Title: A Re-evaluation of the Role of hCTR1, the human high affinity Cu transporter in Pt-Drug Entry into Human Cells.

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Abbreviations: cDDP, cisplatin; hCTR1, human copper transporter 1; Cu, copper; Ag, silver; CBDCA, carboplatin; L-OHP, oxaliplatin; trans, transplatin; ICP-MS, inductively coupled plasma mass spectrometry; mefs, mouse embryonic fibroblasts; A2780, human ovarian tumor cells; A2780CP, cisplatin resistant human ovarian tumor cells, hek, human embryonic kidney cells; tet, tetracycline; Ab’s, antibodies
ABSTRACT

Cisplatin (cDDP) is an anti-cancer drug used in a number of malignancies including testicular, ovarian, cervical, bladder, lung, head, and neck cancers. Its use is limited by the development of resistance, often rationalized via effects on cellular uptake. It has been claimed that hCTR1, the human high affinity copper transporter, is the major entry pathway for cDDP and related drugs via a mechanism that mimics copper. This is an unexpected property of hCTR1, a highly selective copper (I) transporter. We compared the uptake rates of copper with cDDP (and several analogs) into HEK293 cells over-expressing wild-type or mutant hCTR1, mouse embryonic fibroblasts (mefs) that do or do not express CTR1, and human ovarian tumor cells, sensitive or resistant to cDDP. We have also compared the effects of extracellular copper, which causes regulatory endocytosis of hCTR1, to those of cDDP. We confirm the correlation between higher hCTR1 levels and higher Pt-drug uptake in tumor cells sensitive to the drug. However, we show that hCTR1 is not the major entry route of platinum-drugs and that the copper transporter is not internalized in response to extracellular drug. Our data suggest the major entry pathway for platinum-drugs is not saturable at relevant concentrations and not protein-mediated. Clinical trials have been initiated that depend upon regulating membrane levels of hCTR1. If reduced drug uptake is a major factor in resistance, hCTR1 is unlikely to be a productive target in attempts to enhance efficacy, although the proteins involved in copper homeostasis may play a role.
INTRODUCTION

Platinum drugs, including cisplatin (dichloro-diamino-platinum, or cDDP), have been used since the 1970’s as chemotherapeutic agents for a variety of malignancies, including testicular, ovarian, cervical, bladder, lung, head, and neck cancers (Loehrer and Einhorn, 1984; Zumaeta et al., 2001). One major limitation of cDDP usage is the rapid development of resistance (Giaccone, 2000). cDDP, along with its later developed analogs, act by forming DNA crosslinks. Platinum crosslinks inhibit DNA replication and halt cell division. The DNA repair system is activated and once repair is unsuccessful, apoptosis is activated (Andrews and Howell, 1990; Kelland, 2007). There has been considerable interest in understanding the cellular entry mechanism of platinum drugs, as their effectiveness depends on the amount delivered to the tumor cell.

Copper is an essential micronutrient playing a critical role as a co-factor in a number of cellular enzymes, including SOD, cytochrome c oxidase, among others. Initial studies on the entry of cDDP into yeast cells indicated that entry correlated with the expression of CTR1, the copper transporter in the yeast plasma membrane (Ishida et al., 2002; Lin et al., 2002). The same correlation was seen with hCTR1 in some tumor cells (Song et al., 2004; Zisowsky et al., 2007). Several subsequent studies confirmed this correlation and led to the suggestion that hCTR1, the major high affinity copper-transporter, also mediates platinum-drug uptake (Chen et al., 2012; Kalayda et al., 2012; Larson et al., 2010b; Larson et al., 2009; Pabla et al., 2009).

Elevated copper in the extracellular media causes an internalization of the transporter, with an associated decrease in copper entry, a process that is reversed when copper loads return to normal. This acute regulatory response protects against excessive copper accumulation (Molloy and Kaplan, 2009). It had earlier been reported that copper-induced internalization of
hCTR1 results in degradation of the transporter (Petris et al., 2003). Such internalization and degradation has also been reported following cDDP addition to human ovarian carcinoma cells (Holzer and Howell, 2006). Copper homeostasis in mammalian cells utilizes hCTR1 for entry and ATP7A and ATP7B (copper-activated ATPases) for mediating its exit (Kaplan and Lutsenko, 2009). These ATPases are also responsible for delivering copper to proteins in the secretory pathway. Samimi et al showed in Me32a human fibroblasts that expressed neither ATP7A nor ATP7B were more sensitive to cDDP, copper, and carboplatin than cells transfected with vectors expressing ATP7A or ATP7B (Samimi et al., 2004). Thus transporters involved in copper homeostasis were again associated with cDDP efficacy. There has recently been a clinical trial assuming that increases in membrane levels of hCTR1 would lead to enhanced platinum drug entry and increased drug effectiveness (Fu et al., 2012).

