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Selective Intracellular Release of Copper and Zinc Ions from Bis(thiosemicarbazonato) Complexes Reduces Levels of Alzheimer Disease Amyloid-β Peptide*[S]

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Copper and zinc play important roles in Alzheimer disease pathology with recent reports describing potential therapeutics based on modulation of metal bioavailability. We examined the ability of a range of metal bis(thiosemicarbazonato) complexes (M^{II}(btsc), where M = Cu^{II} or Zn^{II}) to increase intracellular metal levels in Chinese hamster ovary cells overexpressing amyloid precursor protein (APP-CHO) and the subsequent effect on extracellular levels of amyloid-β peptide (Aβ). The Cu^{II}(btsc) complexes were engineered to be either stable to both a change in oxidation state and dissociation of metal or susceptible to intracellular reduction and dissociation of metal. Treatment of APP-CHO cells with stable complexes resulted in elevated levels of intracellular copper with no effect on the detected levels of Aβ. Treatment with complexes susceptible to intracellular reduction increased intracellular copper levels but also resulted in a dose-dependent reduction in the levels of monomeric Aβ. Treatment with less stable Zn^{II}(btsc) complexes increased intracellular zinc levels with a subsequent dose-dependent depletion of monomeric Aβ levels. The increased levels of intracellular bioavailable copper and zinc initiated a signaling cascade involving activation of phosphoinositol 3-kinase and c-Jun N-terminal kinase. Inhibition of these enzymes prevented Aβ depletion induced by the M^{II}(btsc) complexes. Inhibition of metalloproteases also partially restored Aβ levels, implicating metal-driven metalloprotease activation in the extracellular monomeric Aβ depletion. However, a role for alternative metal-induced Aβ metabolism has not been ruled out. These studies demonstrate that M^{II}(btsc) complexes have potential for Alzheimer disease therapy.

Alzheimer disease is the most common form of neurodegenerative disease. The onset of the disease is associated with the formation of senile plaques that are a pathological marker of the disorder. The primary constituent of the plaques is the aggregated peptide β amyloid (Aβ),[3] a 39–43-amino acid peptide derived from amyloid precursor protein (APP). Aβ is generally accepted as being toxic and as such is a key therapeutic target as well as a diagnostic marker. Recent evidence suggests that altered metal homeostasis is a key factor in the etiology of Alzheimer disease (1–5) and other neurodegenerative conditions such as Creutzfeldt-Jakob and Parkinson diseases (6). Both copper and zinc induce aggregation of neurotoxic Aβ (7). In addition, biometals modulate the structure and redox activity of both the parental APP and the prion protein involved in Creutzfeldt-Jakob disease (6, 8). Because of the importance of metals in neurodegenerative diseases, research has been directed toward harnessing the capability of metal-complexing ligands as therapeutic agents (9, 10). Important progress has been made with the cell-permeable ligand clioquinol (cq), which has been shown to inhibit amyloid plaque formation in brains of APP transgenic mice (11). Subsequent early phase IIa clinical trials have been promising (12). However, little is known about the *in vivo* mechanism of action of cq.

In a recent study, we investigated the influence of $M^{II}(cq)_2$ complexes upon Aβ metabolism in cell culture (13). $Cu^{II}(cq)_2$ and $Zn^{II}(cq)_2$ were taken up by the cells, triggering activation of phosphoinositol 3-kinase (PI3K) and subsequent phosphorylation of the downstream target molecules Akt and glycogen synthase kinase 3 (GSK3). Mitogen-activated protein kinases (MAPKs) (such as JNK and extracellular signal-regulated kinase (ERK)) were also activated. This resulted in the up-reg-

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[3] The abbreviations used are: Aβ, amyloid-β peptide; AD, Alzheimer disease; APP, amyloid precursor protein; APP-CHO, Chinese hamster ovary cells overexpressing APP; cq, clioquinol; PI3K, phosphoinositol 3-kinase; GSK3, glycogen synthase kinase 3; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MMP, matrix metalloprotease; btsc, bis(thiosemicarbazone); gtsm, glyoxalbis(*N*(4)-methylthiosemicarbazonato); nc, neocuproine; ICP-MS, inductively coupled plasma mass spectrometry; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; gtse, glyoxalbis(N(4)-ethyl-3-thiosemicarbazone); gtsp, glyoxalbis(N(4)-phenyl-3-thiosemicarbazone); atsm, diacetylbis(N(4)-methyl-3-thiosemicarbazone); atse, diacetylbis(N(4)-ethyl-3-thiosemicarbazone).

ulation of matrix metalloproteases (MMPs) and the degradation of extracellular Aβ (13). These findings demonstrated that cell-permeable metal transporting agents might be developed as therapeutic agents for modulation of Aβ turnover in AD patients. Metal complexes of cq cannot be given directly to patients via oral administration because of the moderate affinity of cq metal complexes (11). As a result cq metal complexes offer little or no control over metal release and retention.

In an attempt to overcome this difficulty, we have been investigating the use of metal complexes of bis(thiosemicarbazone) (btsc) ligands (see Fig. 1). btsc complexes have been investigated as metallodrugs for a number of years and proven to have a broad range of pharmacological activity (14, 15). In particular, recent interest has focused on the use of btsc ligands as vehicles for the selective delivery of radioactive copper isotopes to hypoxic tissue and leukocytes in assessment of their potential as radiopharmaceuticals (16, 17). Cu^{II}(btsc) complexes are stable (log K_A = 18) (17, 18), neutral, low molecular weight compounds capable of crossing cell membranes. In some cases, it has been demonstrated that, once inside cells, Cu^{II} is reduced by intracellular reductants to Cu^{I}, which subsequently dissociates from the ligand (19–21). Other Cu^{II}(btsc) complexes are more resistant to reduction and dissociation and are trapped only in hypoxic cells. This selectivity is remarkably sensitive to the nature of alkyl groups attached to the diimine backbone of the ligand. For example, diacetylbis(*N* (4)-methyl-3-thiosemicarbazonato)copper(II) (Cu^{II}(atsm); see Fig. 1) features two methyl substituents on the backbone and is not released in normal cellular conditions. On the other hand, glyoxalbis(*N* (4)-methyl-3-thiosemicarbazonato)copper(II) (Cu^{II}(gtsm); see Fig. 1) releases copper intracellularly (22, 23). The selective release of copper has been correlated with the Cu^{II}/Cu^{I} reduction potential because Cu^{II}(atsm) is more difficult to reduce than Cu^{II}(gtsm) (by some 160 mV) (23). However, differences in pK_a values, and the stability of the reduced state to dissociation of the metal may also be important (24–26). Importantly, because of the ligand donor set (N_2S_2), these ligands do not bind Ca^{II} with any appreciable affinity and therefore should have no effect on other intracellular metals.

btsc complexes are also capable of transporting Zn^{II} into cells, and the intrinsic fluorescence of certain Zn^{II}(btsc) complexes has been used to probe their intracellular distribution in several lines of cancer cells (27). Zn^{II}, like Cu, is central to a number of cell signal pathways including modulation of *N*-methyl-D-aspartate receptor activity (28), expression of metallothioneins (29, 30) and activation of MAPK-mediated signal transduction pathways (31). It is apparent that both Cu^{II}(btsc) and Zn^{II}(btsc) uptake could have complex effects on downstream metal-mediated cell signaling.

M^{II}(btsc) complexes have a number of properties that make them worthy of investigation as potential therapeutic agents for AD. Several Cu^{II}(btsc) complexes are capable of crossing the blood-brain barrier, and certain examples of the ligands have low toxicity (17, 23, 32). Importantly, the ligands can be modified readily by varying the nature and number of alkyl substituents, and these modifications allow subtle control of subcellular targeting and metal release/retention properties. Therefore, we examined the ability of Cu^{II}(btsc) and Zn^{II}(btsc) complexes to deliver bioavailable metals to APP-CHO cells and how this affected Aβ turnover. Our studies found that intracellular release of zinc or copper from btsc complexes abrogated extracellular levels of Aβ.

EXPERIMENTAL PROCEDURES

General Synthetic Procedures

The general synthetic procedures are described in the supplemental data.

Materials

5-Chloro-7-iodo-8-hydroxyquinoline (cq), Me_2SO, neuocuproine (nc), LY294002, SB203580, and SP600125 were purchased from Sigma-Aldrich. GM6001, MMP inhibitor-I, MMP-2 inhibitor-I, and MMP-9 inhibitor-I were obtained from Merck. Antibodies to total or phospho-specific forms of Akt, JNK, and GSK3 were obtained from Cell Signaling Technology (Beverly, MA).

