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The Regulation of the Human Copper Transporter 1 and its Role in the Cellular Uptake of Cisplatin

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Alison Kay Holzer

Committee in charge:

Prof. Stephen B. Howell, Chair
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2006

The dissertation of Alison K. Holzer is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2006

DEDICATION

To my parents, for instilling in me the idea that hard work and perseverance will get me through

To my grandparents, for teaching me to appreciate all that I have

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ABSTRACT OF THE DISSERTATION

The Regulation of the Human Copper Transporter 1 and Its Role in the Cellular Uptake of Cisplatin

by

Alison Kay Holzer

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2006

Professor Stephen B. Howell, Chair

Cisplatin, a commonly used chemotherapeutic, is limited on it efficacy due to the rapid development of resistance. A common underlying factor in the development of this resistance is a decrease in cellular accumulation of the drug. Recent studies have suggested that the copper transport pathway may be responsible for the uptake and cellular trafficking of cisplatin. The overall goal of the studies described here was to determine whether the Cu plasma membrane transporter hCTR1 plays a role in the cellular accumulation of cisplatin, and if so, whether it influences the sensitivity of cells to this common chemotherapy. This was achieved by using two cellular models, one in which hCTR1 was over expressed and the other in which CTR1 was absent.

Once hCTR1 was identified as an influx transporter of DDP, investigations went on to identify the cellular regulation and trafficking of the protein through the use of chemical inhibitors and dominant negative mutants to block normal

endocytotic and proteasomal pathways. The role that hCTR1 may play in the resistance of human tumors to cisplatin was investigated using cisplatin sensitive and resistant ovarian carcinoma cell lines and the screening of human tumor tissue. The experiments described in the following work demonstrate that hCTR1 plays a role in the transport of cisplatin, as alterations of hCTR1 protein levels correspond to platinum accumulation levels. While hCTR1 does appear to influence cisplatin, the results presented here indicate that hCTR1 does not necessary correlate to drug sensitivity in human cells, as hCTR1 levels were not significantly altered for normal tissue in a majority of human tumors or in sensitive and resistant cell pairs. Finally, the cellular trafficking of hCTR1 depends on the continual cycling of the protein. However, up on exposure to DDP, hCTR1 is rapidly internalized through macropinocytosis and degraded by the proteasome.

Chapter 1

Introduction

Platinum-based chemotherapy

The ability of platinum (Pt) based complexes to function as chemotherapeutic agents was first demonstrated through their ability to prevent bacterial cell division (Rosenberg et al., 1965). This finding led to an entire of field of study examining numerous Pt compounds and their potential anticancer activity. The following sections provide background information on three of the Pt compounds currently used in the clinic.

Cisplatin

Cisplatin (DDP) has a high level of efficacy in a wide range of tumor types, thus making it a widely utilized chemotherapeutic agent (Chu, 1994; Fuertes et al., 2003; Jordan and Carmo-Fonseca, 2000). DDP is delivered intravenously and once in the blood it is bound by plasma proteins, rendering it relatively unreactive. However, once in the cell, the chloride atoms are displaced from the DDP due to the low intracellular chloride concentrations (2-30 mM). At this point the DDP is an activated Pt compound capable of reacting with a multitude of intracellular molecules including nuclear and mitochondrial DNA, RNA, cysteine, histidine and methoinine-containing proteins and phospholipids. Once DDP reaches the nucleus, it binds to DNA forming

adducts. These adducts inhibit transcription and DNA synthesis (Jordan and Carmo-Fonseca, 2000). The primary mechanism of DDP cytotoxicity is through the formation of covalently linked adducts at the N^7 positions of quinine and adenine in DNA. A majority of DNA adducts generated by DDP are 1,2d(GG) and 1,2-d(ApG) intrastrand crosslinks. Interstrand and 1,3-intrastrand crosslinks also occur, but they form only about 10-20% of all adducts. The significant bending of the DNA by the 1,2-intrastrand adducts is believed to contribute to the recognition of DNA adducts by cellular machinery (reviewed in (Chu, 1994; Fuertes et al., 2003; Jordan and Carmo-Fonseca, 2000)).

The hypothesis is that adduct-damaged DNA triggers G2 cell cycle arrest and the subsequent activation of apoptosis (Chu, 1994). Additionally, a recent report suggests that DNA damage by DDP induces two death response pathways, each of which is dependent on p53 or p73-induced cell cycle arrest (Gong et al., 1999). DDP adducts also induce apoptosis through the mismatch repair system. In this system, the mismatch repair protein complex recognizes and binds to the 1,2-dGpG adducts and serves as a DNA damage sensor (reviewed in (Lin et al., 1999)). Cells lacking mismatch repair are less able to detect DDP-induced damage and are thus more resistant to the induction of G2 arrest and apoptosis (Strathdee et al., 1999).

Cisplatin Analogs

While commonly used as a chemotherapeutic agent, DDP exhibits a high level of toxicity, namely nephrotoxicity, ototoxicity, neuropathy and myelosuppression, which limits its effectiveness for use in the clinic. Additionally, resistance to DDP

develops rapidly serving to further limit its use. A wide range of DDP analogs have been created in attempts to circumvent these limitations of DDP; the two most widely used of these analogs are carboplatin (CBDCA) and oxaliplatin (L-OHP).

Carboplatin

Carboplatin, a second generation Pt compound, exhibits a toxicity spectrum than differs from that of DDP. As shown in Figure 1-1, while DDP has 2 chloride leaving groups, CBDCA possesses a 1,1 cyclobutanedicarboxylate leaving group that renders it more stable in aqueous environments, thus slowing the aquation rate and reducing reactivity (Jordan and Carmo-Fonseca, 2000). While CBDCA is a more stable molecule, it has a similar spectrum of activity against human tumors and exhibits cross-resistance with DDP in most tumor types, a feature that limits its efficacy. The reason for this cross-reactivity is most likely do to the fact that both drugs produce the same types of adducts in DNA.

Oxaliplatin

Oxaliplatin (L-OHP) is a third generation platinum drug that belongs to the diaminocyclohexane (DACH) platinum class of compounds. This class has a different spectrum of activity than DDP or CBDCA. The structure of L-OHP is depicted in figure 1-1. L-OHP exhibits less cross-resistance with both DDP than CBDCA. L-OHP is cytotoxic to a broad range of cell lines, including colon, ovarian, and lung cancer (reviewed in (Raymond et al., 2002)). L-OHP has been tested *in vitro* and *in vivo* against DDP-resistant cell lines and tumor models, including human ovarian, lung,

cervix, colon, and leukemia cell lines, and was shown to have cytotoxic activity with a

low incidence of cross-resistance (reviewed in (Raymond et al., 2002)).
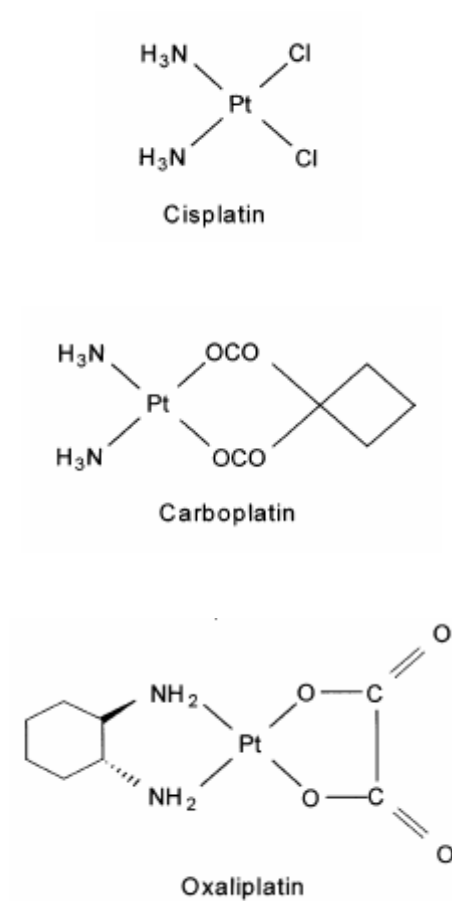
Figure 1-1. Chemical structures of cisplatin, carboplatin and oxaliplatin.

Resistance to Platinum-based chemotherapy

The development of resistance to the platinum based drugs occurs commonly during therapy and is a major cause of treatment failure. Resistance is believed to develop through enrichment for cells that have become less drug-sensitive as a result of spontaneous or drug-induced somatic mutation. In the face of the selective pressure imposed by the chemotherapy, the resistant cells outgrow the sensitive cells to become the dominant population. DDP sensitivity measured *in vitro* using tumor samples or cell lines obtained before and after treatment indicate that the level of resistance that emerges *in vivo* is quite modest at 1.5 - 3-fold (Inoue et al., 1985; Wilson et al., 1987). This is consistent with levels of resistance produced in experimental animals by clinically relevant DDP dose schedules. Prior studies have confirmed that resistance emerges quickly, and that even low level resistance is sufficient to account for the clinical failure of DDP therapy (Andrews et al., 1990).

Several mechanisms that can potentially contribute to resistance have been identified, although the specific 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 (Andrews and Howell, 1990). As depicted in Figure 1-3, DDP is taken up into the cell at the plasma membrane probably by several different transport mechanisms (Gately and Howell, 1993). Uptake has been shown to be altered in DDP resistant cell line models (Andrews et al., 1987; Kelland et al., 1992; Metcalfe et al., 1986; Oldenburg et al., 1994; Teicher et al., 1991; Twentyman et al., 1992; Wallner et

al., 1986; Waud, 1987). How the platinum drugs actually enter the cell is poorly

defined as will be discussed later in this chapter.
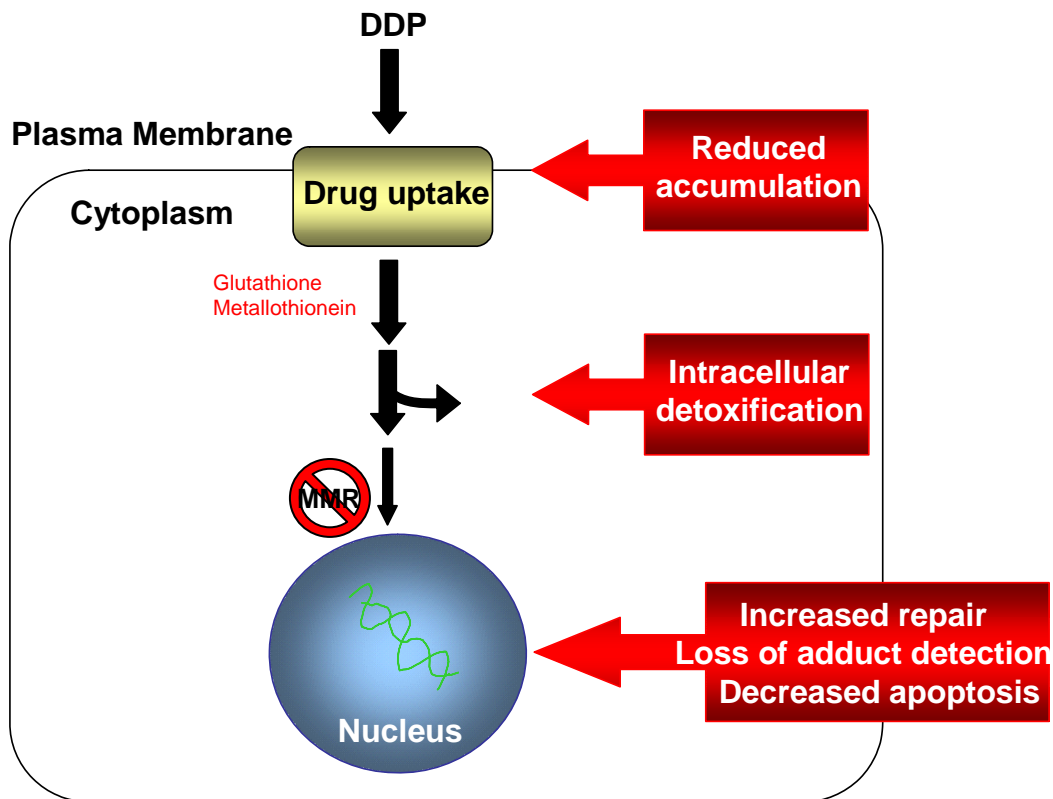
Figure 1-2. Schematic drawing of the major mechanisms of cisplatin resistance. DDP is taken up into the cell at the plasma membrane, and accumulation has been shown to be altered in some DDP resistant cells. Once inside the cell, DDP must avoid detoxification mechanisms such as conjugation with glutathione or binding to metallothionein, both which have been shown to be up-regulated in some DDP resistant cells. Once DDP binds to DNA, several possible mechanisms exist to avoid the triggering of apoptosis cell death, including increased adduct repair, decreased adduct detection and decreased induction of apoptosis. Figure modified from (Fuertes et al., 2003).

Avoidance of intracellular DDP sinks

To date, the ability of DDP to reach the DNA and trigger apoptosis despite the presence of several DDP detoxifying mechanisms can not be explained. Once inside the cell, a fraction of the DDP gains access to the nucleus, avoiding naturally occurring detoxification mechanisms present in the cell. As mentioned previously, DDP will readily bind thiol containing amino acids such as cysteine and methionine. Several proteins with high levels of such amino acids exist within the cell. For example, the cysteine rich protein glutamate-cysteine-glycine tripeptide glutathione is present in the cell in high concentrations (0.5-10 mM) in comparison to any levels physiologically attainable by DDP and its analogs (Fuertes et al., 2003).

Glutathione is another protein that is expressed at levels much higher than DDP within the cell, and upon binding DDP, the glutathione-DDP conjugate is eliminated from the cell by an ATP-dependent glutathione S-conjugate export pump (Ishikawa and Ali-Osman, 1993). }. Some DDP-resistant cells have increased levels of intracellular glutathione (Godwin et al., 1992), suggesting that it may protect DDP treated cells by intercepting the drug before it is able to react with DNA. Another cellular constituent that is believed to be a determinant of Pt-drug sensitivity is metallothionein. This small protein contains an abundance of thiol groups due to its high cysteine content and plays a role in detoxification of many heavy metal ions. Metallothionein is up-regulated in cells resistant to various heavy metals and in some cell lines selected for resistance to DDP (Kasahara et al., 1991; Kelley et al., 1988).

Platinum-drug transport

In order for the Pt drugs to exert their cytotoxicity, they must first gain entry to the cell. DDP enters cells much more slowly than most anticancer agents, and there is evidence suggesting that one component of DDP uptake is mediated by a transport mechanism or channel (Andrews and Albright, 1991; Andrews et al., 1991; Andrews et al., 1988b; Mann et al., 1991). Uptake is temperature, pH and potassium (K^+) ion-dependent (Amtmann et al., 2001; Atema et al., 1993; Endo et al., 2000), and the presence of reducing agents like ascorbate and dithioreitol increase DDP uptake (Chiang et al., 1994; Sarna and Bhola, 1993; Zhang et al., 1994). Impaired drug uptake is a common feature of cells that have acquired resistance to the Pt-based drugs suggesting that the pathways involved in drug uptake and accumulation are likely to play an important role in the development of resistance. Many *in vitro* studies have shown that DDP-resistant cell lines, derived from a wide variety of tumor types, demonstrate decreased DDP accumulation compared to their sensitive counterparts (Andrews et al., 1988a; Kelland et al., 1992; Metcalfe et al., 1986; Oldenburg et al., 1994; Teicher et al., 1991; Twentyman et al., 1992; Wallner et al., 1986; Waud, 1987).

The mechanisms involved in transporting the Pt-containing drugs across the plasma membrane and to the nucleus are not well defined. DDP, CBDCA and L-OHP are all polar molecules and thus do not cross lipid bilayer membranes easily. Previous studies have suggested that one or more transporters are involved in DDP uptake (Andrews and Albright, 1991). As discussed later in this chapter, recent experiments done in yeast suggest that the major copper uptake transporter, CTR1, may be involved in DDP uptake (Ishida et al., 2002; Lin et al., 2002). However, it is important

to note that there are two aspects of drug accumulation to be considered. Impaired Pt drug accumulation is commonly observed in cell lines selected *in vitro* for resistance to DDP, but this decrease may be due to either decreased influx or enhanced efflux. Waud *et al*. (Waud, 1987) examined the rate of DDP uptake in murine leukemia cells, and found a decrease in uptake and adduct accumulation in DDP-resistant cells, but no difference in efflux. Teicher *et al*. (Teicher et al., 1991) also demonstrated a decrease in whole cell and nuclear platinum content in 5 different cell lines selected for DDP resistance following exposures to equimolar concentrations of drug. However, Parker *et al*. (Parker et al., 1991)demonstrated that the DDP-resistant cell line A2780/CP displayed increased drug efflux compared with its parental DDP-sensitive line, accounting for an overall decrease in drug accumulation. To add more complexity to the issue of decreased cellular accumulation, Andrews *et al*. demonstrated an alteration in mitochondrial membrane morphology and potential in the DDP-resistant ovarian carcinoma cell line 2008/C13*5.25, suggesting the importance of active energy-dependent transport of DDP in these cells (Andrews and Albright, 1992). Although there are several other possible mechanisms for acquisition of DDP resistance in a cell, including an increase in DNA repair mechanisms and adduct tolerance (Johnson et al., 1994), and an increase in detoxification secondary to reaction with glutathione or metallothionein (Godwin et al., 1992; Kelley et al., 1988), it is clear that alterations in DDP transport are important in some types of DDP resistance. However, there are numerous potential candidate transporters including heavy metal transporters, gated channels and ATPase transporters and it at time this research was initiated little information was available about exactly which proteins are

involved in the uptake of DDP from the extracellular space and how it is transported

through the cell to the nucleus.

Copper transport

Recent evidence suggests that DDP resistance can also result from the up-

regulation of copper efflux transporters that are also capable of exporting DDP from

the cell (Higashimoto et al., 2003; Kanzaki et al., 2002; Katano et al., 2002; Katano et

al., 2003; Komatsu et al., 2000; Miyashita et al., 2003; Nakayama et al., 2002;

Nakayama et al., 2001; Ohbu et al., 2003). The following sections provide background

information on the importance of copper for proper cellular function and the role of

copper transporters in managing copper homeostasis, and on the role of copper

transporters in mediating the influx and efflux of the Pt drugs.

Background

Copper (Cu) is an essential trace element that is required for the activity of

several critical enzymes, including superoxide dismutase, cytochrome-c oxidase, lysyl

oxidase and dopamine β-hydrolase. Table 1-1 presents a list of several key Cu-

dependent proteins and their functional roles in various cell types. The necessity for

Cu originates in its ability to shift between a reduced Cu(I) and an oxidized Cu(II)

state under physiologic conditions, allowing it to serve as a redox cofactor for a

variety of processes including electron transport and detoxification of reactive oxygen

species (Linder and Hazegh-Azam, 1996). However, that redox potential also make

Cu a very toxic metal if not tightly regulated.

Table 1-1. Copper-dependent proteins

Enzyme	Function
Cu, Zn – superoxide dismutase	Antioxidant
Cytochrome c oxidase	Mitochondrial respiration
Tyrosinase	Melanin production
Lysyl oxidase	Collagen and elastin crosslinking
Ceruloplasmin	Ferroxidase
Hephaestin	Intestinal iron efflux
Dopamine ß-hydroxylase	Catecholamine production
Peptidylglycine-amidating mono-oxygenase	Neuropeptide/peptide hormone processing

Cellular copper transport

Accumulation of Cu in a cell can result in the production of reactive oxidative species that cause severe damage to DNA and other components of the cell including lipids and several proteins. In addition to the damage induced by reactive oxidative species, excess intracellular Cu can also exert toxicity by displacing other metal cofactors from their natural proteins, thereby potentially interfering with their normal cellular function (Pena et al., 1999). To avoid the toxicity of Cu, a complex Cu homeostasis system has evolved that is highly conserved during evolution. It is a highly regulated system of transporters and chaperones that extract Cu from the environment, mediate its transport across cellular membranes and deliver it to Cu-requiring proteins in the cell. The mechanisms involved in preventing accumulation of excess Cu are also extensively regulated. In fact, Cu transport is so tightly regulated, that as Cu(I) enters and moves through the cell, it is handed from one protein to the next through interactions that virtually never allow Cu to be free in the cell (Hamza et al., 1999; Lippard, 1999; Pena et al., 1999; Pufahl et al., 1997).

Figure 1-3 shows a schematic diagram of the current understanding of Cu homeostasis pathways in mammalian cells. It is thought that the process of Cu entry into the cell begins when Cu (II) bound to ceruloplasmin arrives at the cell surface. The Cu(II) is reduced by a cell surface reductase before binding to the plasma membrane transporter named "human copper transporter 1" and abbreviated hCTR1. Once bound to hCTR1, Cu is passively transported across the plasma membrane in an energy independent manner (Amaravadi et al., 1997). hCTR1 hands the Cu to one of several Cu chaperones. As depicted in Figure 1-3, once inside the cell, Cu chaperones, such as hCOX17, HAH1 and CCS, sequester Cu into thiol-rich pockets that protect it against oxidation during delivery to specific cellular compartments including mitochondria, the Golgi and superoxide dismutase (Pena et al., 1999).

ATP7A and ATP7B are Cu transporters that sequester Cu from the cytoplasm into the *trans*-Golgi for loading onto ceruloplasmin and other Cu-requiring enzymes and subsequent export from the cell (Figure 1-3). ATP7A is expressed in many tissues whereas expression of ATP7B is limited to the liver. When Cu levels are low, ATP7A and ATP7B are located primarily in the *trans*-Golgi where they function to incorporate the metal into Cu-dependent enzymes. Upon exposure to increased Cu levels, the proteins relocalize to other sites in the cell, a response that is thought to be important to their ability to limit Cu toxicity. ATP7A relocalizes in part from the *trans*-Golgi to the plasma membrane (Petris et al., 1996). In contrast, ATP7B relocates primarily to intracellular vesicular compartments whose function is not clearly defined but which are presumably involved in the secretory export pathway (reviewed in (Pena

et al., 1999)). ATP7A or ATP7B serve as the major proteins involved in the efflux of

Cu.
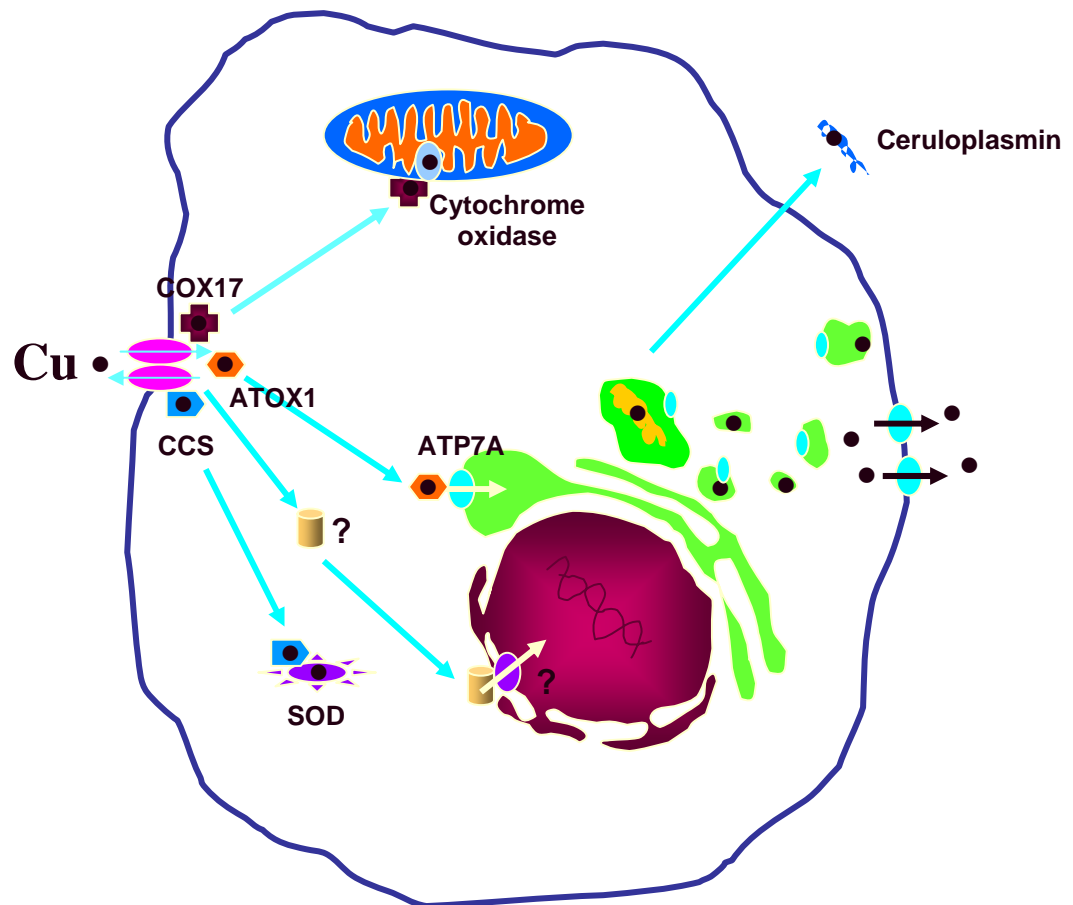
Figure 1-3. Cu uptake, distribution, and export pathways in mammalian cells (redrawn from (Pena et al., 1999)).

Characterization of hCTR1

The human copper transporter 1 (hCTR1) is a very unique protein. It was initially discovered through the screening of a human cDNA expression library in yeast strain lacking *ctr1* and looking for functional complementation (Zhou and Gitschier, 1997). In comparing the resulting protein sequences, hCTR1 was identified as a high affinity Cu transport protein based on transmembrane homology to some previously characterized yeast Cu transporters (Zhou and Gitschier, 1997) and it's ability to rescue *ctr1*$^{-/-}$ yeast. The ability of hCTR1 to influence Cu uptake in human cells was identified by over expressing the protein in human fibroblasts (Moller et al., 2000).

It is initially synthesized as a 28 kDa protein that is modified to a 35 kDa mature form by glycosylation. As shown in Figure 1-4, the genomic sequence of hCTR1 is comprised of 4 exons that code for a 190 amino acid protein. The N terminus of hCTR1 is very histidine and methionine rich. There are also 3 MXXM Cu binding motifs in the first 67 amino acids. hCTR1 shares little sequence homology with non-mammalian plasma membrane copper transport proteins (reviewed in (Petris, 2004)). Deletion of both CTR1 genes in the mouse, which shares 92% sequence identity with hCTR1 (Lee et al., 2001), causes embryo death *in utero* indicating that it is essential during development (Kuo et al., 2001; Zhou and Gitschier, 1997).

Complete DNA Sequence
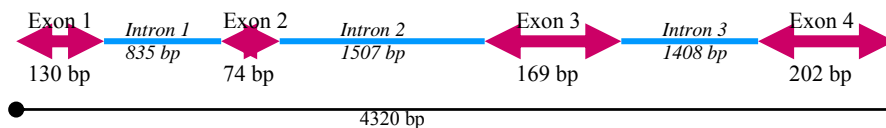
Protein Sequence

1
MDHSHHMGMSYMDSNSTMQPSHHHPTTSASHSHGGGDSSMM**M****MM**TFYF
GFKNVELLFSGLVINTA**GM**AGAFVAVFLLAMFYEGLKIARESLLRKSQVSIR
YNSMPVPGPNGTILMETHKTV**XM**LSFPHLLQTVLHIIQVVISYFLMLIFMFYN
GYLCIAVAAGAGTGYFLFSWKKAVVVDITEHCH*
191

━━ Necessary for Cu transport
━━ Transmembrane Domains

Underlining indicates Extracellular Region   *indicates glycosylated site
Bold lettering indicates intron/exon boundaries

Figure 1-4. Genomic layout and protein sequence of hCTR1.

