

An impaired mitochondrial electron transport chain increases retention of the hypoxia imaging agent diacetylbis(4-methylthiosemicarbazonato)copper^{II}

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Radiolabeled diacetylbis(4-methylthiosemicarbazonato)copper^{II} [Cu^{II}(atsm)] is an effective positron-emission tomography imaging agent for myocardial ischemia, hypoxic tumors, and brain disorders with regionalized oxidative stress, such as mitochondrial myopathy, encephalopathy, and lactic acidosis with stroke-like episodes (MELAS) and Parkinson's disease. An excessively elevated reductive state is common to these conditions and has been proposed as an important mechanism affecting cellular retention of Cu from Cu^{II}(atsm). However, data from whole-cell models to demonstrate this mechanism have not yet been provided. The present study used a unique cell culture model, mitochondrial xenocybrids, to provide whole-cell mechanistic data on cellular retention of Cu from Cu^{II}(atsm). Genetic incompatibility between nuclear and mitochondrial encoded subunits of the mitochondrial electron transport chain (ETC) in xenocybrid cells compromises normal function of the ETC. As a consequence of this impairment to the ETC we show xenocybrid cells upregulate glycolytic ATP production and accumulate NADH. Compared to control cells the xenocybrid cells retained more Cu after being treated with Cu^{II}(atsm). By transfecting the cells with a metal-responsive element reporter construct the increase in Cu retention was shown to involve a Cu^{II}(atsm)-induced increase in intracellular bioavailable Cu specifically within the xenocybrid cells. Parallel experiments using cells grown under hypoxic conditions confirmed that a compromised ETC and elevated NADH levels contribute to increased cellular retention of Cu from Cu^{II}(atsm). Using these cell culture models our data demonstrate that compromised ETC function, due to the absence of O₂ as the terminal electron acceptor or dysfunction of individual components of the ETC, is an important determinant in driving the intracellular dissociation of Cu^{II}(atsm) that increases cellular retention of the Cu.

sodium arsenite | energy metabolism | radiopharmaceutical

PET allows noninvasive imaging to assist in diagnosis of disease and monitoring of therapeutic treatments. The technique relies on administration of a compound radiolabeled with a positron-emitting isotope. Although there are several positron-emitting isotopes of copper that are of interest in the development of copper-based PET tracers (copper-60, copper-62, and copper-64), it is essential to selectively and safely deliver the radioactive copper ion to target tissue. An approach to achieve this goal is to incorporate the radioactive copper ion into a coordination complex. The resulting complex is likely to have distinctly different biodistribution when compared to the use of the simple complex ions in aqueous solution.

A family of ligands derived from the condensation of 1,2-diones with substituted thiosemicarbazides collectively known as bis(thiosemicarbazones) form stable, neutral, and lipophilic complexes with Cu^{II}. These qualities have led to the use of bis(thiosemicarbazones) as delivery vehicles for radioactive copper

isotopes in the development of unique radiopharmaceuticals (1–4). A particular focus is on the use of radiolabeled diacetylbis(4-methylthiosemicarbazonato)copper^{II} [Cu^{II}(atsm)] (Fig. 1) as a hypoxia imaging agent. Hypoxia can be associated with aggressive tumors and the effective delineation of degrees of hypoxia is also of interest to clinicians in assessing stroke and myocardial ischemia.

An early study into the potential of ⁶⁰Cu^{II}(atsm) as a hypoxia imaging agent described selective accumulation of ⁶²Cu^{II}(atsm) in rat hearts subjected to ischemic insult (5). In control mice only 23% of the injected dose of ⁶²Cu^{II}(atsm) was retained in the heart, whereas 81% was retained in hearts subjected to ischemic insult (5). A subsequent in vivo study using dog models of hypoxic myocardium demonstrated the ability to use PET to monitor selective tissue retention of the ^{60/64}Cu from Cu^{II}(atsm) (6), and a preliminary study has indicated the ability to use ⁶²Cu^{II}(atsm) to image the heart in people with coronary heart disease (7). Subsequent studies focused on the potential of radiolabeled Cu^{II}(atsm) as an imaging agent of tumor hypoxia. Radiolabeled ⁶⁰Cu^{II}(atsm) has been investigated as a PET tracer to detect tumor hypoxia in cervical cancer and is currently undergoing human clinical trials (8, 9).

