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Copper induces neuron-sparing, ferredoxin 1-independent astrocyte toxicity mediated by oxidative stress

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Abstract

Copper is an essential enzyme cofactor in oxidative metabolism, antioxidant defenses and neurotransmitter synthesis. However, intracellular copper, when improperly buffered, can also lead to cell death. Given the growing interest in the use of copper in the presence of the ionophore elesclomol (CuES) for the treatment of gliomas, we investigated the effect of this compound on the surround parenchyma – namely neurons and astrocytes *in vitro*. Here we show that astrocytes were highly sensitive to CuES toxicity while neurons were surprisingly resistant, a vulnerability profile that is opposite of what has been described for zinc and other toxins. Bolstering these findings, a human astrocytic cell line was similarly sensitive to CuES. Modifications of cellular metabolic pathways implicated in cuproptosis, a form of copper regulated cell death, such as inhibition of mitochondrial respiration or knock-down of ferredoxin 1 (FDX1), did not block CuES toxicity to astrocytes. CuES toxicity was also unaffected by inhibitors of apoptosis, necrosis or ferroptosis. However, we did detect the presence of lipid peroxidation products in CuES-treated astrocytes, indicating that oxidative stress is a mediator of CuES-induced glial toxicity. Indeed, treatment with antioxidants mitigated CuES induced cell death in astrocytes indicating that oxidative stress is a mediator of CuES-induced glial toxicity. Lastly, prior induction of metallothioneins 1 and 2 in astrocytes with zinc plus pyrithione was strikingly protective against CuES toxicity. As neurons express high levels of metallothioneins basally, these results may partially account for their resistance to CuES toxicity. These results demonstrate a unique toxic response to copper in glial cells which contrasts with the cell selectivity profile of zinc, another biologically relevant metal.

Graphical Abstract

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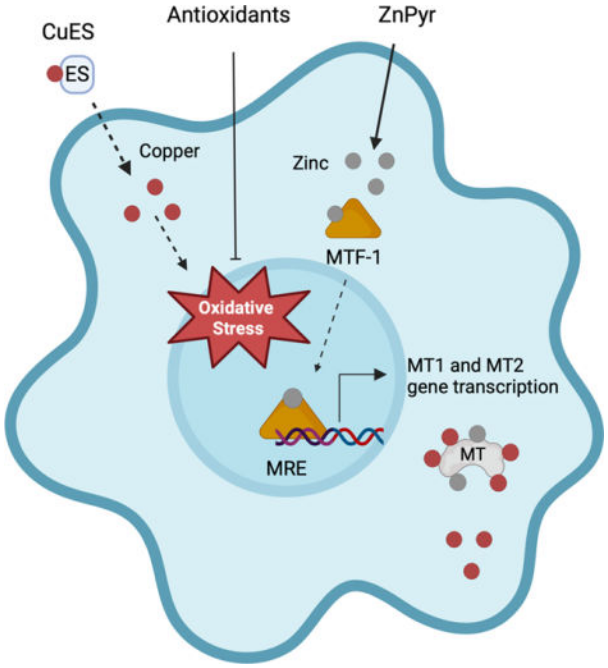

Copper is an essential enzyme cofactor. However, intracellular copper, when improperly buffered, can lead to cell death. Given growing interest in the use of copper in the presence of the ionophore elesclomol (CuES) for the treatment of gliomas, we investigated the effect of this compound on neurons and astrocytes *in vitro*. Here we show that astrocytes were highly sensitive to CuES toxicity while neurons were surprisingly resistant. We found oxidative stress to be a key mediator of CuES toxicity. Indeed, antioxidant treatment mitigated CuES-induced cell death in astrocytes. We also found that induction of metallothioneins (MTs) with zinc pyrithione (ZnPyr) was protective against CuES toxicity. Created with BioRender.com.

Keywords

Metal; Elesclomol; Glia; Cell Death; Free Radicals; Glioma

Introduction

Copper is a catalytic and structural cofactor for cuproenzymes involved in oxidative phosphorylation, iron homeostasis, antioxidant defense, as well as neurotransmitter synthesis (Ruiz *et al.* 2021; Scheiber *et al.* 2014). Copper also serves important biological functions in the brain, including modulation of synaptic transmission (D'Ambrosi and Rossi 2015). The critical role of copper in key physiological processes is evidenced by the fact that the transition metal is an essential micronutrient in virtually all eukaryotes (Festa and Thiele 2011). Furthermore, genetic mutations that result in copper deficiency lead to human disorders with profound clinical consequences (Jaksch *et al.* 2000; Garza *et al.* 2023; Kaler 2013). However, copper is able to redox cycle, allowing it to form toxic free radicals, displace other metals from enzymes, and disrupt proper protein folding (Festa and Thiele 2011). Indeed, the accumulation of intracellular copper is the key driver of

pathology in Wilson's disease (Ala *et al.* 2007). Moreover, copper dyshomeostasis has been linked to neurodegenerative disorders including Alzheimer's disease and Parkinson's disease (Gromadzka *et al.* 2020; Chen *et al.* 2022; Pal *et al.* 2021). Indeed, cells in the central nervous system (CNS) appear to be particularly vulnerable to the toxic effects of copper as highlighted by the neurotoxicity/neurodegeneration observed in animal models of chronic copper intoxication (Pal and Prasad 2015). As such, intracellular concentrations of copper are tightly regulated, with the metal being tightly buffered to attomolar concentrations in the cytoplasm by thiol ligands (Morgan *et al.* 2019), including glutathione and metallothioneins.

A copper-dependent regulated cell death pathway, cuproptosis, was recently described (Tsvetkov *et al.* 2022). Cuproptosis involves the disruption of mitochondrial respiration through increased lipoylation of tricarboxylic acid (TCA) cycle proteins (Tsvetkov *et al.* 2022). In this cell death pathway, lipoylation, a post-translational modification of lysine, is mediated by ferredoxin 1 (FDX1), an iron-sulfur cluster protein involved in electron transfer for a variety of biological processes (Schulz *et al.* 2023). The bulk of research on cuproptosis has involved the copper ionophore elesclomol, which has been previously investigated as a possible anti-neoplastic drug (Yadav *et al.* 2013). Although it has had limited efficacy in clinical trials thus far (O'Day *et al.* 2013; Monk *et al.* 2018), elesclomol is a potent inhibitor of cancer cell proliferation *in vitro* (Hasinoff *et al.* 2015; Nagai *et al.* 2012; Blackman *et al.* 2012) and, as such, the discovery of cuproptosis has reignited interest in copper's potential as a treatment strategy for difficult-to-treat cancers. Indeed, cuproptosis-associated genes have been evaluated as predictors of prognosis and treatment response in glioma *in silico* (Zhang *et al.* 2022; Zhang *et al.* 2023a; Ye *et al.* 2022; Zhu *et al.* 2022). Gliomas are tumors derived from aberrant glial or glial precursor cells and are amongst the most common and most deadly types of primary brain tumor (Ostrom *et al.* 2019; Nicholson and Fine 2021). Unfortunately, no novel pharmaceutical treatments that affect patient survival have been successfully developed for this malignancy since temozolomide was introduced over 15 years ago (Stupp *et al.* 2005; Nicholson and Fine 2021).

While CuES may be effective at targeting glioma and glioblastoma cells, the effect it has on the surrounding parenchyma needs to be considered. An ideal treatment would target tumor cells while leaving the surrounding tissue minimally affected, especially in the brain where treatment such as radiation therapy has been linked to oxidative stress and cognitive dysfunction (Soffietti *et al.* 2023; Yoo *et al.* 2023). Gliomas are intermixed with neurons and astrocytes – cell types that have previously been shown to be vulnerable to copper toxicity and dyshomeostasis (Bulcke *et al.* 2015; Hwang *et al.* 2014; Gromadzka *et al.* 2020; Pal and Prasad 2014).

In this study we sought to investigate the effect of copper in the presence of elesclomol (CuES) on the tumor microenvironment using primary rodent cortical cultures and immortalized human astrocytes. To our surprise, we discovered that astrocytes are extremely sensitive to CuEs toxicity while neurons are strongly resistant to this insult. Importantly, our results indicate that CuES toxicity is not mediated via cuproptosis as it lacks dependence on FDX1. To our knowledge, this is one of a few of a biologically relevant compounds that is selectively lethal to astrocytes while leaving neurons unharmed.

Material and Methods

Compounds

Chemicals: elesclomol (cat. no. S1052), Z-VAD-FMK (cat. no. S7023), necrostatin-1s (cat. no. S8641), deferoxamine mesylate (cat. no. S5742), and ferrostain-1 (cat. no. S7243) were purchased from Selleck Chemicals. Cupric chloride dihydrate (cat. no. C3279), Antimycin A (cat. no. A8674), Sodium azide (cat. no. S8032), EUK-134 (cat. no. SML0743) and pyrroloquinoline quinone (PQQ) (cat. no. D7783) were purchased from Sigma. Dizocilpine hydrogen maleate (MK801) (cat. no. M107) was purchased from Milipore. Primary antibodies for immunocytochemistry: chicken anti-MAP2 (Abcam, cat. no. ab5392, RRID:AB_2138153, 1:5000), rabbit anti-GFAP (Abcam, cat. no. ab7260, RRID:AB_305808, 1:1000), mouse anti-4-hydroxynonenal (12F7) (Invitrogen, cat. no. MA5–27570, RRID:AB_2735095, 1:100). Secondary antibodies for immunocytochemistry: donkey anti-rabbit conjugated with Alexa Fluor 488 (Abcam, cat. no. ab150073, RRID:AB_2636877, 1:1000), goat anti-mouse conjugated with Alexa Fluor 568 (Abcam, cat, no. ab175473, RRID:AB_2895153, 1:1000), goat anti-chicken conjugated with Alexa Fluor 647 (Abcam, cat. no. ab150175, RRID:AB_2732800, 1:1000).

Cerebrocortical Cultures

All procedures involving the use of animals were reviewed and approved by the University of Pittsburgh IACUC (Protocol # 21039053). Primary cortical cultures were prepared from embryonic day 16–17 Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) as previously described (Hartnett *et al.* 1997). Briefly, pregnant rats were housed alone in a standard cage in the University central animal facility for a maximum of three days with free access to food and water and were sacrificed humanely and painlessly by CO_2 inhalation and further exsanguinated via severing of the jugular vein to assure completion of euthanasia. The animals showed no sign of distress during this procedure and perished quickly and painlessly. Embryos of either sex were removed and quickly decapitated, although they likely had also perished before the dissection was performed. Five-10 embryonic cortices from a single littler were pooled and dissociated with trypsin and cells were plated on 12 mm, poly-L-ornithine (PLO) coated glass coverslips in six well plates at a density of 635,000 cells per well. We consider a single dissociation, from one pregnant animal, a biological replicate. Non-neuronal proliferation was inhibited after 14 days *in vitro* (DIV) with 2 μM cytosine arabinsoside. Cultures were utilized at 3–4 weeks *in vitro*. We calculate that approximately 50–70 pregnant rats (approximately 450 embryos) were utilized for these studies as we normally perform one dissociation per week throughout the year. On occasion, a pregnant animal of the wrong gestation age was shipped, and these were excluded from the study (i.e. no cultures were obtained). Otherwise, no cultures were excluded from the study. This primary cortical culture model was chosen as it is a well-validated model in our laboratory and we have previously demonstrated developmental increases in AMPAR and NMDAR subunit expression in these cultures consistent with their *in vivo* development (Ross and Aizenman 2023; Sinor *et al.* 2000).

