Biometals (2011) 24:117–133
DOI 10.1007/s10534-010-9380-7

# Cell cycle arrest in cultured neuroblastoma cells exposed to a bis(thiosemicarbazonato) metal complex

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Received: 17 January 2010 / Accepted: 18 September 2010 / Published online: 8 October 2010


**Abstract** Brain tumors such as neuroblastomas and gliomas are often refractory to current treatments. Development of metal-based drugs may offer an alternative approach due to the ability to deliver radionuclides or cytotoxic metals to the tumor. Previous studies have shown that diacetyl-bis(*N*(4)-methylthiosemicarbazonato)-copper(II) ($Cu^{II}$(atsm)) can selectively target hypoxic tumors and this feature has been utilized for development of imaging and radiotherapy. However, we have recently shown that glyoxal-bis(*N*(4)-methylthiosemicarbazonato)-copper(II) ($Cu^{II}$(gtsm)) can target the brain in animal models of neurodegeneration. Unlike $Cu^{II}$(atsm), $Cu^{II}$(gtsm) is able to release Cu intracellularly under normoxic conditions. Glyoxal-bis(thiosemicarbazones) have reported anticancer effects but little is known about the cellular mechanisms involved. Therefore, in this study, we used protein microarray analysis to investigate the effect of $Cu^{II}$(gtsm) on neuroblastoma cell growth in vitro. Treatment of the human neuroblastoma cell line BE(2)-M17, resulted in cell cycle arrest as assessed by fluorescent activated cell sorting (FACS) analysis. Rapidly arrested growth was not associated with onset of apoptosis. Instead, protein microarray analysis revealed that $Cu^{II}$(gtsm) rapidly and potently reduced cyclin D1 expression, while increasing Kip2 expression. Other changes observed were decreased Cdk7 expression and activation of CHK2. These changes may be associated with the cell cycle arrest. We also observed a potent decrease of total and phosphorylated insulin-like growth factor receptor (IGF-IR) by $Cu^{II}$(gtsm) which is associated with modulation of cyclin D1 expression. Our studies reveal important insights into the potential anticancer activity of $Cu^{II}$(gtsm). Further studies are needed to examine the therapeutic potential of $Cu^{II}$(gtsm) and other bis(thiosemicarbazonato) metal complexes as metallo-drugs for treatment of systemic or brain tumors.

**Keywords** Copper · Cell cycle · Bis(thiosemicarbazone) · Protein microarray

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## Introduction

The wide range of pharmacological activity of bis(thiosemicarbazones) has stimulated continued interest in their synthesis and interactions with biological systems. The carcinostatic action of glyoxal-bis(thiosemicarbazones) was first investigated in 1958 in a study against Sarcoma 180 in Swiss mice (French and Freelander 1958a). A variety of different derivatives were investigated with best results achieved when the compounds were administered orally in the diet at dosages ranging from 50–150 mg/kg for 7 days. Treatment with glyoxal-bis($N^4$-methyl-3-thiosemicarbazone) (gtsm$H_2$, Fig. 1) resulted in a 45% reduction in the tumour weight when compared to untreated controls. In this early investigation the authors suggested "a mechanism of action involving inactivation or translocation of metal ions was possible" (French and Freelander 1958b). Interestingly bis(thiosemicarbazones) derived from 2,3-butanedione such as astm$H_2$ (Fig. 1) were shown to be inactive in this model.

After these pioneering studies on glyoxal-bis(thiosemicarbazones), research efforts shifted to bis(thisoemicarbazones) with a different backbone that were derived from 2-keto-3-ethoxybutyraldehyde (kethoxal). This dione was known to have "a low order" of anti-leukemic activity (French and Freelander 1958b), which led to investigations of the bis(thiosemicarbazone) derivative, kethoxal-bis(thiosemicarbazone) (kts$H_2$, Fig. 1). Studies showed this compound had excellent anti-tumour activity against a number of transplanted rodent tumours (Petering et al. 1964). The mechanism of action of kts$H_2$ was not known but the toxicity and anti-tumour activity was shown to be dependent on nutritional intake, and in particular, copper(II) ($Cu^{II}$) ions in the diet (Petering et al. 1963, 1967). The importance of $Cu^{II}$ to the activity of bis(thiosemicarbazones) was subsequently highlighted by studies in which a combination of $Cu^{II}$ and kts$H_2$ ($Cu^{II}$(kts), Fig. 2) resulted in inhibition of DNA synthesis in sarcoma 180 ascites cells (Booth and Sartorelli 1966; Booth and Sartorelli 1967) and possessed anti-tumour activity against Walker 256 carcinoma in rats (Crim and Harold 1967). An alternative mechanism has been suggested in which $Cu^{II}$(kts) inhibits respiration and ATP production with a concomitant metabolic breakdown, and consequently impaired nucleic acid metabolism (Van Giessen et al. 1973).

On coordinating to $Cu^{II}$, bis(thiosemicarbazone) ligands doubly deprotonate to give neutral bis(thiosemicarbazonato)copper(II) complexes. The resulting complexes are highly lipophilic and membrane permeable (Fig. 2). Upon entering cells, complexes such as $Cu^{II}$(kts) decompose in a process involving reduction of the metal ion to $Cu^{I}$ possibly by thiol residues in the biological milieu. The $Cu^{I}$ dissociates from the bis(thiosemicarbazone) ligand and can subsequently be distributed within the cell (Minkel and Petering 1978). A study of a series of substituted bis(thiosemicarbazonato)copper(II) complexes revealed correlations between their reactivity towards sulfhydryl groups and cytotoxicity, suggesting that biological activity was dependent on the release of the metal ion intra-cellularly following reduction from $Cu^{II}$ to $Cu^{I}$ (Minkel et al. 1978). The reactivity of the complexes correlated directly with their ability to inhibit DNA synthesis and cellular respiration.

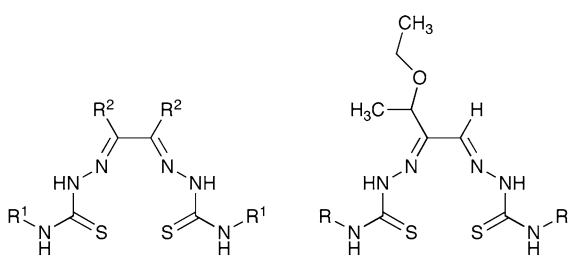


**Fig. 1** The chemical structures of the proligands, $H_2$gtsm, $H_2$gtse, $H_2$atsm, $H_2$kts and $H_2$ktsm

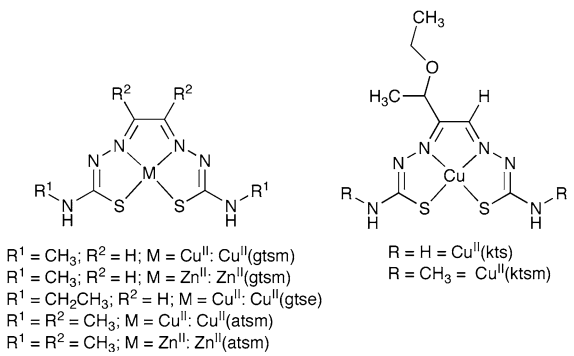


**Fig. 2** The chemical structures of bis(thiosemicarbazonato) copper(II) and zinc(II) complexes

Although bis(thiosemicarbazonato)copper(II) complexes are stable, neutral and lipophilic, subtle changes to the backbone of the ligand can dramatically alter their biological properties (Blower et al. 2003). $Cu^{II}$(atsm) (the ligand is derived from 2,3-butanedione) enters cells and the Cu is retained only in hypoxic (low oxygen concentrations) cells. In contrast, when $Cu^{II}$(gtsm) enters the cell (Fig. 1) the Cu is released and trapped regardless of oxygen concentration. (Fujibayashi et al. 1997; Dearling et al. 1998a, 1998b, 2002; Lewis et al. 1999; Maurer et al. 2002; Vavere and Lewis 2007). The different reduction potentials and subsequent Cu release properties of $Cu^{II}$(atsm) and $Cu^{II}$(gtsm) could account for the reported inactivity of the ligands derived from 2,3,-butanedione ($Cu^{II}$(atsm)) in early studies against Sarcoma 180 in Swiss mice (French and Freelander 1958a).

