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# Sustained Activation of Glial Cell Epidermal Growth Factor Receptor by Bis(thiosemicarbazonato) Metal Complexes Is Associated with Inhibition of Protein Tyrosine Phosphatase Activity

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Bis(thiosemicarbazonato) metal complexes ($M^{II}$(btsc)) have demonstrated potential neuroprotective activity in cell and animal models of Alzheimer's disease (AD). Metal complexes can activate the epidermal growth factor receptor (EGFR), leading to inhibition of amyloid peptide accumulation in neuronal cells. As glial cells also have an important role in modulating neuronal health and survival in AD, we examined the effect of $M^{II}$(btsc) on activity of EGFR in an astroglial cell line. Our findings reveal potent activation of glial EGFR by glyoxalbis(*N*(4)-methylthiosemicarbazonato)$Cu^{II}$] ($Cu^{II}$(gtsm)). Activation of EGFR by $Cu^{II}$(gtsm) involved phosphorylation of multiple tyrosine residues and was mediated by a cognate ligand-independent process involving $M^{II}$(btsc) inhibition of protein tyrosine phosphatase (PTP) activity. EGFR activation resulted in release of growth factors and cytokines with potential modulatory effects on neuronal function. These studies provide an important insight into the mechanism of action of a neuroprotective $M^{II}$(btsc) and provide a basis for future studies into this novel approach to AD therapy.

## Introduction

Metal ions from copper (Cu) and zinc (Zn) have a central role in several major neurodegenerative diseases. These metals are elevated in Alzheimer's disease (AD[a]) neuropil,[1] and both metals contribute to aggregation of amyloid beta (A$\beta$) peptide,[2] promoting extracellular deposition of the protein in the AD brain.[3,4] $Cu^{II}$ reduction to $Cu^{I}$ by A$\beta$ and potentially the amyloid precursor protein (APP) results in generation of neurotoxic free radicals.[5−8] Production of reactive oxygen species (ROS) by aberrant Cu metabolism is believed to contribute to the elevated oxidative damage to proteins and lipids in AD brain.[2,9−11] Cu-mediated ROS generation promotes A$\beta$ cross-linking and aggregation.[10] Cuproproteins are also central to the neuropathology of prion diseases such as Creutzfeldt−Jakob disease. Prion protein ($PrP^{c}$) binds to Cu in at least two different sites, and this may affect its structure, induce aberrant cell signaling, and promote neurotoxicity. In addition, mutations in the Cu/Zn superoxide dismutase (Cu/ZnSOD) result in increased oxidative stress and motor neuron death in amyotrophic lateral sclerosis[12,13] and iron (Fe) may be involved in neurotoxicity in the substantia nigra in Parkinson's disease.[14,15]

Because of the central role of biometals in these disorders, there has been an increasing focus on development of therapies targeting metals in the brain. In 2001, Cherny et al.[16] reported that treatment of AD transgenic mice with the 8-hydroxyquinoline metal ligand, 5-chloro-7-iodo-quinolin-8-ol (clioquinol, CQ),[17] resulted in decreased amyloid plaque deposition in the brain. The proposed mechanism, based on in vitro studies, involved chelation of Cu and/or Zn from extracellular aggregated A$\beta$ peptide, resulting in dissolution of the aggregates and clearance by proteases.[18] Small clinical trials with CQ in AD patients resulted in favorable outcomes, with a subset of patients showing slowed cognitive decline.[19] Unfortunately, further studies were suspended due to potential purification issues with the compound. Recently, another 8-hydroxyquinoline, PBT-2, has also induced improvement in APP/PS1 AD mouse cognition and reduced brain amyloid levels.[20] As with CQ, early clinical testing indicates improved performance by AD patients treated with PBT-2 compared to controls.[21] Again, the proposed mechanism of action is through solubilization of extracellular A$\beta$ deposits in the treated mice and humans. While the authors discussed possible ionophore-related effects of PBT-2, resulting in redistribution of metals in the brain, in vivo supportive data has yet to be reported.

Recently, we proposed a novel mechanism of action for metal ligand induced neurotherapy in AD. Our in vitro studies

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[a] Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; CQ, clioquinol; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGF, epidermal growth factor; HB-EGF, heparin binding-EGF; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinase; GSK3, glycogen synthase kinase 3; IGFBP4, insulin like growth factor-binding protein 4; JNK, c-Jun N-terminal kinase; LIF, leukemia inhibitory factor; $M^{II}$(btsc), metal-bis(thiosemicarbazone); MIP-1$\beta$, macrophage inflammatory protein-1 beta; MMP2, matrix metalloprotease 2; PI3K, phosphoinositol-3-kinase; PS1, presenilin 1; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; STAT, signal transducers and activators of transcription; TGF, transforming growth factor; TIMP2, tissue inhibitor of metalloprotease 2.

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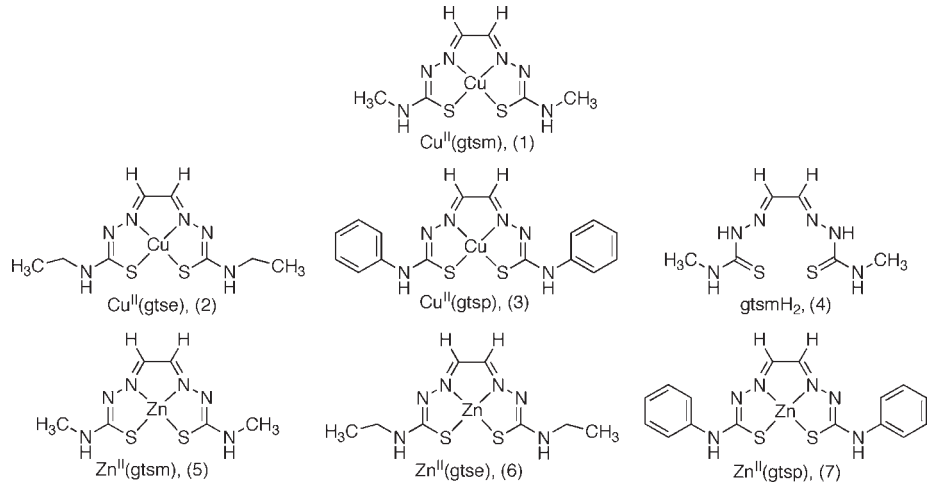


**Figure 1.** Structures of $Cu^{II}$(btsc) and $Zn^{II}$(btsc) complexes used in this study. Structural studies of $Zn^{II}$(btsc) complexes reveal they are often five-coordinate, with solvent (DMSO) occupying the fifth apical site.[62]

demonstrated that CQ complexed to Cu or Zn substantially elevated intracellular levels of these metals, resulting in activation of phosphoinositol-3-kinase (PI3K), phosphorylation of the downstream targets, Akt and GSK3 as well as stimulating ERK activation. This led to increased matrix metalloprotease 2 (MMP2) degradation of extracellular A$\beta$.[22] In subsequent studies, we demonstrated, using bis(thiosemicarbazonato)−metal complexes ($M^{II}$(btsc)), that the biometals (Cu or Zn) must be released intracellularly to induce neurotherapeutic effects.[23,24]

Further studies have shown that $M^{II}$(btsc) have a strong potential as therapeutic agents for treatment of neurodegenerative disorders. Crouch et al.[24] have recently shown that glyoxalbis(*N*(4)-methylthiosemicarbazonato)$Cu^{II}$] **1** ($Cu^{II}$(gtsm)) (Figure 1)[25] delivers Cu into neurons, both in vitro and in vivo, activating PI3K and inhibiting GSK3, resulting in reduction of neurotoxic A$\beta$ trimers and $\tau$-phosphorylation and improving cognition in APP/PS1 AD-like mice. This is the first report that delivering intracellular bioavailable Cu to the brain can induce neurotherapeutic effects in AD and potentially other disorders.[24]

Despite these promising outcomes, the upstream mechanism of action by $M^{II}$(btsc) in vitro or in vivo is not understood. While biometals must be complexed to a lipid permeable carrier ligand[26] and released inside the cell,[23] it is not known if the activation of PI3K-Akt-GSK3 and MAPK (ERK) pathways are mediated by cell membrane receptors or occurs by alternative mechanisms such as direct interaction of downstream proteins with metal or modulation of cellular redox environment. Recently, we reported that $Cu^{II}(CQ)_2$ complexes can activate the epidermal growth factor receptor (EGFR) in epithelial cells and neurons.[27] Activation was mediated by ligand-independent phosphorylation of EGFR by src-kinase and was specific for $Cu^{II}(CQ)_2$ and not Fe or Zn complexes. Significantly, we demonstrated that activation of EGFR by $Cu^{II}(CQ)_2$ resulted in downstream activation of ERK but not PI3K-Akt or JNK and resulted in up regulation of A$\beta$ degradation by metalloproteases.

EGFR activation, either ligand-dependent (i.e., EGF, TGF-$\alpha$) or ligand-independent (i.e., integrin-mediated) has been well characterized due to its important role in cancer cell replication. EGFR is a membrane-associated receptor tyrosine kinase (RTK) and a member of the ErbB family of kinase receptors.[28−30] Activation by cognate ligands (EGF, TGF-$\alpha$, HB-EGF, and others) or ligand-independent activation results in downstream signaling through PI3K, ras-raf-ERK, or JAK-STAT pathways. The consequences of EGFR activation are many and varied, depending on cell-type, stimuli, and co-ordination with other receptors and pathways.[28] Consequences can include modulation of replication, differentiation, and survival. Activation of EGFR in the brain is poorly understood. EGFR is important during development, but few studies have characterized EGFR activation in adult brain.[31−34] Interestingly, reports have shown that EGFR activation in astrocytes is important for neuronal survival and/or neuroprotection.[32] This mechanism may involve astroglial-mediated neuronal support.