Astrocyte Cultures

To generate astrocyte cultures, mixed cortical cultures were exposed to 1 mM kainate (KA) in HEPES-buffered minimal essential media with 0.01% BSA (MHB) for 18–24 hours to eliminate the neuronal component, which comprise 10–20% of cultures at 3–5 weeks *in vitro*, while sparing the astrocytes which comprise 80–90% of cells in this preparation (Rosenberg and Aizenman 1989). Following overnight KA treatment, astrocytes were transferred to fresh MHB solution. For experiments utilizing both mixed cultures and astrocyte cultures, coverslips containing neurons and astrocytes were treated in MHB without KA in parallel to control for manipulation of coverslips. We chose to use this model of astrocyte cultures as opposed to pure astrocyte cultures as it has previously been demonstrated that neurons release soluble factors that regulate the expression of astrocytic proteins (Gegelashvili *et al*. 1997; Swanson *et al*. 1997; Schlag *et al*. 1998) and recent evidence strongly suggests that neurons play a key role in the regulation of genes important in astrocyte metabolism and development (Hasel *et al*. 2017). As such, this model recapitulates key aspects of mature astrocytes *in vivo*.

C6 glioma cells

Rat C6 glioma cells (ATCC, cat. no. CCl-107; RRID:CVCL_0194) were cultured in Ham's F12 Nutrient Mixture with GlutaMAX (Gibco) supplemented with 2.5% fetal bovine serum (Thermo Fisher, cat. no. A4766801) 15% horse serum (heat inactivated) (Thermo Fisher, 26050088), and penicillin (100 U/ml), streptomycin (0.1 mg/ml) and maintained in an incubator at 37°C with 5% CO_2. Cells were split twice a week. For LDH assays, C6 cells were plated at a 188,000 cells per well in PLO-coated 24 well plates; for CellTiter Glo assays, cells were plated at a density of 32,000 cells per well in PLO-coated 96 well plates. The maximum number of passages utilized for these cells was ~25. C6 cells have been extensively used to study glioma both *in vitro* and *in vivo* and are considered by some to be to be "the gold standard" in glioma research [for review, see: (Giakoumettis *et al*. 2018)]. C6 is not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee (ICLAC). To our knowledge, the C6 cell line has not been further analyzed since karyotype analysis was performed on the initial seed stock at ATCC. However, ATCC performs authentication and quality-control tests on all distribution lots of cell lines.

U138-MG glioma cells

Human U138-MG glioma cells (ATCC, cat. no. HTB-16; RRID: CVCL_0020) and were cultured in Eagle's Minimum Essential Medium (ATCC, cat. no. 30-2003) supplemented with 10% fetal bovine serum (Thermo Fisher, cat. no. A4766801), penicillin (100 U/ml), streptomycin (0.1 mg/ml) and maintained in an incubator at 37°C with 5% CO_2. Cells were split one to two times a week. For LDH assays, U138-MG cells were plated at a density of 80,000 cells per well poly-d-lysine (Gibco, cat. no. A3890401) -coated 24 well plates. The maximum number of passages utilized for these cells was ~25. These cells were derived from an individual with grade IV glioblastoma and were included for additional verification of the vulnerability of glioma cells to copper-elesclomol treatment *in vitro* Authentication using short tandem repeat profiling was performed in 2012 and 2016 (Allen *et al*. 2016;

Bady *et al.* 2012). Although similarity was reported between U138-MG and U118-MG, U138-MG is not listed as a commonly misidentified cell line by the ICLAC and ATCC notes that U138-MG and U118-MG are reportedly derived from two separate individuals with similar STR patterns.

Immortalized Human Astrocytes

Immortalized human fetal astrocytes (hTert) (Applied Biological Materials, cat. no. T0281) were cultured on poly-D-lysine (PDL) (Gibco, cat. no. A3890401) coated flasks in Prigrow IV medium (Applied Biological Materials, cat. no. TM004) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 ng/mL human epidermal growth factor (EGF) (Gibco, cat. no. PHG0323), and 100 U/mL penicillin, 100 μg/mnL streptomycin (Sigma). Cells were split twice a week and seeded at a density of 40,000 cells per well onto PDL-coated 24 well plates for LDH assays and 14,000 cells per well onto PDL-coated 96-well plates for CellTiter Glo assays. The maximum number of passages utilized for these cells was ~20. This immortalized astrocyte cell line was generated from primary human astrocytes that are representative of cortical astrocytes and have been previously used as a cell model of normal human astrocytes in multiple studies investigating glioma and glioblastoma (Agnihotri *et al.* 2019; Erkan *et al.* 2014; Baskin *et al.* 2015). These immortalized human astrocytes are not listed as a commonly misidentified cell line by the ICLAC. STR analysis was performed by Applied Biological Materials on this immortalized cell line for comparison to its parental primary cells, however it is not clear what year this analysis was completed.

Cell Treatments

All experimental paradigms were performed in MHB unless otherwise stated. Cells were exposed to indicated concentrations of copper elesclomol (CuES) overnight or for a two-hour pulse. For CuES pulse experiments, cells were exposed to CuES for 2 hours and then rinsed with MHB. Cells were then incubated overnight in fresh MHB before toxicity/viability measurements. All experiments using drugs to modify CuES toxicity, except for zinc pyrithione (ZnPyr), included a one-hour pre-incubation period before the addition of CuES. Drugs remained in their respective treatment groups overnight before toxicity or viability assays were conducted. For zinc pre-conditioning experiments, cortical cultures were simultaneously exposed to 1 mM KA and 20 μM zinc in the presence of 250 nM pyrithione. Following overnight incubation with KA and ZnPyr, coverslips were moved to fresh MHB before treatment with CuES.

siRNA Treatment

Mixed cortical cultures were treated with 1 μM Acell rat *FDX1* SMARTPool siRNA (Horizon, cat. no. E-089545-00-0020) for 48 hours in DMEM-Glutamax supplemented with 2% FBS and 31 mM HEPES. Sister cultures were treated in parallel with 1 μM Acell non-targeting control siRNA (Horizon, cat. no. D-001910-10-20). For initial confirmation of *FDX1* knockdown, RNA was collected after 48 hours. For CuES toxicity experiments, siRNA treated coverslips were transferred to MHB after 48 hours and exposed to CuEs overnight. RNA was collected from sister cultures treated identically to confirm the persistence of knockdown.

Toxicity Assays

The toxicity of treatment conditions was assessed using a using a commercially available colorimetric lactate dehydrogenase (LDH) activity assay kit (Sigma, cat. no. MAK066). Following experimental treatment, medium was collected for LDH assays as this enzyme is rapidly released from cells following damage to the plasma membrane (Aras *et al.* 2008). Toxicity is represented by increased OD450 values. A minimum of three experiments from separate culture dates were performed, each in triplicate.

Viability Assays

The CellTiterGlo Luminescent Cell Viability Assay (Promega, cat. no. G7570) was used to assess viability following copper-ionophore treatment in experiments utilizing EUK-134 and PQQ, as these compounds interfered with the enzymatic reaction utilized in the LDH assay. Experiments were performed according to the manufacturer's directions. Viability was calculated as percent of control luminescence values due to variability between experiments in absolute luminescence values. A minimum of three experiments from separate culture dates were performed, each in triplicate, for primary cultures and from three separate split dates for cell lines.

Immunocytochemistry

Coverslips were fixed with 4% paraformaldehyde/4% sucrose for 20 minutes, washed three times with PBS, and were then blocked and permeabilized with 0.05% Triton-X with 10% normal goat serum in PBS for 45 minutes. Coverslips were then incubated with primary antibodies at 4°C overnight. The following day, cells were incubated with secondary antibodies for one hour at room temperature before mounting with Fluoromount-G mounting medium (Thermo Fisher, cat. no. 00-4958-02). Three to four random fields of view were imaged from each coverslip (60x) on a Nikon A1R laser scanning confocal.

Quantitative Polymerase Chain Reaction

Total RNA was isolated from cortical cultures using the TRIzol Plus RNA Purification Kit (Invitrogen, cat. no., 12183555). RNA samples were eluted in RNAse free H_2O and approximately 500 nanograms of RNA from each sample was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, cat. no. 1708891). Quantitative PCR (qPCR) was performed using the following primers: β-actin sense (FW): 5′-ACTCTTCCAGCCTTCCTTC-3′; β-actin antisense (RV): 5′- ATCTCCTTCTGCATCCTGTC-3′; FDX1 sense (FW): 5'-TCGATGGATTTGGTGCGTGT-3'; FDX1 antisense (RV): 5'-CAGGCACACGGACAGTCATA; MT1 sense (FW): 5'-CACCGTTGCTCCAGATTCAC-3'; MT1 antisense (RV): 5'- GCAGCAGCACTGTTCGTCAC-3'; MT2 sense (FW): 5'- GCAGCAGCACTGTTCGTCAC-3'; MT2 antisense (RV): TGCACTTGTCCGAAGCCTCT-3'. qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, cat. no. 1725271) and a CFX96 Touch Real-Time PCR detection system (Bio-Rad). The qPCR reactions for each sample were run in triplicate and all targets were normalized to β-actin.

Statistics

Data are presented as means ± SEM. The number of experiments noted for each experiment represents biological replicate. All statistical analyses were performed using GraphPad Prism 9 (GraphPad, RRID:SCR_002798). Normality was assessed by the Shapiro-Wilk test. The ROUT method was used to identify outliers. This method uses nonlinear regression and an adapted false discovery rate to identify multiple outliers by comparing whether data points are far enough from the predicted model to be considered outliers (Motulsky and Brown 2006),. No outliers were identified. For comparison of two sample means, a two-tailed *t*-test was used. A one sample t-test was used for analysis of normalized values (control matched to 1). For comparison of more than two sample means, a one-way analysis of variance (ANOVA) with Sidak's or Dunnet's test for multiple comparisons was used (specified in figure legends). For analysis of LDH values in experiments comparing cultures with neurons and astrocytes to astrocytes alone, a two-way ANOVA with Sidak's test for multiple comparisons was used to compare the effect of drug treatments between sample groups. Although no formal power analysis was performed, samples sizes were determined *a priori* based on a large number of previous studies utilizing our cell culture models to examine cell death (Aizenman *et al*. 2000; Hartnett *et al*. 1997; Aras *et al*. 2008). No blinding was performed.

