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Research Paper

The role of p53 in the trafficking of copper-64 to tumor cell nuclei

Martin Eiblmaier, Laura A. Meyer and Carolyn J. Anderson*

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Abbreviations: BSA, bovine serum albumin; CB-TE2A , 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylene diamine tetraacetic acid; EGFR, epidermal growth factor receptor; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PET, positron emission tomography; SDS, sodium dodecyl sulfate; SPECT, single photon emitted computed tomography; SSTr2, somatostatin receptor subtype 2; TETA, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; TLC, thin-layer chromatography; Y3-TATE, tyrosine-3 octreotate; WT, wild type

Key words: copper-64, nuclear localization, targeted radiotherapy, tumor cell nuclei, p53, copper chaperone

Copper-64 ($T_{1/2}$ = 12.7 h; β^+: 17.8%, β^-: 41%) has applications in both positron emission tomography (PET) imaging and targeted radiotherapy of cancer. 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. Here, we report an investigation on the potential role of the tumor suppressor protein p53 in trafficking ^{64}Cu to tumor cell nuclei. Two EGFR expressing human colorectal cell lines (HCT 116 +/+ and HCT 116 -/-) that are positive or negative for p53 expression respectively, were used to compare internalization and nuclear localization of [^{64}Cu]copper acetate and of ^{64}Cu-DOTA-cetuximab, a monoclonal anti-EGFR antibody. [^{64}Cu]copper acetate uptake into cells was similar between the two cell lines during a 24 h time course. In contrast, the uptake of [^{64}Cu]copper acetate in the nuclei of HCT 116 +/+ cells was significantly higher than in HCT 116 -/- cells ($p < 0.0001$) at 24 h. There was no difference in receptor binding, receptor-mediated internalization, and efflux of ^{64}Cu-DOTA-cetuximab between the two HCT 116 cells lines. However, nuclear localization of ^{64}Cu-DOTA-cetuximab showed increased uptake in the nuclei of HCT 116 +/+ cells as early as 4 h. These data demonstrate that ^{64}Cu is delivered to tumor cell nuclei in a p53 positive cell line in significantly greater amounts than in p53 negative cells by both non-specific and receptor-mediated uptake mechanisms.

Introduction

There has been increasing interest in the use of copper radiopharmaceuticals for PET imaging and/or targeted radiotherapy of cancer. Copper-64 ($T_{1/2}$ = 12.7 h; β^+: 0.655 MeV; 17.8%; β^-: 0.573 MeV; 41%) emits both β^+ and β^- radiation for PET and therapy applications, while ^{67}Cu ($T_{1/2}$ = 62 h; 100% β^-: 0.576 MeV max) is a β^- emitter that has shown success in radioimmunotherapy of non-Hodgkin's lymphoma in tumor-bearing mice[1-3] and in humans.[4,5] We previously observed that ^{64}Cu-labeled tumor-targeting agents showed enhanced therapeutic efficacy of an internalizing ^{64}Cu-labeled anti-colorectal carcinoma mAb, ^{64}Cu-labeled 1A3, in a tumor-bearing hamster model compared to ^{90}Y- or ^{131}I-labeled mAbs in the same animal model.[6,7] We also evaluated the ^{64}Cu-labeled somatostatin analogs octreotide and Y3-TATE as therapeutic agents, and although we did not observe complete tumor remissions, we did see tumor growth inhibition at relatively low radiation doses to the tumors (465-600 cGy).[8,9]

Particularly in the area of receptor-targeted therapy agents,[10,11] the intracellular fate of the radiopharmaceuticals following binding to cell surface receptors has been a topic of interest.[12,13] It was previously demonstrated that ^{111}In from ^{111}In-DTPA-octreotide (Octreoscan®), an imaging agent clinically approved for somatostatin-receptor positive tumors in the United States and Europe, showed uptake in tumor cell nuclei.[12] This suggests a possible mechanism for the therapeutic efficacy of this Auger electron-emitting radiopharmaceutical. We demonstrated that ^{64}Cu from ^{64}Cu-TETA-octreotide was significantly localized in the nuclei of AR42J rat pancreatic tumor cells in cell culture, whereas ^{111}In from ^{111}In-DTPA-octreotide was localized to tumor cell nuclei in much smaller amounts.[14] Although ^{64}Cu only emits 2 Auger electrons per decay, it has been reported that the recoil energy from the transmutation of this radionuclide in the vicinity of DNA can cause lethal damage to the cell.[15] These data suggested that delivery of ^{64}Cu to the cell nuclei may enhance the therapeutic effect of beta-emitting, tumor targeting copper radiopharmaceuticals.

Because copper can readily form oxygen radicals, free copper essentially does not exist in vivo.[16] Several chaperones have been identified that transport copper to various organelles in mammalian cells (for a review on this subject, see ref. 17). Copper is transported into the cell via hCtr1 (human copper transporter). Several chaperones serve to traffic copper between cellular compartments. Cox17 carries copper to the mitochondria for incorporation into

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cytochrome c. Copper can also be incorporated into nascent Cu/Zn SOD via the copper chaperone CCS. The Wilson disease P-type ATPase receives copper from HAH1 in the trans-golgi-network, where copper binds to ceruloplasmin during synthesis. Metallothionein is thought to play a role as a copper storage pool within the cell.[18] To date, no chaperones have been identified that traffic copper to the cell nucleus. There are proteins common in tumors and other tissues that may play a role in the transport of copper to the nucleus. The tumor suppressor protein p53 is a critical cellular mediator of the response to oxidative stress and genotoxic damage. Upon activation, p53 accumulates in the nucleus where it transcriptionally activates the expression of a number of proteins. There is in vitro evidence that copper may displace zinc in the p53 structure.[19]