Because of the potential importance of glial cell EGFR activation in neuroprotection, we have examined $M^{II}$(btsc) (Figure 1) stimulation of EGFR in an astroglial cell line. Our findings reveal differential activation of glial EGFR by both Cu and Zn btsc complexes. Activation of EGFR occurred through ligand-independent phosphorylation of EGFR tyrosine residues, 845, 1068, and 1045, however, in contrast to $Cu^{II}(CQ)_2$, EGFR phosphorylation by the $M^{II}$(btsc), compound **1** did not appear to require src kinase activation but was instead mediated by $M^{II}$(btsc) inhibition of protein tyrosine phosphatase (PTP) activity. Activation of EGFR by compound **1** amplified downstream phosphorylation of PI3K-Akt-GSK3, ERK, and JNK and resulted in release of growth factors and cytokines. Furthermore, we found that compound **1** inhibited tissue inhibitor of MMP2 (TIMP-2), which may explain how metal complexes promote activation of MMP2. These studies provide an important insight into the mechanism of action of neuroprotective $M^{II}$(btsc) and provide a basis for future studies into this novel approach to AD therapy.

## Results

**Activation of Glial EGFR by Cu−btscs.** Initially, we confirmed that the U87MG-EGFR cell line overexpressed the EGFR protein when compared to nontransfected cells. Western blotting of U87MG-EGFR cell lysates with antibody against total EGFR revealed substantially higher EGFR expression compared to nontransfected cells (see Figure 2). We then examined if compound **1** treatment induced activation of a specific cytotoxic response. Our

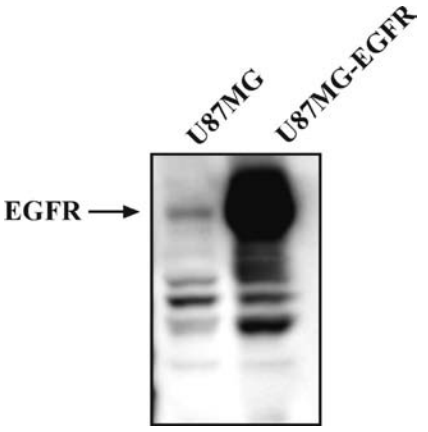


**Figure 2.** U87MG-EGFR glial cells express high levels of total EGFR. Wild-type (nontransfected) and U87MG cells stably transfected with EGFR (U87MG-EGFR) were Western blotted for total EGFR. Stably transfected cells revealed relatively higher levels of EGFR compared to nontransfected cells.

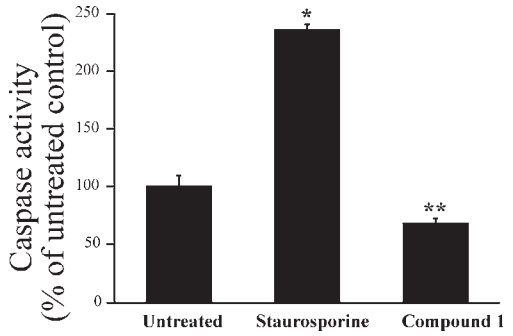


**Figure 3.** Compound **1** did not induce a specific pro-apoptotic response in U87MG-EGFR cells. Cultures of U87MG-EGFR glial cells were treated for 5 h with 25 $\mu$M compound **1** and cell lysates examined for caspase 3 activity with an enzymatic assay. Compound **1** induced a significant decrease in caspase 3 activity compared to untreated control (** $p < 0.005$). Staurosporine (1 $\mu$M) treatment of cells was included as a positive control for caspase 3 activation and revealed a significant elevation in caspase 3 activity compared to untreated controls (* $p < 0.0001$).

studies have shown that $Cu^{II}$(btsc) can affect endo- and exocytotic pathways (unpublished observations), making the use of conventional viability assays unsuitable due to their reliance on uptake and/or exclusion of compounds. Therefore, we measured whether compound **1** induced glial apoptosis by assaying caspase 3 activity in cell lysates after treatment with 25 $\mu$M compound **1** for 5 h. Figure 3 shows that compound **1** inhibited caspase 3 activity, and this is consistent with caspase 3 and 8 activity observed in neuronal cell lines treated with compound **1** (data not shown). These findings indicated that compound **1** treatment did not induce a specific apoptotic response in treated glial cells during the treatment period.

We next established that compound **1** (our prototype $M^{II}$(btsc)) induced activation of EGFR. U87MG-EGFR cells were treated with 25 $\mu$M compound **1** or DMSO as vehicle control for 5 h, and EGFR phosphorylation (at tyrosine 1068) was determined by Western blot. Figure 4A shows that EGFR tyrosine phosphorylation and therefore receptor activation occurred on treatment with compound **1**. The specificity of this effect was confirmed using a blocking peptide for phospho-tyrosine 1068 that substantially reduced the detection of tyrosine 1068 (Figure 4A).

Full activation of EGFR and induction of subsequent downstream signaling events is dependent upon the phosphorylation of specific tyrosine residues within different domains of the receptor (i.e., tyr845, tyr992, tyr1045, and tyr1068). Phosphorylation of each of these tyrosine residues was determined by Western blot in the U87MG-EGFR cells after treatment with 25 $\mu$M compound **1** or DMSO for 5 h. Figure 4B shows that tyr845, tyr1045, and tyr1068 were substantially phosphorylated upon treatment with compound **1** compared to vehicle control while little change in phosphorylation of tyr992 was observed. The reason for the latter could reflect the fact that tyr992 may be more susceptible to subtle oxidative damage associated with $Cu^{II}$, which can inhibit phosphorylation.[35] These findings indicated that compound **1** induced phosphorylation at multiple sites and was likely to have complex effects on EGFR-mediated signaling.

To determine if the effect of compound **1** on EGFR activation was cell-type specific, we examined receptor activation in different cell lines. Cells were treated with 25 $\mu$M compound **1**, $gtsmh_2$ ("free" ligand), $Cu^{II}$ alone, or vehicle control (DMSO) for 5 h. $Cu^{II}(CQ)_2$ was included as a positive control as $Cu^{II}(CQ)_2$ has previously been shown to induce activation of EGFR in multiple cell types.[27] Compound **1** induced robust activation of EGFR in the U87MG-EGFR glial cells line as shown in Figure 4A−C and in HeLa epithelial cells (Figure 4D) but not the SY5Y human neuroblastoma cell line (Figure 4E). Interestingly, $Cu^{II}(CQ)_2$ complexes induced activation of EGFR in HeLa cells (as previously reported, Price et al.[27]) but did not activate glial EGFR. The reason for this is not known. Substantial activation of EGFR was not observed in any of the cell lines with $Cu^{II}$ or $gtsmh_2$ alone.

Next, we examined the sensitivity of EGFR activation by compound **1**. A serial dilution of compound **1** was performed on U87MG-EGFR cells for 5 h. Figure 5A revealed that exposure to 10 $\mu$M, or higher concentrations, of compound **1** induced potent activation of EGFR compared with vehicle control.

To gain a further insight into the mechanism of EGFR stimulation by btsc compounds, the time-course of receptor phosphorylation was examined. This was determined by treating cells with 25 $\mu$M compound **1** for 0.5, 1, 2, 4, or 6 h, and EGFR phosphorylation was determined by Western blot. Figure 5B shows that EGFR phosphorylation by compound **1** was rapid and peaked at 4 h post treatment. Interestingly, we previously found that $Cu^{II}(CQ)_2$ complexes induced delayed activation of EGFR with phosphorylation not occurring until more than 4 h post-treatment.[27]

Structurally different $Cu^{II}$(btsc) induce diverse effects on metal uptake and A$\beta$ levels.[23] We therefore investigated the ability of structurally different $Cu^{II}$(btsc) to promote EGFR phosphorylation. U87MG-EGFR cells were treated for 5 h with 25 $\mu$M compound **1**, glyoxalbis(*N*(4)-ethylthiosemicarbazonato)$Cu^{II}$] **2** ($Cu^{II}$(gtse)),[36]or glyoxalbis(*N*(4)-phenylthiosemicarbazonato)$Cu^{II}$] **3** $Cu^{II}$(gtsp),[23] metal-free ligand glyoxalbis(*N*(4)-methylthiosemicarbazonato) **4** ($gtsmh_2$)[25] (Figure 1), or vehicle control. Compounds **1** and **2**, but not **3**, were very potent activators of EGFR (Figure 5C).

These findings correlated closely with the effects of the same $Cu^{II}$(btsc) on Cu uptake and modulation of A$\beta$ levels seen in APP-CHO cells.[23] In that study, compounds **1** and **2** induced a substantial decrease in extracellular A$\beta$, while

