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TITLE PAGE

Regulation of CTR2 Expression by Copper and Cisplatin in Human Ovarian Carcinoma Cells

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The abbreviations used are: CTR1, copper transporter 1, SLC31A1; CTR2, copper transporter 2, SLC31A2; DDP, cisplatin; CBDCA, carboplatin; ICP-MS, inductively coupled plasma mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectroscopy.

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ABSTRACT

Down-regulation of CTR1 reduces uptake and sensitivity whereas down-regulation of CTR2 enhances both. DDP triggers the rapid degradation of CTR1 and thus limits its own accumulation. We sought to determine the effect of DDP and Cu on the expression of CTR2. Changes in CTR1 and CTR2 mRNA and protein levels in human ovarian carcinoma 2008 cells and ATOX1$^{+/+}$ and ATOX1$^{-/-}$ mouse embryo fibroblasts in response to exposure to DDP and Cu were measured by qRT-PCR, western blot analysis and deconvolution microscopy. DDP triggered rapid degradation of CTR1 in 2008 human ovarian cancer cells. However, it increased the expression of CTR2 mRNA and protein levels. Expression of CTR2 was heavily modulated by changes in intracellular Cu concentration; Cu depletion produced rapid disappearance of CTR2 whereas excess Cu increased the level of CTR2 protein. This increase was associated with an increase in CTR2 mRNA and prolongation of CTR2 half-life. Consistent with prior observations that shRNAi-mediated knockdown of CTR2 enhanced DDP uptake and tumor cell kill, reduction of CTR2 by Cu starvation also enhanced DDP uptake and cytotoxicity. Comparison of the ability of Cu and DDP to modulate expression of CTR1 in ATOX1$^{+/+}$ and ATOX1$^{-/-}$ indicated that ATOX1 participates in the regulation of CTR2 expression. Unlike CTR1, the expression of CTR2 is increased rather than decreased by DDP. Therefore, these two Cu transporters have opposite effects on DDP sensitivity. CTR2 expression is regulated by Cu availability via the Cu-dependent regulator ATOX1.

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INTRODUCTION

The copper (Cu) transporter 1 (CTR1) plays a central role in the transport of the platinum-based chemotherapeutic drug cisplatin (DDP) (Ishida et al., 2002; Larson et al., 2009; Lin et al., 2002). DDP accumulation is markedly reduced in cells in which both alleles of *CTR1* have been knocked out, and re-expression of CTR1 in $CTR1^{-/-}$ cells restores DDP accumulation (Larson et al., 2009). Consistent with its regulation of cellular accumulation, loss of CTR1 results in resistance to the cytotoxic action of DDP both *in vitro* and *in vivo* (Blair et al., 2009; Larson et al., 2009). Recently the second Cu transporter found in mammalian cells, copper transporter 2 (CTR2), has been shown to affect the cellular accumulation of DDP in a manner opposite to that of CTR1 (Blair et al., 2009). Cells in which the expression of CTR2 has been knocked down ($CTR2^{kd}$) accumulate 2-3-fold more DDP than wild type parental cells (Blair et al., 2009) and this increase is associated with a large increase in the cytotoxicity of the drug (Blair et al., 2009). Furthermore, the effect of the loss of CTR2 on DDP uptake and sensitivity is independent of the expression of CTR1 (Blair et al., 2009).

Ctr2 in the yeast *S. cerevisiae*, and the *S. pombe* ortholog Ctr6, are associated with vacuoles with the C-terminal tail oriented toward the cytosol (Bellemare et al., 2002; Rees et al., 2004). Ctr2 delivers Cu to various chaperones by releasing Cu from intercellular vacuolar stores under conditions of Cu starvation (Kampfenkel et al., 1995; Portnoy et al., 2001; Rees et al., 2004). When Ctr2 is mutated so that it mislocalizes to the plasma membrane it can mediate Cu influx in a manner similar to Ctr1 (Rees et al., 2004). Less is known about the function of mammalian CTR2. In mammalian cells, CTR2 is reported to be localized to late endosomes and lysosomes, although it also has been found on the plasma membrane in some cells {van den Berghe, 2007 #9392). As in yeast, mammalian CTR2 has been shown to increase Cu influx in

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cells in which it localizes to the plasma membrane (Bertinato et al., 2008), although its affinity for Cu is less than that of CTR1 (Bertinato et al., 2008; van den Berghe et al., 2007).

ATOX1 is a Cu chaperone that accepts Cu from CTR1 and delivers it to the Cu efflux pumps ATP7A and ATP7B that transfer Cu into the secretory pathway (Xiao and Wedd, 2002). ATOX1 expression has been linked to the regulation of several Cu proteins such as ATP7B, SOD1 and CCS (Jeney et al., 2005; Lutsenko et al., 2003; Miyayama et al., 2009). Recently, ATOX1 was found to act as a Cu-dependent transcription factor that regulates the expression of cyclin D1, SOD1(Itoh et al., 2008; Itoh et al., 2009; Muller and Klomp, 2008).

Cu and DDP can quickly down-regulate the expression of CTR1 (Holzer and Howell, 2006). Within 15 minutes of DDP exposure nearly all CTR1 is removed from the plasma membrane in many types of cells in a process that involves macropinocytosis, ubiquitination and subsequent degradation by the proteosome (Holzer and Howell, 2006; Jandial et al., 2009). However, within ~30 min following the removal of DDP, the amount of plasma membrane CTR1 returns to normal (Holzer and Howell, 2006). Currently, very little is known about how CTR2 is regulated. Given its similarity to CTR1 with respect to structure and Cu transport, it is likely that CTR2 is also regulated by DDP and the availability of Cu.

In the current study, we sought to determine how the expression of CTR2 is affected by Cu and DDP in both 2008 human ovarian and mouse embryo fibroblast cells. We report here that CTR2 mRNA and protein levels increase when mammalian cells are exposed to either Cu or DDP. Additionally, we demonstrate that CTR2 protein is rapidly degraded when cells are starved for Cu. Similar to the effect of knocking down CTR2 expression using shRNAi, the loss of CTR2 caused by depletion of cellular Cu leads to increased DDP accumulation and cytotoxicity. We also report that CTR2 is found not only on intracellular membranes but also in

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the nucleus and that exposure to either Cu or DDP increases the level of CTR2 in the nucleus as well as the cytoplasm. Finally, we present evidence that the regulation of CTR2 by Cu status is mediated by ATOX1.

MATERIALS AND METHODS

Drugs and reagents. Platinol AQ was a gift from Bristol-Myers Squibb (Princeton, NJ); it contains DDP at a concentration of 3.33mM in 0.9 percent NaCl. $CuSO_4$ and BCS were purchased from Sigma-Aldrich (St. Louis, MO). The drugs were diluted into OptiMEM Reduced Serum Media (Gibco, 31985-070) to produce final concentrations. Bradford reagent was purchased from BioRad Laboratories, Inc. (Hercules, CA), sulforhodamine B was obtained from Sigma-Aldrich (St. Louis, MO) and 0.4 percent sulforhodamine B (w/v) was solubilized in 1 percent (v/v) acetic acid solution.

Cell types, culture and engineering. Parental mouse embryonic fibroblasts containing wild-type alleles of ATOX1 ($ATOX1^{+/+}$) and an isogenic line in which both copies of ATOX1 had been somatically knocked out ($ATOX1^{-/-}$) were a generous gift from Dr. J.D. Gitlin (Washington University, St. Louis, MO) (Hamza et al., 2003). Ovarian carcinoma 2008 cells were obtained from Dr. Phillip Disaia (Disaia et al., 1972).

Cell Survival Assay. Cell survival following exposure to increasing concentrations of drugs was assayed using the sulforhodamine B assay system (Monks et al., 1991). Five thousand cells were seeded into each well of a 96-well tissue culture plate. Cells were incubated overnight at 37°C, 5 percent CO_2 and then exposed to varying drug concentrations in 200 µl complete medium. Cells were allowed to grow for 5 days, after which the media was removed, the protein precipitated with 50 percent trichloroacetic acid and stained using 100 µl of 0.4 percent

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sulforhodamine B in 1 percent acetic acid at room temperature for 15 minutes. Following washing, the absorbance of each well at 515 nm was recorded using a Versamax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). All experiments were repeated at least three times using three cultures for each drug concentration.