Functional Studies

A schematic diagram of hCTR1 is presented in Figure 1-5. hCTR1 is thought

to possess 2 metal binding domains that play a role in scavenging copper under

conditions of copper starvation (Eisses and Kaplan, 2002). Several mutational studies

have been done to elucidate the components of hCTR1 that are necessary for this

function as shown in Figure 1-6. Two of the methionines in the extracellular domain

and 2 methionines in the second transmembrane region have been shown to be

essential for Cu transport (Eisses and Kaplan, 2002).
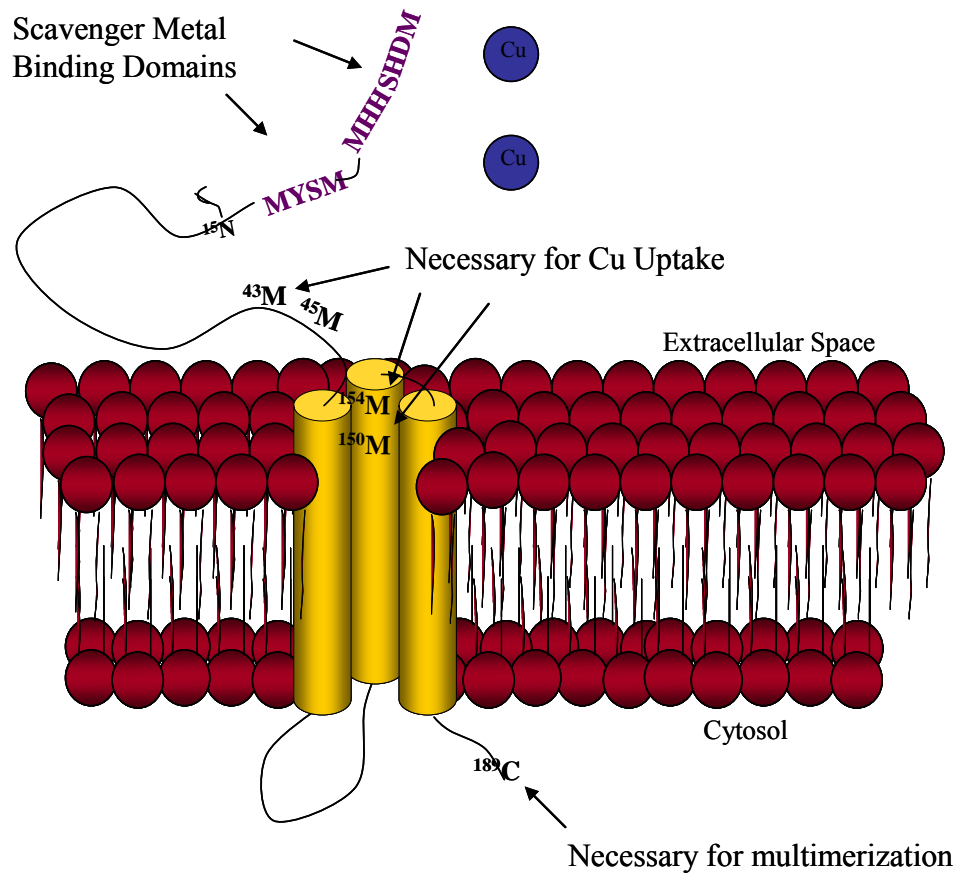
Figure 1-5. Schematic drawing of hCTR1 protein. The yellow cylinders represent the 3 transmembrane domains. The amino acids necessary for Cu transport and noted, as are other essential aspects of the protein.
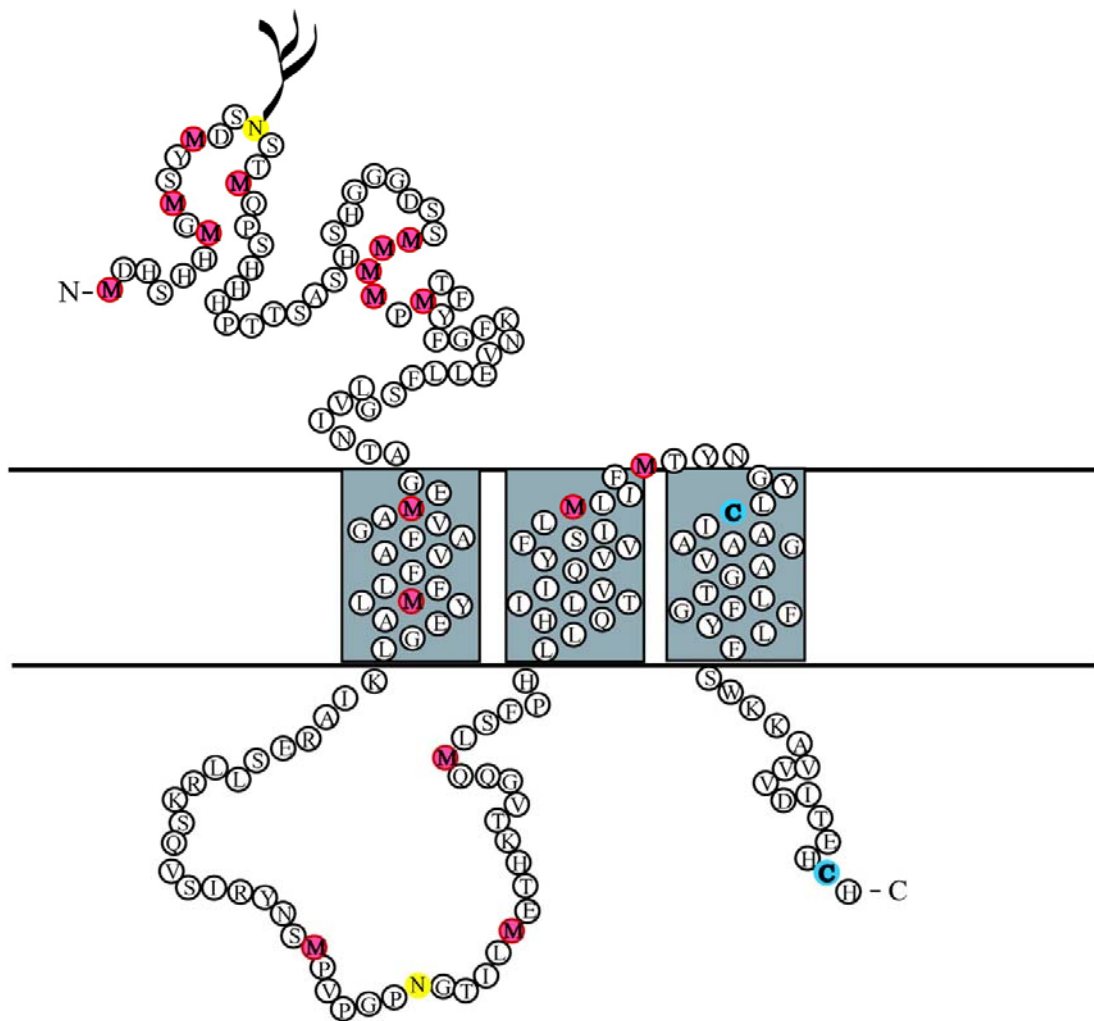
Figure 1-6. Schematic of hCTR1 depicting amino acids that have been altered in mutational studies. The amino acid sequence of hCTR1 is depicted in hypothesized structure with mutated amino acids shown in pink, yellow and blue. Adapted from (Eisses and Kaplan, 2002).

hCTR1 is highly specific for Cu transport. Cu transport by hCTR1 was found to be dependent on time, Cu concentration, extracellular pH and extracellular K^+ level (Lee et al., 2002a). The binding of hCTR1 to several other heavy metals, including zinc, iron, magnesium, cadmium and silver (Ag) has been indirectly tested through competition studies measuring ^{64}Cu uptake in the presence of other heavy metals. Among these the only other heavy metal that has been identified as a possible substrate for hCTR1 is Ag. However, these studies used the decrease of Cu uptake in the presence of the other metal as an indicator of substrate specificity (Lee et al., 2002a). To date, there are no published reports on the ability of hCTR1 to transport other metals.

Cellular localization and organization

hCTR1 has 3 transmembrane regions and is thought to exist in the membrane as a homotrimer (Lee et al., 2000). The N-terminus is localized extracellularly and interacts with itself to form a multimer (Klomp et al., 2003). The cellular location of hCTR1 seems to vary among cell types. While it is commonly found on the plasma membrane, in some types of cells it is also located in the perinuclear and Golgi regions (Eisses and Kaplan, 2002; Klomp et al., 2002; Lee et al., 2002a; Petris et al., 2003). hCTR1 may relocalize from the plasma membrane to vesicles upon exposure to Cu (Petris et al., 2003). However, there are conflicting reports regarding the effect of Cu exposure on subcellular localization of hCTR1(Eisses and Kaplan, 2005; Klomp et al., 2002), and the Cu-induced trafficking of hCTR1 has not been well-defined.

Copper transporters involved in Pt-drug pharmacology

The idea that either influx or efflux Cu transporters might mediate DDP resistance was introduced by Komatsu *et al.* who reported that, in prostate carcinoma cells, DDP resistance was associated with increased expression of ATP7B (Komatsu et al., 2000). Forced over-expression of ATP7B in a human cell line resulted in resistance to both Cu and DDP. The cell line expressing exogenous ATP7B also accumulated less DDP over time and this was coupled with increased efflux of the drug suggesting that ATP7B may serve to regulate the concentration of DDP in the cell (Komatsu et al., 2000). The results of this initial study indicating that ATP7B affects DDP accumulation have now been confirmed by reports from several other laboratories (Safaei and Howell, 2005).

Perhaps the most convincing evidence that Cu transporters play a role in DDP resistance was the observation that human ovarian carcinoma cells selected for DDP resistance *in vitro* are cross-resistance to Cu and *vice versa*, and that ATP7A is over-expressed at the protein level in some DDP-resistant ovarian carcinoma cell lines (Katano et al., 2002). Furthermore, the 3 DDP-resistant sublines examined exhibited decreased accumulation of both DDP and Cu confirming prior reports that cells that over-express ATP7B accumulate decreased amounts of Cu (La Fontaine et al., 1998). Increased expression of ATP7B was found to render ovarian carcinoma cell lines more resistant to both DDP and its analog CBDCA (Katano et al., 2003). These studies suggest that over-expression of ATP7B modulates the pharmacology of DDP and renders cells resistant to the drug.

Additional studies have investigated expression of ATP7A in human tissue and in cell culture systems. Samimi *et al.* demonstrated that altering the expression of ATP7A modulated sensitivity to DDP and its analogs CBDCA and L-OHP, and that the changes in sensitivity were accompanied by proportional changes in the cellular pharmacology of these agents in both murine fibroblast and human ovarian carcinoma cell lines (Samimi et al., 2004). In a direct clinical correlate, immunohistochemical analysis of ATP7A protein levels in normal and malignant tissues demonstrated that ATP7A expression occurs frequently in tumors and that ovarian carcinoma patients whose tumors became enriched for ATP7A-expressing cells during Pt drug-based chemotherapy had a worse survival than those who did not, suggesting that these *in vitro* results are directly relevant to the clinical use of the Pt drugs (Samimi et al., 2003). These results provided the first indication that ATP7A and ATP7B can modulate sensitivity to the clinically important Pt drugs.

The primary uptake transporter for Cu, CTR1, has also been implicated as a determinant of DDP sensitivity and pharmacology (Ishida et al., 2002; Lin et al., 2002). Studies from several laboratories have demonstrated that loss of CTR1 function in *Saccharomyces cerevisiae* results in reduced accumulation of DDP and resistance to its cytotoxic effects; loss of yCTR1 has similar effects on the uptake of CBDCA and L-OHP (Ishida et al., 2002; Lin et al., 2002). Additionally, exposure to DDP causes degradation and delocalization of CTR1 in yeast similar to the effects of Cu (Ishida et al., 2002). These results suggest that DDP uptake and therefore sensitivity are mediated by CTR1; however, these studies remained to be expanded to human models at the beginning of this body of work.

Summary

The commonly used chemotherapeutic agent DDP is limited in its effectiveness due to the frequent development of resistance during treatment. There is now substantial evidence that the Cu exporters ATP7A and ATP7B control efflux, and that both are important determinants of the sensitivity of tumor cells to the cytotoxic effects of these agents. At the time this work started there was also evidence to indicate that in yeast the major Cu influx transporter CTR1 influenced the uptake of the Pt drugs. The available information is consistent with the hypothesis that the Pt drugs enter and exit from the cell using transporters and chaperones that evolved to mediate Cu homeostasis.

Hypothesis

Prior studies of the role of Cu efflux transporters ATP7A and ATP7B indicated that they are mediators of the cellular pharmacology of DDP and determinants of ssensitivity to the cytotoxic effect of this drug. It was the objective of this research to define the role of the human copper transporter 1 (hCTR1) as a transporter of Pt based drugs and as a determinant of DDP resistance. The hypothesis tested was that the human copper transporter hCTR1 is a major influx transporter for DDP and its clinically important analogs, and that defects in the function of this transporter contribute to the impaired uptake of DDP found in cancers with acquired DDP resistance.

Acknowledgments

Chapter 2

Expression of hCTR1 in ovarian carcinoma cells

Introduction

The goal of the experiments described in this chapter was to determine whether hCTR1 influences the cellular pharmacology and cytotoxicity of cisplatin. The first step in achieving this goal was the establishment of an ovarian carcinoma cell line that exogenously expresses high levels of hCTR1 through the stable transfection of hCTR1 cDNA in a mammalian expression vector. Once the over expressing and empty vector cell lines established, the cellular pharmacology of Cu and DDP was characterized. The first step in this characterization was to validate the functionality and localization of the exogenously expressed hCTR1 as determined by Cu uptake. Once the system was verified, studies investigating the cellular pharmacology of cisplatin were performed, including the whole cell and DNA accumulation of cisplatin and sensitivity to Cu and DDP treatment.

Results

Characterization of ovarian carcinoma cells engineered to over-express hCTR1.

The ovarian carcinoma cell line A2780 was transfected with either an empty vector (A2780/EV cells) or a vector containing the hCTR1 cDNA under the control of a CMV promoter (A2780/hCTR1 cells). Figure 1 shows that the level of the 28 kDa

form of the hCTR1 protein was 20-fold higher in the A2780/hCTR1 cells as

determined by Western blot analysis on denaturing gels.
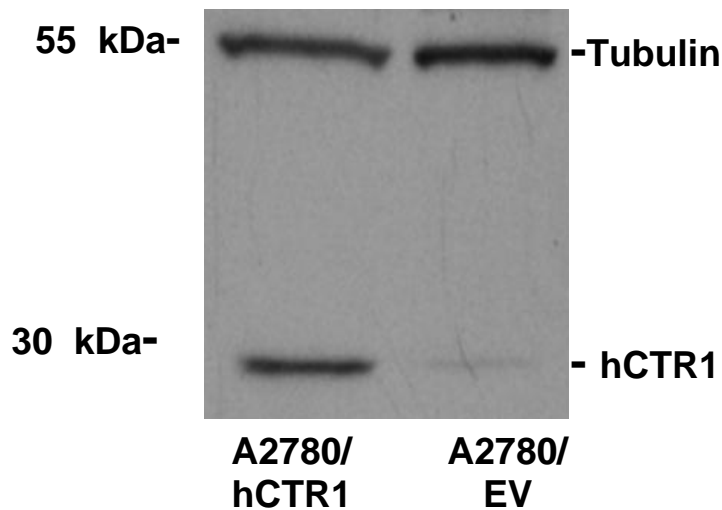
Figure 2-1. hCTR1 expression in A2780/EV and A2780/hCTR1 cells. Western blot
analysis of hCTR1 expression. hCTR1 is detected at 28 kDa while tubulin is detected
at 55 kDa.

To document that the exogenously expressed hCTR1 was correctly localized to

membrane structures in the cell, the distribution of hCTR1 in the A2780/hCTR1 cells

was compared to that of endogenous hCTR1 in the parental A2780 cells using

immunoflourescent staining and deconvoluted confocal digital microscopy. As shown

in Figure 2, although the A2780/hCTR1 cells contained much more total hCTR1, the

distribution of hCTR1 in the transfected cells mimicked the localization and

distribution observed for endogenous hCTR1 in the parental cells indicating that the

exogenously expressed hCTR1 was correctly localized to both the plasma membrane

and intracellular membranous structures. As also shown in Figure 2, exposure of the

A2870/hCTR1 cells to 100 µM Cu for 5 min caused extensive redistribution of

hCTR1 as has previously been reported (Guo et al., 2004; Petris et al., 2003).
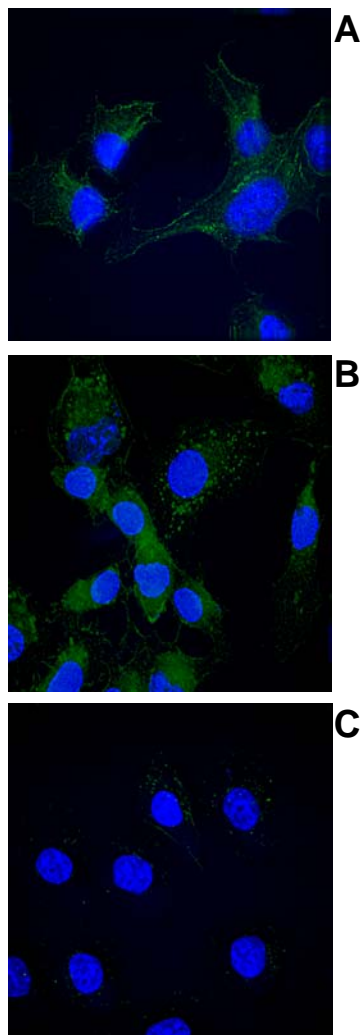
Figure 2-2. **Subcellular localization of hCTR1 in A2780/EV and A2780/hCTR1 cells.** Panel A, A2780 cells; panel B, A2780/hCTR1 cells; panel C, A2870/hCTR1 cells exposed to 100 µM Cu for 5 min. hCTR1 was visualized using a rabbit anti-hCTR1 antibody and a goat anti-rabbit-FITC conjugated secondary. Hoescht 33342 dye was used to label the nucleus. Each image is representative of 3 images taken from each of 3 independent experiments. Images are normalized to the autofluorescence of unstained A2780/EV cells and cells stained with only the secondary antibody.

Effect of hCTR1 expression on the cellular pharmacokinetics of Cu.

The amount of Cu present in cells when they are grown in medium containing no added Cu is a sensitive measure of the extent to which Cu homeostasis has been perturbed. Figure 3A shows that the steady-state level of Cu was 6.5-fold higher in the A2780/hCTR1 than in the A2780/EV cells (23.9 ± 6.9 ng Cu/mg protein vs 3.7 ± 1.18 ng Cu/mg protein). This result suggests that the exogenous hCTR1 expressed from the transfected vector is functional and that it augments the effect of endogenous hCTR1 on the influx of Cu. To document this effect further, the A2870/EV and A2780/hCTR1 cells were exposed to 2 uM ^{64}CuSO$_4$ for time periods varying from 0 to 24 hrs, washed thoroughly and the cell-associated ^{64}Cu was quantified by γ counting. After a 5 min exposure to ^{64}Cu the Cu accumulation in the A2780/hCTR1 cell line was 13.7-fold higher than in the A2870/EV cells. As shown in Figure 3B, after 5 min the A2780/hCTR1 cells had accumulated 7.4 ± 0.71 (SEM) pmole Cu/mg protein while the empty vector transfected A2780/EV cells had accumulated only 0.54 ± 0.56 (SEM) pmole Cu/mg protein. At all time point up to 24 hrs the uptake in the A2780/hCTR1 cells exceeded than in the A2780/EV cells. By 24 hrs, at which time accumulation of ^{64}Cu had reached steady-state in the A2780/EV cells, the A2780/hCTR1 cells contained 17-fold more Cu (40.8 ± 3.2 (SEM) pmole Cu/mg protein vs 2.37 ± 0.07 (SEM) pmole Cu/mg protein). Thus, the exogenously expressed hCTR1 not only localized correctly to membraneous structures in the cell, it also was functional in modulating the cellular pharmacology of Cu.
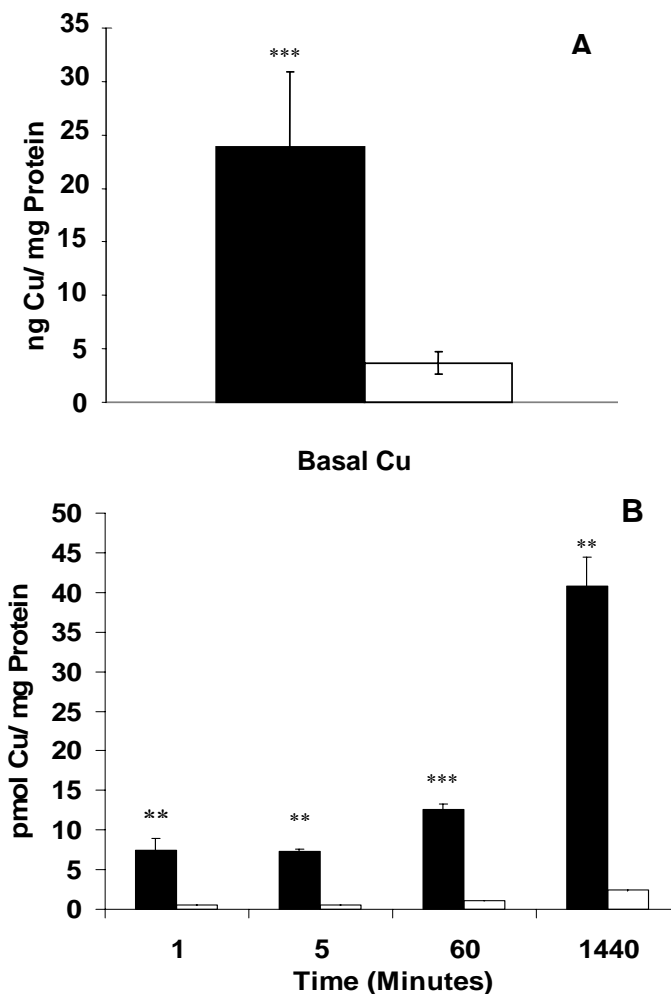
Figure 2-3. Effect of increased hCTR1 expression on steady-state Cu content and ^{64}Cu accumulation. Panel A, pmol Cu present in A2780/hCTR1 and A2780/EV cell lines under basal conditions. Closed bar, A2780/hCTR1 cells; Open bar, A2780/EV cells. Each bar represents the mean of 3 independent experiments, each performed with sextuplet cultures. Panel B, accumulation after 1, 5, 60 and 1,440 min of exposure to ^{64}CuSO$_4$; Closed bars, A2780/hCTR1 cells; open bars, A2780/EV cells. Each bar represents the mean of 3 independent experiments each performed with sextuplet cultures. ** $p < 0.005$, ***$p < 0.0005$ for comparison of accumulation in A2780/EV versus A2780/hCTR1 cells. Vertical bars, SEM.

Effect of hCTR1 expression on the cellular pharmacokinetics of DDP.

The effect of increased expression of hCTR1 on the uptake of DDP was determined by exposing the A2780/EV and A2780/hCTR1 cells to 2 µM DDP, a concentration attained in the plasma of patients receiving standard doses of the drug, and quantifying the total cellular Pt at various time points by inductively-coupled plasmon mass spectroscopy. As shown in Figure 2-4A, following a 1 hr exposure to 2µM DDP, the A2780/hCTR1 cells had accumulated 165 ± 3 (SEM) pg Pt/mg protein whereas the A2780/EV cells had accumulated 113 ± 26 (SEM) pg Pt/mg protein. Thus at this time point the hCTR1 over-expressing cells contained 46 % more Pt. Figure 2-4A shows that after a 24 hr exposure the A2780/hCTR1 cells had accumulated 55 % more Pt than the A2780/EV cells. Even initial uptake was increased in the hCTR1 transfected cells. Following a 4 minute exposure to 2 µM DDP, the A2780/hCTR1 transfected line had accumulated 81 % more Pt than the empty vector transfected line (134 ± 8 pg Pt/mg protein vs 74 pg Pt/ mg protein, respectively) (Figure 2-4B). Thus the increased expression of hCTR1 in the A2780/hCTR1 cells resulted in increased accumulation of both Cu and DDP; however, the magnitude of the effect was much greater for Cu than for DDP. This suggests that DDP is not as good a substrate for hCTR1 as Cu, and that there are substantial differences in the ability of this transporter to mediate the influx of these two compounds.
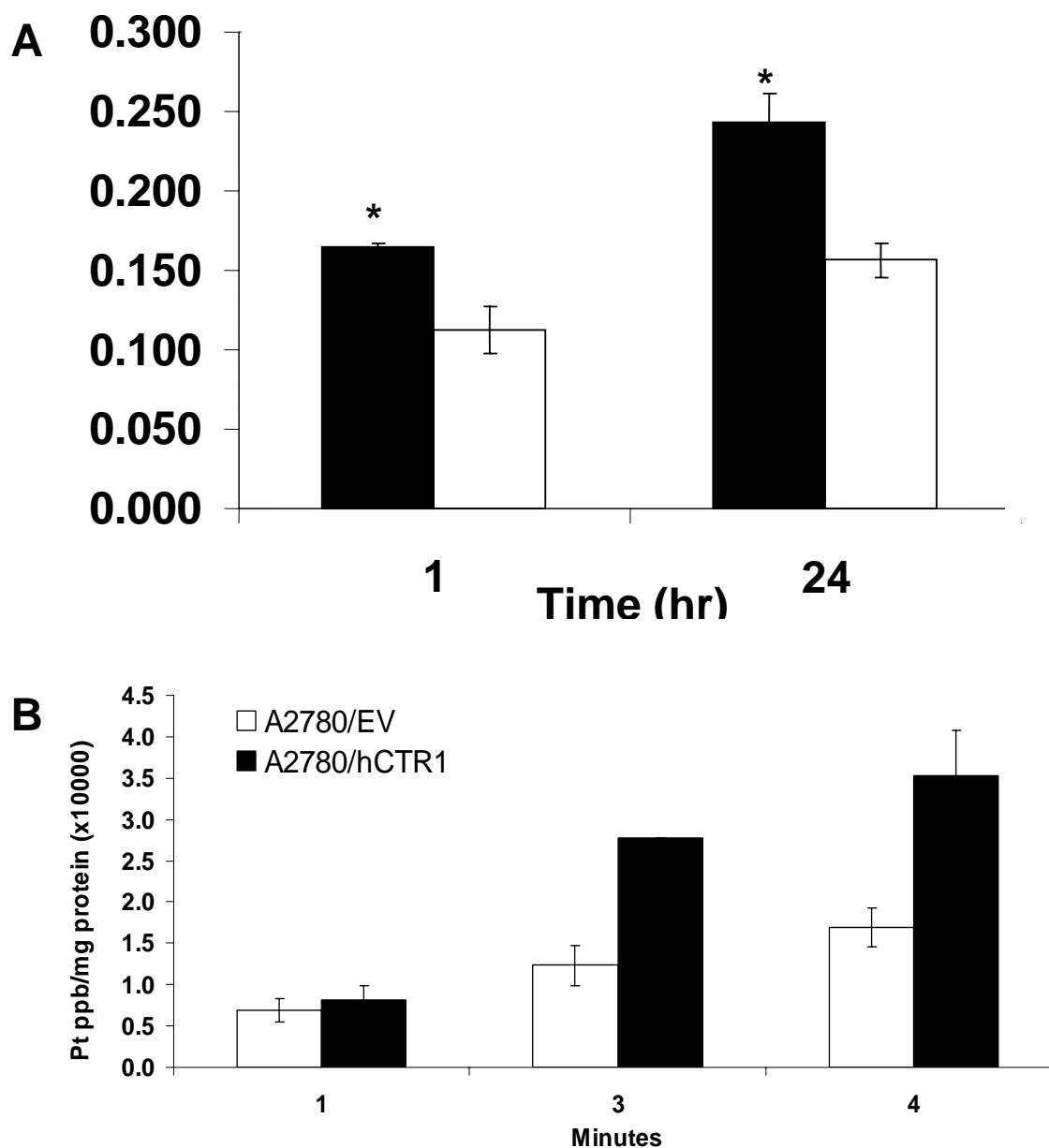
Figure 2-4. Effect of increased hCTR1 expression on long DDP accumulation. Panel A; accumulation was measured after 1 and 24 hrs of exposure to 2 uM DDP. Closed bars, A2780/hCTR1 cells; open bars, A2780/EV cells. Panel B; accumulation was measured after 1, 3 and 4 min of exposure to 2 μM DDP. Closed bars, A2780/hCTR1 cells; open bars, A2780/EV cells. Each bar represents the mean of 3 independent experiments each performed with triplcate cultures. *p <0.03 for comparison of accumulation in A2780/EV versus A2780/hCTR1 cells. Vertical bars, SEM.

Effect of hCTR1 expression on sensitivity to the cytotoxic effects of Cu and DDP.

      The cytotoxicity of both Cu and DDP is a function of how much of the compound enters the cell. However, DDP toxicity is additionally proportional to the amount of drug entering the cell that actually forms adducts in DNA. Based on the observation that expression of exogenous hCTR1 increased the whole cell uptake of Cu and DDP, one might anticipate enhanced sensitivity to the cytotoxic effect of both agents, and since the magnitude of the increase in Cu was substantially greater than DDP, one might expect a greater effect on sensitivity to Cu than DDP. Growth rate as a function of Cu or DDP concentration was determined for each of the cell lines under conditions of continuous drug exposure. As shown in Figure 2-5A, the IC_{50} for Cu was only 6 % lower in the A2780/hCTR1 cells (160.7 ± 5.7 (SEM) µM) that for the A2780/EV cells (171.2 ± 12.0 (SEM) µM). Likewise, as shown in Figure 2-5B, the IC_{50} for DDP was only 1.8 % lower for the A2870/hCTR1 cells (7.17 ± 0.23 (SEM) µM) than for the A2780/EV cells (7.30 ± 0.053 (SEM) µM). Thus, a large change in the level of expression of hCTR1, while it was associated with a clear increase in Cu and DDP uptake, produced only a very small change in sensitivity to either compound. This suggests that much of the Cu and DDP entering the A2780/hCTR1 cells is not reaching key targets in the cell.
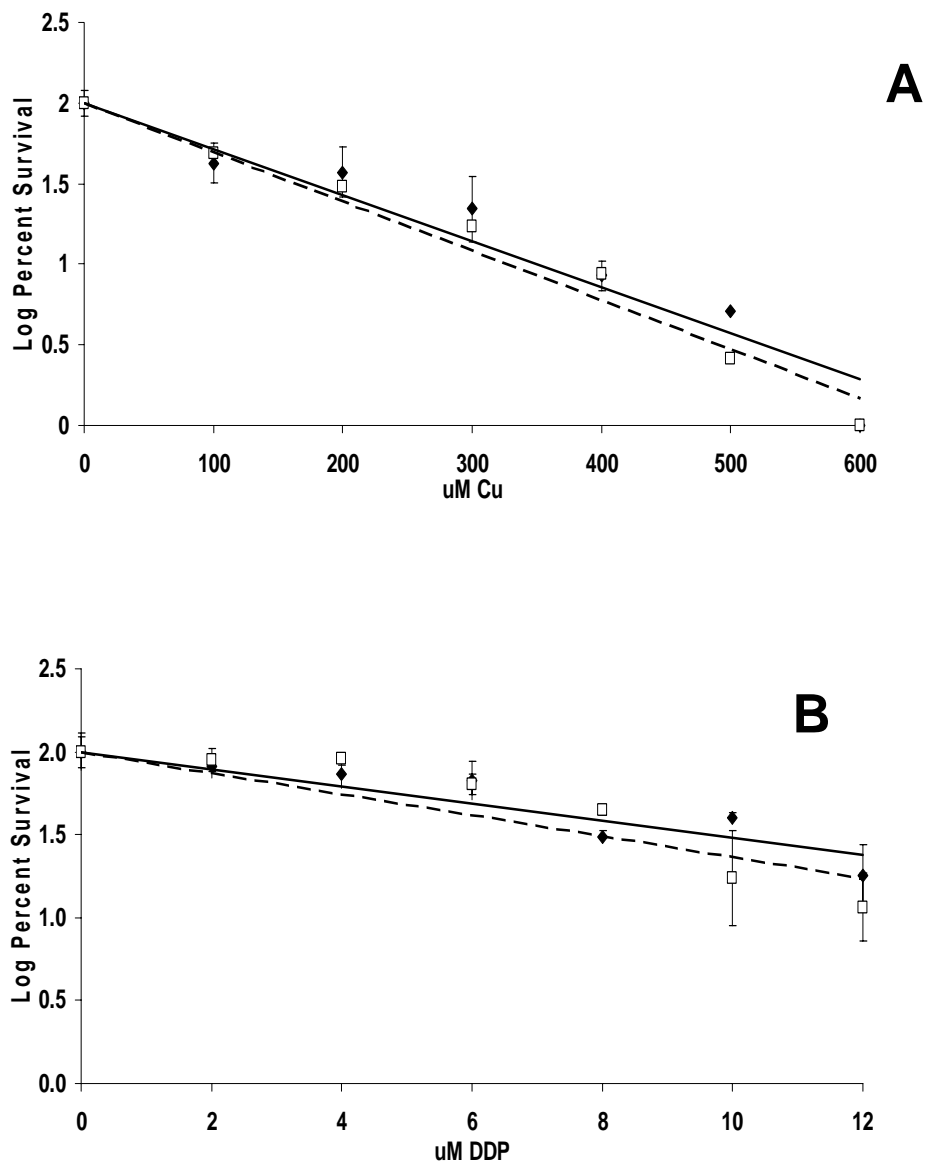
Figure 2-5. Effect of Cu and DDP on cell growth. A, log of percent surviving cells after 7 days of continuous exposure to Cu. B, percent survival after 7 days of continuous exposure to DDP. Solid line, A2780/EV (♦) cells; dashed line, A2780/hCTR1 (□) cells. Each point represents the mean of 3 independent experiments each performed with 6 cultures. Vertical bars, SEM.