Results

Primary rodent neurons and astrocytes are differentially vulnerable to copper-elesclomol induced toxicity

A growing number of *in silico* studies have found that gliomas may be susceptible to cuproptosis (Zhang *et al*. 2023b; Ye *et al*. 2022; Zhu *et al*. 2022). In support of these results, we found that that glioma cell lines are sensitive to copper in the presence of elesclomol (CuES) *in vitro* (Fig. S1). As gliomas are generated within the brain parenchyma, an optimal treatment would selectively target glioma cells while leaving the surrounding tissue uninjured. However, no investigations to date have examined the effect of CuES on healthy neurons and astrocytes. Therefore, we assessed the effect of CuES on the glioma microenvironment, using mixed cerebrocortical cultures containing neurons and astrocytes to evaluate the susceptibility of these cell types to this treatment. We found that overnight treatment with concentrations at or above 2 μM copper in the presence of 1 μM elesclomol resulted in significant cytotoxicity as evidenced by increased LDH release (Fig. 1A). Strikingly, phase contrast microscopy revealed the presence of healthy-appearing neurons surrounded by swollen astrocytes in CuES-treated cultures (Fig. 1B). This finding was surprising in light of the significant body of evidence demonstrating that neurons are exquisitely sensitive to toxicity induced by transition metals, particularly zinc, while astrocytes are generally resistant to this cell stress (Dineley *et al*. 2000; Choi *et al*. 1988). Indeed, exposing the cerebrocortical cultures to overnight treatment with 10–30 μM zinc along with 250 nM of the zinc ionophore pyrithione (ZnPyr) resulted in a significant elevation in LDH release (Fig. 1C) and phase contrast microscopy confirmed that neurons were primarily affected by this treatment, while astrocyte morphology remained intact (Fig. 1D).

The LDH values reported in the previous experiments represent the total LDH released by both neurons and astrocytes present in our cultures. Therefore, to determine whether astrocytes and neurons were differentially vulnerable to CuES and ZnPyr treatment, we next assessed the relative contributions of each cell type to CuES- and ZnPyr-induced LDH release by comparing cultures containing both neurons and astrocytes to those containing astrocytes alone. To achieve astrocyte cultures lacking neurons (astrocyte cultures), mixed cerebrocortical cultures were treated overnight in media containing 1 mM kainate, as previously described (Aizenman *et al.* 2000), which causes widespread neuronal death without affecting the viability of astrocytes (Prieto and Alonso 1999; Qu *et al.* 2003; Aizenman *et al.* 2000). Following kainate or vehicle treatment, cultures were treated with increasing concentrations of CuES or ZnPyr overnight. As suggested by our observations noted above, astrocytes in the mixed cultures contributed the majority of the LDH signal following CuES treatment. In cultures containing neurons and astrocytes, significant toxicity was seen at concentrations at or above 3 μM CuES while in cultures containing astrocytes alone, significant toxicity was observed at concentrations at or above 2 μM CuES (Fig. 2A). These data demonstrate that astrocytes are the primary cell type affected by CuES in the mixed cultures. To confirm the differential sensitivity of neurons and astrocytes to zinc and copper toxicity, we used the same experimental paradigm described above to assess the relative contributions of neurons and astrocytes to zinc-ionophore induced LDH release. These experiments confirmed that astrocytes are relatively resistant to zinc-induced toxicity while neurons remain highly sensitive (Fig. 2B). Indeed, while mixed cultures showed significant toxicity at or above concentrations of 20 μM Zn^{2+} in the presence of 250 nM pyrithione, no concentration of ZnPyr tested elicited significant LDH release in astrocyte cultures lacking neurons, underscoring the differential vulnerability of neurons and astrocytes to zinc ionophore and copper ionophore induced toxicity.

Intriguingly, our results suggested that not only are neurons more resistant to CuES toxicity but that they may protect astrocytes against CuES mediated injury. Elesclomol concentration response experiments demonstrated that in cultures containing both neurons and astrocytes in the presence of 2 μM Cu^{2+}, 200–300 nM ES was necessary to induce significant elevations in LDH signal (Fig. 2C.). However, as little as 50 nM elesclomol was sufficient to induce significant cytotoxicity in astrocyte cultures (Fig. 2D), demonstrating increased sensitivity in the absence of neurons. Moreover, in our initial experiment comparing the vulnerability of neurons and astrocytes to CuES toxicity, described above, (Fig. 2A), a two-way repeated measures ANOVA revealed an interaction between the kainate treatment and CuES ($F(4,16)$, $p=0.0086$), reflecting the possibility that astrocyte cultures may be more sensitive to CuES toxicity in the absence of neurons.

Given that a growing body of literature suggests that changes in astrocyte cytoarchitecture are reflective of astrocyte dysfunction and may contribute to brain pathology (Lazic *et al.* 2022; Villablanca *et al.* 2023), we examined astrocyte morphology following CuES using glial fibrillary acid (GFAP) staining. We found that astrocytic architecture was disrupted at concentrations as low as 2 μM Cu^{2+} in the presence of elesclomol concentrations as low as 30 nM, even when neurons were present in culture (Fig. 2E), further emphasizing the exquisite sensitivity of astrocytes to CuES. In contrast, most neurons had preserved architecture at 2 μM Cu^{2+}/200 nM ES, as revealed by microtubule-associated protein 2

(MAP2) staining (Fig. 2F). Very infrequently (<10% of fields viewed), a few neurons were observed to have dendritic beading following CuES treatment – a phenotype often observed with excitotoxicity (Olney *et al.* 1979; Hasbani *et al.* 1998; Weilinger *et al.* 2016). As dendritic beading was only observed at the highest concentrations of CuES utilized and appeared to correspond with areas of substantial astrocyte damage, we hypothesized that this phenotype was a result of a failure of damaged astrocytes to buffer excess glutamate in the cultures. Indeed, extracellular glutamate has been shown to accumulate in astrocyte-poor neuronal cultures resulting in neuronal injury (Rosenberg 1991) and impairment of the glutamate transporter GLT-1, predominantly, but not exclusively, expressed in astrocytes (Petr et al., 2015) leads to neuronal death that can be rescued by NMDA receptor antagonism (Kawahara *et al.* 2002; Wang *et al.* 1998). To test whether the observed dendritic beading was caused by excitotoxicity or by CuES toxicity, cultures were treated with the NMDA receptor antagonist MK801. Co-treatment with 10 μM MK801 in the presence of CuES abrogated the observed beading suggesting that this form of neuronal injury was a result of excitotoxicity rather than a direct effect of CuES treatment.

Immortalized human astrocytes are also sensitive to CuES treatment

Given the unexpected finding that primary rat cortical astrocytes are selectively vulnerable to CuES-induced toxicity, we next validated these findings in an additional astrocyte model. For these experiments, we utilized an immortalized human astrocyte cell line which allowed us not only to test another cellular model of astrocytes, but also determine whether human astrocytes are also sensitive to copper ionophore toxicity. Indeed, a concentration response curve demonstrated that human astrocytes are also exquisitely sensitive to copper ionophore toxicity showing a significant increase in LDH signal at 2 μM Cu^{2+} in the presence of 100 nM elesclomol (Fig. 3A). Much like we observed in the C6 glioma cells (Fig. S1), sublethal concentrations of CuES induced a dramatic change in morphology in our immortalized human astrocyte, with a similar loss of spindle-like appearance and the rounding of cells (Fig. 3B). Thus, human astrocytes are also sensitive to copper ionophore induced cell death suggesting that susceptibility to CuES toxicity is not restricted to primary rat cortical astrocytes.

CuES-induced astrocyte death is not mediated via cuproptosis

Having established that astrocytes are vulnerable to CuES treatment, we next aimed to determine whether the selective astrocyte cell death was mediated by the recently described cuproptosis pathway, which involves FDX1-mediated lipoylation and subsequent dysfunction of TCA cycle proteins, (Tsvetkov *et al.* 2022; Li *et al.* 2022a). As a previous study has shown that shifting metabolism away from mitochondrial oxidative respiration and towards glycolysis by inhibiting oxidative phosphorylation is protective against cuproptosis (Tsvetkov *et al.* 2022), we first treated astrocyte cultures with the complex III inhibitor, antimycin A. Astrocytes were pre-treated for one hour with 300 nM antimycin A before the addition of CuES and then incubated overnight with both drugs. LDH assays revealed that inhibition of complex III was not protective against CuES induced cell death (Fig. 4A). As sodium azide, a complex IV inhibitor, has been shown to be a potent inhibitor of mitochondrial respiration, we also tested whether this drug could attenuate CuES-induced cell death. Using the same treatment paradigm as described for antimycin A, we found that

300 μM sodium azide was also ineffective at rescuing astrocytes from CuES toxicity (Fig. 4B). Thus, inhibition of mitochondrial respiration, an important target of the cuproptosis pathway, is ineffective at attenuating CuES-induced death in astrocytes.

Having determined that inhibiting oxidative phosphorylation was insufficient to rescue astrocytes from CuES, we next turned our attention to FDX1, an iron sulfur protein that is a key modulator of cuproptosis (Tsvetkov *et al.* 2022) and asked whether knockdown of this protein would be protective against CuES toxicity as previous experiments demonstrated that *FDX1* knockdown was sufficient to confer resistance against cuproptosis (Tsvetkov *et al.* 2022). Given that our knockdown protocol required us to use cultures containing both neurons and astrocytes to avoid an extended incubation period in serum-free media, we first determined via qPCR whether *FDX1* was abundant in both cell cultures. To do so, we compared *FDX1* expression in cultures containing neurons and astrocytes to those containing astrocytes alone. We found no significant difference between *FDX1* expression between these cultures (Fig. S2A), consistent with the fact that neurons make up a relatively small percentage of cells in our mixed cerebrocortical cultures. These data suggested that if knockdown (KD) were successful in mixed cultures, we would have achieved KD largely in the astrocytic component. We next tested whether we could KD *FDX1* in our cultures using siRNA. Indeed, 48 hours following siRNA treatment, we observed a significant reduction in *FDX1* expression compared to the non-targeting control (NTC) siRNA (Fig. S2B). Having demonstrated that *FDX1* was expressed in our primary cultures and could be knocked down, we treated cultures with siRNA for 48 hours after which time cells were treated overnight with CuES. LDH results demonstrated that *FDX1* KD did not attenuate CuES-induced toxicity (Fig. 4C). Of note, qPCR was also performed on sister cultures which demonstrated that this lack of rescue could not be attributed to a lack of KD as cultures treated with *FDX1* siRNA showed greater than a 90% reduction in *FDX1* expression compared to the NTC (Fig. 4D). These experiments demonstrate that *FDX1* is not a modulator of copper ionophore-induced toxicity in our cultures and that, importantly, cuproptosis does not mediate the observed astrocyte cell death.