Western blotting. Whole-cell lysates were dissolved in lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 10 mM Tris, pH 7.4) and were subjected to electrophoresis on 4 to 15 percent gels using ~30 µg of protein per lane. Protein levels were first determined by Bradford assay (Bio-Rad, Richmond, CA). A Bio-Rad trans-blot system was used to transfer the proteins to Immobilin-P membranes (Millipore, Billerica, MA). Blots were incubated overnight at 4°C in 4 percent, dry, nonfat milk in Tris-buffered saline (150 mM NaCl, 300 mM KCl, 10 mM Tris, pH 7.4, 0.01 percent Tween 20). Blots were incubated with primary antibody for 1 h at room temperature. A horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was dissolved in 4 percent milk in the Tris-buffered saline buffer and incubated with the blot for 1 h at room temperature. After four 5-min washes, blots were exposed to the PIERCE ECL reagent (Thermo Scientific, Wilmington, DE) and detected on X-ray films (HyBlot CL; Denville Scientific, Inc. Metuchen, NJ). The primary antibodies used for Western Blot analysis were: anti-CTR2 (provided by Dr. Jesse Bertinato), anti-CTR2 (Novus Biologicals, Littleton, CO) anti-tubulin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and anti-laminin B1 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA).

qRT-PCR. CTR2 mRNA levels were measured using a qRT-PCR method of detection of relative amounts of first-strand cDNA. cDNA was generated from mRNA isolated using Trizol (Invitrogen, Carlsbad, CA). Purified mRNA was converted to cDNA using Oligo$(dT)_{20}$ priming and the SuperScript III First-Strand Kit (Invitrogen, Carlsbad, CA). qRT-PCR was performed on

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a Bio-Rad MyIQ qPCR machine (Hercules, CA). The forward and reverse primers used were: mCTR2 forward – tccaggtagtcatcagct; mCTR2 reverse – tggcagtgctctgtgatgtc; ß-actin forward – aggtgacagattgcttctg; ß-actin reverse – gctgcctcaacacctcaac; Gapdh forward – tcaccaccatggagaaggc; Gapdh reverse – gctaagcagttggtggtgca; mATOX1 forward - ctgggaggagtggagttcaa ; mATOX1 - gccaaggtaggaaacagcct. Reactions were prepared using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), according to manufacturer's recommendations. Samples were prepared in quadruplicate with three independent sample sets being analyzed. Analysis was done using the Bio-Rad iQ5 system software (Hercules, CA).

Measurement of drug accumulation into whole cells. *Cells* were grown to 90 percent confluence in T-150 tissue culture flasks. Cells were then harvested using trypsin; 7.5 x 10^5 cells were placed into each well of 6-well tissue culture plates and allowed to grow overnight in 2.5 ml of media at 37°C in 5 percent CO_2. The next day, medium was removed by aspiration and the cells either pre-treated with $CuSO_4$, BCS or untreated control media for 1h. The media was then removed and the cells were exposed to 500 µl of Cisplatin-containing OptiMEM medium (Invitrogen, Carlsbad, CA) at 37°C for either 0 or 60 minutes, after which the drug-containing medium was removed, the plates were washed three times with ice-cold PBS and were then placed on ice. In the case of the time zero samples, the drug-containing medium was aspirated within 15 sec of the start of drug exposure. 214 µl of concentrated (50-70 percent) nitric acid was added to each well and the plate was incubated overnight at room temperature. The following day the acid was moved into Omni-vials (Wheaton, Millville, NJ) and incubated at room temperature overnight to thoroughly dissolve all cellular debris. The following day, the nitric acid was diluted with 3 ml of buffer (0.1 percent Triton X-100, 1.4 percent nitric acid, 1 ppb In in ddH_2O). Pt concentration was measured using a Perkin-Elmer Element 2 ICP-MS located at

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the Analytical Facility at Scripps Institute of Oceanography at the University of California, San Diego. As a method of normalization, total sulfur was measured using a Perkin-Elmer ICP-OES, also located at SIO at UCSD. Samples that were previously prepared for the ICP-MS were then introduced into the ICP-OES where total µg of sulfur was measured. All data presented are the means of at least three independent experiments each performed with six wells per concentration tested.

Measurement of CTR2 Half-life. 2008 cells were pre-incubated with $CuSO_4$ and BCS for 1 h as previously described and were then exposed to 100 µg/mL cyclohexamide for 0, 5, 10, 20, 30 or 45 min. Cells were then washed 3 times with PBS and lysates were harvested for western blotting as previously described.

Nuclear Fractionation. Cells were grown to ~90% confluence in 10 mm dishes, harvested and nuclei were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce) following the manufacturer's protocols.

Deconvolution Microscopy. Cells were grown on 8-well microscope chamber slides (Waltham, MA). Upon reaching ~60% confluence, the media was removed from each chamber. The chambers were then treated with either 300 µl DMEM RS media alone (Invitrogen, Carlsbad, CA) or DMEM RS containing either 200µM $CuSO_4$, 100 µM BCS or 30 µM DDP for 1 h. Following 1h drug exposure, the media was removed and the slide was treated then washed with PBS in triplicate. Cells were fixed with 3.7% formalin in PBS for 30 min followed by three 10 min PBS washes. Cells were then permeablized with 0.3% Triton X in PBS followed by three 1 min PBS washes. The slides were blocked for 1 h with 5% BSA in PBS and then treated with 20 µM anti-CTR2 antibody (provided by Dr. Jesse Bertinato) overnight at 4ºC followed by three 10 min PBS washes. The slides were then exposed to 1:1000 anti-Rabbit Texas Red for 1 h,

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washed 3 times in PBS and viewed using a Deltavision deconvolution microscope (Applied Percision, Inc. Issaquah, Washington). Other primary/secondary antibodies used include: anti-Nuclear Pore Complex (NPC) proteins 414 (Abcam, Cambridge, MA); anti-mouse FITC secondary antibody (Invitrogen, Eugene, OR).

Proteosomal Degradation Assay. 2008 cells were plated on chamber slides and treated with 50 nM bortezomib (BTZ) for 4 h or left as untreated controls. During the last hour of BTZ exposure, selected wells were treated with 100 μM BCS. The cells were then fixed and prepared for microscopy as previously described.

Statistical Analysis. All data were derived from at least 3 independent experiments, and presented with the standard error from the mean (SEM). Statistical comparisons were performed using a two-tailed t-test with the assumption of unequal variance.

RESULTS

Regulation of CTR2 Expression by Cu and DDP. Human ovarian carcinoma 2008 cells were exposed to 200 μM $CuSO_4$ for 1 h following which mRNA and cellular proteins were isolated to assess the effect of Cu on CTR2 expression. At 1 h, the level of CTR2 mRNA had increased by 2.4-fold ($p = 0.01$) as measured by qRT-PCR (Figure 1A), and this was accompanied by a 2.4-fold ($p = 0.02$) increase in CTR2 protein as quantified by western blot analysis (Figure 1B). To confirm that the availability of Cu affects CTR2 levels, the 2008 cells were treated with 100 μM BCS for 1 h to deplete intracellular Cu. As shown in Figures 1C and 1D, this brief period of Cu starvation produced a 29% decrease in CTR2 mRNA ($p = 0.052$) but a more marked 90% decrease in CTR2 protein level ($p = 0.0006$).

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To determine whether DDP was able to regulate CTR2 expression in a manner similar to that of Cu, cellular CTR2 mRNA and protein levels were measured following exposure of the 2008 cells to 30 μM DDP for 1 h. DDP treatment increased the CTR2 mRNA level by 1.4-fold ($p = 0.057$) (Figure 1A) and produced a similar 1.4-fold increase in protein level ($p = 0.063$) (Figure 1B). Thus, at the concentration tested, DDP caused only modest changes in CTR2 mRNA and protein expression compared to those produced by Cu alone when measured at 1 h.

To further document the ability of Cu and DDP to regulate the expression of CTR2 at the protein level, 2008 ovarian carcinoma cells were treated for 1 h with either 200 μM $CuSO_4$, 100 μM BCS, 30 μM DDP or drug-free control media and were then stained with an anti-CTR2 antibody and a fluorescently tagged secondary antibody and visualized by deconvolution microscopy. As shown in Figure 1C, exposure to 200 μM $CuSO_4$ greatly increased the cellular level of CTR2 protein, and Cu starvation produced by a 1 h treatment with 100 μM BCS resulted in near total loss CTR2 protein. In contrast, exposure to 30 μM DDP for 1 h produced only a modest increase in CTR2 staining.