To examine this question further, the extent of DDP adduct formation in DNA was assessed by measuring the platinum content of DNA extracted from A2780/EV and A2780/hCTR1 cells following a 1 hr exposure to 2 μM DDP. As shown in Figure 2-6, despite the fact that forced expression of hCTR1 in the A2780/hCTR1 cells increased whole cell DDP accumulation, there was no difference in the extent of DNA adduct formation. Thus, the hCTR1-mediated enhanced DDP uptake delivers this compound to one or more compartments in the cell from which it does not have ready access to its major cytotoxic target, and the same thing appears to be true for Cu.
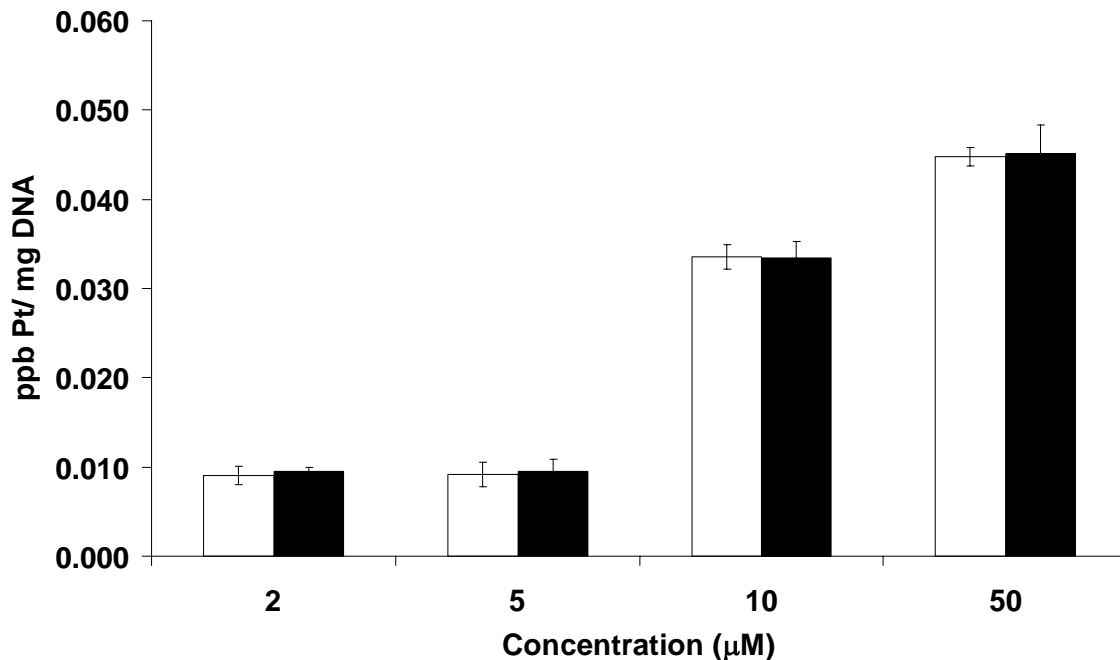
Figure 2-6. Accumulation of Pt in DNA following 1 hour exposure to 2, 5, 10 and 50 μM DDP. Closed bar, A2780/hCTR1; open bar, A2780/EV. Each bar represents the mean of 3 independent experiments each performed with triplicate cultures. Vertical bars, SEM.

Discussion

The results of these studies demonstrate that the level of expression of the Cu influx transporter hCTR1 influences the cellular accumulation of DDP as well as that of Cu in human ovarian carcinoma cells, but that the enhanced cellular uptake of these compounds is not accompanied by a significant change in sensitivity to their cytotoxic effect or the delivery of DDP to DNA. These observations introduce the novel concept that, while hCTR1 enhances DDP uptake into the cell, it delivers it to cellular compartments from which it does not have ready access to key cytotoxic targets. This idea is supported by previous studies showing that exogenous expressed hCTR1 relocalizes from the plasma membrane to vesicles upon Cu exposure (Guo et al., 2004), and that exposure to DDP produces a similar effect on both endogenous and exogenously expressed hCTR1 (Holzer et al., 2004a).

The A2780/hCTR1 cells expressed a 20-fold higher level of hCTR1 as detected by Western blot analysis, and the exogenously expressed hCTR1 corrected localized to membrane structures as demonstrated by high resolution immunofluorescent analysis of its subcellular distribution in the A2780/EV and A2780/hCTR1 cells. The functionality of the exogenous hCTR1 was demonstrated by the observation that it relocalized in response to Cu exposure, and that its expression in the A2780/hCTR1 cells was accompanied by substantial changes in the cellular pharmacology of Cu. The basal steady-state level of Cu in the A2780/hCTR1 cells was increased by 6.5-fold, and these cells exhibited a marked increase in the uptake rate for ^{64}Cu. Thus, exogenous hCTR1 was functional in A2780 cells and augmented

their ability to internalize the small amounts of Cu found in standard tissue culture medium and the rate of Cu accumulation.

The results of the current study suggest that Cu is also a better substrate for hCTR1 than Cu in human cells as the effect of increasing hCTR1 expression was substantially greater for Cu than for DDP. Nevertheless, hCTR1 clearly increased the cellular uptake of DDP as well as Cu, and the magnitude of this effect is well within the range where, if the intracellular drug had ready access to DNA, it would be expected to produce increased cell kill (Katano et al., 2002).

Materials and Methods

Drugs and Reagents.

Platinol-AQ containing DDP at a concentration of 3.3 mM, was a gift from Bristol-Myers Squibb (Princeton, NJ). It was stored in the dark at room temperature, and a 100 uM stock solution was created by dilution 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 Company (Hercules, CA). The generation of the rabbit polyclonal antibodies against amino acids 1 through 67 of hCTR1 used for immunofluorescence is described elsewhere (Klomp et al., 2002). Fluorescein isothiocyanate-conjugated goat anti-rabbit 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 antibody used for Western blotting was generated by immunizing rabbits with a peptide containing amino acids 2-22 of the N-terminus of hCTR1 by

Biocarta Inc. (San Diego, CA). Horseradish peroxidase-conjugated goat anti- rabbit secondary antibody was purchased from Amersham Pharmacia (Piscataway, NJ). All other chemicals and reagents were obtained from Fisher Scientific (Tustin, CA).

Cell Lines and Vectors.

The A2780 ovarian carcinoma cell line (Hamilton et al., 1985) was grown in RPMI 1640 media containing 10% fetal bovine serum at $37^{o}C$ in 5% CO_2. A pcDNA3.1 vector containing full length hCTR1 cDNA and expressing a geneticin resistance marker constructed as previously described (Moller et al., 2000), was generously provided by Dr. Lisbeth Birk Moller. Cells were transfected with either hCTR1/pcDNA3.1 or empty vector (EV) 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 generation.

Western Blot Analysis.

Cells from an 80% confluent T75 flask were trypsinized and recovered by centrifugation at $4^{o}C$ following which they were frozen at $-20^{o}C$ for 1 hr and then thawed on ice. The pellet was resuspended in 100 µL buffer (250 mM sucrose, 10mM Tris-HCl pH 7.4, 1 µg/ml antipain, 1µg/ml pepstatin, 1 µg/ml leupeptin and 20 µg/ml phenylmethanesulfonylflouride) and homogenized using a dounce homogenizer for 1min. The suspension was centrifuged for 15 min at 500 x g at $4^{o}C$ to remove nuclei and large particulate matter (Deutscher, 1990), and the protein concentration of the resulting supernatant was determined. Samples containing 100 µg protein were boiled prior to electrophoresis in a 4-20% SDS-PAGE gel and transfer to nitrocellulose

membranes (Bio-Rad Co.) via electrophoresis for 30 min at 200 volts using a Transblot SD apparatus (Bio-Rad Co). Membranes were blocked in 5% milk in Tris buffered saline for 1 hr at room temperature. Blots were incubated at 4ºC overnight with anti-hCTR1 antibody diluted 1:1000 and mouse anti-tubulin (Sigma Products, St. Louis, MO) diluted 1:20,000 in 5% milk in Tris buffered saline. Membranes were then washed 3 times with the same buffer containing 0.05% Tween-20 and incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies for 1 hr at room temperature. Prior to detection membranes were again washed 3 time with Tris buffer saline containing 0.05% Tween-20 and detection was performed using the enhanced chemiluminescence western detection system from Amersham-Pharmacia (Piscataway, NJ) according to manufacturer's instructions.

Fixation and staining.

Cells were grown in T75 flasks until 80% confluent and then harvested by trypsinization and pelleted by centrifugation in 10 % RPMI 1640 with 10 % fetal bovine serum. The resulting pellets were resuspended in 5ml of 10 % RPMI 1640 with 10 % fetal bovine serum and 100 µL of cells were added to each well of a 24 well plate. Prior to the addition of cells, a 0.16 mm thick coverslip and 300 µL of 10 % RPMI 1640 containing 10 % fetal bovine serum and 200 µM bathocuprione disulphonic acid were placed in each well. Bathocuprione disulphonic acid was added to chelate any Cu in the media ensuring that Cu could not affect DDP binding. Once cell growth on the coverslips reached 80 % confluency at approximately 48 hrs after plating, the cells were exposed to 10 % RPMI 1640 with 10 % fetal bovine serum containing Cu for 5 minutes. After the specified duration of exposure, the coverslips

were placed at 4º C and washed 3 times with phosphate buffered saline and then fixed in 3.7 % formaldehyde in the same buffer at room temperature for 30 minutes. Subsequent staining and washing were performed as previously described (Klomp et al., 2002).

Measurement of ^{64}Cu accumulation.

Cu uptake measurements were made using cells grown in medium lacking geneticin to 80% confluent in the 35 mm wells of a 6 well plate. Following addition of pre-warmed media containing 2μM ^{64}CuSO$_4$ the plates were incubated at 37º C on 5 % CO$_2$ for 0 to 24 hrs. At the end of the incubation period the plates were placed on ice and rinsed 3 times with 6 ml ice cold PBS. Cell lysis buffer (0.1 % Triton-X, 1 % SDS in PBS) in a volume of 500 μL was added to the wells, and the lysate was harvested by scraping the dish twice before it was transferred to tubes for gamma counting on a (Gamma 5500B, Beckman Coulter, Fullerton, CA). Six cultures were harvested for each time point. Lysates from a set of identical cultures not exposed to ^{64}CuSO$_4$ were used to measure protein concentration.

Drug Sensitivity Assay.

Six-well plates were seeded with 3,000 cells per well and 24 hrs later the medium was replaced with medium containing various concentrations of either DDP or Cu. After 7 days of continuous exposure, cultures were trypsinized and stained with trypan blue. The live cell number per well was determined by counting cells that excluded trypan blue with a hemocytometer. Each drug concentration had 6 samples per cell line and the experiment was repeated 3 times.

Pt accumulation assays.

Cells were plated in 145 cm^2 dishes in media without any drugs. Once plates are 80 % confluent, 2 µM DDP was added and plates were incubated at 37º C in 5% CO2 for various period of time. The plates were then placed on ice and rinsed 3 times with 3 ml ice cold phosphate buffered saline. Cells were harvested by scraping followed by centrifugation for 10 min at 3000 rpm and 4º C. The resulting pellet was dissolved in 70 % nitric acid at 65º C for at least 2 hours. Samples were diluted to 5 % nitric acid with water containing 1 ppb indium and 0.1 % Triton-X. Pt content was determined using inductively-coupled plasmon mass spectroscopy (ICP-MS) (Element2; Perkin Elmer Life Sciences) on an instrument available through the Analytical Facility at the Scripps Institute of Oceanography. Lysates from a set of identical cultures suspended in 0.1 % Triton-X and 1 % SDS in phosphate buffer saline were used to measure protein concentration.

Measurement of basal copper levels.

Cells were grown in 145 cm^2 dishes until 80% confluent following which they were washed 3 times with ice cold phosphate buffered saline, harvested by scraping with a rubber policeman, and pelleted at 3000 rpm for 10 min at 4ºC. The pellet was resuspended in 70% nitric acid at 65ºC for a minimum of 2 hours. Following dilution of the nitric acid to 5%, Cu content was assayed using an inductively coupled plasma optical emission spectroscopy (ICP-OES) apparatus (model 3000DV; PerkinElmer Life Sciences) available at the Analytical Facility at the Scripps Institute of Oceanography.

Statistics.

Tests of significance used a two-sided paired Student's *t* test with the assumption of unequal variance; *p* values of <0.05 were considered significant. All p values are for the comparison of the values for the A2780/EV versus the A2780/hCTR1 cells.

Acknowledgements

Chapter 3

Uptake of Pt based therapies in CTR1$^{-/-}$ murine embryonic fibroblasts

Introduction

A preliminary study suggested that the cellular accumulation of DDP was impaired in embryo fibroblasts established from mice in which both mCTR1 alleles had been disrupted (Ishida et al., 2002). The studies presented in Chapter 2 demonstrated that over-expression of hCTR1 in human ovarian carcinoma cells enhanced DDP uptake (Holzer et al., 2003). However, this turned out not to be a consistent finding in other cell models. Whereas increased hCTR1 expression in human small cell lung cancer cells (Song et al., 2004) was reported to enhance the uptake of DDP, CBDCA and L-OHP, over-expression of hCTR1 in human squamous carcinoma cells was reported to have no effect on DDP uptake (Beretta et al., 2004). Such forced over-expression of hCTR1 is toxic to human cells, and it is not clear that it functions normally at such high intracellular levels as evidenced by the finding that increased CTR1 expression and DDP uptake into the whole cell is accompanied by minimal changes in sensitivity to the cytotoxic effect of the drug and DNA adduct formation (Holzer et al., 2004b).

The overall goal of the experiments described in this chapter was to refine understanding of how mCTR1 modulates the cellular pharmacology of the Pt-containing drugs. The experiments were performed using an isogenic pair of mouse

embryo fibroblasts, one of which lacks both alleles of mCTR1. The first step in this study was to verify the uptake phenotype of these cell lines by studying the cellular pharmacology of ^{64}Cu. Once that was completed, the effect of mCTR1 expression on the cellular pharmacology and cytotoxicity of DDP and its analogs was examined.

Results

Effect of the loss of CTR1 on Cu accumulation.

Previous studies have shown that CTR1 plays a substantial role in the cellular accumulation of Cu. To verify the influence of CTR1 on Cu uptake, and validate this cell line model for the study of the Pt-containing drugs, CTR1$^{+/+}$ and CTR1$^{-/-}$ cells were exposed to a 2 μM ^{64}Cu for 1 hour and then washed extensively before the accumulation of Cu was measured by γ counting. This concentration of Cu is near K$_m$ of 1 μM reported for murine CTR1 (Lee et al., 2002b). As shown in Table 1, the cells lacking CTR1$^{-/-}$ accumulated only 2% as much Cu as the CTR1$^{+/+}$ cells. Thus, as previously reported (Lee et al., 2002b), loss of CTR1 markedly impaired the influx of Cu in these

Table 3-1. Accumulation of Cu, DDP, CBDCA and L-OHP in CTR1$^{+/+}$ and CTR1$^{-/-}$ cells*.

Drug	Whole cell accumulation		Accumulation Ratio**	p Value
	CTR1$^{+/+}$	CTR1$^{-/-}$		
Cu (CPM ^{64}Cu/ mg protein)	244.77 ± 41.40	5.80 ± 2.29	0.057 ± .02	1.36 x 10^{-10}
DDP (ng Pt/mg protein)	2.98 ± 0.61	0.58 ± 0.29	0.196 ± .19	0.0085
CBDCA (ng Pt/mg protein)	5.0 ± 0.42	2.3 ± 0.56	0.456 ± .19	0.0033
L-OHP (ng Pt/mg protein)	4.81 ± 0.3	4.1 ± 0.57	0.849 ± .09	0.148
*Values are mean ± SEM, N = 5 **CTR1$^{-/-}$/CTR1$^{+/+}$				

Effect of the loss of CTR1 on DDP, CBDCA and L-OHP accumulation.

The accumulation of the Pt-containing drugs was quantified by measuring the Pt content of the whole cell following a 1 h exposure to equitoxic concentrations of DDP, CBDCA or L-OHP. As shown in Table 1, when exposed to 2 μM DDP for 1 h, the CTR1$^{-/-}$ cells accumulated only 20% as much Pt as the CTR1$^{+/+}$ cells. Following a 1 h exposure to 12 μM CBDCA the CTR1$^{-/-}$ cells accumulated only 46% as much Pt as the CTR1$^{+/+}$ cells. In contrast, following a 1 h exposure to 6 μM L-OHP there was no discernable difference in Pt content of the CTR1$^{-/-}$ and CTR1$^{+/+}$ cells. These results indicate that CTR1 contributes importantly to the accumulation of DDP but that it is less involved in mediating the uptake of CBDCA and plays no role in the accumulation of L-OHP when measured at the end of a period of drug exposure that mimics the typical residence time of free Pt drugs in human plasma following intravenous injection.

Effect of the loss of CTR1 on sensitivity to the growth-inhibitory effects of Cu, DDP, CBDCA and L-OHP.

As the CTR1$^{+/+}$ and CTR1$^{-/-}$ cells do not form discrete colonies when cultured on plastic, the effect of loss of CTR1 function on sensitivity to the cytotoxic effect of each drug was determined by quantifying inhibition of proliferation during a 72 h exposure to increasing concentrations of drug. Figure 1 depicts inhibition of growth as a function of drug concentration. The CTR1$^{-/-}$ cells were 1.4-fold more resistant to Cu than their wild type counterparts. Interestingly, loss of CTR1 produced a larger degree of resistance to DDP than to Cu; cells lacking CTR1 were 3.2-fold resistant to DDP (0.416 CI). The CTR1$^{-/-}$ cells were only 2.0-fold CBDCA and in a set of 4 experiments this difference did not reach statistical significance. There was only a 1.7-fold difference in sensitivity to L-OHP between the two types of cells which was not statistically significant. Thus, consistent with the effect on the loss of CTR1 on drug uptake, loss of CTR1 produced different degrees of resistance to DDP, CBDCA and L-OHP. It is interesting to note that while there was a marked difference in the uptake of ^{64}Cu, the difference in Cu IC$_{50}$ was not great. In contrast, for cells treated with DDP, there was a closer correspondence between the reduction in uptake and the increase in the IC$_{50}$.
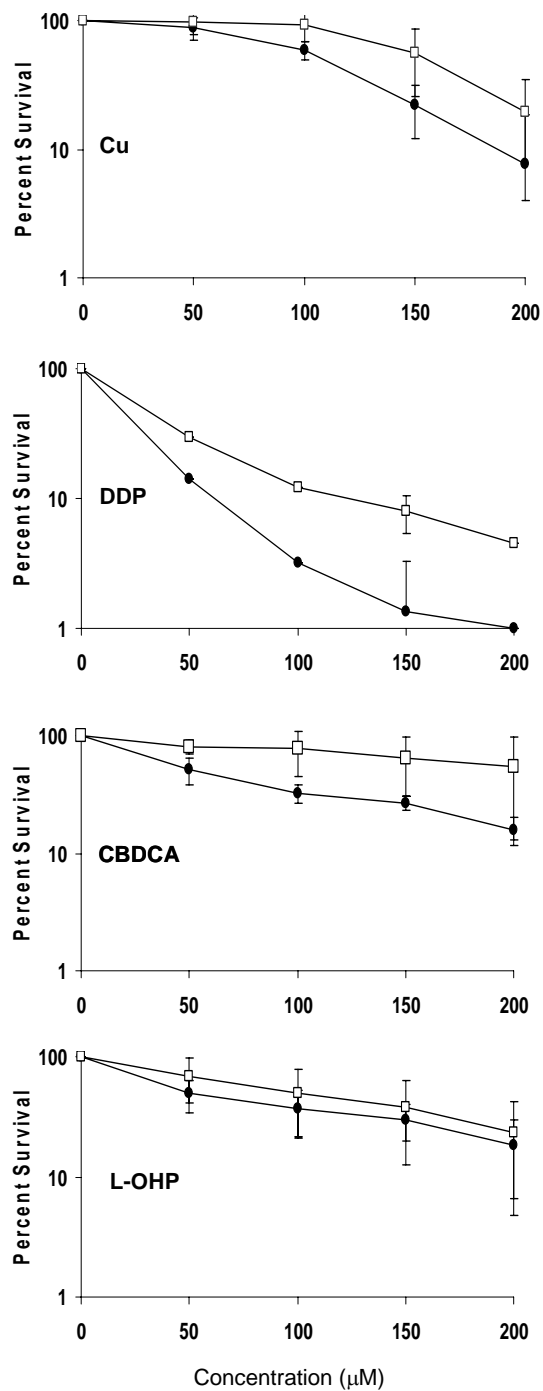
Figure 3-1. Inhibition of the growth of CTR1$^{+/+}$ and CTR1$^{-/-}$ cells by DDP, CBDCA and L-OHP. Each curve is a plot of the percent of cells surviving after a 72 hr continuous exposure to drug. (◆), CTR1$^{+/+}$ cells; (□), CTR1$^{-/-}$ cells. Each point represents the mean of 3 independent experiments each performed with 6 replicate cultures. Vertical bars, SEM. Where SEM bars are missing they are smaller than the symbol.

Discussion

The isogenic pair of $CTR1^{+/+}$ and $CTR1^{-/-}$ cells used in this study provided a powerful model in which to refine understanding of the role of CTR1 in the influx of the Pt-containing chemotherapeutic agents. As previously reported (Lee et al., 2002b), the accumulation of Cu was found to be markedly reduced in the $CTR1^{-/-}$ cells when they were exposed to low concentrations of Cu for 1 h. Accumulation in the $CTR1^{-/-}$ cells was just 2% of that in the $CTR1^{+/+}$ cells. Loss of CTR1 function had a smaller effect on the uptake of DDP. When cells were exposed to a DDP concentration that approximates the peak level attained in human plasma, accumulation in the $CTR1^{-/-}$ cells was 20% of that in the wild type cells. In contrast, loss of CTR1 had much less effect on the accumulation of CBDCA and no effect on the uptake of L-OHP.

In the mammalian cells, the extent to which Pt drug uptake was impaired in the $CTR1^{-/-}$ was closely linked to the extent to which sensitivity to the cytotoxic effect of the drug was reduced. This suggests that the accumulation differences detected at the end of a 1 h drug exposure is a good surrogate measure of the total amount of drug reaching critical intracellular targets even when cells were exposed for 72 h as they were in the cytotoxicity assay. In contrast, in the case of Cu there was a discrepancy between the effect on accumulation and on cytotoxicity. Knockout of CTR1 inhibited accumulation of Cu to a much greater extent than DDP; however, the impact on sensitivity to Cu was less than it was for DDP. The explanation for this likely relates to the fact that, in the case of Cu, the uptake studies were performed at a concentration ~60-fold lower than the IC_{50} whereas in the case of the Pt drugs the concentrations were at the IC_{50}.

The fact that deletion of CTR1 did not completely eliminate DDP accumulation indicates that CTR1 is not the only route by which DDP enters mammalian cells. This mirrors the situation for Cu; even though loss of CTR1 reduced Cu uptake by 98%, and this transporter is essential for embryonic viability, Cu nevertheless enters the cell in amounts sufficient to sustain growth. To date, not other transporter has definitively been shown to mediate the influx of Cu in mammalian cells (Lee et al., 2002b). Likewise, loss of CTR1 function reduced DDP uptake to only 20% of control, indicating that there is yet another mechanism by which DDP enters cells that has not yet been characterized

The results reported here establish that CTR1 regulates the cellular accumulation of DDP and CBDCA but not L-OHP. This indicates that L-OHP must enter the cell by a different, and as of yet, unknown mechanism. The concept that these drugs have different influx transporters is consistent with their different spectrum of action against various types of human cancer. Identification of the major L-OHP influx transporter may inform the design of additional Pt drugs that are even better substrates for the transporter than L-OHP.

Materials and Methods

Reagents.

Refer to methods described in chapter 2 for DDP, $CuSO_4$ and ^{64}Cu. CBDCA was purchased from Sigma (St. Louis, MO) and a stock solution was prepared at 10 mM in water. L-OHP was a generous gift from Sanofi Pharmaceuticals (Malvern, PA). A stock solution was prepared at 10 mM in water.

Cell lines.

The murine embryonic fibroblasts, originally isolated from day 7 embryos and immortalized using SV40 large T antigen, were cultured as described previously (Lee et al., 2002b). Cells were grown in 20% fetal bovine serum, 2 mM glutamine, 1X non-essential amino acids, 55 μM 2-mercaptoethanol, 50 mg/L uridine and 110 mg/ml pyruvate.

Measurement of ^{64}Cu accumulation.

Refer to methods described in Chapter 2.

Pt accumulation assays.

Cells were plated in 6-well plates in media without any drugs. Once the cultures were 80 % confluent, 2 μM DDP, 12 μM CBDCA or 6 μM L-OHP was added and plates were incubated at 37º C in 5% CO_2 for 1 h. The plates were then placed on ice and wells were rinsed 3 times with 3 ml ice cold phosphate buffered saline. Cells were harvested by dissolving them in 70% nitric acid at 65ºC for at least 2 hours. Samples were diluted to 5% nitric acid with water containing 1 ppb indium and 0.1% Triton-X. Pt content was determined using inductively-coupled plasmon mass spectroscopy (ICP-MS) (Element2; Perkin Elmer Life Sciences) on an instrument available through the Analytical Facility at the Scripps Institute of Oceanography. Lysates from a set of identical cultures suspended in 0.1% Triton-X and 1% SDS in phosphate buffered saline were used to measure protein concentration.

Drug sensitivity assay.

Six-well plates were seeded with 1,000 cells per well and 24 h later the medium was replaced with medium containing various concentrations of either DDP,

CBDCA, L-OHP or Cu. After 3 days of continuous exposure, cultures were trypsinized and stained with trypan blue. The number of live cells per well was determined by counting cells that excluded trypan blue with a hemocytometer. Each drug concentration was tested in 6 cultures per cell line and each experiment was repeated 3 times.

Statistics.

Refer to methods described in Chapter 2.

Acknowledgements

Chapter 4

The subcellular localization and expression level of hCTR1 following cisplatin exposure

Introduction

The previous two chapters have established that hCTR1 influences the cellular accumulation of DDP. However, the mechanistic details of how hCTR1 mediates DDP uptake are unknown. Regulation of the level of CTR1 protein is believed to occur primarily by post-transcriptional mechanisms (Lee et al., 2000). In yeast, exposure to elevated levels of Cu triggers both endocytosis and degradation as separate events (Ooi et al., 1996). Recent reports suggest that both endocytosis and protein degradation are also important to the amount of hCTR1 expressed in the plasma membrane of mammalian cells as well (Petris et al., 2003). In order 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. The overall goal of the experiments in this chapter was to study the effect of clinically relevant concentrations of DDP on the subcellular localization of hCTR1 protein in human ovarian carcinoma cells. The results indicate that DDP very rapidly triggers the loss of both endogenous and exogenously expressed hCTR1 in ovarian carcinoma cells, and that this has functional consequences for the uptake of Cu. We conclude that DDP down-regulates the level of its own major influx trasporter.

Results

Western blot analysis of the effect of DDP on hCTR1.

In order 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 Figure 4-1, a 5 min exposure to 2 μM 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 min. When exposed to 2 μM DDP, a clear decrease was detectable even at 1 min. Figure 4-1 shows that even a 5 min exposure to 0.5 μM DDP was effective in reducing the hCTR1 level. As seen in Figure 4-1, the loss of hCTR1 was manifested by disappearance of the 28 kDa band; there was no smearing of the bands suggestive of degradation of the protein. Similar results were obtained with a A2780 subline stably transfected with a vector expressing hCTR1 from a cytomegalovirus promoter, indicating that DDP triggered the disappearance of both endogenous and exogenously expressed hCTR1 (see Figure 2-2). 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 μM Cu for 5 min. As shown in Figure 1, Cu caused a decrease in hCTR1 protein level at 5 min although the reduction was less than that produced by 2 μM DDP. The decrease in Cu-treated cells was similar to that reported by Petris *et al.* (Petris et al., 2003).

A

55 kDa -					Tubulin
28 kDa -					hCTR1
DDP Concentration	-	0.5 µM	1.0 µM	2 µM	-
Cu Concentration	-	-	-	-	100 µM
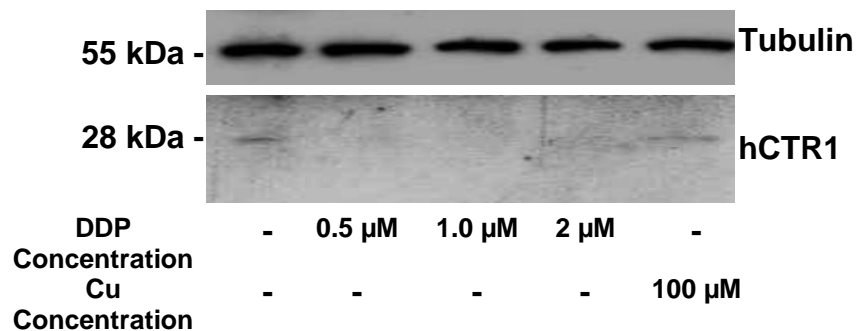
B
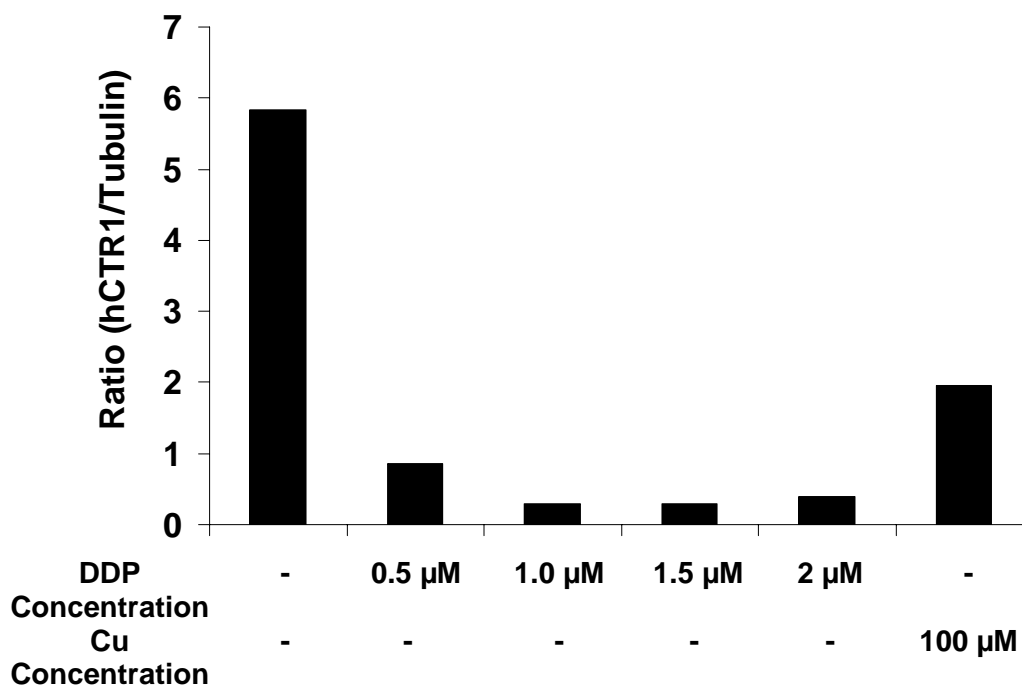
Figure 4-1. Western blot analysis of the effect of DDP and Cu on the level of endogenous hCTR1 in A2780 cells. A2780 ovarian carcinoma cells were treated with 0.5, 1.0, 1.5 or 2.0 µM DDP or 100 µM Cu for 5 minutes. Panel A, denaturing Western blot showing the level of tubulin and hCTR1 (representative of 5 separate experiments). Panel B, densitometric analysis showing level of hCTR1 relative to the tubulin lane loading control.

