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Mechanisms Controlling the Cellular Accumulation of Copper Bis(thiosemicarbazonato) Complexes

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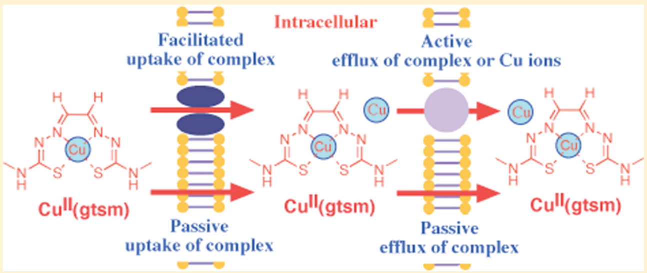
ABSTRACT: Copper (Cu) bis(thiosemicarbazonato) metal complexes [Cu^{II}(btsc)s] have unique tumor-imaging and treatment properties and more recently have revealed potent neuroprotective actions in animal and cell models of neurodegeneration. However, despite the continued development of Cu^{II}(btsc)s as potential therapeutics or diagnostic agents, little is known of the mechanisms involved in cell uptake, subcellular trafficking, and efflux of this family of compounds. Because of their high lipophilicity, it has been assumed that cellular accumulation is through passive diffusion, although this has not been analyzed in detail. The role of efflux pathways in cell homeostasis of the complexes is also largely unknown. In the present study, we investigated the cellular accumulation of the Cu^{II}(btsc) complexes Cu^{II}(gtsm) and Cu^{II}(atsm) in human neuronal (M17) and glial (U87MG) cell lines under a range of conditions. Collectively, the data strongly suggested that Cu^{II}(gtsm) and Cu^{II}(atsm) may be taken into these cells by combined passive and facilitated (protein-carrier-mediated) mechanisms. This was supported by strong temperature-dependent changes to the uptake of the complexes and the influence of the cell surface protein on Cu accumulation. We found no evidence to support a role for copper-transporter 1 in accumulation of the compounds. Importantly, our findings also demonstrated that Cu from both Cu^{II}(gtsm) and Cu^{II}(atsm) was rapidly effluxed from the cells through active mechanisms. Whether this was in the form of released ionic Cu or as an intact metal complex is not known. However, this finding highlighted the difficulty of trying to determine the uptake mechanism of metal complexes when efflux is occurring concomitantly. These findings are the first detailed exploration of the cellular accumulation mechanisms of Cu^{II}(btsc)s. The study delineates strategies to investigate the uptake and efflux mechanisms of metal complexes in cells, while highlighting specific difficulties and challenges that need to be considered before drawing definitive conclusions.



■ INTRODUCTION

The investigation of metal-based compounds for use in disease therapy and their application in diagnostic imaging is well established. The bis(thiosemicarbazone) family of ligands has been investigated for several decades for biological application after the carcinostatic activity of glyoxalbis(thiosemicarbazones) was first established for Sarcoma 180 in Swiss mice in the 1950s.[1] Additional studies focused on the antitumor and antileukemic activity of bis(thiosemicarbazones) that were synthesized with a range of different chemical backbone structures.[2,3] Importantly, the role of copper (Cu^{II}) ions in the activity of these compounds was established in these early studies.[4,5] This was confirmed in subsequent reports demonstrating that kethoxalbis(thiosemicarbazone) ($ktsH_2$) complexed to Cu^{II} had potent anticancer properties.[4,6−9]

Subtle changes to the backbone of the ligand can dramatically alter the biological properties of bis(thiosemicarbazonato)-copper(II) complexes [Cu^{II}(btsc)], despite otherwise being stable, lipophilic, and neutral compounds.[10] When Cu^{II} is coordinated to bis(thiosemicarbazone), the ligand undergoes double deprotonation, forming the resulting neutral metal complex. The ligand derived from 2,3-butanedione, diacetylbis-(N4-methylthiosemicarbazonato)copper(II) [Cu^{II}(atsm) = $Cu^{II}\mathbf{L^1}$, Figure 1], is cell-permeable; in "normal" cells, the complex largely remains intact and appears to have some propensity to wash out of the cells, but under low oxygen conditions (hypoxia), the copper is retained within the cell, leading to selective accumulation in hypoxic cells. The unique hypoxia trapping mechanism has led to the application of $Cu^{II}\mathbf{L^1}$ radio-labeled with positron-emitting isotopes of copper as a diagnostic imaging agent of hypoxia.[11] In contrast, when cell-permeable copper(^{II}) glyoxalbis[N(4)-methylthiosemicarbazonato] [Cu^{II}(gtsm) $Cu^{II}\mathbf{L^2}$, Figure 1] enters the cells, the reducing environment encountered in most cells is sufficient to elicit a reduction of Cu^{II} to Cu^{I}, leading to the release of the metal ion from the btsc ligand.[12] Cu^{I} is then thought to be trapped in the cell irrespective of cellular oxygen concentration.[13−18] The differences in the cellular

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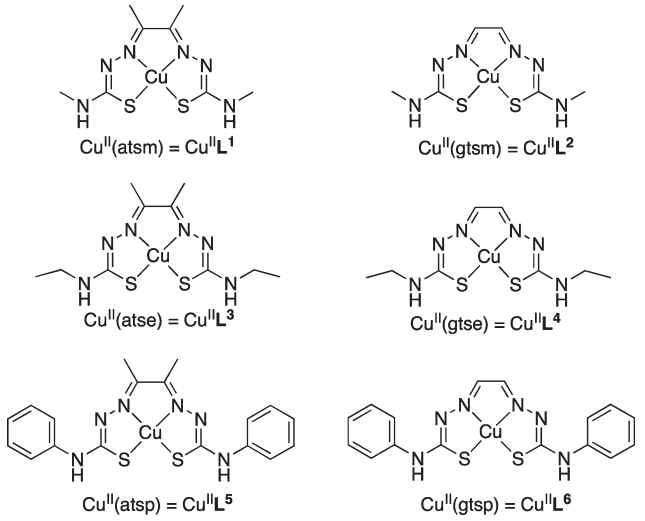

Figure 1. Chemical structures of $Cu^{II}L^{1-6}$.

behavior of $Cu^{II}L^{1}$ and $Cu^{II}L^{2}$ can be related to the $Cu^{II/I}$ reduction potential. The two methyl electron-donating groups on the backbone of $Cu^{II}L^{1}$ lower the $Cu^{II/I}$ couple by 160 mV.[12,14−16,19]

The potential use of Cu^{II}(btsc) complexes as novel therapeutic agents for the treatment of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) is currently under investigation because of the complex metal-related pathogenesis of these diseases. Both $Cu^{II}L^{1}$ and $Cu^{II}L^{2}$ can cross the blood-brain barrier[20] and have demonstrated significant potential as effective therapies for neurodegeneration based on cell and animal studies. The Cu-releasing complex $Cu^{II}L^{2}$ activated a phosphoinositol-3-kinase (PI3K)-dependent signaling pathway, resulting in the downstream regulation of Alzheimer's amyloid-β (Aβ) peptide accumulation in cell lines.[21] Subsequent studies revealed that the treatment of an AD mouse model (APP/PS1) with $Cu^{II}L^{2}$ decreased the levels of trimeric forms of Aβ and phosphorylation of the microtubule-associated protein τ together with significant cognitive improvement.[22] These effects were mediated through $Cu^{II}L^{2}$ modulation of the same PI3K-dependent signaling pathways as those identified in neuronal cell lines.[22] In addition, $Cu^{II}L^{1}$ exhibits a potent therapeutic effect in animal models of PD and ALS (unpublished observations), although the exact mechanisms of action are still under investigation.

Despite evidence of their therapeutic potential for the treatment of cancer and neurodegeneration, the cellular uptake and accumulation patterns of Cu^{II}(btsc) complexes remain largely unknown. Understanding these mechanisms is critical for the development of effective therapeutics possessing minimal toxicity and selective cell and organelle targeting. From the few studies on the cellular uptake and metabolism of metal complexes, it has become apparent that cell uptake can proceed in a myriad of complex ways. For example, europium (Eu^{III}) and terbium (Tb^{III}) macrocyclic metal complexes appear to enter cells by macropinocytosis.[23−26] In contrast, the uptake of ruthenium (Ru^{II}) complexes is linked to iron (Fe) transport mechanisms,[27] while a combination of passive uptake, endocytosis, and copper-transporter 1 (Ctr1)-mediated entry into the cells has been reported for the anticancer drug cisplatin ($[PtCl_2(NH_3)_2]$[28]). Very early studies of Cu^{II}(btsc)s proposed that they diffuse across cell membranes based on their high lipid solubility.[7] A subsequent report found that when radioisotopic Cu^{II} was combined with the 1,5-bis(thiosemicarbazone) ligand, accumulation was at least partially temperature-sensitive in leukemia cells,[29] indicating that an active or protein-mediated uptake mechanism may be responsible for delivery of the Cu into the cell. A later study emphasized that the accumulation of $Cu^{II}L^{1}$ was dependent on cell-specific factors such as the oxygenation status and differential expression of Cu-transport proteins but did not present a well-defined uptake mechanism.[30] Moreover, there is currently no substantial information available on how Cu^{II}(btsc)s are trafficking through and effluxed from cells and how this affects the overall cellular accumulation of Cu. A great deal of research is evidently needed to determine how Cu^{II}(btsc)s are trafficked by different cells and tissues.