Recently ⁶²Cu^{II}(atsm) has been used as a PET imaging agent in a single subject with mitochondrial myopathy, encephalopathy, and lactic acidosis with stroke-like episodes (MELAS) (10) and 15 people with Parkinson's disease (11). Unlike the previous studies that emphasized Cu^{II}(atsm) as an agent to image hypoxic tissue, the studies in patients with MELAS and Parkinson's disease described selective accumulation of Cu from Cu^{II}(atsm) in the disease-affected tissue as a method to image regionalized oxidative stress (10, 11). Oxidative stress and hypoxia are both consistent with a cellular environment in which the abundance of cellular reductants relative to O₂ is increased above normal conditions, raising the possibility that factors conducive to a perturbed reductant:O₂ balance are a more important determinant of cellular accumulation of the Cu from Cu^{II}(atsm) than merely oxygen concentrations or oxidative stress per se. Cell-free studies and experiments using isolated subcellular components support this possibility but have not demonstrated feasibility in whole-cell studies. As a consequence, the cellular conditions that control

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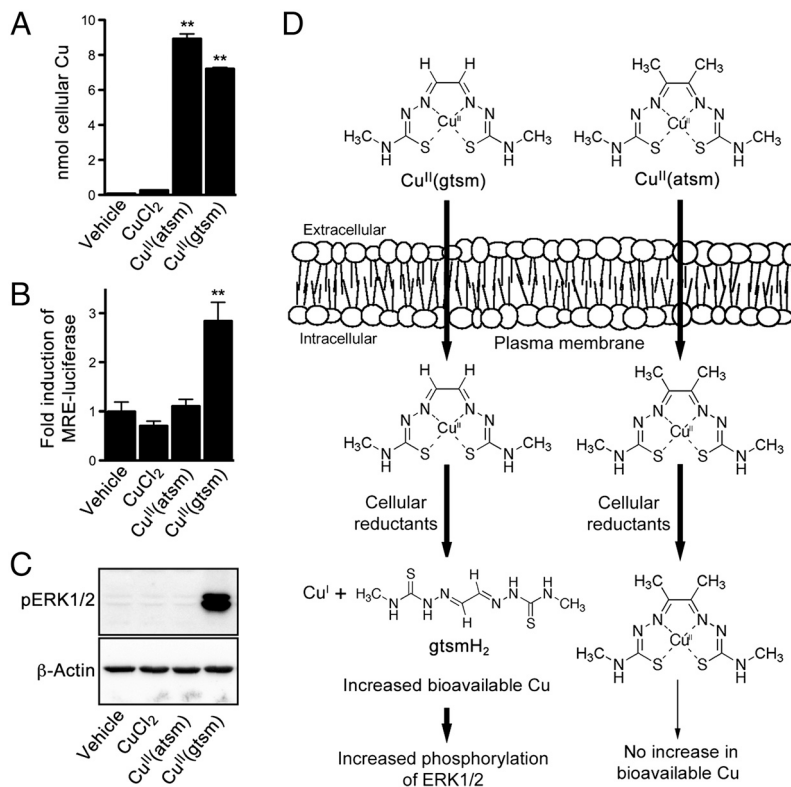


Fig. 1. Comparative effects of CuCl₂, Cu^{II}(atms), and Cu^{II}(gtsm). (A) SH-SY5Y cells treated with 10 μM CuCl₂, Cu^{II}(atms), or Cu^{II}(gtsm) for 1 h before analyzing cells for Cu content by ICP-MS, which does not differentiate between intact Cu^{II}(atms)/Cu^{II}(gtsm) and Cu that has dissociated from the respective bis(thiosemicarbazone) scaffolds. (B) Before treating with 500 nM CuCl₂, Cu^{II}(atms), or Cu^{II}(gtsm) for 6 h, SH-SY5Y cells were transfected with MRE-luciferase construct. Increased bioavailable Cu within the cell upregulates expression of the luciferase reporter, induction of which is measured by luminescence. (C) SH-SY5Y cells treated with 10 μM CuCl₂, Cu^{II}(atms), or Cu^{II}(gtsm) for 1 h were analyzed for phosphorylated ERK1/2 (pERK1/2). β-Actin levels are shown as a control. (D) Schematic illustrating that although Cu^{II}(atms) and Cu^{II}(gtsm) both enter the cell, only Cu^{II}(gtsm) is sensitive to the activity of cellular reductants under normal conditions. Increased ERK1/2 phosphorylation is responsive to increases in bioavailable Cu within the cell. Vehicle represents cells treated with the DMSO vehicle used to prepare the Cu compounds. Data are mean values ± SEM, *n* = 6–12. ***P* < 0.01 compared to vehicle-treated cells (ANOVA with Tukey's posttest).

selective retention of radioactive Cu in disease-affected tissues remain incompletely understood (4). The present study aimed to delineate the role of impaired energy metabolism, oxidative stress, hypoxia, and mitochondrial electron transport chain (ETC) function cellular retention of Cu from Cu^{II}(atms).

Results

Cu^{II}(atms) is Resistant to Intracellular Dissociation Under Normal Cellular Conditions.

