

Cisplatin Rapidly Down-regulates Its Own Influx Transporter hCTR1 in Cultured Human Ovarian Carcinoma Cells

Alison K. Holzer,¹ Kuniyuki Katano,¹
Leo W. J. Klomp,² and Stephen B. Howell¹

¹Department of Medicine and the Rebecca and John Moores Cancer Center, University of California at San Diego, La Jolla, California; and ²Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, The Netherlands

ABSTRACT

Purpose: Cisplatin (DDP)-resistant cells commonly exhibit reduced drug accumulation. Previous studies have shown that the major copper (Cu) influx transporter CTR1 controls the uptake of DDP in yeast and mammalian cells. The goal of this study was to examine the effect of Cu and DDP on the level and subcellular localization of hCTR1 protein in human ovarian carcinoma cells.

Experimental Design: Cultured human ovarian carcinoma A2780 cells were exposed to DDP and Cu, and the effect on hCTR1 was determined using Western blot analysis and confocal digital deconvolution microscopy.

Results: Loss of hCTR1 was triggered by DDP exposure in a concentration and time-dependent manner. Exposure to 0.5 $\mu\text{mol/L}$ DDP for 5 minutes reduced hCTR1 levels and exposure to DDP concentrations $\geq 2 \mu\text{mol/L}$ caused almost complete disappearance. The loss of hCTR1 was observed within 1 minute of the start of exposure to 2 $\mu\text{mol/L}$ DDP. Treatment of cells with 100 $\mu\text{mol/L}$ Cu for 5 minutes produced a smaller effect. Pretreatment of cells with 2 $\mu\text{mol/L}$ DDP for 5 minutes resulted in a 50% decrease in ⁶⁴Cu uptake, demonstrating that the DDP-induced loss of hCTR1 detected by Western blot analysis and imaging was functionally significant.

Conclusions: DDP down-regulated the amount of its major influx transporter in cultured human ovarian carcinoma cells in a concentration- and time-dependent manner. The effect was observed at DDP concentrations within the range found in the plasma of patients being treated with

DDP, and it occurred very quickly relative to the half-life of the drug.

INTRODUCTION

Cisplatin (DDP) is an important chemotherapeutic agent, but the development of resistance during therapy is common. Several mechanisms that can contribute to resistance have been identified, although the molecular changes that produce the stable DDP-resistant phenotype have not been well defined. Tumor cell death is dependent upon the amount of drug that enters the cell, the amount that enters the nucleus and reacts with DNA, how tolerant the cell is to lesions in the DNA, and how effectively the cell removes these lesions (1, 2). Although the development of resistance is thought to be multifactorial, impaired drug uptake is the single most consistently identified feature of DDP-resistant cells *in vitro* and *in vivo* (reviewed in refs. 1 and 3). The pathways by which DDP enters and exits from cells are poorly defined, but many DDP-resistant cells have a defect in the initial influx of drug. DDP enters cells much more slowly than most other classes of anticancer agents, and the currently available evidence suggests that one component of DDP uptake is mediated by a transport mechanism or channel (3).

Each of the factors known to affect the uptake of DDP also influence the uptake of copper (Cu; refs. 4 and 5). Cu uptake is mediated by a highly conserved transporter that is part of a pathway that is regulated by extracellular Cu level. Cu (I) enters the cell via specific transporters and is then sequestered by chaperones that deliver it to enzymes through direct protein-protein interactions between the chaperone and the Cu-requiring target. High-affinity Cu uptake occurs primarily via the membrane-spanning protein hCTR1. This transporter is highly conserved, and hCTR1 complements the function of yeast CTR1 and restores growth to *ctr1* Δ yeast mutants (6). The murine CTR1 shares 92% sequence homology with hCTR1, and deletion of both murine alleles results in embryonic lethality, indicating that CTR1 is essential for development (7, 8). Cu transport requires 2 critical methionine residues at positions 43 and 45 in the NH₂-terminal extracellular domain and 2 methionine residues in the second transmembrane region (9). hCTR1 also seems to possess two metal binding domains that play a role in scavenging Cu under conditions of Cu starvation (9). Cu transport by hCTR1 was found to be dependent on extracellular Cu concentration, extracellular pH, and extracellular potassium ion concentration (10). The cellular location of hCTR1 varies among cell types. Although it is commonly found on the plasma membrane, in some cell lines, it is also found in the perinuclear and Golgi regions of the cell (9, 11–13). In some cell types, hCTR1 seems to relocalize from the plasma membrane to vesicles upon exposure to Cu, although there are conflicting reports on this point (11, 12).