In the present study, we investigated the accumulation of $Cu^{II}L^{1}$ and $Cu^{II}L^{2}$ in neuronal and glial cell lines under a range of conditions. Collectively, the data suggested that $Cu^{II}L^{1}$ and $Cu^{II}L^{2}$ may be taken into these cells by combined passive and facilitated mechanisms. We found no evidence to support a role for Ctr1 in accumulation of the compounds. Importantly, our findings also demonstrated that Cu from both $Cu^{II}L^{1}$ and $Cu^{II}L^{2}$ was effluxed rapidly from cells through active mechanisms. Whether this was in the form of released ionic Cu or as an intact metal complex is not known. This finding emphasizes the importance of considering efflux as well as uptake mechanisms when studying the accumulation of metal complexes in the cells. This detailed exploration of the cellular accumulation mechanisms of Cu^{II}(btsc)s reveals valuable insight into their metabolism that may be exploited for drug development and optimization.

EXPERIMENTAL SECTION

Materials. $Cu^{II}L^{1}$,[16,31,32] $Cu^{II}L^{2}$,[16,32,33] $Cu^{II}L^{3}$,[34] $Cu^{II}L^{4}$,[32,33] $Cu^{II}L^{5}$,[21] and $Cu^{II}L^{6}$ [21] were synthesized as previously described. Cytochalasin D, nocodazole, methyl β-cyclodextrin (MβCD), filipin, cycloheximide, and paraquat were purchased from Sigma (Sydney, New South Wales, Australia). Antisera to epidermal growth factor receptor (EGFR) and superoxide dismutase 1 (SOD1) were purchased from Cell Signaling Technology (Danvers, MA) and Abcam (Waterloo, New South Wales, Australia), respectively.

Methods. *Cell Culture.* The cells used in this study were human-derived U87MG glioblastoma, BE (2)-M17 neuroblastoma cell lines, Ctr1 knockout [Ctr1(−/−)], and Ctr1 wild-type [Ctr1(+/+)] fibroblasts. Ctr1(−/−) and Ctr1(+/+) fibroblasts were a kind gift from Dr. Sharon La Fontaine, Deakin University, Victoria, Australia. Cells were passaged and maintained in DMEM plus 10% FCS (Ctr1 cells), DMEM/F12 plus 10% FBS (U87MG cells), or OptiMem plus 10% FCS (M17 cells). Cells were grown in 5% CO_2 at 37 °C and passaged at 1:5−1:10 (U87MG and M17 cells) or 1:10−1:20 (Ctr1 fibroblasts) until 70−90% confluency.

Treatment with Cu^{II}(btsc)s. Cells were grown in 6-well plates or 100 mm dishes until ∼80% confluent. Cu^{II}(btsc) complexes were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and applied to a serum-free medium at the indicated concentrations and times. Metal ion solutions (10 mM) were prepared in dH_2O. Where indicated, cells were pretreated with inhibitors of endocytosis/subcellular trafficking or protein synthesis at given concentrations (cytochalasin D, nocodazole, MβCD, filipin, or cycloheximide) each as 10 mM stock solutions in DMSO. Some cultures were also preincubated at 4 °C prior to the addition of Cu^{II}(btsc)s as described in the Results section. Energy depletion was induced in some cultures by treatment with 100 μM paraquat in Hank's Balanced Salt Solution. As a positive control for the

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effect of temperature-dependent analysis, cells were treated with Fe-loaded transferrin (20 μM) plus additional ferric ammonium citrate (FAC; 20 μM). To remove cell surface proteins, cultures were pretreated with 0.1% (w/v) Pronase for 20 min at 4 °C prior to a 10 min exposure of cells to Cu^{II}(btsc)s. Control cultures were treated with the vehicle (DMSO) alone.

Cell Fractionation. Cell fractionation of U87MG cells was performed to determine the distribution of Cu^{II}(btsc)s in cytosol and the membrane. Cell fractionation was performed according to the Q Proteome cell compartment kit (QIAGEN, Victoria, Australia) instructions. All protein fractions were stored at −20 °C until analysis by inductively coupled plasma mass spectrometry (ICP-MS) and Western blot.

Cell Harvesting for Immunoblotting. Cells were harvested into Phosphosafe Extraction Buffer (Merck Biosciences, San Diego, CA) containing a protease inhibitor cocktail (Roche Diagnostics) and stored at −80 °C until use.

Western Blot Analysis of the Protein Expression. Cell lysates prepared in Phosphosafe Extraction Buffer at equal protein concentrations were mixed with a sodium dodecyl sulfate (SDS) electrophoresis sample buffer and separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE) tris(glycine) gels. Proteins were transferred to poly(vinylidene difluoride) membranes and blocked with a 4% skim milk solution in phosphate buffered saline with Tween 20 (PBST) before immunoblotting for total or phosphospecific proteins. EGFR and SOD1 were detected using polyclonal antisera (1:1000) against each protein. Secondary antiserum was rabbit-HRP at 1:5000 dilution. Blots were developed using GE Healthcare ECL Advance Chemiluminescence and imaged on a Fujifilm LAS3000 imager (Berthold, Bundoora, Australia).

ICP-MS. ICP-MS of metal levels was performed as reported previously.[21] Cell pellets collected for metal analysis by ICP-MS were resuspended in 50 μL of concentrated nitric acid (Aristar, BDH) and left to digest overnight. Samples were then heated for 20 min at 90 °C to complete the digestion. The volume of each sample was reduced to approximately 40−50 μL after digestion, and then 1 mL of 1% (v/v) nitric acid diluent was added to each cell sample. Measurements were made using a Varian UltraMass ICP-MS instrument under operating conditions suitable for routine multielement analysis. The instrument was calibrated using 0, 10, 50, and 100 parts per billion (ppb) of a certified multielement ICP-MS standard solution (ICP-MS CA12-1, Accustandard) prepared in 1% (v/v) nitric acid for Cu, Fe, Zn, and Mn. A certified internal standard solution containing 100 ppb of yttrium (Y 89) via a T piece was used as an internal control (ICP-MS- IS-MIX1-1, Accustandard). The results were expressed as micromole per liter (μmol/L) concentrations of metal. The concentrations of Cu and other metals were calculated as micrograms of metal per milligram of protein based on the protein concentration of parallel cultures.

Atomic Absorption Spectroscopy (AAS). Cell pellets collected for metal analysis by AAS were resuspended in 500 μL of concentrated ultrapure nitric acid (Aristar, BDH), along with 0.5−2.5 mL of dH_2O. Samples were then heated in a heating block for 3 h at 65 °C. Measurements were made using a Varian SpectrAA 800 instrument under operating conditions suitable for routine multielement analysis (furnace). Several blanks and multiple Cu standards were routinely measured with each run. The results were expressed as micromole per liter (μmol/L) concentrations of metal. The concentrations of Cu and other metals were calculated as micrograms of metal per milligram of protein based on the protein concentration of parallel cultures.

Adenosine Triphosphate (ATP) Assay. For a crude determination of the energy difference between samples, 100 μL of an ATP assay mix (Sigma, New South Wales, Australia; diluted 1:100 with an ATP assay dilution buffer) per replicate was added to an Eppendorf tube, swirled gently, and allowed to stand at room temperaturefor 3 min. Following this, 100 μL of the sample was added to the same tube, mixed quickly, and added to the 96-well assay plate. The plate was placed in the WALLAC Victor^2 plate reader set at luminescence, and the light emitted from the plate was measured. For the ATP assay, the luminescence intensity correlated with the ATP activity. The results were expressed as a percentage change in the ATP levels compared to DMSO (100% ATP).

Statistical Analysis. All data described in graphical representations are mean ± standard error of the mean unless stated from a minimum of three experiments. The results were analyzed using a two-tailed Student's *t* test.