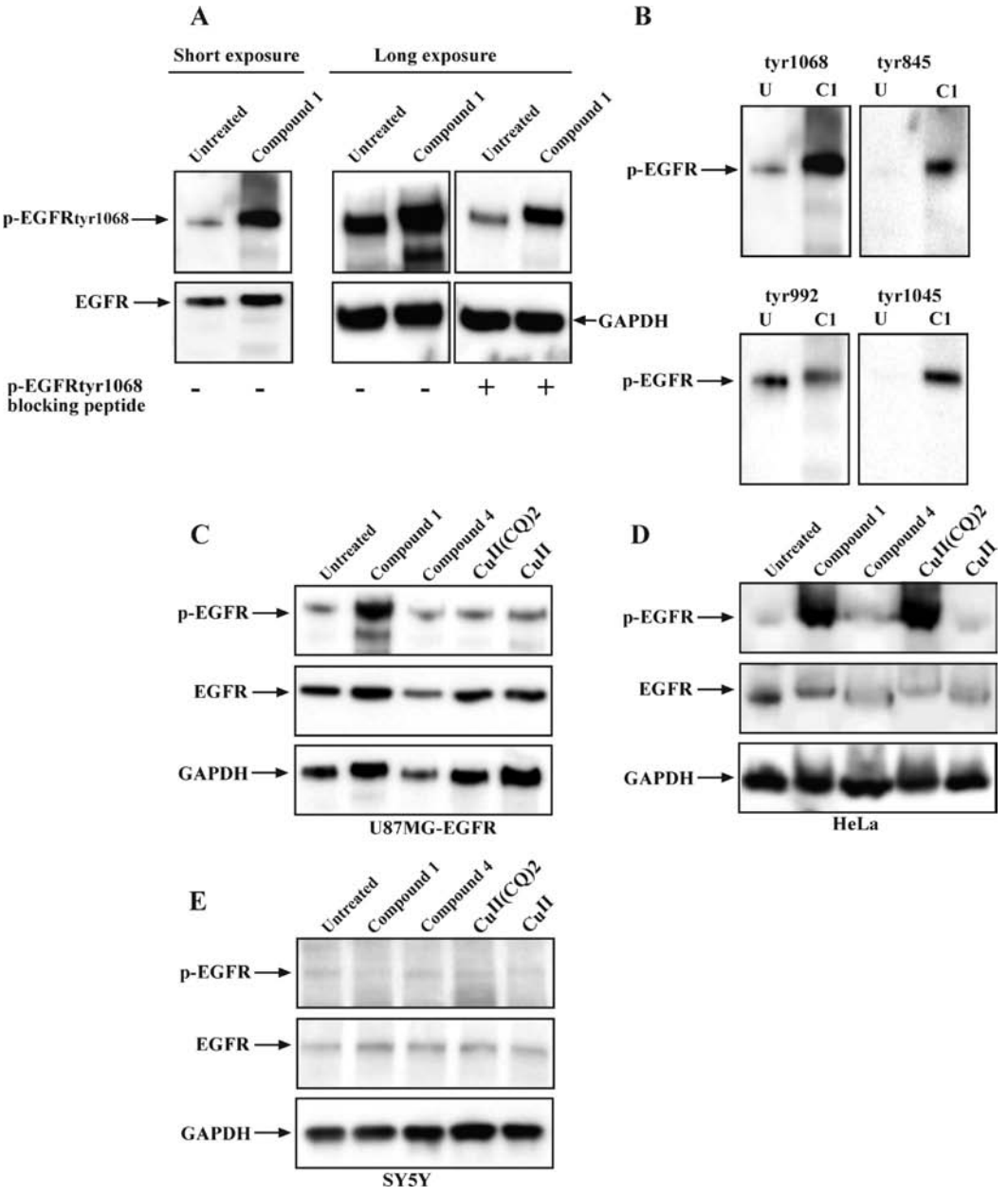


**Figure 4.** Compound **1** induced activation of EGFR. (A) U87MG-EGFR glial cells were treated with 25 $\mu$M compound **1** for 5 h and activation of EGFR was determined using antisera to phospho-tyr1068. The images revealed that compound **1** induced robust EGFR activation after a short exposure of the blotting membrane (~10 s). After a longer exposure (~30 s), several lower molecular weight bands of unknown origin were observed and were potentially degradation products of EGFR. A longer exposure in the presence of a p-tyr1068 blocking peptide significantly decreased the observable signal for p-tyr1068. (B) Compound **1** treated glial lysates were probed for phospho-tyr1068, 845, 992, or 1045. Compound **1** treatment induced significant phosphorylation of tyr1068, 845, and 1045 but not 992. U = untreated, C = compound **1** treated. (C−E) U87MG-EGFR, HeLa, or SY5Y cells respectively were treated with compound **1**, **4**, $Cu^{II}(CQ)_2$, or $Cu^{II}Cl_2$ and probed for phosphorylation of EGFR tyr1068. Compound **1** induced phosphorylation of EGFR in U87MG-EGFR cells and HeLa cells but not SY5Y cells. CQ-$Cu^{II}$ induced activation of EGFR in HeLa cells but not U87MG-EGFR cells.

compound **3** had a limited effect. The reason for these effects may have been related to the level of Cu uptake induced by the different $Cu^{II}$(btsc). In the study by Donnelly et al.,[23] the loss of A$\beta$ induced by each $M^{II}$(btsc) correlated with the level of Cu uptake in APP-CHO cells. We therefore investigated whether activation of EGFR in U87MG-EGFR cells correlated with Cu uptake. This was performed by measuring Cu uptake after treatment with $Cu^{II}$(btsc) for 5 h. However, while compound **2** induced a high level of uptake, compound **1** and **3** induced relatively lower levels of uptake (Figure 5D). It may be possible that Cu levels must reach a certain threshold to activate EGFR, and this was obtained with $Cu^{II}$(gtse) and compound **1** but not **3**. However, this requires further investigation. Alternatively, the structural differences in the substituents in the *N*4 position of ligands may affect delivery of Cu to a critical cellular compartment necessary for EGFR activation, compound **3** has a phenyl substituent compared to the alkyl substituents of compounds **1** and **2**.

**Activation of EGFR by Compound 1 is Not Mediated through Binding of Cognate Ligands.** EGFR can be activated by several processes including oxidative stress-mediated

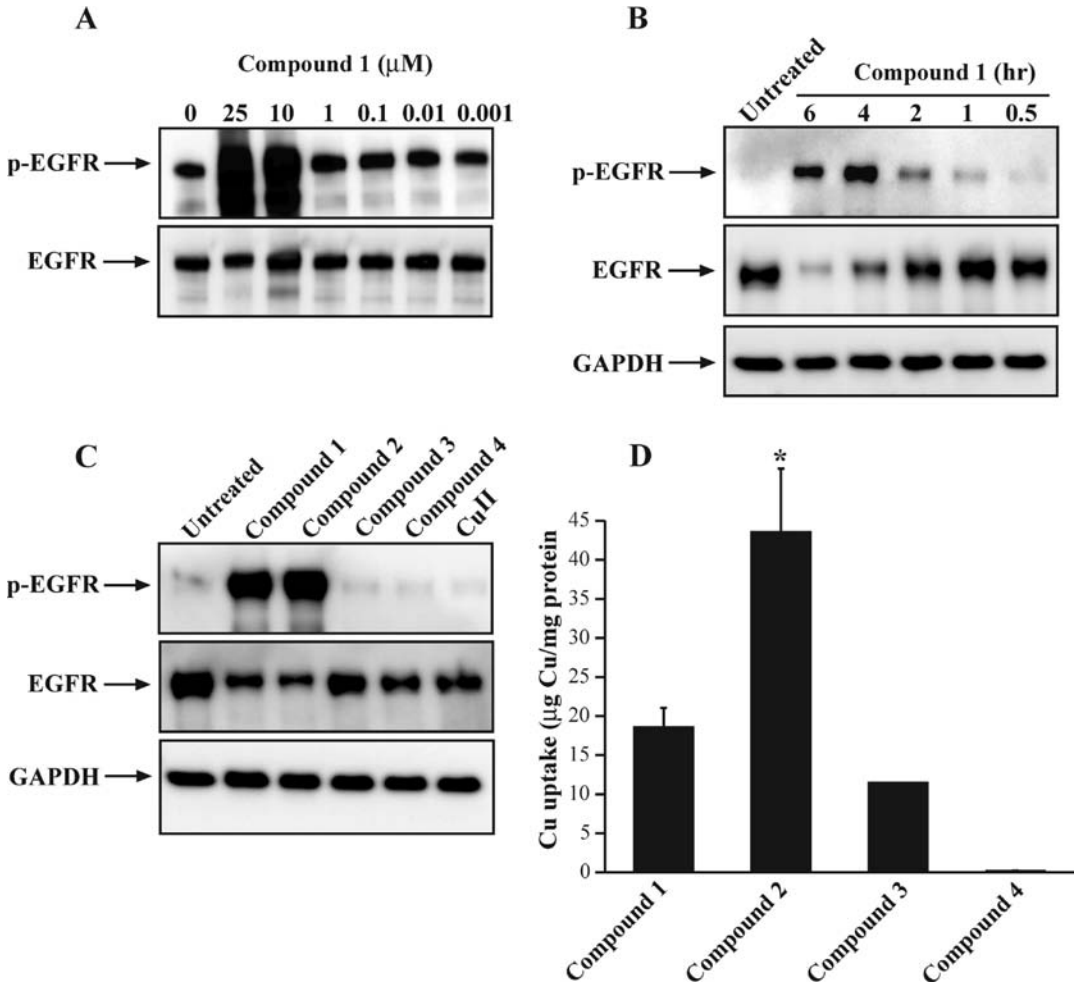


**Figure 5.** Activation of EGFR by $Cu^{II}$(btsc) was rapid and dependent on the structure of the btsc ligand. (A) U87MG-EGFR glial cells were treated with 0.001−25 $\mu$M compound **1** for 5 h and lysates were examined for activation of EGFR. A slight increase in EGFR phosphorylation was observed at 1 $\mu$M compound **1**, but robust activation was induced by 10 $\mu$M compound **1**. (B) Glial cells were treated with 25 $\mu$M compound **1** for 0.5−6 h and cell lysates were examined for activation of EGFR. Modest activation was evident at 0.5 h after activation and peaked at 2−4 h post-treatment. (C) Treatment of glial cells with different $Cu^{II}$(btsc) (25 $\mu$M) for 5 h resulted in potent activation by compound **1** and compound **2** and weak activation by compound **3**. Changes to the expression of total EGFR were evident in $Cu^{II}$(btsc) treated cells, however, this was not a consistent finding and did not correlate with the level of EGFR phosphorylation (compare total EGFR for compound **2** and **3** with phospho-EGFR for the same). (D) Cells were treated with 25 $\mu$M compound **1** for 5 h and levels of Cu were determined in cell lysates by ICP-MS and converted to $\mu$g Cu/mg protein. Negligible Cu was observed with $gtsmh_2$. Compound **2** induced relatively higher Cu uptake compared to compounds **1** or **3** * $p < 0.05$ compared to compound **1**.

phosphorylation of tyrosine residues.[37,38] As Cu is a potent redox active metal ion with the potential to induce oxidative stress, we needed to establish whether Cu-mediated oxidative stress following compound **1** treatment was the mechanism of EGFR activation. To achieve this, we compared EGFR activation induced by compound **1** to the effects of various $Zn^{II}$(btsc). Zn is a nonredox active metal and therefore is unlikely to induce direct oxidative stress-mediated EGFR activation.