Methods

APP-transfected Chinese Hamster Ovary Cells—APP-CHO cells were generated by expressing the 695-amino acid APP cDNA in the pIRESpuro2 expression vector (Clontech, Mountain View, CA) as described previously (13). Transfected cells were maintained in RPMI 1640 medium supplemented with 1 mM glutamine and 10% fetal bovine serum (all from Invitrogen) selected and maintained using 7.5 μg/ml puromycin (Sigma-Aldrich).

Exposure of Cells to M^{II}(btsc)—APP-overexpressing cells were passaged at a ratio of 1:6 and grown in 6- or 12-well plates for 2–3 days before experiments. The cells were treated when ~90% confluent. M^{II}(btsc), cq, and nc were prepared as 10 mM stock solutions in Me_2SO and added to serum-free RPMI medium supplemented with puromycin as above. Basal metal levels in the medium were 0.5 and 1.3 μM for copper and zinc, respectively, as determined by inductively coupled plasma mass spectrometry (ICP-MS). Additional metals were added where indicated, and medium was briefly mixed by aspiration prior to addition to cells. The control cultures were treated with vehicle (Me_2SO) alone. Inhibitors of Akt, (LY294002), JNK (SP600125), p38 (SB203580), or metalloproteases (GM6001, MMP inhibitor I, MMP-2 inhibitor-I, and MMP-9 inhibitor-I) were prepared as 10 mM stock solutions in Me_2SO and added at the indicated concentrations. The cultures were incubated for 6 h, and conditioned media were taken for measurement of Aβ levels by ELISA. For immunoblotting, the cells were harvested into Phosphosafe extraction buffer (Novagen) containing protease inhibitor mixture (Calbiochem) and stored at −80 °C until use. Alternatively, the cells were washed three times with PBS and harvested for analysis of metal levels by ICP-MS.

Inductively Coupled Plasma Mass Spectrometry—The cells were treated with M^{II}(btsc) for 6 h and washed three times with Chelex 100-treated PBS, pH 7.4. The cells were scraped into PBS, an aliquot was taken for protein determination (Protein Microassay; Bio-Rad), and the remaining cells were collected by centrifugation at 14,000 rpm for 2 min in a Hermle Microfuge (Labnet, Woodbridge, NJ). The metal levels were determined in cell pellets by ICP-MS as described previously and converted to fold increase in metal compared with untreated controls (37).

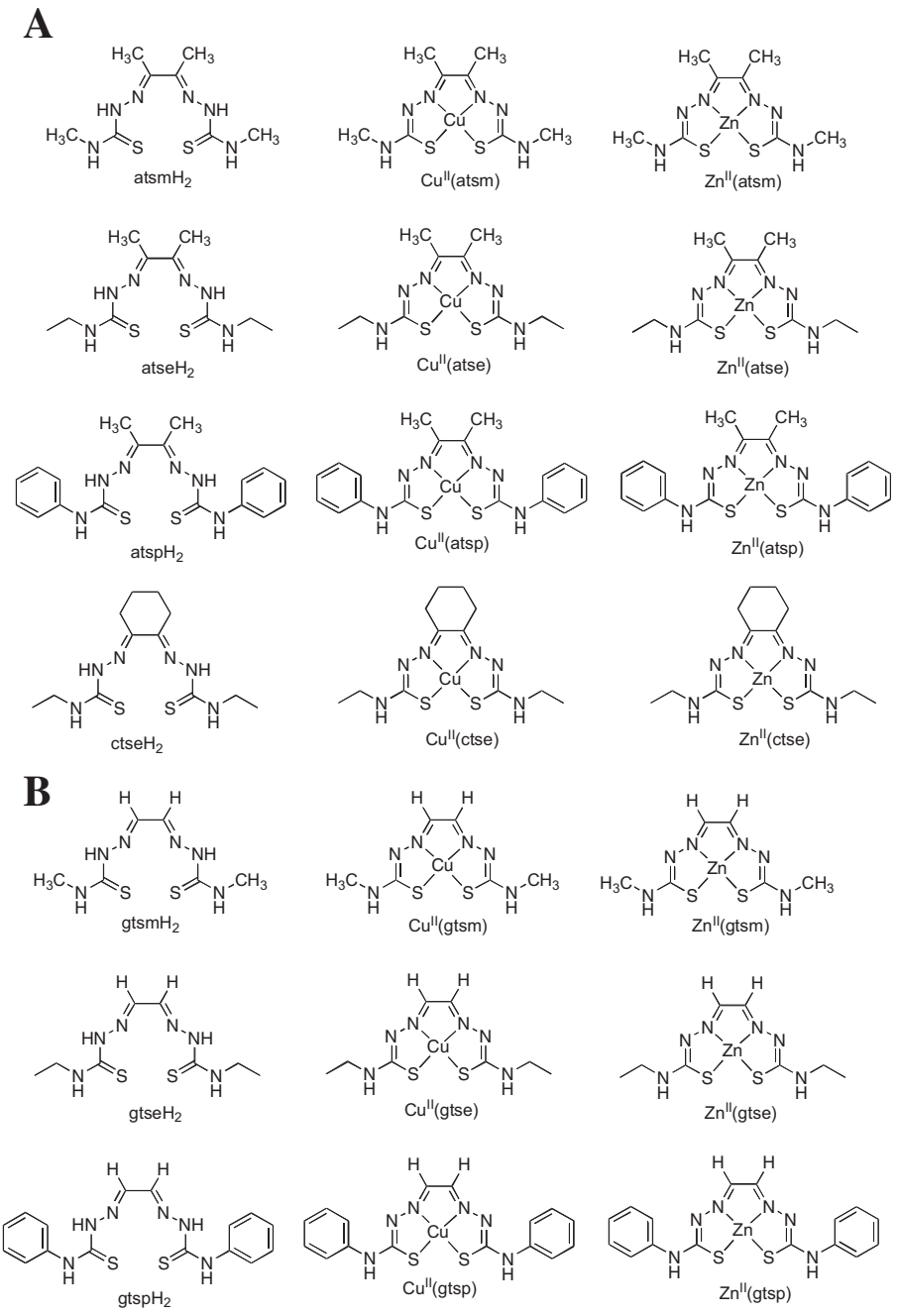

FIGURE 1. *A*, the library of ats ligands and M^{II}(ats) (M = Cu^{II} or Zn^{II}) complexes tested. *B*, the library of gts ligands and M^{II}(gts) derivatives (M = Cu^{II} or Zn^{II}) tested.

Degradation of Synthetic Aβ1–40—Human Aβ1–40 was purchased from the W. Keck Laboratory (Yale University, New Haven, CT) and dissolved in Me_2SO at 1 mg/ml. The dissolved peptide was further diluted into Chelex 100-treated dH_2O at 100 ng/ml before addition to M17 neuroblastoma cell cultures in serum-free medium at 10 ng/ml without aging as previously reported (13). After 6 h (with or without the addition of 25 μM CuGTSM), medium was collected, and the remaining Aβ1–40 levels were determined by ELISA.

Double Antibody Capture ELISA for Aβ Detection—Aβ levels were determined in culture medium using the DELFIA® Double Capture ELISA. 384-well plates (Greiner, Frickenhausen, Germany) were coated with monoclonal antibody G210 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) for Aβ1–40 detection. The plates were washed with PBS containing 0.05% Tween (PBST) and blocked with 0.5% (w/v) casein. Biotinylated monoclonal antibody WO2 (epitope at Aβ5–8) and culture medium or Aβ peptide standards were added (50 μl) to each well and incubated overnight at 4 °C. The plates were washed with PBST and streptavidin-labeled Europium (PerkinElmer Life Sciences) was added. The plates were washed, enhancement solution (PerkinElmer Life Sciences) was added, and the plates were read in a WALLAC Victor^2 plate reader with excitation at 340 nm and emission at 613 nm. Aβ1–40 peptide standards and samples were assayed in triplicate. The values obtained from the triplicate wells were used to calculate the Aβ concentration (expressed as ng/ml) based on the standard curve generated on each plate.