It is currently unclear how the Cu is subsequently metabolized upon release from the ligand. Upon binding to intracellular Cu proteins, the Cu may be sequestered to particular subcellular compartments with a range of downstream actions. We recently reported that $Cu^{II}$(gtsm), but not $Cu^{II}$(atsm), activated phosphoinositol-3-kinase (PI3 K)-dependent signaling resulting in downstream regulation of Alzheimer's amyloid peptide accumulation (Donnelly et al. 2008). Subsequently, we demonstrated that treatment of an Alzheimer's disease mouse model with $Cu^{II}$(gtsm) resulted in cognitive improvement associated with altered metabolism of amyloid processing and microtubule tau protein phosphorylation (Crouch et al. 2009). These effects were mediated through $Cu^{II}$(gtsm) modulation of kinase signaling including PI3 K, glycogen synthase kinase 3 beta (GSK3$\beta$) and extracellular signal-regulated kinase (ERK). In contrast, $Cu^{II}$(atsm) had no effect on the Alzheimer's disease model. We have also shown that $Cu^{II}$(gtsm) can induce sustained activation of epidermal growth factor receptor through inhibition of protein tyrosine phosphatase activity (Price et al. 2009). In unpublished studies we have found that $Cu^{II}$(gtsm) efficiently delivers Cu to the brain in mice with $Cu^{II}$(gtsm) elevating brain Cu levels by greater than 3% as compared to $CuCl_2$ which increased levels by less than 1% (Fodero-Tavoletti et al. 2010).

Due to the brain uptake of $Cu^{II}$(gtsm) and the potential for Cu to modulate cell growth through altered cell signaling mechanisms, we investigated the ability of $Cu^{II}$(gtsm) to inhibit neuroblastoma growth in vitro. Treatment of the human neuroblastoma cell line BE(2)-M17 resulted in cell cycle arrest as assessed by fluorescent activated cell sorting (FACS) analysis. Rapidly arrested growth was not associated with early onset of apoptosis. Instead, protein array analysis revealed that $Cu^{II}$(gtsm) rapidly and potently reduced cyclin D1 expression, while increasing Kip2 expression. We also observed a potent decrease of insulin-like growth factor receptor (IGF-IR) by $Cu^{II}$(gtsm) and this receptor has been demonstrated to modulate cyclin D1 expression. Our studies reveal important insights into the potential anticancer activity of $Cu^{II}$(gtsm).

## Materials and methods

### Materials

Ascorbate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Sydney, Australia). $Mn^{(III)}$tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP), PD153035, SB600125, LY294002 and PD98059 were obtained from Merck Biosciences (Melbourne, Victoria, Australia). Antibodies to cyclins D1, D2 and E, Kip2/p57, total and phospho-insulin-like growth factor-I receptor (IGF-IR) $\beta$ (Tyr1135/1136), phospho-CHK2, Cdk7 and total and phospho-epidermal growth factor receptor (EGFRtyr1068) were obtained from Cell Signaling Technology (Beverly, MA, USA).

### Methods

#### Chemical synthesis

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 $d_6$-DMSO.

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 × 150 mm, 5 μm), 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 nm, 254 nm and 275 nm.

The compounds $H_2$gtsm (Gingras et al. 1962; Beraldo et al. 1998), $Cu^{II}$(gtsm) (Gingras et al. 1962; Beraldo et al. 1998), $Zn^{II}$(gtsm) (Donnelly et al. 2008), $H_2$gtse (Beraldo et al. 1998), $Cu^{II}$(gtse) (Dearling et al. 2002; Blower et al. 2003), $H_2$atsm (Gingras et al. 1962) and $Zn^{II}$(atsm) (Cowley et al. 2002; Cowley et al. 2005) were all synthesised 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, whilst the copper complexes were analysed by mass spectrometry and reverse phase HPLC.

## Cell culture

The cell line used in this study was the human neuroblastoma BE(2)-M17. Cells were maintained in OptiMem plus 10% FBS and passaged at a dilution of 1:6 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 (Donnelly et al. 2008). Control cultures were treated with vehicle (DMSO) alone. Where stated, inhibitors of EGFR (PD153035), MEK-ERK pathway (PD98059), JNK (SB600125) or phosphoinositol-3-kinase (LY294002) were prepared as 10 mM stock solutions in DMSO and added at indicated concentrations. The antioxidant, ascorbate and free radical scavenger, MnTMPyP were added to cultures at 1 mM and 200 μM 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-proteins. Polyclonal antisera against cyclins, Kip2 or growth factor receptors were used at 1:1000 followed by secondary antiserum (goat anti-rabbit-HRP) at 1:10,000. 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.

## Clontech Antibody Microarray 500 for the determination of altered protein expression in $Cu^{II}$(gtsm) treated neurons

M17 cells were grown in 100 mm dishes and treated with $Cu^{II}$(gtsm) (25 μM) for 5 h. Cell extracts were prepared and analyzed using the Clontech Antibody Microarray 500 as previously reported (Du et al. 2008b). Raw fluorescence data from the microarray experiments were analyzed were curated using Genepix Pro v.6.1 and analyzed using the statistical R platform v2.4.1 (Du et al. 2008b). Array features were selected for quality and sufficient signal above background. Features that failed the quality control were not included in further statistical analysis. Normalized data for each microarray experiment was carried out using a global Lowess regression on log transformed expression ratios (M) and average intensities (A) of array features after local background subtraction. The normalized microarray results were obtained from the two dye-swap arrays using the 'linear models for microarray data' (LIMMA) software package v2.9.1 (Smyth et al. 2005). To identify potential protein–protein interactions induced by $Cu^{II}$(gtsm), we analyzed the top 50 proteins with altered expression of the array data set ($Cu^{II}$(gtsm) compared to untreated) for binding interactions using the program Pathway Studio™ (Ariadne Genomics), which uses Resnet 5 database (Du et al. 2008b). We selected protein–protein binding interactions only to narrow down the amount of information obtained and have a discernable image that we could interpret (Du et al. 2008b).

## Caspase-3 and 8 assays

The caspase-3 and caspase-8 colorimetric assays were performed according to kit instructions (R&D

Systems, MN, USA). Briefly, cells treated with $Cu^{II}$(gtsm) for 5 h were collected at 250×*g* for 10 min. The supernatant was removed and cells were lyzed with 25 µl cold lysis buffer per $1 \times 10^6$ cells. Cells were then incubated on ice for 10 min, and centrifuged at 10,000×*g* for 1 min. In a 96-well plate, 50 µL of the supernatant was added to each reaction well, along with 50 µL of the 2× reaction buffer 3 containing 10 µL of 1 M dithiothreitol (DTT). Caspase-3 colorimetric substrate was added (5 µL) to each reaction well and the plate was incubated overnight before reading in the WALLAC Victor$^2$ plate reader at $\lambda = 405$ nm.

### p53 Activity ELISA

p53 activity in treated cells was determined using the TransAM$^{TM}$ p53 transcription factor (ELISA) assay kit (Active Motif). Cells were collected after being treated for 5 h and pelleted at 720×*g* for 3 min. Pelleted cells were resuspended in 50 µL of complete lysis buffer. The cell suspension was centrifuged at 10,000×*g* for 5 min at 4°C, then the supernatant (cell extract) collected and stored at −80°C until used to determine p53 activity. The assay was performed as per manufacturer's instructions. The absorbance was determined within 5 min at $\lambda = 450$ nm on a WALLAC Victor$^2$ plate reader.

### Fluorescence-activated cell sorting (FACS) analysis

$Cu^{II}$(gtsm)-treated cells were collected after 5 h treatment and washed twice in suspension buffer (PBS + 2% FBS). Cell pellets were resuspended in 1 ml suspension buffer and 3 ml of absolute cold ethanol was added. Cells were stored at −20°C. Cell samples in suspension buffer/ethanol were centrifuged at 4000×*g* for 5 min, the supernatant removed, and the cell pellet washed twice with PBS. Washed cells were resuspended in 1 ml of propidium iodide (PI) solution (50 µg/mL PI in PBS) and mixed thoroughly. Fifty micro liters RNase A stock solution (10 µg/mL RNase A in PBS) was added followed by incubation at 4°C for 1 h. FACS analysis was performed on a FACSCalibur flow cytometer (BD). Data analysis was performed using CellQuest Pro software.