In addition to the possibility that ^{64}Cu might bind to p53 itself, there is also evidence that p53 facilitates the transport of other copper-based proteins to the nuclei of tumor cells. Azurin is a cupredoxin protein secreted by *Pseudomonas aeruginosa*. It has been shown to have cytotoxic effects on J774 macrophages and human breast cancer cells.[20,21] The induction of apoptosis by azurin is independent of its redox activity and involves complex formation with p53 and translocation to the nucleus.[22-24]

Here we report on the transport of ^{64}Cu to the nuclei of HCT116 cells lines that are either p53 WT or p53 negative (herein referred to as HCT 116 +/+ and HCT 116 -/-). We evaluated [^{64}Cu]copper acetate, which will bind to proteins in serum and be taken up by cells via the hCtr1 transporter, as well as the EGFR antibody ^{64}Cu-DOTA-cetuximab, which will be delivered to the EGFR-positive HCT 116 cell lines by a receptor-mediated pathway. Cetuximab was the first monoclonal antibody targeted against the ligand-binding site of EGFR to be approved by the FDA for the treatment of patients with EGFR-expressing, metastatic colorectal carcinoma.[25-27] We demonstrate a potential pathway for the trafficking of copper (in the form of ^{64}Cu) to the nuclei of tumor cells via the tumor suppressor protein, p53.

Results

Internalization and nuclear uptake of [^{64}Cu]copper acetate. HCT 116$^{+/+}$ and HCT 116$^{-/-}$ cells were incubated with [^{64}Cu]copper acetate over a 24 h time course to measure internalization. ^{64}Cu accumulated in both lines over time and reached approximately 20% ID/mg at 24 h. Minimal difference was observed between the two cell lines (Fig. 1A). p53 status thus had no apparent influence on transport of ^{64}Cu via the hCtr1 transporter. The difference in nuclear uptake of ^{64}Cu, however, was quite significant (Fig. 1B). At the end of the 24 h time course, ^{64}Cu accumulation in the nuclei was two-fold higher in HCT 116 +/+ cells compared HCT 116 -/- cells (8.26 ± 0.13 vs. 4.05 ± 0.07% ID/mg cellular protein, $n = 3$, $p < 0.0001$). Even though uptake of ^{64}Cu from [^{64}Cu]copper acetate was not influenced by p53 status, transport of this radiometal to the nucleus was significantly higher in the p53 expressing cell line.

To test for the integrity of the HCT 116 cell lines utilized in this study, which have been passaged for several decades, and to ensure the absence of clonal artifacts, HCT 116$^{+/+}$ cells were treated with p53 siRNA (short interfering RNA, silencing RNA) to downregulate p53 mRNA and p53 protein. After three days, these cells were exposed to 25 µCi of [^{64}Cu]copper acetate, and cell nuclei were isolated and measured for ^{64}Cu activity. Nuclear uptake was significantly lower in HCT 116$^{+/+}$ cells treated with p53 siRNA (0.126 ± 0.008% ID/mg, $n = 3$) compared to untreated HCT 116 $^{+/+}$ cells (0.252 ± 0.003% ID/mg, $n = 2$, $p < 0.01$). There was no statistical significance between down-regulated HCT 116$^{+/+}$ cells and HCT 116$^{-/-}$ cells (0.165 ± 0.033% ID/mg, $n = 2$, $p = 0.23$). Thus, downregulation of p53 mRNA and protein in HCT 116$^{+/+}$ cells led to nuclear localization of ^{64}Cu more similar in quantity to HCT 116$^{-/-}$ cells than to HCT 116$^{+/+}$ cells. This control experiment reduces the probability that clonal artifacts were responsible for the ^{64}Cu nuclear uptake patterns observed in the two HCT 116 clones.

Synthesis and receptor binding of ^{64}Cu-DOTA-cetuximab. Next, experiments were performed to determine whether this difference would be observed when ^{64}Cu enters the cells via a receptor-mediated pathway. HCT 116 cells express EGFR, and we chose ^{64}Cu-DOTA-cetuximab as a vector for bringing ^{64}Cu into HCT 116 cells. Cetuximab is a humanized anti-EGFR monoclonal antibody and has been approved by the FDA for treatment of EGFR-positive metastatic colorectal cancer and advanced head-and-neck cancer. DOTA is a well-characterized chelator for copper, and it is known that ^{64}Cu can dissociate from the ^{64}Cu-DOTA complex in vivo.[32] ^{64}Cu-DOTA-cetuximab was produced with a specific activity of 10 –15 µCi/µg [0.37–0.56 MBq/µg; 1.5–2.3 nCi/fmol (560–860 Bq/fmol)]. Initial radiochemical purity ranged from 75–100%. For yields < 95%, ^{64}Cu-DOTA-cetuximab was challenged with 5 µl of 10 mM EDTA to complex free ^{64}Cu. ^{64}Cu-DOTA-cetuximab was then isolated with a miniature size exclusion column, resulting in final radiochemical purities ranging from 98 - 100%. Saturation receptor binding experiments with ^{64}Cu-DOTA-cetuximab and isolated HCT 116 +/+ and HCT 116 -/- cell membranes showed that there was no significant difference in the binding characteristics between the two cell lines (Fig. 2). The dissociation constant (K_d) was 0.95 ± 0.26 nM for HCT 116$^{+/+}$ cells and 1.13 ± 0.22 nM for HCT 116$^{-/-}$ cells. These values are comparable to dissociation constants reported for unmodified cetuximab, which range from 0.15–1.2 nM.[33,34] Maximum EGF receptor densities (B_{max}) were very similar as well: 1,950 ± 180 fmol/mg for HCT 116$^{+/+}$ cells, and 2,180 ± 150 fmol/mg for HCT 116$^{-/-}$ cells. It was important to demonstrate that EGF receptor numbers were comparable for both cell lines and were not dependent on their p53 status in order to exclude the possibility that a difference in receptor numbers would confound the results of internalization, nuclear uptake, and efflux studies with ^{64}Cu-DOTA-cetuximab.