Western Blot Analysis of Protein Expression and Phosphorylation—Cell lysates prepared in Phosphosafe buffer were mixed with electrophoresis SDS sample buffer (Novex) and separated on 12% Novex SDS-PAGE Tris-glycine gels. The proteins were transferred to polyvinylidene difluoride membranes and blocked with milk solution in TBST before immunoblotting for total or phospho-specific proteins. For detection of signal transduction molecules, the membranes were probed with polyclonal antisera against JNK or phospho-JNK, Akt or phospho-Akt, GSK3α, or phospho-GSK3α/β all at 1:5000. Secondary antiserum was goat anti-rabbit horseradish peroxidase at 1:10,000. Blots were developed using Amersham Biosciences ECL Advance Chemiluminescence and imaged on a GeneGnome Chemiluminescence Imager (Syngene, Cambridge, UK). We found that the expression of total levels of kinases (Akt and JNK) was unaffected by metal uptake in APP-CHO cells. In contrast, actin, tubulin and other proteins normally used for equalizing protein loading were found to be altered depending on metal levels.[4] Therefore, equal sample loading and protein

[4] P. S. Donnelly, A. Caragounis, T. Du, K. M. Laughton, I. Volitakis, R. A. Cherny, R. A. Sharples, A. F. Hill, Q.-X. Li, C. L. Masters, K. J. Barnham, and A. R. White, unpublished observations.

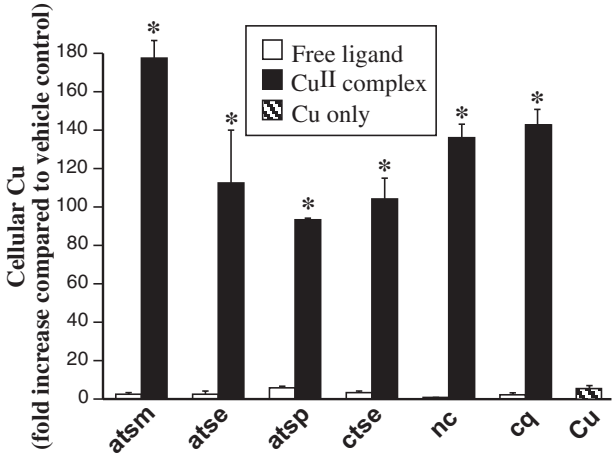

FIGURE 2. **Copper levels in treated APP-CHO cells.** APP-CHO cells were treated with the free ligands ($btscH_2$) or with Cu^{II}(btsc) complexes (25 μM) for 6 h. The cells were also treated with nc or cq (25 μM) alone and with equimolar $CuCl_2$ for 6 h. The metal levels were measured in washed cell pellets by ICP-MS and calculated as fold increases compared with vehicle-treated controls. All of the metal complexes induced significant increases in cellular copper levels ($p < 0.01$ for all copper complexes).

transfer was assessed by the consistency of total kinase protein levels on immunoblots rather than unrelated proteins.

Statistical Analysis—The data are the means ± S.E. from at least three separate experiments unless stated. The results were analyzed using the analysis of variance test.

RESULTS AND DISCUSSION

Cellular Uptake of Cu^{II}(btsc) Complexes—APP-CHO cells were treated for 6 h with the four Cu(btsc) complexes (1–50 μM) shown in Fig. 1*A*, all of which feature dialkyl backbones. Typical results are shown in Fig. 2. Significant increases were observed in intracellular copper levels when compared with treatment with the free ligands or with Cu^{2+} alone. The highest levels were induced by treatment with Cu^{II}(atsm), where a 177 (±9)-fold increase in copper levels was detected compared with untreated control cells. This corresponded to cellular copper levels of 4.5 and 800 ng/mg protein for control and Cu^{II}(atsm)-treated cells, respectively. The other three Cu^{II}(btsc) complexes provided 90–115-fold increases in copper levels and were comparable with those achieved by treatment with other cell-permeable complexes $Cu^{II}(nc)_2$ and $Cu^{II}(cq)_2$ (Fig. 2) (13).

Redox Stable Cu^{II}(btsc) Complexes Do Not Affect Secreted Aβ Levels—We have reported that copper uptake induced by $Cu^{II}(cq)_2$ resulted in lower levels of secreted Aβ from APP-CHO cells (13). Therefore, we examined whether Cu^{II}(btsc) complexes also affected Aβ levels by measuring extracellular Aβ1–40 levels in the culture medium from the treated cells (13). Treatment with the free ligands ($btscH_2$) and the Cu^{II}(btsc) complexes had no significant effect on the level of Aβ1–40 (Fig. 3). In contrast, treatment with $Cu^{II}(cq)_2$ and $Cu^{II}(nc)_2$ resulted in a dose-dependent reduction in the levels of secreted Aβ1–40 in the culture medium, confirming our previous study of $Cu^{II}(cq)_2$ (13). A small decrease in total APP expression was observed consistent with cq-treated APP-CHO cells (13). However, this was insufficient to account for the loss of Aβ (80–90%). The form of decreased extracellular Aβ

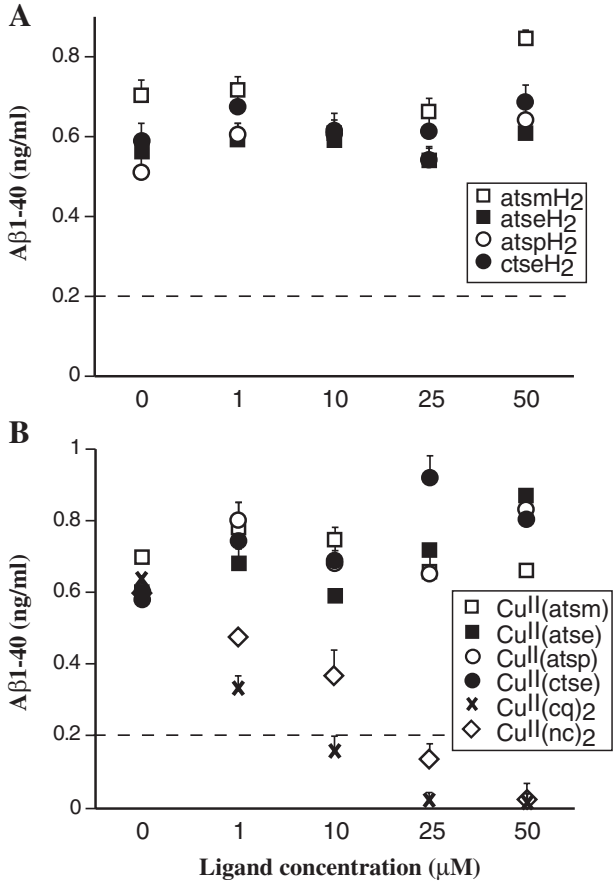

FIGURE 3. **ELISA analysis of secreted Aβ1–40 levels in APP-CHO cell cultures treated with metal ligands (1–50 μM) for 6 h.** *A*, free ligands ($btscH_2$). No significant change was apparent. *B*, complexes Cu^{II}(btsc), $Cu^{II}(nc)_2$, or $Cu^{II}(cq)_2$. Cu^{II}(btsc) did not significantly inhibit secreted levels. $Cu^{II}(nc)_2$ and $Cu^{II}(cq)_2$ did significantly inhibit levels at all concentrations tested ($p < 0.01$). The *dotted lines* represent the detection limits of the assays.

detected by ELISA was monomeric. This was supported by the loss of monomeric Aβ in Cu^{II}(gtsm)-treated cell culture medium by Western blot and is consistent with our previous report on cq inhibition of extracellular monomeric Aβ levels in APP-CHO cells (13). Whether oligomers are also affected by Cu^{II}(gtsm) is not known because these were not observed by Western blot, and the ELISA does not detect aggregated Aβ. However, because there was a substantial loss of monomeric Aβ (~90%), this is likely to preclude formation of higher oligomeric forms.

The lack of effect of Cu^{II}(btsc) complexes (featuring two alkyl substituents on the backbone of the ligand) on Aβ turnover may be related to their high thermodynamic stability ($\log K_A = 18$) and their resistance to reductively assisted transchelation reactions (for example Cu(atsm): $E_{1/2}(Cu^{II}/Cu^{I}) = -0.59$ V *versus* SCE) (23, 33, 34). These properties would promote retention of structural integrity inside the cell, ensuring that the copper is not bioavailable. In comparison, less stable cell-permeable complexes such as $Cu^{II}(nc)_2$ and $Cu^{II}(cq)_2$ ($\log K_A = 9$) are susceptible to dissociation of the metal from the ligand inside the cell (11). Dissociation would allow the copper to become bioavailable and, as reported previously (13), to participate in metal-mediated cell signaling pathways that ultimately result in degradation of Aβ.