Several lines of evidence indicate that hCTR1 is important

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Requests for reprints: Stephen B. Howell, Department of Medicine 0058, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0058. Phone: 858-822-1110; Fax: 858-822-1111; E-mail: showell@ucsd.edu.

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to the cellular pharmacology of the Pt-containing drugs. There are many similarities between the uptake of DDP and Cu. Both are highly polar, and their uptake is influenced by factors known to affect transporter-mediated processes (3, 14). Furthermore, acquired DDP resistance is often accompanied by resistance to other metalloids (15, 16) and specifically by resistance to Cu (17, 18). Perhaps the most convincing evidence is the fact that knockout of CTR1 in yeast and mammalian cells markedly reduces DDP uptake and increases resistance to DDP cytotoxicity (19, 20). Loss of CTR1 function in yeast also impairs uptake of several DDP analogs including carboplatin, oxaliplatin, and AM0473 (19).

The mechanistic details of how hCTR1 mediates DDP uptake are unknown. We report here a study of the effect of DDP on the level of hCTR1 protein in human ovarian carcinoma cells that indicates that DDP very rapidly triggers the loss of endogenously and exogenously expressed hCTR1 in ovarian carcinomas and that this has functional consequences for the uptake of Cu. We conclude that DDP down-regulates the level of its own major influx transporter.

MATERIALS AND METHODS

Drugs and Reagents. Platinol-AQ was a gift from Bristol-Myers Squibb (Princeton, NJ). The clinical formulation containing 3.33 mol/L DDP was kept in the dark at room temperature. A 100 mol/L stock was created by diluting the drug in 0.9% NaCl. Cu in the form of cupric sulfate was obtained from Fisher Scientific (Tustin, CA). Protein concentration was measured using Bradford's Reagent from Bio-Rad, Inc. (Hercules, CA). The generation of the rabbit polyclonal antibodies against amino acids 1 through 67 of hCTR1 used for immunofluorescence is described elsewhere (12). Fluorescein isothiocyanate-conjugated goat antirabbit antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Hoechst 33342 dye for nuclear staining was purchased from Molecular Probes (Eugene, OR). The polyclonal rabbit anti-hCTR1 antibodies used for Western blotting was generated by immunizing rabbits with a peptide containing amino acids 2 to 22 of the NH₂ terminus of hCTR1 by Biocarta, Inc. (San Diego, CA). Horseradish peroxidase-conjugated goat antirabbit secondary antibody was purchased from Amersham Biosciences (Piscataway, NJ). All other chemicals and reagents were obtained from Fisher Scientific. ⁶⁴CuSO₄ was purchased from the Mallinckrodt Institute of Radiology, Washington University Medical School (St. Louis, MO).

Cell Lines. The A2780 and 2008 ovarian carcinoma cell lines (21, 22) were grown in RPMI 1640 containing 10% fetal bovine serum at 37°C in 5% CO₂. A2780 cells were engineered to overexpress hCTR1 by transfecting them with a pcDNA3.1 vector containing full-length hCTR1 cDNA and expressing a Geneticin resistance marker constructed as described previously (23) that was generously provided by Dr. Lisbeth Birk Moller (John F. Kennedy Institute, Glostrup, Denmark). Cells were transfected with either hCTR1/pcDNA3.1 or empty vector using LipofectAMINE (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Transfected cells were selected with 500 µg/mL Geneticin. Surviving clones were combined to create a multiclonal population.