U87MG-EGFR cells were treated with 25 $\mu$M compounds **1**, **5** ($Zn^{II}$(gtsm)), **6** ($Zn^{II}$(gtse)), **7** ($Zn^{II}$(gtsp)) (Figure 1),[23] $Zn^{II}Cl_2$ alone, or vehicle control for 5 h, and EGFR activation was determined by Western blot. Figure 6A shows that compound **7** was able to induce increased phosphorylation of EGFR relative to vehicle control. However, compared to compound **1**, the activation of EGFR by compound **7** was weak. No activation of EGFR was seen with compounds **5** and **6** or $Zn^{II}$ alone. These data demonstrated that compound **1** is more potent at activating EGFR than $Zn^{II}$(btsc) and therefore could involve Cu-mediated activation.

We examined this further by adding 1 mM ascorbate and 200 $\mu$M Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP)[39] to cells treated with 25 $\mu$M compound **1** or **7**. Ascorbate is a known antioxidant and MnTMPyP acts as a cell permeable free radical scavenger. Figure 6B shows that the addition of ascorbate or MnTMPyP had no effect on the ability of compound **1** to induce activation of EGFR, suggesting that activation of EGFR by compound **1** does not involve altered redox activity. Similarly, ascorbate and MnTMPyP had no effect on the ability of compound **7** to induce weak EGFR phosphorylation. These data provide strong evidence that activation of EGFR by compound **1** did not involve oxidative stress. However, Cu could still alter the redox environment or redox state of a protein without inducing oxidative stress or free radicals.

Next, we examined whether compound **1** induced activation through increased release of cognate ligands such as EGF or HB-EGF. Conditioned medium collected from compound **1**-treated U87MG-EGFR cells was collected and tested on the RayBio human growth factor antibody

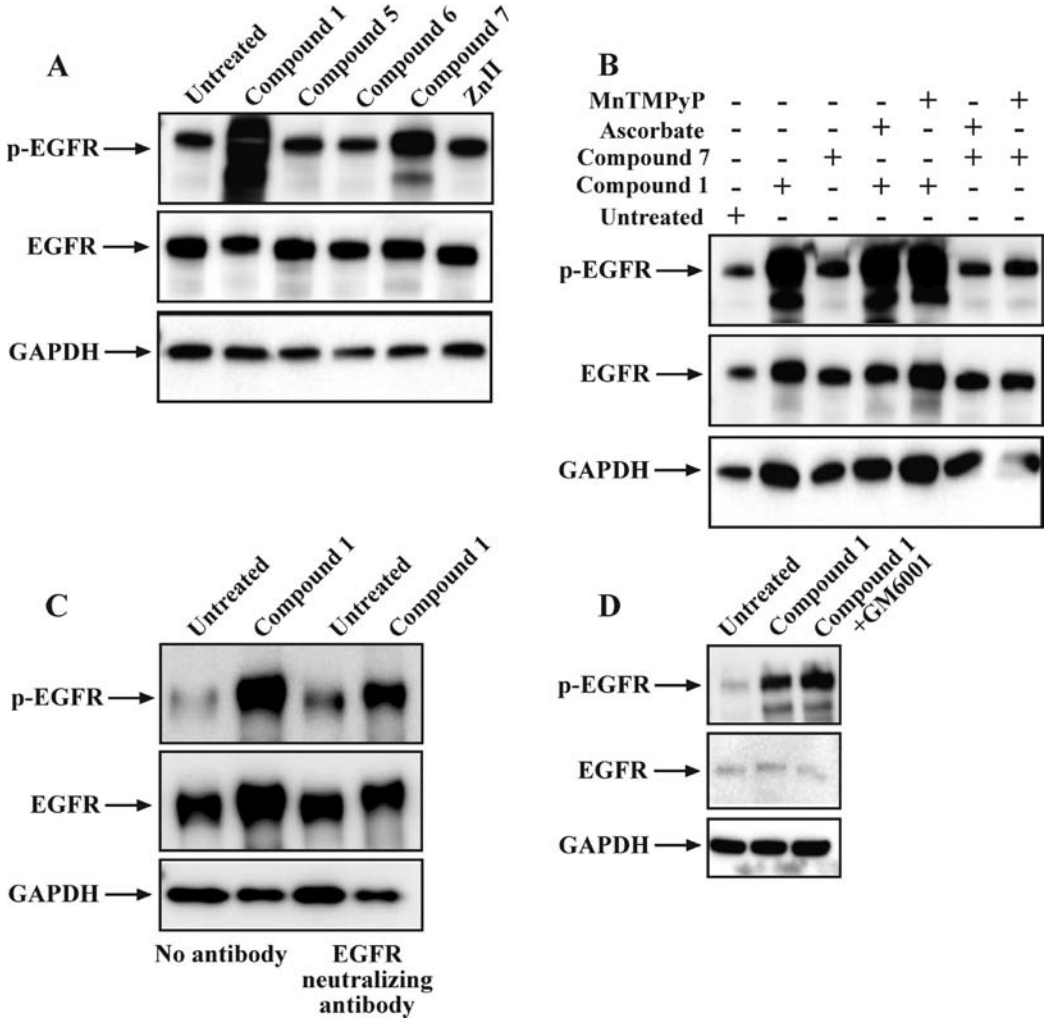


**Figure 6.** Activation of EGFR by $M^{II}$(btsc) was cognate ligand-independent. (A) U87MG-EGFR glial cells were treated with 25 $\mu$M $Cu^{II}$ or $Zn^{II}$(btsc) for 5 h and cell lysates were examined for activation of EGFR. Compound **7** induced relatively weak activation of EGFR while other $Zn^{II}$(btsc) had no effect. (B) Glial cells were treated with compound **1** or **7** with or without 1 mM ascorbate and 200 $\mu$M MnTMPyP. Cotreatment with the antioxidant or free radical scavenger had no effect on phosphorylation of EGFR. (C) Glial cells were treated with compound **1** and an EGFR neutralizing antibody. The antibody had no effect on activation of the receptor by EGFR. (D) Glial cells were treated with compound **1** and the broad-spectrum metalloprotease inhibitor, GM6001 to inhibit release of soluble cognate ligands. GM6001 did not inhibit EGFR activation by compound **1**.

**Table 1.** Protein Array Analysis of Soluble Ligand Release from Compound **1**-Treated U87MG-EGFR Glial Cells

| protein | untreated | compound **1** treated (% change compared to untreated) | *P* value (compared to untreated) | compound **1** + PD153035 | *P* value (compared to compound **1** alone) |
|---|---|---|---|---|---|
| EGF | 100 ± 7 | 100 ± 6 | 1.0 | na | na |
| HB-EGF | 100 + 6 | 53 ± 17 | 0.07 | na | na |
| TGFα | 100 ± 6 | 52 ± 22 | 0.14 | na | na |
| MIP-1$\beta$ | 100 ± 9 | 140 ± 4 | 0.002 | 86 ± 6 | 0.001 |
| thrombo[a] | 100 ± 9 | 151 ± 7 | 0.001 | 88 ± 6 | 0.0002 |
| PDGF | 100 ± 2 | 138 ± 8 | 0.006 | 117 ± 7 | 0.08 |
| IGF-BP4 | 100 + 3 | 126 ± 3 | 0.0001 | 106 ± 5 | 0.005 |
| LIF | 100 ± 2 | 119 ± 3 | 0.001 | 93 ± 4 | 0.0006 |
| TGF-$\beta$2 | 100 ± 18 | 148 ± 5 | 0.01 | 88 ± 5 | 0.0001 |
| TIMP-2 | 100 ± 14 | 16 ± 3 | 0.0001 | 7 ± 4 | 0.08 |

[a] *Thrombopoetin*

array. Table 1 shows that there was no increase in expression of three EGFR ligands (EGF, HB-EGF, or TGFα) in compound **1**-treated cultures when compared to controls.

These results indicated that EGFR activation by compound **1** did not involve binding of cognate ligands, however, additional soluble ligands not examined with the protein array may have been involved in modulating receptor activity (e.g., amphiregulin). Therefore, we further examined if cognate ligand interaction with EGFR was involved in its activation by $Cu^{II}$(btsc). This was performed using neutralizing antisera to the receptor. U87MG-EGFR cells were treated with 25 $\mu$M compound **1** or vehicle control for 5 h with or without anti-EGFR neutralizing antibody (10 $\mu$g/mL). Figure 6C shows that the neutralizing antibody