Cu(gtsm) Induces Loss of Secreted Aβ—To investigate the hypothesis that dissociation of copper from the ligand is required to decrease the levels of secreted Aβ, cells were treated with Cu^{II}(gtsm), Cu^{II}(gtse), and Cu^{II}(gtsp), analogues of Cu^{II}(atsm), Cu^{II}(atse) and Cu^{II}(atsp), respectively. These analogues lacked the two methyl groups on the diimine backbone of the ligand (Fig. 1*B*). The complexes still exhibit a high thermodynamic stability but are more susceptible to reduction to Cu^{I} (for example Cu(gtsm): $E_{1/2}(Cu^{II}/Cu^{I}) = -0.43$ V). The ligands exhibit a lower affinity for Cu^{I} and are more likely to dissociate under the reducing conditions of the normal intracellular environment (21, 23, 33).

Treatment of APP-CHO cells with Cu^{II}(gtsm), Cu^{II}(gtse), and Cu^{II}(gtsp) (25 μM each) induced 216 (±2)-, 251 (±18)-, and 109 (±19)-fold increases in cellular Cu, respectively, compared with untreated controls (Fig. 4*A*). This result was expected for the cell-permeable copper complexes (*cf.* Fig. 2), but now there was a dose-dependent reduction in the extracellular levels of monomeric Aβ1–40 (Fig. 4*B*). Treatment of APP-CHO cells with Cu^{II}(gtsm), Cu^{II}(gtse), and Cu^{II}(gtsp) reduced Aβ1–40 levels to 15, 16, and 38% of untreated controls, respectively (Fig. 4*B*). Small reductions in monomeric Aβ1–40 levels were seen with the free ligands $gtsmH_2$ and $gtspH_2$ (Fig. 4*B*). We speculate that this could be due to the formation of the Cu^{II} and/or Zn^{II} complexes from those metals available in the culture medium (0.5 copper and 1.3 μM zinc). The metal-free $gtsmH_2$ is capable of binding free or loosely bound (bioavailable) Cu^{II} or Zn^{II} in the medium and transporting this metal into the cell. However, additional studies will be required to determine whether this process occurs and is sufficient to account for the level of Aβ1–40 reduction observed in $gtsmH_2$-treated cultures. Alternative interactions between the free ligand and cells or Aβ1–40 also need to be investigated. Further studies revealed a dose-dependent reduction in the extracellular levels of monomeric Aβ1–40 upon treatment with 1–50 μM Cu^{II}(gtsm) (Fig. 4*C*). A significant reduction in Aβ1–40 levels was observed with 1 μM Cu^{II}(gtsm), and this was further reduced to below the detection limit of the assay at the 25 and 50 μM concentrations (Fig. 4*C*). These results show that minor modification of the btsc backbone can crucially modify the chemical and biological behavior of the complex. The modifications still allowed Cu^{II}(gtsm), Cu^{II}(gtse), and Cu^{II}(gtsp) to transport copper into cell (as for Cu^{II}(atsm)) but, once inside, appear to permit reductively assisted dissociation of the copper from the ligand to give elevated levels of intracellular bioavailable copper.

Zn^{II}(btsc) Complexes Induce Loss of Secreted Aβ—We also examined the effect of Zn^{II}(btsc) complexes on metal uptake and Aβ turnover. The cells were treated with four complexes (Fig. 1*A*; 25 μM), and this resulted in significant increases in the intracellular zinc levels as measured by ICP-MS (Fig. 5*A*). Zn^{II}(atsm) and Zn^{II}(atse) induced 8.2 (±0.25)- and 9.8 (±0.9)-fold increases in cellular zinc levels, respectively, a result consistent with increased levels induced by the other cell-permeable complexes $Zn^{II}(cq)_2$ and $Zn^{II}(nc)_2$ (Fig. 5*A*) (13). The lower stabilities of the Zn^{II}(btsc) complexes ($\log K_A = 7$) inferred that they were more susceptible to intracellular transchelation than their copper analogues and therefore elevate levels of bioavail-

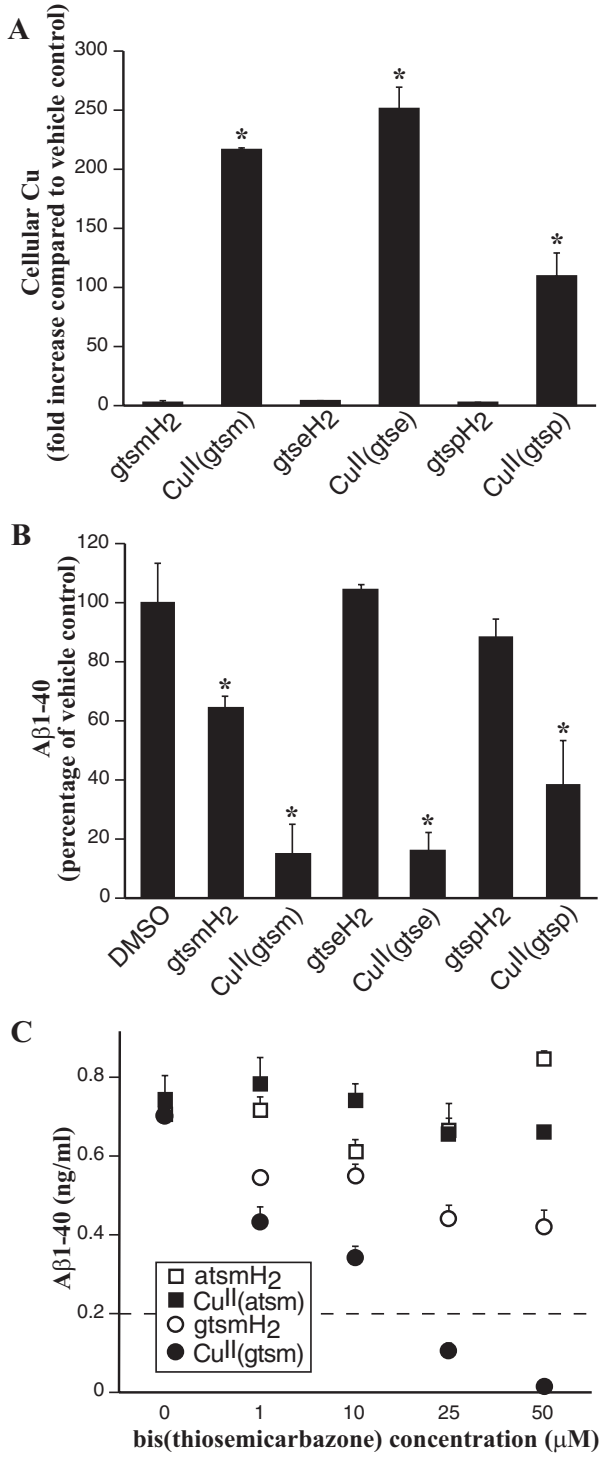

FIGURE 4. **Cu^{II}(gtsm), Cu^{II}(gtse), and Cu^{II}(gtsp) inhibit secreted Aβ1–40 in APP-CHO cell cultures.** *A*, cells were treated with 25 μM free ligands ($gtsmH_2$, $gtseH_2$, or $gtspH_2$) or copper complexes (Cu^{II}(gtsm), Cu^{II}(gtse), or Cu^{II}(gtsp)) for 6 h. Cellular copper levels were determined in cell pellets by ICP-MS. All of the copper ligands significantly increased cellular copper levels ($p < 0.01$). *B*, Aβ1–40 levels were determined by ELISA. Cu^{II}(gtsm), Cu^{II}(gtse), and Cu^{II}(gtsp) significantly inhibited Aβ1–40 levels compared with $gtsmH_2$, $gtseH_2$, and $gtspH_2$, respectively ($p < 0.01$, compared with free ligands). *C*, dose response effects of Cu^{II}(gtsm) on secreted Aβ1–40 in APP-CHO cell cultures. The cells were treated with 1–50 μM free ligand ($gtsmH_2$) or the copper complexes Cu^{II}(atsm) and Cu^{II}(gtsm) for 6 h. Aβ1–40 levels were determined by ELISA. Cu^{II}(gtsm) significantly inhibited Aβ1–40 levels compared with $gtsmH_2$, $atsmH_2$, and Cu^{II}(atsm). ($p < 0.01$, Cu^{II}(gtsm) compared with $gtsmH_2$ at all concentrations tested). The *dotted line* represents the detection limit of the assay.