RESULTS

$Cu^{II}L^1$ and $Cu^{II}L^2$ Revealing Substantial Membrane Localization. This study began with an examination of the broad cellular distribution of Cu from the Cu^{II}(btsc)s after a short incubation (Figure 2). The localization of Cu from $Cu^{II}\mathbf{L^1}$, $Cu^{II}\mathbf{L^2}$ (25 μM), or DMSO as the vehicle control was examined in U87MG glioblastoma cells and M17 neuroblastoma cells following a 10 min treatment. The cells were separated into membrane and cytosolic fractions, and the metal content of these fractions was determined. The samples were also analyzed by Western blot to determine the relative purity of the separated fractions (Figure 2). Similar levels of Cu from $Cu^{II}\mathbf{L^2}$ were distributed between membrane and cytosolic fractions in both cell lines (Figure 2 A). In contrast, U87MG cells treated with $Cu^{II}\mathbf{L^1}$ for 10 min revealed the metal complex to be at a high concentration in the membrane fraction with a comparatively low concentration of $Cu^{II}\mathbf{L^1}$ present in the cytosolic fraction (Figure 2D). A longer treatment time of 1 h indicated a shift of $Cu^{II}\mathbf{L^1}$ from the membrane fraction into the cytosolic fraction, suggesting that $Cu^{II}\mathbf{L^1}$ may take longer to pass through the cell membrane because of its highly lipophilic nature (log *P* of 1.48 compared with 0.84 for $Cu^{II}\mathbf{L^2}$; Figure 2D).[16] This is consistent with other reports on $Cu^{II}\mathbf{L^1}$ suggesting that its lipophilic structure would cause it to rapidly associate with the cell membrane.[35] This difference in the compound lipophilicity indicates an initial basis for differences in the way cells may metabolize $Cu^{II}\mathbf{L^1}$ and $Cu^{II}\mathbf{L^2}$.

The data shown in Figure 2, particularly the data for $Cu^{II}\mathbf{L^1}$, indicate that exposure to Cu^{II}(btsc)s involves an initial interaction with the cell membrane. To examine the nature of this Cu^{II}(btsc)−membrane interaction in greater detail, Pronase was used to remove cell surface proteins prior to Cu^{II}(btsc) treatment. Pronase is a commercially available mixture of proteinases capable of digesting cell surface proteins.[36] The pretreatment of cells with Pronase is therefore a useful tool for examining the genuine cellular accumulation of metal complexes across the cell membrane compared to complexes adventitiously associated with the surface. The U87MG and M17 cells were treated with 0.1% (w/v) Pronase for 20 min at 4 °C prior to a 10 min exposure of the cells to Cu^{II}(btsc)s (25 μM). Control cells not pretreated with Pronase were also kept at 4 °C for 20 min to control for the reduced temperature. Pronase-treated and control cell lysates of both cell lines were probed for the ubiquitous cell surface protein EGFR to ensure that the Pronase removed cell surface proteins. Western blot data shown in Figure 2F,H demonstrate efficacy of the Pronase treatment. Pronase pretreatment led to a significant decrease in the cellular concentration of Cu in both cell types (Figure 2E,G), suggesting a role for cell surface proteins in the association of Cu^{II}(btsc)s with these cells.

Temperature-Dependent Cu^{II}(btsc) Accumulation in Neuronal and Glial Cell Lines. To examine whether the accumulation

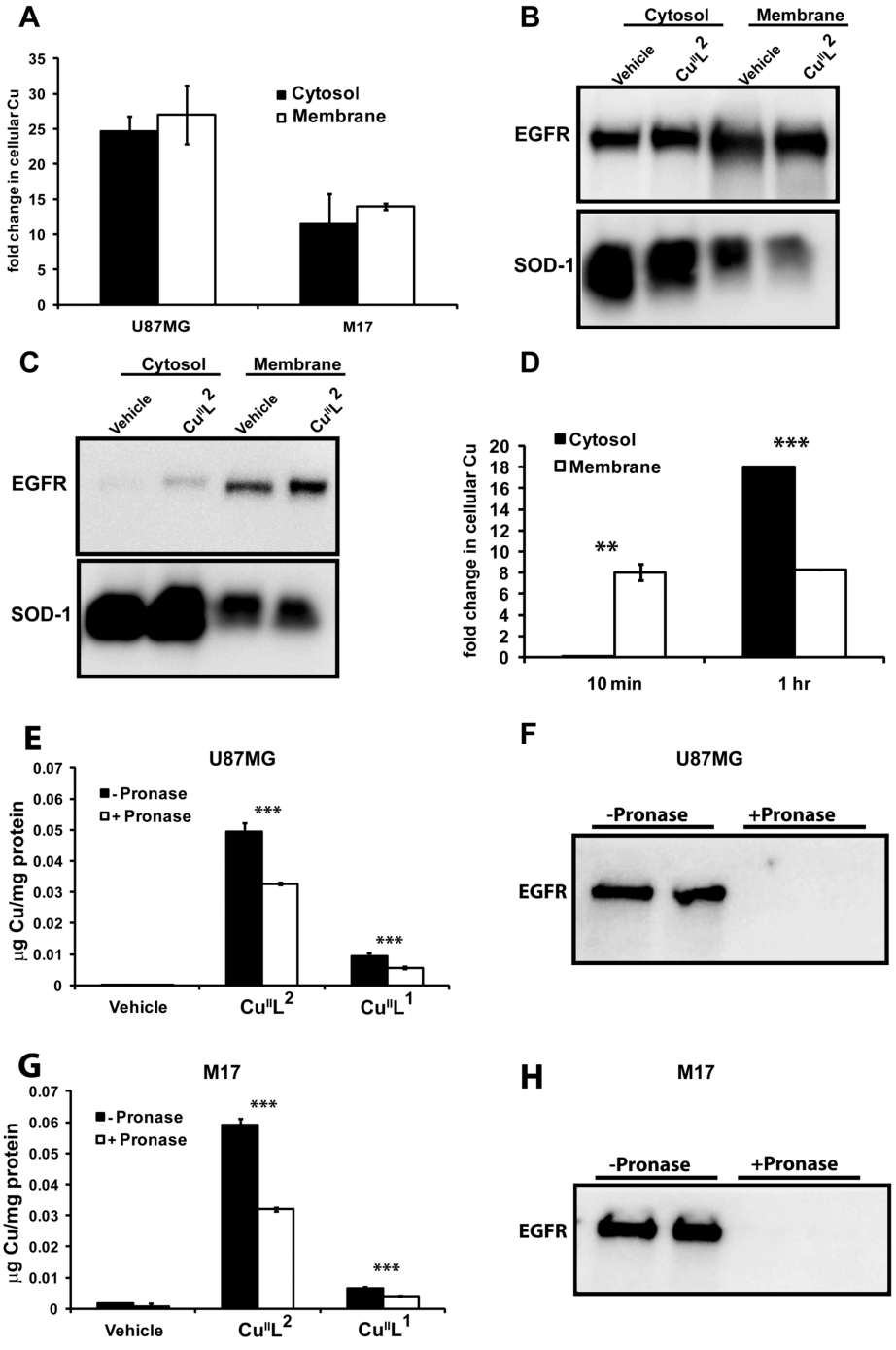

Figure 2. Membrane localization of Cu^{II}(btsc)s. Cells treated with 25 μM $Cu^{II}L^{2}$ (A and B; 10 min) or $Cu^{II}L^{1}$ (C and D; 10 min and 1 h) were fractionated and the Cu concentrations of the fractions measured by ICP-MS. The Cu levels are shown as fold increase over the corresponding fractions from DMSO-treated cells. Enrichment of the membrane and cytosolic fractions in U87MG (B) and M17 cells (C) using the described fractionation procedure was confirmed by Western blot analysis of appropriate proteins (EGFR as a membrane marker and SOD1 as a cytosolic marker). ** ($p < 0.01$) and *** ($p < 0.001$) denote the statistical significance between the cytosol and membrane fractions. Cells were pretreated with Pronase to strip cell surface proteins and then exposed to Cu^{II}(btsc)s (25 μM) for 10 min before measuring the Cu content of the cells by ICP-MS in U87MG (E and F) and M17 cells (G and H). Western blots of U87MG (F) and M17 (H) cells probed for the cell surface protein EGFR after Pronase treatment showed removal of the EGFR protein from the cell surface [***, $p < 0.001$ between Pronase-pretreated and -untreated samples exposed to Cu^{II}(btsc)s].

of Cu^{II}(btsc)s is active or passive, temperature-dependent analysis was performed. Active uptake is inhibited at low temperature, while passive uptake should not be affected. As a positive control for these experiments, the temperature-dependent cellular internalization of the Fe-loaded transferrin protein was examined. Cellular internalization of transferrin is known to occur via an active, energy-dependent, receptor-mediated endocytosis pathway.[37−39] The uptake of Fe-loaded transferrin protein should therefore be suppressed at 4 °C. A significant decrease in the cellular concentration of Fe was observed in the glial U87MG cells at 4 °C