Western Blot Analysis. Cells were grown in T75 flasks until 80% confluent and then exposed to DDP, Cu, or medium alone for various times and at various concentrations. After exposure, cells were harvested by trypsinization and pelleted by centrifugation at 4°C. The pellets were frozen at -20°C for 1 hour, then thawed on ice, resuspended in 100 µL of homogenizing buffer [250 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 1 µg/mL aprotinin, 1 µg/mL pepstatin, 1 µg/mL leupeptin, and 20 µg/mL phenylmethanesulfonylfluoride], and homogenized using a Dounce homogenizer for 1 minute. The suspensions were centrifuged for 15 minutes at 500 × g at 4°C, and the protein contents of the supernatant were measured. Supernatant samples containing 100 µg of protein were boiled before electrophoresis in a 4 to 20% SDS-PAGE gel. Transfer to nitrocellulose membranes (Bio-Rad Co.) was performed electrophoretically for 30 minutes at 200 volts using a Transblot SD apparatus (Bio-Rad Co). Membranes were blocked with 5% milk in Tris-buffered saline for 1 hour at room temperature. Blots were incubated at 4°C overnight with anti-hCTR1 antibodies diluted 1:1000 or with anti-Na/K⁺ATPase diluted 1:1000 (Novus Biologicals, Littleton, CO) and mouse antitubulin (Sigma Products, St. Louis, MO) diluted 1:20,000 in 5% milk in Tris-buffered saline. Membranes were then washed three times with Tris-buffered saline containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated antimouse and antirabbit antibodies for 1 hour at room temperature. Before detection, membranes were again washed three times with Tris-buffered saline containing 0.05% Tween 20, and detection was performed using the enhanced chemiluminescence Western detection system from Amersham Biosciences according to manufacturer's instruction.

Fixation and Staining. Cells were grown in T75 flasks until 80% confluent, then harvested by trypsinization, and pelleted by centrifugation in 10% RPMI 1640 with 10% fetal bovine serum. The resulting pellets were resuspended in 5 mL of 10% RPMI 1640 with 10% fetal bovine serum, and 100 µL of cells were added to each well of a 24-well plate. Before the addition of cells, a 0.16-mm-thick coverslip and 300 µL of 10% RPMI 1640 containing 10% fetal bovine serum and 200 µmol/L bathocuprione disulphonic acid were placed in each well. Bathocuprione disulphonic acid was added to chelate any Cu in the medium ensuring that Cu could not affect DDP binding. Once cell growth on the coverslips reached 80% confluency at approximately 48 hours after plating, the cells were exposed to 10% RPMI 1640 with 10% fetal bovine serum containing Cu or DDP for 0 to 5 minutes. After the specified duration of exposure, the coverslips were placed at 4°C, washed three times with PBS, and then fixed in 3.7% formaldehyde in the same buffer at room temperature for 30 minutes. Subsequent staining and washing were performed as described previously (12).

Measurement of ⁶⁴Cu Accumulation. Cu uptake measurements were made using cells grown to 80% confluency in the 30-mm wells of a 6-well plate. After addition of prewarmed medium containing 2 µmol/L ⁶⁴CuSO₄, the plates were incubated at 37°C in 5% CO₂ for 5 minutes. At the end of the incubation period, the plates were placed on ice and rinsed three times with 3 mL of ice-cold PBS. Cell lysis buffer (0.1% Triton-X and 1% SDS in PBS) in a volume of 500 µL was added to the wells, and the lysate was harvested by scraping the