It is important to note that our treatment paradigm differed from the paradigm used in the characterization of cuproptosis by Tsvetkov et al. (2022) as we exposed cells to CuES overnight rather than a much shorter, 2-hour pulse. To pursue the possibility that treatment duration might influence mechanism, we next treated astrocytes for 2 hours with CuES and then incubated them in fresh media overnight. Similar to what we observed with the overnight exposure, pulsed CuES treatment led to cell death as evidenced by increased LDH release (Fig. S3A). Phase contrast microscopy revealed once again the presence of healthy appearing neurons surrounded by swollen astrocytes (Fig. S3B). To determine whether cell death induced by this shorter CuES exposure was mediated by cuproptosis, we treated astrocytes with the complex III inhibitor antimycin A and the complex IV inhibitor sodium azide, as described above. Once again, we found no difference in toxicity between groups exposed to CuES in the presence or absence of inhibitors of oxidative phosphorylation (Figs. S3C, S3D). These results suggest that both short and long exposure to CuES triggers a cell death pathway in astrocytes but not neurons that is distinct from cuproptosis.

Having established that astrocytes undergo copper-ionophore triggered cell death in a manner distinct from cuproptosis, we next turned our attention to other known, regulated cell death pathways such as apoptosis, necroptosis, and ferroptosis. Although there are some reports that elesclomol induces apoptosis in cancer cells and that copper and/or elesclomol can trigger ferroptosis (Xue *et al.* 2023; Gao *et al.* 2021; Hasinoff *et al.* 2015), we found that the pan-caspase inhibitor Z-VAD-FMK (100 μM), the necroptosis inhibitor necrostatin-1s (Nec-1s) (20 μM), and the iron chelator deferoxamine (DFO) (50 μM) all failed to abrogate cell death in astrocytes exposed to CuES both overnight ($F(1.316, 3.949)=17.26$, repeated measures one-way ANOVA, $p=0.0131$, Šídák post-hoc, CuES vs Z-VAD-FMK, $p=0.5793$; CuES vs Nec-1s, $p=0.5840$; CuES vs DFO, $p=0.1559$, n=4 biological replicates) and for a 2-hour pulse ($F(1.125, 3.374)=9.081$, repeated measures one-way ANOVA, $p=0.0482$, Šídák post-hoc, CuES vs Z-VAD-FMK, $p=0.9686$; CuES vs Nec-1s, $p=0.7346$; CuES vs DFO, $p>0.9999$, n=4 biological replicates). Given recent evidence that copper can contribute to ferroptosis independent of iron accumulation (Xue *et al.* 2023), we also tested the ferroptosis inhibitor ferrostatin-1 (Fer-1) (1 μM) on astrocytes exposed to CuES overnight. As we observed with DFO treatment, 1 μM Fer-1 failed to rescue cells ($F(3,9)=4.547$, repeated-measures one-way ANOVA, $p=0.0013$, Šídák post-hoc, control vs CuES, $p=0.0170$; CuES vs CuES + Fer-1, $p=0.8260$, n=4 biological replicates). Concentrations as high as 30 μM Fer-1 also did not rescue astrocytes from CuES toxicity, strongly suggesting that ferroptosis is not the cell death pathway mediating CuES induced death in astrocytes.

Copper-ionophore treatment induces oxidative stress in astrocytes

Given that neither cuproptosis nor other canonical regulated cell death pathways appear to mediate CuES-induced death in astrocytes, we evaluated whether oxidative stress may be involved in CuES toxicity due to copper's ability to redox cycle between the Cu^{2+} and Cu^{+} state and induce reactive oxygen species (Valko *et al.* 2016). In cancer cells, elesclomol has been shown to transport copper to the mitochondria where redox cycling of copper results in the production of mitochondrial reactive oxygen species (ROS) (Nagai *et al.* 2012). Additionally, there is evidence that copper is able to bind to and form a complex with glutathione, and that this complex is capable of generating superoxide radicals (Speisky *et al.* 2009). Therefore, we assessed whether CuES treatment resulted in lipid peroxidation, an oxidative protein modification that can be triggered by reactive oxygen and nitrogen species (Li *et al.* 2022b; Murphy *et al.* 2022). We chose to look at 4-hydoxy-2-nonenal (4-HNE) as it is both an indicator of oxidative damage as well as a mediator of oxidative stress in cells due to its reactive nature (Li *et al.* 2022b). An increase in 4-HNE staining in glial fibrillary acid protein (GFAP)-stained astrocytes was observed when cultures containing neurons and astrocytes were exposed to 2 μM Cu in the presence of 200 nM elsesclomol, consistent with an increase in oxidative stress. (Fig. 5A).

While these findings strongly suggested that CuES treatment was sufficient to induce oxidative stress in astrocytes, whether this stress mediated CuES-induced cell death was still unknown. Therefore, to determine whether attenuation of oxidative stress would also attenuate CuES-induced cell death, we treated astrocytes with the superoxide dismutase (SOD)-catalase mimic, EUK-134, which has been shown to catalytically eliminate both superoxide and hydrogen peroxide (Doctrow *et al.* 2002; Sharpe *et al.* 2002), before and

during exposure to CuES. Remarkably, 30 μM EUK-134 was able to restore viability by over 40% in astrocytes treated overnight with CuES and by approximately 70% in astrocytes exposed to a CuES pulse (Fig. 5B). Thus, reactive species are a significant mediator of CuES-induced astrocyte cell death. Given evidence that redox cycling of copper may contribute to CuES-induced ROS generation (Nagai *et al.* 2012), we next investigated whether pyrroloquinoline quinone (PQQ), a naturally occurring redox cofactor that has been shown to act as an antioxidant through suppression of reactive oxygen species (Zhang and Rosenberg 2002), could also rescue astrocytes from CuES-induced death. In contrast to our findings with EUK-134, we found that 50 μM PQQ failed to protect astrocytes from CuES treatment (Fig. 5C). Given evidence that manganese-salen compounds, such as EUK-134, protect against mitochondrial oxidative stress (Melov *et al.* 2001; Hinerfeld *et al.* 2004), these results suggest that CuES induced oxidative stress may be mitochondrially generated.

As our prior experiments confirmed that immortalized human astrocytes are also sensitive to CuES toxicity, we next assessed whether oxidative stress mediated their susceptibility to copper ionophore treatment, as it did in primary rodent astrocytes. Indeed, treatment with EUK-134 rescued viability of immortalized human astrocytes in a concentration dependent manner with 10 μM EUK-134 restoring viability to control levels (Fig. 5D). Unlike what we observed in primary cultures, however, treatment with PQQ also restored viability of CuES treated immortalized astrocytes (Fig. 5E). Together, these results suggest that oxidative stress is a common mechanism underlying astrocyte cell death in response to CuES treatment.

Given that antioxidant treatment protected both primary rodent astrocytes and immortalized human astrocytes from CuES-induced death, we tested whether treatment with EUK-134 or PQQ attenuated the sensitivity of C6 glioma cells against CuES toxicity to determine whether antioxidant treatment could serve as a glioprotective strategy without inhibiting the potential effectiveness of this therapeutic treatment. However, both compounds rescued C6 glioma cells from CuES mediated cell death in a concentration dependent manner (Figs. S4A, 4B) Moreover, 10 μM EUK-134 was able to restore the viability of C6 glioma cells to close to 100% (Fig. S4A) of control cells while 50 μM PQQ restored viability to approximately 70% of control cells (Fig S4B). Thus, oxidative stress appears to be a major factor in CuES-induced glioma cell death as well as in healthy astrocytes.

Zinc preconditioning is protective against CuES toxicity

Although our experiments established that CuES-induced oxidative stress mediates cell death in astrocytes, they did not reveal why neurons are able to survive this insult – especially since astrocytes are thought to have more robust systems for buffering oxidative stress as compared to neurons (Bell *et al.* 2015; Muyderman *et al.* 2007; Dringen *et al.* 2015). However, neurons are highly enriched in metallothionein 3 (MT3) (Aschner *et al.* 1997), which can bind copper with high affinity (Calvo *et al.* 2018; Mehlenbacher *et al.* 2022). Moreover, previous research has shown most of MT3 in our neuronal preparation exists in its apo-form (Aras *et al.* 2009; Aras and Aizenman 2011) allowing it to bind excess metal. Although there is some data to support low levels of MT3 expression in astrocytes (Hidalgo *et al.* 2001), these cells primarily express metallothionein 1 (MT1) and

metallothionein 2 (MT2) (Aschner *et al.* 1997). Importantly, MT1 and MT2 are expressed at low levels basally but their expression can be substantially induced by zinc (Silva *et al.* 2023). Therefore, we hypothesized that MT expression may buffer excess cytosolic copper and partially account for the differential sensitivity of neurons and astrocytes to CuES mediated injury.

To test whether the induction of MTs could protect astrocytes from CuES toxicity, we preconditioned astrocyte cultures with 20 μM Zn^{2+} in the presence of 250 nM Pyr during kainate treatment, conditions that are not injurious to our astrocyte cultures (Fig. 3B). We first confirmed that overnight treatment with 20 μM ZnPyr was sufficient to increase expression of MT1 and MT2 in astrocyte cultures via qPCR, which was indeed the case (Fig. 6A). Having established that this concentration of ZnPyr induced a significant increase in MT1 and MT2 gene expression, we next treated astrocytes with CuES following an overnight exposure to ZnPyr. Remarkably, ZnPyr preconditioning completely abrogated CuES-induced toxicity and restored the LDH signal to that of the control group (Fig. 6B). Together these data suggest that upregulation of MTs are protective against CuES induced cell death and injury and that the differing basal levels of MTs in neurons and astrocytes may explain their differential sensitivity to CuES toxicity. It is important to note that a higher concentration of zinc is needed to induce neuronal death than the concentration of copper used here to induce astrocyte death (Fig. 2A, 2B), likely due to the need to overcome the buffering actions provided by MT3.