Internalization and nuclear uptake of ^{64}Cu-DOTA-cetuximab. These experiments were performed in the same manner as described for [^{64}Cu]copper acetate. Figure 3A shows the uptake of ^{64}Cu-DOTA-cetuximab into both HCT 116 cell lines. As was the case with [^{64}Cu]copper acetate, internalization was similar in both lines. However, uptake did not increase between the 4 h and 24 h time point. It is possible that the receptor-mediated uptake of ^{64}Cu-DOTA-cetuximab was saturated at 4 h, whereas non-specific uptake of [^{64}Cu]copper acetate increased over the 24 h time course. At 24 h the total amount of internalized ^{64}Cu-DOTA-cetuximab was 43.7 ± 1.3% ID for HCT 116$^{+/+}$ cells vs. 42.2 ± 3.3% ID for HCT 116$^{-/-}$ cells ($P = 0.68$, $N = 6$, data not shown). As expected from similar K_d and B_{max} values for the interaction of ^{64}Cu-DOTA-cetuximab and the EGF receptor on the two HCT 116 cell lines, a difference in receptor-mediated endocytosis of ^{64}Cu-DOTA-cetuximab could not be observed.

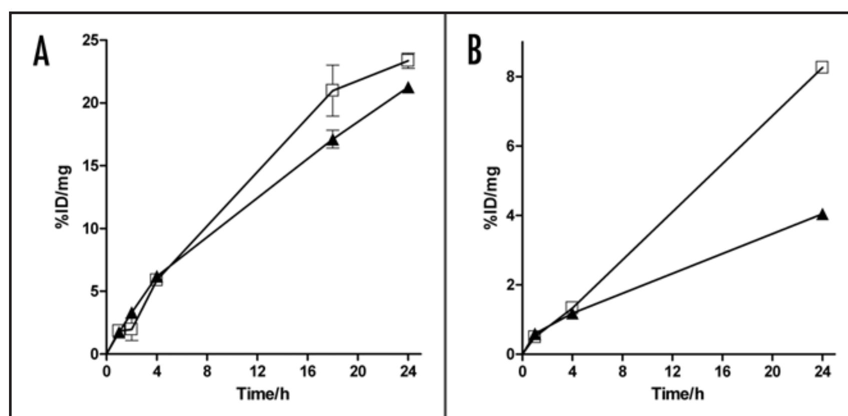

Figure 1. Internalization (A) and nuclear localization (B) of [^{64}Cu]copper acetate in HCT 116 cell lines positive (□) and negative (▲) for p53 (N = 3) in %ID/mg.

When isolated nuclei were measured for ^{64}Cu activity after incubation with ^{64}Cu-DOTA-cetuximab (Fig. 3B), numbers were higher in HCT 116$^{+/+}$ cells compared to HCT 116$^{-/-}$ cells (0.56 ± 0.05% vs. 0.36 ± 0.03% of internalized activity/mg cellular protein at 24 h, $P < 0.001$, N = 6). Those values correspond to 10.3 ± 1.0% and 7.0 ± 0.5% of internalized ^{64}Cu-DOTA-cetuximab (P = 0.01, N = 6, data not shown). In contrast to the analogous experiment with [^{64}Cu]copper acetate, when a significant difference had not been seen at early time points, more ^{64}Cu from ^{64}Cu-DOTA-cetuximab appeared in the nuclei of HCT 116$^{+/+}$ cells as early as 1 h, with the difference being statistically significant at 4 h (0.75 ± 0.09% vs. 0.47 ± 0.05% of internalized activity/mg, p = 0.02, n = 6).

Efflux of ^{64}Cu-DOTA-cetuximab. We hypothesize that ^{64}Cu dissociates from ^{64}Cu-DOTA-cetuximab and is complexed by a different protein before entering the cell nucleus. It is possible that this new complex also translocates to other cell compartments or leaves the tumor cell. To rule out that the observed difference in nuclear uptake between the two HCT 116 cell lines resulted from a variation in release of ^{64}Cu into the medium, ^{64}Cu efflux was monitored for 20 h after an uptake period of 4 h. Figure 4 shows the efflux profiles of both cell lines. Both cell lines retained comparable amounts of ^{64}Cu from internalized ^{64}Cu-DOTA-cetuximab throughout the time course, with the exception of increased efflux of ^{64}Cu from HCT 116$^{-/-}$ cells at 4 h. This time point may be an outlier, and the overall profiles suggest that the difference in ^{64}Cu efflux between HCT 116 cell lines is minimal.

Discussion

Copper is one of the most important trace metals in humans, being surpassed in abundance only by iron and zinc.[35] Therefore, mechanisms for copper uptake, transport and excretion exist within all living cells. Molecular imaging and targeted radiotherapy can take advantage of these mechanisms, e.g., by developing strategies to deliver radioactive isotopes like ^{64}Cu or ^{67}Cu to target cells. Due to its cytotoxicity, however, copper exists in biological systems only in a chelated species, bound by small molecules or proteins. Many of these copper binding proteins have been identified, such as the ubiquitous copper transporter hCtr1, or the metallochaperone CCS, which delivers the metal to the copper redox enzyme superoxide dismutase.[36] Copper also exists in the cell nucleus and can interact with DNA; however, it is not known how copper enters the nucleus or which proteins may assist in its transport.