### 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 students' *t*-test.

## Results

To examine the effect of $Cu^{II}$(gtsm) on growth of human neuroblastoma cells, we treated M17 cultures with $Cu^{II}$(gtsm) (25 µM) for 5 h and measured cell

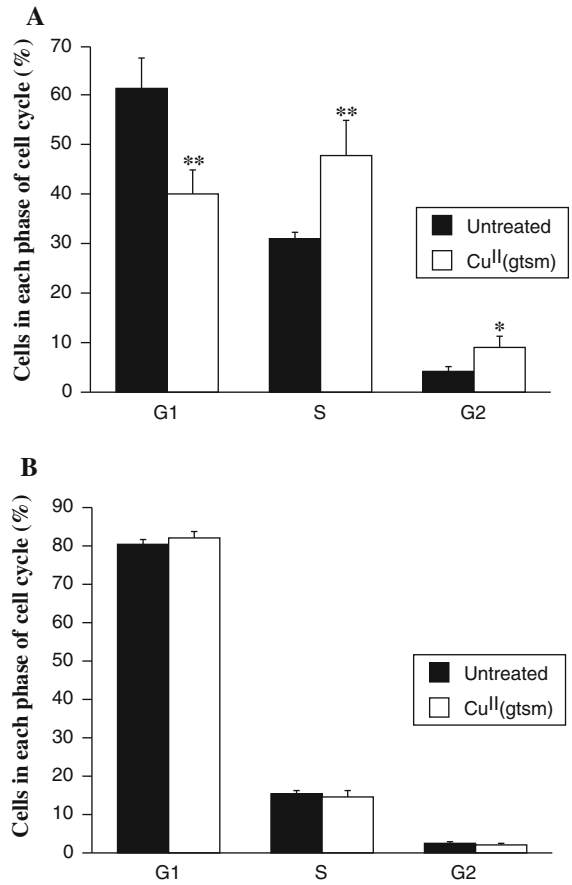


**Fig. 3** **a** M17 cells were treated with 25 µM $Cu^{II}$(gtsm) for 5 h and cells analyzed for cell cycle progression using PI and FACS. $Cu^{II}$(gtsm) treatment significantly reduced the proportion of cells in the G1 phase and increased the proportion of cells in the S and G2 phase (*$P < 0.05$, **$P < 0.005$). **b:** M17 cells were treated with 0.5 µM $Cu^{II}$(gtsm) for 24 h and cells analyzed for cell cycle progression using PI and FACS. $Cu^{II}$(gtsm) treatment did not alter the proportion of cells in any phase of the cell cycle

cycle progression by FACS analysis. Treatment with $Cu^{II}$(gtsm) significantly decreased the proportion of cells in the G1 phase (from approximately 60–40%) concomitantly with an increase in the proportion of cells in the S (from 30 to 48%) and G2 (from 5 to 9%) phases (Fig. 3a). These findings are consistent with arrested cell cycle progression from treatment with a Cu complex, copper(I) N-heterocyclic carbene complex, (1,3-bis(2,4,6-trimethylphenyl)imidaolin-2-ylidene) chlorocopper(I) (Teyssot et al. 2009). To determine if a longer exposure to $Cu^{II}$(gtsm) also affected the cell cycle in the same manner, we treated cells for 24 and 48 h with 0.5 μM $Cu^{II}$(gtsm). This concentration was used as higher levels of $Cu^{II}$(gtsm) were found to induce significant cell death after prolonged exposure. Treatment with the 0.5 μM $Cu^{II}$(gtsm) had no significant effect on cell cycle after the longer exposure (Fig. 3b). These results indicate that cell cycle arrest occurs at higher doses of $Cu^{II}$(gtsm) which precedes cytotoxic effects.

We then determined if the apparent cell cycle arrest was associated with neurotoxic effects or apoptosis. M17 cells were treated with $Cu^{II}$(gtsm) (25 μM, 5 h) and cell survival was determined with the LDH release assay. No significant decrease in cell survival was observed after treatment with $Cu^{II}$(gtsm) for 5 h (Fig. 4a). To measure potential induction of apoptosis, caspase 3 (effector caspase) and caspase 8 (initiator caspase) activity was determined in $Cu^{II}$(gtsm) treated cells. As shown in Fig. 4b and c, treatment with $Cu^{II}$(gtsm) actually significantly decreased caspase 3 and caspase 8 actvity. In addition, we measured the effect of $Cu^{II}$(gtsm) on p53 activation as this is central to Cu-dependent apoptotic cell death. However, treatment with $Cu^{II}$(gtsm) significantly decreased p53 activity in M17 cells after 5 h (Fig. 4d). Together, these data strongly suggest that cell cycle arrest induced by $Cu^{II}$(gtsm) is not directly associated with cytotoxic/apoptotic effects within the exposure period examined.

To determine how $Cu^{II}$(gtsm) mediated cell cycle arrest, we used protein array analysis of treated cultures. M17 cells were exposed to $Cu^{II}$(gtsm) (25 μM) or DMSO vehicle control for 5 h and cell lysates were examined for altered protein expression using the Clontech Antibody 500 microarray. The top 50 (top 10%) of altered proteins in $Cu^{II}$(gtsm) treated cells are listed in Table 1. To confirm that the changes observed using the array were reproducible by another method, we examined the expression of some proteins altered in the $Cu^{II}$(gtsm) treated cells using Western blot. Proteins were examined from Table 1, and revealed the same direction of change (increased or decreased) as observed using the protein array (Fig. 4e–f). This level of reproducibility was consistent with our previous finding of approximately 80% concordance in protein changes detected by the array and Western blot (Du et al. 2008b). Due to the large number of proteins with altered expression induced by $Cu^{II}$(gtsm), we analyzed the data using Pathway Studio™ as previously reported (Du et al. 2008b). This allowed potential protein–protein interactions to be examined and pathways mapped. We found that several key hubs were identified using Pathway Studio™ analysis, including RAF1, STAT3 and the cell cycle proteins cyclin D (CCND3) and cyclin E (CCNE1) (Fig. 5). Given the potent ability of $Cu^{II}$(gtsm) to inhibit cell cycle progression, we further examined metabolism of cyclins in treated cultures. M17 cells treated with $Cu^{II}$(gtsm) revealed an almost complete loss of detectable cyclin D1 and cyclin D2, although no change was observed to cyclin E (Fig. 6a).

We then further characterized the effect of $Cu^{II}$(gtsm) on cyclin D1 expression. Dose-response analysis revealed that relatively low concentrations (0.1–25 μM) of $Cu^{II}$(gtsm) significantly inhibited cyclin D after 5 h (Fig. 6b). A noticeable decrease in cyclin D1 was observed with 2 μM $Cu^{II}$(gtsm) while 5 μM (or higher concentrations) of $Cu^{II}$(gtsm) completely depleted detectable cyclin D1 (Fig. 6b).