The chemistry and cellular metabolism of the bis(thiosemicarbazone)copper(II) complexes is dependent on the backbone substituents of the ligand. The electron donating methyl groups on the atms ligand lower the Cu^{II}/Cu^I reduction potential for Cu^{II}(atms) when compared to glyoxalbis[N(4)-methylthiosemicarbazone]Cu^{II} [Cu^{II}(gtsm)] (Fig. 1) (*E*_m = 0.60 mV and *E*_m = 0.44 mV, respectively, versus SCE where *F*c/*F*c⁺ = 0.54 V) (12, 13). Cu^{II}(atms) is therefore more resistant to intracellular reduction of the metal ion and less likely to dissociate under normal cellular conditions. This salient feature of Cu^{II}(atms) compared to Cu^{II}(gtsm) is demonstrated in SH-SY5Y cells treated with 10 μM CuCl₂, Cu^{II}(atms), or Cu^{II}(gtsm) for 1 h. Cells analyzed for Cu content show that both bis(thiosemicarbazone)-Cu^{II} compounds increase cellular Cu levels more efficiently than CuCl₂ (Fig. 1A) (14). However, analysis of cell pellets by inductively coupled plasma (ICP)-MS does not provide information on whether the copper ion has dissociated from the ligand within the cell. Intracellular dissociation of the compounds cells was therefore examined using cells transfected with a metal-responsive element (MRE)-luciferase reporter (15, 16) prior to treating with the bis(thiosemicarbazone)-Cu^{II}

compounds. The MRE-luciferase reporter responds to treatments that induce an increase in cytosolic levels of bioavailable Zn (16). The increased bioavailable Zn activates the metal-responsive transcription factor 1 (MTF-1) that in turn promotes the expression of genes that contain MREs in their promoter region (17, 18). Increasing bioavailable Zn in cells by treating them with high concentrations of ZnCl₂ (40–100 μM for 24 h) promotes expression of the MRE-luciferase construct, as does treating with high concentrations of CuCl₂ (40–100 μM for 24 h) (16). The effect induced by treating with Cu is due to the increased bioavailable Cu within the cell displacing Zn from endogenous metallothioneins (16). When treated with 500 nM Cu^{II}(gtsm) for 6 h SH-SY5Y cells transfected with the MRE-luciferase reporter showed a threefold induction of the reporter (Fig. 1B), indicating the Cu^{II}(gtsm) treatment had increased bioavailable Cu within the cells. By contrast, the same concentration of Cu^{II}(atms), which increased overall Cu content of the cells to a level comparable to the Cu^{II}(gtsm) treatment as determined by ICP-MS (Fig. 1A), did not influence the MRE-luciferase (Fig. 1B), indicating no change to bioavailable Cu in the Cu^{II}(atms)-treated cells. Further, when SH-SY5Y cells were treated with the bis(thiosemicarbazone)-Cu^{II} compounds or the CuCl₂ control under normal cellular conditions, only the Cu^{II}(gtsm) induced an increase in phosphorylation of the signaling kinase ERK1/2. Together with the MRE-luciferase data in Fig. 1B, these data (Fig. 1C) show that cellular responses leading to increased ERK1/2 phosphorylation, induced by treating with bis(thiosemicarbazone)-Cu^{II} compounds, are initiated by the compound increasing bioavailable Cu within the cell.