In the case of ^{64}Cu, nuclear localization may have an additional effect on targeted radiotherapy of cancer. If transported to the nucleus, ^{64}Cu from radiopharmaceuticals may have improved tumor cell killing properties in tumors, in part due to the delivery of a higher radiation dose to nuclear DNA. This extra dose comes from two Auger electrons with energies of 6.5 keV (22.5%) and 840 eV (57.7%), which are emitted by ^{64}Cu. According to the MIRD database,[37] the range for the 6.5 keV electron is less than 1.5 μm in soft tissues, while the range for the 840 eV electron is only 0.05 μm. The diameter of HCT 116 tumor cell nuclei was determined to be 12–13 μm, therefore, nuclear localization is clearly necessary to bring tumor cell DNA in range of these Auger electrons. Another potential mode of cell killing is caused by the recoil energy associated with the transmutation of ^{64}Cu nuclei to stable ^{64}Ni (β^+ decay) or ^{64}Zn (β^- decay). Apelgot and colleagues proposed that this mode of cell killing may be important if the decays occur within the DNA molecule itself, as DNA is highly sensitive to changes in its structure.[15]

We previously demonstrated that ^{64}Cu localized to the nuclei from ^{64}Cu-TETA-octreotide in AR42J rat pancreatic tumor cells,[38] and from ^{64}Cu-TETA-Y3-TATE in A427-7 human non-small cell lung carcinoma cells.[39] ^{64}Cu-CB-TE2A-Y3-TATE is a very stable copper complex, and no nuclear accumulation has been observed with this somatostatin analogue over 24 h.[39] This finding supports the hypothesis that ^{64}Cu must dissociate from its chelator before entering the nucleus. Given the lack of free copper ions in living systems, a yet unidentified binding partner involved in copper trafficking to the nucleus almost certainly exists.

We present research indicating that the tumor suppressor protein p53 is directly or indirectly involved in the process of copper transport to tumor cell nuclei. ^{64}Cu administered as [^{64}Cu]copper acetate and ^{64}Cu-DOTA-cetuximab entered the nuclei of HCT 116$^{+/+}$ cells, which express p53, in significantly greater amounts than was the case with HCT 116$^{-/-}$ cells (Figs. 1B and 3B), which lack both p53 alleles. In the case of [^{64}Cu]copper acetate, the metal is transported into the tumor cells via the hCtr1 transporter. ^{64}Cu-DOTA-cetuximab, a monoclonal chimeric antibody against the EGF receptor, enters the cell by receptor-mediated endocytosis. Different uptake mechanisms may account for the observation that the uptake of ^{64}Cu-DOTA-cetuximab into HCT 116$^{+/+}$ nuclei was significantly increased after 4 h of exposure to the radiopharmaceutical (Fig. 3B), whereas a significant increase of [^{64}Cu]copper acetate uptake occurred only at 24 h (Fig. 1B). Control experiments showed that uptake of [^{64}Cu]copper acetate (Fig. 1A) and ^{64}Cu-DOTA-cetuximab (Fig. 3A), as well as receptor binding (Fig. 2) and efflux of ^{64}Cu-DOTA-cetuximab (Fig. 4) are not significantly different between p53 positive and negative cell lines. Thus, increased trafficking of ^{64}Cu into HCT 116$^{+/+}$ cell lines is not confounded by increased uptake or reduced efflux.

p53 protein is maintained at very low levels in the absence of cellular stress.[40] It can be activated by many conditions such as

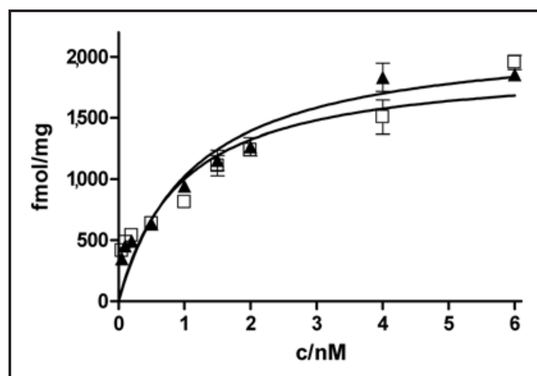

Figure 2. Saturation receptor binding of ^{64}Cu-DOTA-cetuximab to cell membranes isolated from HCT 116 cell lines positive (□) and negative (▲) for p53. Dissociation constants (K_d, 0.95 ± 0.26 nM vs. 1.13 ± 0.22 nM) and maximum receptor densities (B_{max}, 1,950 ± 180 fmol/mg vs. 2,180 ± 150 fmol/mg) were similar for HCT 116 +/+ and HCT 116 -/- cell lines, respectively (N = 3).

DNA damage, hypoxia, chromosomal aberrations, telomere shortening and others, whereupon it accumulates in the nucleus,[41] acting as a sequence-specific transcriptional regulator. Copper toxicity is correlated with apoptotic cell death, and translocation of p53 from the cytosol into the nucleus has been shown to occur in cells treated with 200 µM copper supplied as cupric sulfate.[42] Our study shows that presence of p53 in turn increases the amount of ^{64}Cu that is transported to the nucleus of HCT 116 cells. Copper is capable of displacing zinc from metal-binding sites in proteins and has been shown to bind to p53 in vitro.[19] Thus one possibility is that p53 itself is responsible for the increased transport of ^{64}Cu into the nuclei of HCT $116^{+/+}$ cells. We are currently examining another option, namely that one of the many binding partners of p53 complexes ^{64}Cu and is transported to the nucleus together with p53. One promising candidate is metallothionein, which is involved in the intracellular storage of metal ions like zinc and copper, and which has been shown to physically interact with p53 by co-immunoprecipitation with both anti-p53 and anti-metallothionein antibodies.[43] A third potential mechanism involves proteins from genes whose expression is upregulated by p53 after exposure to the radiocopper. Oligonucleotide array studies in neurons identified several p53 target genes which are differentially regulated after exposure to copper.[44] Genes involved in cell cycle arrest (e.g., *p21*, *reprimo*, *stathmin*, *Tp53INP1*) and apoptotic processes (e.g., *IGFBP-6*, *PUMA*), as well as heat-shock proteins (e.g., Hsp70, Hsp 27) were identified; however, their potential role in copper transport has not yet been examined. Finally, it is possible that HCT $116^{+/+}$ cells express a protein spectrum fundamentally different from HCT $116^{-/-}$ cells in quality and quantity even in the ground state before exposed to any external stressor like radiation. This implies that the transcription of an as of now unknown copper-binding protein would be activated by basal levels of p53.