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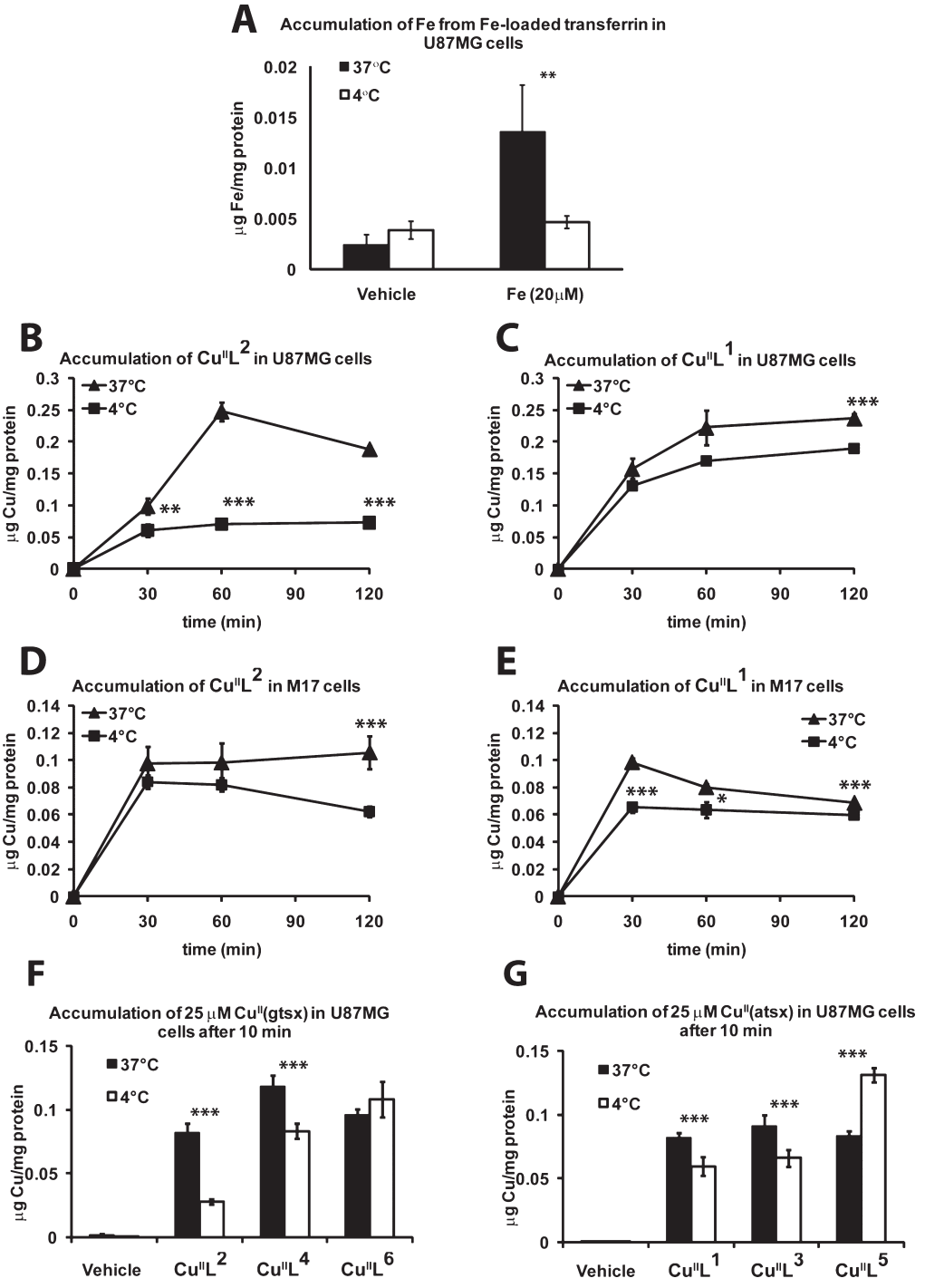

Figure 3. Effects of temperature on the accumulation of Cu from Cu^{II}(btsc)s in U87MG cells (A and B) and M17 cells. As a positive control for temperature effects, cells were cotreated with Fe-loaded transferrin (20 μM) plus additional FAC (20 μM) at 37 or 4 °C for 30 min. The Fe content of the cells was then measured (A). A significant decrease in the Fe accumulation was observed at 4 °C. Cells were treated with $Cu^{II}L^2$ in U87MG cells (B) and M17 cells (D) or $Cu^{II}L^1$ in U87MG cells (C) and M17 cells (E) (25 μM) at 37 or 4 °C for the indicated time periods, and the Cu content of the cells was measured. Temperature-dependent effects were observed for both $Cu^{II}L^1$ and $Cu^{II}L^2$ in each cell type. Cells were treated with $Cu^{II}L^2$, $Cu^{II}L^4$, and $Cu^{II}L^6$ (25 μM) (F) or $Cu^{II}L^1$, $Cu^{II}L^3$, and $Cu^{II}L^5$ (25 μM) (G) at 37 or 4 °C for 10 min, and then the cellular Cu was measured. A decrease in the accumulation of Cu at 4 °C was observed for $Cu^{II}L^2$ and $Cu^{II}L^4$ but not $Cu^{II}L^6$. The same pattern of temperature effects was observed for $Cu^{II}L^1$, $Cu^{II}L^3$, and $Cu^{II}L^5$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; 4 °C compared to 37 °C).

compared to 37 °C (Figure 3A) following treatment with Fe-loaded transferrin.

U87MG and M17 cells were exposed to $Cu^{II}L^1$ and $Cu^{II}L^2$ (25 μM) at 37 or 4 °C for 30, 60, or 120 min. The accumulation of Cu from $Cu^{II}L^1$ and $Cu^{II}L^2$ was significantly inhibited at 4 °C compared to 37 °C after 30 min in the U87MG cell line (Figure 3B,C). A similar but less robust temperature-dependent effect on the Cu^{II}(btsc) accumulation was generally observed in

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the M17 cell line (Figure 3D,E). This effect persisted through 120 min in both cell types. This temperature-dependent activity was consistent with a role for active or facilitated uptake of the metal complexes in these cell lines. An important caveat with these experiments is that changes in the membrane fluidity through prolonged incubation at 4 °C rather than genuine inhibition of the uptake mechanism cannot be ruled out. To minimize potential changes to the membrane fluidity, the accumulation of Cu from Cu^{II}(btsc)s at a shorter incubation time of 10 min in the U87MG cell line was investigated. Structurally related Cu^{II}(btsc)s with the same ligand backbone (Figure 2) were compared with $Cu^{II}\mathbf{L^1}$ and $Cu^{II}\mathbf{L^2}$ to determine if the temperature-dependent accumulation varied with different compounds (Figure 3F,G). Altered accumulation due to temperature effects on the membrane fluidity would affect all compounds tested. The data showed that, after only 10 min of incubation at 4 °C (compared to 37 °C), there was a significant decrease in the accumulation of Cu from $Cu^{II}\mathbf{L^1}$ and its closely related analogue Cu^{II}(gtse) ($Cu^{II}\mathbf{L^4}$, Figure 1), which has ethyl substituents in the N4 position of the ligand, but not Cu^{II}(gtsp) ($Cu^{II}\mathbf{L^6}$, Figure 1), which has phenyl substituents in the N4 position and is significantly more lipophilic than Cu^{II}(gtsm/e) (Figure 3F). Likewise, $Cu^{II}\mathbf{L^1}$ and Cu^{II}(atse) ($Cu^{II}\mathbf{L^3}$, Figure 1) showed decreased accumulation at 4 °C after 10 min but increased accumulation of Cu^{II}(atsp) ($Cu^{II}\mathbf{L^5}$, Figure 1) (Figure 3G). The discrepancy between the effect of decreased temperature on the accumulation of $Cu^{II}\mathbf{L^6}$ and $Cu^{II}\mathbf{L^5}$ when compared to all other Cu^{II}(btsc)s tested, together with the short 10 min treatment period, strongly supported an active or facilitated-uptake process in the glial cells rather than an altered membrane fluidity. Increased accumulation of $Cu^{II}\mathbf{L^5}$ at 4 °C was unexpected, and the reason is not known, but the rapid active efflux of this particular complex (inhibited at 4 °C) is a possibility.

Cu^{II}(btsc) Accumulation Is Not Saturated at Low Concentrations in U87MG Cells. The data from Pronase and temperature-dependent analysis suggested a possible role for an energy-requiring transport protein in the cellular uptake of Cu from $Cu^{II}\mathbf{L^1}$ and $Cu^{II}\mathbf{L^2}$. To examine this further, dose-dependent uptake was assessed. Passive diffusion requires no binding step and no specific membrane protein, and therefore saturation of the membrane should not occur until relatively high concentrations are reached.[40] By contrast, transporter-mediated uptake should become saturated at relatively low concentrations because of the limited availability of receptor molecules on the cell surface.