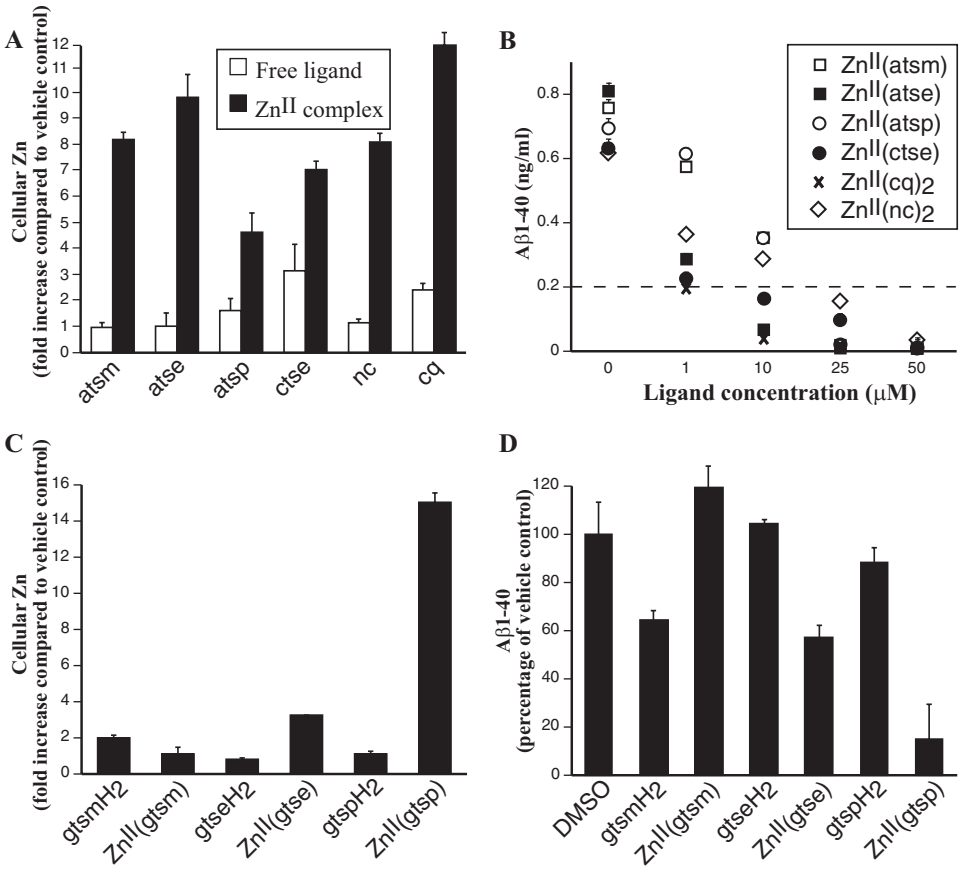

FIGURE 5. *A*, zinc levels in APP-CHO cells treated with Zn^{II}(btsc). APP-CHO cells were treated with free (btsc) ligand or Zn^{II}(btsc) (25 μM) for 6 h. The cells were also treated with nc or cq (25 μM) alone and with equimolar $ZnCl_2$ for 6 h. After 6 h the metal levels were determined in cell pellets by ICP-MS. The metal levels were calculated as fold increase compared with vehicle-treated controls. All of the metal ligands induced significant increases in cellular zinc levels ($p < 0.01$ for all zinc complexes. *B*, Zn^{II}(btsc) inhibited secreted Aβ1–40 levels in APP-CHO cells. The cells were treated with Zn^{II}(btsc), $Zn^{II}(nc)_2$, or $Zn^{II}(cq)_2$ for 6 h, and secreted Aβ1–40 levels were determined by ELISA. All of the complexes significantly inhibited secreted Aβ1–40 levels at all of the concentrations tested ($p < 0.01$ for all data points except 1 μM Zn^{II}(atsp) ($p < 0.05$)). The *dotted line* represents the detection limit of the assay. *C*, zinc uptake induced by Zn^{II}(gtsm), Zn^{II}(gtse), and Zn^{II}(gtsp). APP-CHO cells were treated with free (btsc) ligand or Zn^{II}(gtsm), Zn^{II}(gtse), or Zn^{II}(gtsp) (25 μM). After 6 h the metal levels were determined in cell pellets by ICP-MS. Zn^{II}(gtsm) did not induce a significant increase in cellular zinc levels. Zn^{II}(gtse) induced a small but significant increase in zinc levels ($p < 0.05$), whereas Zn^{II}(gtsp) substantially increased cellular zinc ($p < 0.01$). *D*, Aβ1–40 levels in APP-CHO cells treated with Zn^{II}(gtsm), ZnII(gtse), and Zn^{II}(gtsp). The cells were treated with Zn^{II}(btsc) for 6 h, and secreted Aβ1–40 levels were determined by ELISA. Zn^{II}(gtsm) did not reduce Aβ1–40 levels. Zn^{II}(gtse) modestly reduced Aβ1–40, whereas Zn^{II}(gtsp) strongly decreased Aβ1–40 levels. ($p < 0.01$). The *dotted line* represents the detection limit of the assay.

able zinc within the cells. The elevated zinc levels correlated with a reduction in the extracellular levels of monomeric Aβ1–40 (Fig. 5*B*). The concentration of Aβ1–40 in the medium of untreated cells was 0.6–0.8 ng ml^{-1} and was reduced to less than 0.2 ng ml^{-1} (the detection limit) following treatment with 25 μM of Zn^{II}(btsc). The different Zn^{II}(btsc) complexes exhibited detectable differences in the dose-dependent reduction of Aβ1–40 levels. Treatment with Zn^{II}(atse) and Zn^{II}(ctsc) resulted in greater reductions at a lower dose (1 μM) compared with the complexes Zn^{II}(atsm) and Zn^{II}(atsp) (Fig. 5*B*). This could reflect different binding affinities or different subcellular localization (which would initiate different metal-mediated cell signaling pathways).

We also examined the effect of zinc homologues of the gtsm, gtse, and gtsp ligands (Fig. 5*C*). Interestingly, Zn^{II}(gtsm) had no significant effect on cellular zinc levels (1.1 ± 0.3-fold), possibly because of instability of the complex (Fig. 5*C*). Zn^{II}(gtse) induced a small but significant increase in zinc levels (2.6 ± 0.03-fold), whereas Zn^{II}(gtsp) had a greater effect, increasing cellular zinc by 15 ± 0.5-fold. The zinc uptake corresponded to effects of these complexes on monomeric Aβ1–40 levels. Zn^{II}(gtsm) did not decrease Aβ levels in treated cultures, whereas Zn(gtse) had a modest effect (57 ± 5% of controls), and Zn^{II}(gtsp) greatly reduced Aβ1–40 to 15 ± 14% of controls (Fig. 5*D*). Further support for the link between metal uptake and loss of Aβ1–40 was obtained through our studies on transmetallation reactions and temperature-dependent uptake of M^{II}(btsc) (see supplemental information).

M(btsc) Complexes Activate PI3K- and JNK-dependent Pathways Resulting in Metalloprotease-mediated Degradation of Secreted Aβ1–40—We have shown previously that cell-permeable metal complexes can induce activation of PI3K-Akt-GSK3 and MAPK pathways resulting in up-regulation of MMP activity and degradation of secreted Aβ (13). APP-CHO cells were treated with M^{II}(btsc) (10 μM; 6 h), and the cell lysates were examined for activation of PI3K and MAPK signal pathways. Cu^{II}(atsp) and Cu^{II}(atse) did not induce activation of PI3K (Akt phosphorylation) or JNK (Fig. 6*A*), consistent with the lack of effect on Aβ1–40 levels by these complexes (Fig. 3). In contrast, treatment with Zn^{II}(atse) or Zn^{II}(atsm) induced activation of Akt and JNK (Fig. 6*A*). Activation of PI3K-Akt by Zn^{II}(atse) also induced downstream phosphorylation (deactivation) of GSK3 and increased GSK3 expression (Fig. 6*B*). These results were indistinguishable from the effects of $Cu^{II}(cq)_2$ on APP-CHO cells (13). Interestingly, Zn^{II}(atsp) did not induce detectable activation of Akt or JNK (Fig. 6*A*), although a small increase in GSK3 expression was observed (Fig. 6*B*). Because Zn^{II}(atsp) also induced a reduction in secreted Aβ1–40 levels, it may affect different cell signaling pathways. Alternatively, Zn^{II}(atsp), which is the only zinc complex tested that possesses an aromatic substituent, could directly interfere with detection of some phospho-proteins, consistent with the observed increase in GSK3 expression but poor detection of phospho-GSK3 (Fig. 6*B*).