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, both denaturing and non-denaturing gels were used in all experiments. Identical results were observed when the cell lysates were run under either condition (Figure 4-1 and 4-2).
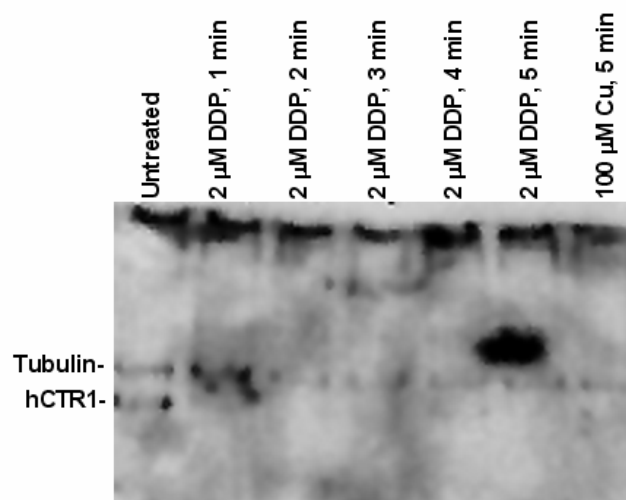
Figure 4-2. Non-Denaturing Western blot analysis of the effect of DDP and Cu on the level of endogenous hCTR1 in A2780 cells. A2780 ovarian carcinoma cells were treated 2.0 µM DDP for 1, 2, 3, 4, or 5 min or 100 µM Cu for 5 minutes.

Further, full length hCTR1 was expressed in *E. coli* and this purified fragment of hCTR1 was incubated with either 100 µM Cu or 2 µM DDP for 5 min and analyzed on both non-denaturing and denaturing Western blots (Figure 4-3). 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 (Figure 4-3).
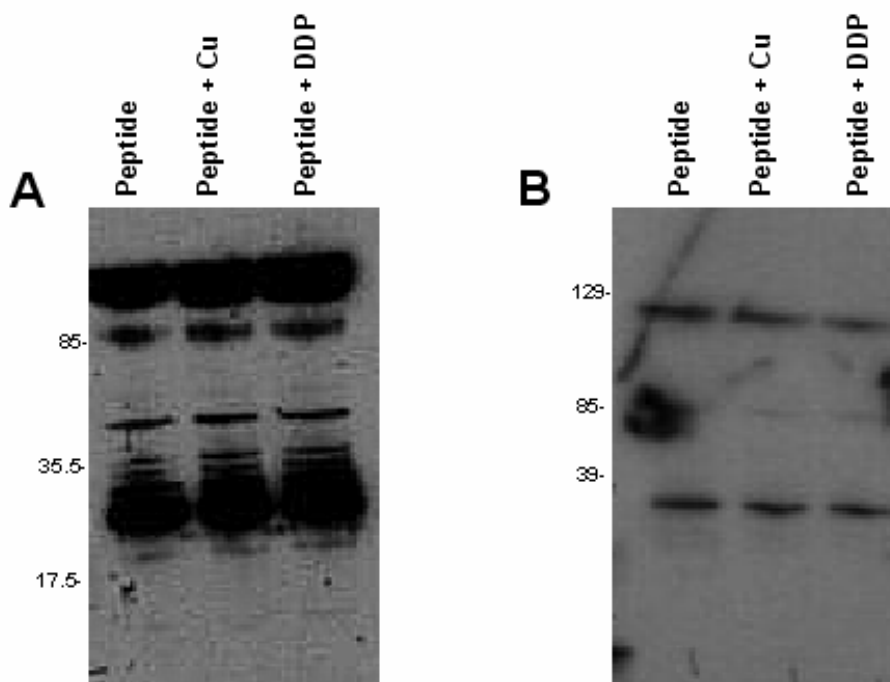
Figure 4-3. Western blot analysis of the effect of DDP and Cu on hCTR1 peptide.
A construct containing hCTR1 was expressed and partially purified from *e.coli*. The peptide was exposed to 100 μM Cu or 2 μM DDP for 5 min prior to loading on a 4-15% gradient gel and was run in denaturing or non-denaturing conditions. Panel A; Western blot of hCTR1 peptide under denaturing conditions, Panel B; Non-denaturing western blot.

Additionally, cell extracts were obtained from untreated cells and then incubated with 100 μM Cu or 2 μM DDP for 5 min before being subjected to denaturing and non-denaturing Western blot analysis. The antibody to hCTR1 recognized hCTR1 in untreated cellular extracts and those treated with Cu or DDP equally well (Figure 4-4). The retention of signal under all of these situations suggests that the loss of hCTR1 signal on the Western blots following DDP exposure is due to the disappearance of the protein and not an inability of the antibody to recognize hCTR1 following DDP or Cu exposure.
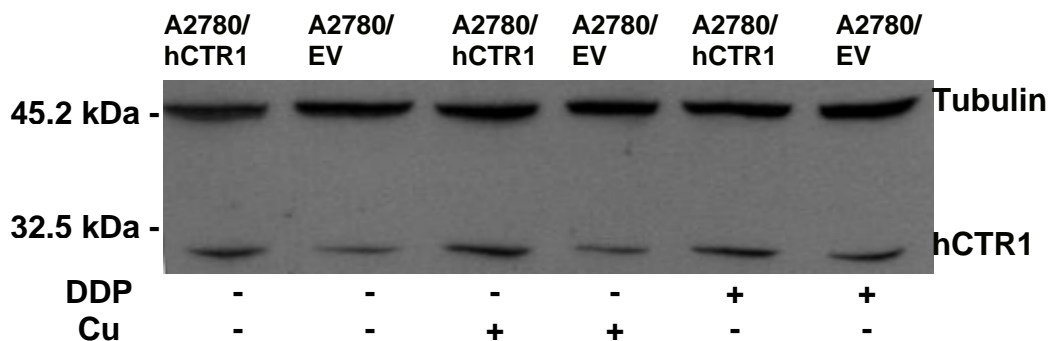
Figure 4-4. Western blot of cell lysates incubated with Cu or DDP. Cell lysates from A2780/hCTR1 and A2780/EV transfected ovarian carcinoma cells were incubated with either 100 μM Cu or 2 μM DDP for 5 min before being subjected to denaturing Western blot analysis.

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 cell 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 Figure 4-5, no difference in the amount of Na/K$^+$ATPase was observed following exposure to 0.5 to 2 μM DDP by Western blot analysis of either denatured or non-denatured lysates. Thus, the disappearance of hCTR1 following exposure to low concentrations of DDP is specific for hCTR1 in comparison to Na/K$^+$ATPase.
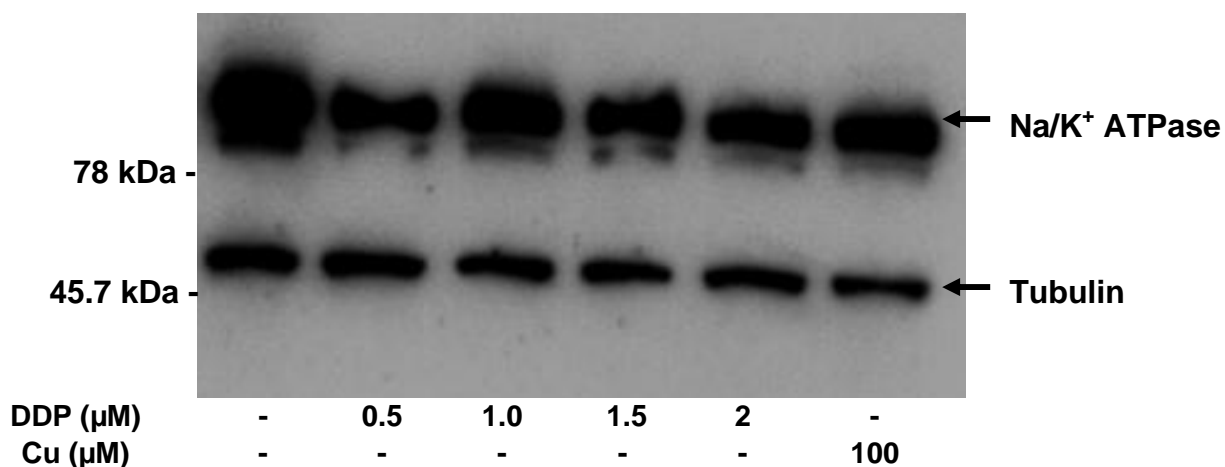
Figure 4-5. Denaturing Western blot analysis of Na/K⁺ATPase in lysates isolated from cells incubated with DDP or Cu (representative of 3 separate experiments). A2780 ovarian carcinoma cells were treated with 0.5, 1.0, 1.5 or 2.0 µM DDP or 100 µM Cu for 5 minutes.

Confocal image analysis of the effect of DDP on hCTR1.

The cellular location of hCTR1 varies among cell types when determined by immunohistochemical staining. While it is commonly found on the plasma membrane, in some cells it is also found in the perinuclear and Golgi regions of the cell (Eisses and Kaplan, 2002; Klomp et al., 2002; Lee et al., 2002a; Petris et al., 2003). One group has reported that hCTR1 relocalizes from the plasma membrane to intracellular vesicles upon exposure to Cu (Petris et al., 2003). 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 N-terminal region of hCTR1 and imaged by deconvolution confocal microscopy. As shown in Figure 4-3, 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 0.5µM DDP for 5 min resulted in a reduction in hCTR1 staining and exposure to 1.0 µM 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 µM DDP for varying times up to 5 min. This concentration of DDP was found to cause marked disappearance of hCTR1 staining by 1 min. As a control, A2870 cells were exposed to 100 µM Cu for up to 5 min. Cu also produced a decrease in the level of hCTR1 at the plasma membrane at 5 min, 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 over-express hCTR1 (Figure 4-6 and Figure 2-2). To further rule out the possibility of epitope masking or loss of the N terminal domain of hCTR1, 2008 ovarian carcinoma cells were exposed to 2 µM DDP for 5 min and were stained using an antibody generated to the C terminal end of hCTR1. The results of the confocal imaging studies using both an N terminal antibody and a C terminal antibody confirm the Western blot analysis and indicate that DDP produces a very rapid and major reduction in hCTR1 level in human ovarian carcinoma cells (See Figure 4-6 and 5-4).

A

B
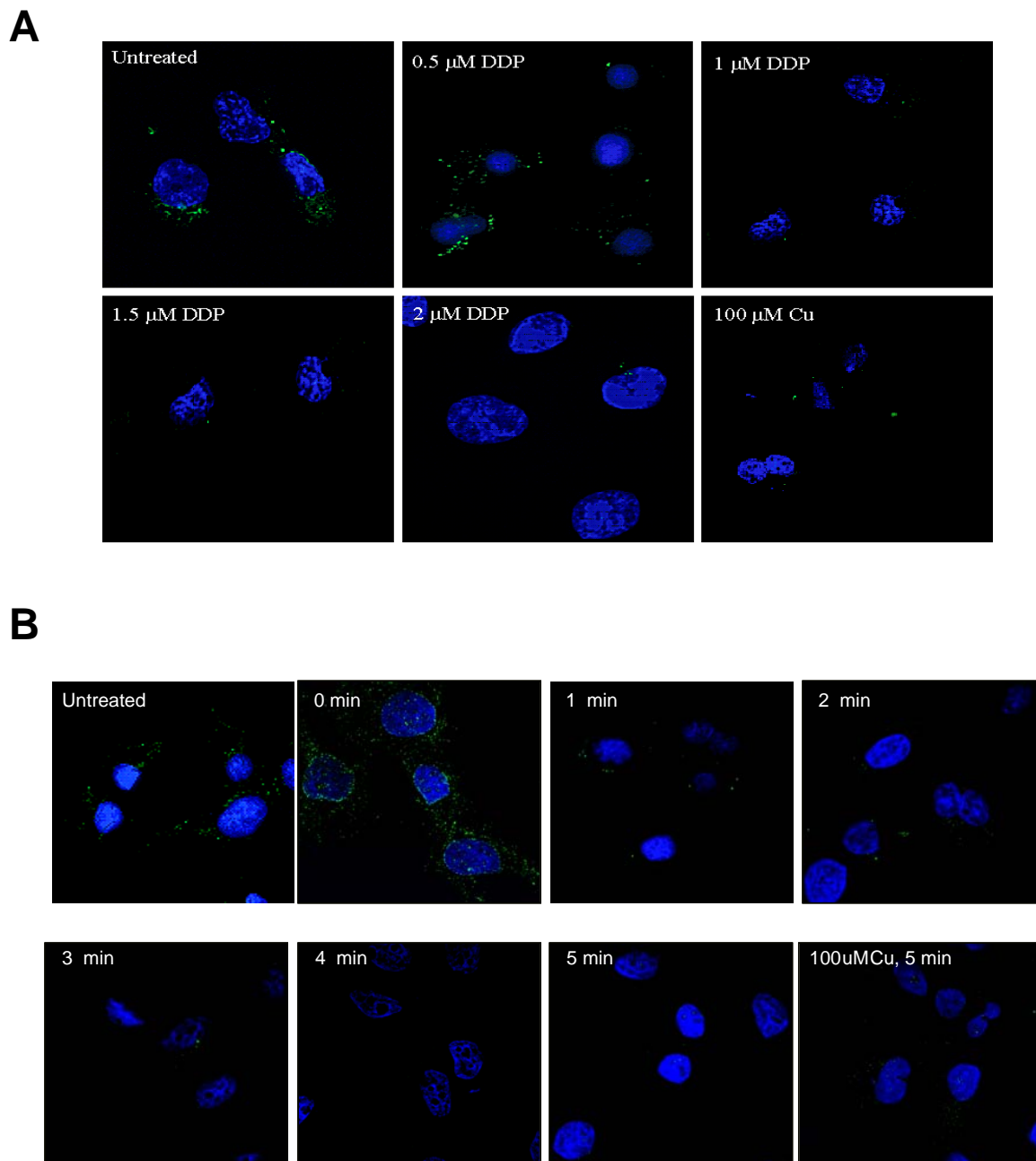
Figure 4-6. Confocal microscopic analysis of the effect of DDP on hCTR1in A2780 cells. Panel A, A2780 cells were exposed to 0, 0.5, 1.0, 1.5, or 2.0 µM DDP or 100 µM Cu for 5 min. Panel B, A2780 cells were exposed to 2 µM DDP for 0, 1, 2, 3, 4 or 5 min or 100 µM Cu for 5 minutes. hCTR1 was visualized using the rabbit anti-hCTR1 antibody and a goat anti-rabbit-FITC conjugated secondary. Hoescht 33342 dye was used to label the nucleus. Each image is representative of 3 images taken from each of 3 independent experiments. Images are normalized to the autofluorescence of unstained A2780 cells and cells stained with only the secondary antibody.

Effect of DDP exposure on Cu uptake and *vice versa*.

Since 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 ^{64}Cu would also be reduced. A2780 cells were exposed to 2 µM DDP for 5 min, washed, and then exposed to 2 µM ^{64}Cu for the subsequent 5 min. This concentration of 2 µM ^{64}Cu was chosen based on the reported K_m for Cu of 1.7 µM (Lee et al., 2002a). As shown in Figure 4-7A, the untreated cells accumulated 23 ± 2 pmole Cu/mg protein (mean $\pm$ SEM), whereas the DDP treated cells accumulated only 11.5 ± 0.6 pmole Cu/mg 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 done regulated little DDP was taken up, A2780 cells were exposed first to 100 µM Cu for 1 hr and then to 2 µM DDP for 30 min. Figure 4-7B shows that the untreated cells accumulated 4.179 ± 0.712 pmole Pt/mg protein (mean $\pm$ SEM), whereas the Cu treated cells accumulated only 2.491 ± 0.246 pmole Ptu/mg 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 %.
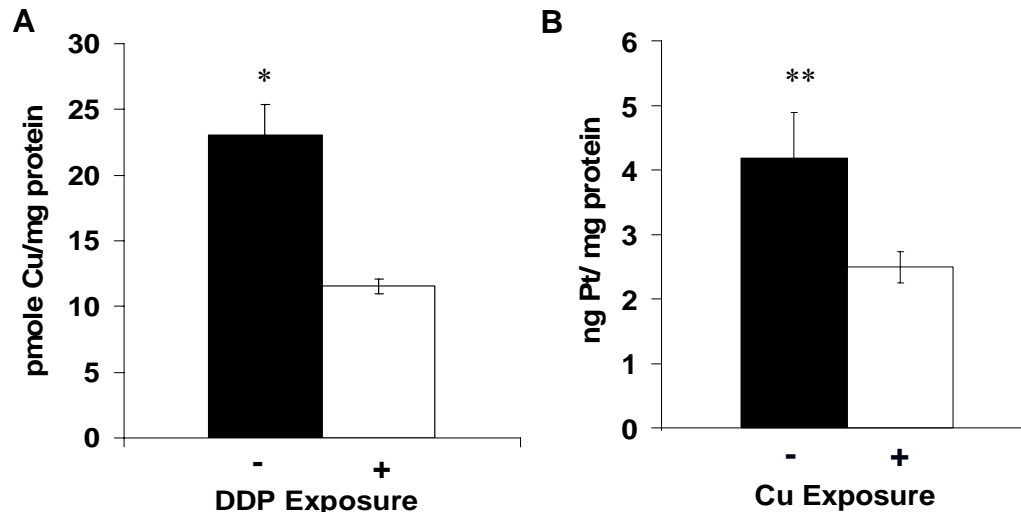
Figure 4-7. Effect of pretreatment with DDP on the cellular accumulation of ^{64}Cu and *vice versa*. Panel A, A2780 ovarian carcinoma cells were treated with 2 μM DDP for 5 min, washed and then exposed to with 2 μM ^{64}CuSO$_4$ for 5 min. Panel B, A2780 ovarian carcinoma cells were treated with 100 μM Cu for 1 hr, washed and then exposed to 2 μM DDP for 30 min. Each bar represents the mean of 4 independent experiments, each performed with sextuplet cultures. *p<0.0008, **p<0.05. Vertical bars, SEM.

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 appears to have different effects in different experimental systems.

Klomp *et al*. (Klomp et al., 2002) reported that 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.* (Petris et al., 2003) 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 immunoflourescent confocal microscopy and by Western blot analysis of both denatured and non-denatured 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 where it is in a native conformation and when it is denatured and lacks tertiary structure. Furthermore, when the purified N-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 N-terminus did not change in a manner that blocked or hid the antibody recognition site. Finally, the decrease in Cu uptake following treatment of cells with DDP provides evidence that the function of hCTR1 was in fact diminished by prior exposure to DDP.

Materials and Methods

Drugs and Reagents.

Refer to methods in Chapter 2. The polyclonal anti-rabbit antibody was generated by immunizing rabbits with a peptide containing amino acids from the C-terminus of hCTR1 by Novus Biologicals (Littleton, CO).

Cell Lines.

The A2780 and 2008 ovarian carcinoma cell lines (Disaia et al., 1972; Hamilton et al., 1985) were grown in RPMI 1640 media containing 10% fetal bovine serum at 37°C in 5% CO_2. 2780 cells were engineered to over-express hCTR1 by transfecting them with a pcDNA3.1 vector containing full length hCTR1 cDNA and expressing a geneticin resistance marker constructed as previously described (Moller et al., 2000) 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 (EV) 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 media alone for various times and at various concentrations. Following exposure cells were harvested by trypsinization and pelleted by centrifugation at 4º C. The pellets were frozen at -20º C for 1 hr and then thawed on ice and resuspended in 100

μL of homogenizing buffer (250 mM sucrose, 10 mM Tris-HCl pH 7.4, 1 μg/ml antipain, 1 μg/ml pepstatin, 1 μg/ml leupeptin and 20 μg/ml phenylmethanesulfonylflouride) and homogenized using a Dounce homogenizer for 1 min. The suspensions were centrifuged for 15 min at 500 g at 4º C, and the protein contents of the supernatant were measured. Supernatant samples containing 100 μg protein were boiled prior to electrophoresis in a 4-20 % SDS-PAGE gel. Transfer to nitrocellulose membranes (Bio-Rad Co.) was performed electrophoretically for 30 min at 200 volts using a Transblot SD apparatus (Bio-Rad Co). Membranes were blocked with 5 % milk in Tris buffered saline for 1 hr at room temperature. Blots were incubated at 4º C overnight with anti-hCTR1 antibodies diluted 1:1000 or with anti-Na/K$^+$ATPase diluted 1:1000 (Novus Biologicals, Littleton, CO) and mouse anti-tubulin (Sigma Products, St. Louis, MO) diluted 1:20,000 in 5 % milk in Tris buffer saline. Membranes were then washed 3 times with Tris buffered saline containing 0.05 % Tween-20 and incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies for 1 hr at room temperature. Prior to detection, membranes were again washed 3 times with Tris buffered saline containing 0.05 % Tween-20 and detection was performed using the enhanced chemiluminescence western detection system from Amersham-Pharmacia (Piscataway, NJ) according to manufacturer's instruction.

Fixation and staining.

Cells were grown in T75 flasks until 80% confluent and then harvested by trypsinization and pelleted by centrifugation in 10 % RPMI 1640 with 10 % fetal bovine serum. The resulting pellets were resuspended in 5ml of 10 % RPMI 1640 with

10 % fetal bovine serum and 100 µL of cells were added to each well of a 24 well plate. Prior to the addition of cells, a 0.16 mm thick coverslip and 300 µL of 10 % RPMI 1640 containing 10 % fetal bovine serum and 200 µM bathocuprione disulphonic acid were placed in each well. Bathocuprione disulphonic acid was added to chelate any Cu in the media ensuring that Cu could not affect DDP binding. Once cell growth on the coverslips reached 80 % confluency at approximately 48 hrs 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 4º C and washed 3 times with phosphate buffered saline and then fixed in 3.7 % formaldehyde in the same buffer at room temperature for 30 minutes. Subsequent staining and washing were performed as previously described (12).

Measurement of ^{64}Cu accumulation.

Refer to methods described in Chapter 2.

Chapter 5

Mechanism of hCTR1 internalization and degradation

Introduction

The previous chapters have established that hCTR1 influences DDP uptake by ovarian carcinoma cells, and that exposure to DDP triggers the rapid disappearance of hCTR1 from the cell. However, the mechanism of this loss is unknown. Recent reports also suggest that both endocytosis and protein degradation are also important to the amount of hCTR1 expressed in the plasma membrane of mammalian cells as well (Petris et al., 2003). In order to determine the mechanism of this loss, digital deconvolution microscopy of cells stained with antibodies directed at both ends of the protein and western blot analysis were utilized. Treatment of 2008 cells with DDP in combination with inhibitors of various endosomal pathways indicated that hCTR1 was internalized primarily by macropinocytosis. Expression of transdominant negative forms of dynamin I and Rac demonstrated that loss of hCTR1 was not dependent on pathways regulated by either of these proteins. DDP-induced loss of hCTR1 was blocked by the use of proteosome inhibitors. hCTR1 was rapidly lost from the cell when new protein synthesis was inhibited with cyclohexamide. hCTR1 levels recovered within ~30 min following removal of either DDP or cyclohexamide. This study demonstrates the hCTR1 is rapidly synthesized and degraded under steady-state conditions, and confirms that DDP triggers the rapid loss of hCTR1 from ovarian

carcinoma cells at clinically relevant concentrations. The results indicate that DDP-induced loss of hCTR1 involves internalization from the plasma membrane by macropinocytosis followed by proteasomal degradation. Since hCTR1 is a major determinant of early DDP uptake, prevention of its degradation offers a potential approach to enhancing tumor sensitivity.

Results

Effects of chemical inhibitors of endocytosis on hCTR1 loss.

The level of expression of hCTR1 in 2008 human ovarian carcinoma cells was monitored using confocal digital deconvolution microscopic examination of cells immunohistochemically stained with an antibody specific for an epitope in the N-terminal end of hCTR1. Cells were exposed to either Texas Red-labeled 70 kDa neutral dextran alone or in combination with 2 μM DDP for 15 min prior to staining. Neutral dextran of this molecular weight enters cells via a macropinocytotic process and the uptake of this molecule served as a control (Araki et al., 1996; Oliver et al., 1984)}. As shown in panel A of Figure 5-1, in 2008 cells hCTR1 was found in both the plasma membrane and in internal membranes of the cell. As demonstrated by comparing panels 5-1A and B, exposure of 2008 cells to DDP for 15 min caused complete disappearance of the green fluorescent hCTR1 signal but had no effect on the uptake of dextran as evidenced by persistence of the red signal. The DDP-induced loss of hCTR1 was not just due to re-localization from the plasma membrane to an interior cellular compartment as the hCTR1 signal disappeared from both sites. As we have previously reported (Holzer et al., 2004a), it was also not due to masking of the

epitope detected by the antibody. The same results were obtained when DDP-treated cells were stained with an antibody recognizing the C-terminal end of hCTR1 (Figure 5-2).

In order to determine the mechanism by which DDP triggered the loss of hCTR1, we used both chemical inhibitors and expression of transdominant negative variants of proteins known to inactivate endocytotic pathways. A number of unique endocytotic pathways are now known, each of which depends on somewhat different protein complexes to manage the process of vesicle formation and release from the plasma membrane (reviewed in (Conner and Schmid, 2003)). These pathways can be disabled by agents belonging to a variety of different chemical classes, although none of the drugs known to inhibit these pathways are entirely specific for any one endocytotic mechanism. Four agents widely used to study endocytotic pathways were selected for this study including amiloride, cytocholasin D, nystatin and methyl-β-cyclodextrin. Using propidium iodide uptake in 2008 cells as evidence of toxicity, a concentration-survival curve was determined for each endosomal pathway inhibitor and a concentration that produced inhibition of endocytosis in the absence of discernable cytotoxicity was identified (Figure 5-3). Among the drugs that inhibit endocytosis amiloride is one of the more specific agents in that it inhibits macropinocytosis without blocking other forms of pinocytosis or endocytosis (Riezman et al., 1997). As shown in panel C of Figure 5-4, exposure of 2008 cells to 5 mM amiloride for 15 min markedly reduced the uptake of neutral dextran indicating that macropinocytosis was effectively inhibited. As shown in panel D, amiloride also largely blocked the DDP-induced loss of hCTR1. Figure 5-4A provides confirmation

by Western blot analysis that a 15 min exposure to 2 μM DDP induced complete disappearance of hCTR1 from 2008 membranes and that this effect of DDP was almost entirely blocked by concurrent exposure to amiloride. This result was further confirmed using an antibody to the C-terminal end of hCTR1 which demonstrated the same effect of amiloride (Figure 5-2). This result identifies macropinocytosis was being an important step in the down-regulation of hCTR1 expression.

Additional experiments were performed with cytocholasin D, a drug that inhibits both macro- and micropinocytosis (Riezman et al., 1997). As shown in panel C of Figure 5-1, as was the case with amiloride, cytocholasin D effectively blocked the uptake of neutral dextran. Comparison of panels E and F demonstrates that it also partially blocked the DDP-induced loss of hCTR1 although the effect was of a smaller magnitude than that of amiloride. This was confirmed on Western blot analysis and through use of the antibody to the C-terminal rather than N-terminal end of hCTR1 (Figure 5-2 and 5-6). Thus, two drugs that target macropinocytosis, presumably by different mechanisms, both blocked DDP-induced loss of hCTR1 providing further evidence for the importance of this pathway in at least the initial steps in hCTR1 degradation.
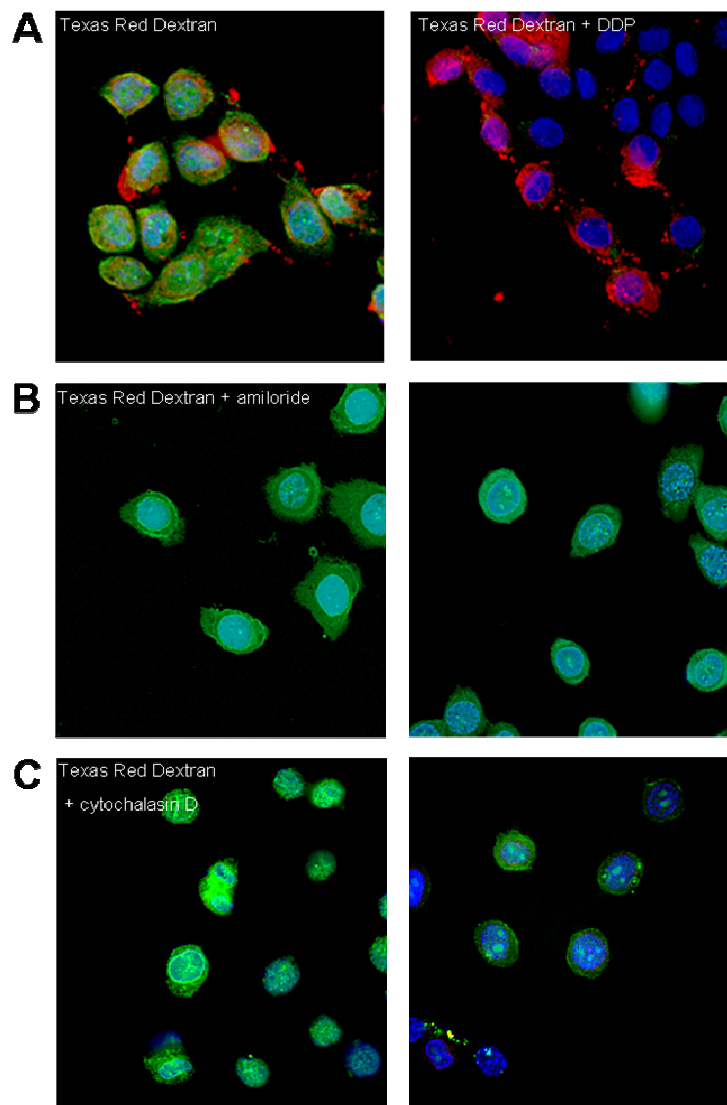
Figure 5-1. Confocal microscopic analysis of the effect of endocytotic pathway inhibitors amiloride and cytochalasin D on DDP-induced loss of hCTR1 from human ovarian carcinoma 2008 cells. In each case, following the indicated exposures, cells were stained for hCTR1 (green) using an antibody generated to the N terminus of hCTR1; Hoescht 33342 dye was used to label the nucleus (blue). Panel A, untreated control cells incubated with Texas Red-labeled dextran alone (red); panel B, cells incubated with Texas Red-labeled dextran and exposed to 2 µM DDP for 15 min; panel C, cells incubated with Texas Red-labeled dextran and 5 mM amiloride for 15 min; panel D, cells incubated with Texas Red-labeled dextran, 5 mM amiloride and then 2 µM DDP for 15 min; panel E, cells incubated with Texas Red-labeled dextran and 10 µM cytocholasin D for 30 min; panel F, cells incubated with Texas Red-labeled dextran, 10 µM cytocholasin D for 30 min and then exposed to 2 µM DDP for 15 min.

Figure 5-2. Confocal microscopic analysis of the effect of endocytotic pathway inhibitors amiloride and cytochalasin D on DDP-induced loss of hCTR1 from human ovarian carcinoma 2008 cells. In each case, following the indicated exposures, cells were stained for hCTR1 (green) using an antibody generated to the C terminus of hCTR1; Hoescht 33342 dye was used to label the nucleus (blue). Panel A, untreated control cells incubated with Texas Red-labeled dextran alone (red); panel B, cells incubated with Texas Red-labeled dextran and exposed to 2 μM DDP for 15 min; panel C, cells incubated with Texas Red-labeled dextran and 5 mM amiloride for 15 min; panel D, cells incubated with Texas Red-labeled dextran, 5 mM amiloride and then 2 μM DDP for 15 min; panel E, cells incubated with Texas Red-labeled dextran and 10 μM cytocholasin D for 30 min; panel F, cells incubated with Texas Red-labeled dextran, 10 μM cytocholasin D for 30 min and then exposed to 2 μM DDP for 15 min.