The time course of the changes induced in the level of CTR2 protein by Cu, DDP and BCS were examined following a 1 h exposure to each agent. In response to $CuSO_4$ the increase in CTR2 peaked at the end of the 1 h exposure and then subsequently decreased to a nadir of 22% of control before returning to basal levels at 24 h (Figure 2A). The marked decrease in CTR2 produced by BCS gradually resolved once the drug was removed at 1 h and basal levels were attained by 24 h (Figure 2B). As shown in Figure 2C, following exposure to DDP the CTR2 protein level continued to increase over the ensuing 5 h reaching a peak of 2.6-fold at 6 h from the start of drug exposure but had returned to baseline by 24 h. Thus, a 1 h exposure to each

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of the three agents produced a perturbation in CTR2 protein level lasting out to at least 6 h from the start of drug exposure before resolving by 24 h.

Effect of Cu and DDP on CTR2 Half-life. To determine whether the increase in CTR2 level following DDP and Cu treatment was due to increased protein stability, 2008 cells were treated with cyclohexamide to block new CTR2 synthesis and the CTR2 level was determined as a function of time in the presence or absence of either DDP or Cu. The data presented in Figures 3A and B demonstrates that both Cu and DDP increased CTR2 stability. The half-life of CTR2 was 14.53 ± 2.2 min in the absence of either Cu or DDP. The addition of 30 μM DDP for 1 h increased CTR2 half-life to 22.67 ± 0.7 min, or by 1.6-fold ($p = 0.016$). Exposure to Cu increased the half-life to 42.56 ± 4.0 min, or by 3.1-fold ($p = 0.001$). Thus, Cu and DDP can regulate that expression of CTR2 at the post-transcriptional level.

Cu Starvation Enhances DDP Uptake and Sensitivity. Our previous studies in mouse embryo fibroblasts indicated that knockdown of mCTR2 enhanced the cellular accumulation and cytotoxicity of DDP (Blair et al., 2009). To determine whether reduction of CTR2 expression mediated by Cu depletion produced the same effect, and whether this occurred in human tumor cells, 2008 ovarian carcinoma cells were treated with either 200 μM $CuSO_4$, 100 μM BCS or drug-free control media for 1 h and then exposed to increasing concentrations of DDP for 15 min. The platinum content of the cells was then measured by ICP-MS. The data presented in Figure 4A indicates that pre-treatment with Cu did not significantly change the whole cell accumulation of DDP at 15 min. However, pre-treatment with BCS increased whole cell uptake at 15 min by 2.2-fold ($p<0.0003$).

To assess the effect of Cu exposure or depletion on the cytotoxicity of DDP, the cells were treated with either 200 μM $CuSO_4$, 100 μM BCS or drug-free control media for 1 h and the

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effect on growth rate over the ensuing 5 days was assessed using a sulforhodamine B assay. Figure 4B shows that pre-treatment with Cu did not significantly change the sensitivity of the 2008 cells to DDP. The mean (± SEM) IC_{50} was 100.4 ± 4.5 μM in the absence of Cu pre-treatment and 101.8 ± 0.9 μM when the cells were pre-treated with Cu. However, as shown in Figure 4C, BCS-mediated Cu starvation significantly increased sensitivity to DDP by 2.0-fold. The mean (± SEM) IC_{50} value was 100.4 ± 4.5 μM in the absence of BCS pre-treatment and 49.1 ± 14.2 μM with BCS pre-treatment ($p = 0.03$). Thus, similar to the effect of knocking down mCTR2 in mouse embryo fibroblasts, depletion of intracellular Cu enhanced both the cellular accumulation and cytotoxicity of DDP in 2008 human ovarian carcinoma cells. While this result is consistent with the concept that the effect of BCS is due to CTR2 depletion, it leaves open the possibility that the effect of BCS is mediated via up-regulation of CTR1 rather than depletion of CTR2. However, previous studies have shown that forced expression of CTR1 above its basal level had little effect on DDP cytotoxicity, and BCS only increases CTR1 levels by a modest amount (Holzer et al., 2004b).

Cu Starvation Reduces Nuclear CTR2 Levels. As shown in Figure 1C, CTR2 was found abundantly in the nucleus as well as the cytoplasm of 2008 cells. To determine whether nuclear as well as cytoplasmic levels of CTR2 were modulated by Cu and DDP, 2008 cells were pre-treated with 200 μM $CuSO_4$, 30 μM DDP, 100 μM BCS or drug-free control media for 1 h and stained with antibodies against CTR2 and the nuclear pore complex (NPC) proteins Figure 5A shows that CTR2 (red) was partially localized within the nucleus, appearing as discrete foci randomly distributed throughout the nucleus and not clearly associated with nucleoli. There appears to be no co-localization of CTR2 with the nuclear envelope (green). Three dimensional

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images derived from z-stack sections confirmed that CTR2 is present within the nucleus of these cells (Figure 5A).

Exposure of 2008 cells to 30 μM DDP for 1 h produced a small increase in nuclear CTR2 level (Figure 5A). Exposure to Cu produced a more pronounced increase without changing the distribution of CTR2 within the nucleus itself, and causing only a small change in the ratio of cytoplasmic to nuclear staining. Depletion of Cu with BCS caused almost complete disappearance of CTR2 from the nucleus as well as from other compartments of the cell. To assess the effect of Cu and DDP further, nuclei were isolated and subjected to western blot analysis. As shown in Figure 5B, CTR2 was readily detected in the nuclear fraction of 2008 cells. Exposure to 30 μM DDP for 1 h increased the amount of nuclear CTR2 by 25% ($p<0.05$). When the cells were treated with 200 μM $CuSO_4$, the total nuclear CTR2 increased by 40% ($p< 0.03$). Consistent with its effect on the level of CTR2 in the whole cell, exposure to 100 μM BCS completely eliminated CTR2 from the nuclear fraction. Cytosolic CTR2 mimicked the whole cell response; DDP and Cu increased cytosolic CTR2 by 1.3-fold and 2.4-fold, respectively (Figure 5B). Interestingly, as assessed by both immunofluorescent staining and western blot analysis, the effect of DDP and Cu on nuclear CTR2 was muted in comparison to their effect on cytosolic CTR2.

ATOX1 Influences the Expression of CTR2. If CTR2 levels are regulated by the availability of intracellular Cu, then defects in the network of Cu chaperones that control the distribution of Cu might be expected to modulate CTR2 levels as well. This question was addressed using a mouse embryo fibroblast subline in which both alleles of ATOX1 had been knocked out ($ATOX1^{-/-}$). In these cells there is a failure to deliver Cu to the secretory pathway for export from the cell and their steady-state Cu level is 3.2-fold higher than in the parental cells

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(Hamza et al., 2003). The parental ATOX1$^{+/+}$ and ATOX1$^{-/-}$ cells were treated with 200 μM $CuSO_4$, 30 μM DDP, 100 μM BCS or drug-free control media for 1 h and were then fixed and stained with antibody against CTR2. As shown in Figure 6A, loss of ATOX1 greatly increased the steady state level of CTR2. However, this change in steady state level did not appear to alter the subcellular localization of CTR2 protein (red). Cu and DDP mediated up-regulation of CTR2 protein was similar in the ATOX1$^{+/+}$ cells to that observed in 2008 cells. Interestingly, the level of CTR2 protein in ATOX1$^{-/-}$ cells did not significantly change when cells were treated with DDP and Cu. Furthermore, whereas exposure to 100 μM BCS markedly reduced CTR2 expression in ATOX1$^{+/+}$ cells, BCS failed to down-regulate CTR2 expression in the ATOX1$^{-/-}$ cells. The requirement for ATOX1 was further examined by western blot analysis. The data presented in Figure 6B confirm that the CTR2 protein level in untreated ATOX1$^{-/-}$ cells was 3.1-fold higher than in untreated ATOX1$^{+/+}$ cells ($p = 0.003$). Again, while DDP or Cu exposure affected CTR2 level in ATOX1$^{+/+}$ cells in a similar fashion to that in 2008 cells, DDP and Cu did not significantly alter CTR2 protein expression in ATOX1$^{-/-}$ cells. Exposure to BCS down-regulated the expression of CTR2 in the ATOX1$^{+/+}$ cells but failed to do so in the ATOX1$^{-/-}$ cells. BCS pre-treatment of the ATOX1$^{+/+}$ cells caused a near total loss of CTR2 protein (12.5%, $p < 0.0001$) whereas in the ATOX1$^{-/-}$ cells there was no discernable difference in expression.