The cellular Cu concentration in U87MG cells treated for 30 min with increasing concentrations of $Cu^{II}\mathbf{L^2}$ (0.1−50 μM) is shown in Figure 3A. The accumulation of $Cu^{II}\mathbf{L^2}$ at lower concentrations displayed a linear increase, and the accumulation did not reach a plateau, even at the relatively high concentration of 50 μM. This was inconsistent with a role for an active or facilitated transport protein. Further examination of the cellular metal accumulation at concentrations beyond 50 μM (Figure 4B) showed that the accumulation did not saturate until the concentration of $Cu^{II}\mathbf{L^2}$ in the media reached 125 μM. This concentration of $Cu^{II}\mathbf{L^2}$ was unlikely to represent the saturation point for an active or facilitated transport carrier protein. Instead, this probably represents saturation of the membrane and/or equilibration of the complex content between extracellular and intracellular environments. A dose−response of $Cu^{II}\mathbf{L^1}$ was also performed to determine whether accumulation of this metal complex was saturable. At the concentrations tested (1−75 μM for 30 min), we did not observe any saturation for the accumulation of $Cu^{II}\mathbf{L^1}$ (Figure 4C), suggesting that $Cu^{II}\mathbf{L^1}$ accumulation is also likely to be predominantly by passive diffusion. The lack of saturation could also be at least partly accounted for by changes to the cellular Cu-trafficking pathways induced by the increasing Cu load, including altered efflux. Given the substantial interaction of $Cu^{II}\mathbf{L^1}$ with the membrane fraction, as shown in Figure 1, much of this Cu may have also been membrane-associated rather than cytosolic. Because these dose−response data are inconsistent with the Pronase pretreatment data (Figure 2E,G) and temperature-dependent accumulation data (Figure 3), it raises the possibility that Cu^{II}(btsc)s are taken up by multiple processes. This may involve a facilitated or active transport process in addition to passive diffusion, with the latter becoming predominant at higher concentrations.

Energy Depletion Leads to the Accumulation of Cu^{II}(btsc)s in U87MG Cells. The role for energy in cell accumulation of metal complexes was investigated to provide further insight because of the conflicting data presented in the temperature-dependent and dose−response studies above. In contrast to passive and facilitated diffusion, active carrier-mediated transport is dependent on the availability of ATP. Uptake mechanisms dependent on ATP are inhibited under conditions of cellular energy depletion. The effect of energy depletion on the cellular accumulation of $Cu^{II}\mathbf{L^1}$ and $Cu^{II}\mathbf{L^2}$ in U87MG cells was examined at two different time points. Energy depletion was achieved by growing cells in serum-free Hanks Balanced Salts Solution (HBSS) combined with 100 μM paraquat for 48 h. The HBSS treatment starves the cells of potential carbohydrate sources of energy production (e.g., glucose for glycolytic ATP production), and paraquat inhibits mitochondrial oxidative phosphorylation, which could be supported by the mobilization of internal sources of fuel for ATP production (e.g., glycogen). We have shown that concentrations of paraquat up to 1000 μM have do not affect cell survival but impair mitochondrial respiration (data not shown). The levels of ATP in energy-depleted cells are shown in Figure 4D, confirming that HBSS and paraquat effectively deplete cells of ATP by approximately 70% when compared to cells grown in normal culture media. When these energy-depleted cultures were treated with $Cu^{II}\mathbf{L^1}$ for 10 min or 4 h, subsequent measurements of cellular Cu revealed a significant increase in Cu inside the cell compared to control cells (Figure 4E,F). Interestingly, this was indicative of the role for energy production in rapid efflux of the metal complex or Cu from $Cu^{II}\mathbf{L^1}$-treated cells rather than an effect on uptake. There was no substantial change in the cellular Cu concentration for $Cu^{II}\mathbf{L^2}$ at 10 min in energy-depleted cells compared to control cells (Figure 4E); however, at the 4 h time point, an accumulation of Cu for $Cu^{II}\mathbf{L^2}$ in energy-depleted cells similar to that for $Cu^{II}\mathbf{L^1}$ (Figure 4F) was observed. The data for $Cu^{II}\mathbf{L^2}$ suggest that energy is required for efflux of the Cu either from the ligand or as a complex, particularly at the later time point but probably had a small role in uptake. The observation that energy depletion did not inhibit $Cu^{II}\mathbf{L^1}$ or $Cu^{II}\mathbf{L^2}$ accumulation compared to vehicle control was consistent with energy-independent facilitated and/or passive uptake.

Inhibition of Endocytosis and Trafficking Can Enhance the Accumulation of Cu^{II}(btsc)s. Active uptake of molecules can also occur via carrier-mediated transport or endocytosis.[41] To assess whether these processes could contribute to the accumulation of Cu^{II}(btsc)s, U87MG and M17 cells were pretreated with inhibitors that modulate the endocytic uptake and/or trafficking of endosomes. Cytochalasin D reportedly inhibits endocytic processes by disrupting actin.[42] Pretreating U87MG

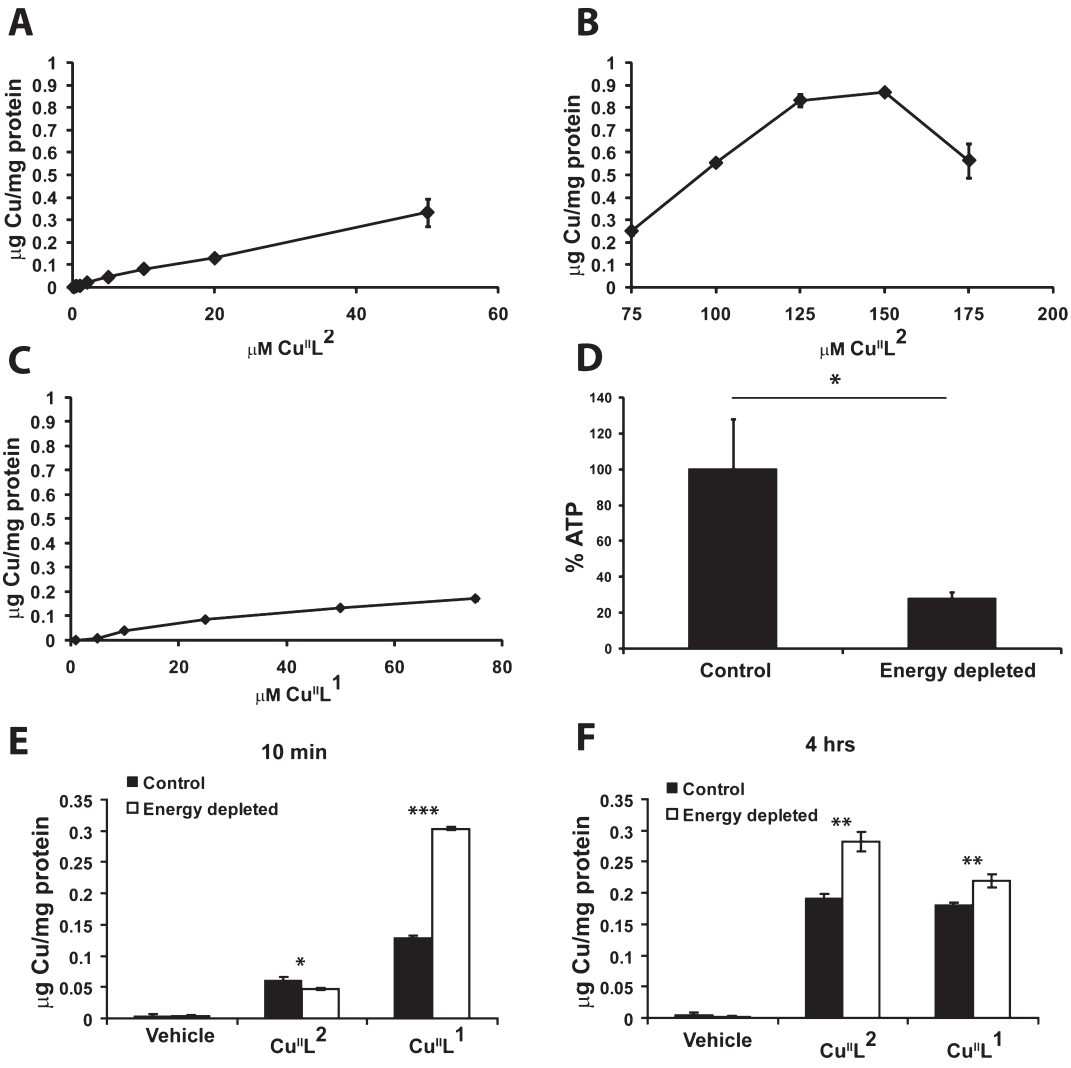

Figure 4. Dose−response and energy-dependent accumulation of Cu^{II}(btsc)s. Cells were exposed to increasing concentrations of $Cu^{II}L^2$ (A and B) or $Cu^{II}L^1$ (C) for 30 min, and the cellular Cu content was then measured. To examine the energy dependence, cells were treated with 100 μM paraquat in HBSS for 48 h and energy levels determined by ATP assay (D). The Cu content of the cells was then measured under normal or energy-depleted conditions after treatment with Cu^{II}(btsc) (25 μM) for 10 min (E) or 4 h (F). Significant changes were observed in the Cu accumulation during energy depletion (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; compared to control, non-energy-depleted cells).

cells with cytochalasin D (10 μM) prior to the treatment with $Cu^{II}L^1$ or $Cu^{II}L^2$ had no effect on the cellular Cu levels (Figure 5A and Table 1). In M17 cells, cytochalasin D had no effect on cellular Cu after the $Cu^{II}L^2$ treatment, although a small but significant increase in the cellular accumulation of Cu from $Cu^{II}L^1$ was observed (Figure 5B and Table 1).