Although there is experimental evidence that the proteins involved in copper homeostasis, the uptake and efflux transporters and cellular copper chaperones, may interact with platinum-containing drugs, most of the evidence that identifies hCTR1 as the major cDDP entry pathway relies upon correlative relationships rather than demonstrated causality (Rabik and Dolan, 2007). It is surprising that a highly selective transporter, such as hCTR1, that has been shown to transport only Ag and Cu (I) ions would also be capable of mediating cDDP transport. cDDP has a diameter of 9.57Å (Hilder and James, 2007) while Cu (I) in aqueous solution has a diameter of about 3.6Å (Blumberger et al., 2004). cDDP is over two times larger than copper and therefore highly unlikely to fit within the narrow pore of hCTR1, which has an estimated diameter at the entrance of less than 8Å (De Feo et al., 2007).

We have carried out a detailed analysis of the relationship between hCTR1, copper uptake, and cDDP (and several analogs) uptake in HEK cells that over-express hCTR1, in mefs
that do or do not express CTR1, and in human ovarian tumor cells that are sensitive or resistant to cDDP. We have compared the responses of hCTR1 in these cells to cDDP with their responses to copper, and have examined the effects of varied hCTR1 levels on copper and cDDP entry rates and also the consequences of altering hCTR1 expression on the rates of copper and platinum-drug entry. We provide evidence that the major entry pathway into human cells for platinum-containing anti-cancer drugs is not hCTR1, and is most likely not protein-mediated. Our data have considerable significance for future approaches to improve platinum-drug efficacy.
MATERIALS and METHODS

Cell Culture. Cells were maintained in a humidified incubator at 37ºC under 5% CO2 atmosphere and passaged every 3-5 days. HEK293 Flp-In™ T-Rex™ (Invitrogen), Mefs +/+ and -/- cells (a generous gift from Dr Dennis Thiele, Duke University), and ovarian carcinoma A2780CP and A2780 cells (Fox Chase Cancer Center, Jenkintown, PA) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals), and 25mM Hepes.

HEK 293 Flp-In cells containing tetracycline-regulated N-terminal FLAG –tagged hCTR1 were created as previously described (Maryon et al., 2007). The cells were transfected with hCTR1 construct using Lipofectamine 2000 (Invitrogen). Transfected cells were selected in 12 μg/ml blastocidin S (RPI Corp.) and 400 μg/ml hygromycin (Invitrogen). Resistant colonies were pooled and tested for tetracycline-regulated expression. Cells were cultured in media containing 1 μg/ml tetracycline for 48 hrs before harvesting. Mutants of hCTR1 were created as previously described (Maryon et al., submitted for publication).

Biotinylation of Surface hCTR1. Biotinylation of cells were carried out using the cell-impermeable, thiol-cleavable Sulfo-NHS-SS-biotin (Pierce) reagent to label cell surface proteins at 4ºC as described previously (Molloy and Kaplan, 2009). Samples were separated by SDS-PAGE, and hCTR1 was detected using either an anti-hCTR1 or anti-FLAG antibody by Western blot analysis as described below.

SDS-PAGE and Western Blot Analysis. Protein samples were separated using protocol described in our earlier studies (Zimnicka et al., 2007) For detection of hCTR1 protein, the following
primary antibodies were used: rabbit anti-C terminus hCTR1 antibody at 1:4000 dilution (Maryon et al., 2007) or mouse anti-Flag antibody (GenScript) followed by anti-rabbit IgG horseradish peroxidase conjugate (GE Healthcare) or anti-mouse HRP (Thermo Scientific). Western blot signals were obtained using SuperSignal West reagents (Pierce) and the intensity of the Western signals was measured using chemiluminescent imaging with the ChemiDoc XRS (BioRad) and quantitated using QUAN-TITY ONE Version 2.6.2 software (Bio-Rad). Loading controls were either alpha subunit of the Na, K ATPase or beta-catenin, as indicated in the Figures. It should be pointed out that commercially available antibodies against hCTR1 have proven to be of limited value for following hCTR1 in western blot analysis. Several of these show either multiple bands or bands at about 25kD, the mass of the unglycosylated precursor, but not 35kD, the mature product. Any antibody used against hCTR1, which has poor antigenicity need to be validated against recombinant proteins and also in cells that do and do not express hCTR1 along with effects of deglycosylation on apparent mass.