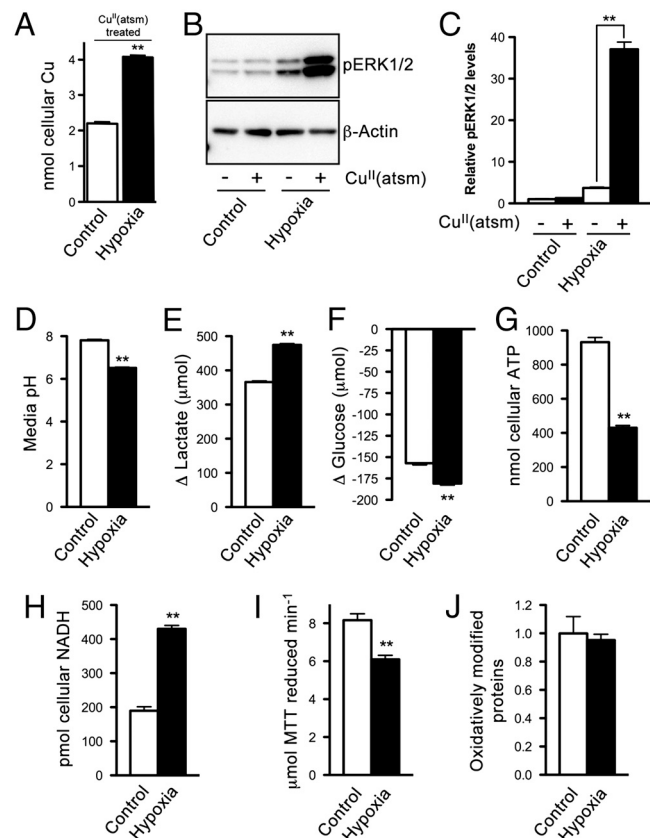


Fig. 2. Effects of hypoxia on energy metabolism and cellular responses to $\text{Cu}^{\text{II}}(\text{atism})$ in SH-SY5Y cells. (A) ICP-MS analysis of cellular Cu in normoxic control cells and hypoxic cells after treating with $10 \mu\text{M}$ $\text{Cu}^{\text{II}}(\text{atism})$ for 1 h. (B) Western blotting image showing $\text{Cu}^{\text{II}}(\text{atism})$ promotes phosphorylation of ERK1/2 (pERK1/2) in hypoxic cells. β -Actin levels are shown as a control. (C) Densitometry analysis of Western blotting results. (D) pH of media collected from cells after 2 d in culture. (E) Lactate produced and (F) glucose consumed by cells over 2 d in culture. (G) ATP and (H) NADH content of control cells and hypoxic cells. (I) Reduction of MTT mediated by cell lysates collected from control and hypoxic cells. (J) Relative content of oxidatively modified proteins in control cells and hypoxic cells. Data are mean values \pm SEM, $n = 3$ –6. Values shown in A and E–I are expressed per milligram cellular protein. $P < 0.01$ compared to control cells (t test) except for C where $**P < 0.01$ compared to vehicle-treated cells (ANOVA with Tukey's posttest).

Hypoxia Impairs Energy Metabolism and Promotes Cellular Retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$. To examine the cellular conditions involved in hypoxia-selective retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$, SH-SY5Y cells were subjected to hypoxic conditions prior to treating with $\text{Cu}^{\text{II}}(\text{atism})$ ($10 \mu\text{M}$). Hypoxia increased the levels of cellular Cu in $\text{Cu}^{\text{II}}(\text{atism})$ -treated cells (Fig. 2A) suggesting intracellular dissociation of Cu from $\text{Cu}^{\text{II}}(\text{atism})$, presumably following reduction of $\text{Cu}^{\text{II}}/\text{Cu}^{\text{I}}$, and sequestration of Cu^{I} by intracellular ligands. Supporting this mechanism, treating control (normoxic) cells with $\text{Cu}^{\text{II}}(\text{atism})$ did not alter levels of phosphorylated ERK1/2, but treating hypoxic cells with $\text{Cu}^{\text{II}}(\text{atism})$ significantly increased levels of phosphorylated ERK1/2 (Fig. 2B and C).

To elucidate the mechanisms through which hypoxia promotes cellular retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$ a number of analyses were performed to compare hypoxic and control cells. Hypoxic conditions decreased the pH of the media (Fig. 2D) a result likely to be the consequence of increased reliance of the hypoxic cells on glycolytic energy production. Increased glycolysis is supported by data showing the hypoxic cells generated more lactate (Fig. 2E) and consumed more glucose (Fig. 2F) compared to control cells. Glycolysis is a less efficient means of ATP production compared to a functional ETC and the data in Fig. 2G show hypoxic cells

contained less ATP. These ATP data are consistent with hypoxic conditions preventing normal function of the ETC by decreasing availability of the terminal electron acceptor O_2 . An accumulation of NADH, the primary source of electrons for the ETC, also indicates decreased ETC function within hypoxic cells (Fig. 2H).

NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase have been reported to contribute to the reduction of $\text{Cu}^{\text{II}}(\text{atism})$ in subcellular fractions from tumor cells (19). To assess reductase activity in SH-SY5Y cells, cell extracts were incubated with the generic reductase substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Cell extracts from hypoxic cells were less effective at reducing MTT compared to extracts from control cells (Fig. 2I), so it is unlikely that increased activity of reductases is responsible for the increased retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$ in hypoxic cells (Fig. 2A). Further, an analysis of oxidatively modified proteins in SH-SY5Y cells revealed an absence of hypoxia-induced oxidative damage in this cell culture model of hypoxia (Fig. 2J).

Mitochondrial Xenocybrid Cells Show Increased Cellular Retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$. Data presented in Fig. 2 indicate impaired function of the mitochondrial ETC increases cellular retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$. To directly test ETC function in increased retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$, without hypoxia being a confounding factor, the cellular retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$ was examined in mitochondrial xenocybrid cells. ATP production via the ETC is dependent on the coordinated assembly of protein subunits encoded by nuclear DNA and mitochondrial DNA to form functional multisubunit protein complexes of the ETC. Because of a series of chemical and genetic manipulations (20), xenocybrid cells contain nuclear DNA and mitochondrial DNA from two separate species. This genetic mismatch between the nuclear and mitochondrial encoded subunits compromises the ability of the nuclear and mitochondrial encoded subunits to form multisubunit protein complexes. The result is that normal transfer of electrons through the ETC is impaired. The xenocybrid cells used in our study contained nuclear DNA from *Mus musculus* and mitochondrial DNA from *Rattus norvegicus* (21). After treating with $\text{Cu}^{\text{II}}(\text{atism})$ ($10 \mu\text{M}$) for 1 h, xenocybrid cells contained more Cu compared to $\text{Cu}^{\text{II}}(\text{atism})$ -treated control cells (Fig. 3A). The $\text{Cu}^{\text{II}}(\text{atism})$ -induced increase in phosphorylated ERK1/2 in the xenocybrid cells (Fig. 3B and C) indicated the increased cellular retention of Cu in the xenocybrid cells involved intracellular dissociation of the compound to increase bioavailable Cu. Supporting this mechanism, xenocybrid cells transfected with the MRE-luciferase reporter displayed a 2.5-fold induction in MRE-luciferase in response to $\text{Cu}^{\text{II}}(\text{atism})$ (Fig. 3D). By contrast, control cells transfected with the same MRE-luciferase reporter were not affected by the $\text{Cu}^{\text{II}}(\text{atism})$ treatment (Fig. 3D).