Discussion

In this report we demonstrate that primary rodent and immortalized human astrocytes are sensitive to cell death induced by CuES treatment. Surprisingly, neurons in primary culture were highly resistant to this insult. This finding is in striking contrast to the selective vulnerability of neurons to the redox-inert metal zinc, when compared to astrocytes (Dineley *et al.* 2000). An extensive investigation into pathways underlying copper ionophore induced toxicity in astrocytes revealed that, in contrast to the recently described cuproptosis pathway (Tsvetkov *et al.* 2022), CuES mediated toxicity to astrocytes in mixed cultures is FDX1-independent and is not attenuated by treatment with inhibitors of oxidative phosphorylation. Furthermore, astrocytes cannot be rescued by established inhibitors of apoptosis, ferroptosis, or necroptosis. However, consistent with previous research that has found that treatment with elesclomol, likely complexed with endogenous copper, can result in the generation of ROS (Kirshner *et al.* 2008; Nagai *et al.* 2012; Blackman *et al.* 2012), we have demonstrated that CuES treatment results in oxidative stress in primary rodent astrocytes and that antioxidant treatment rescues primary astrocytes, immortalized human astrocytes, and C6 glioma cells from CuES induced cell death. Importantly, we discovered that primary astrocytes can also be rescued following zinc pyrithione preconditioning, likely via the induction of metallothioneins 1 and 2, which could buffer excess intracellular copper. Interestingly, the presence of neurons in the cultures seemed to exert a protective effect on surrounding astrocytes.

One possible explanation for the divergence between Tsvetkov et al.'s (2022) findings and the results presented here is that cells that are heavily reliant on mitochondrial respiration

for energy production are most vulnerable to cuproptosis (Tsvetkov *et al.* 2022). Astrocytes, however, are more metabolically flexible, utilizing oxidative phosphorylation, glycolysis, and β-oxidation of fatty acids, amongst other pathways, for energy production (Hertz *et al.* 2007; Harders *et al.* 2023; Juaristi *et al.* 2019; Arend *et al.* 2019). Of note, a recent study found proliferating astrocytes in culture are not dependent on oxidative phosphorylation for growth or survival (Silva *et al.* 2023). Moreover, respiration deficient astrocytes can survive by glycolysis *in vivo* (Supplie et al. 2017). Therefore, astrocytes may naturally shift to glycolysis in the presence of stimuli that compromise mitochondrial respiration. Furthermore, copper itself has been shown to stimulate glycolytic flux in astrocytes (Bulcke and Dringen 2015; Scheiber and Dringen 2011). Thus, it is possible that CuES treatment drives astrocytes further towards glycolysis rendering them less reliant on mitochondrial energy production and therefore less likely to be susceptible to cuproptotic cell death mediated by FDX1-dependent TCA cycle dysfunction, which would account for the failure of complex III and IV inhibitors to ameliorate astrocyte death in our cultures. Additionally, this lack of reliance on TCA cycle function would partially account for the lack of attenuation of CuES toxicity we observed with *FDX1* knockdown. It is important to note that copper was also found to bind to lipoylated TCA cycle proteins resulting in their oligomerization and proteotoxic stress, which was reversed by *FDX1* knockout (Tsvetkov *et al.* 2022; Tsvetkov *et al.* 2019). The lack of rescue by *FDX1* knockdown in our cultures thus indicates that proteotoxic stress may not be a major contributor to CuES induced cell death in astrocytes. Finally, it is important to consider that the concentration of elesclomol found to elicit cuproptosis in various cell lines was 40 nM (Tsvetkov *et al.* 2022; Tsvetkov *et al.* 2019) whereas this concentration of the copper ionophore was insufficient to induce cell death in our cell cultures. Therefore, the higher concentrations of elesclomol needed in our investigation may trigger the alternative cell death-inducing pathway described here.

We found that oxidative stress, rather than perturbed mitochondrial metabolism, is a primary mediator of copper ionophore induced cell death in astrocytes. Not only did astrocytes in primary culture show increased levels of 4-HNE, an end product of lipid peroxidation (Murphy *et al.* 2022), but antioxidant treatment attenuated CuES-induced cell death in primary astrocytes, human immortalized astrocytes, and C6 glioma cells. The antioxidant EUK-134, a SOD-catalase mimic, rescued all three cell types from CuES mediated cell death, demonstrating that oxidative stress is a common mechanism underlying CuES-induced cytoxicity in astrocyte-like cells in our studies. To our surprise, PQQ, an essential nutrient and free radical scavenger due its ability to redox cycle (Stites *et al.* 2000), rescued human immortalized astrocytes and C6 glioma cells from CuES mediated cell death, but had no effect on the viability of primary astrocyte cultures. It is worth noting that while EUK-134 restored viability to nearly control levels in CuES treated cell lines, it had a less dramatic effect in primary cultured astrocytes. Thus, there may be an additional mechanism mediating CuES induced death in primary cultures that EUK-134 but not PQQ was able to partially overcome. Although the mechanism underlying the differential rescue of PQQ in these cell types is unclear, it is worth further investigation as potential differences in the type or location of reactive species production could be exploited for targeted treatment of gliomas while leaving healthy tissue unperturbed. For example, catalase has been shown to have the ability to scavenge peroxynitrite (Gebicka and Didik 2009), a reactive nitrogen

species, while PQQ can prevent the formation of peroxynitrite but cannot protect against its toxicity once formed (Zhang and Rosenberg 2002). Despite the evidence presented here that oxidative stress mediates CuES induced cell death in astrocytes, an important limitation of this study is that the types of reactive species induced by CuES treatment and where within the cell the formation of these species occurs are still open questions.

Given neurons' reliance on mitochondrial metabolism (Zheng *et al.* 2016; Wong-Riley 1989; Halim *et al.* 2010), it is all the more surprising that neurons are not vulnerable to cuproptosis, and that it is astrocytes rather than neurons that are selectively vulnerable to injury induced copper. One clue to a potential mechanism underlying the resistance of neurons to CuES toxicity comes from our ZnPyr preconditioning experiments, which showed that ZnPyr treatment before CuES exposure completely protected astrocytes from copper ionophore induced cell death. Importantly, overnight exposure significantly upregulated *MT1* and *MT2* expression, consistent with a large body of data demonstrating that increases in intracellular free zinc drive metallothionein upregulation (Andrews 2000; Heuchel *et al.* 1994; Radtke *et al.* 1993). As noted previously, astrocytes primarily express low levels of the MT1 and MT2 while neurons are highly enriched in MT3. Importantly, studies have demonstrated that MT3 may exist predominantly in its apo – or non-metal bound – form (Yang *et al.* 2001; Aras *et al.* 2009) and is able to bind redox active free Cu^{2+} (Meloni *et al.* 2007). Thus, high levels of non-metal bound MT3 in neurons may buffer the increase in intracellular copper facilitated by elesclomol and prevent the metal from redox cycling, protecting neurons from copper-induced increases in oxidative species. Although MT3 has been shown to have a high affinity for copper (Calvo *et al.* 2018; Artells *et al.* 2014; Mehlenbacher *et al.* 2022), it is worth noting that in *in vitro* preparations, when apo-MT2 is added to solutions containing copper-bound proteins, it is able to extract Cu^{+} from them, indicating a high affinity for the metal (Banci *et al.* 2010). Additionally, previous research has shown that induction of MT1 and MT2 via zinc treatment protects astrocytes from methylmercury-induced cytotoxicity (Aschner *et al.* 1998; Rising *et al.* 1995), indicating that increased MT expression is protective against a variety of metals. Although we posit that the protective effect of MTs is mediated through its metal binding functions, research also suggests that these proteins are protective against, and can even scavenge, free radicals such as superoxide and hydroxyl radicals (Thornalley and Vašák 1985; Ruttkay-Nedecky *et al.* 2013; Chubatsu and Meneghini 1993; Uchida *et al.* 2002). Therefore, it is also possible that MT-facilitated rescue of astrocytes is mediated through their ability to mitigate oxidative stress, which we found to drive CuES induced cell death.

The findings described here could have significant implications for the potential treatment of gliomas with copper elesclomol therapy. Firstly, we have demonstrated in our proof-of-concept experiments that glioma cells are sensitive to CuES induced cell death although this treatment results in oxidative stress induced cell death in healthy astrocytes as well. Although proliferating glioma cells are often highly glycolytic, a number of studies have found that the TCA cycle is intact in some tumor cells and cancer stem cells (Dekker *et al.* 2020; Datta *et al.* 2021; DeBerardinis *et al.* 2007). Therefore, metabolic profiling of gliomas may allow for targeted therapy of tumors most susceptible to this treatment modality. The range of metabolic phenotypes of cancer cells, and glioma cells in particular, may explain the large number of *in silico* studies linking cuproptosis-related gene expression to glioma

prognosis (Zhang *et al*. 2022; Zhang *et al*. 2023b; Ye *et al*. 2022; Zhu *et al*. 2022). Higher FDX1 expression has been linked to a higher pathological tumor grade as well as overall poorer prognosis in multiple *in silico* investigations of glioma and glioblastoma (Zhang *et al*. 2023b; Guowei *et al*. 2023). However, given the importance of FDX1 in the regulation of mitochondrial metabolism and cuproptosis, these tumors may be most susceptible to CuES treatment. Together, these studies highlight the importance of combining molecular studies with *in silico* investigations to improve treatment outcomes.

The finding that metallothionein induction is protective against copper ionophore induced cytotoxicity may also have important implications for the treatment of gliomas. Malignant gliomas have been found to express higher levels of MT genes and higher expression has been associated with a more aggressive tumor type and poorer patient prognosis (Masiulionytė *et al*. 2019; Mehrian-Shai *et al*. 2015). *In vitro*, loss of MT1E and MT2A expression sensitized human glioblastoma cells to disulfiram treatment (Corsello *et al*. 2020), which is particularly relevant to the data presented here as disulfiram is another copper ionophore (Kannappan *et al*. 2021). Therefore, gliomas with lower MT expression are more likely to respond favorably to CuES treatment and MTs may represent a new target for anti-neoplastic therapeutics. In conclusion, we have demonstrated that glioma cells and astrocytes are highly susceptible to CuES induced cell death while neurons are resistant to this insult. We have also demonstrated that this cytoxicity is FDX1-independent and mediated by oxidative stress. Additionally, we have shown that induction of metallothionein gene expression may protect against CuES toxicity.