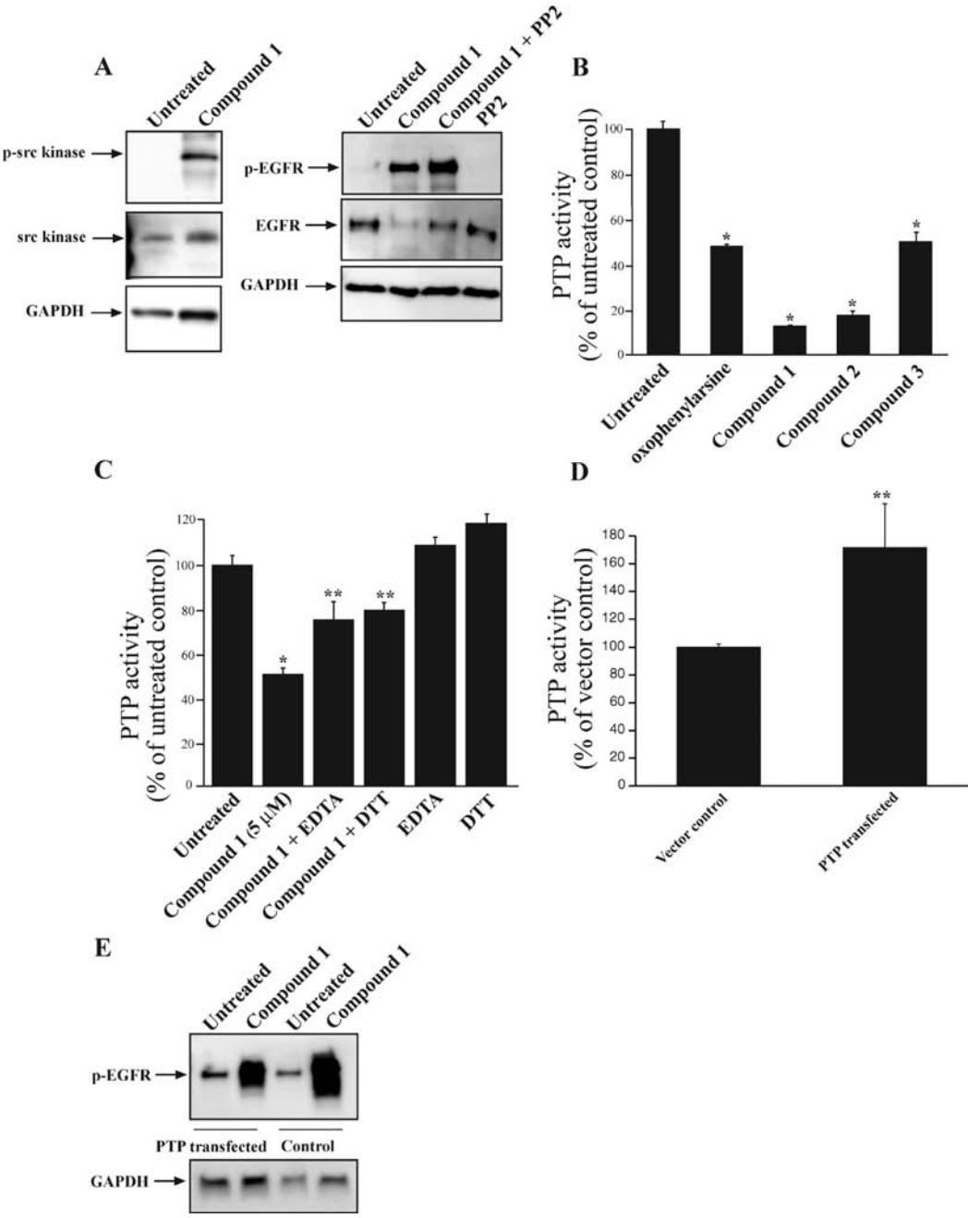


**Figure 7.** Activation of EGFR correlated with inhibition of PTP. (A) U87MG-EGFR glial cells were treated with 25 $\mu$M compound **1** for 5 h and cell lysates were examined for activation of src-kinase. Compound **1** induced phosphorylation of src-kinase, however, inhibition of activation using 25 $\mu$M PP2 did not affect EGFR activation. (B) Glial cells were treated with 25 $\mu$M compounds **1**−**3** or the positive control oxophenylarsine (3 $\mu$M) and cell lysates examined for PTP activity. Each of the $Cu^{II}$(btsc) induced potent inhibition of PTP activity with the level of inhibition broadly correlating with the level of EGFR activation induced by each $Cu^{II}$(btsc). * $p < 0.002$, 0.001, 0.001, and 0.006 for oxophenylarsine, compounds **1**−**3** as compared to untreated control. (C) Glial cells were treated with 5 $\mu$M compound **1** for 5 h and cell lysates treated for a further 30 min with 0.1 mM EDTA or 10 mM DTT. Treatment of lysates with either EDTA or DTT significantly inhibited the loss of activity induced by compound **1**. * $p < 0.005$ compared to untreated control, ** $P < 0.05$ and $P < 0.01$ for EDTA and DTT cotreatment compared to compound **1** alone. (D) Cells were transfected with PTP1b or vector control and PTP activity determined in cell lysates. Transfection of U87MG-EGFR cells with PTP1b significantly increased PTP activity. ** $p < 0.01$. (E) PTP1b or control cells were treated with compound **1** (25 $\mu$M) for 5 h and activation of EGFR was examined in cell lysates. Transfection with PTP1b substantially reduced the level of EGFR phosphoryation.

did not block phosphorylation of EGFR by compound **1**, indicating that compound **1**-mediated EGFR activation occurs through a cognate ligand-independent mechanism. Further confirmation of this was obtained by treating cells with 100 $\mu$M galardin (GM6001), a broad-spectrum inhibitor of metalloproteases that release cognate ligands from the cell surface.[40] Figure 6D shows that the addition of GM6001 had no effect on the ability of compound **1** to induce phosphorylation of EGFR, providing further evidence that EGFR activation by compound **1** was mediated by a cognate ligand-independent mechanism.

**Compound 1 Inhibits Protein Tyrosine Phosphatase Activity Resulting in Sustained Activation of EGFR.** In our previous study on $Cu^{II}(CQ)_2$ induced activation of EGFR, we found

that src-kinase activation was responsible for phosphorylation of EGFR.[27] We examined this here, and while src-kinase was activated by compound **1** (Figure 7A), its inhibition with 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine (PP2) (a src-kinase inhibitor) had no effect on EGFR activity after treatment with compound **1**[41] (Figure 7A).

Protein tyrosine phosphatases (PTPs) oppose the action of kinases by removing the phosphate from the phosphorylation sites of tyrosine kinases such as EGFR.[38,42] The ability of compound **1** and other $M^{II}$(btsc) to affect PTP activity as a possible mechanism of EGFR activation was investigated by treating U87MG-EGFR cells with compounds **1**−**3** (all at 25 $\mu$M) or DMSO control. Oxophenylarsine (3 $\mu$M) was used as a positive control to show the inhibition of PTP. A RediPlate 96 EnzCheck tyrosine phosphatase assay was performed on the treated cell lysates and showed that all treatments substantially inhibited PTP activity (Figure 7B). Compounds **1** and **2** induced a greater level of inhibition than compound **3**, which corresponded to the level of EGFR activation by these complexes. The results of the PTP activity assay and its correlation with the relative EGFR activation efficacy of the different $M^{II}$(btsc) strongly support the hypothesis that the inhibition of PTP by metals could cause sustained activation of EGFR.

We therefore investigated whether inhibition of PTP by the complexes was due to interaction of metal with the phosphatase. To achieve this, U87MG-EGFR cells were treated with compound **1** for 5 h, and cell lysates were then incubated with $EDTA^{4-}$ (0.1 mM) to remove metals from PTP or DTT (10 mM) to reverse oxidation of PTP by the metal. The lysates were subsequently examined for PTP activity as above. Initially, we found that $EDTA^{4-}$ or DTT had no effect on the level of PTP inhibition by 25 $\mu$M compound **1** (data not shown). However, given the potent inhibitory effect of compound **1** on PTP at 25 $\mu$M, we believe that the protein may have become irreversibly oxidized[43], and therefore we were unable to restore activity with $EDTA^{4-}$ and DTT. To confirm this, we treated glial cells with a lower concentration of compound **1** (5 $\mu$M) that induced less potent inhibition of PTP activity (approximately 52% of control activity for 5 $\mu$M compared to 13% activity induced by 25 $\mu$M compound **1**) and again treated lysates with $EDTA^{4-}$ or DTT. We found that in this case, treatment with $EDTA^{4-}$ or DTT partially restored PTP activity (from 52 to 76% or 80% for EDTA or DTT, respectively) after treatment with compound **1** (Figure 7C). The fact that $EDTA^{4-}$ and DTT can restore activity at lower concentrations of compound **1** suggests inhibition is most likely to be occurring via the Cu coordinating to the active site of PTP. However, at higher concentrations, other effects maybe dominating the inhibition.

The PTP inhibition most likely results in sustained activation of EGFR as previously reported.[38] To provide further support for this, we transfected U87MG-EGFR cells with PTP1b, which has been reported to dephosphorylate EGFR.[44] Transfection with PTP1b resulted in an increase in PTP1b activity by approximately 170% compared to vector control (Figure 7D). Subsequent treatment of transfected cells with compound **1** for 5 h revealed a substantial (but incomplete) reduction in EGFR phosphorylation in PTP1b transfected cells (Figure 7E). This supported the role for PTP inhibition by $Cu^{II}$(gtsm) in sustained EGFR phosphorylation.

**EGFR Activation by Compound 1 Modulates Downstream Activation of Akt, ERK, GSK3, and JNK.** EGFR activation can lead to the modulation of several downstream pathways. These include PI3K, ras/raf and STAT pathways. We have shown previously that Cu complexes activate PI3K and ras/raf pathways with neuroprotective effects.[22,26,27] We therefore examined if activation of EGFR by compound **1** resulted in downstream effects on these pathways. To examine this, U87MG-EGFR cells were treated for 5 h with 25 $\mu$M compound **1** or DMSO and the activation of different kinases involved in these pathways was determined by Western blot. Figure 8 shows that compound **1** was able to induce the phosphorylation of JNK (Figure 8A), GSK3$\beta$ (Figure 8B), and ERK (Figure 8C) but not Akt (Figure 8D) in the glial cells. On the basis of these data, it appeared that compound **1** did not induce P13K activation and, in fact, inhibited activation. The observed increase in phosphorylation of GSK3, which is often associated with PI3K activation, however, suggested that another mechanism was responsible for phosphorylation of GSK3. Alternatively, PI3K activation may have been transient. In addition, the increased phosphorylation of JNK and ERK in the presence compound **1** suggested that the ras/raf pathway was activated by compound **1** (as these kinases are known to be implicated in this pathway).

To confirm the involvement of EGFR in activation of these kinases, we treated U87MG-EGFR cells with 25 $\mu$M compound **1** alone, compound **1** plus 10 $\mu$M 4-[(3-Bromophenyl)amino]-6,7-dimethoxyquinazoline (PD153035) (EGFR inhibitor),[45] or 10 $\mu$M PD153035 alone. Treatment with PD153035 blocked activation of EGFR (Figure 8E). PD153035 treatment partially decreased compound **1**-induced phosphorylation of JNK, GSK3, and ERK (Figure 8A−C). This suggested that while the activation of EGFR did regulate the activation of these downstream kinases, their regulation is also potentially being controlled by other receptor pathways not examined in this study. Alternatively, inhibition of phosphatase activity associated with compound **1** could lead to higher than expected phosphorylation of MAP kinases in the presence of PD153035. Future studies will be needed to address these possibilities.