Consistent with the hypoxic SH-SY5Y cells, the xenocybrid cells acidified their culture media (Fig. 3E) because of increased glycolytic activity (Fig. 3F and G). The xenocybrid cells also contained lower levels of ATP (Fig. 3H) and elevated levels of NADH (Fig. 3I). Also, consistent with the mechanism of selective Cu retention in $\text{Cu}^{\text{II}}(\text{atism})$ -treated hypoxic cells, the increased retention of Cu in $\text{Cu}^{\text{II}}(\text{atism})$ -treated xenocybrid cells did not involve increased reductase activity (Fig. 3J).

Oxidative Stress in the Absence of an Impaired ETC does not Promote Cellular Retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$. Oxidative modification of proteins was elevated in the xenocybrid cells (Fig. 3K). Unlike the hypoxic SH-SY5Y cells, the xenocybrid cells contained an impaired ETC in the presence of available O_2 . The availability of O_2 as an electron acceptor together with electron leakage from the impaired ETC of the xenocybrid cells is the most likely source of the reactive oxygen species (ROS) that will give rise to the increase in oxidative damage observed in xenocybrid cells (Fig. 3K). Accordingly, elevated retention of Cu from $\text{Cu}^{\text{II}}(\text{atism})$ in the

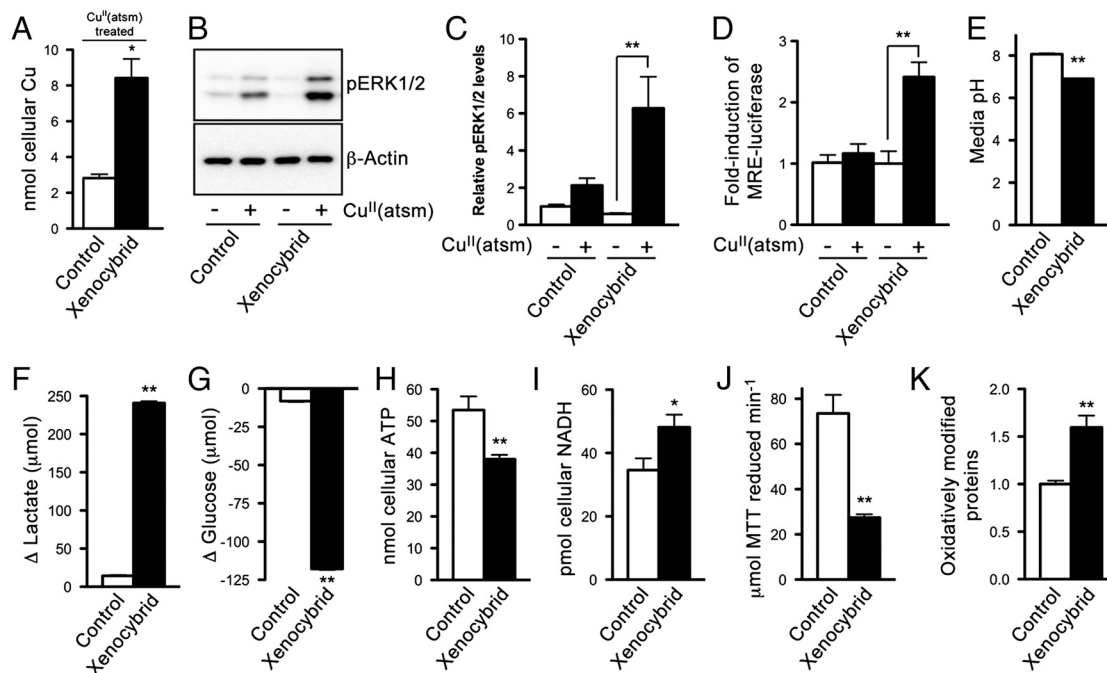


Fig. 3. Effects of a genetically impaired mitochondrial electron transport chain on energy metabolism and cellular responses to Cu^{II}(atism). (A) ICP-MS analysis of cellular Cu in control cells and xenocybrid cells after treating with 10 μM Cu^{II}(atism) for 1 h. (B) Western blotting image showing Cu^{II}(atism) promotes phosphorylation of ERK1/2 (pERK1/2) in xenocybrid cells. β-Actin levels are shown as a control. (C) Densitometry analysis of Western blotting results. (D) Before treating with 500 nM Cu^{II}(atism) for 6 h, cells were transfected with MRE-luciferase construct. Treatment-induced increases in bioavailable Cu within the cell upregulates expression of the luciferase reporter, induction of which is measured by luminescence. (E) pH of media collected from cells after 6 d in culture. (F) Lactate produced and (G) glucose consumed by cells over 6 d in culture. (H) ATP and (I) NADH content of control cells and xenocybrid cells. (J) Reduction of MTT mediated by cell lysates collected from control cells and xenocybrid cells. (K) Relative content of oxidatively modified proteins in control cells and xenocybrid cells. Data are mean values ± SEM, *n* = 3–6. Values in A and E–J are expressed per milligram cellular protein. **P* < 0.05/***P* < 0.01 compared to control cells (*t* test) except for C and D where **P* < 0.01 compared to vehicle-treated cells (ANOVA with Tukey's posttest).

xenocybrid cells supports the notion that Cu^{II}(atism) may be used to image regions of oxidative stress (10, 11). To determine whether oxidative stress in the absence of an impaired ETC could promote cellular retention of Cu from Cu^{II}(atism) we pretreated SH-SY5Y cells with the glutathione synthesis inhibitor buthionine sulfoximine (BSO) (22) before exposing to Cu^{II}(atism). Pretreatment with BSO increased oxidative modification of proteins in SH-SY5Y cells (Fig. S1A), but did not promote cellular retention of the Cu from Cu^{II}(atism) (Fig. S1B). In addition, exposing BSO-treated cells to Cu^{II}(atism) did not alter levels of phosphorylated ERK1/2 (Fig. S1 C and D). These results provide further support that Cu^{II}(atism)-mediated increases in bioavailable Cu within the cell, detectable indirectly by changes to the phosphorylation state of ERK1/2, are needed to promote cellular retention of the Cu from Cu^{II}(atism).