To determine if the effect of $Cu^{II}$(gtsm) was specific, we compared it to the activity of structural analogues, $Cu^{II}$(gtse) and $Cu^{II}$(atsm) (Fig. 1). The cellular metabolism of bis(thiosemicarbazonato)copper(II) complexes is largely dependent on the substitutents of the diimine backbone of the ligand. Both $Cu^{II}$(gtse) and $Cu^{II}$(gtsm) are derived from a gyloxal and have two hydrogen atoms on the backbone of the ligand. In this respect, their $Cu^{II}$/$Cu^{I}$ reduction potentials are similar and they are expected to behave in a similar fashion with respect to intracellular reduction of the metal centre and subsequent release from the ligand (Fujibayashi et al. 1997; Dearling et al. 1998a 2002; Xiao et al. 2008). We have shown previously that $Cu^{II}$(gtse) and $Cu^{II}$(gtsm) have similar effects on amyloid levels and kinase activity in cell culture (Donnelly et al. 2008). In comparison, $Cu^{II}$(atsm) has two eletron

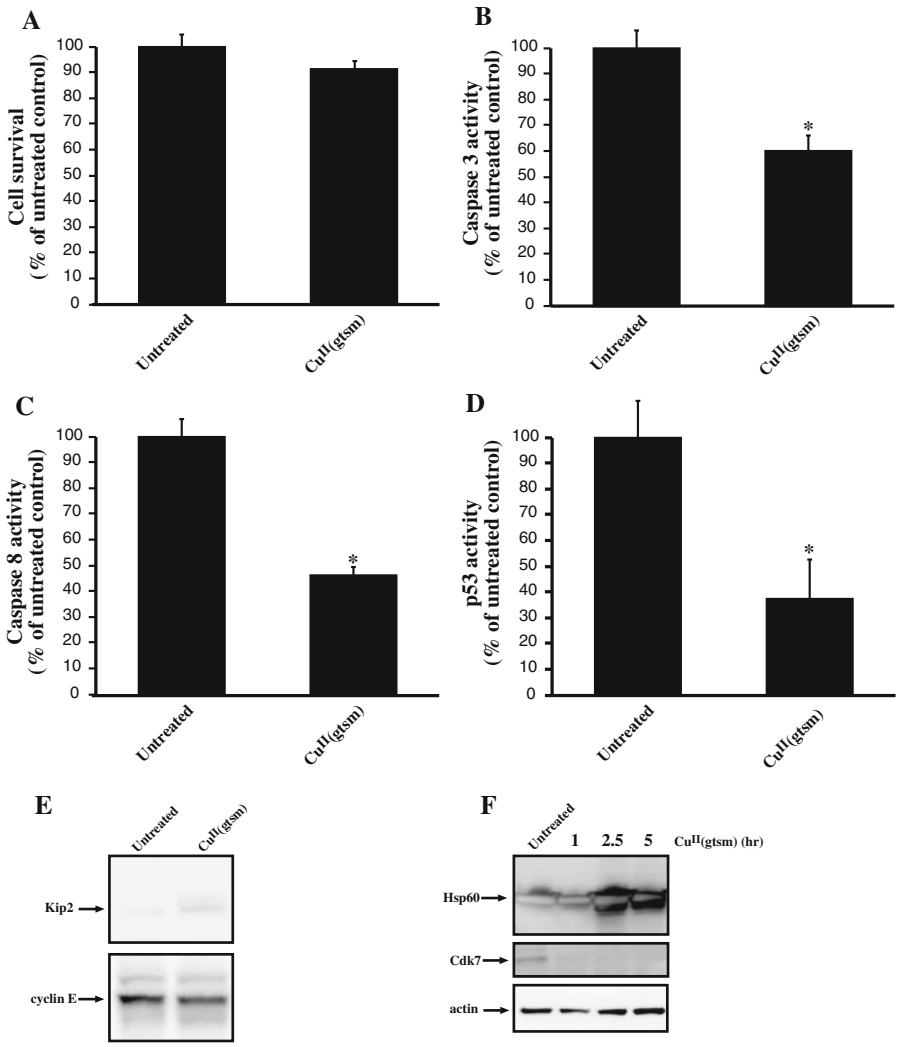


**Fig. 4** M17 cells were treated with 25 μM $Cu^{II}$(gtsm) for 5 h and **a** Cell survival was determined using the LDH assay. No significant change in cell survival was observed, **b** Caspase 3 activity was determined and revealed a significant decrease in activity in $Cu^{II}$(gtsm) treated cells compared to untreated controls, **c** Caspase 8 activity was determined and revealed a significant decrease in activity compared to untreated controls and **d** p53 activity was determined and revealed a significant decrease in activity compared to untreated controls. (*$P < 0.005$). **e** Western blot analysis of proteins showing altered expression in the protein array analysis. As shown in the array, Kip2 was elevated after 5 h exposure to $Cu^{II}$(gtsm). In contrast, no change was observed in cyclin E expression. **f** Western blot analysis of proteins showing altered expression in the protein array analysis. As shown in the array analysis, Hsp60 was up-regulated upon treatment with $Cu^{II}$(gtsm) and Cdk7 expression was decreased

donating methyl groups on the diimine backbone of the ligand. These methyl substituents result in a 160 mV shift in the $Cu^{II}/Cu^{I}$ reduction potentials to a more negative value (Fujibayashi et al. 1997). Consequently $Cu^{II}$(atsm) is more resistant to intracellular reduction of the metal centre and largely retains its structural integrity inside cells at normal oxygen levels (Fujibayashi et al. 1997). As $Cu^{II}$(atsm) does not readily release Cu under normoxic condition, treatment with this M-btsc was included to determine if the effects of $Cu^{II}$(gtsm) were mediated through increased Cu bioavailability.

Bis(thiosemicarbazonato)zinc(II) complexes are neutral and membrane permeable but have relatively modest stability where compared to their copper analogues ($K_a = 10^8$). As such, it was anticipated

**Table 1** Top 50 proteins with altered expression in $Cu^{II}$(gtsm) treated M17 cells compared to untreated controls

| Protein name | SwissProt[a] | % Change[b] |
|---|---|---|
| Cyclin-dependent kinase inhibitor 1C (p57, Kip2) | P49918 | 212.8274 |
| Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulatory factor 1 | Q9JJ19 | 178.7285 |
| Fatty acid synthase | P49327 | 162.43 |
| SH2-B homolog | Q9NRF1 | 189.7091 |
| Glutamate-ammonia ligase (glutamine synthase) | P15104 | 207.7271 |
| Heat shock 60 kDa protein 1 (chaperonin) | P10809 | 141.7078 |
| Cyclin D-type binding-protein 1 | Q9NYH3 | 166.569 |
| Laminin, beta 3 | Q13751 | 155.995 |
| Signal transducer and activator of transcription 3 (acute-phase response factor) | P40763 | 135.0242 |
| CSE1 chromosome segregation 1-like (yeast) | P55060 | 159.4124 |
| Potassium voltage-gated channel, subfamily H (eag-related), member 6 | Q9H252 | 133.6511 |
| SFRS protein kinase 1 | Q12890 | 158.9545 |
| Splicing factor, arginine/serine-rich 2 | Q01130 | 144.5551 |
| Synaptophysin | P08247 | 137.6407 |
| Cytochrome c, somatic | P00001 | 170.5398 |
| Fusion, derived from t(12;16) malignant liposarcoma | P35637 | 135.7355 |
| Microtubule-associated protein, RP/EB family, member 3 | Q9UPY8 | 142.4348 |
| BMX non-receptor tyrosine kinase | P51813 | 148.2358 |
| Glycogen synthase kinase 3 beta | P49841 | 183.236 |
| Lysosomal-associated membrane protein 1 | P11279 | 78.72793 |
| SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 | P51531 | 148.3298 |
| Topoisomerase (DNA) I | P11387 | 143.3965 |
| MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*) | P49959 | 130.5844 |
| Phosphodiesterase 5A, cGMP-specific | O76074 | 147.1014 |
| Chemokine (C–C motif) ligand 2 | P13500 | 127.9082 |
| OLF-1/EBF associated zinc finger gene | O08961 | 171.4558 |
| Solute carrier family 9 (sodium/hydrogen exchanger), isoform 1 (antiporter, Na+/H+, amiloride sensitive) | P19634 | 155.1324 |
| Cyclin E1 | P24864 | 74.91239 |
| Transcription factor Dp-1 | Q14186 | 134.1607 |
| WAS protein family, member 1 | Q92558 | 194.2671 |
| v-raf-1 murine leukemia viral oncogene homolog 1 | P04049 | 125.8995 |
| Caspase 6, apoptosis-related cysteine protease | P55212 | 78.90746 |
| Nuclear mitotic apparatus protein 1 | Q14980 | 137.5435 |
| RAB4A, member RAS oncogene family | P20338 | 140.5653 |
| Traf2 and NCK interacting kinase | Q9UKE5 | 132.1555 |
| CDC37 cell division cycle 37 homolog (S. cerevisiae) | Q16543 | 160.1664 |
| Protein kinase C, alpha | P17252 | 79.87938 |
| Translin-associated factor X | Q99598 | 154.2394 |
| Ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast) | P50550 | 75.33066 |
| Special AT-rich sequence binding protein 1 (binds to nuclear matrix/scaffold-associating DNA's) | Q01826 | 127.635 |