In summary, the data presented here demonstrate a potential mechanism for increased toxicity of ^{64}Cu radiopharmaceuticals for targeted radiotherapy of cancer involving the tumor suppressor protein p53. Copper-64 radiopharmaceuticals may show enhanced localization of ^{64}Cu in tumor cell nuclei in tumors that express WT p53 compared to p53 null or p53 mutant expressing tumor cells. This increased localization of ^{64}Cu in the nuclei may cause increased DNA damage to cells, and therefore improved tumor cell killing. In addition, we have identified a role for p53 in the transport of copper into the cell nucleus.

Materials and Methods

Materials. 1,4,7,10-Tetraazacyclododecane-1,4,7-tris(t-butyl acetate)-10-acetic acid mono (N-hydroxysuccinimide ester) [DOTA-mono-NHS-tris(tBu) ester] was purchased from Macrocyclics (Dallas, TX). Centricon 100 concentrators were purchased from Amicon Inc. (Beverly, MA). All chemicals used were purchased from Aldrich Chemical Co. (Milwaukee, WI). All solutions were made using distilled deionized water (Milli-Q; ~18 MΩ resistivity). Monoclonal antibody cetuximab (C225) was kindly provided by ImClone Systems (New York, NY). HCT 116 +/+ and HCT 116 -/- cell lines were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University). A Branson Sonifier cell disrupter, and a Sorvall RC2-B centrifuge were used in the receptor binding experiments. Size-exclusion HPLC used in conjugation and purification of radiolabeled conjugate was accomplished on a Superose 12 HR 10/300 column (Amersham Biosciences, Uppsala, Sweden) with a Waters (Milford, MA) 2487 dual λ absorbance detector and an Ortec Model 661 (EG&G Instruments, Oak Ridge, TN) radioactive detector. The mobile phase was 20 mM HEPES, 150 mM NaCl, pH 7.3 eluted at a flow rate of 0.5 ml/min. Millenium 32 software (Waters, Milford, MA) was used to quantify chromatograms by integration. A Beckman 8000 automated well-typed gamma counter (Fullerton, CA) was used to measure internalization and nuclear uptake samples. Copper-64 was produced as previously reported on a biomedical cyclotron CS-15 at Washington University School of Medicine.[28]

Preparation of ^{64}Cu-DOTA-cetuximab. DOTA was conjugated to cetuximab in 0.1 M Na_2HPO_4 (pH 7.5) using an adaptation of the method described by Lewis et al.[29] Cetuximab (2 mg/ml) was washed with 0.1 M Na_2HPO_4 (pH 7.5) and concentrated using a Centricon 100. DOTA-mono-NHS-tris(tBu) ester was dissolved in 0.1 M Na_2HPO_4 (pH 7.4) and pH was adjusted to 7.4 by adding 0.1 M NaOH. An aliquot of this solution was added to the concentrated cetuximab in a molar ratio of DOTA-mono-NHS-tris(tBu) ester : cetuximab = 90 : 1, followed by incubation at 4°C overnight with end-over-end rotation. The conjugate was then transferred to a Centricon 100, diluted to 2.0 ml with 0.1 M ammonium citrate (pH 5.5), and centrifuged. This procedure was repeated to remove small molecule reactants and the conjugate was collected from the membrane. Purity and concentration of the resulting conjugate were determined by size-exclusion HPLC, and DOTA-cetuximab was stored at 4°C until needed.

Radiolabeling of DOTA-cetuximab with ^{64}Cu was carried out by adding approximately 100 µg of the conjugate to 0.5–2 mCi (19 –74 MBq) $^{64}CuCl_2$ in 0.1 M ammonium citrate buffer, pH 5.5, followed by a 1 h incubation at 40°C. The radiochemical purity of the resulting ^{64}Cu-DOTA-cetuximab was determined by size-exclusion HPLC and radio-TLC. If necessary, ^{64}Cu-DOTA-cetuximab was challenged with 5 µl of 10 mM EDTA to complex free ^{64}Cu. ^{64}Cu-DOTA-cetuximab was then isolated with a Zeba™ Desalt Spin column (0.5 ml, Pierce, Rockford, IL) to obtain radiochemical purities > 95%.

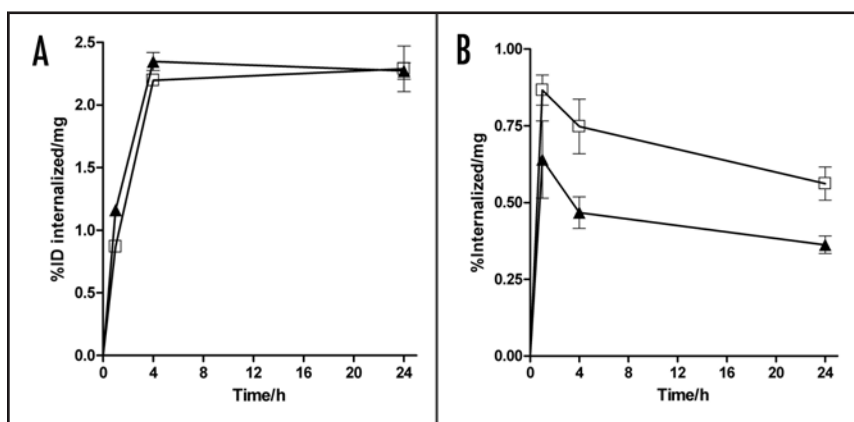

Figure 3. (A) %ID/mg of ^{64}Cu-DOTA-cetuximab internalized in HCT 116 cell lines positive (□) and negative (▲) for p53. (B) Percentage of internalized activity localized to the nuclear fraction of HCT 116 cells per mg (N = 6 with two independent ^{64}Cu productions).