Acidification of the Cell Culture Media in the Absence of an Impaired ETC does not Alter Cellular Responses to Cu^{II}(atism). Conditions conducive to an impaired ETC promote upregulation of alternate ATP producing pathways, as indicated in both the hypoxic cells (Fig. 2 E and F) and the xenocybrid cells (Fig. 3 F and G) by data showing lactate production and glucose consumption are increased. A consequence of the increased glycolysis is increased acidification of the cell culture media (Fig. 2D, Fig. 3E). To test whether acidification of the culture media contributed to cellular retention of the Cu from Cu^{II}(atism), lactic acid was titrated into the media of SH-SY5Y cells prior to treating with Cu^{II}(atism). Lactic acid was added to the media until the pH reached 6.5, as per the media of hypoxic cells (Fig. 2D). Decreasing the media pH in the absence of an impaired ETC did not promote cellular retention of Cu from Cu^{II}(atism) (Fig. S2A), nor did it increase bioavailable Cu within the Cu^{II}(atism)-treated cells as indicated by unaltered levels of phosphorylated ERK1/2 (Fig. S2 B and C).

Inhibiting the Tricarboxylic Acid (TCA) Cycle does not Alter Cellular Responses to Cu^{II}(atism). Impairment of the ETC in hypoxic and xenocybrid cells leads to decreased levels of ATP (Fig. 2G, Fig. 3H) and an accumulation of NADH (Fig. 2H, Fig. 3I). To differentiate between impaired ETC function and an overall impediment to ATP production in cellular retention of Cu from Cu^{II}(atism), cells were pretreated with the TCA cycle inhibitor NaAsO₂ (23). Treatment with NaAsO₂ decreased ATP levels (Fig. S3A) and moderately increased Cu levels in Cu^{II}(atism)-treated cells (Fig. S3B). However, there was no indication of increased bioavailable Cu in Cu^{II}(atism)-treated cells due to the NaAsO₂, as indicated indirectly by the lack of changes to phosphorylated ERK1/2 (Fig. S3 C and D).

Discussion

After 2-keto-3-ethoxybutyraldehydebis(thiosemicarbazonato)Cu^{II} [Cu^{II}(kts)] enters cells the Cu is reduced from Cu^{II} to Cu^I and dissociates from the ligand; H₂kts diffuses back out of the cell while the Cu is, at least initially, retained inside the cell (24, 25). Increased cellular retention of Cu from bis(thiosemicarbazonato) Cu^{II} compounds is dictated, in part, by intracellular reduction of the Cu followed by Cu dissociation from the ligand inside the cell and there is a correlation between Cu^{II}/Cu^I reduction potential and hypoxia selectivity (13, 26, 27). An elevated Cu content was evident in Cu^{II}(atism)-treated hypoxic and xenocybrid cells compared to their relevant Cu^{II}(atism)-treated control cells (Fig. 2A, Fig. 3A), and this increase in Cu content was paralleled by increases in ERK phosphorylation (Fig. 2 B and C, Fig. 3 B and C). Increased phosphorylation of cell-signaling kinases after treating with bis(thiosemicarbazonato)Cu^{II} compounds is dependent on the relative Cu^{II}/Cu^I reduction potential of the compounds (28, 29). Increased kinase phosphorylation in response to bis(thiosemicarbazonato)Cu^{II} treatment is therefore likely to

involve intracellular dissociation of the compound giving rise to increased intracellular levels of bioavailable Cu.