**Table 1** continued

| Protein name | SwissProt[a] | % Change[b] |
|---|---|---|
| CrmA | P07385 | 145.6679 |
| Chromatin assembly factor 1, subunit A (p. 150) | Q9UJY8 | 131.0856 |
| Caspase 9, apoptosis-related cysteine protease | P55211 | 133.6423 |
| Interleukin 5 (colony-stimulating factor, eosinophil) | P05113 | 150.9449 |
| Epoxide hydrolase 1, microsomal (xenobiotic) | P07099 | 80.00455 |
| Thyroid hormone responsive (SPOT14 homolog, rat) | Q92748 | 82.40283 |
| Cyclin-dependent kinase 7 (MO15 homolog, *Xenopus laevis*, cdk-activating kinase) | P50613 | 78.72028 |
| V-abl Abelson murine leukemia viral oncogene homolog 1 | Q13691 | 151.4634 |
| Cyclin D3 | P30281 | 77.25587 |
| RAD50 homolog (*S. cerevisiae*) | Q92878 | 180.4905 |

Data obtained from Clontech Antibody Microarray 500analysis of cell lysates

[a] Swiss Prot protein identification number

[b] % change in protein expression in $Cu^{II}$(gtsm) treated cells compared to untreated cells

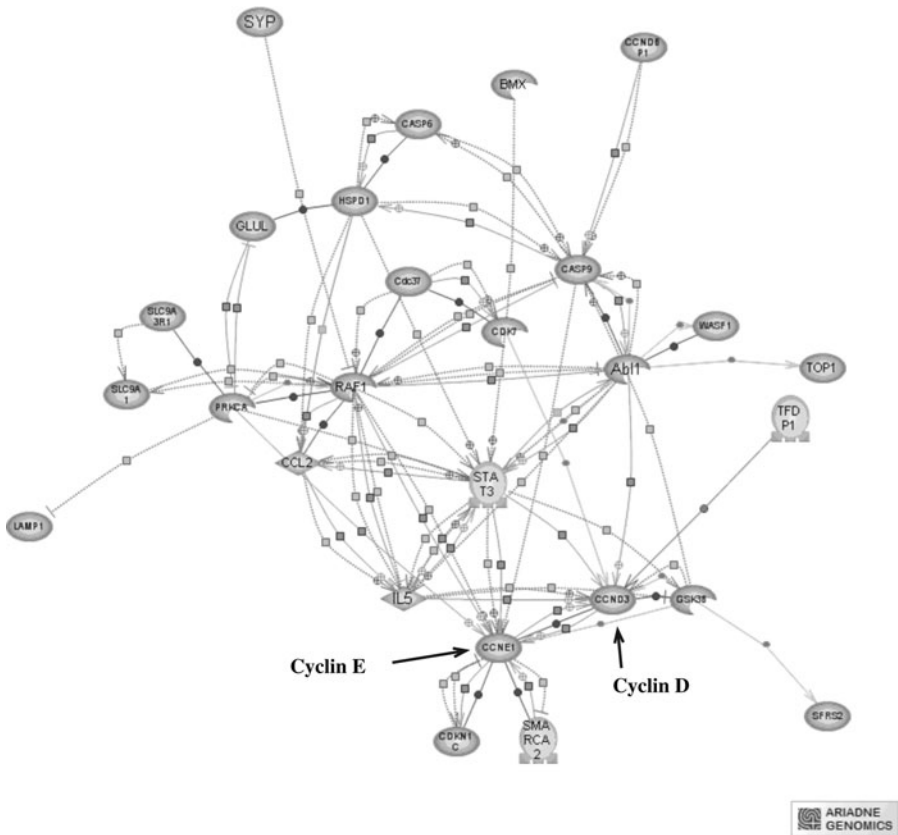


**Fig. 5** M17 cells were treated with 25 μM $Cu^{II}$(gtsm) for 5 h and expression of proteins was determined using the Clontech Antibody Microarray 500. The top 50 proteins with altered expression in $Cu^{II}$(gtsm) compared to untreated controls were further analyzed for protein–protein interactions using the software program, Pathway Studio™ linked to resnet 5 database. Predicted protein–protein interactions revealed a large number of interactions (hubs) associated with several proteins including cyclin E (CCNE1) and cyclin D (CCND3) as well as STAT3 and RAF1. Proteins of interest are indicated by ovals. Protein–protein interactions are shown by small squares

that the zinc complexes, $Zn^{II}$(gtsm) and $Zn^{II}$(atsm) would deliver Zn(II) intracellularly (Donnelly et al. 2008) and were used to determine if the effect was Cu-specific. After treatment with the complexes for 5 h (25 μM), Western blot analysis revealed that, as expected, $Cu^{II}$(gtse) induced an identical effect to $Cu^{II}$(gtsm) (loss of cyclin D1) (Fig. 6b). $Cu^{II}$(atsm) did not inhibit cyclin D1 expression, strongly suggesting

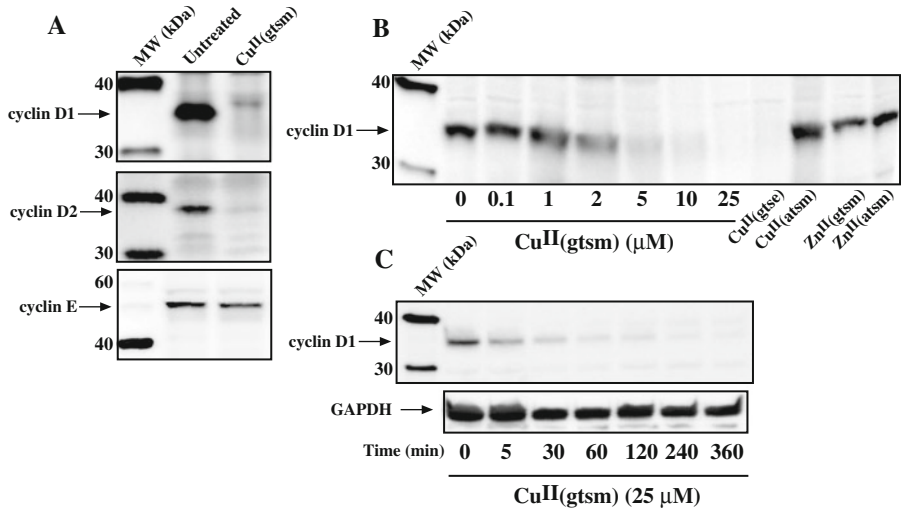


**Fig. 6** M17 cells were treated with 25 μM $Cu^{II}$(gtsm) for 5 h and **a** cell lysates were immunoblotted for cyclin D1, D2 and E. Decreased expression of cyclins D1 and D2 were observed in $Cu^{II}$(gtsm) treated cells but no change to cyclin E was seen. **b** Cells were treated with increasing concentrations of $Cu^{II}$(gtsm) for 5 h or with 25 μM $Cu^{II}$(gtse), $Cu^{II}$(atsm), $Zn^{II}$(gtsm) or $Zn^{II}$(atsm) and cell lysates immunoblotted for cyclin D1. Concentrations of 2 μM $Cu^{II}$(gtsm) or higher lead to a significant decrease in cyclin D1 expression. $Cu^{II}$(gtse) also inhibited cyclin D1 expression while $Cu^{II}$(atsm), and the Zn complexes had no effect. **c** Cells were treated for 0–360 min with 25 μM $Cu^{II}$(gtsm) and cell lysates were immunoblotted for cyclin D1. A significant decrease in cyclin D1 expression was observed after only 5 min exposure and continued to rapidly decline

that the effect is mediated through release of Cu intracellulary by $Cu^{II}$(gtsm) (or $Cu^{II}$(gtse)) rather than through binding of the complex to a membrane receptor. The lack of effect by either Zn complex demonstrated that the effect was specific for Cu.

Time-course analysis of $Cu^{II}$(gtsm) treatment was also performed and revealed a rapid decrease in expression of cyclin D1. An obvious decrease was observed after 5 min of treatment with $Cu^{II}$(gtsm) (25 μM) (Fig. 6c). The level of cyclin D1 progressively decreased until an almost complete lack of detection at 4 h after treatment.