64Cu Uptake. The day before the assay, cells were plated at 0.6 x 10^6 cells/ml into each well of a 3 x 4 tissue culture plate in DMEM containing 10% fetal bovine serum and incubated at 37 ºC. The following day, fresh media containing 64Cu (Isotrace Technologies Inc.) was added and was incubated for 1hr at 37 ºC. Copper uptake rates were then measured as previously described (Zimnicka et al., 2011). All transport determinations were carried out in triplicate. The amount of protein per well was determined after radioisotope decay, and the copper uptake/well calculated as pmol of Cu/mg protein/hr, the average of triplicate wells was determined for each treatment.
Pt-drug Uptake. One day prior to the assay, cells were plated at 10.8 x 10⁶ cells/ml in 10-cm tissue culture plates in DMEM containing 10% fetal bovine serum and incubated at 37 ºC. The day of the assay, the media was aspirated and cells were incubated in cisplatin, carboplatin, transplatin or oxaliplatin dissolved in DMEM containing 10% fetal bovine serum for 1hr at 37ºC. The uptake was stopped by the addition of stop buffer (Zimnicka et al., 2011). The cells were washed an additional 2 times with stop buffer. Cells were collected and resuspended in 70% nitric acid for 3 hrs. Afterwards, the cells were diluted to 14% nitric acid by the addition of 1ml of water, mixed, centrifuged and the supernatant was submitted for platinum content by inductively coupled plasma mass spectrometry (ICP-MS) at the Chemical Analysis Laboratory in Athens, Ga. The results were expressed as pmol Pt/ milligram protein/ hour.

Drugs. Cis-Platin (Enzo), transplatin (Sigma-Aldrich), carboplatin (TCI), oxaliplatin (Enzo) solutions were freshly prepared for each experiment. Cisplatin and transplatin were dissolved in 100% dimethyl sulfoxide (DMSO); carboplatin and oxaliplatin were dissolved in water.

Cell Fractionation. Fractionation of cells was carried out using a five-step sucrose gradient as previously described (Zimnicka et al., 2007).

siRNA-mediated knock-down of hCTR1. A2780 cells were transiently transfected with siRNA plasmids from Ambion (Life Technologies) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to manufacturer’s instructions. The cells were cultured to 50% confluence and incubated with either siRNA duplexes against CTR1 or siRNA negative control in Opti-MEM medium (Invitrogen). The following day, fresh DMEM media was added. Human
CTR1 knockdown was a pre-designed sequence. Forward target sequence used was as follows:
5'-GCCUAUGACCUUCUACUUUt-3'; Reverse target sequence: 5'-AAAGUAGAAGGUCAUAGGCat-3'.

Statistical analysis: Data are shown as means ± SD. Statistical analysis was performed using
Student’s t-test, and *P* <0.05 was considered statically significant. The data are representative of
means of at least three independent experiments and values are expressed as percentages of total
cell surface hCTR1, to which no copper or cisplatin was added.
RESULTS

HEK cells overexpressing hCTR1.

We have previously developed a line of HEK cells that express hCTR1, the human high affinity copper transporter at the flip-in recombinase site, under the influence of a tetracycline-sensitive promoter (Maryon et al., 2007). When the initial rate of $^{64}$Cu uptake into these cells, grown in the absence or presence of tetracycline, is measured, cells grown in the presence of the antibiotic show a higher (about 8-10-fold, see Figure 1A) rate. However, when the rate of cDDP entry is determined in these two sets of cells, there is no difference in the rate of uptake of the platinum drug (Figure 1B). These data suggest that elevated rates of copper entry correlate with an increase in the expression level of hCTR1 while the rate of cDDP entry is not altered by over-expression of hCTR1. The increase in hCTR1 expression has been previously documented (Zimnicka et al., 2011).