Nystatin binds to cholesterol in cell membranes and disrupts the formation and trafficking of caveolae. Cholera toxin enters cells largely via the calveolar pathway and is used as a marker of pathway flux (Riezman et al., 1997). As shown by comparing panels A and C of Figure 5-4, a 30 min exposure to 30 μg/ml nystatin successfully blocked caveolar-mediated endocytosis as demonstrated by the lack of Alexa 555-labeled cholera toxin B accumulation in the nystatin-treated 2008 cells. However, as shown by comparing panels C and D in Figure 5-4, blocking this pathway failed to prevent DDP-induced loss of hCTR1 from 2008 cells. As shown in Figure 5-6B, the failure of nystatin to prevent down-regulation of hCTR1 expression was confirmed by Western blot analysis; staining with antibody to the C-terminal end of hCTR1 produced the same result (Figure 5-5).

Methyl-β-cyclodextrin is another compound that binds to cholesterol in membranes and blocks both caveolin and clathrin-mediated endocytosis (Rodal et al., 1999; Subtil et al., 1999). Transferrin is a well-recognized substrate for the clathrin-mediated pathway (Riezman et al., 1997). As shown by a comparison of panels E and G of Figure 5-4, a 30 min exposure to 3.5 mM methyl-β-cyclodextrin markedly reduced the accumulation of Alexa 546-labeled. However, as shown in panel H, it failed to block DDP-induced loss of hCTR1. As shown in Figure 6B, this result was confirmed by Western blot analysis; it was further confirmed by showing that the same result was obtained with an antibody directed to a C-terminal hCTR1 epitope (Figure 5-5). Taken together, the results of the nystatin and methyl-β-cyclodextrin experiments indicate that clathrin and caveolin mediated endocytosis does not play a significant role in the DDP-induced disappearance of hCTR1.
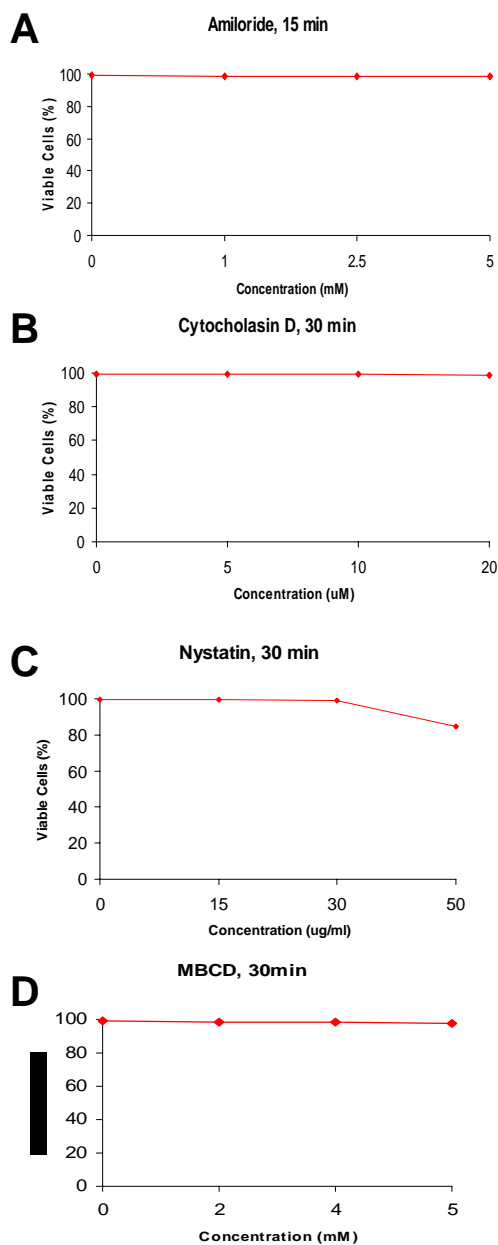
Figure 5-3. Cell viability following exposure to chemical inhibitors. 2008 ovarian carcinoma cells were exposed to varying concentrations of chemical inhibitors and cell viability following treatment was determined by propidium iodide staining. Panel A; 10 min exposure to 0, 1, 2.5, 5 mM amiloride, Panel B; 30 min exposure to 0, 5, 10, 20 uM cytocholasin D, Panel C; 30 min exposure to 0, 15, 30, 50 ug/ml nystatin, Panel D; 30 min exposure to 0, 2, 3.5, 5 mM methyl-B-cyclodextrin. Each experiment was perform in triplicate with n=3. Error bars represent SEM.
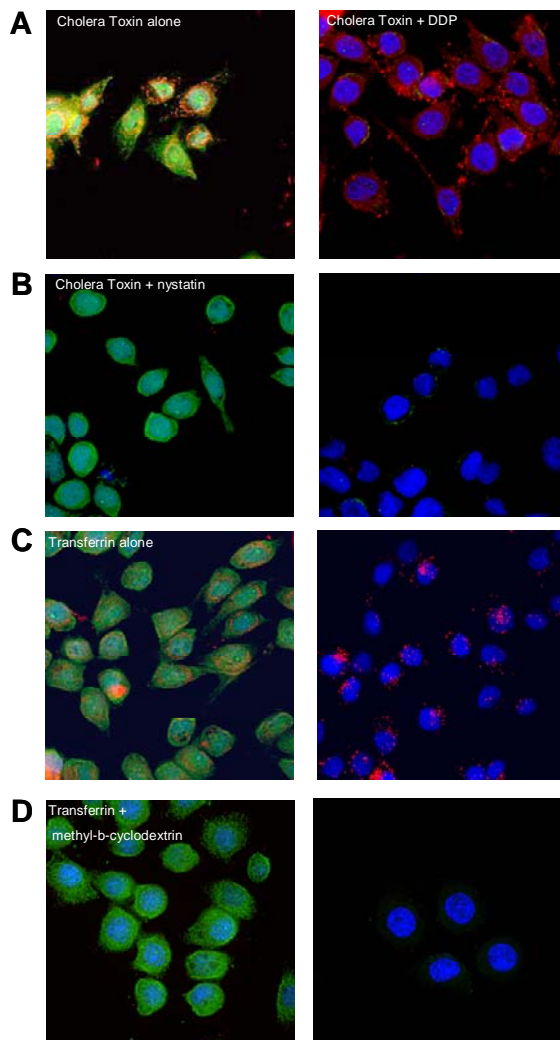
Figure 5-4. Confocal microscopic analysis of the effect of endocytotic pathway inhibitors nystatin and methyl-β-cyclodextrin on DDP-induced loss of hCTR1. In each case, following the indicated exposures, cells were stained for hCTR1 (green) using an antibody generated to the N terminus of hCTR1; Hoescht 33342 dye was used to label the nucleus (blue). Panel A, untreated control cells incubated with Alexa 555-labeled cholera toxin B alone (red); panel B, cells incubated with Alexa 555-labeled cholera toxin B and 2 μM DDP for 15 min; panel C, cells incubated with Alexa 555-labeled cholera toxin B and exposed to 30 μg/ml nystatin for 30 min; panel D, cells incubated Alexa 555-labeled cholera toxin B and 30 μg/ml nystatin for 30 min and then 2 μM DDP for 15 min; panel E, untreated control cells exposed to Alexa 546 labeled transferrin (red); panel F, cells exposed to Alexa 546 labeled transferrin and 2 μM DDP for 15 min; panel G, cells exposed to 3.5 mM methyl-β-cyclodextrin for 30 min followed by Alexa 546 labeled transferrin; panel H, cells exposed to 3.5 mM methyl-β-cyclodextrin for 30 min followed by Alexa 546 labeled transferrin and then 2 μM DDP for 15 min.

Figure 5-5. Confocal microscopic analysis of the effect of endocytotic pathway inhibitors nystatin and methyl-β-cyclodextrin on DDP-induced loss of hCTR1. In each case, following the indicated exposures, cells were stained for hCTR1 (green) using an antibody generated to the C terminus of hCTR1; Hoescht 33342 dye was used to label the nucleus (blue). Panel A, untreated control cells incubated with Alexa 555-labeled cholera toxin B alone (red); panel B, cells incubated with Alexa 555-labeled cholera toxin B and 2 μM DDP for 15 min; panel C, cells incubated with Alexa 555-labeled cholera toxin B and exposed to 30 μg/ml nystatin for 30 min; panel D, cells incubated Alexa 555-labeled cholera toxin B and 30 μg/ml nystatin for 30 min and then 2 μM DDP for 15 min; panel E, untreated control cells exposed to Alexa 546 labeled transferrin (red); panel F, cells exposed to Alexa 546 labeled transferrin and 2 μM DDP for 15 min; panel G, cells exposed to 3.5 mM methyl-β-cyclodextrin for 30 min followed by Alexa 546 labeled transferrin; panel H, cells exposed to 3.5 mM methyl-β-cyclodextrin for 30 min followed by Alexa 546 labeled transferrin and then 2 μM DDP for 15 min.

Figure 5-6. Western blot analysis of the effect of chemical inhibitors of endocytosis on hCTR1 loss following exposure to 2 μM DDP for 15 min. Ovarian carcinoma 2008 cells were treated as described in Figure 5-1. Panel A, denaturing Western blot showing the expression of tubulin and hCTR1 following exposure amiloride alone or in combination with DDP. Panel B, denaturing Western blot showing the expression of tubulin and hCTR1 following exposure to nystatin or methyl-β-cyclodextrin alone or in combination with DDP

Effects of dominant negative proteins on endocytosis of hCTR1.

Cells engineered to express dominant negative forms of dynamin I or Rac1 were used to further test the dependence of DDP-induced loss of hCTR1 on the various endocytotic pathways. Dynamin I is essential for the pinching off of vesicles from the plasma membrane in many forms of endocytosis including caveolin and clathrin-mediated endocytosis as well as some clathrin and caveolin-independent endocytotic processes and phagocytes (Conner and Schmid, 2003). The expression of a dominant negative form of dynamin I, K44A, disables all forms of endocytosis except for macropinocytosis. In this study we used HeLa cells transfected with a tetracycline-repressible vector expressing the K44A variant of dynamin I bearing an HA tag as previously reported (Damke et al., 1994). K44A dynamin I expression was induced by removing tetracycline for 48 h and then one aliquot of cells was exposed to 2 μM DDP for 15 min while the another was left untreated. In order to identify those cells in which the transdominant negative dynamin I was successfully induced, both aliquots were exposed to Alexa546-labeled transferrin prior to incubation with DDP; cells that express the K44A mutant do not accumulate transferrin. The cells were then fixed and stained for hCTR1. As shown in panels A and B of Figure 5-7, DDP induced the loss of hCTR1 in both cells that accumulated Alexa546-labeled transferrin and those that did not indicating that loss of hCTR1 was mediated by a dynamin I-dependent process. To further demonstrate specificity, those cells in which the K44A dynamin I was expressed were directly identified by staining with an antibody for the HA tag appended to the mutant protein. Comparison of panels C and D in Figure 5-7 show that DDP induced the loss of CTR1 in both HA-positive and HA-negative cells.

The loss of hCTR1 was verified by western blot and staining with an antibody toward the C terminus of hCTR1 (Figure 5-8 and 5-9). These results provide strong evidence that dynamin I-dependent mechanisms are not required for DDP-induced down-regulation of hCTR1.

Pinocytosis and other forms of endocytosis require the activity of the Rac Tapes, and these endocytotic pathways can be inhibited by expressing a dominant negative form of Rac (Burridge and Wennerberg, 2004). The 2008 ovarian carcinoma cells were transiently transfected with a vector expressing the N17rac1 dominant negative Rac bearing a my tag at the N-terminal end (Ridley et al., 1992). They were then exposed to Texas Red-labeled 70 kDa dextran to permit identification of those cells in which pinocytosis and endocytosis had been successfully disabled by expression of the dominant negative Rac1; one aliquot was also exposed to DDP while a control aliquot was not. Both sets of cells were then stained for hCTR1. A comparison of panels E and F in Figure 5-7 indicates that DDP-induced loss of hCTR1 in cells expressing the Rac1 N17 dominant negative vector and therefore not accumulating Texas Red labeled 70 kDa neutral dextran. Further specificity was afforded by staining the DDP-treated and untreated cells for both hCTR1 and expression of the my tag on the dominant negative Rac. Panels G and H demonstrate that DDP successfully down-regulated hCTR1 in both cells that expressed the mutant Rac and those that did not. The loss of hCTR1 staining with an antibody toward the C terminus of hCTR1 (Figure 5-8). Due to poor transfection efficiencies, no detectable difference in hCTR1 protein level was observed via Western blot (Figure 5-9). These

results support the conclusion that the loss of hCTR1 provoked by DDP is not

dependent on a mechanism that requires Rac.
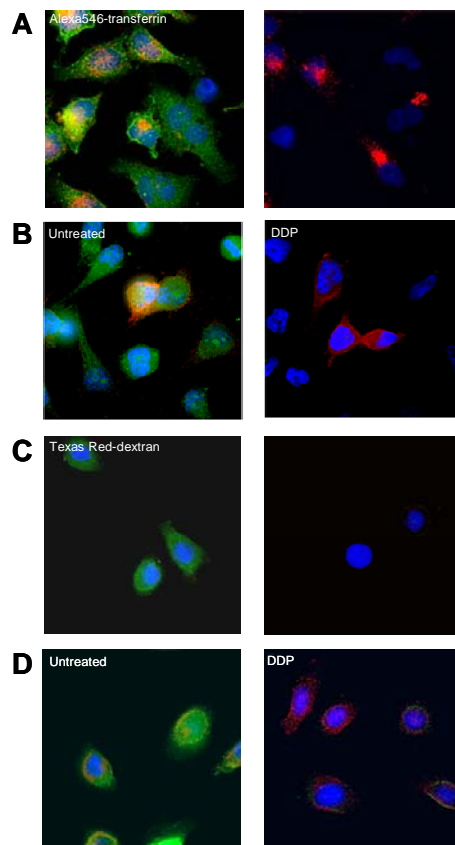
Figure 5-7. Confocal microscopic analysis of the effect of dominant negative dynamin I and Rac on DDP-induced loss of hCTR1 loss. Anti-HA or anti-my antibodies were used to identify the subset of cells in the population that expressed the K44A or Rac dominant negative mutant. In each case, following the indicated exposures, cells were stained for hCTR1 (green) using an antibody toward the N terminus; Hoescht 33342 dye was used to label the nucleus (blue). Panel A, K44A transfected control cells exposed to Alexa 546-labeled transferrin (red) to document inhibition of clathrin and caveolin-mediated endocytosis; panel B, cells exposed to Alexa 546-labeled transferrin for 30 min and then with 2 μM DDP for 15; panel C, cells stained for the expression of the HA-tagged K44A dominant negative mutant (red); panel D, cells exposed to 2 μM DDP for 15 min and stained for expression of the HA-tagged K44A dominant negative mutant; panel E, cells transiently transfected with Rac1 N17 dominant negative vector and incubated with Texas Red-labeled 70 kDa neutral dextran to document inhibition of pinocytosis; panel F, cells exposed to 2 μM DDP for 15 min following a transient transfection with Rac1 N17 dominant negative vector and incubation with Texas Red-labeled 70 kDa neutral dextran to document inhibition of pinocytosis; panel G, cells transiently transfected with Rac1 N17 dominant negative vector and stained for expression of the my tagged Rac1 N17 dominant negative (red); panel H, cells exposed to 2 μM DDP for 15 min following a transient transfection with Rac1 N17 dominant negative vector and stained for expression of the my taggedRac1 N17 dominant negative (red).
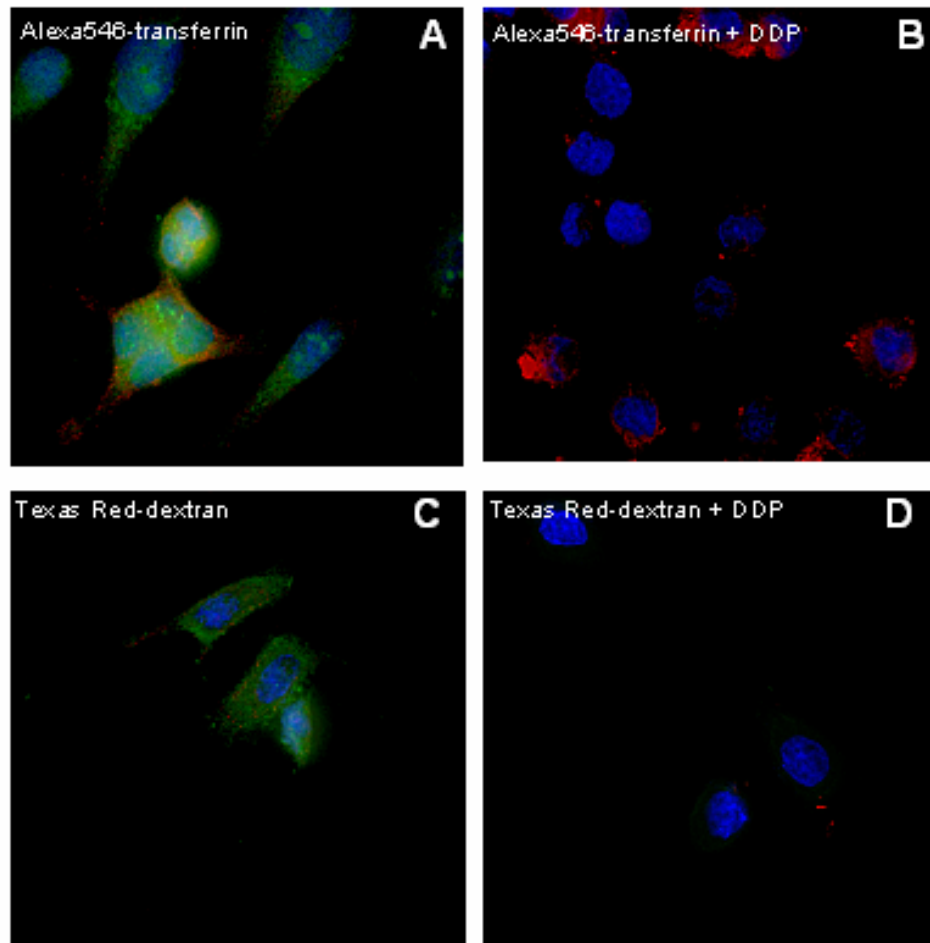
Figure 5-8. Confocal microscopic analysis of the effect of dominant negative dynamin I and Rac on DDP-induced loss of hCTR1 loss. In each case, following the indicated exposures, cells were stained for hCTR1 (green) using an antibody toward the C terminus; Hoescht 33342 dye was used to label the nucleus (blue). Panel A, K44A transfected control cells exposed to Alexa 546-labeled transferrin (red) to document inhibition of clathrin and caveolin-mediated endocytosis; panel B, cells exposed to Alexa 546-labeled transferrin for 30 min and then with 2 µM DDP for 15; panel C, cells transiently transfected with Rac1 N17 dominant negative vector and incubated with Texas Red-labeled 70 kDa neutral dextran to document inhibition of pinocytosis; panel D, cells exposed to 2 µM DDP for 15 min following a transient transfection with Rac1 N17 dominant negative vector and incubation with Texas Red-labeled 70 kDa neutral dextran to document inhibition of pinocytosis.
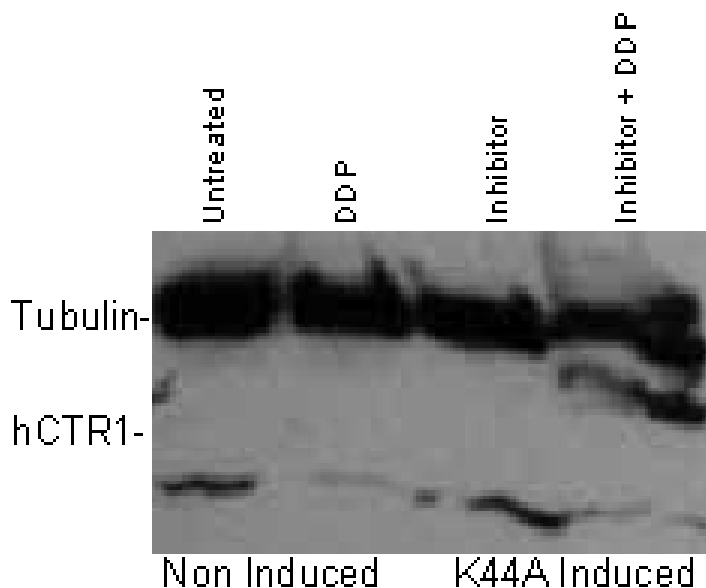
Figure 5-9. Western blot analysis of the effect of dominant negative K44A expression on endocytosis of hCTR1 loss following exposure to 2 μM DDP for 15 min. Denaturing Western blot of HeLa cells transfected with Dynamin I K44A dominant negative mutant showing the expression of tubulin and hCTR1. Cells had tetracycline removed to induce K44A expression for 48 hours prior to exposure to 2 μM DDP for 15 min.

Effect of proteasomal inhibition of hCTR1 loss.

The results presented above identify macropinocytosis as the most important endocytotic pathway involved in the down-regulation of hCTR1 induced by clinically attainable concentrations of DDP. Plasma membrane proteins internalized by this route are frequently degraded in lysosome (reviewed in (Peters and von Figura, 1994)). However, some proteins internalized via macropinocytosis and phagocytosis undergo proteasomal degradation (Johannes and Lamaze, 2002; Zhou et al., 2002). In order to determine the contribution of proteasomal degradation to the loss of hCTR1, 2008 cells were incubated with 2 μM DDP in the presence or absence of several

different proteosome inhibitors at concentrations shown not to produce significant cytotoxicity based on propidium iodide viability staining (Figure 5-10). The concentration of inhibitor selected was the lowest concentration that successfully blocked proteasomal degradation while maintaining cell viability.

As shown in panels C and D of Figure 5-11, inhibition of the trypsin and chymotrypsin-like activities of the 20S proteasome with lactacystin prevented the loss of hCTR1 following DDP exposure. Likewise, inhibition of only the chymotrypsin-like portion of the 20S proteosome with proteasome inhibitor 1 also blocked the loss of hCTR1. Inhibition of the 26S portion of the proteasome responsible for degrading ubiquitin-conjugated proteins with MG132 also blocked the loss of hCTR1 upon exposure to 2 μM DDP. To verify these results, and to ensure that the drugs were not just altering the epitope recognized by the N-terminal antibody, all immunoflourscent microscopic results were verified using an antibody that recognized an epitope on the C-terminal end of hCTR1 (Figure 5-12). The results were also verified by Western blot analysis as shown in Figure 5-13. Thus, although some component of hCTR1 degradation may occur in lysosomes, the majority of the rapid loss of hCTR1 from the cell appears to occur by proteosomal degradation.
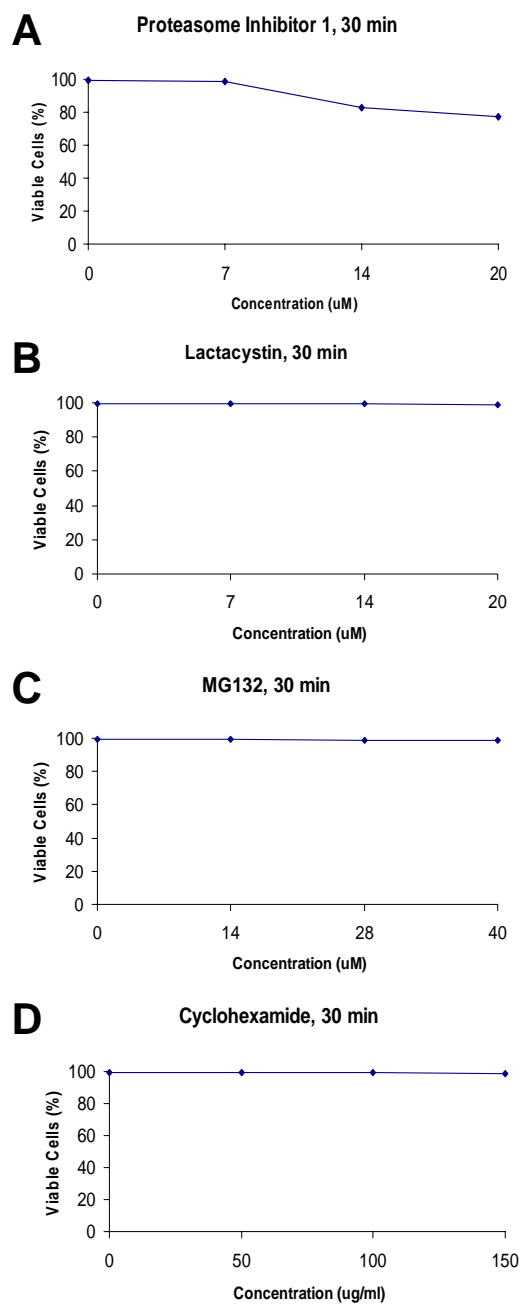
Figure 5-10. Cell viability following exposure to proteasomal inhibitors and a protein synthesis inhibitor. 2008 ovarian carcinoma cells were exposed to varying concentrations of chemical inhibitors and cell viability following treatment was determined by propidium iodide staining. Panel A; 30 min exposure to 0, 7, 14, 20 uM proteasome inhibitor 1, Panel B; 30 min exposure to 0, 7, 14, 20 uM lactacystin, Panel C; 30 min exposure to 0, 14, 28, 40 uM MG132, Panel D; 30 min exposure to 0, 50, 100, 150 μg/ml cyclohexamide. Each experiment was perform in triplicate with n=3. Error bars represent SEM.
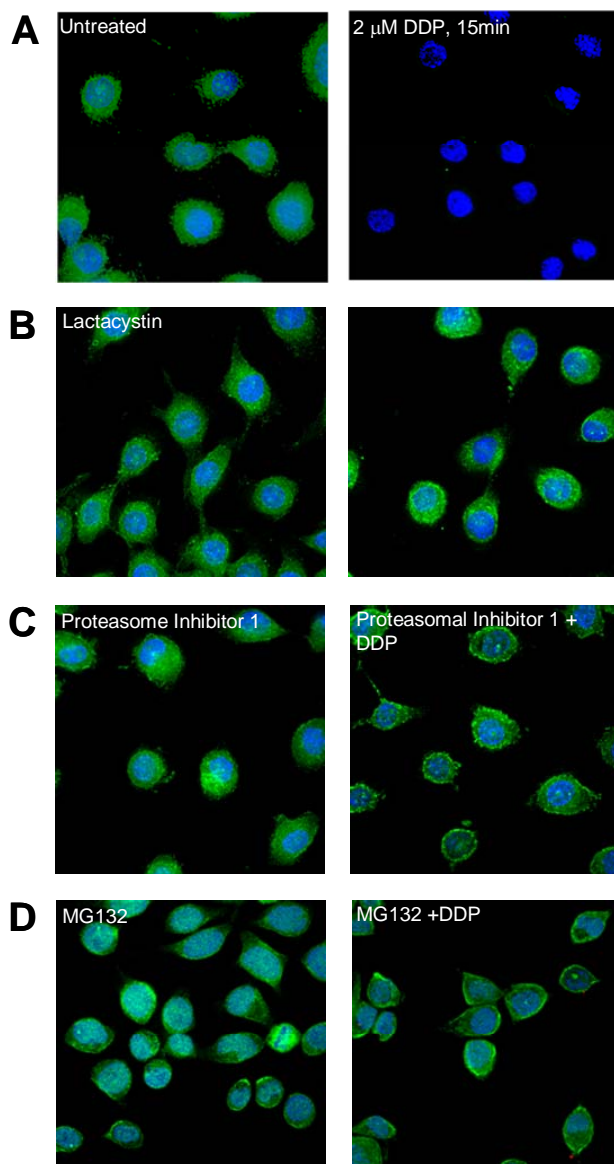
Figure 5-11. Confocal microscopic analysis of the effect of proteasomal inhibitors on DDP-induced down-regulation of hCTR1 in ovarian carcinoma 2008 cells. In each case, following the indicated exposures, cells were stained for hCTR1 using an antibody toward the N terminus (green); Hoescht 33342 dye was used to label the nucleus (blue). Panel A, untreated control cells; Panel B, control cells exposed to 2 µM DDP for 15 min; Panel C, cells pretreated with 20 µM lactacystin for 30 min; panel D, cells pretreated with 20 µM lactacystin for 30 min followed by exposure to 2 µM DDP for 15 min; panel E, cells pretreated with 7 µM proteasome inhibitor 1 for 30 min; panel F, cells pretreated with 7 µM proteasome inhibitor 1 for 30 min followed by exposure to 2 µM DDP for 15 min; panel G, cells pretreated with 40 µM MG132 for 30 min; panel H, cells pretreated with 40 µM MG132 for 30 min followed by exposure to 2 µM DDP for 15 min.

Figure 5-12. Confocal microscopic analysis of the effect of proteasomal inhibitors on DDP-induced down-regulation of hCTR1 in ovarian carcinoma 2008 cells. In each case, following the indicated exposures, cells were stained for hCTR1 using an antibody toward the C terminus (green); Hoescht 33342 dye was used to label the nucleus (blue). Panel A, untreated control cells; Panel B, control cells exposed to 2 μM DDP for 15 min; Panel C, cells pretreated with 20 μM lactacystin for 30 min; panel D, cells pretreated with 20 μM lactacystin for 30 min followed by exposure to 2 μM DDP for 15 min; panel E, cells pretreated with 7 μM proteasome inhibitor 1 for 30 min; panel F, cells pretreated with 7 μM proteasome inhibitor 1 for 30 min followed by exposure to 2 μM DDP for 15 min; panel G, cells pretreated with 40 μM MG132 for 30 min; panel H, cells pretreated with 40 μM MG132 for 30 min followed by exposure to 2 μM DDP for 15 min.

DDP   -   +   -   +   -   +   -   +

Tubulin-

hCTR1-

Untreated   Lactacystin   Proteasome   MG 132
Inhibitor I

Figure 5-13. Denaturing western blot analysis of the effect of proteasomal inhibitors on DDP-induced hCTR1 loss. Human ovarian carcinoma 2008 cells were treated as described in Figure 4. The blot shows the expression of tubulin and hCTR1 following exposure to lactacystin, proteasome inhibitor I, or MG132 alone or in combination with DDP.

Recovery of hCTR1 following DDP and cyclohexamide exposure.