Identifying methods of glioprotection is likely a critical step in order to move CuES treatment from *in vitro* studies to pre-clinical models of glioma as well as to clinical studies. While the finding that neurons are spared from CuES toxicity is promising, the detrimental effect of this compound on astrocytes should not be overlooked. Although astrocytes, unlike neurons, are mitotic and theoretically could recover from this insult, oxidative stress has been associated with astrocyte senescence and reduced proliferation (Bitto *et al*. 2010; Cohen and Torres 2019). These findings may have negative therapeutic implications for the potential use of CuES in the treatment of gliomas. In addition to reducing proliferation, astrocyte senescence has been associated with an increase in neurotoxic secretions which could damage surrounding neurons unaffected by CuES treatment (Limbad *et al*. 2020; Pertusa *et al*. 2007). In fact, we observed limited excitotoxic injury to neurons as a result of astrocytic damage following CuES treatment. In addition to oxidative stress, aging has been found to contribute to astrocyte senescence (Cohen and Torres 2019) – a point that is of particular importance in glioblastoma, a very aggressive glioma, as the incidence of this tumor type increases with age (Ostrom *et al*. 2019). Therefore, damage by CuES to healthy astrocytes may have detrimental and long-lasting consequences. Learning how to protect astrocytes from CuES, or how to increase the selectivity of copper toxicity for glioma cells may have important therapeutic benefits and improve long-term positive patient outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability:

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

List of Abbreviations

4-HNE	4-hydoxy-2-nonenal
AA	Antimycin A
BSA	Bovine serum albumin
CuES	Copper-elesclomol
MK801	Dizocilpine hydrogen maleate
DIV	Days *in vitro*
DFO	Deferoxamine mesylate
ES	Elesclomol
Fer-1	Ferrostatin-1
FDX1	Ferredoxin-1
FBS	Fetal Bovine Serum
GFAP	Glial fibrillary acid protein
ICLAC	International Cell Line Authentication Committee
LDH	Lactate Dehydrogenase
KA	Kainate
MAP2	Microtubule-associated protein 2
MT	Metallothionein
Nec-1s	Necrostatin-1s
NTC siRNA	Non-targeting control siRNA
OD	Optical density
PLO	Poly-L-ornithine
PQQ	Pyrroloquinoline quinone
ROS	Reactive oxygen species

RRIDs	Research Resource Identifiers
NaAz	Sodium Azide
TCA cycle	Tricarboxylic acid cycle
qPCR	Quantitative polymerase chain reaction
ZnPyr	Zinc pyrithione

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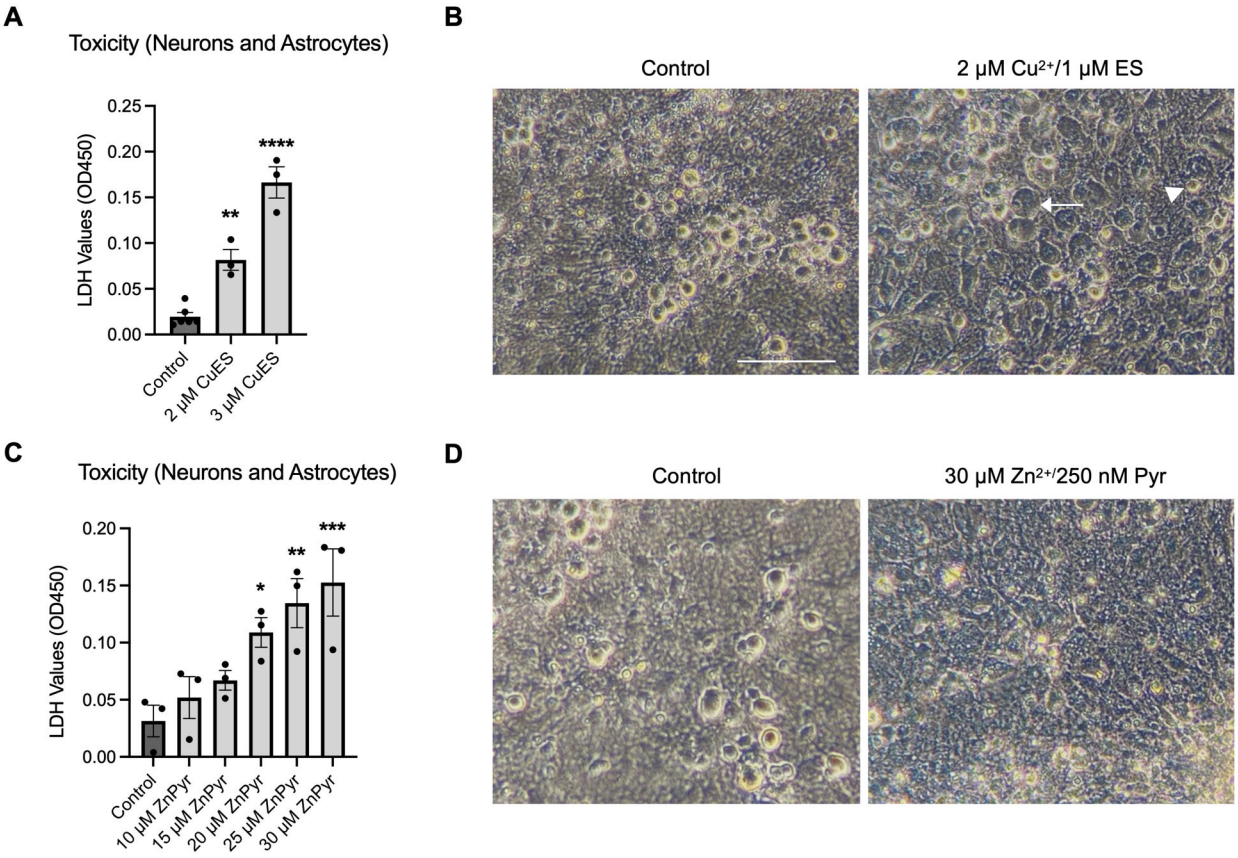

Figure 1. Copper and zinc toxicity appear to target different cell populations in mixed neuronal glial cultures.

(A) LDH assay showing concentration-dependent copper toxicity in the presence of 1 μM elesclomol in cerebrocortical cultures ($F(2,9)=62.82$, one-way ANOVA, $p<0.0001$, Dunnet post-hoc, control vs 2 μM CuES, $p=0.0021$; control vs 3 μM CuES, $p<0.0001$, n=3 biological replicates). (**B)** Phase contrast images of cerebrocortical cultures following overnight treatment with CuES, demonstrating swollen glia (arrow), but seemingly healthy-appearing, phase-bright neurons (arrowhead). **C.** LDH assay showing concentration-dependent zinc toxicity in the presence of 250 nM of the zinc ionophore, pyrithione ($F(5,12)=6.672$, one-way ANOVA, $p=0.0034$, Dunnet post-hoc, control vs 20 μM ZnPyr, $p=0.0477$; control vs 25 μM ZnPyr, $p=0.0085$, control vs 30 μM ZnPyr, $p=0.0026$, n=3 biological replicates). **D.** Phase contrast images of cerebrocortical cultures following overnight treatment with ZnPyr showing a lack of phase bright neurons, but seemingly healthy glia. Scale bar is 100 μM.

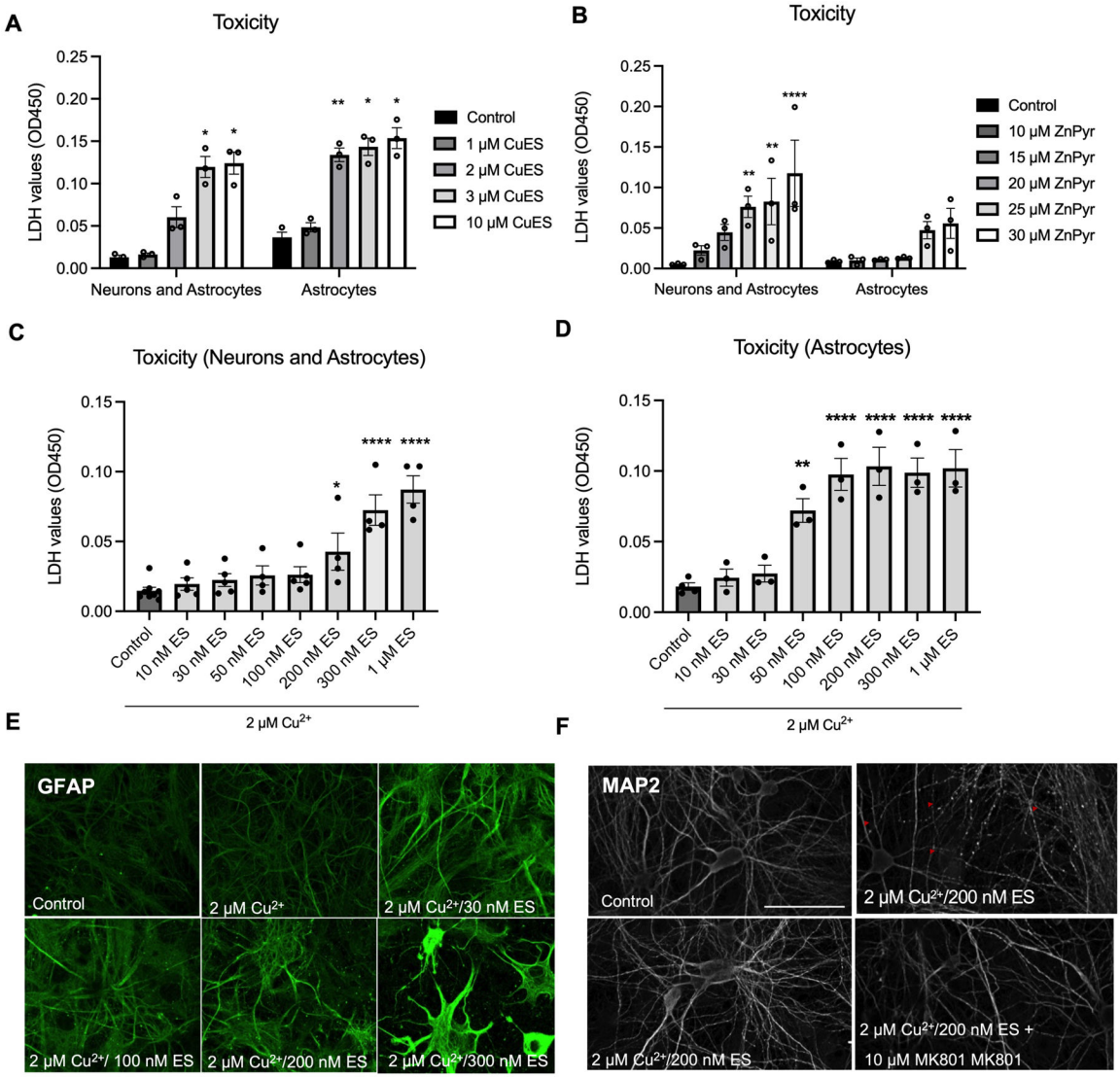

Figure 2. Astrocytes are selectively vulnerable to CuES toxicity and neurons in co-culture provide some protection against this insult.