Nocodazole is an alternative inhibitor of endocytic pathways that acts via microtubule depolymerization.[38] Nocodazole can affect the endocytic uptake as well as the cellular trafficking of endosomes and efflux of compounds. A result similar to that of the experiments with cytochalasin D was observed for the Cu levels in U87MG and M17 cells pretreated with nocodazole prior to treatment with $Cu^{II}L^2$ and $Cu^{II}L^1$ (Figure 5C,D, respectively, and Table 1). The pretreatment of cells with nocodazole (20 μM) for 2 h before a 10 min exposure to Cu^{II}(btsc)s (25 μM) results in significant increases in the cellular accumulation of Cu following the addition of $Cu^{II}L^2$ and $Cu^{II}L^1$ to U87MG cells (Figure 5C and Table 1). In the M17 cell line, the nocodazole pretreatment did not impair the accumulation of Cu from $Cu^{II}L^2$ but significantly increased the accumulation of $Cu^{II}L^1$ (Figure 5D and Table 1). These data support the results from the energy depletion studies, indicating that Cu^{II}(btsc)s are not being taken up by active endocytosis but that efflux of the metal ion or metal complex is dependent upon active processes.

Two additional chemical inhibitors of endocytosis were examined for their effects on the cellular metal complex accumulation. MβCD is reported to sequester cholesterol in cell membranes and can potentially affect endocytosis. U87MG and M17 cells pretreated with MβCD (5 mM) for 1 h demonstrated small but significant increases in the cellular accumulation of Cu following treatment with $Cu^{II}L^1$ (Figure 5E,F, respectively, and Table 1). In contrast, the cellular Cu levels from $Cu^{II}L^2$ in U87MG and M17 cells were either decreased or unchanged, respectively. A similar pattern was observed for $Cu^{II}L^2$ in U87MG and M17 cells after pretreatment with filipin (5 μg/L) for 5 min, another potential endocytosis inhibitor that disassembles membrane caveolae.[43] The accumulation of Cu from $Cu^{II}L^2$ was either unaffected or decreased with filipin

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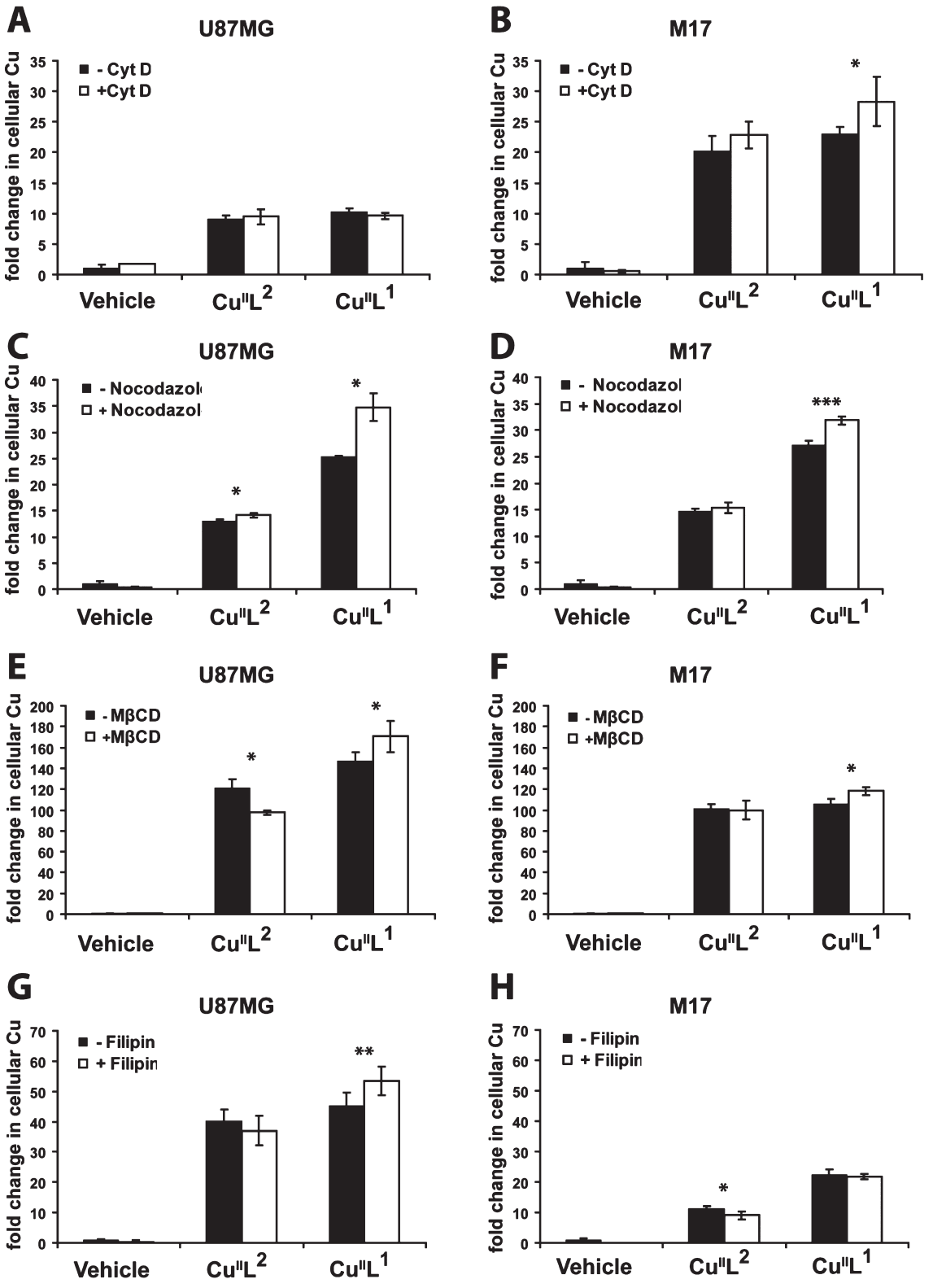

Figure 5. Effects of endocytosis inhibitors on the Cu content of U87MG and M17 cells treated with Cu^{II}(btsc)s. U87MG and M17 cells were pretreated with cytochalasin D (10 μM) for 30 min (A and B), nocodazole (20 μM) for 2 h (C and D), MβCD (5 mM) for 2 h (E and F), or filipin (5 μg/L) for 30 min (G and H) before the addition of Cu^{II}(btsc)s (25 μM) for a further 10 min. The cellular Cu content is expressed as fold increase in the cellular Cu relative to vehicle-treated cells plus or minus inhibitor pretreatment (determined from Cu/mg of protein). Significant changes in the Cu content were observed with some inhibitors (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; compared to cells without inhibitor treatment).

in U87MG and M17 cells (Figure 5G,H, respectively, and Table 1). The Cu accumulation pattern for $Cu^{II}L^{1}$ following filipin pretreatment also reflected that of the MβCD data in both cell lines. Overall, these data were generally consistent

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Table 1. Summary of the Effect of the Pharmacological Inhibitors on the Accumulation of Cu^{II}(btsc)s in U87MG and M17 Cells

		effect on the cellular Cu accumulation			
		U87MG glial cells		M17 neuroblastoma cells	
inhibitor	target	$Cu^{II}L^1$	$Cu^{II}L^2$	$Cu^{II}L^1$	$Cu^{II}L^2$
cytochalasin D	disrupts actin filaments	no effect	no effect	increase	no effect
nocodazole	promotes microtubule depolymerization	increase	no effect	increase	no effect
MβCD	sequesters membrane cholesterol	increase	no effect	increase	no effect
filipin	binds cholesterol	increase	no effect	no effect	no effect
cycloheximide	protein synthesis	increase	increase	increase	increase