Cyclin D1 is a binding partner for cyclin-dependent kinase 4/6 (cdk4/6) and is a key component of cdk4/6-mediated cell cycle progression (Kim and Diehl 2009). Cyclin D1 promotes cdk4/6-induced phosphorylation of retinoblastoma protein (Rb) to form phospho-Rb, which promotes cell cycle progression (Gladden and Diehl 2005). A decrease in cyclin D1 therefore can result in cell cycle arrest. Similarly, increased kip2 expression (modulator of cdk4/6 activity) can also inhibit cdk4/6 with the same effect on Rb (Wang et al. 2007). To examine the effect of $Cu^{II}$(gstm) on these components of cell cycle progression, we measured kip2 expression. As shown in Table 1, protein array analysis indicated that kip2 expression was elevated by $Cu^{II}$(gtsm) treatment. This was subsequently confirmed by Western blot of M17 cells treated with $Cu^{II}$(gtsm) (25 μM) for 5 h (Fig. 7a). We found that while there was an early decrease in cyclin D1 expression (5 min after exposure to $Cu^{II}$(gtsm) Fig. 6c), kip2 was unchanged until 4 h after treatment (Fig. 7a), at which point it significantly increased in expression. This finding indicated that there are separate effects of $Cu^{II}$(gtsm) on kip2 and cyclin D1. Although we examined M17 cells for altered Rb phosphorylation by decreased cyclin D1, we were not able to show clearly detectable levels of Rb in these cells. While these data show that $Cu^{II}$(gtsm) affects both cyclin D1 and Kip2 levels, both important elements in controlling cell cycle, the changes shown here in expression are consistent with G1 arrest rather than S or G2 arrest as found in this study. Therefore, it seems most likely that the affects of $Cu^{II}$(gtsm) on cyclin D1 and Kip2 are parallel events and that an alternative mechanism is responsible for the arrest at S and/or G2 phase.

One possible mechanism for cell cycle arrest at S or G2 stage is through DNA damage. Cells treated with $Cu^{II}$(gtsm) were examined for increased levels of

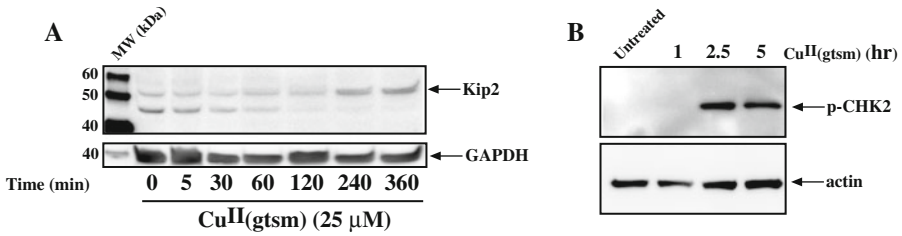


**Fig. 7** **a** M17 cells were treated with 25 μM $Cu^{II}$(gtsm) for 0–360 min and the level of Kip2 was measured in cell lysates. A significant increase in Kip2 levels was observed after 240 min of treatment. **b** Treatment of M17 cells with CuII(gtsm) induced phosphorylation of CHK2 (p-CHK2)

DNA damages markers phosphorylated ATR, CHK2. Although no consistent effect on ATR was observed, an increase in phospho-CHK2 was evident by 2.5 and 5 h exposure to $Cu^{II}$(gtsm) (Fig. 7b). This finding is consistent with the cell cycle arrest observed at 5 h treatment with $Cu^{II}$(gtsm). Down-regulation of Cdk7 has also been associated with cell cycle arrest at the G2 phase. We confirmed that treatment with $Cu^{II}$(gtsm) significantly decreased Cdk7 expression (Fig. 4f). Together with DNA damage, this is another possible mechanism of cell cycle arrest by $Cu^{II}$(gtsm).

As $Cu^{II}$(gtsm) had a potent and rapid effect on cyclin D1 expression, we then further investigated the possible mechanisms involved in this process. Firstly, as increased Cu bioavailability has the potential to induce reactive oxygen species and increased oxidative stress (supported by the DNA damage marker, phospho-CHK2), we examined whether this could contribute to decreased cyclin D1. Cells were treated with $Cu^{II}$(gtsm) with and without the free radical scavenger, MnTMPyP and the antioxidant, ascorbate for 5 h. The loss of cyclin D1 induced by $Cu^{II}$(gtsm) was not altered by co-treatment with MnTMPyP and ascorbate (Fig. 8a), strongly suggesting that Cu-mediated free radical production was not involved in the effect of $Cu^{II}$(gtsm) on cyclin D1.

As we have shown that $Cu^{II}$(gtsm) can activate kinases such as PI3 K, ERK and JNK (Donnelly et al. 2008; Crouch et al. 2009), we examined whether the activation of these kinases may be involved in loss of cyclin D1 expression. Co-treatment of cells with specific inhibitors of PI3 K (LY294002), p38 (1203580), JNK (SB600125) and MEK-ERK pathway (PD98059) and $Cu^{II}$(gtsm), however, had no effect on loss of cyclin D1 levels (Fig. 8b). Inhibition of kinase phosphorylation with the inhibitors was confirmed (data not shown). Similarly, inhibition of the epidermal growth factor receptor (EGFR) (which can be activated by $Cu^{II}$(gtsm)) using PD153035, did not affect cyclin D1 expression (Fig. 8b). We also examined expression of kinases that may be involved in modulation of cyclin D1, including ERK and Akt (target of PI3 K). Figure 8c shows that $Cu^{II}$(gtsm) induced activation of ERK but inhibited activation of PI3 K. Inhibition of ERK phosphorylation using the MEK inhibitor PD98059 did not affect cyclin D1 expression (Fig. 8b) indicating that ERK activation was not responsible for loss of cyclin D1. However, as PI3 K-Akt activation has been reported to enhance cyclin D1 levels (Ramljak et al. 2000), the loss of PI3 K-Akt activity in $Cu^{II}$(gtsm)-treated cells could lead to loss of cyclin D1.

Cyclin D1 expression can be controlled through activity of the insulin-like growth factor receptor (IGF-IR) and downstream modulation of PI3 K-Akt (Shukla and Gupta 2009). As we have found that $Cu^{II}$(gtsm) and other metal-ligand complexes can modulate EGFR activity (Price et al. 2009), we decided to examine if $Cu^{II}$(gtsm) also affected IGF-IR activity or expression. Treatment of cells with $Cu^{II}$(gtsm) significantly decreased expression of both total and phosphorylated forms of IGF-IR in M17 cells (Fig. 8d). This decrease in IGF-IR activity was a relatively specific effect as $Cu^{II}$(gtsm) simultaneously induced phosphorylation of EGFR in the same cultures (Fig. 8d). We therefore further examined the potential role of IGF-IR inhibition in decreased cyclin D1 expression. Treatment of cells with 100 ng/ml of recombinant IGF induced a small increase in levels of phosphorylated IGF-IR, without change to total IGF-IR (Fig. 8e). No significant increase in cyclin D1 was observed during the treatment, suggesting that basal IGF-IR levels may be sufficient to maintain optimal cyclin D1 expression in these cultures and