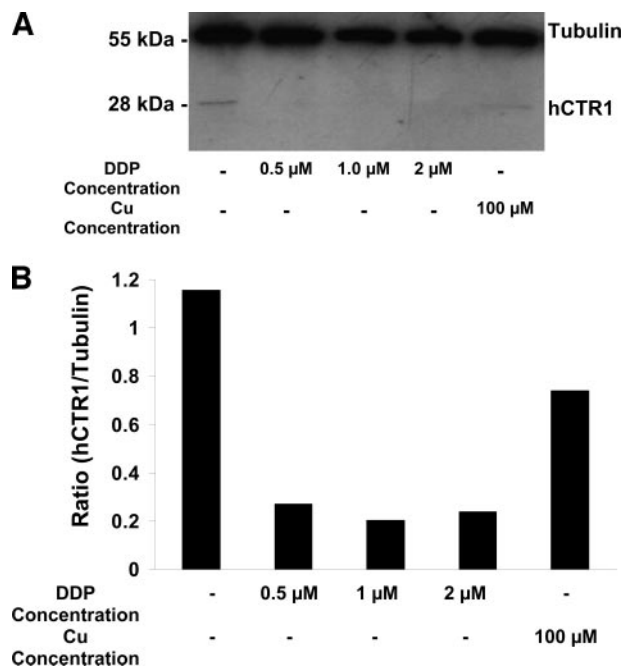


Fig. 1 Western blot analysis of the effect of DDP and Cu on the level of endogenous hCTR1 in A2780 cells. A2780 ovarian carcinoma cells were treated with 0.5, 1, 1.5, or 2 μ mol/L DDP or 100 μ mol/L Cu for 5 minutes. **A**, denaturing Western blot showing the level of tubulin and hCTR1 (representative of five separate experiments). **B**, densitometric analysis showing level of hCTR1 relative to the tubulin lane loading control.

dish twice before it was transferred to tubes for γ counting on a Beckman Gamma 5500B. Six cultures were harvested for each time point. Lysates from a set of identical cultures not exposed to $^{64}\text{CuSO}_4$ were used to measure protein concentration. For cells that were pretreated with 2 μ mol/L DDP, normal medium was replaced with medium containing 2 μ mol/L DDP for 5 minutes. Cells were then rinsed once with warm medium, and the $^{64}\text{CuSO}_4$ -containing medium was added.

RESULTS

Western Blot Analysis of the Effect of Cisplatin on hCTR1. Regulation of the level of CTR1 protein is believed to occur primarily by post-transcriptional mechanisms (24). In yeast, exposure to elevated levels of Cu triggers both endocytosis and degradation as separate events (4). Recent reports suggest that endocytosis and protein degradation are also important to the amount of hCTR1 expressed in the plasma membrane of mammalian cells as well (11). To determine whether DDP alters the level of hCTR1, the level of endogenous hCTR1 in membrane-enriched samples was measured in cultured human ovarian carcinoma A2780 cells before and after the start of exposure to DDP. As shown in Fig. 1, a 5-minute exposure to 2 μ mol/L DDP produced a marked reduction in hCTR1 protein level. Additional experiments were done to define the minimum concentration of DDP that reduced the hCTR1 level when measured at 5 minutes. When exposed to 2 μ mol/L DDP, a clear decrease was detectable even at 1 minute (data not shown). Fig.

1 shows that even a 5-minute exposure to 0.5 μ mol/L DDP was effective in reducing the hCTR1 level. As seen in Fig. 1, the loss of hCTR1 was manifested by disappearance of the M_r 28,000 band; there was no smearing of the bands suggestive of degradation of the protein. Similar results were obtained with the 2008 ovarian carcinoma cell line and with a A2780 subline stably transfected with a vector expressing hCTR1 from a cytomegalovirus promoter, indicating that DDP triggered the disappearance of endogenously and exogenously expressed hCTR1 (data not shown). Thus, at concentrations found in the plasma of patients treated with DDP, this drug triggers the rapid disappearance of hCTR1 from human ovarian carcinoma cells. A similar but less marked effect was observed when cells were treated with 100 μ mol/L Cu for 5 minutes. As shown in Fig. 1, Cu caused a decrease in hCTR1 protein level at 5 minutes, although the reduction was less than that produced by 2 μ mol/L DDP. The decrease in Cu-treated cells was similar to that reported by Petris *et al.* (11).

To document that the disappearance of hCTR1 on Western blots was due to a decrease in the protein rather than masking of the epitopes recognized by the antibody, denaturing and nondenaturing gels were used in all experiments. Identical results were observed when the cell lysates were run under either condition. Furthermore, the extracellular domain of hCTR1 containing amino acids 1 to 67 was expressed in *Escherichia coli*, and this purified fragment of hCTR1 was incubated with either 100 μ mol/L Cu or 2 μ mol/L DDP for 5 minutes and analyzed on denaturing and nondenaturing Western blots. There was no diminution in the ability of the anti-hCTR1 antibody to recognize epitopes on the extracellular domain after exposure to either Cu or DDP (data not shown). Additionally, cell extracts were obtained from untreated cells and then incubated with 100 μ mol/L Cu or 2 μ mol/L DDP for 5 minutes before being subjected to denaturing and nondenaturing Western blot analysis. The antibody to hCTR1 recognized hCTR1 in untreated cellular extracts and those treated with Cu or DDP equally well (data not shown). The retention of signal under all of these situations suggests that the loss of hCTR1 signal on the Western blots after DDP exposure is due to the disappearance of the protein and not an inability of the antibody to recognize hCTR1 after DDP or Cu exposure.