To determine how ATOX1 influences CTR2 mRNA levels, CTR2 expression was quantified by qRT-PCR in the ATOX1$^{+/+}$ and ATOX1$^{-/-}$ cells with and without drug pre-treatment. Consistent with the protein expression data, the steady-state level of CTR2 mRNA in ATOX1$^{-/-}$ cells was 3.5-fold higher than that in theATOX1$^{+/+}$ cells (Figure 6C). The magnitude of the change in CTR2 mRNA produced by exposure to 200 μM $CuSO_4$ (1.8-fold increase, $p = 0.03$), 30 μM DDP (1.6-fold increase, $p<0.04$) or 100 μM BCS (32% decrease, $p<.07$) in

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ATOX1$^{+/+}$ cells was similar to that observed in 2008 cells. Interestingly, while the CTR2 protein level did not significantly change in the ATOX1$^{-/-}$ cells in response to DDP or Cu exposure, CTR2 mRNA expression in ATOX1$^{-/-}$ cells changed in a manner similar to that observed in ATOX1$^{+/+}$ and 2008 cells. Exposure to 200 μM $CuSO_4$ led to a 1.9-fold increase in CTR2 mRNA ($p<0.01$) and exposure to 30 μM DDP led to a 1.4-fold increase in CTR2 mRNA ($p = 0.02$). Cu starvation due to 100 μM BCS treatment resulted in a 43% decrease in CTR2 mRNA expression.

DISCUSSION

The level of expression of CTR2 is a major determinant of sensitivity to the cytotoxic effect of DDP (Blair et al., 2009). To study the regulation of CTR2 we measured the effect of Cu, DDP and Cu starvation on CTR2 mRNA and protein expression using drug concentrations at which CTR2 knockdown is known to produce phenotypic effects (Blair et al., 2009; Holzer and Howell, 2006; Holzer et al., 2004a). Treatment of 2008 human ovarian carcinoma cells with 200 μM $CuSO_4$ for 1 h led to a large 2.4-fold increase in both CTR2 mRNA expression and protein level. On the other hand, starving the 2008 cells of Cu by treating with 100 μM BCS for 1 h depleted CTR2 mRNA and reduced the protein to nearly undetectable levels. Similar results were obtained in mouse embryo fibroblast cells indicating that these phenomena are not artifacts of a single cell line. Thus, Cu regulates CTR2 expression and the close relationship between the changes in mRNA and protein levels support the conclusion that this regulation occurs, at least partially, at the transcriptional level. Having previously shown that the expression of CTR2 has a large effect on both the accumulation and cytotoxicity of DDP (Blair et al., 2009), it was of interest to determine whether DDP also modulates the expression of this protein. Exposure to 30

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μM DDP for 1 h increased CTR2 mRNA expression and protein levels by 1.4-fold. This indicates that, at a concentration known to be cytotoxic, DDP also up-regulated CTR2, and thus that DDP induces the expression of a protein that limits its own uptake into tumor cells. It is noteworthy that the two Cu transporters, CTR1 and CTR2, have opposite effects on the cellular accumulation of DDP, with CTR1 enhancing uptake and CTR2 limiting uptake (Blair et al., 2009; Larson et al., 2009), and that DDP and Cu have opposite effects on the level of expression of these two transporters. DDP causes rapid degradation of CTR1 (Holzer and Howell, 2006; Jandial et al., 2009) whereas at the same concentration it produces a modest increase in the expression of CTR2.

Although the perturbations in CTR2 protein level produced by all three agents resolved by 24 h, there were differences in the pattern of recovery among them. Cu produced a biphasic response with an initial increase at 1 h followed by a substantial decrease at 6 h indicating a secondary response perhaps reflecting a compensatory response to the changes in Cu homeostasis produced by the initial CTR2 elevation. In contrast, following a 1 h exposure to BCS there was a gradual return to basal levels without a secondary overshoot. DDP produced a substantially more prolonged rise in CTR2 than Cu, perhaps reflecting the fact that a large fraction of DDP that enters the cell resides in compartments from which it is not readily effluxed.

To determine whether Cu and DDP additionally regulate CTR2 by altering its degradation, CTR2 half-life and proteosomal degradation was measured following Cu exposure or starvation. Cyclohexamide inhibits new protein production, and the disappearance of a given protein during cyclohexamide exposure is commonly used to estimate its half-life. Using this approach, the half-life of CTR2 under basal conditions was found to be ~14.5 min. Following exposure to 200 μM $CuSO_4$ for 1 h, the half-life of CTR2 increased to ~42.5 min whereas

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following exposure to 30 μM DDP it increased to ~22.7 min. Although it is possible that cyclohexamide itself perturbed the half-life of CTR2 in the absence of Cu or DDP exposure, it is evident that that both Cu and DDP increase CTR2 half-life. This indicates that the increase in the level of CTR2 protein following exposure to Cu or DDP is due in part to an effect on CTR2 degradation. This was confirmed by showing that the degradation of CTR2 was blocked by the proteosome inhibitor bortezomib. It is of interest that the degradation of CTR1 by DDP and Cu is also mediated by proteosomal degradation (Holzer and Howell, 2006; Jandial et al., 2009). Thus, both CTR1 and CTR2 depend on the proteosome for degradation despite that fact that the signals that trigger down-regulation differ. CTR1 is known to become polyubiquitinated in response to the signal that triggers its degradation (DDP exposure) (Safaei et al., 2009); whether CTR2 becomes polyubiquinated in response to its degradation signal (Cu starvation) remains to be determined. The results reported here support the conclusion that CTR2 is regulated both transcriptionally and post-transcriptionally by Cu availability and exposure to DDP. Caution is required with regard to our data on transcriptional regulation since we have not determined whether there is an effect of Cu or DDP on CTR2 mRNA stability. In addition, the results do not permit a quantitative assessment of which is the dominant determinant of CTR2 protein levels.

Knockdown of CTR2 expression using RNA inference results in a large increase in the cellular accumulation and cytotoxicity of DDP (Blair et al., 2009). In addition, increased CTR2 levels have been identified as a potential marker of DDP resistance (Blair et al., 2009). Since Cu starvation leads to a rapid and near total loss of CTR2, and Cu exposure quickly up-regulates CTR2 levels, it was of interest to determine whether these Cu-induced changes in CTR2 also affect DDP accumulation and cytotoxicity. Whole cell Pt levels were measured by ICP-MS, and the extent of cell kill by SRB assay, after a 15 min exposure to DDP that was preceded by

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treatment with either Cu or BCS. Cu pretreatment did not significantly alter Pt uptake or enhance cytotoxicity; however, BCS pretreatment led to a 2.2-fold increase in whole cell Pt accumulation and a 2-fold increase in cytotoxicity. These results suggest that DDP accumulation is increased by depletion of CTR2 irrespective of how this was attained although they do not exclude the possibility that Cu starvation also alters the expression of other DDP transporters. These data suggest that Cu chelators might be used to enhance tumor sensitivity to the Pt-containing drugs.