If hCTR1 is important for the entry of cDDP, and copper and cDDP interact in a similar fashion with hCTR1, it would be expected that cells expressing mutants of hCTR1 that show elevated rates of copper entry would also show enhanced cDDP entry rates. We have recently discovered that truncations at the intracellular C-terminus of hCTR1 produce hCTR1 mutants with greatly enhanced (about 10-fold) Vmax values for copper entry (Maryon et al., submitted for publication). The entry of copper into cells expressing the tr179 mutant that lacks the final 11 amino acid residues of hCTR1 has a much higher rate (about 15-fold) than cells expressing wild-type hCTR1. However, the rates of cDDP entry of these two sets of cells are identical (Figure 1C) and the same as cells not over-expressing hCTR1 (Figure 1B). Thus, it is likely that cDDP is not transported by hCTR1 in the same manner as copper in these cells.
It has previously been shown that when cells are exposed to elevated copper levels, one mechanism to protect the cells against copper toxicity is a lowering of the entry rate. This is accomplished by a copper-dependent endocytosis of hCTR1 (Molloy and Kaplan, 2009; Petris et al., 2003) and associated decrease in copper entry (Molloy and Kaplan, 2009). This phenomenon is illustrated in the data of Figure 1D, where cell-surface biotinylation has been employed to determine the plasma membrane level of hCTR1. Clearly the amount of hCTR1 in the HEK plasma membranes falls when the cells are incubated with excess copper; and when copper is removed, the transporter returns to the surface (Figure 1D). In these same cells, there is no internalization of the transporter in response to high levels of extracellular cDDP (Figure 1D). This is in contrast to results reported in human ovarian carcinoma cells (Holzer and Howell, 2006; Holzer et al., 2004a) and in agreement with previous studies in HEK293 cells (Guo et al., 2004; Pabla et al., 2009) and renal proximal tubules cells as well studies performed in mice (Pabla et al., 2009). Previous studies have suggested that only a brief exposure (5-15 minutes) to cDDP causes rapid internalization and degradation of hCTR1 (Holzer and Howell, 2006; Holzer et al., 2004a; Larson et al., 2009). We exposed tetracycline-induced HEK cells to 50 μM cDDP for 1, 5, 10, 30, and 180 minutes. No reduction of surface hCTR1 (ie internalization) was evident (Figure 1E). We also exposed cells to 3 μM cDDP for the same times, with the same results (data not shown). These results suggest cDDP does not cause internalization of hCTR1, and thus cellular entry of cDDP is unlikely to be via the endocytosis of hCTR1.

Mouse embryonic fibroblasts (mefs).

When the rate of cDDP entry into cells that do not express CTR1 (mefs -/-) are compared with similar cells that endogenously express the copper transporter (mefs +/+), it is apparent that
the presence or absence of CTR1 has no influence on the rate of cDDP entry (see Figure 2A). Furthermore, the presence of copper in the medium did not inhibit the rate of cDDP entry (Figure 2A). It has previously been shown that the copper entry rate into the -/- mefs is about 25% -30% of the +/- cells via an unidentified pathway (Eisses and Kaplan, 2002; Lee et al., 2002; Zimnicka et al., 2011). Thus it seems unlikely that CTR1 in these cells plays a significant role in cDDP entry. We have also examined the entry rates of several analogs of cDDP that are in clinical use. The absence or presence of CTR1 does not alter their rates of uptake (Figure 2B).

It is apparent from these studies that CTR1 does not play a major role in mediating platinum-drug entry into these model cells. However, the most relevant issue is whether or not hCTR1 plays a role in cDDP entry into tumor cells. We have extended our studies on cDDP entry to tumor cells of relevance to these issues.

Ovarian tumor cell lines.