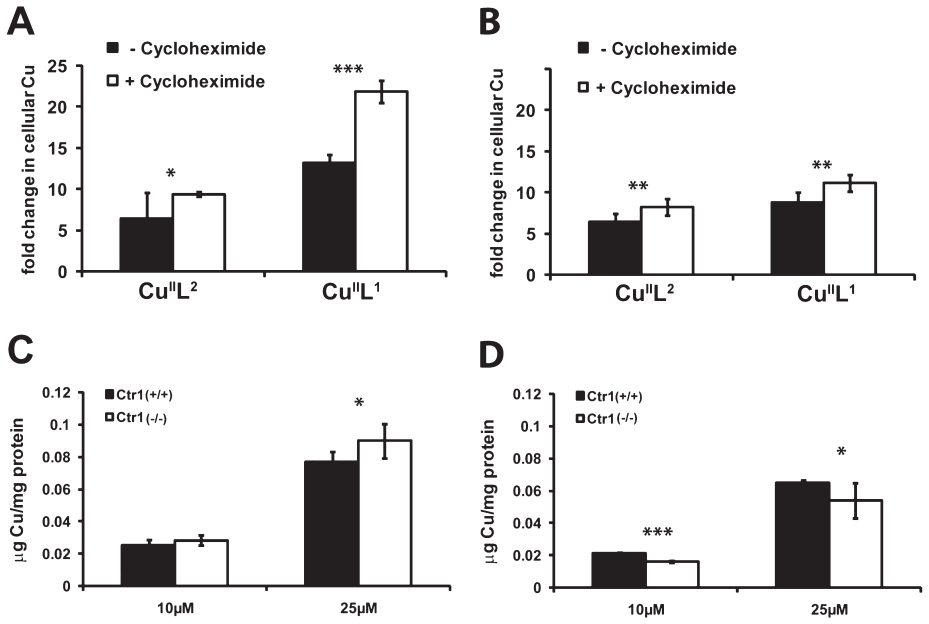

Figure 6. Role of protein synthesis and the Ctr1 expression on Cu^{II}(btsc) accumulation. Cells were pretreated for 24 h with cycloheximide (100 μM) before the addition of $Cu^{II}L^2$ (A) or $Cu^{II}L^1$ (B) (25 μM) for 10 min, and the cellular Cu content was measured. The Cu levels are shown as fold increase over corresponding DMSO-treated cells. Significant changes to the Cu accumulation were observed (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; compared to samples not pretreated with cycloheximide). Ctr1(−/−) and Ctr1(+/+) mouse fibroblasts were treated with two concentrations of $Cu^{II}L^2$ (A) and $Cu^{II}L^1$ (B) for 10 min, and the Cu concentration in the cells was measured. Some small but significant changes were observed [*, $p < 0.05$; ***, $p < 0.001$; compared to Ctr1(+/+) cells].

with the results obtained with cytochalasin D, nocodazole, and energy depletion.

The results revealed complex cell-specific handling of Cu^{II}(btsc)s, which was further affected by minor structural changes to the chemical structure of the btsc ligand. Generally, the accumulation of $Cu^{II}L^1$ was significantly increased by pretreatment with the various endocytosis/trafficking inhibitors, except for cytochalasin D in U87MG cells and filipin in M17 cells, which had no effect. In contrast, except for MβCD, none of the inhibitors had any effect on the $Cu^{II}L^2$ uptake or accumulation. The MβCD effect could be related to membrane cholesterol changes affecting passive or facilitated diffusion across the cell membrane due to the altered membrane fluidity. The overall minimal effect on the uptake by these inhibitors is consistent with the earlier data that broadly supports passive uptake processes. The accumulation of $Cu^{II}L^1$ after treatment with inhibitors also supports an active efflux mechanism of the complex probably involving a specific secretory pathway.

Inhibition of the Protein Synthesis Affects the Accumulation of Cu^{II}(btsc)s. The data from our experiments indicated a potential role for passive and possibly facilitated diffusion for uptake of the Cu from Cu^{II}(btsc)s and an active efflux mechanism. Both facilitated transporter uptake and active efflux would potentially be dependent on the protein synthesis. To test this, cycloheximide was used to block protein synthesis in the U87MG and M17 cells (Figure 6A,B). Cells were pretreated with cycloheximide (100 μM) for 6 h before they were exposed to Cu^{II}(btsc)s (25 μM) for a further 10 min. Cycloheximide pretreatment caused a significant increase in the accumulation of Cu delivered by $Cu^{II}L^1$ and $Cu^{II}L^2$ in both cell lines, indicating that the synthesis of proteins required for the efflux of Cu^{II}(btsc)s (either as ionic Cu or as a complex) was inhibited by cycloheximide (Figure 6). The increased accumulation of cellular Cu due to the cycloheximide treatment was therefore consistent with the energy depletion data in Figure 3 and the trafficking inhibitor data in Figure 5, which supported active efflux of the metal complexes, particularly $Cu^{II}L^1$. Although no effect was seen on

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the uptake, this is not inconsistent with facilitated uptake as de novo protein synthesis may not be necessary. The results overall indicated that modulating the cell metabolism using different techniques, e.g., temperature or pharmacological inhibitors, could simultaneously affect both uptake and efflux processes. Because Cu^{II}(btsc)s may be rapidly trafficked from the cell, it is technically challenging to delineate the processes experimentally.

Cu^{II}(btsc) Accumulation Is Not Dependent on Ctr1. Ctr1, a facilitated-uptake protein, has been demonstrated to have a role in the cellular accumulation of the chemotherapeutic platinum-based compounds cisplatin, carboplatin, and oxaloplatin.[43] Because the data suggested that the uptake of Cu^{II}(btsc)s could be partially mediated by a temperature-dependent carrier, the Ctr1 Cu uptake protein was investigated for a potential role in this uptake. Knockout of the Ctr1 gene in yeast and mouse embryonic fibroblasts has been shown to significantly reduce the accumulation of cisplatin.[44,45]

Genetically modified Ctr1-deficient murine fibroblasts [Ctr1(−/−)] were used to examine the role of Ctr1 in the accumulation of Cu from Cu^{II}(btsc)s. The results in Figure 5C show that knockout of the Ctr1 gene in the fibroblasts led to a small increase in Cu in $Cu^{II}\mathbf{L}^{2}$-treated cells. In contrast, there was a small decrease in the accumulation of Cu following $Cu^{II}\mathbf{L}^{1}$ treatment in the Ctr1-deficient cells compared to wild-type [Ctr1(+/+)] cells (Figure 6B). When compared to the effect of low temperature on the uptake of Cu^{II}(btsc)s in U87MG and M17 cells (Figure 3), the effect of removing functional Ctr1 on the Cu content in $Cu^{II}\mathbf{L}^{1}$-treated fibroblasts was relatively mild. A caveat worth noting here is that the accumulation profiles of Cu^{II}(btsc)s in the U87MG, M17, and Ctr1-deficient fibroblasts may be different. Attempts to inhibit Cu^{II}(btsc) uptake in U87MG and M17 cells by cotreatment with cisplatin showed no significant changes in the Cu^{II}(btsc) accumulation (data not shown), supporting the finding that Ctr1 is not involved in Cu^{II}(btsc) uptake.

DISCUSSION

The initial interest in the biological activity of Cu^{II}(btsc) metal complexes focused on potential clinical applications as anticancer agents; the ligands have been used as delivery vehicles for radioactive copper isotopes in the design of diagnostic or therapeutic radiopharmaceuticals.[8] More recent studies have identified Cu^{II}(btsc) complexes as having neuroprotective effects in cell and animal models of neurodegeneration.[21,22,46] Despite continued interest in the pharmacological activity of Cu^{II}(btsc) complexes for 50 years, little is known on the mechanisms of cellular uptake, intracellular trafficking, and efflux mechanisms. A detailed understanding of how these metal complexes are trafficked by cells will guide translation into clinically applied therapeutics with specific cell and organelle targeting as well as acceptable toxicity profiles. This study sought to clarify some of the basic mechanisms involved in the cellular accumulation of Cu^{II}(btsc) complexes by measuring the cellular metal content in neuronal and glial cell lines following treatment with Cu^{II}(btsc) complexes under a variety of conditions. Our key findings were as follows: (i) Temperature-dependent analysis suggested a role for active (or facilitated) transport of Cu^{II}(btsc)s into U87MG and M17 cells (Figure 3); (ii) dose−response studies were consistent with passive accumulation of Cu^{II}(btsc)s in U87MG cells (Figure 4A−C); (iii) energy-depletion analysis indicated that Cu^{II}(btsc) uptake was passive but that efflux may be active in U87MG cells (Figure 4E,F); (iv) the treatment of U87MG and M17 cells with pharmacological inhibitors of subcellular trafficking pathways generally led to the accumulation of $Cu^{II}\mathbf{L}^{1}$, further supporting active afflux (Figure 5); (v) inhibition of protein synthesis indicated that efflux of Cu^{II}(btsc)s was protein-mediated (Figure 6A,B).