To determine whether the DDP-induced loss of hCTR1 was specific to hCTR1 and not due to a general loss of membrane proteins, the lysates from DDP- and Cu-treated cells were also probed with an antibody to Na/K⁺ATPase. The Na/K⁺ATPase is known to localize to the plasma membrane and thus serves as a good control to exclude the possibility that DDP triggers degradation or endocytotically mediated membrane clearance of other intrinsic proteins. As shown in Fig. 2, no difference in the amount of Na/K⁺ATPase was observed after exposure to 0.5 to 2 μ mol/L DDP by Western blot analysis of either denatured or nondenatured lysates. Thus, the disappearance of hCTR1 after exposure to low concentrations of DDP is specific for hCTR1 in comparison with Na/K⁺ATPase.

Confocal Image Analysis of the Effect of Cisplatin on hCTR1. The cellular location of hCTR1 varies among cell types when determined by immunofluorescent staining. Although it is commonly found on the plasma membrane, in some cells, it is also found in the perinuclear and Golgi regions of the

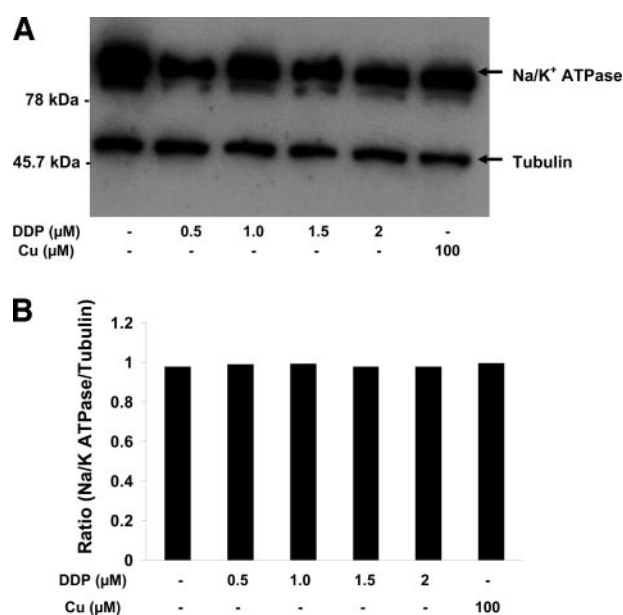


Fig. 2 Denaturing Western blot analysis of Na/K⁺ATPase in lysates isolated from cells incubated with DDP or Cu (representative of three separate experiments). A2780 ovarian carcinoma cells were treated with 0.5, 1, 1.5, or 2 μmol/L DDP or 100 μmol/L Cu for 5 minutes. A, Western blot; B, densitometric analysis of the Western blot in A.

cell (9, 11–13). One group has reported that hCTR1 relocalizes from the plasma membrane to intracellular vesicles upon exposure to Cu (11). To investigate the localization of endogenous hCTR1 in the cultured ovarian carcinoma cells, the A2780 and 2008 ovarian carcinoma cell lines were stained with an antibody specific for the NH₂-terminal region of hCTR1 and imaged by deconvolution confocal microscopy. As shown in Fig. 3, in exponentially growing A2780 cells, hCTR1 was found associated with the plasma membrane and with vesicular structures scattered throughout the perinuclear region. Exposure of A2780 cells to 0.5 μmol/L DDP for 5 minutes resulted in a reduction in hCTR1 staining, and exposure to 1 μmol/L caused nearly complete loss of all hCTR1 staining. To establish a time course for the loss of hCTR1 staining, A2780 cells were exposed to 2 μmol/L DDP for varying times up to 5 minutes. This concentration of DDP was found to cause marked disappearance of hCTR1 staining by 1 minute. As a control, A2870 cells were exposed to 100 μmol/L Cu for up to 5 minutes. Cu also produced a decrease in the level of hCTR1 at the plasma membrane at 5 minutes, although the decrease was not as dramatic. Similar effects on hCTR1 plasma membrane levels in response to exposure to DDP and Cu were also observed in human ovarian carcinoma 2008 cells and A2780 cells molecularly engineered to overexpress hCTR1 (data not shown). The results of the confocal imaging studies confirm the Western blot analysis and indicate that DDP produces a very rapid and major reduction in hCTR1 level in human ovarian carcinoma cells.

