S-M., Xue, L-Y., Friedman, L. R., and Oleinick, N. L. Copper ion-mediated sensitization of nuclear matrix attachment sites to ionizing radiation. Biochemistry, *32:* 6214–6219, 1993.
14. Duncan, J. R., Stephenson, M. T., Wu, H. P., and Anderson, C. J. Indium-111-diethylenetriaminepentaacetic acid-octreotide is delivered *in vivo* to pancreatic, tumor cell, and hepatocyte lysosomes. Cancer Res., *57:* 659–671, 1997.
15. McCarthy, D. W., Shefer, R. E., Klinkowstein, R. E., Bass, L. A., Margenau, W. H., Cutler, C. S., Anderson, C. J., and Welch, M. J. The efficient production of high specific activity Cu-64 using a biomedical cyclotron. Nucl. Med. Biol., *24:* 35–43, 1997.
16. Zinn, K. R., Chaudhuri, T. R., Buchsbaum, D. J., Mountz, J. M., and Rogers, B. E. Simultaneous evaluation of dual gene transfer to adherent cells by γ-ray imaging. Nucl. Med. Biol., *28:* 135–144, 2001.
17. Li, W. P., Lewis, J. S., Srinivasan, A., Schmidt, M. A., and Anderson, C. J. Copper-64/61 and iodine-125-labeled DOTA-D-TYR1-octreotate: a new somatostatin analaog for labeling with metals and halogens. J. Label. Compd. Radiopharm., *44* (Suppl. 1)*:* S948–S950, 2001.
18. Morton, P. A., Zacheis, M. L., Giacoletto, K. S., Manning, J. A., and Schwartz, B. D. Delivery of nascent MHC class II-invariant chain complexed to lysosomal compartments and proteolysis of invariant chain by cysteine proteases precedes peptide binding in B-lymphoblastoid cells. J. Immunol., *154:* 137–150, 1995.
19. Lewis, J. S., Lewis, M. R., Srinivasan, A., Schmidt, M. A., Wang, J., and Anderson, C. J. Comparison of four ^{64}Cu-labeled somatostatin analogs *in vitro* and in a tumor-bearing rat model: evaluation of new derivatives for PET imaging and targeted radiotherapy. J. Med. Chem., *42:* 1341–1347, 1999.
20. de Jong, M., Bernard, B. F., de Bruin, E., van Gameren, A., Bakker, W. H., Visser, T. J., Maecke, H. R., and Krenning, E. P. Internalization of radiolabelled [DTPA0]octreotide and [DOTA0, Tyr3]octreotide: peptides for somatostatin receptor-targeted scintigraphy and radionuclide therapy. Nucl. Med. Comm., *19:* 283–288, 1998.
21. Bass, L. A., Lanahan, M. V., Duncan, J. R., Erion, J. L., Srinivasan, A., Schmidt, M. A., and Anderson, C. J. Identification of the soluble *in vivo* metabolites of indium-111-diethylenetriaminepentaacetic acid-D-Phe^{1}-octreotide. Bioconj. Chem., *9:* 192–200, 1998.
22. Chiu, S-M., Zue, L-Y., Friedman, L. R., and Oleinick, N. L. Differential dependence on chromatin structure for copper and iron ion induction of DNA double-strand breaks. Biochemistry, *34:* 2653–2661, 1995.
23. Trumbore, C. N., Ehrlich, R. S., and Myers, Y. N. Changes in DNA conformation induced by γ irradiation in the presence of copper. Radiat. Res., *155:* 453–465, 2001.
24. Glerum, D. M., Shtanko, A., and Tzagoloff, A. Characterization of *COX17*, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. J. Biol. Chem., *271:* 14504–14509, 1996.
25. Koichiro, K., Tsumori, K., Ohmasa, Y., Takahashi, Y., and Munekata, E. The expression of Cox17p in rodent tissues and cells. Eur. J. Biochem., *267:* 6699–6707, 2000.
26. Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., and Di Lisa, F. Mitochondria and cell death. Eur. J. Biochem., *264:* 687–701, 1999.

Subcellular Localization of Radiolabeled Somatostatin Analogues: Implications for Targeted Radiotherapy of Cancer

Mu Wang, Amy L. Caruano, Michael R. Lewis, et al.

Cancer Res 2003;63:6864-6869.