Immunocytochemical and deconvolution microscopic examination confirmed the results of the qRT-PCR and western blot analyses with respect to the ability Cu and DDP to increase, and BCS to deplete, CTR2 in the 2008 ovarian cancer cells. Similar effects were also observed in mouse embryo fibroblasts. The immunocytochemical analysis disclosed two additional findings of interest. First, neither Cu nor DDP produced a major change in the subcellular localization of CTR2. Second, a substantial amount of CTR2 was present in the nucleus, a novel observation. This observation was confirmed in several cell lines using two independently derived CTR2 antibodies. When co-stained with an antibody to a nuclear envelope protein, and particularly when the images were reconstructed in 3 dimensions, it was clear that a fraction of CTR2 resides inside the nucleus. One possible reason why nuclear localization was not previously detected is that prior studies of CTR2 localization relied primary on cells transfected with exogenous CTR2 containing a C-terminal tag that may have interfered with nuclear trafficking (Bertinato et al., 2008; van den Berghe et al., 2007). The nuclear CTR2 was found in discrete randomly distributed sites that yielded a punctuate pattern similar to that of p-bodies. Like its effect on the level of CTR2 in the cytosolic and membrane-bound fractions, DDP and Cu treatment increased the amount of CTR2 within the nucleus; however, this increase appeared to be less dramatic than

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in the rest of the cell. These observations were confirmed by western blot analysis of nuclear fractions. The mechanism by which CTR2 reaches the nucleus and how nuclear levels are regulated is unknown and is to be the subject of further studies. However, there are now several examples of transmembrane proteins that function at the plasma membrane, including EGFR (Liao and Carpenter, 2007) and CD44 (Lee et al., 2009), that are also trafficked to the nucleus where they participate in transcriptional regulation. Therefore, it is feasible that CTR2 is trafficked to the nucleus in a similar manner and that CTR2 participates in transcriptional activation of Cu-responsive genes either directly or by regulating nuclear Cu. The exact role CTR2 plays in the nucleus as well as the mechanism by which it traffics to the nucleus are important questions for future investigation

ATOX1 is the Cu chaperone that transfers Cu to the efflux transporters ATP7A and ATP7B. ATOX1 has also recently been shown to be a Cu-dependent transcription factor capable of activating the expression of cyclin D1 and SOD1 (Itoh et al., 2008; Muller and Klomp, 2008). Analysis of the CTR2 promoter region with the Genomatix software suggested that it contains several potential ATOX1 binding motifs. This raised the possibility that the regulation of CTR2 mRNA expression by Cu, DDP and Cu starvation could be due to different transcriptional activation by ATOX1. Under steady-state conditions the $ATOX1^{-/-}$ cells were found to express 3.5-fold more CTR2 mRNA and 3.1-fold more protein than $ATOX1^{+/+}$ cells. This suggests that ATOX1 may play a role in inhibiting CTR2 expression under basal conditions either at the transcriptional or post-translational level. It is possible that the observed increase in CTR2 in $ATOX1^{-/-}$ cells is due to changes in the copper levels rather than direct ATOX1 transcriptional regulation. Consistent with this concept, $ATOX1^{-/-}$ cells have been reported to have higher endogenous Cu levels than $ATOX1^{+/+}$ cells. Furthermore, the magnitude of the changes in CTR2

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mRNA in response to excess Cu, DDP or starvation were similar in the ATOX1$^{+/+}$ and ATOX1$^{-/-}$ cells indicating that ATOX1 was not required for these perturbations and therefore is unlikely to be directly regulating CTR2 transcription. ATOX1 has been reported to directly regulate CTR1 proteosomal degradation stimulated by DDP and Cu (Safaei et al., 2009).While not essential for the changes in mRNA level, ATOX1 was essential to the ability of Cu starvation to down-regulate CTR2 protein. When cells are starved for Cu, CTR2 is quickly degraded by the proteosome. It is likely therefore that the interaction of ATOX1 with Cu is necessary for the stabilization of CTR2, and the apo-form of ATOX1 is necessary for CTR2 degradation. Figure 6D presents a model of how CTR2 might be regulated by ATOX1. ATOX1 appears to be necessary for the Cu-dependent regulation of CTR2 at the post-transcriptional level either through direct interaction with CTR2 or a downstream effect on a pathway that controls CTR2 stability. We suggest that ATOX1 in the apo-conformation, is necessary for the degradation of CTR2, as evidenced by the fact that both ATOX1 and copper starvation are necessary for post-transcriptional regulation of CTR2. Presence of the unbound form of ATOX1 is needed for CTR2 degradation, since both Cu and loss of ATOX1 stabilize CTR2 protein.

In summary, CTR2 has a large effect on DDP accumulation and sensitivity in human ovarian carcinoma cells. As such, study of its protein regulation remains tremendously important. CTR2 levels are regulated by the availability of Cu as well as by DDP at the transcriptional and post-transcriptional level. How CTR2 traffics to the nucleus, its role in the nucleus and how ATOX1 controls CTR2 expression remain worthy of further investigation in an effort to identify strategies by which the effectiveness of DDP can be enhanced.

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REFERENCES

Bellemare DR, Shaner L, Morano KA, Beaudoin J, Langlois R and Labbe S (2002) Ctr6, a vacuolar membrane copper transporter in Schizosaccharomyces pombe. *J Biol Chem* 277:46676-46686.

Bertinato J, Swist E, Plouffe LJ, Brooks SP and L'Abbe M R (2008) Ctr2 is partially localized to the plasma membrane and stimulates copper uptake in COS-7 cells. *Biochem J* 409:731-740.

Blair BG, Larson CA, Safaei R and Howell SB (2009) Copper transporter 2 regulates the cellular accumulation and cytotoxicity of Cisplatin and Carboplatin. *Clin Cancer Res* 15:4312-4321.

Disaia PJ, Sinkovics JG, Rutledge FN and Smith JP (1972) Cell-mediated immunity to human malignant cells. *Am J Obstet Gynecol* 114:979-989.

Hamza I, Prohaska J and Gitlin JD (2003) Essential role for Atox1 in the copper-mediated intracellular trafficking of the Menkes ATPase. *Proc Natl Acad Sci USA* 100:1215-1220.

Holzer AK and Howell SB (2006) The internalization and degradation of human copper transporter 1 following cisplatin exposure. *Cancer Res* 66:10944-10952.

Holzer AK, Katano K, Klomp LW and Howell SB (2004a) Cisplatin rapidly down-regulates its own influx transporter hCTR1 in cultured human ovarian carcinoma cells. *Clin Cancer Res* 10:6744-6749.

Holzer AK, Samimi G, Katano K, Naerdemann W, Lin X, Safaei R and Howell SB (2004b) The copper influx transporter human copper transport protein 1 regulates the uptake of cisplatin in human ovarian carcinoma cells. *Mol Pharmacol* 66:817-823.

Ishida S, Lee J, Thiele DJ and Herskowitz I (2002) Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proc Natl Acad Sci USA* 99:14298-14302.

Itoh S, Kim HW, Nakagawa O, Ozumi K, Lessner SM, Aoki H, Akram K, McKinney RD, Ushio-Fukai M and Fukai T (2008) Novel role of antioxidant-1 (atox1) as a copper dependent transcription factor involved in cell proliferation. *J Biol Chem* 283:9157-9167.

Itoh S, Ozumi K, Kim HW, Nakagawa O, McKinney RD, Folz RJ, Zelko IN, Ushio-Fukai M and Fukai T (2009) Novel mechanism for regulation of extracellular SOD transcription and activity by copper: role of antioxidant-1. *Free Radic Biol Med* 46:95-104.

Jandial DD, Farshchi-Heydari S, Larson CA, Elliot GI, Wrasidlo WJ and Howell SB (2009) Enhanced delivery of cisplatin to intraperitoneal ovarian carcinomas mediated by the effects of bortezomib on the human copper transporter 1. *Clinical Cancer Research* 15:553-560.

Jeney V, Itoh S, Wendt M, Gradek Q, Ushio-Fukai M, Harrison DG and Fukai T (2005) Role of antioxidant-1 in extracellular superoxide dismutase function and expression. *Circ Res* 96:723-729.

Kampfenkel K, Kushnir S, Babiychuk E, Inze D and Van Montagu M (1995) Molecular characterization of a putative Arabidopsis thaliana copper transporter and its yeast homologue. *J Biol Chem* 270:28479-28486.

Larson CA, Blair BG, Safaei R and Howell SB (2009) The role of the mammalian copper transporter 1 in the cellular accumulation of platinum-based drugs. *Mol Pharmacol* 75:324-330.

Lee JL, Wang MJ and Chen JY (2009) Acetylation and activation of STAT3 mediated by nuclear translocation of CD44. *J Cell Biol* 185:949-957.

Liao HJ and Carpenter G (2007) Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression. *Mol Biol Cell* 18:1064-1072.

Lin X, Okuda T, Holzer A and Howell SB (2002) The copper transporter CTR1 regulates cisplatin uptake in saccharomyces cerevisiae. *Mol Pharmacol* 62:1154-1159.