Two ovarian cancer cell lines were examined: A2780 and A2780CP, that were sensitive or resistant to cDDP, respectively (see Methods). It is clear that there is a significant difference in the expression levels of hCTR1 in the plasma membrane of these cells. The sensitive cells have a higher level of hCTR1 than the resistant cells (about 2 fold see Figures 3A, 3B). This agrees with previous correlations that led to the suggestion that hCTR1 played a major role in cDDP entry, as the resistant line would take up less cDDP than sensitive tumor cells. Rates of copper transport were measured and resistant tumor cells had a 2-3 fold lower rate of copper uptake when compared to sensitive tumor cells (Figure 3C), in keeping with their lower expression of hCTR1. When cDDP entry rates are compared in the two cell lines, the resistant cells take up cDDP at a significantly slower rate (Figure 3D), which correlates with the hCTR1 expression levels.
Exposure of the tumor cells to excess copper causes an internalization of plasma membrane hCTR1 (see Figures 4A, 4B) as in HEK 293 cells (Figure 1D, 1E). Exposure of the tumor cells to cDDP (20 µM or 50 µM) did not cause internalization of the transporter, just as had been observed in the HEK cells, emphasizing that hCTR1 does not respond in tumor cells or in normal cells in the same manner to excess cDDP and to excess copper. However in contrast to the HEK cells, removal of copper from the external medium did not result in the return of hCTR1 to the plasma membrane of the ovarian tumor cells (Figure 4C) as is observed in the acute regulatory mechanism seen in HEK cells. This enabled a further experiment to be performed in this cell system. If hCTR1 is internalized following exposure to elevated copper (~50%) and if hCTR1 is responsible for cDDP entry, then cDDP entry should be inhibited by a prior Cu treatment of the ovarian cells. This protocol was applied to the ovarian cells. It can be seen that although about 50% of the plasma membrane hCTR1 is internalized following copper treatment, cDDP uptake is unaffected (Figure 4D).

In an experiment to directly alter hCTR1 expression in tumor cells, we employed siRNA of hCTR1 in the A2780 ovarian carcinoma cells. Figure 5A and 5B shows we achieved ~80% knockdown of plasma membrane hCTR1. In the cells transiently transfected with hCTR1 siRNA, the rate of copper transport was reduced by ~50% (Figure 5C). However, cDDP uptake was not altered (Figure 5D).

The mechanism of cDDP entry.

In earlier studies in Ehrlich ascites tumor cells, based on the use of non-specific chemical reagents it was suggested that cDDP entry might occur by a non-protein-mediated pathway (Gale et al., 1973). The most likely being a partition-diffusion mechanism, where an uncharged or lipophilic molecule partitions into the membrane and diffuses down its concentration gradient to
emerge into the cytosol. One of the characteristics of such a mechanism is that it would be non-saturating (since a limited number of sites are not involved). Furthermore, interactions between proteins and substrates are expected to show some stereospecific discrimination. This is often seen where D but not L isomers are recognized by enzymes or transporters (Narawa et al., 2007). We have tested both of these expectations.

In Figure 6B (see also Figure 3D), the concentration-dependence of cDDP uptake into ovarian tumor cells has been examined. Over a wide range of concentrations uptake is approximately linear and there is no sign of saturation. Although D and L isomers of cDDP are not available, the essential elements of such enantiomers are retained by comparison of cDDP (or cisplatin) and the non-therapeutic analog, transplatin (Figure 6A). The concentration-dependence of the uptakes of these two analogs is identical (Figure 6B).
**DISCUSSION**

Correlations between hCTR1 expression and drug entry in yeast and tumor cells (Ishida et al., 2002; Lin et al., 2002; Song et al., 2004) led to the idea that the copper transporter mediated cDDP entry. The basis for clinical trials (Fu et al., 2012) of agents that might influence responsiveness to cDDP is that cDDP is transported like copper, via a pore formed by the transporter (Larson et al., 2010b). It was also suggested that extracellular cDDP, like copper, causes internalization of hCTR1 (Holzer and Howell, 2006; Holzer et al., 2004a). Thus, hCTR1 would be down-regulated in response to cDDP and further uptake would be reduced, leading to resistance. Our studies provide strong evidence that, although the proteins involved in copper homeostasis influence cellular cDDP levels, hCTR1 does not play a major role in cDDP entry. The most probable entry mechanism is via a non-protein-mediated pathway.

cDDP uptake in normal eukaryotic cells.

hCTR1 does not play a major role in cDDP uptake in HEK and mef cells based on the following: (a) over-expression of hCTR1 increases copper entry, but not cDDP (Figures 1A, 1B); (b) cells lacking CTR1 (-/- mefs) show the same rates of cDDP entry as cells (+/+ mefs) with hCTR1 (Figures 2A, 2B); (c) cells overexpressing hyperactive hCTR1 mutants have identical rates of cDDP uptake to cells not over-expressing hCTR1 (Figure 1C).