Effect of Cisplatin Exposure on Cu Uptake. Because hCTR1 is the major Cu influx transporter, if exposure to DDP reduces the level of this protein, one would expect that the initial rate of uptake of ⁶⁴Cu would also be reduced. A2780 cells were

exposed to 2 μmol/L DDP for 5 minutes, washed, and then exposed to 2 μmol/L ⁶⁴Cu for the subsequent 5 minutes. This concentration of 2 μmol/L ⁶⁴Cu was chosen based on the reported *K_m* for Cu of 1.7 μmol/L (13). As shown in Fig. 4A, the untreated cells accumulated 23 ± 2 pmol Cu per milligram of protein (mean ± SEM), whereas the DDP treated cells accumulated only 11.5 ± 0.6 pmol Cu per milligram of protein. Thus, exposure of the A2780 cells to even a very low concentration of DDP for a short period of time was sufficient to produce a 50% reduction in the initial rate of Cu uptake. The effect of exposure to Cu on the uptake of DDP was also examined. Because Cu was less effective at down-regulating hCTR1, and once hCTR1 was down regulated little DDP was taken up, A2780 cells were exposed first to 100 μmol/L Cu for 1 hour and then to 2 μmol/L DDP for 30 minutes. Fig. 4B shows that the untreated cells accumulated 4.179 ± 0.712 pmol Pt per milligram of protein (mean ± SEM), whereas the Cu-treated cells accumulated only 2.491 ± 0.246 pmol Pt per milligram of protein. Thus, exposure of A2780 cells to Cu at a concentration shown by digital confocal microscopy to cause disappearance of hCTR1 also reduced Pt accumulation uptake by 40%.

DISCUSSION

The results of this study provide strong evidence for an interaction between DDP and hCTR1 that triggers the disappearance of hCTR1 from the cell, thus indicating that DDP down-regulates its own major influx transporter. DDP causes reduction of hCTR1 levels at concentrations well within the range of those found in the plasma of cancer patients receiving therapy with this drug, and the effect of DDP was substantially greater than that produced by exposure to Cu at a 50-fold higher concentration. How either DDP or Cu modulates the level and trafficking of hCTR1 is not yet clear. Cu seems to have different effects in different experimental systems. Klomp *et al.* (12) reported that neither Cu starvation followed by Cu exposure nor an increase in extracellular Cu concentration was capable of triggering the degradation of endogenous hCTR1 in HeLa, BeWo, or Caco 2 cells. Conversely, Petris *et al.* (11) reported that exogenous hCTR1 was rapidly cleared from the plasma membrane of HEK293 cells and degraded upon exposure to Cu.

Multiple lines of evidence indicate that DDP triggers the actual degradation of hCTR1 rather than simply destroying the epitopes with which the anti-hCTR1 polyclonal antibody reacts. The fact that loss of protein was observed by both immunofluorescent confocal microscopy and by Western blot analysis of both denatured and nondenatured protein suggests that hCTR1 is rapidly degraded. If the loss of signal were due to masking of the antigenic sequence, either to due cleavage of the protein or an alteration in its conformation, then one would not expect to observe a comparable loss of protein under circumstances in which it is in a native conformation and when it is denatured and lacks tertiary structure. Furthermore, when the purified NH₂-terminal portion of the molecule, against which the antibody was generated, was exposed to either DDP or Cu, there was no loss of signal on Western blot analysis, indicating that the conformation of the NH₂ terminus did not change in a manner that blocked or hid the antibody recognition site. Finally, the decrease in Cu uptake after treatment of cells with DDP pro-