**Activation of EGFR by Compound 1 Induces Release of Growth Factors and Cytokines.** Finally, we examined whether activation of EGFR by compound **1** induced release of growth factors and/or cytokines that could potentially modulate neuronal function. U87MG-EGFR cells were treated with compound **1** for 5 h and conditioned medium examined for release of growth factors and cytokines using the RayBio human growth factor array as above. Media from cultures cotreated with compound **1** and the EGFR inhibitor, PD153035, were also examined in parallel. We found that treatment with compound **1** resulted in EGFR-dependent elevation in levels of macrophage inflammatory protein-1$\beta$ (MIP-1$\beta$), thrombopoetin, platelet-derived growth factor (PDGF), insulin-like growth factor-binding protein 4 (IGF-BP4), leukemia inhibitory factor (LIF), and tumor growth factor $\beta$2 (TGF-$\beta$2) (Table 1). No other significant EGFR-dependent changes to cytokines or growth factors were observed using these arrays. Whether any of these factors has a role in the beneficial effects of compound **1** in vivo remains to be determined. However, this study demonstrates that compound **1** is able to elicit activation of growth factor and cytokine release from glia via activation of EGFR. Furthermore, we also found that

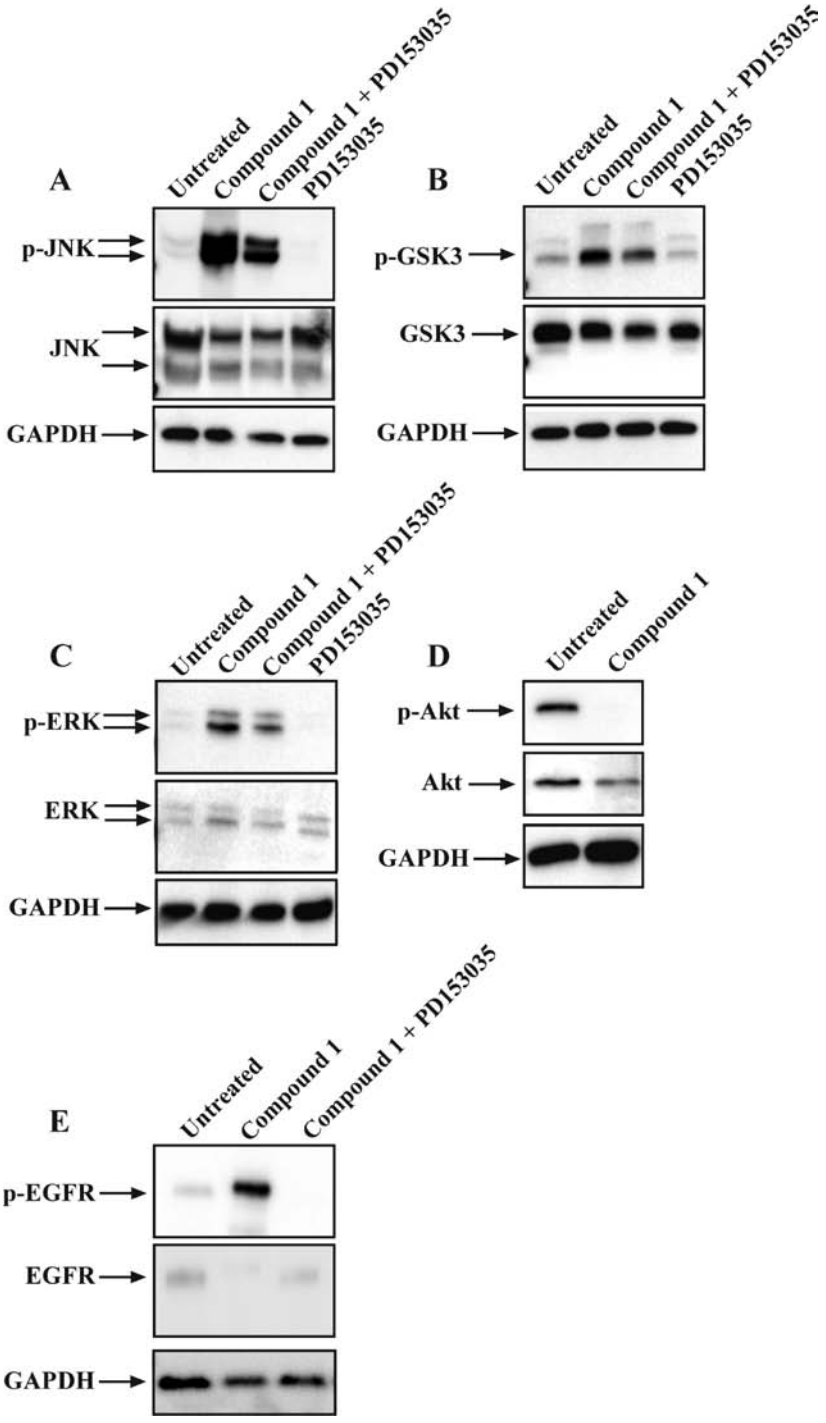


**Figure 8.** Activation of EGFR modulated downstream kinase activity. (A−D) U87MG-EGFR glial cells were treated with 25 $\mu$M compound **1** with or without 10 $\mu$M PD153035 (EGFR inhibitor) for 5 h and activation of kinases was determined in cell lysates. Compound **1** induced robust activation of JNK (A), GSK3 (B), and ERK (C) but inhibited Akt (D). Cotreatment with PD153035 partially inhibited the activation of JNK, GSK3, and ERK. (D) Treatment of glial cells with compound **1** and PD153035 demonstrated that the EGFR inhibitor blocked phosphorylation of EGFR tyr1068.

compound **1** induced a substantial decrease in the levels of tissue inhibitor of metalloprotease 2 (TIMP-2) in conditioned medium (Table 1). However, this was not due to EGFR activation, as PD153035 decreased the levels further. Our studies reveal that sustained stimulation of EGFR in glial cells by compound **1** is mediated through PTP inhibition and can result in modulation of potentially important cytokines and growth factors through EGFR and non-EGFR-mediated pathways.

## Discussion

$M^{II}$(btsc) and other metal complexes have shown promising results as a potential AD therapeutic agent in both cell culture and animal studies.[22−24] The $Cu^{II}$(btsc) class of compounds are neutral, membrane permeable complexes, with good stability in vivo, and are known to be capable of crossing the blood−brain barrier.[46−49] Once the complex, such as compound **1** encounters the reducing environment found inside cells the metal ion reduces from $Cu^{II}$ to $Cu^{I}$ and

dissociates from the ligand, becoming bioavailable. In vitro models have demonstrated a potent effect of $Cu^{II}$(btsc) on degradation of extracellular A$\beta$ peptide.[23] This effect was mediated through $M^{II}$(btsc)-induced elevation in bioavailable metal, followed by activation of PI3K-Akt-GSK3 and MAPK pathways, leading to increased MMP2 degradation of secreted A$\beta$. These effects were observed in APP-transfected epithelial cells as well as neuronal cells. Subsequent studies demonstrated potent inhibition of $\tau$ phosphorylation, depletion of A$\beta$ trimers, and improvement of short-term memory in APP/PS1 mice treated with compound **1**.[24] In the animal studies, GSK3$\beta$ and MAPK phosphorylation correlated closely with activation of these pathways in neuronal cells in vitro. Interestingly, Malm et al. reported analogous activation of PI3K and inhibition of GSK3$\beta$, resulting in cognitive improvement in APP/PS1 mice treated with the metal ligand, pyrrolidine dithiocarbamate (PDTC).[50] It is not yet known whether the same signal pathways are also activated in AD transgenic mice treated with 8-hydroxyquinoline derivatives, although Grossi et al. recently reported that CQ induced Akt phosphorylation in TgCRND8 mice.[20,51] This is interesting, as $M^{II}$(btsc) delivers bioavailable metal to the brain, whereas PDTC, CQ, and PBT2 would need to acquire the metal from elsewhere prior to delivering it into cells, possibly from A$\beta$ plaques, which contain metal ions in the AD brain.[1]

We are now investigating the activation processes involved in metal complex mediated neurotherapeutic effects. Price et al.[27] recently reported that CQ−Cu complexes activated EGFR, resulting in downstream ERK phosphorylation and increased degradation of A$\beta$. In this study, we have now shown that compound **1** and additional $M^{II}$(btsc) also induce EGFR activation although through a different mechanism than that of $Cu^{II}(CQ)_2$. For compound **1**, we demonstrated that EGFR activation was cognate ligand-independent (as supported by data shown in Figure 6C), resulting from metal-mediated inhibition of PTP activity (thus preventing dephosphorylation of activated EGFR). The inhibitory action of $M^{II}$(btsc) on PTP may be due to either Cu/Zn binding to, or oxidation of, the active site cysteine residue as inhibition could be restored by metal chelation or protein thiol reduction. The inactivation of active-site cysteine of PTP by $Cu^{II}$ has been demonstrated in cell-free assays by Kim et al.[52] Whether compound **1** can induce similar deactivation of the active-site cysteine in recombinant PTP1b will depend on the ability of Cu from the metal−ligand complex to interact with the cysteine residue under cell-free conditions. As the complex is stable and Cu is released only under intracellular reducing conditions,[46−49] manipulation of the cell-free assay conditions may be required to mimic the intracellular Cu release and subsequent Cu−cysteine interaction. Such studies however, would provide a valuable insight into the inhibitory action of compound **1** on PTP1b. The ability of oxidative damage to inhibit PTP activity has also been reported previously and our findings that EDTA and DTT can restore activity (Figure 7C) are in concordance with previous studies.[38,53−55] The fact that higher concentrations of compound **1** induced irreversible PTP inhibition is also consistent with previous reports that PTP can be irreversibly damaged through oxidation.[43,56]

While the present study did not investigate the effect of EGFR activation by $M^{II}$(btsc) on A$\beta$ metabolism, our data showed that there is a close correlation between EGFR activation and the known effects of $Cu^{II}$(btsc) on A$\beta$ homeostasis. In the study by Donnelly et al., compounds **1** and **2** induced greater uptake of Cu than Cu-GTSP, which subsequently resulted in a more substantial decrease in A$\beta$ in APP-CHO cells.[23] Here we show that compounds **1** and **2** produced stronger activation of EGFR than compound **3** (Figure 5C), suggesting that increased EGFR activation may lead to more potent effects on downstream inhibition of A$\beta$. These studies also demonstrated that structurally different $Cu^{II}$(btsc) have a different efficacy at inducing metal uptake and activating EGFR. The structural differences in the $M^{II}$(btsc) studied, in particular the number of alkyl groups attached to the diimine backbone of the ligand, created marked differences in their chemical properties.[23] These differences can include cell-type selectivity, distribution, and altered reduction potential, which in turn affects the ability of the ligand to deliver and release metal into the cell and activate EGFR.[23] Further to this, compound **1** (but not diacetylbis-(*N*(4)-methylthiosemicarbazonato)-copper(II), $Cu^{II}$(atsm)) was shown to improve cognitive performance of APP/PS1 mice,[24] indicating that subtle differences in chemical structure of the $M^{II}$(btsc) compounds can also substantially affect the therapeutic potential of these compounds.