Previous studies in mefs, HEK cells and yeast yielded varied conclusions. In HEK cells no increase in cytotoxicity to cDDP was seen when hCTR1 was over-expressed (Rabik et al., 2009). Other studies in HEK and other renal cells claimed that siRNA-induced decrease in hCTR1 resulted in a decrease in Pt uptake (Pabla et al., 2009). However, questions remain on the Ab used to quantitate hCTR1 (see internalization, below). In previous studies using mef’s, in
contrast to our results, higher uptake of Pt was seen in cells expressing hCTR1 than in -/- cells (Larson et al., 2010a; Larson et al., 2010b; Larson et al., 2009). These studies make several interesting points: most of the over-expressed hCTR1 protein was not delivered to the plasma membrane; there is a lack of correlation between cDDP uptake and cytotoxicity; truncation of the first 45 amino acids of hCTR1 do not inhibit cDDP uptake (Larson et al., 2010b), and replacement of Met residues 150, 154 (Larson et al., 2010a) increase cDDP uptake and decrease Cu uptake. The authors conclude that if hCTR1 is responsible for cDDP uptake, its interactions with cDDP are different from those with Cu. Cytotoxic effects seen in those studies are observed following very brief (15 seconds or 5 minutes) exposures to cDDP. It is difficult to conclude that surface binding phenomena are separated from uptake. The results of the present work suggest that if hCTR1 does interact with cDDP it can play only a minor role, if any in its cellular accumulation. Studies in yeast were the first to show the correlation between copper transporter and cDDP entry (Ishida et al., 2002). However, yeast ctr1 and hCTR1 have only 39% identity and yeast protein is considerably larger. cDDP transport was characterized in ctr1-expressing strains and Km values for copper and cDDP were reported of 129µM and 140µM respectively (Lin et al., 2002). The values for copper are considerably higher than the 3-5µM reported for hCTR1 (Eisses and Kaplan, 2002; Liang et al., 2009; Zimnicka et al., 2007) and 5µM for yeast ctr1 reported previously (Dancis et al., 1994).

cDDP uptake in tumor cells.

Our observations on cDDP entry into tumor cells replicate findings in normal cells: (a) copper treatment which lowers surface hCTR1 does not affect cDDP entry (Figures 4C, 4D); (b) lowering hCTR1, by 80% using siRNA (Figures 5A, 5B), reduces copper entry (Figure 5C), but
not cDDP uptake (Figure 5D); (c) absence of internalization of hCTR1 on exposure to cDDP (Figures 4A, 4B).

In ovarian carcinoma cells (A2780), Holzer et al achieved twenty-fold over-expression of hCTR1 and only 30-50% increase in cDDP accumulation (Holzer et al., 2004b). They also reported cDDP treatment of cells decreased hCTR1 and copper uptake. Subsequently, they confirmed these observations, and interestingly that increased cDDP accumulation was not associated with increased cytotoxicity (Holzer et al., 2004b). In contrast, it was recently reported on the same cells, in agreement with our findings, that increased membrane levels of hCTR1 had no effect on cDDP accumulation and cDDP treatment did not decrease hCTR1 levels (Kalayda et al., 2012). Similarly, in cervix squamous carcinoma cells (A431), hCTR1 overexpression (3-4 fold) had no effect on cDDP accumulation (Beretta et al., 2004). In tumor cells, the concentration-dependence of cDDP uptake has been reported. In agreement with our results in A2780 cells, uptake is linear up to 100 µM cDDP in 2008 cells (Andrews et al., 1988), in Ehrlich ascites cells (Gale et al., 1973) and in A2780 and HeLa cells (Zisowsky et al., 2007). In small cell lung carcinoma cells a Km of about 15 µM for cDDP uptake was reported (Liang et al., 2009) a value that is considerably lower (at least an order of magnitude) than reported in other cells.

The entry mechanism of cDDP.

It seems likely that most of cDDP entry into tumor cells is mediated via a non-protein-mediated pathway. This is supported by our observations that over a wide range of concentrations no saturation of uptake is seen (Figure 3D, 6B), and that the cis and trans isomers of cDDP have identical uptake rates (Figure 6B). Our data support entry being predominantly via
solubility-diffusion through the membrane (Gately and Howell, 1993). Other candidates have been proposed (OCT2, Na\(^+\) K\(^+\) ATPase, and SLC family members) (Ahmed et al., 2009; Filipski et al., 2009; Koepsell and Endou, 2004). Our observations suggest they are not primary players.

Internalization of hCTR1 in response to cDDP.