In the present study cells transfected with the MRE-luciferase reporter construct were used to monitor cellular conditions that affect the intracellular dissociation of Cu from Cu^{II}(atsm). Although the initial event required to upregulate expression of the luciferase reporter gene is Cu release from the atsm ligand, the MRE component of the construct only detects an increase in bioavailable Zn, which occurs subsequent to Cu displacement of Zn from endogenous metallothioneins (16). Observed changes to ERK1/2 phosphorylation (Fig. 2 *B* and *C*, Fig. 3 *B* and *C*) are likely to be the consequence of multiple overlapping cell signaling cascades in which increased Zn bioavailability plays a promiscuous but central role. Zn bioavailability regulates multiple components of cell-signaling cascades (30–32). Collectively, the changes to ERK1/2 phosphorylation in Cu^{II}(atsm)-treated hypoxic cells (Fig. 2 *B* and *C*) and xenocybrid cells (Fig. 3 *B* and *C*), together with data indicating that increased ERK1/2 phosphorylation in Cu^{II}(atsm) or Cu^{II}(gtsm)-treated cells is only detectable in cells that also show increased expression of the MRE-luciferase reporter (Fig. 1*B*, Fig. 3*D*), indicate that increased cellular retention of Cu from Cu^{II}(atsm) requires an initial intracellular dissociation of the Cu from the bis(thiosemicarbazone) ligand. Although the mechanisms by which increased bioavailable Cu within the cell leads to increased ERK1/2 phosphorylation remain to be fully elucidated, the inhibition of phosphatase activity is a possibility (33). Phosphatase inhibition may involve direct inhibition by Cu or involve Cu-mediated displacement of Zn from metallothioneins, as both Cu and Zn are capable of inhibiting phosphatase activity (30, 34).

Hypoxic cells and xenocybrid cells possess an impaired mitochondrial ETC. The origin of impaired ETC function in hypoxic cells is decreased availability of the terminal electron acceptor O₂. The cell culture model of hypoxia showing increased retention of Cu from Cu^{II}(atsm) (Fig. 2*A*) is consistent with increased retention of radiolabeled Cu^{II}(atsm) in hypoxic tumors and myocardial ischemia, but these data cannot eliminate the possibility that decreased ETC function is responsible for the increased cellular retention of the Cu. The use of xenocybrid cells enabled us to determine the effects of impaired ETC function without needing to deprive the cells of O₂ or to expose them to toxic exogenous inhibitors of the ETC, such as rotenone (1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-*b*]furo[2,3-*h*]chromen-6-one) or paraquat (1,1'-Dimethyl-4,4'-bipyridinium dichloride). Consistency in the data shown for hypoxic cells (Fig. 2) and the mitochondrial xenocybrids (Fig. 3) provides strong support for an impaired mitochondrial ETC in cellular retention of Cu from Cu^{II}(atsm).

A mechanism in which cellular retention of Cu from Cu^{II}(atsm) is promoted by cellular conditions that increase NADH within the cell is a possibility. The present experiments show that conditions conducive to sustained (or potentially accelerated) metabolic flux through the TCA cycle in the presence of an impaired ETC drives accumulation of NADH (Fig. 2*H*, Fig. 3*I*). An accumulation of NADH has been proposed to facilitate reduction of Cu^{II}(atsm) (5), and the accumulation of NADH shown in hypoxic cells and xenocybrid cells is likely to provide the increased cellular reduction potential required to promote reduction of Cu in Cu^{II}(atsm) from Cu^{II} to Cu^I, thereby promoting subsequent intracellular dissociation of Cu^I from the ligand and increased cellular retention of Cu from Cu^{II}(atsm). In an earlier study submitochondrial particles from mouse brains were exposed to Cu^{II}(atsm), then levels of Cu^{II}(atsm) reduction measured using electron spin resonance spectrometry (5). Cu^{II}(atsm) reduction by the submitochondrial particles was promoted by NADH, and although isolated mitochondria supported only marginal (3.4%) reduction of Cu^{II}(atsm), the reduction was increased to 14.7% when the mitochondria were treated with