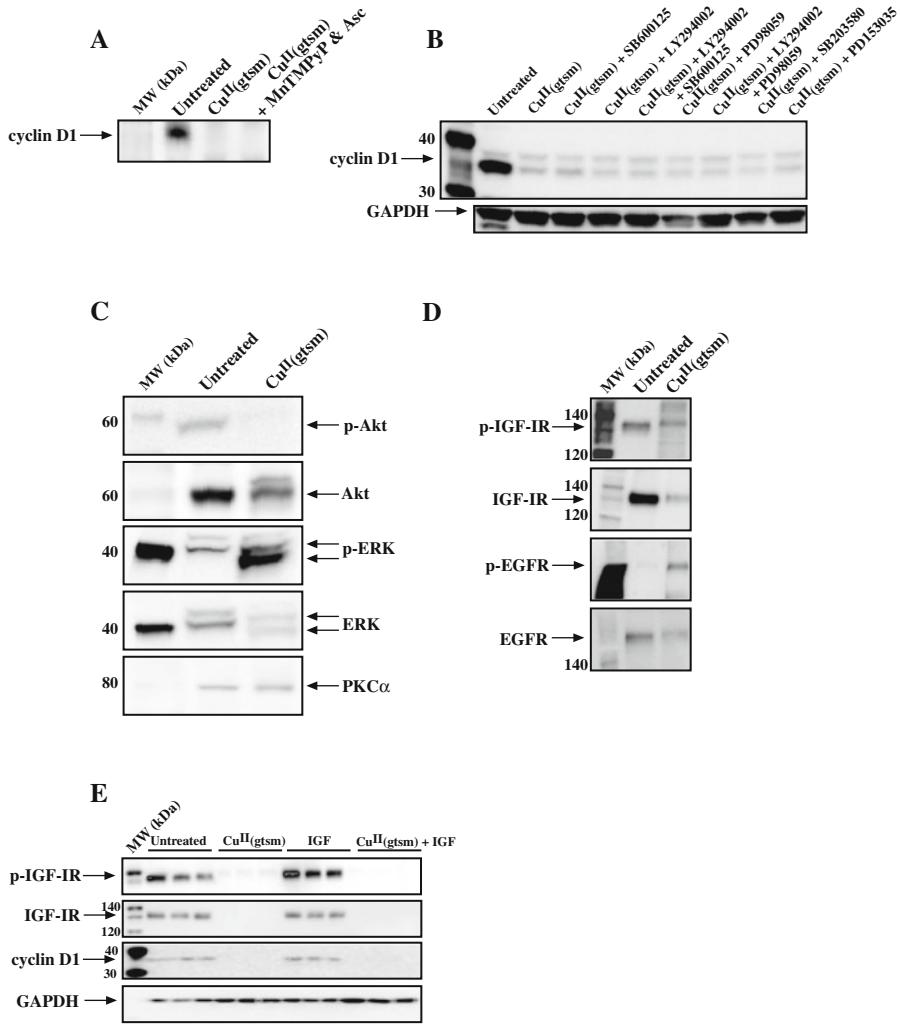


**Fig. 8** **a** M17 cells were treated with 25 μM $Cu^{II}$(gtsm) for 5 h with or without addition of the antioxidant, ascorbate (1 mM) and the free radical scavenger, MnTMPyP (200 μM). Treatment with ascorbate and MnTMPyP had no effect on the loss of cyclin D1 expression induced by $Cu^{II}$(gtsm). **b** Cells were treated with 25 μM $Cu^{II}$(gtsm) for 5 h with or without inhibitors of JNK (SB600125), PI3 K (LY294002), MEK-ERK pathway (PD98059), p38 (SB203580) or EGFR (PD153035) or combinations of LY294002 and SB600125 and LY294002 and PD98059. All inhibitors were used at 20 μM except PD153035 (1 μM). Treatment with the inhibitors had no effect on the loss of cyclin D1 induced by $Cu^{II}$(gtsm). **c** Cells were treated with $Cu^{II}$(gtsm) and lysates immunoblotted for total and phospho-Akt (Akt and p-Akt), total and phospho-ERK (ERK and p-ERK) and total PKCα as a control. $Cu^{II}$(gtsm) induced increased p-ERK levels but decreased total and p-Akt. **d** Cells were treated with $Cu^{II}$(gtsm) and lysates immunoblotted for total and phosphorylated IGR-IR and EGFR. $Cu^{II}$(gtsm) induced a decrease in total expression and concordant levels of phospho-IGF-IR but increased phosphorylation of EGFR. **e** Cells were treated with IGF (100 ng/ml) overnight and then with $Cu^{II}$(gtsm) (25 μM) for 5 h and cell lysates immunoblotted for IGF-IR and cyclin D1. IGF treated modestly increased IGF-IR phosphorylation without altering total IGF-IR. No significant increase in cyclin D1 was seen by IGF treatment. In the presence or absence of IGF treatment, $Cu^{II}$(gtsm) robustly inhibited IGF-IR total and phosphorylated protein expression and inhibited cyclin D1 expression

overstimulation does not rapidly increase cyclin D1 expression. In parallel, we co-treated cells with $Cu^{II}$(gtsm) and found that $Cu^{II}$(gtsm) completely abolished detectable IGF-IR expression and simultaneously blocked cyclin D1 expression. We also co-treated cultures with $Cu^{II}$(gtsm) and the proteasome inhibitor, MG132 (20 μM) for 5 h, to examine if loss of cyclin D1 was due to increased proteasomal-mediated degradation. However, $Cu^{II}$(gtsm) still caused reduced IGF-IR and cyclin D1 detection in the

presence of MG132, indicating that enhanced proteasomal degradation of either IGF-IR or cyclin D1 was unlikely to have a role in loss of these proteins induced by $Cu^{II}$(gtsm) (data not shown). However, our findings are still consistent with the hypothesis that inhibition of IGF-IR by $Cu^{II}$(gtsm) may result in loss of cyclin D1 expression and this could be mediated through loss of PI3 K-Akt activity (Fig. 8c). However, further studies are needed to determine how $Cu^{II}$(gtsm) inhibits IGF-IR and to definitively link decreased IGF-IR with decreased cyclin D1.

## Discussion

Copper(II) complexes of bis(thiosemicarbazones) have been investigated as metal based drugs for close to 50 years. Initial research had focused on the anti-cancer activity of $H_2$gtsm and these early pioneering studies had identified a possible role of metal ion chelation in the biological activity and in particular $Cu^{II}$ ions (French and Freelander 1958b). Later studies focused on "the pre-formed" $Cu^{II}$ chelate ($Cu^{II}$(kts), Fig. 2) which was shown to be active against cancer cells in vitro and in vivo (Booth and Sartorelli 1967; Crim and Harold 1967). Assessing the mechanistic action of metal-drugs in cancer and non-cancer cells is vital to allow further development of this class of compound as anti-cancer agents. Given recent developments in the area of protein microarray analysis and fluorescent cell sorting analysis we attempted to provide further mechanistic insights into the biological activity of $Cu^{II}$(gtsm).

In this study, we examined the effect of the M-btsc, $Cu^{II}$(gtsm) on neuroblastoma cells in culture. We show that $Cu^{II}$(gtsm) can induce cell cycle arrest in neuroblastoma cells. Interestingly, we found that while $Cu^{II}$(gtsm) induced rapid inhibition of cell cycle activity (5 h), at this time point, no cytotoxic effects were observed. This was supported by our data showing no LDH release, reduced caspase 3 and caspase 8 activities and decreased p53 activity after 5 h treatment with $Cu^{II}$(gtsm). The reduction in caspase activity may be linked to decreased p53 expression in the presence of Cu. Cu can modulate the expression of p53 at the transcriptional level. However, the data clearly supports a lack of early apoptotic pathway induction by $Cu^{II}$(gtsm). In contrast, reports on the anti-cancer effects of alternative Cu-ligand complexes, including pyrrolidine diothiocarbamate (PDTC)-Cu and clioquinol (CQ)-Cu have described strong cytotoxic action (Chen et al. 2005; Daniel et al. 2005; Ding et al. 2005; Yu et al. 2007; Zhang et al. 2008). The reason for this is likely to be that continual exposure to the lipid soluble complexes for longer periods results in substantial cytotoxic effects. This was supported by the fact that prolonged exposure of cells with high concentrations of $Cu^{II}$(gtsm) also induced cytotoxic effects. In our study, we specifically examined the early effect of $Cu^{II}$(gtsm) on neuroblastoma cells and observed no cytotoxicity or apoptosis. Interestingly, Ding et al. (Ding et al. 2008) found that CQ-Zn induced significant cell death (cell lysis) as assessed by LDH release at 6 h using 10 μM CQ and 50 μM Zn. In contrast, 25 μM $Cu^{II}$(gtsm) for 5 h had no effect on LDH release in M17 neuroblastoma cells, suggesting that Zn complexes may be more toxic than Cu complexes.