Treatment of cells with Cu^{II}(gtsm) (25 μM) reduced secreted monomeric Aβ1–40 levels and also induced phosphorylation of Akt, JNK, and GSK3 (Fig. 6*C*), whereas treatment with the

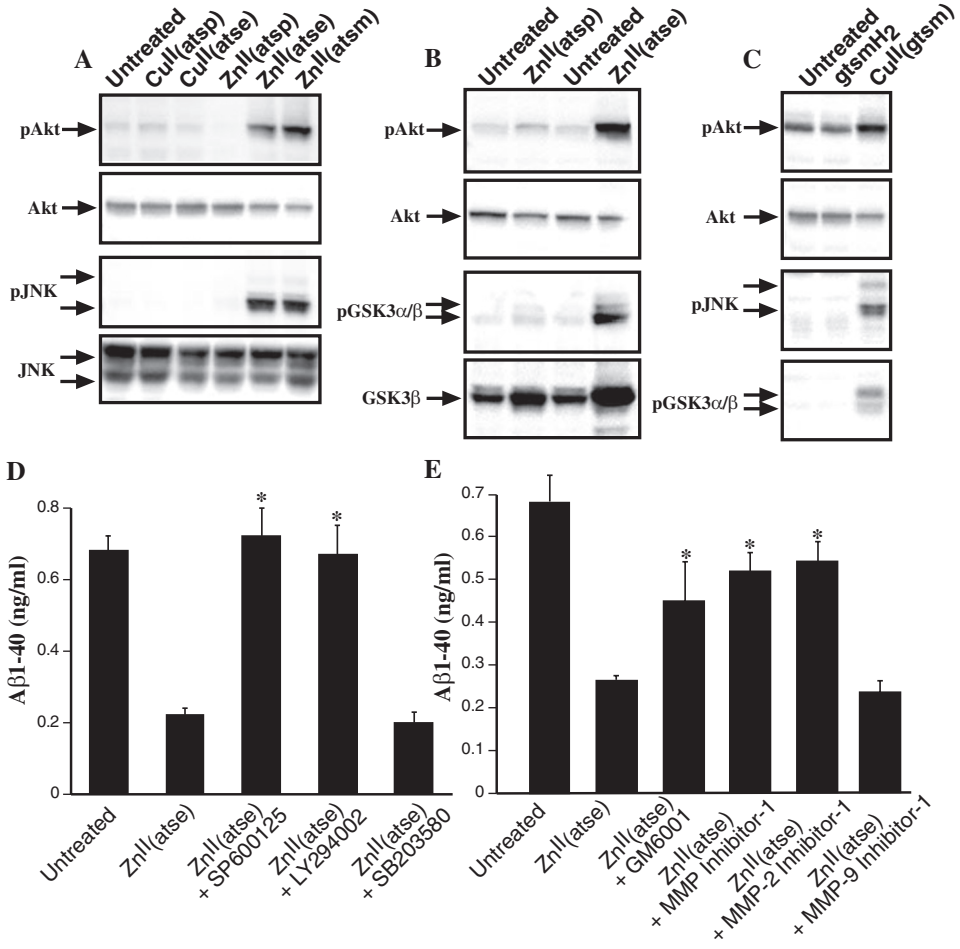

FIGURE 6. **Cell signal activation by M^{II}(btsc).** *A*, APP-CHO cells were exposed to 25 μM M^{II}(btsc) for 6 h. Western blotting of cell lysates revealed that both Zn^{II}(atse) and Zn^{II}(atsm) induced activation of JNK and Akt, whereas Cu^{II}(atse) and Cu^{II}(atsp) had no effect. No activation of Akt or JNK was observed using Zn^{II}(atsp). *B*, APP-CHO cells were treated with 25 μM Zn^{II}(atsp) or Zn^{II}(atse) for 6 h. Western blotting revealed that Zn^{II}(atse) activated Akt and induced phosphorylation of GSK3, whereas Zn^{II}(atsp) had no effect. Both Zn^{II}(atse) and Zn^{II}(atsp) increased the expression of total GSK3. *C*, APP-CHO cells were treated with 25 μM $gtsmH_2$ or Cu^{II}(gtsm) for 6 h. Western blotting revealed that only Cu^{II}(gtsm) induced activation of Akt and JNK. Cu^{II}(gtsm) also induced phosphorylation of GSK3. *D*, APP-CHO cells were treated with 25 μM Zn^{II}(atse) for 6 h with or without inhibitors of JNK (SP600125), Akt (LY294002), or p38 (SB203580). Aβ1–40 levels in conditioned medium were measured by ELISA. Co-treatment of cells with SP600125 or LY294002 and Zn^{II}(atse) significantly increased the extracellular Aβ1–40 levels compared with Zn^{II}(atse) alone ($p < 0.01$). SB203580 had no effect on Aβ1–40 levels. *E*, APP-CHO cells were treated with 25 μM Zn^{II}(atse) with or without broad spectrum MMP inhibitors (GM6001 and MMP inhibitor-I), MMP-2 inhibitor-I, or MMP-9 inhibitor-I. Aβ1–40 levels were determined in conditioned medium by ELISA. Co-treatment of cells with Zn^{II}(atse) and GM6001, MMP inhibitor-I, or MMP-2 inhibitor-1 significantly increased the extracellular levels of Aβ1–40 compared with Zn^{II}(atse) alone ($p < 0.01$). MMP-9 inhibitor-I had no significant effect on Aβ1–40 levels.

free ligand $gtsmH_2$ did not. Because both Cu^{II}(atsp) and Cu^{II}(atse) did not activate these pathways (Fig. 6*A*) and had no effect on secreted Aβ1–40 levels, the data provide further support for the importance of PI3K-Akt-GSK3 and JNK activation in metal ligand-mediated inhibition of Aβ. To further substantiate this hypothesis, APP-CHO cells were treated with Zn^{II}(atse) (10 μM) with and without specific inhibitors of PI3K (LY294002), JNK (SP600125), or p38 (SB203580) (25 μM of each). These inhibitors blocked activation of PI3K, JNK, and p38, respectively, in APP-CHO cells treated with $Cu^{II}(cq)_2$ (13). Treatment with both LY294002 and SP600125 blocked the effect of treatment with Zn^{II}(atse) and prevented the loss of Aβ1–40 (Fig. 6*D*). Treatment with SB203580 had no effect (Fig. 6*D*) and was consistent with our recent finding that p38 activation is not involved in loss of Aβ1–40 induced by metal complexes (13).

The mechanisms by which metals activate these pathways is not known, but given the relatively large changes in intracellular metals involved, it is unlikely to involve direct enzymatic effects of copper or zinc Previous studies have demonstrated that MMP enzymes can degrade both human-derived and synthetic Aβ (35), whereas MMP expression is altered in the brains of AD patients (36). We have shown that $Cu^{II}(cq)_2$ complexes induced PI3K- and JNK-mediated up-regulation of MMPs, and the increased MMP activity resulted in enhanced degradation of secreted Aβ (13). To substantiate this, the effect of broad spectrum MMP inhibitors (GM6001, MMP inhibitor-I, and MMP-2 inhibitor-I) on Aβ levels was examined in cultures exposed to Zn^{II}(atse) (10 μM). Co-administration of each inhibitor (25 μM) with Zn^{II}(atse) increased significantly the level of secreted Aβ after 6 h when compared with treatment with Zn^{II}(atse) alone (Fig. 6*E*). In contrast, MMP-9 inhibitor-I had no effect on Aβ levels. These results are consistent with previous studies (13) showing that broad spectrum and MMP-2 inhibitors prevented a loss of secreted Aβ induced by $Cu^{II}(cq)_2$ and strongly support a role for elevated MMP activity in reductions in the levels of secreted Aβ induced by treatment with Zn^{II}(atse) and possibly other M^{II}(btsc) complexes.

We have also reported recently that treatment of APP-CHO cells with alternative metal complexes had no effect on cellular Aβ levels or APP processing (37). In the present study, no intracellular Aβ was observed (data not shown), consistent with previous reports (13, 37) that loss of extracellular Aβ is most likely induced by increased MMP synthesis. However, the oligomeric state of the secreted Aβ in our cultures has not been fully investigated. It is also possible that altered metal levels in the medium or cells could promote formation of higher oligomeric states of Aβ including dimers, trimers, etc. Because these are not routinely detectable by our Western blot or ELISA techniques, we cannot rule out the metal-mediated aggregation of Aβ as a factor contributing to the loss of detectable monomeric Aβ in the conditioned medium. Further studies will be needed to address this possibility.