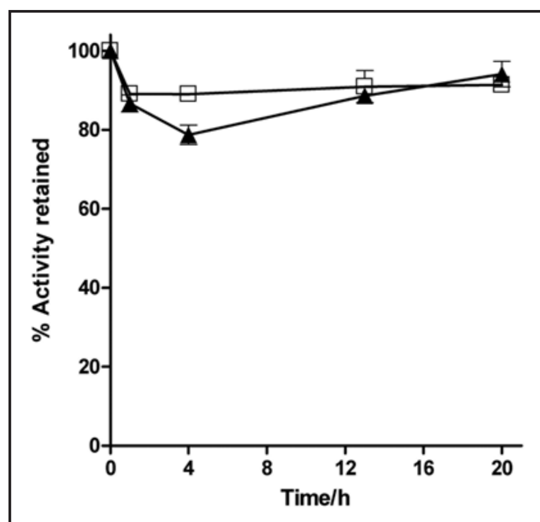

Figure 4. Efflux of ^{64}Cu-DOTA-cetuximab from HCT 116 cell lines positive (□) and negative (▲) for p53. Efflux is expressed as the percentage of retained activity, where the activity at t = 0 (when the radioactive media is removed) is 100%.

Binding affinity of ^{64}Cu-DOTA-cetuximab. Cell membranes from HCT $116^{+/+}$ and HCT $116^{-/-}$ cell lines for binding assays were prepared as previously described.[30] Assays were performed on a 96-well Multiscreen® Durapore filtration plate (Millipore, Billerica, MA) using methods previously described with some modifications.[31] Membranes were diluted in binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2 \cdot 6\ H_2O$, 0.1% BSA, 0.5 μg/ml aprotinin, 200 μg/ml bacitracin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A), and 10 μg of membrane protein were used per well. Increasing concentrations (0.05 nM–6 nM) of ^{64}Cu-DOTA-cetuximab were added to membranes to measure total binding, and non-specific binding was determined by conducting the assay in the presence of an excess (2 μM) of cetuximab. After incubation at RT for 2 h, the medium was removed with a vacuum manifold and the membranes were washed twice with 200 μl binding buffer. OptiPhase 'Super-Mix' (PerkinElmer, Boston, MA) (25 μl) was added to each well, and bound activity was measured with a 1450 Microbeta liquid scintillation and luminescence counter (Perkin Elmer, Boston, MA). Specific binding was obtained by subtraction of non-specific binding from total binding. Maximum binding capacities (B_{max}) were estimated from non-linear curve fitting of specific binding versus the concentration of ^{64}Cu-DOTA-cetuximab using GraphPad Prism (San Diego, CA).

Internalization and efflux of [^{64}Cu]copper acetate and ^{64}Cu-DOTA-cetuximab in HCT 116 cell lines. HCT 116 +/+ or HCT 116 -/- cells were seeded in 6-well plates containing growth medium (McCoy's 5a medium with 1.5 mM L-glutamine adjusted to contain 2.2 g/l sodium bicarbonate, 90%; fetal bovine serum, 10%) and were incubated at 37°C, 5% CO_2 until 80% confluent. On the day of the assay, the medium was aspirated and 3 ml fresh growth medium was placed in each well. For determination of nonspecific internalization in the cetuximab experiments, one set of wells was incubated with unlabeled cetuximab (375 μg/100 μl) at 37°C, 5% CO_2 for 10 min to block EGF receptors. [^{64}Cu]copper acetate (25 μCi/10 μl) or ^{64}Cu-DOTA-cetuximab (3.6 μCi, 0.38 μg/15 μl) were added to all of the wells and incubated at 37°C, 5% CO_2 for 1, 4 or 24 h. At each time point, radioactive medium was aspirated, and the plate was washed twice with 2 ml PBS. To collect the surface-bound fraction, each well was treated with 20 mM sodium acetate in PBS (pH 3.0) and was incubated at 4°C for 10 min followed by a second 20 mM sodium acetate in PBS (pH 3.0) wash without incubation, which was pooled with the first rinse. The cellular fraction was solubilized in 0.1% SDS. All of the fractions were counted for radioactivity with a 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, and normalized to protein content.

For efflux experiments, cells were seeded in 6-well plates and allowed to adhere overnight. Cells were fed 3 ml fresh growth media on the morning of the experiment, ^{64}Cu-DOTA-cetuximab was added to each well, and cells were incubated at 37°C, 5% CO_2 for 4 h. The radioactive medium was removed and the cells were washed twice with ice cold PBS. Fresh growth medium (3 ml) was added to each well, and the cells were incubated for 1, 4 or 20 h. At each time point, the media was removed and the cells were washed twice with ice cold PBS. The cells were solubilized in 0.1% SDS, transferred to a microfuge tube, and counted for radioactivity on the gamma counter.

Nuclear localization of [^{64}Cu]copper acetate and ^{64}Cu-DOTA-cetuximab in HCT 116 cell lines. For isolation of HCT 116 cell nuclei, the procedure of Wang et al.[14] was used. The experiments were performed in T-175 flasks containing approximately 5 x 10^7 cells at the beginning of the time course. ^{64}Cu-DOTA-cetuximab (4.0 μCi, 0.33 μg/20 μl) was added, and 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 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 CSK buffer without Triton X-100, and centrifuged

at 560 g at 4°C for 5 min. The supernatant was discarded and the nuclear pellet was counted with a gamma counter. Aliquots of nuclei were assayed qualitatively for purity by fluorescence microscopy after staining with a 1:10 dilution of FITC (3 μg/ml) and propidium iodide (7 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 internalized into whole cells, and was corrected for the yield of nuclei.