Collectively, the data indicated a central role for passive diffusion but also a potential role for the facilitated uptake of Cu^{II}(btsc)s across the cell membrane in both cell lines. The latter was supported by the fact that uptake was temperature-dependent but did not require energy. In addition, the data supported rapid energy-dependent $Cu^{II}\mathbf{L}^{1}$ efflux (either efflux of Cu released from $Cu^{II}\mathbf{L}^{1}$ or efflux of the intact $Cu^{II}\mathbf{L}^{1}$ complex) in glial cells. The efflux of Cu associated with $Cu^{II}\mathbf{L}^{2}$ did not appear to exhibit the same characteristics as $Cu^{II}\mathbf{L}^{1}$ at early time points, which may be due to the differential release of Cu from the gtsm ligand compared to the atsm ligand under normal cellular redox conditions.[13,35] It is likely that Cu is largely released from $Cu^{II}\mathbf{L}^{2}$ under reducing conditions and bound by endogenous cellular metallochaperones such as Atox1, metallothionein, and glutathione, leaving the gtsm ligand to be degraded or effluxed from the cell. Cu released from $Cu^{II}\mathbf{L}^{2}$ may be effluxed at a later time point after passing through normal cellular Cu trafficking pathways, e.g., ER, Golgi, and excreted via ATP7a. Conversely, Cu from the $Cu^{II}\mathbf{L}^{1}$ complex is less likely to dissociate in the cell under normal reducing conditions, and the intact complex may therefore be rapidly effluxed from the cell possibly via a secretory vesicle-dependent pathway. This may explain the relatively rapid accumulation of Cu^{II}(btsc)s (especially $Cu^{II}\mathbf{L}^{1}$) with inhibition of the energy levels (Figure 4E,F), trafficking inhibitors (Figure 4), and protein synthesis inhibition (Figure 6A,B). However, further work will be required to clarify this and will require an investigation of how metallochaperones and secretory vesicle pathways modulate Cu^{II}(btsc) trafficking.

Importantly, our results suggest that there is perhaps no singular mechanism of uptake or efflux for Cu^{II}(btsc)s, but rather multiple pathways may be involved, and these are likely to be dependent on the cell type, concentration, and chemical structure of Cu^{II}(btsc). This complexity was highlighted by the observation that $Cu^{II}\mathbf{L}^{2}$ exhibited a lack of saturable uptake consistent with simple passive diffusion but was also temperature-dependent, even at 10 min, which could support a role for facilitated uptake. This was supported by the observation that $Cu^{II}\mathbf{L}^{5}$ and $Cu^{II}\mathbf{L}^{6}$ had different temperature-dependent uptake profiles compared to the other Cu^{II}(btsc)s tested, which would not be expected if the temperature effect was simply due to altered membrane fluidity. In addition, the removal of cell surface proteins using the enzyme Pronase led to reduced accumulation of Cu in cells, suggesting that some uptake may be protein-mediated. Interestingly, the differences observed in the Cu^{II}(btsc) accumulation studies in the Ctr1(−/−) embryonic fibroblasts did not support a major role for this Cu import protein. This is in contrast to reports from other researchers, which have demonstrated that knockout of the Ctr1 gene in yeast and mouse embryonic fibroblasts can significantly decrease accumulation of the platinum-based complex, cisplatin, in these models.[44,45] This would indicate that Ctr1 is not simply a carrier for metal complexes per se.

It is possible that the metal complexes are taken up through a mixture of passive and facilitated diffusion processes in a biphasic manner. The biphasic uptake of Cu in Menkes lymphoid cell lines has been reported, with evidence for both passive and facilitated uptake.[47] The uptake of metal complexes investigated

in this study could proceed in a similar way. An alternative proposal is that Cu^{II}(btsc) uptake is only passive but is exquisitely sensitive to temperature-mediated changes to the cell membrane. There is currently no literature to support this; however, while our studies supported a temperature-dependent, protein-mediated uptake mechanism, the saturation (dose−response) data were not consistent with protein-mediated uptake. Although, it should be noted that continued uptake by simple passive diffusion at higher concentrations of complexes could mask parallel uptake mediated by a protein.

The complexities of the pathways exploited by some metal complexes for cell entry were highlighted in a recent review.[41] These included the uptake of Eu^{III} and Tb^{III} metal complexes by macropinocytosis and the Fe-transport pathway-mediated uptake of ruthenium (Ru^{II}) complexes.[27] In addition, multiple and complex accumulation mechanisms proposed for cisplatin, which has been used clinically for decades, continue to be disputed.[28] Arguments for passive diffusion and facilitated or carrier-mediated uptake by numerous solute carriers have all been proposed as the mechanism for cisplatin uptake.[28] These studies provide a useful parallel to the work presented here and highlight the difficulties associated with delineating the uptake and efflux mechanisms that may influence metal-complex accumulation. In this study, the complexity was further enhanced by the observation that $Cu^{II}\mathbf{L^1}$ and $Cu^{II}\mathbf{L^2}$ revealed different uptake and accumulation profiles under various conditions and that efflux can potentially occur rapidly, complicating measurements designed to probe the uptake. Relatively minor structural changes to Cu^{II}(btsc)s also affected the accumulation. This was highlighted by the fact that $Cu^{II}\mathbf{L^5}$ and $Cu^{II}\mathbf{L^6}$ revealed different accumulations during temperature-dependent studies compared to the $Cu^{II}\mathbf{L^2}$/$Cu^{II}\mathbf{L^4}$) and $Cu^{II}\mathbf{L^1}$/$Cu^{II}\mathbf{L^3}$) complexes. Interestingly, differences between the phenyl and methyl/ethyl derivatives were identified in our previous studies on Cu^{II}(bstc)s. Both $Cu^{II}\mathbf{L^2}$ and $Cu^{II}\mathbf{L^4}$ are potent activators of the EGFR membrane protein in the U87MG cell line.[46] This effect was related to the potent inhibition of the protein tyrosine phosphatase activity by $Cu^{II}\mathbf{L^2}$ and $Cu^{II}\mathbf{L^4}$, but $Cu^{II}\mathbf{L^6}$ has a substantially weaker effect.[46] These studies were consistent a greater decrease in extracellular $A\beta_{1-40}$ with $Cu^{II}\mathbf{L^2}$ and $Cu^{II}\mathbf{L^4}$ compared to $Cu^{II}\mathbf{L^6}$.[21]

The complexity of the accumulation profiles of Cu^{II}(btsc)s is also likely to be influenced by the cell type examined including the presence of factors that could modulate the uptake and efflux (e.g., different rates of protein turnover or secretory pathways, different organelle activities, etc.) as well as structural differences across molecules. For instance, neuronal cells lack endocytic caveolae found at the cell membrane,[48,49] which may influence how these cells import metal complexes, as well as other molecules. Additionally, it has been reported that cultured astrocytes take up Cu more effectively than cultured neurons,[50] which may at least, in part, reflect the comparatively high metallothionein and glutathione contents of these (astrocyte?) cells.[50,51] The accumulation of Cu following treatment with Cu^{II}(btsc) varies between glial-derived U87MG cells and the neuroblastoma M17, and this is supported by studies investigating the uptake of radio-labeled $Cu^{II}\mathbf{L^1}$. The metabolism of $Cu^{II}\mathbf{L^1}$ is cell-line-dependent, as well as dependent upon the cellular oxygenation conditions.[30] Cell-specific differences in the accumulation profiles of Cu^{II}(btsc)s may also help to explain discrepancies within the small body of literature that exist on the metabolism of this family of metal complexes.

An additional finding of interest was the observation that $Cu^{II}\mathbf{L^1}$ was present within the cell membrane at a higher concentration when compared to the concentration in the cytosol at an early time point (10 min) in U87MG cells (Figure 2D). Examination at a later time point revealed that the $Cu^{II}\mathbf{L^1}$ concentration in the cytosol had substantially increased (Figure 2D). These data are consistent with previous reports that the highly lipophilic nature of $Cu^{II}\mathbf{L^1}$ means that the molecule is likely to show strong affinity for the hydrophobic cell membrane, which may delay its movement into the cytosol.[52,53] This was also consistent with preliminary data on radio-labeled $Cu^{II}\mathbf{L^1}$ uptake into CHO cells (unpublished observations), which suggested that $Cu^{II}\mathbf{L^1}$ remains predominantly cell-surface-bound, although results using different cell types require careful interpretation. This is interesting because it suggests that the pharmacological actions demonstrated by $Cu^{II}\mathbf{L^1}$ in various neurodegenerative disease models may be mediated at the cell surface level, at least in some cell types. Further work is needed to determine the full implications of this for the actions of $Cu^{II}\mathbf{L^1}$ in disease models of neurodegeneration.

In summary, this study is the first comprehensive examination of the cellular accumulation of Cu^{II}(btsc) complexes and revealed intriguing results supporting passive and potentially facilitated components to the uptake process together with active efflux. These findings provide a valuable contribution to the understanding of Cu^{II}(btsc) trafficking, particularly the influence of concurrent uptake and efflux processes on the total cell Cu accumulation. Future studies are needed to gain a greater insight into the complex nature of Cu^{II}(btsc) trafficking in neuronal and glial cells as well as tumors. These studies are important for the continued development of metal-complex-based therapies for neurodegeneration and cancer.

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