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

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ABSTRACT

Copper-64 (T1/2 = 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 64Cu by focusing on subcellular distribution of the somatostatin analogues 64Cu-labeled 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid-octreotide (64Cu-TETA-OC) and 111In-labeled diethylenetriaminepentaacetic acid-octreotide (111In-DTPA-OC). Cell uptake and organelle isolation studies were conducted on 64Cu-TETA-OC and 111In-DTPA-OC. Nuclear localization of 64Cu and 111In from 64Cu-TETA-OC and 111In-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 64Cu-TETA-OC was incubated with AR42J cells for 4 h, it was found that the nuclear localization of 64Cu 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 64Cu from [64Cu]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 64Cu from 64Cu-TETA-OC is different from that for 64Cu from [64Cu]cupric acetate. The amount of 64Cu from 64Cu-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 64Cu to the cell nucleus and mitochondria may contribute to cell killing with 64Cu 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 111In-DTPA-OC, 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 111In 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 64Cu (T1/2 = 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 111In and 64Cu are summarized in Table I. 64Cu-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 64Cu-labeled mAb, 64Cu-BAT-2IT-1A3, in a tumor-bearing rodent model compared with published studies of 131I- or 90Y-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, 64Cu 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 64Cu-TETA-OC compared with 111In-DTPA-OC, because radiolabeled somatostatin analogues are known to be internalized (14). Other investigators have observed the localization of 111In to the nuclei of tumor cells after incubation with 111In-DTPA-OC (3, 4), and a comparison of 64Cu-TETA-OC with this agent is warranted. Most importantly, determining the extent of the nuclear localization of 64Cu from 64Cu-TETA-OC will be a first step in determining whether the delivery of 64Cu radiopharmaceuticals to the nuclei of tumor cells has implications for cancer therapy.

MATERIALS AND METHODS

Materials. High specific activity 64CuCl2 was produced from enriched 64Ni targets on a CS-15 cyclotron at Washington University (St. Louis, MO) as described previously (15). Indium-111 chloride (111InCl3) 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× tripsin–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.
Ultracentrifuge (Spinco Division, Palo Alto, CA) was used for the isolation of mitochondria (3,000 g/11,000 g). 5.5) were added to 1.30 acetate (pH 5.5). The reaction mixture was incubated at room temperature for 20 min, 4°C. A Sorvall Superspeed R2-LB-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 Diagnostics 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).

46Cu-cupric acetate as described previously (5). Briefly, 1–11 mCi (37–407 MBq) of 46Cu-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 46Cu-TETA-OC was purified with a C18 SepPak cartridge. DTPA-OC (1 μg; Mallinkrodt, Inc.) was labeled with 111InCl 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 46Cu-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 t-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. 46Cu-TETA-OC (300,000 cpm/10 μl; specific activity = 440 μCi/μg; 4 μmol 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) 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.

**Isolation of AR42J Nuclei after Administration of 46Cu-TETA-OC or 111In-DTPA-OC.** 111In-DTPA-OC or 46Cu-TETA-OC was added to AR42J cells (1 × 107 cells/flask). To maintain a molar ratio of somatostatin receptors: SST2R 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 Bmax (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 MgCl2, and 10 mM PIPES (pH 6.8)] and were incubated on ice for 2 min. Cell lysates were centrifuged at 560 g × 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 × 4°C for 5 min. The supernatant was discarded and the postnuclear 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 nuclei was determined by the gamma count divided by gamma counts associated with whole cells, and was corrected for the yield of nuclei.

**Subcellular Fractionation.** 111In-DTPA-OC or 46Cu-TETA-OC (0.15–0.20 pmol) was added to AR42J cells (1 × 107 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 disrupted by nitrogen cavitation (200 psi, 10 min). Nuclei and monolysed cells were pelleted by centrifugation (400 g for 2 min, 4°C). The supernatant was then centrifuged at 3,000 g × 20 min at 4°C for 30% 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 × 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-hexamidase (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 46Cu-TETA-OC and 111In-DTPA-OC were >95% as determined by radio-thin-layer chromatography. The specific activity for 46Cu-TETA-OC ranged from 1497 to 16,467 mCi/μmol (55,389–498,501 MBq/μmol) and for 111In-DTPA-OC was 1507–13,563 mCi/μmol (55,759–501,831 MBq/μmol).

**Internalization of 46Cu-TETA-OC in AR42J Cells.** We previously showed that 46Cu-TETA-OC and other 46Cu-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 46Cu-TETA-OC (Fig. 2). Internalization of 46Cu-TETA-OC (4 pmol) by AR42J cells (1 × 105) increased over time. Internalization was significantly higher at 24 h (6.89 ± 1.42% of initial cell number and the collected nuclei using a Coulter Counter. The percentage in the cell nuclei was determined by the gamma count divided by gamma counts associated with whole cells, and was corrected for the yield of nuclei.
of total activity added per 1 × 10⁶ 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 ⁶⁴Cu-TETA-OC is internalized.