In order to investigate the kinetics of the recovery of hCTR1 after DDP exposure, the time course of the reappearance of hCTR1 was monitored in 2008 cells following a 15 min exposure to 2 μM DDP. Both the absolute level and the subcellular localization were monitored by immunoflourescent microscopy (Figure 5-14A) and Western blotting (Figure 5-14B). hCTR1 protein levels returned to normal approximately 30 min after the removal of DDP indicating the synthesis of new hCTR1 protein is rapid. This conclusion was verified by blocking new hCTR1 synthesis with 100 μg/ml cyclohexamide. As shown in Figures 5-15A and 5-15B, a 30 min exposure to cyclohexamide reduced hCTR1 to undetectable levels, but normal levels were again achieved approximately 30 min after removal of the inhibitor. The loss of hCTR1 was verified by western blot and staining with an antibody toward the

C terminus of hCTR1 (Figure 5-14B,5-15 B and 5-16). These results indicate that hCTR1 is rapidly re-synthesized suggesting rapid turnover under steady-state conditions of synthesis and degradation.
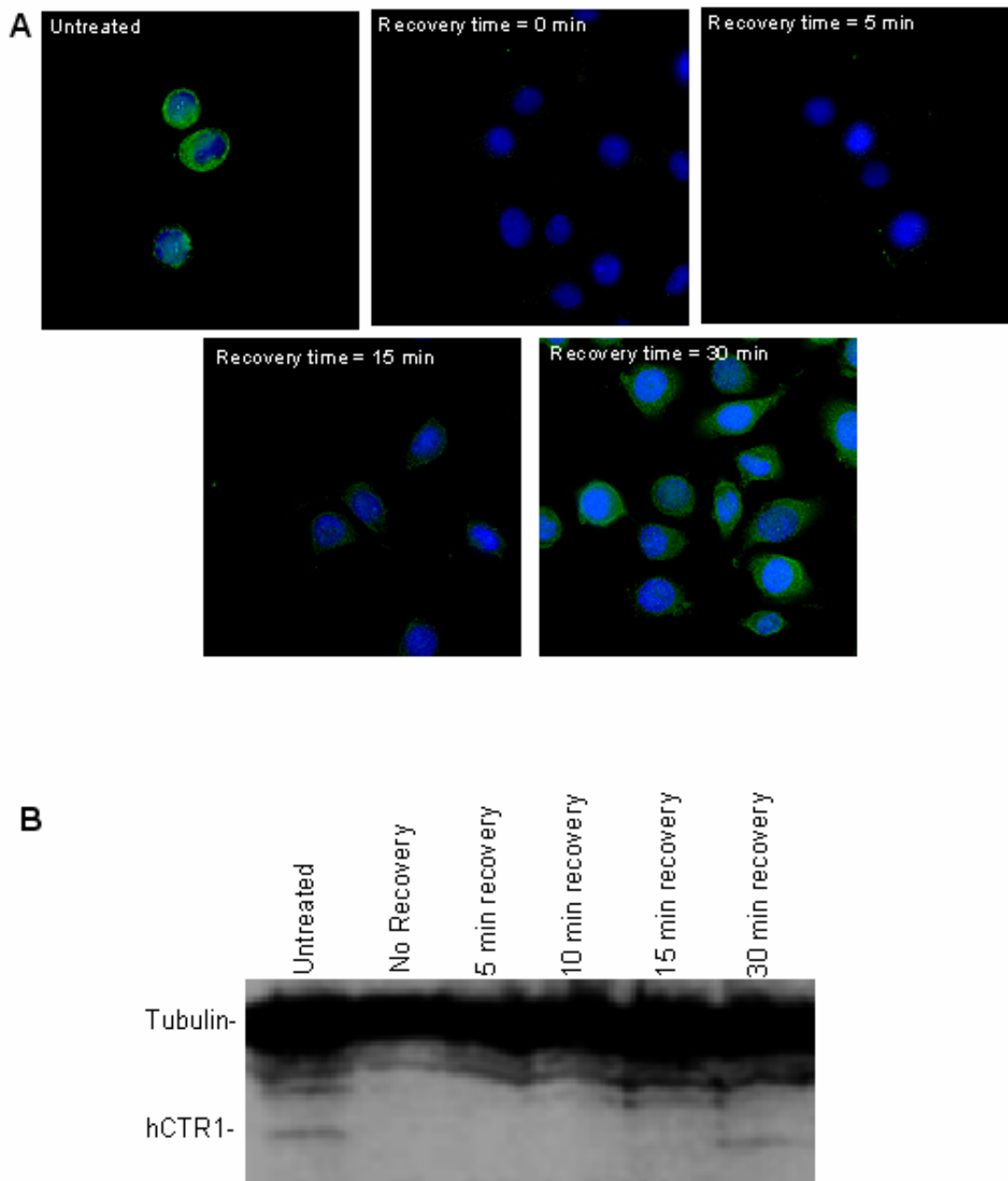
Figure 5-14. Confocal microscopic analysis of the recovery of hCTR1 following DDP exposure. Panel A, 2008 ovarian carcinoma cells were exposed to 2 μM DDP for 15 min. Following removal of the DDP cells were allowed to recover for 0, 5, 15 or 30 min prior to fixation. Cells were then stained for hCTR1 (green) using an antibody to the N terminus and with Hoescht 33342 to label the nucleus (blue). Panel B, 2008 ovarian carcinoma cells were exposed to 2 μM DDP for 15 min following which they were allowed to recover for 0, 5, 10, 15 or 30 min prior harvesting. A denaturing Western blot was probed for the presence of tubulin and hCTR1.
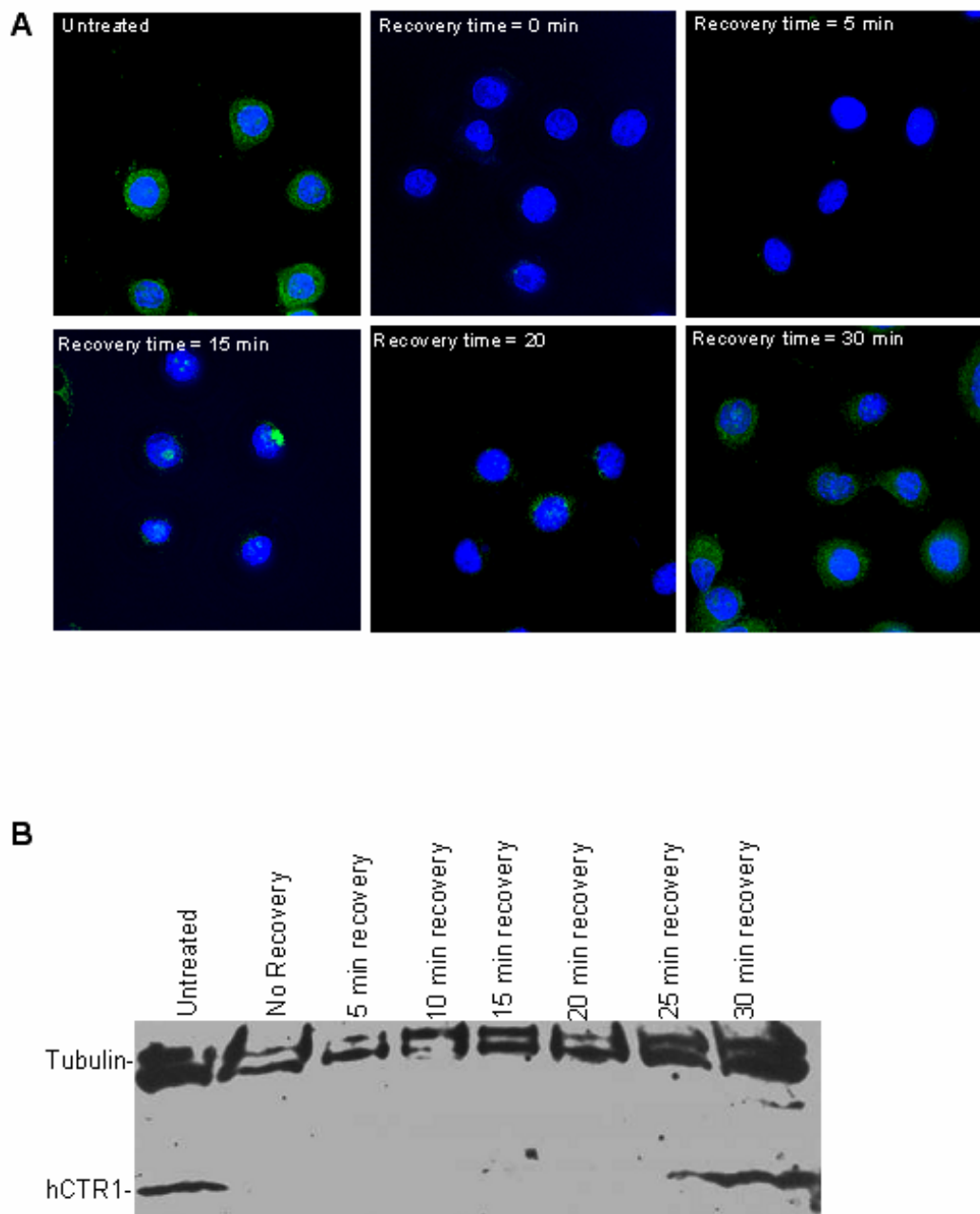
Figure 5-15. Confocal microscopic analysis of the recovery of hCTR1 following cyclohexamide exposure. Panel A, 2008 ovarian carcinoma cells were exposed to 100 μg/ml cyclohexamide for 30 min and then allowed to recover for 0, 5, 15, 20 or 30 min prior to fixation. Cells were then stained for hCTR1 (green) using an antibody to the N terminus and with Hoescht 33342 to label the nucleus (blue). Panel B, 2008 ovarian carcinoma cells were exposed to 100 μg/ml cyclohexamide for 30 min following which there were allowed to recover for 0, 5, 10, 15, 20, 25 or 30 min prior harvesting. A denaturing Western blot was probed for the presence of tubulin and hCTR1.
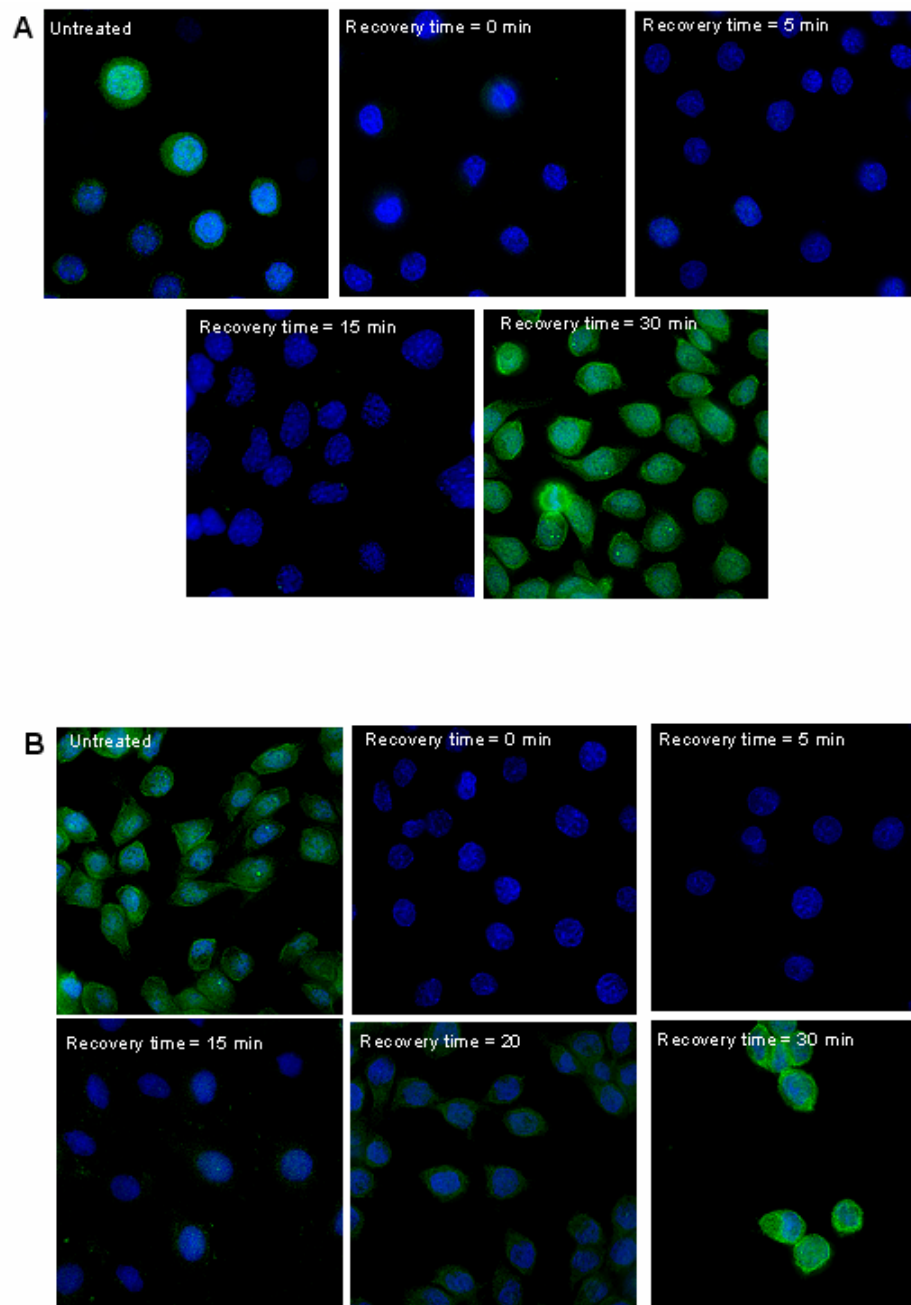
Figure 5-16. Confocal microscopic analysis of the recovery of hCTR1 following DDP and cyclohexamide exposure. Panel A, 2008 ovarian carcinoma cells were exposed to 2 μM DDP for 15 min and then allowed to recover for 0, 5, 15 or 30 min prior to fixation. Cells were then stained for hCTR1 (green) using an antibody to the C terminus and with Hoescht 33342 to label the nucleus (blue). Panel B, 2008 ovarian carcinoma cells were exposed to 100 μg/ml cyclohexamide for 30 min and then allowed to recover for 0, 5, 15, 20 or 30 min prior to fixation. Cells were then stained for hCTR1 (green) using an antibody to the C terminus and with Hoescht 33342 to label the nucleus (blue).

Discussion

hCTR1 is important not only because it is the major Cu influx transporter but because it also mediates a significant component of the cellular accumulation of DDP. As one step toward identifying how CTR1 transports DDP, we sought to determine the mechanism by which hCTR1 is degraded following brief exposure to DDP using several different strategies for inhibiting endocytotic pathways and proteosomal degradation. The results support the conclusion that the initial steps of DDP-induced loss of hCTR1 from human ovarian carcinoma cells is mediated by a process that is inhibited by amiloride and cytochalasin D but not by nystatin or methyl-β-cyclodextrin and is not dependent on either dynamin I or the Rac1 GTPase. Among the various endocytotic pathways currently defined, this pattern of inhibition most closely matches the process of macropinocytosis.

As currently understood, macropinocytosis occurs through the formation of large primary endocytotic vesicles in regions of membrane ruffling. While the specific mechanisms involved and controls on the process are not clearly defined, it is thought to be regulated by the small GTPase ARF6 and may be dependent on phospholipase Cγ activity. There is evidence that phosphatidylinositol (4,5)-bisphosphate is a regulator of the rate of macropinocytosis (Nichols and Lippincott-Schwartz, 2001). However, whether macropinocytosis is triggered by interaction with molecules external to the cell or is a byproduct of membrane turnover and cytoskeletal activity is currently a matter of debate (Nichols and Lippincott-Schwartz, 2001). Unlike endosomes generated by clathrin and caveolar-mediated endocytosis, macropinosomes

do not fuse into lysosomes (Wadia et al., 2004) suggesting that their cargo is not degraded by lysosomal enzymes (Johannes and Lamaze, 2002). The observation that proteosome inhibitors blocked DDP-induced loss of hCTR1 is consistent with this concept. Several different proteasome inhibitors markedly reduced loss of hCTR1 indicating that proteasomal degradation accounts for the vast majority of hCTR1 breakdown and disappearance.

The specificity controls included in these studies provide strong evidence that the observed disappearance of hCTR1 from the cell following DDP and Cu exposure was not an artifact. Two separate antibodies directed at opposite ends of the protein detected the same time course of the loss and recovery of hCTR1 in immunoflourescent studies. Yet another antibody, capable of detecting hCTR1 on western blots, also demonstrated a similar temporal pattern. Thus, it is unlikely that loss of hCTR1 was due to epitope masking. In addition, DDP did not appear to cause a general disturbance in the function of endocytotic pathways as there was no disturbance of the cellular accumulation of transferrin, cholera toxin B or dextran in the DDP-treated cells.

The studies reported here suggest that hCTR1 is a rapidly cycling protein whose steady-state levels are controlled at the protein level. They are consistent with the concept that, in the absence of excessively high Cu levels or DDP exposure, hCTR1 resides largely in membranes where it is protected from proteosomal degradation. The rapid loss of hCTR1 when new protein synthesis is inhibited suggests that basal rates of macropinocytosis internalize hCTR1 at a relatively high rate and make it accessible to degradation by the proteasome. Both brief exposure to

DDP and inhibition of new protein synthesis with cyclohexaminde rapidly reduced the level of hCTR1 to below the limit of detection. The recovery of hCTR1 levels over the ensuing ~30 min following either exposure to DDP or cyclohexaminde indicates that hCTR1 is rapidly re-synthesized and trafficked to its normal cellular membrane locations. The available data supports the idea that both Cu and DDP markedly increase the rate of hCTR1 internalization by macropinocytosis thus enhancing proteosomal degradation, although on a molar basis DDP is far more effective than Cu at triggering this effect.

Materials and Methods

Drugs and Reagents.

Platinol-AQ was a gift from Bristol-Myers Squibb (Princeton, NJ). The clinical formulation containing 3.33 mM DDP was kept in the dark at room temperature. A 100 μM stock was created by diluting the drug in 0.9 % NaCl. Protein concentration was measured using Bradford's Reagent from Bio-Rad Inc. (Hercules, CA). The generation of the rabbit polyclonal antibody against amino acids 1 through 67 of hCTR1 used for immunofluorescence is described elsewhere (Klomp et al., 2002) The polyclonal anti-rabbit antibody was generated by immunizing rabbits with a peptide containing amino acids from the C-terminus of hCTR1 by Novus Biologicals (Littleton, CO). Fluorescein isothiocyanate-conjugated goat anti-rabbit antibody was obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Hoechst 33342 dye for nuclear staining was purchased from Molecular Probes (Eugene, OR). Transferrin-Alexa 546 and 70 kDa dextran-Texas Red were purchased from Molecular

Probes (Eugene, OR). The polyclonal rabbit anti-hCTR1 antibody used for Western blotting was generated by immunizing rabbits with a peptide containing amino acids 2-22 of the N-terminus of hCTR1 by Biocarta Inc. (San Diego, CA). Horseradish peroxidase-conjugated goat anti-rabbit secondary was purchased from Amersham Pharmacia (Piscataway, NJ). All other chemicals and reagents were obtained from Fisher Scientific (Tustin, CA).

Cell Lines and vectors.

The 2008 ovarian carcinoma cells (Disaia et al., 1972; Hamilton et al., 1985) were grown in RPMI 1640 media containing 10% fetal bovine serum at 37°C in 5% CO_2. The HeLa cells engineered to express an inducible K44A dominant negative mutant were used as described elsewhere (Damke et al., 1994). Ovarian carcinoma 2008 cells were transiently transfected with the Rac dominant negative N17rac1 vector using LipofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturers instructions.

Western Blot Analysis.

Refer to methods in Chapter 4.

Deconvolution Digital Immunofluorescent Microscopy.

Cells were grown in a T75 flask until 80% confluent and then harvested with trypsin and pelleted by centrifugation in RPMI 1640 with 10% fetal bovine serum. The resulting pellet was resuspended in 5 ml of RPMI 1640 containing 10% fetal bovine serum and 100 μL of cells were added to each well of a 24 well plate. Prior to the addition of cells, a 0.16 mm thick coverslip and 300 μL of RPMI 1640 containing 10% fetal bovine serum were placed in each well. Once cells became 80% confluent

on the coverslip, at approximately 48 hrs after plating, the cells were exposed to RPMI 1640 with 10% fetal bovine serum containing either a chemical inhibitor or media alone for 30 min (excluding amiloride which was added for 15 min) followed by the addition of 2 μM DDP and either transferrin-Alexa546 (25 μg/ml) or Texas Red-labeled neutral dextran 70 (100 μg/ml) for 15 minutes. After the specified duration of exposure, the coverslips were placed at 4ºC and washed 3 times with phosphate buffered saline and then fixed in 3.7 % formaldehyde in the same buffer at room temperature for 30 minutes. The images in each panel of the figures in this paper are representative of 3 images taken from each of 3 independent experiments. Images are normalized to the autofluorescence of unstained 2008 cells and cells stained with only the secondary antibody.

Propidium Iodide Staining.

2008 ovarian carcinoma cells were grown to 80% confluency in 30 cm plates. Cells were then exposed to a range of inhibitors at varying concentrations for 30 min. The inhibitors and concentrations used are as follows; Nystatin (0, 15, 30, 50 μg/ml), methyl-B-cyclodextrin (0, 2, 3.5, 5 mM), Cytocholasin D (0, 5, 10, 20 uM), Cyclohexamide (0, 50, 100, 150 μg/ml), Lactacystin (0, 7, 14, 20 uM), Proteasome Inhibitor 1 (0, 7, 14, 20 uM) and MG132 (0, 14, 28, 40 uM). Cells were exposed to Amiloride (0, 1, 2.5, 5 mM) for 15 min. Following exposure cells were rinsed with PBS, trypsinized, and pelleted. The resulting pellet was rinsed with PBS and repelleted. The pellet was resuspended in 500 uL of 0.1% bovine serum albumin in PBS followed by the addition of 5 uL of Propidium Iodide (10 μg/ml). Membrane viability as determined by propidium iodide uptake was measured by FACS scanning.

Repeated freezing-thawing and hydrogen peroxide treatment were used as positive controls. Each inhibitor concentration was performed in triplicate. The concentration used for each inhibitor was based on the highest inhibitor concentration that maintained membrane viability.

Acknowledgements

Chapter 6

hCTR1 in DDP sensitive and resistant ovarian carcinoma cell lines and ovarian tumor samples

Introduction

To date, the expression and function of hCTR1 with respect to Cu homeostasis has been studied primarily in non-cancerous cell lines. As previously mentioned hCTR1 plays a role in the transport of clinically relevant chemotherapeutic agents making an examination of hCTR1 expression in fully malignant cells important. While the localization of hCTR1 in has been documented in some cell lines, little is known about the protein expression level and function of hCTR1 in normal and malignant human tissues. Further, while a handful of studies have examined the role of plasma membrane copper transport proteins in the uptake of platinum-containing drugs, little is known about the role of CTR1 in the development of resistance to these agents.

As an initial step towards understanding the potential clinical significance of hCTR1 expression, studies were undertaken of its expression in isogenic pairs of DDP-sensitive and resistant ovarian carcinoma cell lines, and in tumor samples obtained from patients treated with platinum-based chemotherapy regiments.

Results

DDP-sensitive and resistant ovarian carcinoma cell lines.

Six well-established human ovarian carcinoma cell lines were repeatedly exposed to increasing concentrations of DDP to develop sublines that had acquired varying degrees of resistance to DDP. Thereafter they were maintained in the absence of additional drug exposure. Colony formation assays were used to determine the sensitivity of the 6 pairs of DDP-sensitive and resistant cell lines to the cytotoxic effect of DDP. The results, presented in Table 6-1, indicate that the difference in DDP sensitivity, as measured by the ratio of IC_{50} values, ranged from 1.6-fold for the 2008 – 2008/C13*5.25 pair to 11.9-fold for the A2780-A2780/CP70 pair. Impaired accumulation of DDP in the 2008/C13*5.25, A2780/CP and IGROV-1/CP sublines has previously be documented (Katano et al., 2002).

Table 6-1. DDP IC_{50} values as determined by colony formation assay.

Parental cells	IC_{50}, μM*	Resistant cells	IC_{50}, μM*	Fold-resistant
2008	0.70	2008/C13*5.25	1.10	1.6
A2780	0.21	A2780/CP70	2.50	11.9
HEY	0.76	HEY C2	4.60	6.1
IGROV-1	0.30	IGROV-1/CP	1.08	3.6
KF28	0.18	KFr13	0.40	2.2
UCI 107	1.00	UCI CPR	8.00	8

*Continuous drug exposure

hCTR1 expression in DDP-sensitive and resistant cell lines.

In an effort to define the role hCTR1 as a determinant of sensitivity to the cytotoxic effect of DDP, the expression of hCTR1 was assessed in the 6 isogenic pairs of ovarian carcinoma cell lines. All 4 coding exons of hCTR1 were sequenced and no mutations were found in any of the 12 cell lines examined. As shown in Figure 6-1, on Western blot analysis hCTR1 expression was detectable in only 2 of the 6 parental ovarian carcinoma cell lines. Based on the fact that prior studies had shown that 3 of the DDP-resistant sublines exhibit reduced uptake of DDP and Cu (Katano et al., 2002), one might have expected to find a reduction of hCTR1 expression in the resistant sublines. However, this was observed in only 1 of the 2 cases where the parental line expressed hCTR1 (UCI107/CPR); in the other case there was no change in hCTR1 level. In addition, while the parental HEY line did not express a detectable amount of hCTR1, its expression in the DDP-resistant HEY/C2 subline was just enough to be detectable. Thus, in this set of 6 pairs of DDP-sensitive and resistant cell lines it was not possible to reliably determine the frequency with which hCTR1 expression was reduced in resistant cells, but there was little suggestion of a consistent change in hCTR1 expression in the resistant cells.
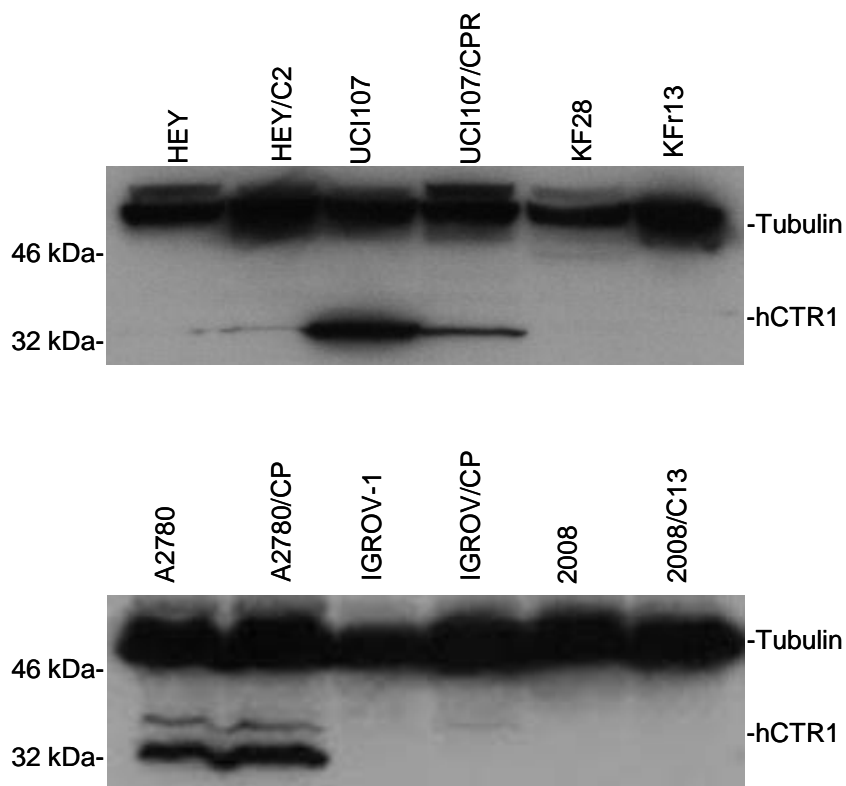
Figure 6-1. Western blot analysis of hCTR1 expression in DDP-sensitive and resistant ovarianc carcinoma cell lines. Blots were co-stained with polyclonal anti-hCTR1 and an antibody to tubulin to provide a loading control.

Functionality of hCTR1 in DDP-sensitive and resistant cell lines.

The initial rate of uptake of ^{64}Cu was used to determine the relative functionality of hCTR1 in each member of the paired DDP-sensitive and resistant ovarian cell lines. Initial uptake was defined as the uptake rate between 0 and 15 minutes when cells the cells were exposed to 2 μM Cu. As shown in Table 6-2, the whole cell accumulation of ^{64}Cu following 15 min of ^{64}Cu exposure varied widely from 4.1 to 57.7 pmol Cu/ mg protein among the 6 parental cell lines. In one pair (UCI107 vs UCI107/CPR) there was no difference in ^{64}Cu uptake between the DDP-

sensitive and DDP-resistant cells. In 3 pairs (2008 vs 2008/C13, HEY vs HEY/C2, KF28 vs KFr13), ^{64}Cu uptake was statistically significantly higher in the DDP-resistant subline, and in 2 pairs (IGROV-1 vs IGROV-1/CP and A2780 vs A2780/CP) it was significantly lower. It is interesting to note that the parental cell line with the highest expression of hCTR1 on Western blot analysis was the one with the highest initial ^{64}Cu uptake rate. However, there was otherwise no clear association of initial ^{64}Cu uptake and hCTR1 expression. No discernable association was detected between ^{64}Cu initial uptake rate and sensitivity to DDP, as the resistant lines did not consistently accumulate more or less DDP as shown in Table 6-2.

Table 6-2. Comparison of copper uptake in isogenic pairs of DDP sensitive and resistant ovarian carcinoma cell lines.

DDP Sensitive Lines		DDP Resistant Sublines		
Cell Line	pmol Cu/ mg protein	Cell Line	pmol Cu/ mg protein	Ratio (Resistant/sensitive)
2008	10.3 ± 1.98	2008/C13	$20.0 \pm 2.5^*$	1.9
A2780	57.7 ± 4.6	A2780/CP	$13.5 \pm 3.6^*$	0.2
HEY	5.7 ± 0.4	HEY/C2	$14.0 \pm 1.6^*$	2.5
KF28	4.1 ± 1.28	KFr13	$9.3 \pm 1.1^*$	2.3
IGROV-1	12.9 ± 0.2	IGROV-1/CP	$8.6 \pm 0.5^*$	0.6
UCI107	6.0 ± 0.8	UCI107/CPR	7.6 ± 0.5	1.3

* $p < 0.05$

Cellular localization of hCTR1 in DDP-sensitive and resistant cells.

Another factor that could influence the accumulation of both Cu and DDP in the DDP-sensitive and resistant cell lines is the subcellular localization of hCTR1. The cellular localization of hCTR1 is known to vary among cell lines (Eisses and Kaplan,

2002; Klomp et al., 2002; Lee et al., 2002a; Petris et al., 2003). The level of hCTR1 present at the plasma membrane could affect the initial uptake of ^{64}Cu or DDP thus explaining the accumulation differences among isogenic cell lines. Using digital confocal immunoflourescent microscopy hCTR1 localization was examined in all 6 of the isogenic pairs. As shown in Figure 6-2, the cellular localization of hCTR1 did not clearly differ between the parental and DDP-resistant sublines of any of the cell pairs. Thus, the acquired DDP-resistant phenotype was not associated with a major change in the subcellular distribution of CTR1. There was no detectable difference in the distribution of hCTR1 between the different parental DDP-sensitive cell lines suggesting that hCTR1 localization does not vary substantially among ovarian carcinoma cell lines.
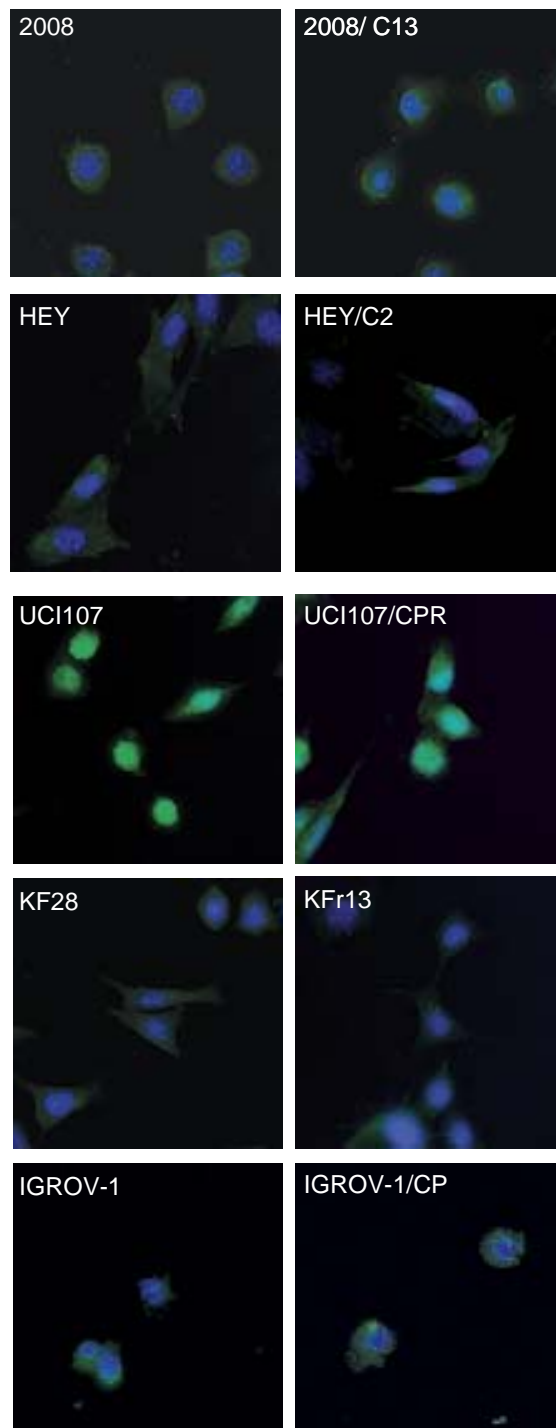
Figure 6-2. Cellular localization of hCTR1 in isogenic cell lines. Cells were fixed, permeablized and stained with an anti-hCTR1 antibody (green) and Hoescht nuclear dye (blue).