Study of hCTR1 has been hampered by limitations of many of the available Ab’s. For example, a very interesting study of the effects of mutations on cDDP uptake in SCLC’s (Liang et al., 2009) shows an apparent mass of 25 kDa for both wild-type and the N15Q mutant. It has been shown that N15Q reduces the mass of endogenous protein by about 10 kDa (Eisses and Kaplan, 2002). hCTR1 can appear as multiple bands above 35 kDa, due to the presence of stable monomers and dimers (Eisses and Kaplan, 2002; Maryon et al., 2007) which complicates analysis. These issues make it important that Ab’s used show the appropriate behavior in Western blots: ie shift to lower mass on enzymatic deglycosylation (or with a non-glycosylated mutant), do not appear in cells that do not express hCTR1 or disappear on down-regulation with siRNA. In HEK cells, we do not observe any hCTR1 internalization with cDDP, although extracellular copper causes internalization. This confirms previous findings in HEK cells with cDDP using epitope-tagged hCTR1 and an anti-myc Ab (Guo et al., 2004). cDDP has been claimed to cause internalization and degradation in A2780 cells (Holzer et al., 2004b) but has been recently disputed (Kalayda et al., 2012), in agreement with the present work. Similarly, in yeast, relocalization of ctr1 following cDDP treatment was not observed (Sinani et al., 2007).

Platinum-drugs and copper homeostasis.
cDDP interacts with several proteins in copper homeostasis. Increased expression of ATP7A and ATP7B has been associated with cDDP resistance (Katano et al., 2002; Komatsu et al., 2000; Rabik and Dolan, 2007). cDDP also binds to the copper chaperone, Atox1, destabilizing Atox1, resulting in unfolding and aggregation. Atox1 was suggested as a potential candidate for cDDP resistance (Palm et al., 2011). Further work is needed to clarify the interactions between platinum drugs and proteins in copper homeostasis. Such interactions are significant as lowering cell copper alters tumor cell sensitivity to platinum-drugs (Ishida et al., 2010). The observations of Holzer (Holzer et al., 2004b) that separate cytotoxic effects of cDDP from uptake, suggest that a more complete understanding of the fates of cDDP following its initial interactions with the cell surface would be of great value. Inhibition of proteasomal activity enhances delivery of cDDP in ovarian carcinoma cells and promises significant clinical potential, but its basis is also not yet clear (Jandial et al., 2009). The recent observation that copper is involved in growth factor kinase cycling in tumor cells introduces a new complexity (Tsai et al., 2012), and recent studies suggest important interactions between ATP7A, hCTR1, Atox1 and the PDGF receptor in cell activation (Ashino et al., 2010).

The present studies focus attention on the proteins involved in copper homeostasis as important contributors to the complexity of the cellular pharmacology of the widely-used Pt-drugs. It seems likely that interactions among the proteins involved in copper homeostasis play a major role in deciding the fate and effectiveness of these drugs, following cellular entry through a pathway that is not related to the entry mechanisms utilized by copper.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Ivy, Kaplan

Contributed new reagents:

Performed data analysis: Ivy, Kaplan

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FOOTNOTES

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FIGURE LEGENDS

**Figure 1.** Copper uptake, cDDP uptake and cell surface expression of hCTR1 in HEK 293 cells. HEK 293 cells over-expressing hCTR1 were induced with tetracycline 48 hrs prior to experiment. (A) $^{64}$Cu uptake was measured in cells incubated with 5 µM or 10 µM copper for 30 minutes in media. (B, C) cDDP uptake was measured in cells incubated with 30 µM or 100 µM cDDP for 5 hrs. (D) Tetracycline-induced cells were incubated with 100 µM copper for 30 min, 100 µM copper and then washed 3 times with media and placed back in media for 30 min, or 50 µM cDDP for 30 min. (E) Tetracycline-induced HEK cells were incubated with 100 µM copper for 1 hr, or 50 µM cDDP for 1, 5, 10, 30, or 180 mins. (D, E) Cells were biotinylated and the biotinylated protein was analyzed on western blots. hCTR1 protein was detected using an anti-Flag antibody and the protein levels were normalized to Beta catenin, loading control.

**Figure 2.** Platinum uptake rates in Mefs.

A. Mefs (+/+) and (-/-) were incubated with 30 µM cDDP or 30 µM cDDP and 100 µM copper for 5 hrs. (B) Mefs (+/+) and (-/-) were incubated with 30 µM cDDP, carboplatin, transplatin or oxaliplatin for 5 hrs.