Our studies identify cell cycle arrest as a potent and primary event in neuroblastoma cells treated with $Cu^{II}$(gtsm). This can have significant advantages when developing anticancer drugs as inhibition of proliferation rather than broad spectrum cytotoxicity can allow targeting of cancerous cells. Whether the cytotoxic action of Cu-ligand complexes is directly associated with cell cycle arrest or are separate pathways is not yet clear. Previous reports on anti-cancer activity of Cu complexes such as CQ–Cu suggest that inhibition of proteasome activity is an important process in anti-cancer toxicity (Chen et al. 2005; Daniel et al. 2005; Ding et al. 2008). In addition, we reported previously that CQ-Cu can induce anti-cancer action through TNFα release from macrophages (Du et al. 2008a). These studies suggest that Cu-complexes have diverse actions on cancer cells, which can culminate in cell cycle arrest and/or cytotoxicity. Importantly, we found that longer treatment with 0.5 μM $Cu^{II}$(gtsm) had reduced cytotoxicity and did not affect the cell cycle suggesting that while high doses may have potential for anticancer therapy, prolonged lower doses can be used for alternative therapies without adverse effect on cell viability or cycle.

Our studies also demonstrated that the Cu must be made bio-available for anti-cancer activity. Treatment

of cells with $Cu^{II}$(atsm) which does not release Cu under normoxic conditions had no effect on cyclin D1 expression, which was rapidly inhibited by $Cu^{II}$(gtsm). This precludes the likelihood that $Cu^{II}$(gtsm) and other complexes induce their actions through binding as a complex to membrane receptors. While $Cu^{II}$(atsm) is widely studied as a hypoxia imaging agent (where it releases Cu due to hypoxic conditions) (Lewis et al. 2008), $Cu^{II}$(gtsm) could be used to target non-hypoxic cancer cells. Our studies also suggest that under hypoxia in tumours, $Cu^{II}$(atsm) (which would then release Cu) could act in an analogous manner to $Cu^{II}$(gtsm).

Interestingly, we also found that Zn-btsc had no effect on cyclin D1 expression. Both these Zn compounds ($Zn^{II}$(atsm) and $Zn^{II}$(gtsm)) readily release Zn intracellularly due to their relatively low dissociation constants compared to the Cu-btscs. We have reported previously that both Zn and Cu-btsc can activate PI3 K and ERK in cell cultures, suggesting similar mechanisms of action (Donnelly et al. 2008). However, we also recently found that Cu-btsc were significantly more potent at activating the EGFR receptor than Zn-btsc (Price et al. 2009). In the present study, no effect of Zn-btsc were observed suggesting that Cu-btsc are metabolized through different intracellular pathways. This is supported by Pascu et al. (2008) who have shown that $Zn^{II}$(atsm) targets to the mitochondria in IGROV cells. Localization to structures identified as lysosomes was also reported in different cancer cell lines (Pascu et al. 2008). Our own studies have found diverse subcellular processing of different btsc (unpublished observations). The lack of effect of Zn-btsc on cyclin D1 expression may be explained by the weak activity of Zn-bstc for membrane receptor modulation (unpublished observations). The lack of effect by Zn-btsc could also be explained by the fact that Cu but not Zn directly induces free radical generation. However, addition of a free radical scavenger and antioxidant had no effect on loss of cyclin D1 expression and this is consistent with our previous findings that ROS has no role in other effects of Cu-ligand complexes (Price et al. 2008, 2009).

Our findings in this study suggest that the potent effect of $Cu^{II}$(gtsm) on cyclin D1 expression may be through inhibition of IGF-IR expression/activity. Previous studies have shown that IGF-IR activity is important for maintaining cyclin D expression and cell cycle progression (Wilsbacher et al. 2008) and that this can be mediated by IGF-IR-dependent PI3 K-Akt activity (Banudevi et al. 2010). Also, aberrant metabolism of IGF-IR is associated with a number of cancers including prostate, colorectal, breast and brain (Coulter et al. 2008; Ma et al. 2010; Kaulfuss et al. 2009; Ryan et al. 2009). We found that $Cu^{II}$(gtsm) was a potent inhibitor of IGF-IR expression in the presence or absence of IGF and that this effect was associated with loss of PI3 K expression and activation. Concomitant with decreased IGF-IR expression we observed a potent inhibition of cyclin D1 expression. Although studies involving IGF-IR knockdown will be necessary to confirm this effect, the data are consistent with $Cu^{II}$(gtsm) mediated inhibition of cyclin D1 expression through inhibition of IGF-IR and downstream PI3 K activity. Interestingly, Banudevi et al. (2010) has also reported metal-mediated inhibition of IGF-IR, Akt and cyclin D1 expression in cancer cells. However, the effects they reported were induced by Zn while we found no effect of Zn-btsc complexes in this study. This could represent differences in cell type or delivery of Zn. It is not yet known how $Cu^{II}$(gtsm) induces loss of either IGF-IR or cyclin D1. We found that inhibition of the proteasome with MG132 did not alter either IGF-IR or cyclin D1 levels and the rapid loss of cyclin D1 (within 5 min of $Cu^{II}$(gtsm) treatment) means that changes to protein transcription are probably unlikely. It is possible that non-proteasome degradation could target cyclin D1 or IGF-IR, however, extensive studies will be required to determine if this is the case.

Kip2 inhibits cdk4/6 activity and inhibits cell cycle progression through hypophosphorylation of Rb (Wang et al. 2007). Increased kip 2 expression may result in cell cycle arrest (Chen et al. 2003; Xia et al. 2006). In this study we found that $Cu^{II}$(gstm) elevated Kip2 expression after several hours. $Cu^{II}$(gtsm) rapidly deceased cyclin D1 expression after only 5 min. However, as these changes are consistent with G1 arrest rather than S or G2 as observed here, they may be a parallel process rather than the cause of cell cycle arrest. Our finding that $Cu^{II}$(gtsm) activated the DNA damage marker, CHK2 may indicate that arrest at G2 stage could be mediated from DNA damage. Interestingly, Herman-Antosiewicz et al. (Herman-Antosiewicz et al. 2007) demonstrated that activation

of CHK2 and S phase arrest was associated with changes in a number of cell cycle proteins including a decrease in cyclin D1. Additionally, we found a reduction in cdk7 expression that is also consistent with arrest at G2 phase. Cdk7 has also been associated with cell cycle arrest and changes to cyclin D1 (Wang et al. 2007). Considerable studies will be required to tease out the exact order of events and which mechanisms are crucial for arrest, cyclin D1 inhibition and DNA damage. The complexity of this process is supported by the fact that the protein array data and subsequent pathway analysis revealed changes to many proteins that are associated with the cell cycle.

Finally, our work showed the utility of protein array technology combined with protein–protein interaction analysis as a powerful tool for identifying critical changes in cell function induced by potential drug compounds. The array and pathway study combination provided valuable insight into aberrant cell cycle processes and altered signal mechanisms induced by $Cu^{II}$(gtsm) on neuroblastoma cells. As previously reported by us (Du et al. 2008b), we have found strong concordance between protein array data and Western blot or ELISA analysis (70–80% of altered proteins show analogous effects in each method) (Du et al. 2008b). This concordance precludes the need to do multiple, expensive array studies and provided that the analysis of data is limited to the top 10% of altered proteins, a high level of confidence can be applied to the altered protein expression. Follow up with Western blot or other techniques for protein expression are likely to be positive. False positive results with the array are most often due to the presence of cross-reactive proteins (also a problem with immunocytochemical studies) rather than false detection of elevated protein per se. The power of the protein array is in the identification of pathways and networks that are altered rather than identification of single proteins. In this study, we successfully used the Clontech protein microarray to identify groups of proteins that may be associated with altered cell cycle progression induced by $Cu^{II}$(gtsm).

In summary, we have demonstrated that the M-btsc, $Cu^{II}$(gtsm) induces a rapid and potent change to important cell cycle proteins associated with cell cycle progression without concurrent or prior onset of cytotoxic effects. Inhibition of IGF-IR expression by $Cu^{II}$(gtsm) may be responsible for the decreased cyclin D1 expression and subsequent changes to cell cycle activity. These findings may have important implications for development of metal-based bis (thiosemicarbizone) complexes as potential cancer therapeutics.

**Acknowledgments** This work was supported by funding from the National Health and Medical Research Council of Australia and Australian Research Council. We thank Dr Anthony Purcell, Dr Dan Barr and Mr Ken Field for their help with FACS analysis.

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