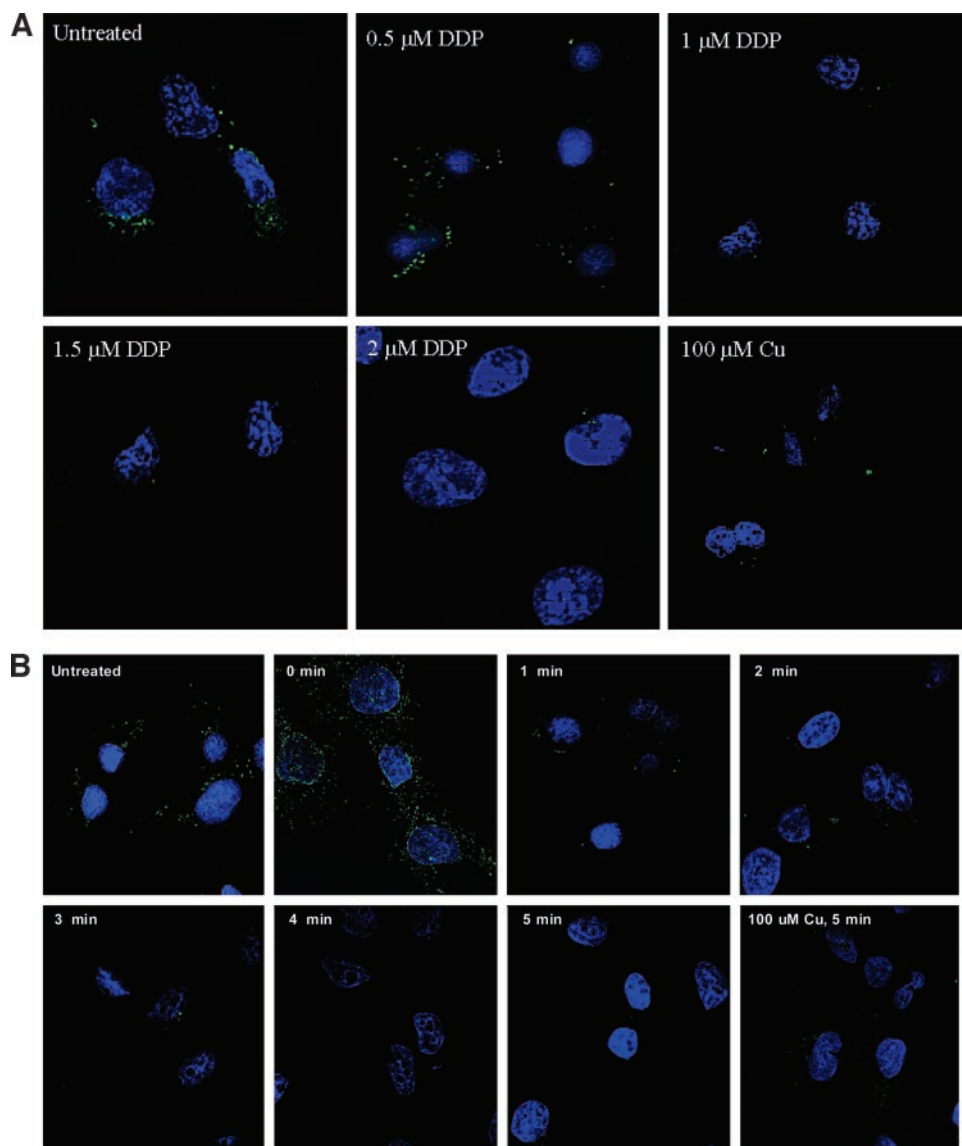


Fig. 3 Confocal microscopic analysis of the effect of DDP on hCTR1 in A2780 cells. **A.** A2780 cells were exposed to 0, 0.5, 1, 1.5, or 2 $\mu\text{mol/L}$ DDP or 100 $\mu\text{mol/L}$ Cu for 5 minutes. **B.** A2780 cells were exposed to 2 $\mu\text{mol/L}$ DDP for 0, 1, 2, 3, 4, or 5 minutes or 100 $\mu\text{mol/L}$ Cu for 5 minutes. hCTR1 was visualized using the rabbit anti-hCTR1 antibody and a goat anti-rabbit FITC-conjugated secondary. Hoechst 33342 dye was used to label the nucleus. Each image is representative of three images taken from each of three independent experiments. Images are normalized to the autofluorescence of unstained A2780 cells and cells stained with only the secondary antibody.