Lutsenko S, Tsivkovskii R and Walker JM (2003) Functional properties of the human copper-transporting ATPase ATP7B (the Wilson's disease protein) and regulation by metallochaperone Atox1. *Ann N Y Acad Sci* 986:204-211.

Miyayama T, Suzuki KT and Ogra Y (2009) Copper accumulation and compartmentalization in mouse fibroblast lacking metallothionein and copper chaperone, Atox1. *Toxicol Appl Pharmacol* 237:205-213.

Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J and Boyd M (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 83:757-765.

Muller PA and Klomp LW (2008) ATOX1: A novel copper-responsive transcription factor in mammals? *Int J Biochem Cell Biol* 41:1233-1236.

Portnoy ME, Schmidt PJ, Rogers RS and Culotta VC (2001) Metal transporters that contribute copper to metallochaperones in Saccharomyces cerevisiae. *Mol Genet Genomics* 265:873-882.

Rees E, Lee J and Thiele D (2004) Mobilization of intracellular copper stores by the Ctr2 vacuolar copper transporter. *J Biol Chem* 279:54221-54229.

Safaei R, Maktabi MH, Blair BG, Larson CA and Howell SB (2009) Effects of the loss of Atox1 on the cellular pharmacology of cisplatin. *J Inorg Biochem* 103:333-341.

van den Berghe PV, Folmer DE, Malingre HE, van Beurden E, Klomp AE, van de Sluis B, Merkx M, Berger R and Klomp LW (2007) Human copper transporter 2 is localized in late endosomes and lysosomes and facilitates cellular copper uptake. *Biochem J* 407:49-59.

Xiao Z and Wedd AG (2002) A C-terminal domain of the membrane copper pump Ctr1 exchanges copper(I) with the copper chaperone Atx1. *Chem Commun Camb*:588-589.

FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Measurement of CTR2 in 2008 cells following 1 h pre-treatment with either 200μM $CuSO_4$, 100 μM BCS, 30 μM DDP or drug-free media. (A) Relative mRNA levels measured by qRT-PCR. (B) Western blot and densitometric analysis of CTR2 protein levels. (C) Representative deconvolution micrographs of 2008 cells stained for CTR2 (red).

Figure 2. Time course of changes produced in CTR2 protein levels by a 1 h exposure to either: (A) 200 μM CuSO4; (B) 100 μM BCS; or (C) 4.5 μM DDP.

Figure 3. Measurement of CTR2 half-life by Western blot. (A) Blot showing lysates extracted from cells at 0, 5, 10, 20, 30 or 45 min following the start of exposure to 100 μg/ml cyclohexamide. Cells were pre-treated with either drug-free media, 200 μM $CuSO_4$ or, 30 μM DDP for 1 h prior to cyclohexamide exposure; (♦), control; (□), DDP pretreatment; (▲), Cu pretreatment. (B) Logarithmic representation of the percent remaining CTR2 protein as a function of time following the start of cyclohexamide treatment. (C) Deconvolution microscopy of 2008 cells following exposure to drug-free control media or 50 nM bortezomib in the presence or absence of 100 μM BCS.

Figure 4. (A) Uptake of DDP flowing 1 h pre-treatment with either 200μM $CuSO_4$, 100 μM BCS, or drug-free media. Whole cell ng Pt accumulation per μg sulfur. B) DDP concentration-survival curves for 2008 cells with (◊) and without (■) pretreatment with 200 μM $CuSO_4$ for 1 h.

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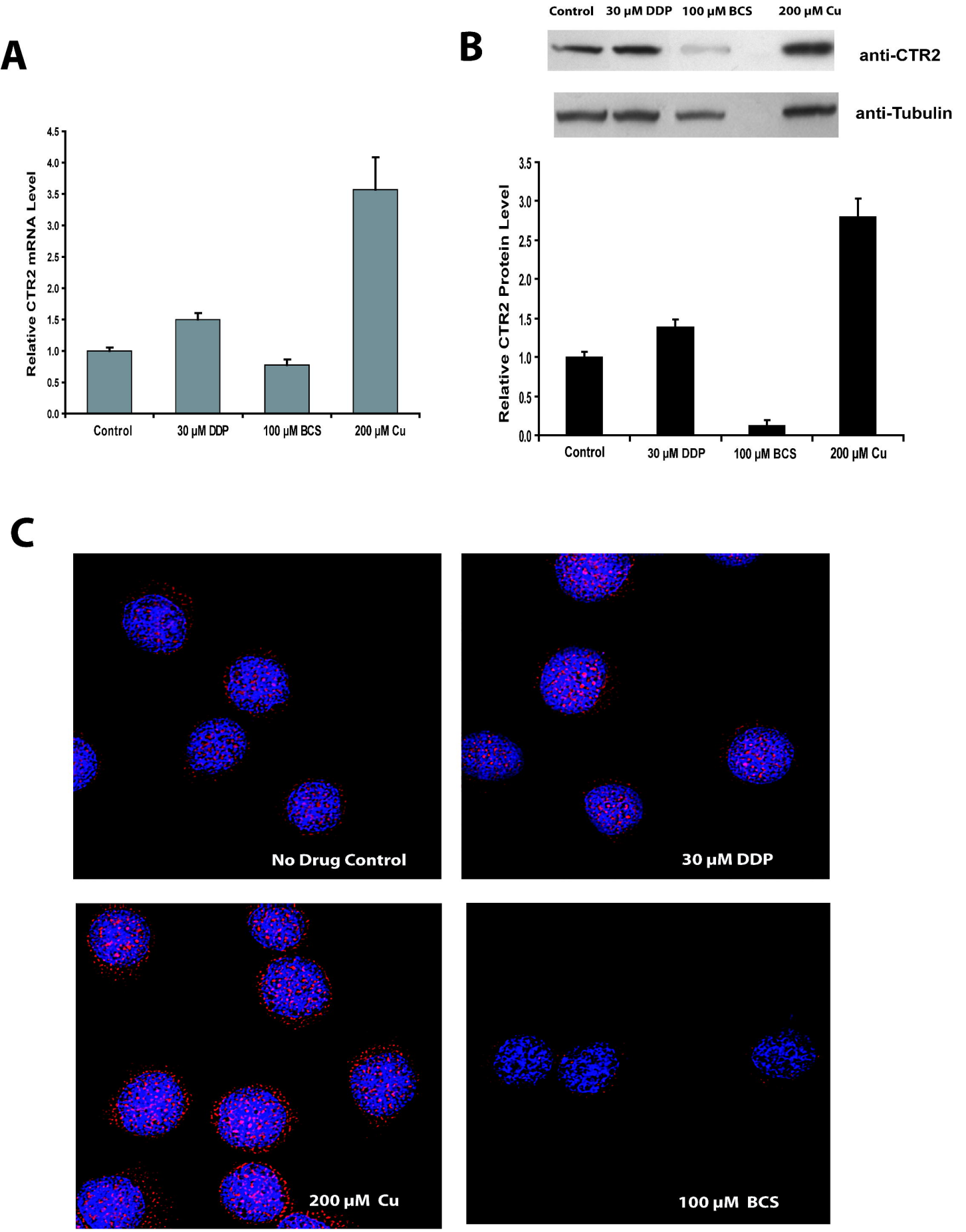
(C) DDP concentration-survival curves for 2008 cells with (◊) and without (■) pretreatment with 100 μM BCS for 1 h.

Figure 5. (A) Deconvolution microscopic images of 2008 cells following 1 h pre-treatment with either 200 μM $CuSO_4$, 100 μM BCS, or drug-free media. Red, CTR2; green, NPC proteins. (B) Western blot analysis of CTR2, tubulin and lamin B in the whole cell, cytosolic and nuclear fractions of 2008 cells. (C) Western blot analysis of CTR2 in the cytosolic and nuclear fractions of 2008 cells flowing 1 h pre-treatment with either 200 μM $CuSO_4$, 100 μM BCS, or drug-free media. Loading controls were anti-tubulin (cytosol) and anti-laminin B1 (nucleus).