Compound **1** was also found to be more potent in activating EGFR than $Zn^{II}$(btsc) (as shown in Figure 6A). Again, this finding correlated closely with findings by Donnelly et al., which showed that the $Zn^{II}$(btsc), compounds **5**−**7** had a limited ability to increase cellular Zn levels and degrade A$\beta$ in APP-CHO cells compared to $Cu^{II}$(btsc) with structurally identical ligand backbones.[23] This evidence supports a direct role for the metal ions derived from the complexes in downstream effects, leading to EGFR activation rather than pharmacological effects of the metal−ligand complex or the ligand alone. In addition, $Cu^{II}(CQ)_2$ complexes were found to be substantially more potent than CQ−Zn complexes at activating EGFR in epithelial cells,[27] further supporting the distinct role or each metal ion in the process, with $Cu^{II}$ being more potent. The reason for the different potency between Cu and Zn complexes is not yet known. It is possible that the activation of EGFR is mediated through release of endogenous Zn from metallothionein (MT). This can be induced by Cu (i.e., from compound **1**) displacing Zn from binding sites on the MT as previously reported,[49,57] and it has been demonstrated that $Cu^{II}$(btsc) can deliver copper to MT. If this mechanism resulted in EGFR activation (rather than from direct interaction of Cu with EGFR), then $Cu^{II}$(btsc) would be expected to have a different effect than $Zn^{II}$(btsc). For example, Zn from $Zn^{II}$(btsc) may be more effectively sequestered intracellularly by MT. Alternatively, the different effects could be related to the alteration of the intracellular reducing environment by $Cu^{II}$(btsc) as compared to relatively redox inert $Zn^{II}$(btsc). However, the latter is not supported by our data, as cotreatment of cells with $Cu^{II}$(btsc) and antioxidants did not affect $Cu^{II}$(btsc)-mediated EGFR activation. Similarly, reducing agents had no effect on EGFR activation or loss of A$\beta$ induced by $Cu^{II}(CQ)_2$.[27] Whether more subtle effects on cellular redox activity occur in response to $Cu^{II}$ compared to $Zn^{II}$ has not been explored. Kim et al.[52] reported that $Cu^{II}$ but not $Zn^{II}$ induced deactivation of PTP (VHR) by interaction with active-site cysteine. $Zn^{II}$ also inhibited PTP activity in the cell-free assays by Kim et al.,[52] but this was found to be through an alternative and undetermined mechanism.

Differential activation by Cu and Zn may also be related to different transmembrane and intracellular metal transport. This could subsequently affect subcellular localization of the metals or complexes (and each metal may be trafficked

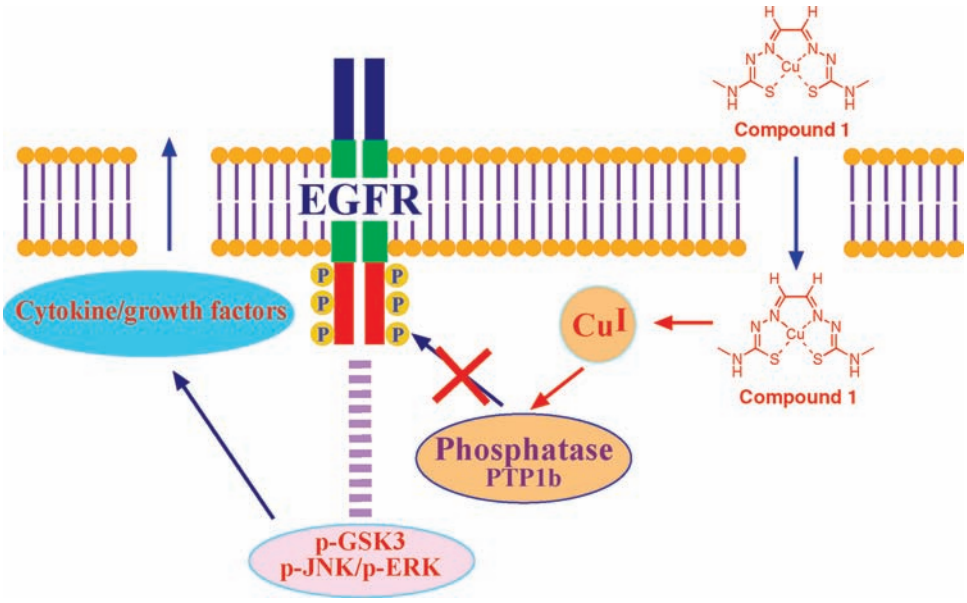


**Figure 9.** Schematic of proposed compound **1**-mediated activation of EGFR. Upon entering the cell, $Cu^{I}$ is released from compound **1** due to the intracellular reducing environment. The $Cu^{I}$ inhibits PTP1b phosphatase activity, leading to sustained phosphorylation and activation of EGFR. This results in increased phosphorylation of GSK3, JNK, and ERK with subsequent effects on levels of cytokines and growth factors released from the cell.

internally through different mechanisms). Although we do not yet know the subcellular distribution of $Cu^{II}$ and $Zn^{II}$ ions in the U87MG-EGFR cells after treatment with the complexes, previous studies have demonstrated that PTPs including PTP1b have specific subcellular localization patterns, including endoplasmic reticulum.[58,59] It will be of interest to determine if localization of the metals is associated with subcellular localization of PTP1b and/or EGFR and how this could modulate RGFR activity by $Cu^{II}$ and $Zn^{II}$-based complexes.

It also remains to be determined how $Zn^{II}$(btsc) affect A$\beta$, $\tau$, and cognition in animal models of AD. The instability of the Zn complexes in the low pH of the alimentary tract precludes the delivery of $Zn^{II}$(btsc) by oral gavage as was used for compound **1**.[24] However, nasal administration or intraperotineal injection may be an effective delivery route and $Zn^{II}$(btsc) may have reduced potential for toxic effects compared to $Cu^{II}$(btsc).

Our previous study demonstrated that $Cu^{II}(CQ)_2$ complexes can activate EGFR in a ligand-independent process involving up-regulation of src activity.[27] While compound **1**-mediated activation of EGFR also involved ligand independent elevation of src activity, this was not the primary mechanism of sustained EGFR activation. EGFR Tyr845 is phosphorylated by src kinase and can result in cognate ligand independent receptor activation.[60,61] Compound **1** was able to induce the phosphorylation of tyr845 on EGFR in the glial cells through increased src activity. However, inhibition of src-kinase did not block EGFR phosphorylation by compound **1** (Figure 7A). Our data strongly suggests an alternative process in which compound **1** and other $M^{II}$(btsc) inhibit the EGFR phosphatase, PTP1b, and this leads to accumulation of activated receptor. This would also explain why $Cu^{II}(CQ)_2$ induced delayed EGFR activation (due to src-kinase activation), but compound **1** induced rapid EGFR phosphorylation (due to rapid PTP inhibition).

Tal et al. previously demonstrated that $Zn^{2+}$ exposure in human airway epithelial cells could inhibit PTP activity and subsequently modulate EGFR.[38] Metals such as Zn (and Cu) can bind to the active site cysteine residue of the PTP. This catalytic cysteine contains a critical thiol group, which can be oxidized by metals or structural changes may be induced, leading to inhibition of the PTP. A role for $M^{II}$(btsc)-mediated oxidation of this thiol group was supported in the present study by demonstrating inhibition of PTP by $M^{II}$-(btsc) and reversal of this using metal chelators or thiol reductants to restore normal thiolate activity of the phosphatase (Figure 7C). Importantly, overexpression of PTP1b, the primary EGFR phosphatase, inhibited EGFR by compound **1** (Figure 7D,E), providing strong support for metal-mediated PTP inhibition in EGFR activation.

Interestingly, $Cu^{II}(CQ)_2$ did not induce measurable loss of PTP activity in HeLa cells.[27] It is uncertain whether this is due to different effects of 8-hydroxyquinoline−metal complexes compared to $M^{II}$(btsc) or different cell types (epithelial and glioma). The structural differences between ligands could affect metal uptake and intracellular delivery. This is supported by studies demonstrating different subcellular localization of specific $M^{II}$(btsc) complexes.[62,63] Similarly, other groups have reported diverse intracellular transport of different $M^{II}$(btsc) complexes in various cell types.[62−66] Further studies are required to map the differences in receptor activation induced by alternate complexes in each cell type.