vides evidence that the function of hCTR1 was in fact diminished by prior exposure to DDP.

It is of interest that DDP was found to be more potent on a molar basis than Cu in its ability to reduce hCTR1 levels and that the magnitude of the reduction was greater after exposure to DDP. This difference in potency and magnitude of effect was observed by both Western blot analysis and confocal microscopy. Treatment with 2 $\mu\text{mol/L}$ DDP for 5 minutes resulted in an 85% decrease in hCTR1 levels, whereas treatment with 100 $\mu\text{mol/L}$ CuSO₄ produced only a 66% decrease in protein level in the same time frame. The NH₂-terminal extracellular region of hCTR1 is rich in MXXM and MXMXXM sequences. These sequences are similar to putative Cu-binding motifs found in other proteins (23) and that are excellent candidates for interaction with DDP as well. However, given the different coordination chemistry of Cu and DDP, it is likely that the way in which these metalloids alter the structure of this region upon

binding is quite different, and this may be the basis for their different effects on hCTR1 degradation and trafficking. Furthermore, the first 25 amino acids of the hCTR1 extracellular domain are also very rich in methionine and histidine residues. Whereas amino acids 40 to 45 of the extracellular domain are essential for Cu transport (25), DDP could be binding to sites in the first 25 amino acids of hCTR1. Binding at this position could result in an altered protein conformation that may elicit a stronger or more rapid down-regulation response.

hCTR1 seems to account for a significant fraction of all of the DDP accumulated by the cell (19, 20). The ability of DDP to cause the disappearance of this transporter indicates that DDP likely rapidly impairs its own ability to enter cells as it impairs the influx of Cu. It is of note that Naguma *et al.* (26) found that Cu can protect mice against DDP toxicity *in vivo*, an affect that may be explained by Cu-mediated down-regulation of hCTR1. Although it is unlikely that hCTR1 is the sole influx transporter

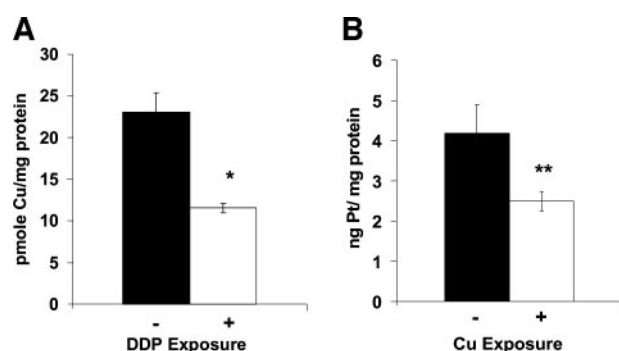


Fig. 4 Effect of pretreatment with either DDP or Cu on subsequent cellular accumulation. **A.** A2780 ovarian carcinoma cells were treated with 2 $\mu\text{mol/L}$ DDP for 5 minutes, washed, and then exposed to with 2 $\mu\text{mol/L}$ $^{64}\text{CuSO}_4$ for 5 minutes. **B.** A2780 cells were treated with 100 $\mu\text{mol/L}$ Cu for 1 hour and then exposed to 2 $\mu\text{mol/L}$ DDP for 30 minutes. Each bar represents the mean of three independent experiments, each performed with sextuplet cultures. *, $P < 0.0008$; **, $P < 0.002$. Vertical bars, SEM.

for DDP, pharmacologic antagonism of its ability to reduce hCTR1 level by inhibition of protein degradation pathways may be the basis for a clinically important strategy for increasing delivery of this important chemotherapeutic agent to tumors.

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