Evaluation of hCTR1 expression in ovarian tumors.

In addition to examining ovarian cell lines, tissue sections from fixed and paraffin-embedded samples of ovarian carcinoma obtained from 54 patients both before and after treatment with platinum-based chemotherapeutic regimens were stained for hCTR1. These samples were from patients treated at the Memorial Sloan-Kettering Cancer Center by Dr. William Hoskins and his colleagues in the era when second look laparotomies were performed to assess the extent of response to primary chemotherapy. Representative staining patterns are shown in Figure 6-3A. Only 61% (30/49) of the pre-treatment samples exhibited any hCTR1 staining. There was no apparent difference in the extent of staining in the samples obtained after primary chemotherapy compared to the tumor sample obtained from the same patient prior to the start of platinum-based treatment; 65% (32/49) of the post-treatment samples exhibited staining. There was no correlation between hCTR1 expression and any of the primary outcome measures including patient survival time, disease progression or type of treatment. Additionally, as shown in Figure 6-3B, there was no significant difference in the distribution of staining intensities before and after treatment with a platinum-based therapy.
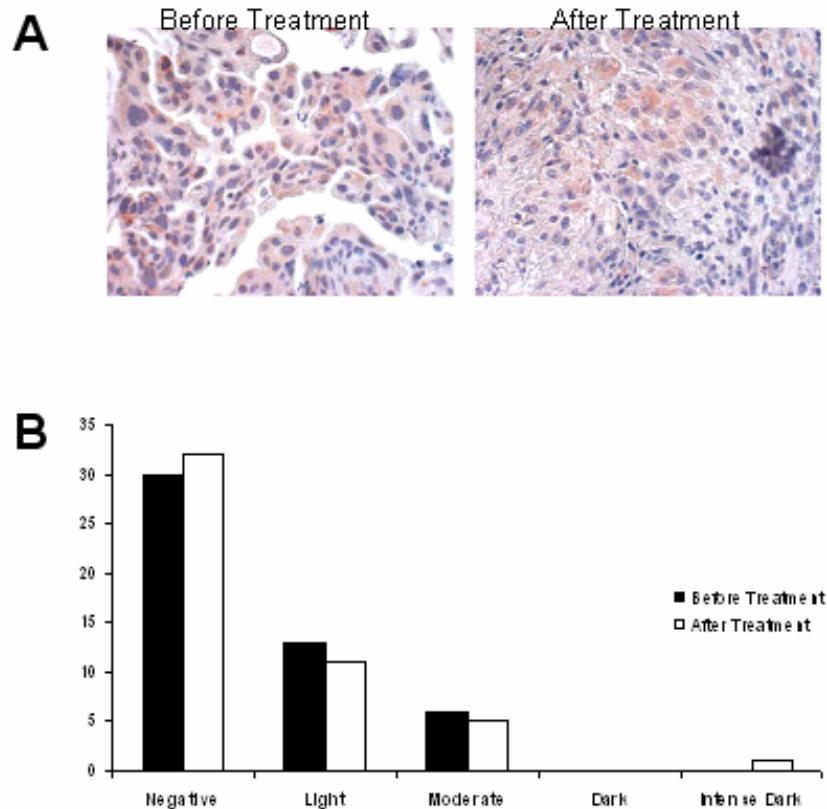
Figure 6-3. Ovarian tumors stained with anti-hCTR1 antibody. *Panel A,* Sample ovarian tumor before (left) and after (right) treatment with platinum based therapy stained with 1:200 dilution of hCTR1. *Panel C,* Graph depicting Staining intensity levels in patients before (closed bars) and after (open bars) treatment.

In a second study of the expression of hCTR1 in patients with ovarian carcinoma, formal application was made to the tissue bank maintained by the Gynecologic Oncology Group (GOG) to obtain sections cut from blocks of fixed paraffin-embedded tumor samples obtained from 16 patients with stage III/IV ovarian cancer treated on GOG protocols who had unusually short survival (< 9 mo) and 18 patients who survived >5 years following primary chemotherapy with a platinum-based regimen. As shown in Figure 6-4B, there was no correlation between hCTR1

expression and length of survival. The overall fraction of samples that stained positively for hCTR1 was less than had been observed in the set of tumors obtained from Memorial Sloan-Kettering Cancer Center; only 48 % (14/29) of the tumor samples from the GOG Tissue Bank stained positively for hCTR1. The most likely explanation for this is the low sample number used for measuring short vs. long term survival. Additionally, the quality of the embedded tissue can dramatically affect the staining intensity. Further, the antibody may not be sensitive enough to pick up small changes in hCTR1 expression, so those samples that do not stain positive for hCTR1 may express hCTR1 at much lower but nevertheless functionally important levels. However, as assessed in this study, there was not discernable difference in the hCTR1 staining pattern between patients with short versus long term survival.
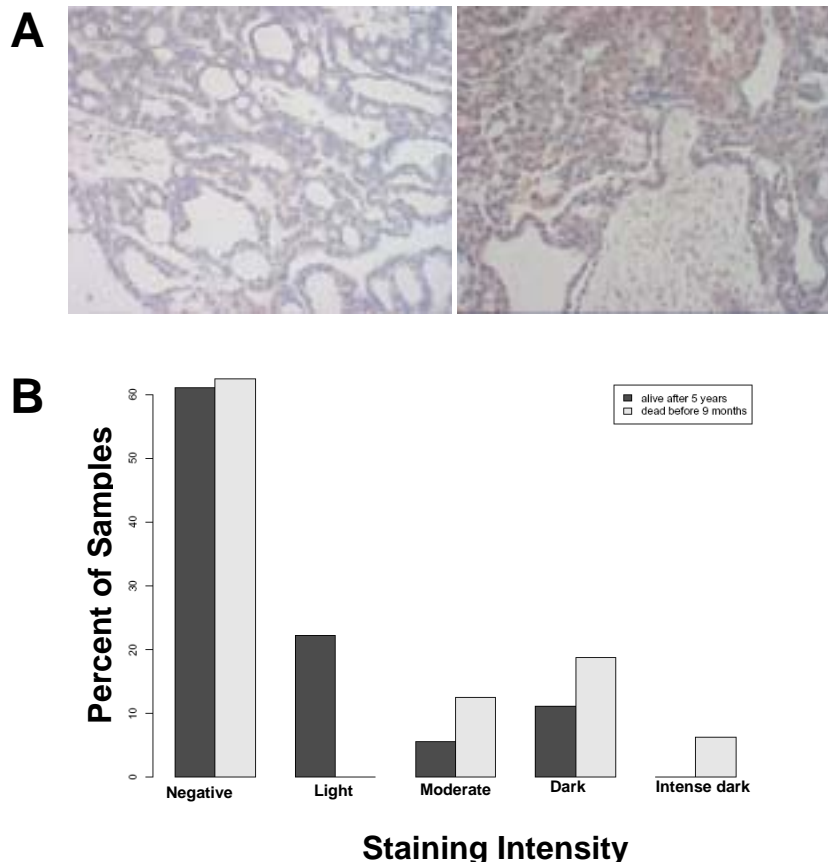
Figure 6-4. Ovarian tumors stained with anti-hCTR1 antibody. Panel A, Ovarian tumors from short and long term survivors stained with 1:200 dilution of anti-hCTR1. Panel B, Graph depicting Staining intensity levels in patients with short vs long term survival rates.

Discussion

In an attempt to determine whether the expression of hCTR1 is altered in tumors that have become resistant to DDP, 6 pairs of isogenic DDP-sensitive and resistant ovarian carcinoma cell lines were screened for mutations in the hCTR1 coding sequence and alterations in hCTR1 protein level and subcellular distribution. None of the 6 resistant sublines contained any mutations in their coding sequence

indicating that this gene is not a frequent target for DDP-induced mutagenesis and that mutations in hCTR1 are not commonly a primary cause of DDP resistance.

The only clear difference among the 6 cell pairs with respect to hCTR1 expression as determined by Western blot analysis was found in the UCI07 pair in which the resistant subline had a decrease in hCTR1 protein level. It is not clear that this decrease was functionally important since it was not associated with a decrease in initial copper uptake rate. Another possible mechanism of DDP resistance stems from the down regulation of hCTR1 following DDP exposure as discussed in previous chapters. An increased rate of hCTR1 down regulation could explain a resistance to DDP. If hCTR1 is down regulated at a faster rate, then it is possible that less DDP would accumulate in the cell leading to less cell death. However, no differences in subcellular localization of hCTR1 were observed between the DDP-sensitive and resistant members of each cell pair suggesting that disturbances of hCTR1 trafficking are also not commonly associated with DDP resistance.

There are several points are of interest with respect to the measurement of initial ^{64}Cu influx rate. First, the influx rate varied more than 10-fold among the 6 DDP-sensitive parental ovarian carcinoma cell lines indicating great heterogeneity in overall Cu homeostatic mechanisms among ovarian carcinoma cell lines, presumably reflecting a similar heterogeneity of ovarian tumors in patients. This heterogeneity may reflect differences in the expression of other Cu transporters. It is also of interest that there was no clear association between initial ^{64}Cu influx rate and the degree of DDP resistance. In 2 of the 6 pairs of cell lines ^{64}Cu uptake was substantially reduced in the DDP-resistant subline, whereas in 3 of the 6 it was actually substantially

increased. Thus, in the majority of cases, selection for acquired DDP-resistance clearly altered overall Cu homeostasis but not in a consistent manner. The copper efflux proteins ATP7A and ATP7B have been shown to influence DDP sensitivity and cellular pharmacology (reviewed in (Safaei and Howell, 2005). In fact, Katano et al. showed increased expression of ATP7A or ATP7B in 3 of the 6 isogenic lines examined here (Katano et al., 2002), including A2780 and A2780/CP, IGROV-1 and IGROV/CP and 2008 and 2008/C13. Given the potential role of the Cu efflux proteins and the lack of evidence suggesting a role for hCTR1, further studies into the mechanism of DDP resistance in the remaining isogenic pairs should involve the examination of ATP7A and ATP7B.

Immunohistochemistry studies revealed that hCTR1 is expressed at detectable levels in 48 – 61% of ovarian tumors prior to initiation of treatment. Neither the initiation level of hCTR1 expression nor the change in expression correlated with patient survival or response to treatment. There was no detectable difference in staining intensity distribution between tumor samples from the same patient obtained before and after treatment with platinum-based therapy. Likewise, in a comparison of hCTR1 in short and long term ovarian tumor survivors, there was no significant difference in hCTR1 protein levels. It is thought that hCTR1 is expressed at low levels within the cell, so it is feasible that there are differences in hCTR1 before and after treatment or in short versus long term survivors, but that immunohistochemistry detection is not sensitive enough to identify changes in those tumors that express low levels of hCTR1. Further, the sample numbers used in the studies reported in this chapter were not large and thus the power to detect differences if actually present was

limited and analysis of additional samples may lend new insight into any alterations in

hCTR1 staining patterns. It is also important to note that normal ovarian tissue also

does not stain intensely for hCTR1 (see results in Chapter 7), so a limited, low

intensity staining pattern in tumor tissue is not unexpected. Given the limitations of

immunohistochemistry to identify protein expression, the best way to definitively

examine hCTR1 expression in future studies is perform Western blots on cell lysates

harvested from large numbers of ovarian tumors where the patients treatment and

survival rate are known.

Both clinical samples and ovarian cells lines lacked significant differences in

hCTR1 levels regardless of their degree of resistance or response to treatment with

platinum-based therapy. This suggests that the steady-state level of hCTR1 is not a

primary determinant of sensitivity to the cytotoxic effect of DDP. Given that DDP

rapidly down-regulates hCTR1 expression, future studies should determine whether

the rate or extent of down-regulation is more closely linked to the sensitivity to DDP

than the steady-state level.

Materials and Methods

Cell Lines. All ovarian carcinoma cell lines (Disaia et al., 1972; Hamilton et

al., 1985) were grown in RPMI 1640 media containing 10% fetal bovine serum at

37^oC in 5% CO_2. A2780 cells were engineered to over-express hCTR1 by transfecting

them with a pcDNA3.1 vector containing full length hCTR1 cDNA and expressing a

geneticin resistance marker constructed as previously described (Moller et al., 2000)

that was generously provided by Dr. Lisbeth Birk Moller (John F. Kennedy Institute,

Glostrup, Denmark). The A2780 ovarian carcinoma cell line over expressing hCTR1

was generated as previously described (Holzer et al., 2004b).

Reagents. Refer to methods of Chapter 2.

Western Blot Analysis. Refer to methods of Chapter 2.

Fixation and staining for fluorescent microscopy. Refer to methods of

Chapter 2.

Measurement of ^{64}Cu accumulation. Refer to methods of Chapter 2.

Tissue Samples. The study population for the Memorial Sloan-Kettering Cancer

Center collected by Dr. William Hoskins consisted of patients with ovarian cancer

who had been entered on one of several different Memorial Sloan-Kettering studies

(patients 1–41) or Southwest Oncology Group study 8835 (patients 42–54). All of the

cases from these two trials in which paired fixed and paraffin-embedded tumor tissues

were available from both before treatment and after at least two cycles of

chemotherapy were included. Each patient had received DDP- or CBDCA-based

chemotherapy as part of her first line treatment, followed by various second- and

third-line treatments. Each patient's age, stage, histological grade, histological

subtype, type of chemotherapy, clinical response eventually attained, and survival

status was available for analysis. Data on the CA 125 level (pre- and post-first-line

treatment) and the number of cycles of treatment received were available for patients

1–41 only. The study population from the Gynecologic Oncology Group consisted of

paraffin-embedded tumor samples obtained from 29 patients with stage III/IV ovarian

cancer treated on GOG protocols who had unusually short survival (< 9mo) or long

term (>60 mo) survival following primary chemotherapy with a platinum-based

regimen.

Immunohistochemical Staining. In preparation for staining, slides were

deparaffinized with three 10 minute soaks in Xylene and hydrated through two 5

minute immersions in graded alcohol (100% ethanol followed by 95 % and then 70 %

ethanol) followed by three rinses with 0.1% Trition-X in PBS. Antigen retrieval was

performed by microwaving the slides twice for 5 minutes in citrate buffer pH 5

followed by 3 washings with 0.1% Trition-X in PBS. Endogenous peroxidases were

blocked by immersion of the slides in 0.3 % hydrogen peroxide for 30 minutes

followed by three 0.1% Trition-X in PBS washings. Immunohistochemical staining

was performed according to the protocol outlined in the Catalyzed Signal

Amplification System (DAKO, Carpinreria, CA, cat. no. K1500). Briefly, blocking of

endogenous biotin was performed by first overlaying the slides with 0.1 % avidin for

15 minutes and washing 3 times with 0.1% Trition-X in PBS. Slides were then

overlayed with 0.01 % biotin for 15 minutes followed by another 3 washes with 0.1%

Trition-X in PBS. Non-specific protein binding was blocked by immersion of the

slides in 1% BSA in PBS for 20 minutes. Slides were incubated with anti-hCTR1

antibody (Novus Biologicals, Littleton, CO) at a dilution of 1:200 in 1% BSA in PBS

overnight at 4°C. As a negative control, parallel arrays were incubated with non-

immune rabbit IgG$_1$ sera (DAKO, prediluted). As a technical positive control, slides

were incubated with anti-vementin antibody (DAKO, prediluted, cat. no V1613).

Further controls were performed using a 1:50 dilution of immunizing peptide (1

mg/ml) to compete out the anti-hCTR1 antibody, resulting in a negative stain.

Additional controls included staining of cell blocks prepared from cell line that lack

hCTR1 expression or which were molecularly engineered to over-express hCTR1.

Acknowledgements

Chapter 7

Expression of hCTR1 in normal and malignant human tissues

Introduction

A major objective is to identify mechanisms that mediate DDP resistance with the hope of preventing or overcoming the therapeutic limitations associated with this and the other Pt-containing chemotherapeutic agents. The studies in Chapters 2 and 3 support the hypothesis that the plasma membrane transporter hCTR1 influences the cellular pharmacology of DDP. These results raise the possibility that hCTR1 may be an important determinant of tumor responsiveness to DDP *in vivo* in cancer patients.

The subcellular location of hCTR1 varies among cell lines from localizing to the plasma membrane to higher expression in the perinuclear region (Eisses and Kaplan, 2002; Klomp et al., 2002; Lee et al., 2002a; Petris et al., 2003). While originally thought to behave as a plasma membrane transport channel, hCTR1 may relocalize from the plasma membrane to vesicles upon exposure to Cu [Petris, 2003 #6471}. However, there are conflicting reports on this point (Klomp et al., 2002; Petris et al., 2003). To date, the localization of hCTR1 in cell lines has been well documented, but little is known about the level of its expression in normal and malignant human tissues. Zhou *et al.* reported that hCTR1 mRNA was detectable by Northern blot analysis in all of 16 major human organs (Zhou and Gitschier, 1997).

There is currently no information about the level of expression of hCTR1 protein in normal or malignant human tissues.

As previously mentioned, the level of hCTR1 expression is important in part because hCTR1 influences the cellular pharmacology of DDP. The observations made in Chapters 2 and 3 indicate that the level of hCTR1 modulates DDP uptake (Holzer et al., 2004b), and those presented in Chapter 4 indicate that DDP interacts with hCTR1 to trigger rapid down-regulation of expression in human ovarian cancer cells (Holzer et al., 2004a). Thus, the level of hCTR1 expression is a candidate biomarker for the prediction of tumor cell sensitivity to the Pt drugs.

This chapter presents the results of an immunohistochemical study of the expression of hCTR1 in the major human organs and many types of malignancies. hCTR1 expression was not universal, but rather limited to only a subset of tissues and often to specific cell types within a tissue. hCTR1 expression was found in malignancies arising from normal tissues that expressed this protein, but not in malignancies originating from tissues that did not.

Results

Characterization of the hCTR1 antibody.

The specificity of the polyclonal antibody generated to the C-terminal end of hCTR1 was documented by staining human cell lines known to express varying levels of hCTR1. Fixed and paraffin-embedded cell blocks were prepared from human ovarian carcinoma A2780 cells and the A2780/hCTR1 subline which had been stably transfected with an hCTR1 expression vector (Holzer et al., 2004b). As shown in

Figure 7-1A, the A2780 cells expressed a moderate level of hCTR1 and staining was blocked by co-incubation of the antibody with the peptide that was used as the immunogen. The A2780/hCTR1 cells expressed a much higher level of hCTR1 and this staining was also eliminated by co-incubation with the immunizing peptide demonstrating the specificity of the antibody. Sections of the cell blocks of these two cell lines were included as controls when staining all the other tissues in this study.

hCTR1 expression in normal human tissues.

Arrays containing a wide range of tissues from various normal organs were stained with the polyclonal hCTR1 antibody to assess the level of hCTR1 expression. The results of the staining are summarized in Tables 7-1 thru 5 and representative images of stained tissues are presented in Figure 7-1B and C and Figure 7-2. hCTR1 staining was found in various parts of the brain; specifically hCTR1 was expressed in the choroid plexus, a subset of the cells in the anterior pituitary, and in the hypothalamus. It was also found in the adrenal medulla and the autonomic ganglia (Figure 7-1B). No hCTR1 expression was observed in any component of the eye. hCTR1 was not expressed in the esophagus, gall bladder, duodenum or small bowel but expression was detected in some samples of normal gastric and colonic epithelium(Figure 7-1C); interestingly no staining was detected in the rectal epithelium.
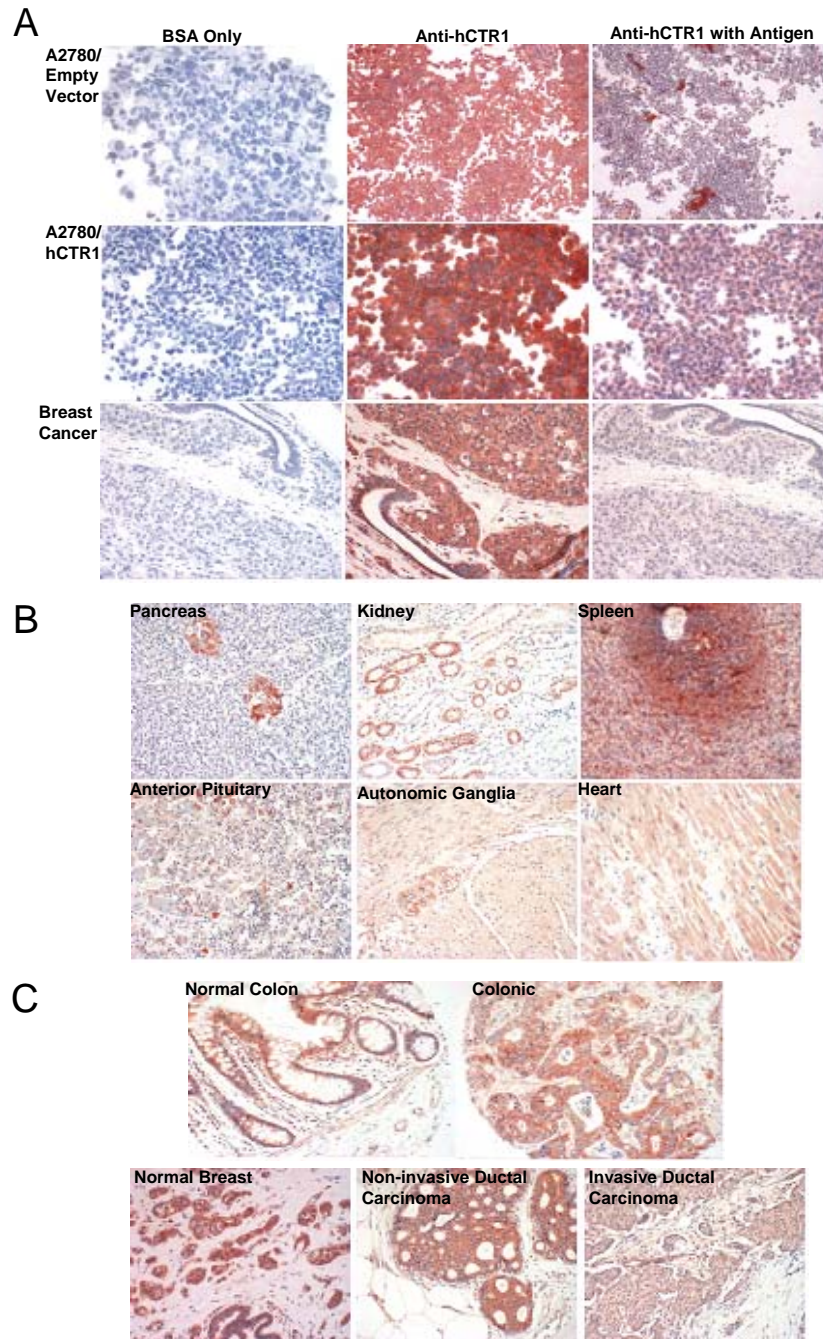
Figure 7-1. Immunohistochemical staining for hCTR1 in normal and malignant human tissues. Panel A,. Staining specificity controls. Cell blocks prepared from A2870 ovarian cancer cells and a subline engineered to express high levels of hCTR1, and a sample of breast cancer, stained with a BSA alone, a 1:200 dilution of anti-hCTR1 antibody or a 1:200 dilution of anti-hCTR1 neutralized by prior addition of the immunizing peptide. Panel B, hCTR1 expression in selected normal human tissues. Panel C, hCTR1 expression in selected normal tissues and malignancies arising from these tissues. All images are at magnification of 200.

The G cells in the gastric mucosa stained strongly in 22 of 25 samples as shown in Figure 6-2. Most samples of normal breast epithelium expressed readily detectable levels of hCTR1 in the ducts and lobular cells (Figure 7-1C). Based on a prior report that hCTR1 mRNA could be detected in the liver by Northern blot analysis (Zhou and Gitschier, 1997), it was anticipated that the liver would stain for hCTR1 but no staining was observed in 13 samples.
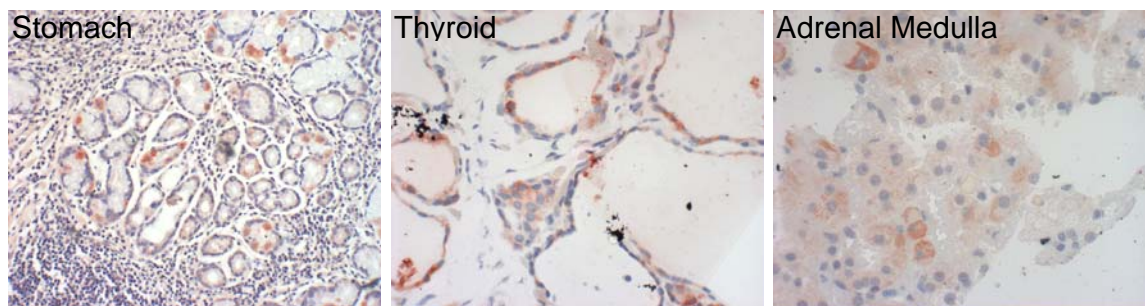
Figure 7-2. hCTR1 expression in normal human tissue possessing enteroendocrine cells. hCTR1 expression in the gastrin cells of the stomach, the C cells of the thyroid and the adrenal medulla. All images are at magnification of 400.

None of the 15 samples of pancreas expressed hCTR1 in the epithelial cells, but all 13 of these samples that included an islet demonstrated strong staining of the islet cells. The islets were stained with additional antibodies specific for α and β cells. As shown in Figure 7-3, the α cells of the pancreatic islet stained positively for hCTR1 (Figure 7-3A), while the β cells did not (Figure 7-3B).
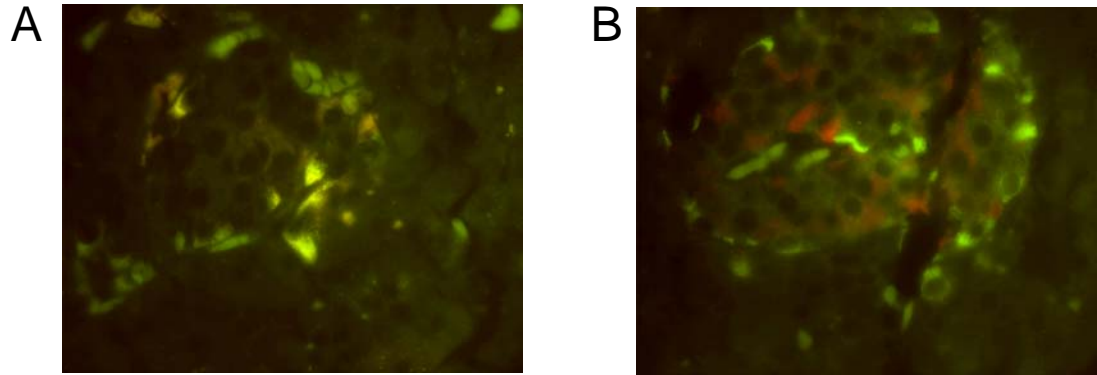
Figure 7-3. hCTR1 expression in cells of the pancreatic islet. Panel A, Human pancreatic islets stained with antibodies specific for hCTR1 (green) and glucagons-containing α cells (red). Yellow indicates co-localization of hCTR1 and glucagon. Panel B, Human pancreatic islets stained with antibodies specific for hCTR1 (green) and insulin-containing β cells (red). Images are at a magnification of 400.

No staining was observed in the larynx or and the only cell types that stained in the lung were the APUD cells of the bronchioles and alveolar macrophages. hCTR1 was diffusely expressed in all 7 samples of splenic tissue, with intense staining of the splenic nodules and the peri-arterial lymphoid sheath (PALS); only 1 of 5 normal lymph nodes demonstrated any staining. hCTR1 was expressed in 5 of 8 samples of cardiac muscle as well as in skeletal muscle. All samples of normal kidney expressed hCTR1 but only in the distal tubular epithelial cells (Figure 7-1B). No staining was observed in any component of the prostate or bladder. hCTR1 was expressed in the epithelial cells in only 1 of 19 thyroid samples; however, in all samples the parafollicular cells, otherwise known as C cells, of the thyroid stained intensely for hCTR1 (Figure 7-2). No staining was observed in the ovarian surface epithelium, but hCTR1 was expressed in oocytes and corpus letea. Among the uterine tissues no

staining was observed in the myometrium or the cervix but hCTR1 expression was found in 2 of the 3 samples of endometrium. No staining was observed in any component of the testis, but hCTR1 was expressed in the seminal vesicle, epididymus, and vas deferens. No staining was observed in the skin, skin appendages, melanocytes or blood vessels including arterioles. Thus, hCTR1 is not uniformly expressed in all tissues, and even within a single tissue its expression is often restricted to a limited number of cell types.

Table 7-1. Summary of hCTR1 expression in various brain regions.

Tissue Type	No. positive/ No. stained
Gray Matter (Cerebral Cortex)	0/3
White Matter (Subcortical)	0/5
Cerebellar Cortex, Purkinje/Gran	$\frac{1}{4}$
Choroid Plexus	3/3
Ependymal Cells	0/3
Globus Pallidus	0/3
Hippocampus	0/3
Meninges	0/3
Motor Neurons (spinal cord)	0/3
Putamen	0/3
Substania Nigra	0/3
Thalamus	0/3
Hypothalamus (Cinereum)	1/1
Anterior Pituitary	2/2
Posterior Pituitary	0/2
Cerebellum	0/5

Table 7-2. Summary of hCTR1 expression in human eye.

Tissue Type	No. positive/ No. stained
Vitreous body	0/1
Sclera	0/1
Retina	0/1
Ciliary Process	0/1
Choroid Membrane	0/1

Table 7-3. Summary of hCTR1 expression in various normal human tissues.