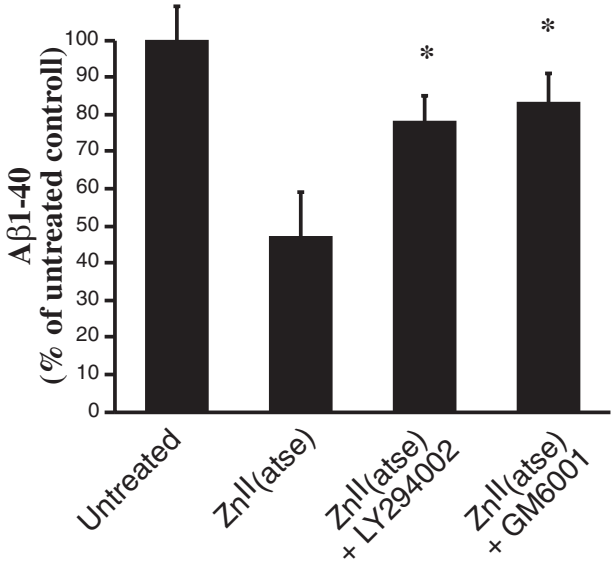

FIGURE 7. **Human M17 neuroblastoma cells were treated with 10 μg/ml Aβ1–40 and 25 μM Zn^{II}(atse) for 6 h.** Measurement of remaining Aβ in conditioned medium revealed a significant decrease in Aβ induced by Zn^{II}(atse). This was abrogated by co-treatment with LY294002 (25 μM) or GM6001 (100 μM). *, $p < 0.01$.

Finally, to confirm that these metal-btsc complexes inhibit Aβ levels in neuronal-like cells, we examined the effect of Zn^{II}(atse) on levels of synthetic Aβ1–40 added to the medium of neuronal-like cells. M17 cells were treated with 10 μg/ml of Aβ1–40 for 6 h with or without 25 μM Zn^{II}(atse). We found that Zn^{II}(atse) treatment induced a significant decrease in the level of Aβ remaining in the conditioned medium (Fig. 7). Co-treatment with the PI3K inhibitor LY294002 or MMP inhibitor GM6001 abrogated this effect, strongly suggesting that the loss of Aβ was mediated through increased MMP levels or activity (Fig. 7).

Concluding Remarks—This study demonstrated that Cu^{II}(btsc) complexes were capable of transporting Cu(II) into APP-CHO cells, and release of metals in the cell resulted in loss of extracellular Aβ. Subtle but crucial modifications of the ligand backbone appear to permit controlled intracellular redox-mediated release of copper from the ligand. The released metal is then able to activate signaling pathways to decrease extracellular Aβ by MMPs (Fig. 8).

A growing body of literature supports a central role for metals in the etiology of AD. Although studies have shown that extracellular metal accumulation may enhance Aβ deposition and toxicity, other studies indicate that copper supplementation by diet or genetic modification can lead to decreased Aβ deposition and improvement in cognition (2, 3). The reason for this paradox is uncertain but may be related to an imbalance between intracellular and extracellular metal homeostasis. Age-related changes may lead to increased efflux of copper or zinc, promoting Aβ accumulation and decreasing activity of copper-dependent enzymes. In this context, restoration of intracellular copper levels could restore the imbalance and promote a decrease in Aβ accumulation. The mechanisms associated with this are not known but could potentially be mediated through increased

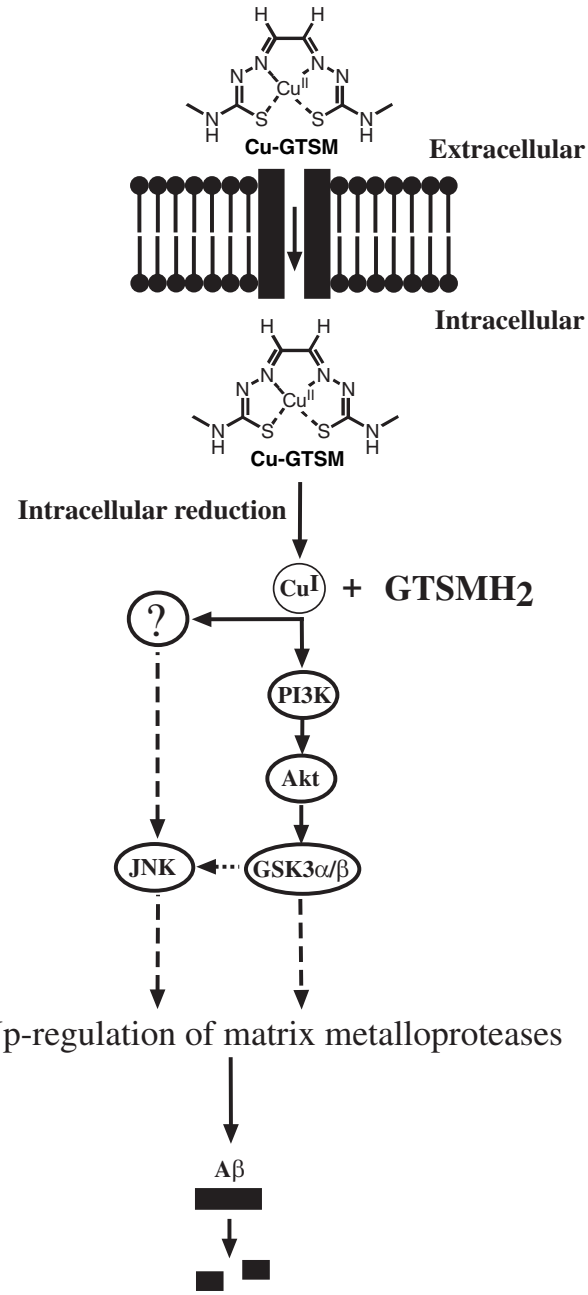

FIGURE 8. **Schematic of proposed mechanism for Cu^{II}(gtsm)-mediated inhibition of amyloid-β peptide levels.** Cu^{II}(gtsm) delivers bioavailable copper to the cell, which subsequently activates PI3K, resulting in phosphorylation of Akt and down-regulation of GSK3 activity. Copper also induces activation of JNK via an unknown pathway. Inhibition of GSK3 and up-regulation of JNK result in elevated matrix metalloprotease activity and increased degradation of Aβ.

Aβ degradation as shown here *in vitro*. However, *in vivo* studies are required to confirm this hypothesis.

Several recent studies have highlighted a potential role for modulating metal levels as a basis for AD therapy. Transgenic AD mice (Tg2576) treated with cq revealed marked decreases in cerebral amyloid load but elevated levels of cerebral copper and zinc (11). Treatment with cq involves administration of a "free ligand" that must sequester a metal from a biological source if it is to activate the pathways outlined previously (13) and in this work. The "cq approach"

involves attenuation of latent metal ion sinks and could result in nondiscriminate binding of essential metal ions in the biological milieu that could have deleterious side effects. Our present work presents a unique and alternative approach. Cell-permeable metal btsc complexes, not free ligands, were utilized, and the metal ion is released inside the cell. Because the complexes are already metal-loaded, there is no requirement for the complexes to strip metals from other sources that may have adverse consequences. The copper complexes are particularly attractive because the high stability of Cu^{II}(btsc) complexes is coupled to the unique ability to control release of the metal ion via subtle changes to the ligand backbone. These changes alter the susceptibility of the complexes to undergo reductively assisted metal ion release, but this release only occurs inside the cell.

In this study, we found that relatively large changes in metal levels (~100–200-fold Cu^{II} and ~10-fold Zn^{II}) were associated with the subsequent biological effects such as kinase activation and loss of monomeric Aβ. Because these metals (Cu^{II} and Zn^{II}) are normally involved in catalytic processes, the requirement for large changes in metal levels is unexpected. The reason for this is unknown but could reflect that fact that the metals may be acting in a noncatalytic manner such as oxidation of cysteine residues on certain proteins. Alternatively, a significant proportion of the metal taken up by the cells could be sequestered by metal binding molecules, thus preventing the interaction of the metal with processes leading to the loss of Aβ. Interestingly, we reported recently that some metal complexes can activate kinase-dependent pathways and alter extracellular Aβ1–40 levels with little or no change in overall cellular metal levels (37). Further investigation is necessary to determine the mechanisms by which altered metal levels result in kinase activation and reduced Aβ1–40 generation.