As an additional control in the HCT 116 cell lines, nuclear uptake of [^{64}Cu]copper acetate was determined in HCT $116^{+/+}$ cells after downregulation of p53 by p53 siRNA, and compared to untreated HCT $116^{+/+}$ and HCT $116^{-/-}$ cells. Cells were seeded in T-75 flasks so that they were 30–40% confluent after 24 h. TransPass™ R1 Transfection Reagent (200 μl; New England BioLabs, Boston, MA) was mixed with 5 ml serum-free medium and incubated for 15 min at RT. p53 ShortCut® siRNA Mix (20 μl; New England BioLabs) was added, followed by another 15 min incubation at RT and addition of 43 ml medium with serum. This mixture was added to HCT $116^{+/+}$ cells (12 ml/flask) and the cells were grown for 48 h under standard conditions. A change to fresh medium was followed by additional 24 h of incubation, and nuclear localization experiments were then conducted as described above.

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References

1. DeNardo GL, Kukis DL, Shen S, Mausner LF, Meares CF, Srivastava SC, Miers LA, DeNardo SJ. Efficacy and toxicity of ^{67}Cu-2IT-BAT-Lym-1 radioimmunoconjugate in mice implanted with human Burkitt's lymphoma (Raji). Clin Cancer Res 1997; 3:71-9.
2. DeNardo GL, Kukis DL, DeNardo SJ, Shen S, Mausner LF, O'Donnell RT, Lamborn KR, Meyers FJ, Srivastava SC, Miers LA. Enhancement of ^{67}Cu-2IT-BAT-LYM-1 therapy in mice with human Burkitt's lymphoma (Raji) using interleukin-2. Cancer 1997; 80:2576-82.
3. Kroger LA, DeNardo GL, Gumerlock PH, Xiong CY, Winthrop MD, Shi XB, Mack PC, Leshchinsky T, DeNardo SJ. Apoptosis-related gene and protein expression in human lymphoma xenografts (Raji) after low dose rate radiation using ^{67}Cu-2IT-BAT-Lym-1 radioimmunotherapy. Cancer Biother Radiopharm 2001; 16:213-25.
4. O'Donnell RT, DeNardo GL, Kukis DL, Lamborn KR, Shen S, Yuan A, Goldstein DS, Carr CE, Mirick GR, DeNardo SJ. A clinic trial of radioimmunotherapy with ^{67}Cu-2IT-Lym-1 for non-Hodgkin's lymphoma. J Nucl Med 1999; 40:2014-20.
5. O'Donnell RT, DeNardo GL, Kukis DL, Lamborn KR, Shen S, Yuan A, Goldstein DS, Mirick GR, DeNardo SJ. ^{67}Copper-2-iminothiolane-6-[p-(bromoacetamido)benzyl]-TET A-Lym-1 for radioimmunotherapy of non-Hodgkin's lymphoma. Clin Cancer Res 1999; 5:3330s-6s.
6. Connett JM, Anderson CJ, Guo LW, Schwarz SW, Zinn KR, Rogers BE, Siegel BA, Philpott GW, Welch MJ. Radioimmunotherapy with a Cu-64-labeled monoclonal antibody: A comparision with Cu-67. Proc Nat Acad Sci USA 1996; 93:6814-8.
7. Connett JM, Buettner TL, Anderson CJ. Maximum tolerated dose and large tumor radioimmunotherapy studies of ^{64}Cu-labeled mAb 1A3 in a colon cancer model. Clin Cancer Res 1999; 5:3207s-12s.
8. Anderson CJ, Jones LA, Bass LA, Sherman ELC, McCarthy DW, Cutler PD, Lanahan MV, Cristel ME, Lewis JS, Schwarz SW. Radiotherapy, toxicity and dosimetry of copper-64-TETA-octreotide in tumor-bearing rats. J Nucl Med 1998; 39:1944-51.
9. Lewis JS, Lewis MR, Cutler PD, Srinivasan A, Schmidt MA, Schwarz SW, Morris MM, Miller JP, Anderson CJ. Radiotherapy and dosimetry of ^{64}Cu-TETA-Tyr^{3}-octreotate in a somatostatin receptor-positive, tumor-bearing rat model. Clin Cancer Res 1999; 5:3608-16.
10. Krenning EP, de Jong M, Kooij PP, Breeman WA, Bakker WH, de Herder WW, van Eijck CH, Kwekkeboom DJ, Jamar F, Pauwels S, Valkema R. Radiolabelled somatostatin analogue(s) for peptide receptor scintigraphy and radionuclide therapy. Ann Oncol 1999; 10(Suppl 2):S23-9.
11. Krenning EP, Kooij PPM, Bakker WH, Breeman WAP, Postema PTE, Kwekkeboom DJ, Oei HY, de Jong M, Visser TJ, Reijs AEM, Lamberts SWJ. Radiotherapy with a radiolabeled somatostatin analogue, ^{111}In-DTPA-Phe^{1}-octreotide: A case history. Ann NY Acad Sci 1994; 733:496-506.
12. Hornick CA, Anthony CT, Hughey S, Gebhardt BM, Espenan GD, Woltering EA. Progressive nuclear translocation of somatostatin analogs. J Nucl Med 2000; 41:1256-63.
13. Janson ET, Westlin JE, Ohrvall U, 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 2000; 41:1514-8.
14. Wang M, Caruano AL, Lewis MR, Meyer LA, Vander Waal RP, Anderson CJ. Subcellular localization of radiolabeled somatostatin analogues: Implications for targeted radiotherapy of cancer. Cancer Res 2003; 63:6864-9.
15. Apelgot S, Coppey J, Gaudemer A, Grisvard J, Guille E, Sasaki I, Sissoeff I. Similar lethal effect in mammalian cells for two radioisotopes of copper with different decay schemes, Cu-64 and Cu-67. Int J Radiat Biol 1989; 55:365-84.
16. Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: The requirement of a copper chaperone for superoxide dismutase. Science 1999; 284:805-8.
17. Field LS, Luk E, Culotta VC. Copper chaperones: Personal escorts for metal ions. J Bioenerg Biomembr 2002; 34:373-9.
18. Prohaska JR, Gybina AA. Intracellular copper transport in mammals. J Nutr 2004; 134:1003-6.
19. Hainaut P, Rolley N, Davies M, Milner J. Modulation by copper of p53 conformation and sequence-specific DNA binding: Role for Cu(II)/Cu(I) redox mechanism. Oncogene 1995; 10:27-32.
20. Yamada T, Goto M, Punj V, Zaborina O, Kimbara K, Das Gupta TK, Chakrabarty AM. The bacterial redox protein azurin induces apoptosis in J774 macrophages through complex formation and stabilization of the tumor suppressor protein p53. Infect Immun 2002; 70:7054-62.
21. Yamada T, Goto M, Punj V, Zaborina O, Chen ML, Kimbara K, Majumdar D, Cunningham E, Das Gupta TK, Chakrabarty AM. Bacterial redox protein azurin, tumor suppressor protein p53, and regression of cancer. Proc Natl Acad Sci USA 2002; 99:14098-103.
22. Punj V, Bhattacharyya S, Saint-Dic D, Vasu C, Cunningham EA, Graves J, Yamada T, Constantinou AI, Christov K, White B, Li G, Majumdar D, Chakrabarty AM, Das Gupta TK. Bacterial cupredoxin azurin as an inducer of apoptosis and regression in human breast cancer. Oncogene 2004; 23:2367-78.
23. Punj V, Das Gupta TK, Chakrabarty AM. Bacterial cupredoxin azurin and its interactions with the tumor suppressor protein p53. Biochem Biophys Res Commun 2003; 312:109-14.
24. Goto M, Yamada T, Kimbara K, Horner J, Newcomb M, Gupta TK, Chakrabarty AM. Induction of apoptosis in macrophages by *Pseudomonas aeruginosa* azurin: Tumour-suppressor protein p53 and reactive oxygen species, but not redox activity, as critical elements in cytotoxicity. Mol Microbiol 2003; 47:549-59.
25. Wong SF. Cetuximab: An epidermal growth factor receptor monoclonal antibody for the treatment of colorectal cancer. Clin Ther 2005; 27:684-94.
26. Saltz LB, Meropol NJ, Loehrer Sr PJ, Needle MN, Kopit J, Mayer RJ. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol 2004; 22:1201-8.
27. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Van Cutsem E. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 2004; 351:337-45.
28. McCarthy DW, Shefer RE, Klinkowstein RE, Bass LA, Margenau WH, Cutler CS, Anderson CJ, Welch MJ. The efficient production of high specific activity Cu-64 using a biomedical cyclotron. Nucl Med Biol 1997; 24:35-43.
29. Lewis MR, Kao JY, Anderson ALJ, Shively JE, Raubitschek A. An improved method for conjugating antibodies with *N*-hydroxysulfosuccinimidyl DOTA. Bioconj Chem 2001; 12:320-4.
30. Parry JJ, Eiblmaier M, Andrews R, Meyer LA, Higashikubo R, Anderson CJ, Rogers BE. Characterization of somatostatin receptor subtype 2 expression in stably transfected A-427 human cancer cells. Mol Imaging 2007; 6:56-67.
31. Sprague JE, Peng Y, Sun X, Weisman GR, Wong EH, Achilefu S, Anderson CJ. Preparation and biological evaluation of copper-64-Tyr^{3}-octreotate using a cross-bridged macrocyclic chelator. Clin Cancer Res 2004; 10:8674-82.
32. Boswell CA, Sun X, Niu W, Weisman GR, Wong EH, Rheingold AL, Anderson CJ. Comparative in vivo stability of copper-64-labeled cross-bridged and conventional tetraazamacrocyclic complexes. J Med Chem 2004; 47:1465-74.
33. Goldstein NI, Prewett M, Zuklys K, Rockwell P, Mendelsohn J. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. Clin Cancer Res 1995; 1:1311-8.