the ETC inhibitor rotenone (5). These data validated previous studies where reduction of the closely related pyruvaldehyde bis(4-methylthiosemicarbazonato)Cu^{II} [Cu^{II}(ptsm)] by mitochondrial preparations was shown to be dependent on NADH and could be induced by treating mitochondrial preparations with inhibitors of the ETC (35, 36). However, none of the previous studies with Cu^{II}(atsm) (5) and Cu^{II}(ptsm) (35, 36) used whole-cell systems to measure whether Cu content of bis(thiosemicarbazonato)Cu^{II}-treated cells was increased by the cellular conditions that elevated NADH levels via impairment of the ETC. Increased cellular retention of Cu from Cu^{II}(atsm) in xenocybrid cells (Fig. 3*A*) are unique data from whole cells showing increased cellular retention of Cu from Cu^{II}(atsm) in cells with an impaired mitochondrial ETC. The data showing elevated ERK1/2 phosphorylation (Fig. 2 *B* and *C*, Fig. 3 *B* and *C*) are consistent with the increased cellular retention of Cu from Cu^{II}(atsm) involving intracellular dissociation of the Cu^{II}(atsm), and the elevated levels of NADH (Fig. 2*H*, Fig. 3*I*) are consistent with elevated reduction potential in cells with an impaired ETC being the mechanism by which Cu^{II}(atsm) reduction is promoted. Our experiments that utilized NaAsO₂ to inhibit TCA cycle activity revealed that after exposing to Cu^{II}(atsm) the overall Cu content of NaAsO₂-treated cells was increased (Fig. S3*B*). However, this increase was moderate compared to the increase observed in hypoxic cells (Fig. 2*A*) and xenocybrid cells (Fig. 3*A*). As per the hypoxic cells (Fig. 2*G*) and the xenocybrid cells (Fig. 3*H*) the ATP content of the NaAsO₂-treated cells was decreased (Fig. S3*A*), but the absence of changes to ERK1/2 phosphorylation (Fig. S3 *C* and *D*) indicated that decreasing ATP production at the level of the TCA cycle did not induce intracellular Cu^{II}(atsm) dissociation. Together, these data indicate cellular retention of Cu^{II}(atsm) as an intact compound may be affected by cellular ATP levels, but enhanced cellular retention of the Cu requires intracellular dissociation of the compound.

Recent studies have described Cu^{II}(atsm) as a PET imaging agent to identify regionalized areas of oxidative stress that can occur when cellular levels of reductants are in excess relative to O₂ (10, 11). Oxidative stress via increased levels of reactive oxygen species will occur when electrons inefficiently handled by the ETC are able to interact directly with O₂. This occurrence is particularly true in conditions where O₂ availability is unaltered as demonstrated by the increased oxidative modification of proteins in the xenocybrid cells (Fig. 3*K*). However, the fundamental requirement for O₂ in the generation of reactive oxygen species means that an excess of reductants relative to O₂ due to decreased O₂ alone, as occurs in hypoxic tissues, will not increase levels of reactive oxygen species or generate oxidative stress. This phenomenon is supported by data showing oxidative modification of proteins is not increased by hypoxia (Fig. 2*J*). Together, these data demonstrate that although selective retention of Cu from Cu^{II}(atsm) will be evident in disease-affected tissues where oxidative stress is the result of a dysfunctional ETC, as occurs in MELAS and Parkinson's disease (37, 38), Cu^{II}(atsm) will not necessarily be suitable for PET detection of generic oxidative stress. Supporting this possibility, the induction of oxidative stress in the absence of any direct impediment to normal function of the ETC (39) did not promote cellular retention of Cu from Cu^{II}(atsm) (Fig. S2).

Cu^{II}(atsm) has excellent potential as a PET imaging agent because of selective retention of the radiolabeled Cu in tissues affected by hypoxia either in tumors or as a result of ischemia. Furthermore, recent studies have supported the application of Cu^{II}(atsm) in imaging MELAS and Parkinson's disease. The present study is unique to use whole cells to examine the mechanisms that govern cellular retention of Cu from Cu^{II}(atsm). The data presented indicate an accumulation of NADH due to impaired activity of the mitochondrial ETC is the primary mechanism that

drives cellular retention of the Cu from Cu^{II}(atsm), and that intracellular dissociation of the compound correlates with increased retention of the Cu. Hypoxic tumors, ischemia, Parkinson's disease, and MELAS are all pathological conditions that involve dysfunction of the mitochondria (40–43). This work suggests the potential for Cu^{II}(atsm) as a PET imaging agent may extend to other mitochondrial diseases.

Materials and Methods

Full details of the materials and methods are presented in the *SI Materials and Methods*. The mitochondrial xenocybrid cells used were *M. musculus* cells (with *M. musculus* nuclear DNA) containing *R. norvegicus* mitochondrial DNA (21). Mitochondrial xenocybrid cells are a unique and powerful tool for studying cells with an endogenous impediment to the ETC and are described in detail elsewhere (20). The cybrids were produced by fusing enucleated

mitochondrial donor cells with mouse ρ^0 cells, which lack mitochondrial DNA (44). Control cells in the xenocybrid experiments were *M. musculus* cybrids containing the same *M. musculus* nuclear DNA but with *M. musculus* mitochondrial DNA. The control cells were therefore cybrids that contained nuclear and mitochondrial DNA from the same species (*M. musculus*) whereas the xenocybrid cells contained nuclear and mitochondrial DNA from two different species (*M. musculus* and *R. norvegicus*, respectively). The xenocybrid cells were previously shown to express defective oxidative phosphorylation complex I, III, and IV activities, and a deficit in ATP generation (4).

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