The data reported here indicate that ⁶⁴Cu-TETA-OC is internalized in AR42J cells by receptor-mediated endocytosis. The amount internalized increased 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 ¹¹¹In-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 ¹¹¹In-DTPA-OC is consistent with the increased internalized activity observed with ⁶⁴Cu-TETA-OC from 1 to 4 h postadministration.

Uptake of ¹¹¹In-DTPA-OC, ⁶⁴Cu-TETA-OC and [⁶⁴Cu]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 ⁶⁴Cu-TETA-OC and ¹¹¹In-DTPA-OC were performed with the radiotracer continuously incubated with cells for various times. In a control experiment, the nuclear localization of [⁶⁴Cu]cupric acetate was also determined. Under these conditions, the nuclear uptake of ⁶⁴Cu from ⁶⁴Cu-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 ⁶⁴Cu from [⁶⁴Cu]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 ⁶⁴Cu had much higher initial uptake in the nucleus (Fig. 4). The uptake of ¹¹¹In 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). ⁶⁴Cu from ⁶⁴Cu-TETA-OC in the cell nuclei showed a 13-fold increase from 1 to 24 h, whereas the amount of ¹¹¹In from ¹¹¹In-DTPA-OC showed a 6-fold increase over the same time period. The nuclear uptake of ¹¹¹In from ¹¹¹In-DTPA-OC was significantly less than ⁶⁴Cu from ⁶⁴Cu-TETA-OC at all time points (P < 0.05).

Another experiment was performed to determine whether the nuclear localization of ⁶⁴Cu and ¹¹¹In 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 ¹¹¹In or ⁶⁴Cu in the cell nucleus. After internalization into AR42J cells and replacement with fresh media, the amount of ⁶⁴Cu from ⁶⁴Cu-TETA-OC and ¹¹¹In from ¹¹¹In-DTPA-OC in the nuclei increased, ⁶⁴Cu to a greater extent than ¹¹¹In (Fig. 5). Interestingly, in the control pulse-chase experiment, levels of ⁶⁴Cu from [⁶⁴Cu]cupric acetate decreased significantly from 4 to 24 h postadministration (20.6 ± 8.7 to

Fig. 2. Internalization of ⁶⁴Cu-TETA-OC (4 pmol) by AR42J cells (1 × 10⁶) 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 ⁶⁴Cu-TETA-OC is internalized.

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 ⁶⁴Cu-TETA-OC, ¹¹¹In-DTPA-OC, or [⁶⁴Cu]cupric acetate (Cu-64-acetate) for 1, 4, or 24 h. The nuclear uptake of ⁶⁴Cu from ⁶⁴Cu-TETA-OC increases significantly from 1 to 24 h. The nuclear uptake after incubation of cells with ¹¹¹In-DTPA-OC is significantly less than that with ⁶⁴Cu-TETA-OC at all time points. The amount of ¹¹¹In from ¹¹¹In-DTPA-OC increases significantly over the time of incubation. The uptake of [⁶⁴Cu]cupric acetate increases significantly from 1 to 24 h.
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 111In was reported to confirm this. In our study, the nuclear uptake was determined by rupturing the organelle, we were unable to conduct reliable quantitative experiments. Fig. 7 demonstrates that the 64Cu from 64Cu-TETA-OC associated with the mitochondrial fraction increased over time, similar to uptake in the cell nucleus.

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

Localization of 64Cu-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 64Cu activity of the Percoll gradient for AR42J cells incubated with 64Cu-TETA-OC for 4 h. After a 4-h incubation, there was minimal 64Cu associated with the fractions containing higher concentrations of cytosolic enzymes; however, there was a significant amount of 64Cu 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 64Cu from 64Cu-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 111In-DTPA-OC localized intact in the nuclei of human neuroblastoma cells that were grown in cell culture; however, no characterization of the chemical form 111In 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 111In activity (3).

Our interest in 64Cu-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 64Cu-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 64Cu-labeled mAb 1A3 in tumor-bearing hamsters (8). Like 64Cu-BAT-2IT-1A3, 64Cu-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 111In-DTPA-OC and 64Cu-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 64Cu than for 111In, most likely because...
was suggested that for therapeutic purposes, a continuous infusion of 64 Cu-TETA-OC from the media suggests the 64 Cu may be removed 24 h (Fig. 4) or in a 4-h pulse followed by removal of the ligand from 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 64 Cu-TETA-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 64 Cu-TETA-OC increased in the nuclei over time, and it was suggested that for therapeutic purposes, a continuous infusion of 111 In-DTPA-OC increased in the nuclei 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 cytochrome 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.

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