34. Naramura M, Gillies SD, Mendelsohn J, Reisfeld RA, Mueller BM. Therapeutic potential of chimeric and murine anti-(epidermal growth factor receptor) antibodies in a metastasis model for human melanoma. Cancer Immunol Immunother 1993; 37:343-9.
35. Sun X, Anderson CJ. Production and applications of copper-64 radiopharmaceuticals. Methods Enzymol 2004; 386:237-61.
36. O'Halloran TV, Culotta VC. Metallochaperones, an intracellular shuttle service for metal ions. J Biol Chem 2000; 275:25057-60.
37. Nuclear decay data in the MIRD format. By: International network of nuclear structure and decay data evaluators (http://www.nndc.bnl.gov/mird/).
38. Wang M, Caruano AL, Lewis MR, Meyer LM, VanderWaal RP, Anderson CJ. Subcellular localization of somatostatin analogues: Implications for targeted radiotherapy of cancer. Cancer Res 2003; 63:6864-9.
39. Eiblmaier M, Andrews R, Laforest R, Rogers BE, Anderson CJ. Nuclear uptake and dosimetry of ^{64}Cu-labeled chelator-somatostatin conjugates in a *SSTr2*-transfected human tumor cell line. J Nucl Med 2007; 48:1390-6.
40. Oren M. Decision making by p53: Life, death and cancer. Cell Death Differ 2003; 10:431-42.
41. Ostrakhovitch EA, Cherian MG. Differential regulation of signal transduction pathways in wild type and mutated p53 breast cancer epithelial cells by copper and zinc. Arch Biochem Biophys 2004; 423:351-61.
42. Narayanan VS, Fitch CA, Levenson CW. Tumor suppressor protein p53 mRNA and subcellular localization are altered by changes in cellular copper in human Hep G_2 cells. J Nutr 2001; 131:1427-32.
43. Ostrakhovitch EA, Olsson PE, Jiang S, Cherian MG. Interaction of metallothionein with tumor suppressor p53 protein. FEBS Lett 2006; 580:1235-8.
44. Vanlandingham JW, Tassabehji NM, Somers RC, Levenson CW. Expression profiling of p53-target genes in copper-mediated neuronal apoptosis. Neuromolecular Med 2005; 7:311-24.