Mixed cortical cultures were first exposed to MHB in the absence or presence of 1 mM kainate (KA) overnight, with the treatment generating neuron-deprived, astrocyte cultures. Cultures-containing coverslips were then transferred to plates containing fresh MHB with various concentrations of CuES or ZnPyr. **(A)** LDH assay comparing toxicity of CuES (1 μM elesclomol) in the presence and absence of neurons. Note that in the presence of neurons, CuES does not induce a significant elevation in LDH until 3 μM copper while in the absence of neurons, CuES induces significant toxicity at 2 μM CuES ($F(4,16)=4.953$, two-way ANOVA, $p=0.00223$ for KA; $p<0.0001$ for Cu^{2+} treatment; $p=0.0086$ for interaction of KA and Cu^{2+} treatments; Sidak's post hoc test; Neurons and astrocytes within group comparisons: control vs 1 μM CuES, $p=0.5596$; control vs. 2 μM CuES, $p=0.1699$; control vs 3 μM CuES, $p=0.0337$; control vs 10 μM CuES, $p=0.0372$; Astrocytes within group comparisons: control vs 1 μM CuES, $p=0.1661$, control vs 2 μM CuES, $p=0.0040$, control vs 3 μM CuES, $p=0.0227$; control vs 10 μM CuES, $p=0.0457$, n=3

biological replicates). **(B)** LDH assay comparing the toxicity of ZnPyr (250 nM elesclomol) in the presence and absence of neurons. When neurons are present, significant LDH is observed beginning at 20 μM ZnPyr while no concentration of ZnPyr tested was toxic in the absence of neurons ($F(5,20)=2.044$, repeated measures two-way ANOVA, $p=0.1047$ for KA, $p<0.0001$, $p=0.1158$ for interaction; Neurons and astrocytes within group comparison: control vs 10 μM ZnPyr, $p=0.8978$; control vs 15 μM ZnPyr, $p=0.2089$; control vs 20 μM ZnPyr, $p=0.0052$; control vs 25 μM ZnPyr, $p=0.0023$; control vs 30 μM ZnPyr, $p<0.0001$; Astrocytes within group comparison: control vs 10 μM ZnPyr, $p>0.9999$; control vs 15 μM ZnPyr, $p>0.9999$; control vs 20 μM ZnPyr, $p=9998$; control vs 25 μM ZnPyr, $p=0.2274$; control vs 30 μM ZnPyr, $p=0.0946$, n=3 biological replicates). **(C)** LDH assay of elesclomol concentration response curve using mixed cerebral cultures demonstrates significant copper (2 μM) toxicity at concentrations at or above 200 nM elesclomol ($F(7,31)=14.25$, one-way ANOVA, $p<0.0001$, Dunnet post hoc, control vs 10 nM ES, $p=0.9939$; control vs 30 nM ES, $p=0.9327$; control vs 50 nM, $p=0.7880$; control vs 100 nM ES, $p=0.6836$; control vs 200 nM ES, $p=0.0295$; control vs 300 nM ES, $p<0.0001$; control vs 1 μM ES, $p<0.0001$, n=4–5 biological replicates) while an **(D)** LDH assay of the same elesclomol concentration curve in astrocyte cultures shows significant copper (2 μM) toxicity beginning at 50 nM elesclomol ($F(7,17)=17.90$, one-way ANOVA, $p<0.0001$, Dunnet post hoc, control vs 10 nM ES, $p=0.9948$; control vs 30 nM ES, $p=0.9650$; control vs 50 nM ES, $p=0.0027$; control vs 200 nM ES, $p<0.0001$; control vs 300 nM ES, $p<0.0001$; control vs 1 μM ES, $p<0.0001$, n=3 biological replicates). **(E)** GFAP staining of mixed cortical cultures treated with CuES shows changes in astrocyte morphology beginning at 30 nM ES and becoming more pronounced at higher concentrations of the ionophore while **(F)** neuronal architecture is preserved, even at much higher ES concentrations. Rarely, dendritic beading was observed (red arrow heads) however this phenotype could be rescued with co-treatment with the NMDA antagonist MK801 suggesting that this neuronal injury was due to a failure of astrocytes to buffer excess glutamate. Scale bars are 100 μM.

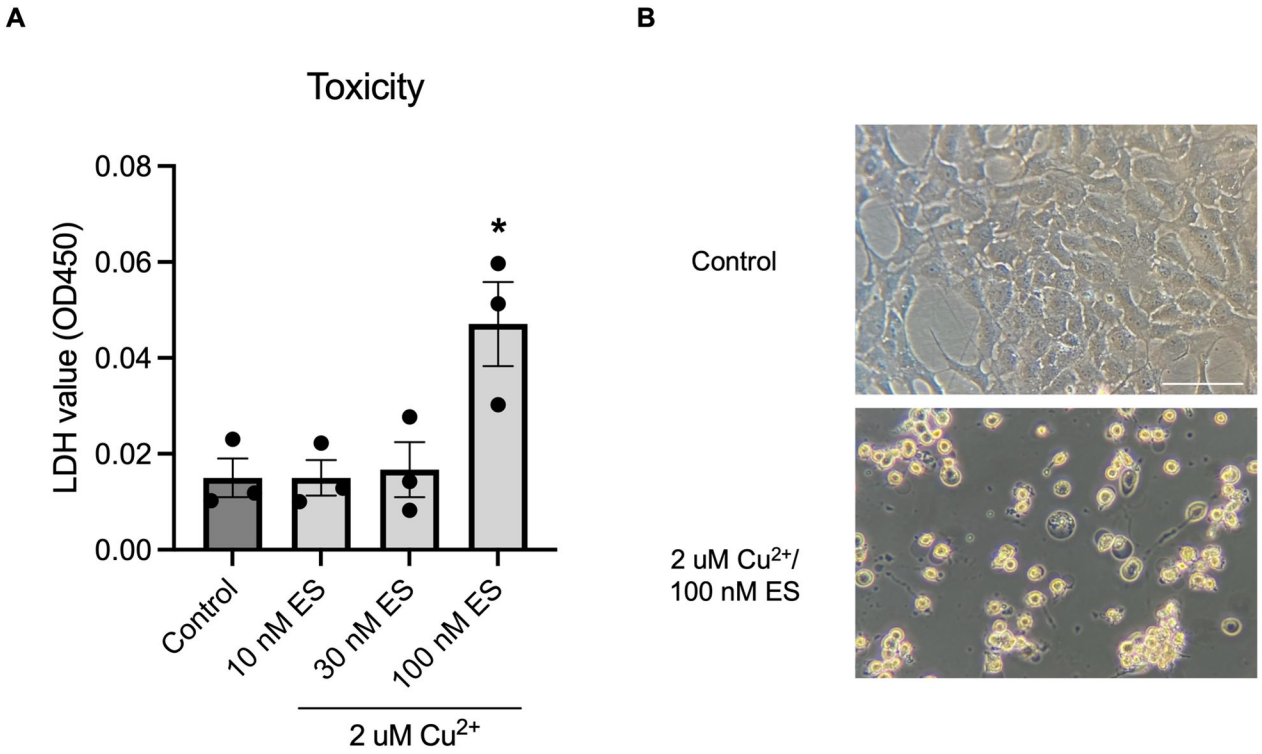

Figure 3. Human immortalized astrocytes are sensitive to copper ionophore induced cell death.
(A) LDH assay showing concentration-dependent copper (2 μM) toxicity with increasing elesclomol concentrations ($F(3, 8)=7.102$, one-way ANOVA, $p=0.0121$, Dunnet post-hoc, control vs 10 nM ES, $p>0.9999$; control vs 30 nM ES, $p=0.9937$; control vs 100 nM ES, $p=0.0127$, $n=3$ biological replicates). (**B)** Phase contrast images of immortalized astrocytes after overnight treatment with CuES showing substantially altered morphology. Scale bar is 100 μM.

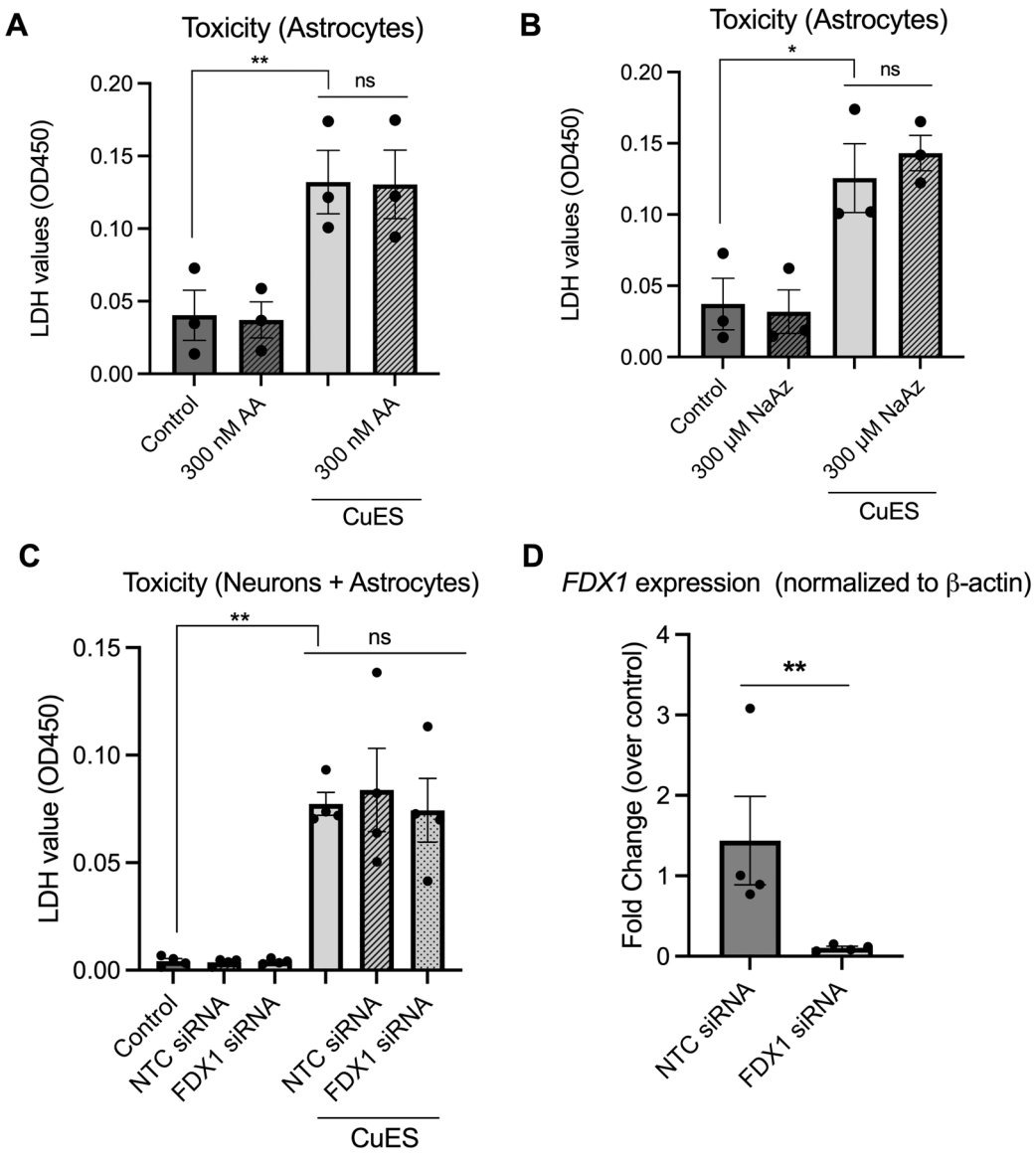

Figure 4. CuES induced astrocyte toxicity is not mediated by cuproptosis.