Figure 6. (A) Deconvolution microscopic images of $ATOX1^{+/+}$ and $ATOX1^{-/-}$ cells following 1 h pre-treatment with either 200 μM $CuSO_4$, 100 μM BCS, or drug-free media. CTR2 is shown in red. (B) Western blot analysis of CTR2 in $ATOX1^{+/+}$ and $ATOX1^{-/-}$ cells flowing a 1 h pre-treatment with either 200 μM $CuSO_4$, 100 μM BCS, or drug-free media. (C) Relative CTR2 mRNA levels measured by qRT-PCR of $ATOX1^{+/+}$ (black) and $ATOX1^{-/-}$ cells (gray). (D) Model of CTR2 regulation by ATOX1.

Figure 1


A
Relative CTR2 mRNA Level
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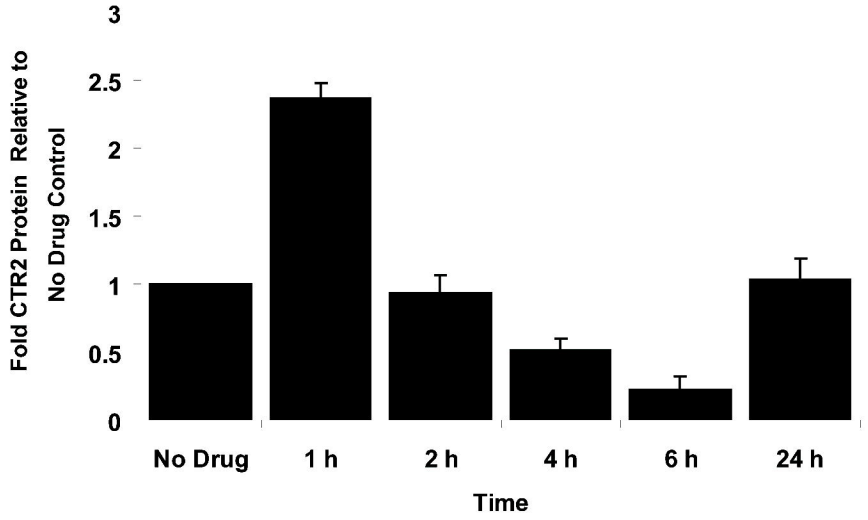
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
30 μM DDP
100 μM BCS
200 μM Cu
B
Control
30 μM DDP
100 μM BCS
200 μM Cu
anti-CTR2
anti-Tubulin
Relative CTR2 Protein Level
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
30 μM DDP
100 μM BCS
200 μM Cu
C
No Drug Control
30 μM DDP
200 μM Cu
100 μM BCS

Figure 2

A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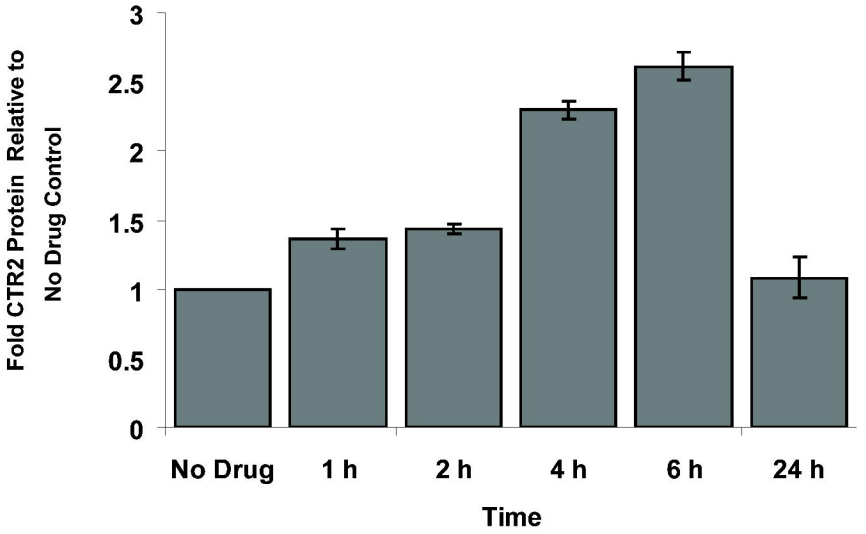
1 h
2 h
4 h
6 h
24 h
Time

B


Fold CTR2 Protein Relative to No Drug Control
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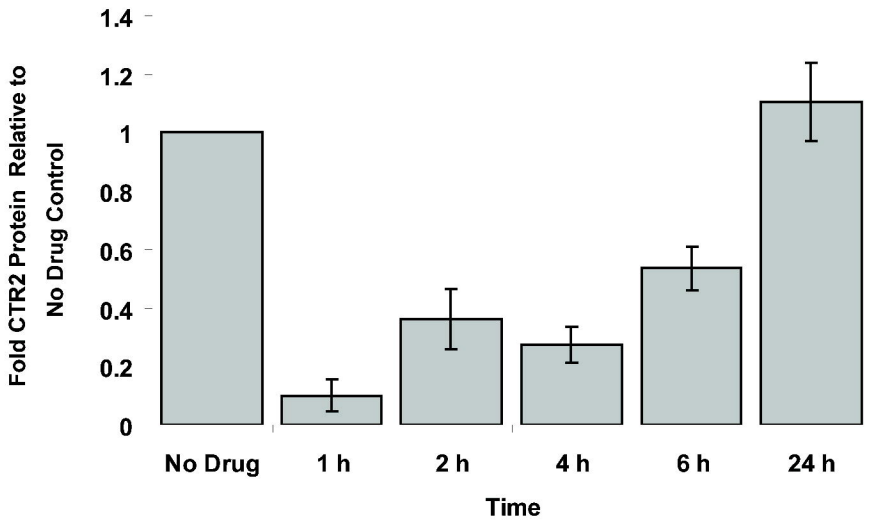
2
1.5
1
0.5
0
No Drug
1 h
2 h
4 h
6 h
24 h
Time

C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1
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0.4
0.2
0
No Drug
1 h
2 h
4 h
6 h
24 h
Time