The activation of EGFR by compound **1** was found to mediate increased release of several cytokines and growth factors by the glial cells (Table 1). As compound **1** has shown neuroprotective effects in vivo, it is possible that one or more of the released factors could be responsible for this neuroprotective activity. However, this remains to be examined and will require demonstration of in vivo EGFR activation and up-regulation of similar soluble factor release. The role of these factors (including MIP-1$\beta$, thrombopoetin, PDGF, IGF-BP4, LIF, and TGF-$\beta$2) in neuroprotective action is not known. It is also possible that increased levels of some cytokines may have antiprotective effects that reduce the efficacy of compound **1** in vivo. Further studies are required

to understand how cytokines and growth factors released by compound **1**-mediated EGFR activation can affect neuronal survival and A$\beta$ levels and $\tau$ phosphorylation in AD models. Our measurement of soluble factors in this study also identified a significant decrease in levels of TIMP-2, the endogenous inhibitor of MMP2[67] after treatment with compound **1** (Table 1). Although this was not mediated by EGFR activation, the significance of this finding may be crucial to the understanding of therapeutic compound **1** in vivo. We have shown that up-regulation of MMP2 by metal complexes results in degradation of soluble A$\beta$.[22,23] While this was shown to involve EGFR activation in epithelial cells,[27] it is possible that MMP2 activity could be modulated by different Cu-dependent mechanisms in glia and/or neurons. The finding of decreased TIMP-2 activity in this study suggests that compound **1** may induce increased MMP2 mediated degradation by inhibiting endogenous TIMP-2 activity. Further studies on TIMP-2 levels in vivo after treatment with compound **1** may help to determine if this mechanism is involved in the reduced A$\beta$.

This study has demonstrated that the potential AD therapeutic compound, compound **1**, is able to potently activate EGFR in glial and other cell types. This activation process is mediated by inhibition of PTP activity, resulting in sustained receptor activation, probably through src kinase stimulation. Structurally different Cu and $Zn^{II}$(btscs) induced variable levels of EGFR activation that correlated with the levels of soluble A$\beta$ inhibition in our previous report.[23] Moreover, we found that compound **1** induces release of several potentially important growth factors and cytokines through activation of EGFR (Figure 9) and inhibits TIMP-2 levels through a non-EGFR mechanism. These studies provide an important advance in the understanding of the cellular mechanism of action of a novel class of neurotherapeutic metal complex. Further studies are warranted to determine if similar mechanisms are involved in animal models of AD treated with $M^{II}$(btsc).

## Experimental Section

**Materials-.** Ascorbate, 5-chloro-7-iodo-8-hydroxyquinoline (CQ), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), ethylene diamine tetraacetic acid ($EDTA^{4-}$), oxophenylarsine, and PD153025 were purchased from Sigma-Aldrich (Sydney, Australia). GM6001, Mn($^{III}$)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP), and PP2 were obtained from Merck Biosciences (Melbourne, Victoria, Australia). Antibodies to total or phospho-specific forms of epidermal growth factor receptor (EGFRtyr845, EGFRtyr992, EGFRtyr1045, and EGFRtyr1068), Akt, c-Jun N-terminal kinase (JNK), c-src kinase (tyr416), glycogen synthase kinase 3 (GSK3), and extracellular signal-regulated kinase (ERK1/2) were obtained from Cell Signaling Technology (Beverly, MA). Neutralizing antibody against the extracellular ligand-binding domain of EGFR was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Bis(thiosemicarbazonato) metal and metal-free ligands including compounds **1**−**7** were synthesized as described previously.[23]

**General Procedures.** Nuclear magnetic resonance (NMR) spectra were acquired on a Varian FT-NMR 500 spectrometer. $^1$H NMR spectra were acquired at 500 MHz, and $^{13}$C NMR spectra were acquired at 125.7 MHz. All NMR spectra were recorded at 25 °C. All chemical shifts were referenced to residual solvent peaks. All spectra were recorded in DMSO-$d_6$.

Mass spectra were recorded on an Agilent 6510 Q-TOF LC/MS mass spectrometer. Reverse phase HPLC traces were acquired using an Agilent 1200 series HPLC system with an Agilent Zorbax Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 $\mu$m), a gradient elution of $H_2O$/$CH_3CN$/0.1% trifluoroacetic acid, 0−100% $CH_3CN$, a 1 mL/min flow rate, and UV spectroscopic detection at 220, 254, and 275 nm.

The compounds: compound **1**,[25,36,68,69] compound **2**,[36,68,69] compound **3**,[23] compound **4**,[25,36,68,69] compound **5**,[23] compound **6**,[23] and compound **7**[23] were all synthesized according to reported procedures. The identity and purity (>95%) of the ligands and zinc complexes were confirmed by NMR spectroscopy, mass spectrometry, and reverse-phase HPLC, while the copper complexes were analyzed by mass spectrometry and reverse-phase HPLC.

**Methods. Cell Culture.** The cell lines used in this study were human neuroblastoma SH-(SY5Y) cell line, human epithelial HeLa cell line, and human astroglial U87MG cell line transfected with the epidermal growth factor receptor (U87MG-EGFR) (a kind gift from Dr. Terrance Johns, Ludwig Institute, Melbourne, Australia). The U87MG-EGFR cells were generated by retroviral transfection of the parental U87MG cell line with human EGFR cDNA.[70] The cell line also expresses endogenous EGFR. Cells were maintained in DMEM plus 5% FBS (HeLa cells), DMEM plus 10% FBS (SY5Y cells), or DMEM/F12 supplemented with 10% FBS and 0.8% G418 (U87MG-EGFR cells). All cells were passaged at a dilution of 1:6 to 1:10 in 5% $CO_2$.

**Exposure of Cells to Metal Complexes.** Cells were grown in 6-well plates or 100 mm dishes for 2−3 days before experiments. $M^{II}$(btsc) were prepared as 10 mM stock solutions in DMSO and added to serum-free medium as previously described.[23] Metal salts ($CuCl_2$ and $ZnCl_2$) were prepared in $dH_2O$ and added at indicated concentrations and medium briefly mixed by aspiration prior to addition to cells. Control cultures were treated with vehicle (DMSO) alone. Where stated, inhibitors of EGFR (PD153035), metalloproteases (GM6001), or c-src kinase (PP2) were prepared as 10 mM stock solutions in DMSO and added at indicated concentrations. Some cultures were also cotreated with anti-EGFR (ligand binding domain) antisera (Santa Cruz) at 10 $\mu$g/mL. The antioxidant, ascorbate, and free radical scavenger, MnTMPyP, were added to cultures at 1 mM and 200 $\mu$M, respectively. For immunoblotting, cells were harvested into Phosphosafe extraction buffer (Novagen) containing protease inhibitor cocktail (Calbiochem) and stored at −80 °C until use.

**Western Blot Analysis of Protein Expression and Phosphorylation.** Cell lysates prepared in Phosphosafe extraction buffer were mixed with electrophoresis SDS sample buffer (Novex) and separated on 12% Novex SDS-PAGE tris-glycine gels. Proteins were transferred to PVDF membranes and blocked with milk solution in PBST before immunoblotting for total or phospho-specific proteins. For detection of signal transduction molecules, membranes were probed with polyclonal antisera against EGFR or phospho-EGFR (tyr1068, tyr992, or tyr845), ERK1/2 or phospho-ERK1/2, GSK3 or phospho-GSK3, JNK or phospho-JNK, c-src kinase or phospho-c-src kinase, and Akt or phospho-Akt at 1:5000. Secondary antiserum was goat anti-rabbit-HRP at 1:10000. Blots were developed using Amersham ECL Advance Chemiluminescence and imaged on a Fujifilm LAS3000 imager (Berthold, Bundoora, Australia). Expression of GAPDH was used as a protein loading control where necessary.

**Inductively Coupled Plasma Mass Spectrometry (ICP-MS).** Cells were treated with $M^{II}$(btsc) for 5 h, and metal levels were determined in cell pellets by ICP-MS as described previously.[23] Changes in metal levels were adjusted to $\mu$g metal per mg protein.

**Growth Factor Array for the Determination of Up-regulation of Growth Factor Release.** Release of human growth factors and cytokines in U87MG-EGFR conditioned media was measured using the RayBio human growth gactor antibody array 1 kit (RayBiotech, Norcross, GA). Slides were incubated in blocking

buffer for 30 min at room temperature (RT) with gentle shaking. One mL of the conditioned media (U87MG-EGFR treated with compound **1** or vehicle control for 5 h) was added per well and incubated for 2 h at RT. The slides were washed five times in wash buffer at RT and primary antibody was added for 2 h at RT. Slides were washed again and HRP-conjugated streptavidin was added for 2 h, followed by addition of detection buffer for 2 min. The membrane was then visualized at 10 s increments in a Fujifilm LAS3000 imager using chemiluminescence.

**Caspase-3 Assay.** The caspase-3 colorimetric assay was performed according to kit instructions (R&D Systems, MN). Briefly, cells treated with varying concentrations of compound **1** for different durations were collected at 250*g* for 10 min. The supernatant was removed and cells were lysed with 25 $\mu$L of cold lysis buffer per $1\times 10^6$ cells. Cells were then incubated on ice for 10 min and centrifuged at 10000*g* for 1 min. To a 96-well plate, 50 $\mu$L of the supernatant was added to each reaction well, along with 50 $\mu$L of the 2× reaction buffer 3 containing 10 $\mu$L of 1 M dithiothreitol (DTT). Then 5 $\mu$L of caspase-3 colorimetric substrate was added to each reaction well and the plate was incubated for 1 h before reading in the WALLAC Victor$^2$ plate reader at 405 nm. The plate was also read following overnight incubation.

**Transient Transfection of PTP1b.** U87MG-EGFR cells were transiently transfected with a plasmid expressing PTP1b. The pJ3H-PTP1b construct (plasmid 8601) was purchased from Addgene. Subsequent transfection-grade plasmid DNA was achieved by large-scale growth, harvesting, and purification using PureLink HiPure Plasmid Midi Kit according to the supplier's instructions (Invitrogen). Transfections were carried out using Fugene HD transfection reagent (Roche Applied Science). Optimization of the supplier's instructions indicated that a Fugene HD:DNA ratio of 6:2 promoted the transfection process.

**Protein Tyrosine Phosphatase Assay.** The phosphatase activity assay was carried out using the RediPlate 96 EnzCheck tyrosine phosphatase assay kit (Invitrogen, Melbourne, Australia) as previously reported.[27]

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

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