All experiments utilized 2 μM copper. Experiments conducted in astrocyte cultures utilized 200 nM ES while those conducted in cultures containing neurons and astrocytes utilized 300 nM ES. Inhibition of mitochondrial respiration with **(A)** 300 nM antimycin A (AA), a complex III inhibitor, is insufficient to rescue CuES toxicity in primary astrocytes ($F(1.052, 2.105)=103.1$, repeated measures one-way ANOVA, $p=0.0080$, Sidak's post hoc, control vs CuES, $p=0.0054$; CuES vs CuES + AA, p=08162, n=3 biological replicates) as is **(B)** inhibition with 300 μM of the complex IV inhibitor sodium azide (NaAz) ($F(1.418, 2.83)=75.11$, repeated measures one-way ANOVA, $p=0.0037$, Sidak post-hoc, control vs CuES, $p=0.0128$; CuES vs CuES + NaAz, $p=0.5748$, n=3 biological replicates). **(C)** Treatment with *FDX1* siRNA does not rescue cultures from CuES toxicity as evidenced by significant LDH release ($F(1.244, 3.733)= 20.55$, repeated measures one-way ANOVA, $p=0.0114$, Sidak post-hoc, control vs CuES, $p=0.0041$; CuES vs CuES + NTC siRNA,

p=0.9713; CuES vs CuES + CuES + FDX1 siRNA, p=0.9914, n=4 biological replicates), which **(D)** cannot be explained by a lack of knockdown in siRNA treated cultures (t(3)=6.143, ratio paired two-tailed t-test, p=0.0087, n=4 biological replicates).

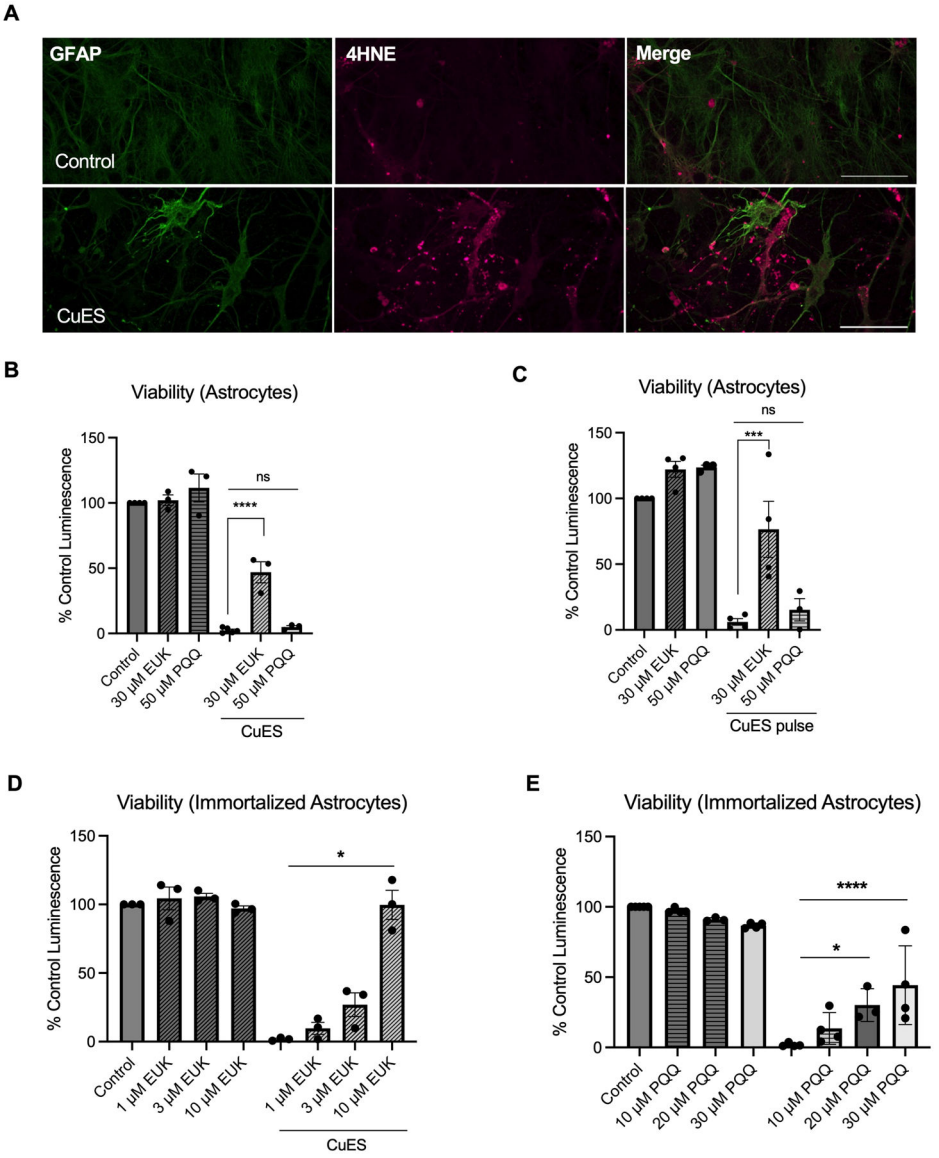

Figure 5. CuES mediates toxicity, in part, through induction of oxidative stress in astrocytes and antioxidant treatment improves viability.

Cultures containing neurons and astrocytes were treated with 2 μM Cu/200 nM ES overnight and were subsequently stained with 4-HNE, a marker of lipid peroxidation. **(A)** Confocal imaging revealed an increase in 4-HNE signal in CuES treated astrocytes as compared to controls strongly indicating increased oxidative stress following copper ionophore treatment. Treatment with the antioxidant EUK134 but not PQQ significantly increased viability of astrocyte cultures exposed to 2 μM Cu/200 nM ES both **(B)** overnight ($F(2,8)=40.29$, one-way ANOVA, $p<0.0001$, Sidak post hoc, CuES vs CuES + 30 μM EUK134, $p<0.0001$; CuES vs 50 μM PQQ, $p=0.84894$, n=3 biological replicates) and **(C)** for a 2-hour pulse ($F(5,16)=24.9$, one-way ANOVA, $p<0.0001$, Sidak post-hoc, CuES vs CuES + 30 μM EUK134, $p=0.0003$; CuES vs CuES + 50 μM PQQ, $p=0.8007$, n=4 biological replicates). In contrast, immortalized human astrocytes exposed to 2 μM Cu/100 nM ES overnight were rescued by treatment with both **(D)** EUK134 ($F(1.338, 2.667)=108.6$, repeated measures

one-way ANOVA, p=0.0029, CuES vs CuES + 1 μM EUK134, p=0.4179; CuES vs CuES + 3 μM EUK134, p=0.2406; CuES vs CuES vs CuES + 10 μM EUK134, p=0.0300, n=3 biological replicates) and **(E)** PQQ ($F(7,23)=48.71$, one-way ANOVA, $p<0.0001$, CuES vs CuES + 10 μM PQQ, p=0.4184; CuEs vs CuES + 20 μM PQQ, p=0.0113; CuES vs CuES + 30 μM PQQ, $p<0.0001$, n=4 biological replicates). Scale bar is 50 μm.

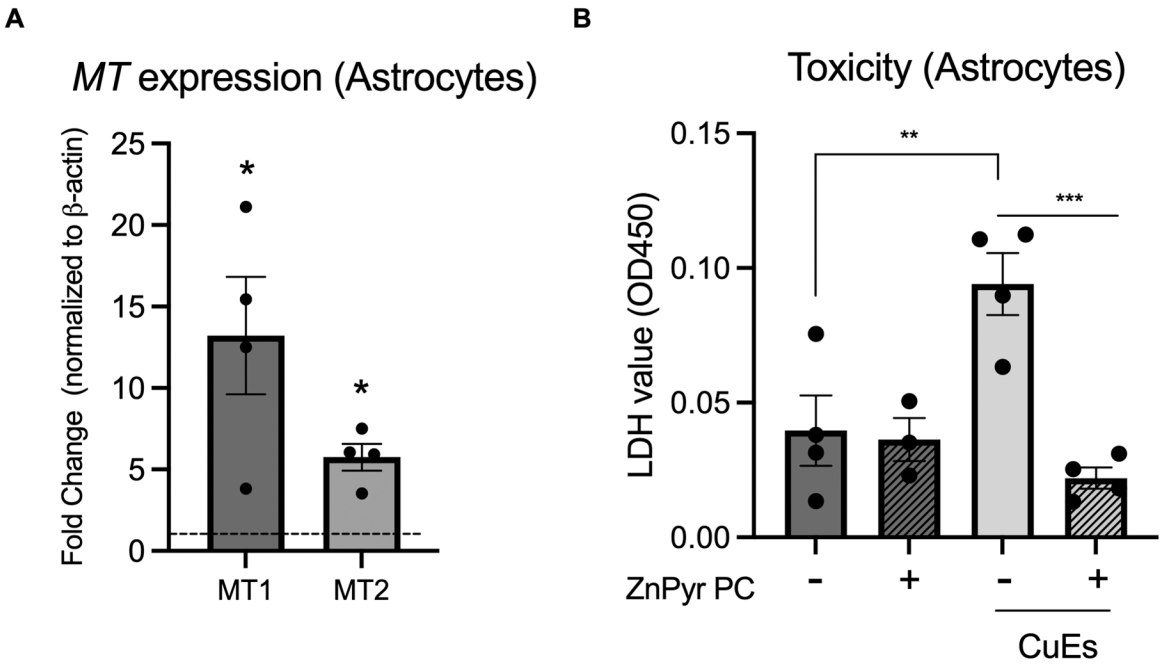

Figure 6. Preconditioning of astrocytes with ZnPyr is protective against CuES induced toxicity.

Prior to exposure to 2 μM Cu^{2+}/200 nM ES, astrocytes were treated overnight with 20 μM Zn^{2+}/250 nM Pyr (ZnPyr PC= ZnPyr preconditioning), **(A)** which significantly increased expression of *MT1* and *MT2* (t(3.387,3), *MT1* fold change vs 1, p=0.0429; t(5.796,3), *MT2* fold change vs 1, p=0.0102, one sample t-test, n=4 biological replicates) and **(B)** completely abrogated CuES induced LDH release (F(3,11)=10.39, one-way ANOVA, p=0.0015, Sidak post hoc, control vs CuES, p=0.0046; CuES vs CuES + ZnPyr PC, p=0.0006, n=3–4 biological replicates).