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Figure 3

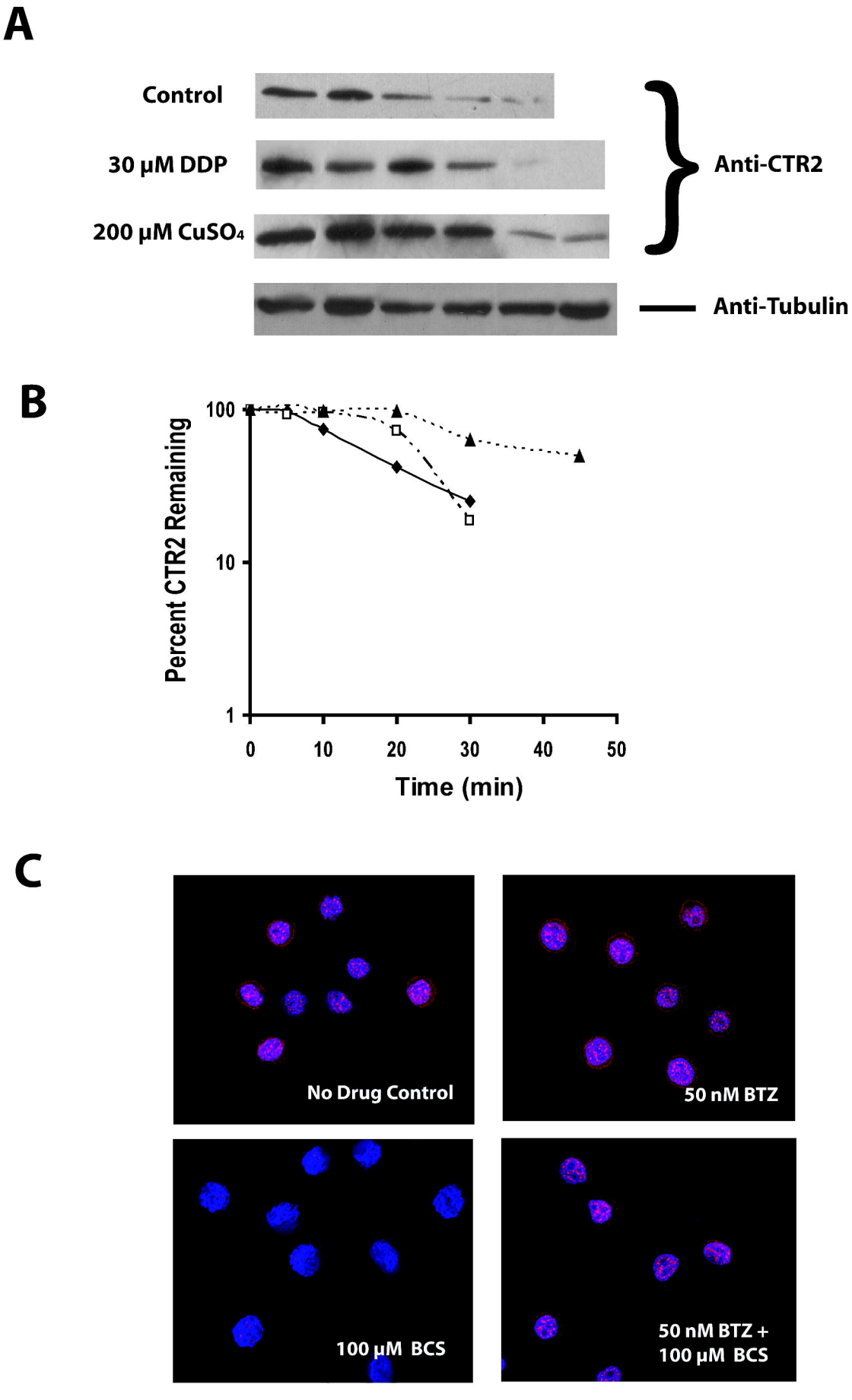
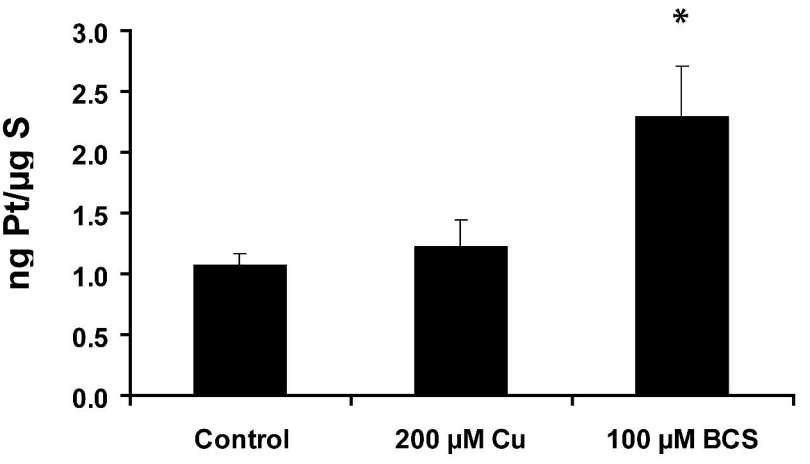


Figure 4

A


*
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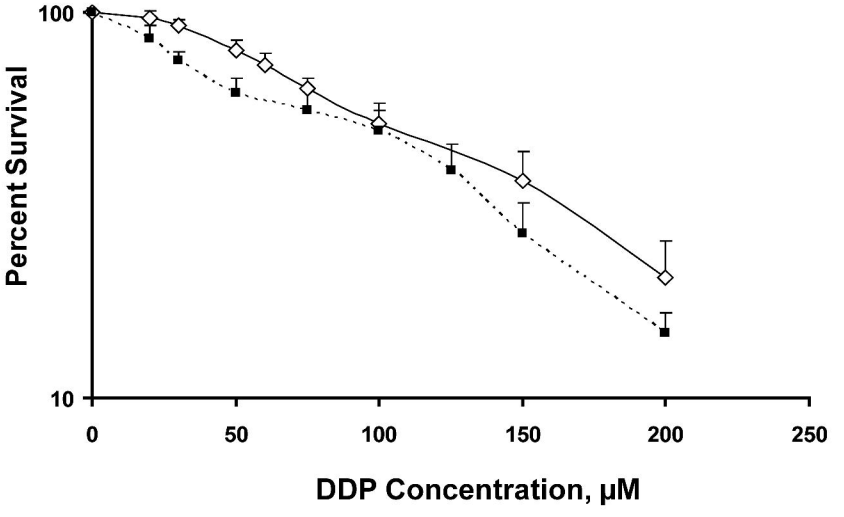
2.0
1.5
1.0
0.5
0.0
ng Pt/µg S
Control
200 µM Cu
100 µM BCS

B


100
10
Percent Survival
0
50
100
150
200
250
DDP Concentration, µM

C

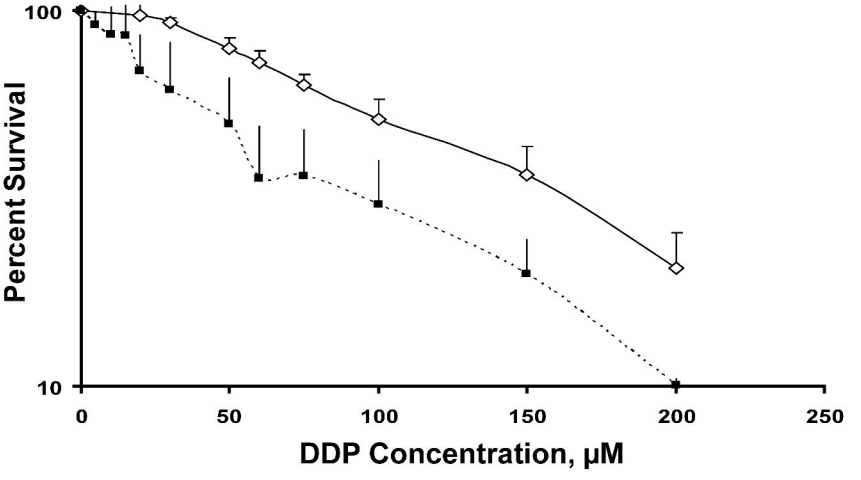

100
10
Percent Survival
0
50
100
150
200
250
DDP Concentration, µM

Figure 5

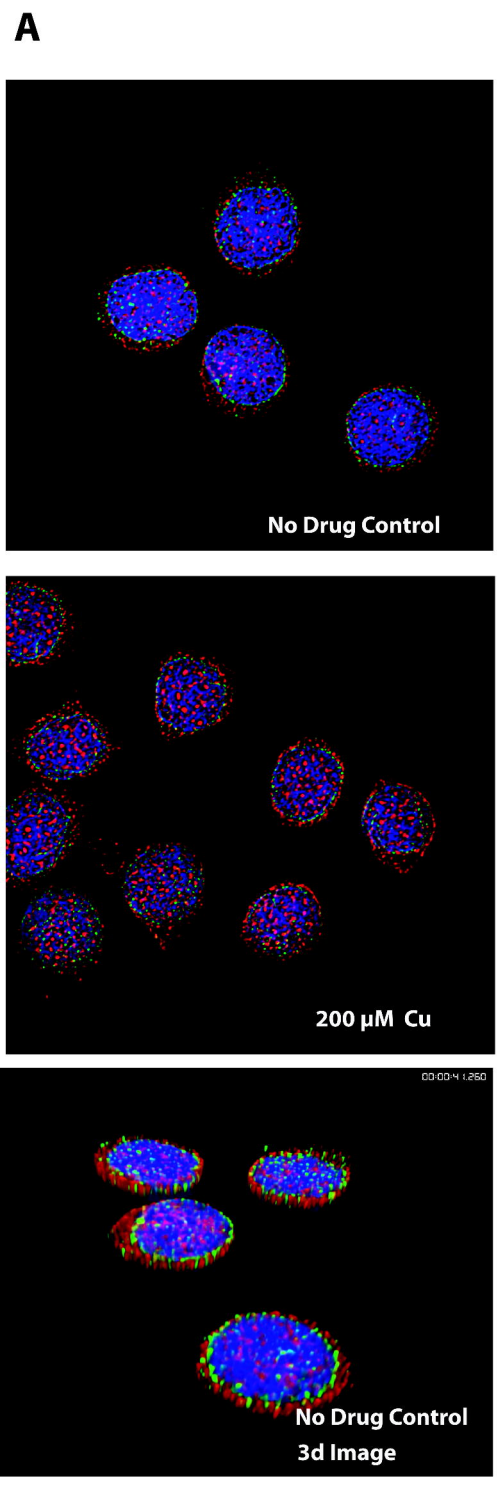


Figure 6