**Figure 3.** hCTR1 protein expression, copper uptake and cDDP uptake in human ovarian carcinoma cells.

A. cDDP-resistant (A2780CP) and cDDP-sensitive (A2780) cells were fractionated and plasma membranes were obtained and analyzed on western blots. hCTR1 was detected using an anti-
CTR1 antibody (rabbit). Na⁺, K⁺ ATPase alpha subunit was the loading control. (B) Biotinylated hCTR1 protein was quantified using Quality One software, and the protein levels were normalized to the Na⁺, K⁺ ATPase alpha subunit. (C) ⁶⁴Cu uptake was measured in cells incubated with 5 µM or 10 µM copper for 30 minutes in media. (D) cDDP uptake was measured in cells incubated with 3 µM, 30 µM, or 100 µM cDDP for 5 hrs in media.

**Figure 4.** Surface expression of hCTR1 and the effects of copper and cDDP.

(A) cDDP-resistant (A2780CP) and cDDP-sensitive (A2780) cells were incubated with 100 µM copper, 20 µM cDDP or 50 µM cDDP for 1 hr. (C) Cells were incubated with 100 µM copper for 30 min or 100 µM Cu and then washed 3 times with media and placed back in media for 30 min. (A, C) Cells were biotinylated and the biotinylated protein was analyzed on western blots using Quality One software. hCTR1 protein was detected using an anti-hCTR1(rabbit) antibody. Na⁺, K⁺ ATPase beta subunit was used as the loading control. (B) Western blot quantitation of figure A. (D) A2780 cells were incubated with 100 µM copper for 1hr, rinsed 3 times with media, and then placed back in fresh media with 30 µM cDDP for 3 hrs.

**Figure 5.** hCTR1 knockdown in A2780 cells.

(A) cDDP-sensitive (A2780) cells were transiently transfected with siRNA duplexes against CTR1 or siRNA negative control in Opti-MEM medium. Cells were biotinylated and the biotinylated protein was analyzed on western blots. hCTR1 protein was detected using an anti-CTR1 antibody (rabbit). (B) Biotinylated hCTR1 protein was quantified using Quality One software, and the protein levels were normalized to the Na⁺, K⁺ ATPase alpha subunit. (B) After transient transfection, cells were incubated with (C) 5 µM copper for 1 hr or (D) 30 µM cDDP
for 3 hrs. In figure (C), Cu uptake is expressed as a % of control (control rate 129.01 pmol Cu/mg protein/hr) and figure (D) cDDP uptake is expressed as a % of control (control rate 92.05 pmol Pt/mg protein/hr).

Figure 6. Cis vs trans cDDP uptake.

(A) cDDP -sensitive (A2780) cells were incubated with 3 µM, 30 µM, or 100 µM cDDP or transplatin for 5 hrs. Platinum content was measured by ICP-MS and shown as pmol Pt/mg protein/hr.
Figure 2

A

pmol Pt/mg protein/hr

Mefs +/+

Mefs -/-

cDDP

cDDP + Cu

B

pmol Pt/mg protein/hr

Mefs +/+

Mefs -/-

cDDP

CBDCA

Trans

L-OHP
Figure 3

A: Western blot analysis showing hCTR1 and NaK Alpha expression in A2780CP and A2780 cell lines. hCTR1 at 35 kDa and NaK Alpha at 100 kDa.

B: Bar graph comparing cell surface hCTR1 expression between A2780CP and A2780 cell lines.

C: Graph showing pmol Cu/mg protein/hour for A2780CP and A2780 cell lines at 5 μM and 10 μM Cu.

D: Graph showing pmol P/mg protein/hour in response to different concentrations of cDDP for A2780CP and A2780 cell lines.
Figure 4

A

<table>
<thead>
<tr>
<th></th>
<th>A2780CP</th>
<th>A2780</th>
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<tbody>
<tr>
<td>cDDP(uM)</td>
<td>- 20 50</td>
<td>- 20 50</td>
</tr>
<tr>
<td>Cu (uM)</td>
<td>100 -  -</td>
<td>100 -  -</td>
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hCTR1: 35 kDa

NaK Beta: 60 kDa

B

Cell surface hCTR1

Control, 100 μM Cu, 20 μM cDDP, 50 μM cDDP

C

Cell surface hCTR1

Control, 100 μM Cu, 100 μM Cu. Media

D

pmol Pt/mg protein/hr

cDDP, Cu, CDDP