Other studies have reported that increased cerebral copper levels (through genetic manipulation or feeding regimes) similarly led to reduced amyloid deposition in transgenic AD mouse models (2, 3). Our findings suggest that amyloid levels may be reversed by increases in intracellular metal levels resulting in up-regulation of Aβ-degrading metalloproteases. Whether similar effects occur *in vivo* is yet to be determined. However, the potential to deliver copper as a complex to inhibit Aβ generation may prove to be a more favorable approach than dietary copper supplementation (3). These characteristics, coupled with our new results showing reduction in levels of Aβ in APP-CHO cells following treatment with Cu^{II}(gts) derivatives and Zn^{II}(btsc) complexes, suggest that these compounds have potential as therapeutic agents for AD.

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REFERENCES

1. Pajonk, F.-G., Kessler, H., Supprian, T., Hamzei, P., Bach, D., Schweickhardt, J., Herrmann, W., Obeid, R., Simons, A., Falkai, P., Multhaup, G., and Bayer, T. A. (2005) *J. Alzheimer's Dis.* **8,** 23–27
2. Phinney, A. L., Drisaldi, B., Schmidt, S. D., Lugowski, S., Coronado, V., Liang, Y., Horne, P., Yang, J., Sekoulidis, J., Coomaraswamy, J., Chishti, M. A., Cox, D. W., Mathews, P. M., Nixon, R. A., Carlson, G. A., St George-Hyslop, P., and Westaway, D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 14193–14198
3. Bayer, T. A., Schaefer, S., Simons, A., Kemmling, A., Kamer, T., Tepest, R., Eckert, A., Schuessel, K., Eikenberg, O., Sturchler-pierrat, C., Abramowski, D., Staufenbiel, M., and Multhaup, G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 14187–14192
4. Sparks, D. L., and Schreurs, B. G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 11065–11069
5. Donnelly, P. S., Xiao, Z., and Wedd, A. G. (2007) *Curr. Opin. Chem. Biol.* **11,** 128–133
6. Brown, D. R., and Kozlowski, H. (2004) *Dalton Trans.* **7,** 1907–1917
7. Ali, F. E. A., Barnham, K. J., Barrow, C. J., and Separovic, F. (2004) *Aust. J. Chem.* **57,** 511–518
8. Ciuculescu, E. D., Mekmouche, Y., and Faller, P. (2005) *Chem. Eur. J.* **11,** 903–909
9. Barnham, K. J., Cherny, R. A., Cappai, R., Melov, S., Masters, C. L., and Bush, A. I. (2004) *Drug. Des. Rev.* **1,** 75–82
10. Curtain, C. C., Barnham, K. J., and Bush, A. I. (2003) *Curr. Med. Chem. Immun. Endo. Metab. Agents* **3,** 309–315
11. Cherny, R. A., Atwood, C. S., Xilinas, M. E., Gray, D. N., Jones, W. D., McLean, C. A., Barnham, K. J., Volitakis, I., Fraser, F. W., Kim, Y.-S., Huang, X., Goldstein, L. E., Moir, R. D., Lim, J. T., Beyreuther, K., Zheng, H., Tanzi, R. E., Masters, C. L., and Bush, A. I. (2001) *Neuron* **30,** 665–676
12. Ritchie, C. W., Bush, A. I., Mackinnon, A., Macfarlane, S., Mastwyk, M., MacGregor, L., Kiers, L., Cherny, R., Li, Q.-X., Tammer, A., Carrington, D., Mavros, C., Volitakis, I., Xilinas, M., Ames, D., Davis, S., Beyreuther, K., Tanzi, R. E., and Masters, C. L. (2003) *Arch. Neurol.* **60,** 1685–1691
13. White, A. R., Du, T., Laughton, K. M., Volitakis, I., Sharples, R. A., Xilinas, M. E., Hoke, D. E., Holsinger, R. M. D., Evin, G., Cherny, R. A., Hill, A. F., Barnham, K. J., Li, Q.-X., Bush, A. I., and Masters, C. L. (2006) *J. Biol. Chem.* **281,** 17670–17680
14. West, D. X., Liberta, A. E., Padhye, S. B., Chikate, R. C., Sonawane, P. B., Kumbhar, A. S., and Yerande, R. G. (1993) *Coord. Chem. Rev.* **123,** 49–71
15. Beraldo, H., and Gambino, D. (2004) *Mini-Rev. Med. Chem.* **4,** 31–39
16. Lewis, J. S., Laforest, R., Buettner, T. L., Song, S.-K., Fujibayahsi, Y., Connet, J. M., and Welch, M. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1206–1211
17. Green, M. A., Klippenstein, D. L., and Tennison, J. R. (1988) *J. Nucl. Med.* **29,** 1549–1557
18. Petering, D. H. (1974) *Biochem. Pharmacol* **23,** 567–576
19. Kraker, A., Krezoski, S., Schneider, J., Minkel, D., and Petering, D. H. (1985) *J. Biol. Chem.* **260,** 13710–13718
20. Anderson, C. J., and Welch, M. J. (1999) *Chem. Rev.* **99,** 2219–2234
21. Adonai, N., Nguyen, K. N., Walsh, J., Iyer, M., Toyokuni, T., Phelps, M. E., McCarthy, T., McCarthy, D. W., and Gambhir, S. S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 3030–3035
22. Fujibayahsi, Y., Taniuchi, H., Yonekura, Y., Ohtani, H., Konishi, J., and Yokoyama, A. (1997) *J. Nucl. Med.* **38,** 1155–1160
23. Dearling, J. L. J., Lewis, J. S., Mullen, G. D., Welch, M. J., and Blower, P. J. (2002) *J. Biol. Inorg. Chem.* **7,** 249–259
24. Blower, P. J., Castle, T. C., Cowley, A. R., Dilworth, J. R., Donnelly, P. S., Labisbal, E., Sowrey, F. E., Teat, S. J., and Went, M. J. (2003) *Dalton Trans.* 4416–4425
25. Cowley, A. R., Dilworth, J. R., Donnelly, P. S., Labisbal, E., and Sousa, A. (2002) *J. Am Chem. Soc.* **124,** 5270–5271
26. Alsop, L., Cowley, A. R., Dilworth, J. R., Donnelly, P. S., Peach, J. M., and Rider, J. T. (2005) *Inorg. Chim. Acta* **358,** 2770–2780
27. Cowley, A. R., Davis, J., Dilworth, J. R., Donnelly, P. S., Dobson, R., Nightingale, A., Peach, J. M., Shore, B., Kerr, D., and Seymour, L. (2005) *Chem. Commun.* **21,** 845–847
28. Frederickson, C. J., Koh, J. Y., and Bush, A. I. (2005) *Natl. Rev. Neurosci.* **6,** 449–462
29. Mocchegiani, E., Bertoni-Freddari, C., Marcellini, F., and Malavolta, M. (2005) *Prog. Neurobiol.* **75,** 367–390
30. Burdette, S. C., and Lippard, S. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 3605–3610

31. Wu, W., Samet, J., Silbajoris, R., Dailey, L. A., Sheppard, D., Bromberg, P. A., and Graves, L. M. (2004) *Am. J. Respir. Cell Mol. Biol.* **30,** 540–547
32. Wada, K., Fujibayahsi, Y., and Yokoyama, A. (1994) *Arch. Biochem. Biophys.* **310,** 1–5
33. Dearling, J. L. J., Lewis, J. S., McCarthy, D. W., Welch, M. J., and Blower, P. J. (1998) *J. Chem. Soc. Chem. Commun.* **22,** 2531–2533
34. Lewis, J. S., Sharp, T. L., Laforest, R., Fujibayahsi, Y., and Welch, M. J. (2001) *J. Nucl. Med.* **42,** 655–661
35. Roher, A. E., Kasunic, T. C., Woods, A. S., Ball, M. J., and Fridman, R. (1994) *Biochem. Biophys. Res. Commun.* **205,** 1755–1761
36. Backstrom, J. R., Lim, G. P., Cullen, M. J., and Tokes, Z. A. (1996) *J. Neurosci.* **16,** 7910–7919
37. Caragounis, A., Du, T., Filiz, G., Laughton, K. M., Volitakis, I., Sharples, R. A., Cherny, R. A., Masters, C. L., Drew, S. C., Hill, A. F., Li, Q.-X., Crouch, P. J., Barnham, K. J., and White, A. R. (2007) *Biochem. J.* **407,** 435–450