Tissue Type	No. positive/ No. stained
Larynx	0/1
Lung	0/26
Bronchioles (APUD cells only)	2/2
Aveolar macrophages	1/1
Gall bladder	0/1
Pancreas, epithelial cells	0/15
Pancreas, islets	13/15
Liver	0/13
Adrenal gland	1/1
Autonomic ganglia	8/8
Kidney (distal tubules only)	19/19
Bladder	0/1
Spleen	7/7
Lymph Node	1/5
Skin	0/2
Thyroid, epithelial cells	1/19
C cells of thryroid	19/19
Cardiac muscle	5/8
Skeletal muscle	1/1
Small muscular arteries	0/1
Placenta	0/2

hCTR1 expression in human malignant tissues.

Tissue arrays containing various types of human tumors were also stained for hCTR1 in order to determine whether there were major differences in expression in the malignancies compared with the normal tissue from which they originated. The results are summarized in Tables 7-3 – 7-5. There were several cases where hCTR1 was expressed in both the normal tissue and its malignant variant, although in most cases the frequency and level of expression was lower in the malignant samples. While hCTR1 was expressed in ~40% of normal colon epithelial samples, no staining was found in 45 colonic adenomas and only 5 of 42 samples of primary colonic adenocarcinoma expressed hCTR1. However, fully 66% (14/21) samples of colon adenocarcinoma metastasic to lymph nodes expressed hCTR1. While hCTR1 was expressed in most samples of normal breast ducts and alveolar cells, and in 12 of 14 samples of ductal carcinoma *in situ*, it was found in only 5 of 14 (35%) of invasive ductal carcinomas and in none of 7 samples of lymph node metastases. This suggests that hCTR1 expression may be lost as breast cancer progresses. hCTR1 expression was found in the all 19 samples of normal renal tubular epithelium but in only 2 of 11 samples of renal cell carcinoma.

Table 7-4. Summary of hCTR1 expression in normal and malignant tissue of digestive tract

Normal Tissue		Malignant Tissue	
Tissue Type	No. positive/ No. stained	Tissue Type	No. positive/ No. stained
Esophagus	0/19	Squamous esophageal carcinoma	0/14
Stomach (fundic mucosa and submucosa)	3/25	Gastric carcinoma	0/29
Fundic G cells	22/25		
Muscularus of fundus	0/25		
Duodenum	0/2		
Small Bowel	0/3		
Appendix	0/1		
Colon (muscle layer)	0/7		
Colon mucosa	16/42	Colonic adenomas	0/45
		Adenocarcinoma T1 or T2	5/21
		Adenocarcinoma T3 or T4	0/21
		Carcinoma metastatic to lymph node	14/21
Rectum	0/17	Rectal carcinoma	0/6

Table 7-5. Summary of hCTR1 expression in normal and malignant breast and reproductive organ tissues.

Normal Tissue		Malignant Tissue	
Tissue Type	No. positive/ No. stained	Tissue Type	No. positive/ No. stained
Breast (Ducts, epithelial cells, lobules)	12/19	Low grade ductal carcinoma in situ (staining in ducts)	7/7
		Grade 3 invasive ductal carcinoma	4/7
		Invasive lobular carcinoma	0/7
		Ductal carcinoma metastatic to lymph nodes	0/7
Ovary epithelium	0/11	Ovarian carcinoma	0/3
Oocytes	4/4		
Corpus luteum	4/4		
Cervix	0/3	Cervical squamous carcinoma	0/20
		Squamous carcinoma of uterus	0/3
Myometrium	0/7		
Endometrium	2/3	Adenocarcinoma of endometrium	0/11
Testes	0/11	Testicular carcinoma	1/1
Seminal vesicle	4/4		
Epididymus	4/4		
Ductus deferens	4/4		
Prostate	0/8	Prostate carcinoma	0/12

hCTR1 staining was evident in only 1 of 5 normal lymph node samples, and it was also found in only a minority of the cases of Hodgkin's disease and non-Hodgkins lymphoma. There were also a number of other types of tumors that expressed hCTR1. Despite the fact that normal melanocytes did not stain, staining was observed in 3 of 22 melanomas. All 3 cases of carcinoid and the 1 case of Ewings sarcoma available stained strongly for hCTR1 as did all 4 cases of undifferentiated carcinoma.

Table 7-6. Summary of hCTR1
in various malignant human tissues.

Tissue Type	No. positive/ No. stained
Hodgkin's disease	1/8
Non-Hodgkin's lymphoma	3/18
Lung Adenocarcinoma	0/13
Melanoma	3/22
Ewings sarcoma	1/1
Carcinoid	3/3
Undifferentiated carcinoma	4/4

There were a number of tissues in which hCTR1 was not expressed in either normal or malignant cells arising from that tissue. This included the rectal epithelium, esophagus, lung, liver, cervix, prostate, and ovary. Although the extent of sampling is limited, there were no cases where hCTR1 expression was clearly higher in the malignant tissue than in the normal tissue from which it arose with the exception of the one case of testicular carcinoma available for study.

Discussion

CTR1 has been identified as the major Cu influx transporter in mammalian cells and studies in which both alleles of mCTR1 have been knocked out have shown it to be essential to murine embryonic development. Given the importance of this protein for development in mice, and the requirement of every human tissue for Cu, one might have expected to find hCTR1 expressed monotonously in all human tissues. However, the current study demonstrated that hCTR1 is detectable by

immunohistochemical staining in only a limited subset of normal tissues, and often in only specific types of cells within the tissue.

It is important to note that many of types of cells that stained most strongly for hCTR1 are of enteroendocrine original, and in many cases they fall into the family of amine precursor uptake and decarboxylation (APUD) cells. Additionally, the APUD cells of the bronchiolar epithelium, parafollicular cells of the thyroid and gastrin (G) cells of the stomach were also found to express high levels of hCTR1 (Ross et al., 2003). The best example is the pancreatic islets, wherein the α cells responsible for secreting glucagon have high levels of hCTR1 expression but the insulin-producing β cells do not. No specific relationship between the unique biochemical features of APUD cells and Cu metabolism has been identified. One possibility is that APUD cells require a specific Cu-dependent enzyme or process to manage the production and secretion of their various hormones and thus secondarily require a lot of Cu to service these enzymes (Petris, 2004). Another is that hCTR1 or Cu itself is involved in a critical step in the vesicle secretory process.

For the most part the frequency and intensity of hCTR1 staining in malignant tissues was found to reflect the levels found in their normal tissue counterparts. Among the normal tissues that did not express hCTR1 the only example of a tumor arising from that tissue that expressed hCTR1 was testicular cancer, and in this study only 1 sample was available. Thus, unlike many other genes, the expression of *hCTR1* does not appear to be frequently up-regulated by the transformation process. Among the tumors arising from normal tissues that expressed hCTR1, only in the case of gastric carcinoma did none of the tumor samples available for study express any

hCTR1. Breast and colon cancers were of particular interest. While hCTR1 was expressed in over 60% of normal breast epithelia, only 18% of invasive breast cancers stained for hCTR1. In the case of colon carcinoma, while the frequency of staining in primary tumors was low, most metastases in lymph nodes stained strongly. Thus, it appears that the host tissue environment of the tumor cell may have a strong influence on the expression of hCTR1.

Consistent with the fact that several types of APUD cells stained strongly in normal tissues, all 3 carcinoid tumors available exhibited stained. Although not clearly of APUD origin, the 1 case of Ewing's sarcoma stained intensely. Currently there are no known histologic markers that distinguish Ewing's sarcoma from related tumors such as adult neuroblastoma, malignant small-cell tumor of the thoracopulmonary region (Askin's tumor), paravertebral small-cell tumors and atypical Ewing's sarcoma. Whether hCTR1 can be used for this purpose will require study of additional cases of Ewing's sarcoma as well other primitive tumors of neuroectodermal origin.

Materials and Methods

Reagents.

Normal and malignant tissue arrays were purchased from Cybri (Frederick, MD), the Cooperative Human Tissue Network (Nashville, TN) and Imgenex (San Diego, CA). Platinol-AQ was a gift from Bristol-Myers Squibb (Princeton, NJ). The polyclonal anti-rabbit antibody used for immunohistochemical staining was generated by immunizing rabbits with a peptide containing amino acids from the C-terminus of hCTR1 by Novus Biologicals (Littleton, CO). Fluorescein isothiocyanate-conjugated

goat anti-rabbit antibody was obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). The guinea pig anti-human insulin and guinea pig anti-human glucagon were obtained from Linco Research Inc (St. Charles, MO).

The anti-guinea pig Texas Red and fluorescien conjugated antibodies were obtained from Vector Laboratories (Burlingame, CA). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was purchased from Amersham Pharmacia (Piscataway, NJ). All other chemicals and reagents were obtained from Fisher Scientific (Tustin, CA).

Immunohistochemical Staining.

In preparation for staining, slides were deparaffinized with three 10 minute soaks in xylene and hydrated through two 5 minute immersions in graded alcohol (100% ethanol followed by 95 % and then 70 % ethanol) followed by 3 rinses with 0.1% Triton-X in PBS. Antigen retrieval was performed by microwaving the slides twice for 5 minutes in citrate buffer pH 5 followed by 3 washings with 0.1% Triton-X in PBS. Endogenous peroxidases were blocked by immersion of the slides in 0.3 % hydrogen peroxide for 30 minutes followed by 3 washings with 0.1% Triton-X in PBS. Immunohistochemical staining was performed according to the protocol outlined in the Catalyzed Signal Amplification System (DAKO, Carpinreria, CA, cat. no. K1500). Briefly, blocking of endogenous biotin was performed by first overlaying the slides with 0.1 % avidin for 15 minutes and washing 3 times with 0.1% Triton-X in PBS. Slides were then overlaid with 0.01 % biotin for 15 minutes followed by another 3 washes with 0.1% Triton-X in PBS. Non-specific protein binding was blocked by immersion of the slides in 1% BSA in PBS for 20 minutes. Slides were incubated with

anti-hCTR1 antibody (Novus Biologicals, Littleton, CO) at a dilution of 1:200 in 1%

BSA in PBS overnight at 4°C. As a negative control, parallel arrays were incubated

with non-immune rabbit IgG$_1$ sera (DAKO, prediluted). As a technical positive

control, slides were incubated with anti-vementin antibody (DAKO, prediluted, cat. no

V1613). Further controls were performed using a 1:50 dilution of immunizing peptide

(1 mg/ml) to compete out the anti-hCTR1 antibody, resulting in a negative stain.

Additional controls included staining of cell blocks prepared from cell lines lacking

hCTR1 expression or which were molecularly engineered to over-express hCTR1.

Staining was scored on a 1 to 5 scale with that number being multiplied by the fraction

of cells in the section that exhibited staining. Tissues with scores above 0.3 were

deemed positive.

Immunofluorescent staining.

In preparation for staining, slides were deparaffinized with three 10 minute

soaks in xylene and hydrated through two 5 minute immersions in graded alcohol

(100% ethanol followed by 95 % and then 70 % ethanol) followed by 3 rinses with

0.1% Triton-X in PBS. Antigen retrieval was performed by microwaving the slides

twice for 5 minutes in citrate buffer pH 5 followed by 3 washings with 0.1% Triton-X

in PBS. Endogenous peroxidases were blocked by immersion of the slides in 0.3 %

hydrogen peroxide for 30 minutes followed by 3 washings with 0.1% Triton-X in

PBS. Immunohistochemical staining was performed according to the protocol outlined

in the Catalyzed Signal Amplification System (DAKO, Carpinreria, CA, cat. no.

K1500). Briefly, blocking of endogenous biotin was performed by first overlaying the

slides with 0.1 % avidin for 15 minutes and washing 3 times with 0.1% Triton-X in

PBS. Slides were then overlaid with 0.01 % biotin for 15 minutes followed by another

3 washes with 0.1% Triton-X in PBS. Non-specific protein binding was blocked by

immersion of the slides in 1% BSA in PBS for 20 minutes. Slides were incubated with

anti-hCTR1 antibody (Novus Biologicals, Littleton, CO) at a dilution of 1:200 in 1%

BSA in PBS overnight at 4^oC. Slides were then washed 3 times with 0.1% Triton-X in

PBS and incubated with a 1:100 dilution of Fluorescein anti-rabbit IgG in 1%

BSA/PBS in 30min in the dark followed by another 3 washes with 0.1% Triton-X in

PBS. Slides were then blocked with 10% Normal horse serum in PBS for 30 min

followed by incubation with either a 1:1000 dilution of anti-insulin antibody (Linco,

St. Charles, MO) or anti-glucagon antibody (Linco, St. Charles, MO) in 10 % normal

horse serum in PBS for 2 hours at room temperature or overnight at 4^oC. Slides were

then rinsed twice for 5 min in PBS. Slides were then incubated at a 1:100 dilution of

Texas Red anti guinea-pig IgG in 10 % normal horse serum in PBS for 30 min

followed by additional 2 washings of 5 min each with PBS. Slides were then mounted

in vector fluorescien and stored in the dark.

Acknowledgements

her assistance in staining. The staining of the pancreatic islets was performed by Dr.

Peter Naredi and Annette Berlund.

Chapter 8

Discussion

Summary

The overall goal of the studies presented in this dissertation was to determine whether the Cu plasma membrane transporter hCTR1 plays a role in the cellular pharmacology of DDP, and if so, whether it influences the sensitivity of cells to the cytotoxic effect of this commonly used chemotherapeutic agent. Once hCTR1 was firmly identified as a DDP influx transporter, these investigations went on to focus on the cellular regulation and trafficking of the protein. The results of the experiments described in this dissertation demonstrate that hCTR1 does play a role in the transport of DDP as forced alterations of hCTR1 protein levels change Pt accumulation. Further, the results suggest that hCTR1 levels are not tightly linked to the DDP resistant phenotype as hCTR1 levels were not significantly altered in a majority of ovarian carcinoma cell lines selected for DDP resistance or in ovarian carcinomas obtained from patients who exhibited resistance to primary Pt-based chemotherapy. Finally, the cellular trafficking of hCTR1 most likely involves the continual cycling of the protein. However, upon exposure to DDP, hCTR1 is rapidly internalized through macropinocytosis and degraded by the proteasome.

Influence of increased hCTR1 levels on copper and DDP uptake.

The results of these studies demonstrate that the level of expression of the Cu influx transporter hCTR1 influences the cellular accumulation of DDP as well as that of Cu in human ovarian carcinoma cells, but that the enhanced cellular uptake of these compounds is not accompanied by a significant change in sensitivity to their cytotoxic effect or the delivery of DDP to DNA. These observations introduce the novel concept that, while hCTR1 enhances DDP uptake into the cell, it appears to deliver it to cellular compartments from which it does not have ready access to key cytotoxic targets. Once brought into the cell by hCTR1, DDP is presumed to be handed to a chaperone, or potential a series of chaperones, that eventually delivery it to the nucleus. While Cu is known to reach the nucleus, the chaperone responsible for transporting Cu to this site has yet to be identified. It is plausible that while an increase in hCTR1 serves to increases the ability of a cell to take up DDP, this is not associated with increased DNA adduct formation because downstream elements of the trafficking system that delivers DDP to the nucleus become saturated.

The increase in hCTR1 expression detected by Western blot analysis in the A2780/hCTR1 cells was accompanied by substantial changes in the cellular pharmacology of Cu. The basal steady-state level of Cu in the A2780/hCTR1 cells was increased by 6.5-fold over A2780 empty vector transfected cells, and these cells exhibited a marked increase in the uptake rate for ^{64}Cu. Thus, exogenous hCTR1 was functional in A2780 cells and augmented their ability to internalize the small amounts of Cu found in standard tissue culture medium and the rate of Cu accumulation. Increased expression of hCTR1 impaired the growth rate of the A2780 cells, but this

did not appear to be due to Cu intoxication since chelation of Cu present in culture medium did not significantly enhance the growth rate of the A2780/hCTR1 cells. The mechanism underlying the slower growth remains unknown.

The decrease in growth rate following increased hCTR1 expression combined with the fact that all of the downstream elements of the copper transport pathway were still at endogenous levels brings into question the validity of using exogenously expressed hCTR1 to study the cellular mechanisms of Cu accumulation. Based on the results presented in this dissertation, it appears that expression of exogenous hCTR1 can mask what is really happening under physiological conditions. A better system in which to study the function of hCTR1 is thus one in which hCTR1 is decreased leaving downstream proteins in excess.

It is also important to note that the examination of the cellular pharmacology of DDP has been a challenge for many years due to the limited sensitivity of atomic absorption spectroscopy and the lack of a readily available radioactive isotope of Pt with a half-life long enough to make it useful for tissue culture studies. This has necessitated the use of DDP concentrations far above those attainable in patients leaving open the question of whether conclusions based on such experiments inform the clinical use of this important drug. As exemplified by the studies reported here, the extraordinary sensitivity of inductively-coupled plasmon mass spectroscopy now offers the opportunity to investigate the cellular pharmacology of DDP at concentrations that mimic those found in the plasma of patients treated with this agent.

Effects of loss of CTR1 on DDP uptake.

Through the course of theses studies, I (Holzer et al., 2004b) and others (Song et al., 2004) have sought to define the role of CTR1 as a transporter of the Pt drugs by transfecting human cells with hCTR1 expression vectors. As mentioned previously, high-level over-expression of hCTR1 in ovarian carcinoma A2780 cells enhanced the accumulation of DDP but this was not accompanied by increased delivery of DDP to DNA or greater cell kill (Holzer et al., 2004b). In combination with the fact that such over-expression of CTR1 reduced cell proliferation even in Cu-depleted medium, this raised concerns about whether this approach provided an accurate picture of the normal role of CTR1. Beretta *et al.* (Beretta et al., 2004) reported that forced over-expression of hCTR1 in cervical squamous cell carcinoma cells did not alter DDP uptake, nor did it change sensitivity to the cytotoxic effect of DDP, suggesting that such high levels of CTR1 did not affect cellular DDP accumulation. In contrast, Song *et al.* (Song et al., 2004) reported that over-expression of hCTR1 in a DDP-sensitive human small cell lung carcinoma cell line increased the accumulation of not only DDP but also CBDCA and L-OHP, and that this was accompanied by enhanced sensitivity to the cytotoxic effect of all 3 drugs. Why forced expression of CTR1 produces different effects in different model systems remains unknown, but Cu homeostasis mechanisms are complex and involve myriad other transporters and chaperones whose expression may vary among tumor cell types. In the studies presented here of the CTR1$^{-/-}$ murine embryonic fibroblasts in which CTR1 was not expressed, there was, as expected, a decrease in DDP accumulation as well as a decrease in cellular sensitivity to DDP. This indeed suggests that exogenous CTR1 expression is not the most

physiologically relevant method for studying the uptake of DDP and other Pt based therapies.

Differences in hCTR1 affinity for Cu and DDP.

Our prior studies of haploid *S. cerevisiae* demonstrated that the CTR1-mediated uptake of Cu was much more rapid than that of DDP, suggesting that Cu is a better substrate for this import pathway than DDP (Lin et al., 2002). Given the high selectivity of hCTR1, as exemplified by its ability to distinguish even between Cu (I) and Cu (II), this is not surprising. However, disruption of the yCTR1 gene produced marked reductions in the uptake of both compounds, suggesting that while DDP may not be a very good substrate, in yeast this transporter accounts for the majority of DDP influx. The results of my studies in mammalian cells also suggest that Cu is a better substrate for hCTR1 than DDP in human cells as the effect of increasing hCTR1 expression was substantially greater for Cu than for DDP. Nevertheless, hCTR1 clearly increased the cellular uptake of DDP as well as Cu, and the magnitude of this effect is well within the range where, if the intracellular drug had ready access to DNA, it would be expected to produce increased cell death (Katano et al., 2002).

One explanation for the larger relative effect of increased hCTR1 expression on Cu than DDP may lie in their relative affinity for the metal binding regions of the protein located in the extracellular domain of the protein. hCTR1 is thought to bind two molecules of Cu at a time (Zhou and Gitschier, 1997) via MXXXM motifs in the N-terminal domain (Eisses and Kaplan, 2002). Assuming that DDP binds to these same motifs, it is likely that the configuration of its binding is different from that of

Cu. It is also possible that DDP and Cu are binding at different regions within the N-terminal domain of hCTR1, as the beginning of the N terminus is very rich in histidine and methionine. Another plausible explanation for the larger relative effect of increased hCTR1 expression on Cu than DDP may be that in human cells other influx transporters account for a larger fraction of total DDP influx than in yeast.

Down-regulation of hCTR1 following DDP exposure.

The results of the studies presented in this dissertaton 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 caused reduction of hCTR1 levels when cells were exposed to 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. During the course of this work, conflicting information emerged on how either DDP or Cu modulates the level and trafficking of hCTR1. Cu was reported to have different effects in different experimental systems. Klomp *et al*. (Klomp et al., 2002) 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.* (Petris et al., 2003) 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 immunoflourescent confocal microscopy using antibodies directed at opposite ends of the protein and by Western blot analysis of both denatured and non-denatured protein suggests that hCTR1 is rapidly degraded. If the loss of signal were due to masking of the antigenic sequence, due to either cleavage of the protein or an alteration in its conformation, then one would not expect comparable loss of protein under circumstances where it is in a native conformation and when it is denatured and lacks tertiary structure. Furthermore, when the purified N-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 N-terminus did not change in a manner that blocked or hid the antibody recognition site. Finally, the decrease in Cu uptake following treatment of cells with DDP provides 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 following exposure to DDP. This difference in potency and magnitude of effect was observed by both Western blot analysis and confocal microscopy. Treatment with 2 μM DDP for 5 min resulted in an 85% decrease in hCTR1 levels, while treatment with 100 μM CuSO$_4$ produced only a 66% decrease in protein level in the same time frame. The N-terminal extracellular region of hCTR1 is rich in MXXM and MXMXXM sequences. These sequences are similar to motifs known to bind Cu

in other proteins, and which 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 methionines and histidines. While amino acids 40 – 45 of the extracellular domain are essential for Cu transport (Puig et al., 2002), 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.

Mechanism of hCTR1 internalization and degradation following DDP exposure.

hCTR1 is important not only because it is the major Cu influx transporter but because it also mediates a significant component of the cellular accumulation of DDP. As one step toward identifying how CTR1 transports DDP, we sought to determine the mechanism by which hCTR1 is degraded following brief exposure to DDP using several different strategies for inhibiting endocytotic pathways and proteosomal degradation. The results support the conclusion that the initial steps of DDP-induced loss of hCTR1 from human ovarian carcinoma cells is mediated by a process that is inhibited by amiloride and cytochalasin D but not by nystatin or methyl-β-cyclodextrin and is not dependent on either dynamin I or the Rac1 GTPase. Among the various endocytotic pathways currently defined, this pattern of inhibition most closely matches the process of macropinocytosis.

As currently understood, macropinocytosis occurs through the formation of large primary endocytotic vesicles in regions of membrane ruffling. While the specific mechanisms involved and controls on the process are not clearly defined, it is thought to be regulated by the small GTPase ARF6 and may be dependent on phospholipase Cγ activity. There is evidence that phosphatidylinositol (4,5)-bisphosphate is a regulator of the rate of macropinocytosis (Nichols and Lippincott-Schwartz, 2001). However, whether macropinocytosis is triggered by interaction with molecules external to the cell or is a byproduct of membrane turnover and cytoskeletal activity is currently a matter of debate (Nichols and Lippincott-Schwartz, 2001). Unlike endosomes generated by clathrin and caveolar-mediated endocytosis, macropinosomes do not fuse into lysosomes (Wadia et al., 2004) suggesting that their cargo is not degraded by lysosomal enzymes (Johannes and Lamaze, 2002). The observation that proteosome inhibitors blocked DDP-induced loss of hCTR1 is consistent with this concept. Several different proteasome inhibitors markedly reduced loss of hCTR1 indicating that proteasomal degradation accounts for the vast majority of hCTR1 breakdown and disappearance.

The specificity controls included in these studies provide strong evidence that the observed disappearance of hCTR1 from the cell following DDP and Cu exposure was not an artifact. Two separate antibodies directed at opposite ends of the protein detected the same time course of the loss and recovery of hCTR1 in immunoflourescent studies. Yet another antibody, capable of detecting hCTR1 on western blots, also demonstrated a similar temporal pattern. Thus, it is unlikely that loss of hCTR1 was due to epitope masking. In addition, DDP did not appear to cause

a general disturbance in the function of endocytotic pathways as there was no disturbance of the cellular accumulation of transferrin, cholera toxin B or dextran in the DDP-treated cells.

The studies reported here suggest that hCTR1 is a rapidly cycling protein whose steady-state levels are controlled at the protein level. They are consistent with the concept that, in the absence of excessively high Cu levels or DDP exposure, hCTR1 resides largely in membranes where it is protected from proteosomal degradation. The rapid loss of hCTR1 when new protein synthesis is inhibited suggests that basal rates of macropinocytosis internalize hCTR1 at a relatively high rate and make it accessible to degradation by the proteasome. Both brief exposure to DDP and inhibition of new protein synthesis with cyclohexaminde rapidly reduced the level of hCTR1 to below the limit of detection. The recovery of hCTR1 levels over the ensuing ~30 min following either exposure to DDP or cyclohexaminde indicates that hCTR1 is rapidly re-synthesized and trafficked to its normal cellular membrane locations. The available data supports the idea that both Cu and DDP markedly increase the rate of hCTR1 internalization by macropinocytosis thus enhancing proteosomal degradation, although on a molar basis DDP is far more effective than Cu at triggering this effect. How either metalloid actually accomplishes this remains unknown. It appears likely that binding to the metal-binding motifs located in the N-terminal extracellular portion of hCTR1 causes a conformational change that marks the protein for loading into macropinosomes, but this has yet to be established.

While the fate of hCTR1 following DDP exposure is now documented, the fate of the DDP that is presumably bound to it or transported by it has yet to be

determined. Previous reports illustrate that hCTR1 does in fact deliver DDP into the cell (Holzer et al., 2004b; Ishida et al., 2002; Song et al., 2004), but where it traffics in the cell and how it reaches the DNA is unknown. The results presented here suggest that while hCTR1 can contribute to the influx of DDP over the first few minutes, since it rapidly disappears from the cell, some other non-hCTR1-dependent uptake process is responsible for the subsequent cellular accumulation of DDP which continues for a number of hours until steady-state levels are attained (Holzer et al., 2004b; Song et al., 2004; Song et al., 2005). In future studies it will be of substantial interest to determine whether amiloride or a proteosome inhibitors can be used to pharmacologically increase DDP uptake into normal tissues that that express large amounts of hCTR1. If amiloride can fix hCTR1 on the cell surface and hCTR1 is behaving as a transport channel for DDP, then it is feasible that increased hCTR1 at the cell surface will result in increased DDP accumulation in the cell, thus increasing the efficacy of DDP.

Comparison of hCTR1 levels and function in DDP sensitive and resistant ovarian carcinoma cell lines.

In an attempt to determine the relevance of hCTR1 in resistance to platinum-based chemotherapy, pairs of sensitive and resistant isogenic cell lines were screened for mutations and alterations in hCTR1 at the genomic, translational and protein level. The only alteration in cell pairs was in the UCI07 pair in which the resistant line had a decrease in hCTR1 protein. This decrease did not significantly influence initial copper uptake. However, the difference in copper uptake was significantly different among the other isogenic cell lines, suggesting that selecting for DDP resistance does

influence the cellular modulation of copper. The copper efflux proteins, ATP7A and ATP7B have been shown to influence DDP sensitivity (reviewed in (Safaei and Howell, 2005)). In fact, Katano et al showed increased ATP7A or ATP7B in three of the 6 isogenic lines examined here (Katano et al., 2002). Further studies into the mechanism of DDP resistance in the remaining isogenic pairs should involve the examination of those proteins.

Expression patterns of hCTR1 in normal and malignant human tissue.

All cells require Cu for the function of key enzymes including cytochrome *c* oxidase, Cu,Zn-superoxide dismutase, lysyl oxidase, dopamine β-hydroxylase, tyrosinase, or multicopper ferroxidases such as ceruloplasmin. Since Cu is a highly polar ion that does not diffuse across cell membranes, transporters are required to bring Cu into the cell. CTR1 has been identified as the major Cu influx transporter in mammalian cells and studies in which both alleles of mCTR1 have been knocked out have shown it to be essential to murine embryonic development. Given the importance of this protein for development in mice, and the requirement of every human tissue for Cu, one might have expected to find hCTR1 expressed monotonously in all human tissues. However, the current study demonstrated that hCTR1 is detectable by immunohistochemical staining in only a limited subset of normal tissues, and often in only specific types of cells within the tissue. Why hCTR1 expression is not detected in all normal tissues is unclear. Low level expression of hCTR1 may be sufficient to serve the needs of most tissues and such low-level expression may not be detectable by immunohistochemical staining. Alternatively, there may be other Cu transporters

that serve the needs of most tissues for Cu and the higher level expression observed in some tissues and types of cells may reflect an unusually high Cu requirement or another non-Cu-related function of hCTR1.

It is important to note that many types of cells that stained most strongly for hCTR1 are of enteroendocrine original, and in many cases they fall into the family of amine precursor uptake and decarboxylation (APUD) cells. Additionally, the APUD cells of the bronchiolar epithelium, parafollicular cells of the thyroid and gastrin (G) cells of the stomach were also found to express high levels of hCTR1 (Ross et al., 2003). The best example is the pancreatic islets, wherein the α cells responsible for secreting glucagon have high levels of hCTR1 expression but the insulin-producing β cells do not. No specific relationship between the unique biochemical features of APUD cells and Cu metabolism has been identified. One possibility is that APUD cells require a specific Cu-dependent enzyme or process to manage the production and secretion of their various hormones and thus secondarily require a lot of Cu to service these enzymes (Petris, 2004). Another is that hCTR1 or Cu itself is involved in a critical step in the vesicle secretory process.

For the most part the frequency and intensity of hCTR1 staining in malignant tissues was found to reflect the levels found in their normal tissue counterparts. Among the normal tissues that did not express hCTR1 the only example of a tumor arising from the tissue that expressed hCTR1 was testicular cancer, and in this study only 1 sample was available. Thus, unlike many other genes, the expression of *hCTR1* does not appear to be frequently up-regulated by the transformation process. Among the tumors arising from normal tissues that expressed hCTR1, only in the case of

gastric carcinoma did none of the tumor samples available for study express any hCTR1. Breast and colon cancers were of particular interest. While hCTR1 was expressed in over 60% of normal breast epithelia, only 18% of invasive breast cancers stained for hCTR1. In the case of colon carcinoma, while the frequency of staining in primary tumors was low, most metastases in lymph nodes stained strongly. Thus, it appears that the host tissue environment of the tumor cell may have a strong influence on the expression of hCTR1.

Consistent with the fact that several types of APUD cells stained strongly in normal tissues, all 3 carcinoid tumors available also stained. Although not clearly of APUD origin, the 1 case of Ewing's sarcoma stained intensely. Currently there are no known histologic markers that distinguish Ewing's sarcoma from related tumors such as adult neuroblastoma, malignant small-cell tumor of the thoracopulmonary region (Askin's tumor), paravertebral small-cell tumors and atypical Ewing's sarcoma. Whether hCTR1 can be used for this purpose will require study of additional cases of Ewing's sarcoma as well other primitive tumors of neuroectodermal origin.

The role of murine CTR1 on the uptake of Pt analogs.

The results reported in chapter 3 establish that CTR1 regulates the cellular accumulation of DDP but not L-OHP. This indicates that L-OHP must enter the cell by a different, and as of yet, unknown mechanism. The concept that these drugs have different influx transporters is consistent with their different spectrum of action against various types of human cancer. Identification of the major L-OHP influx transporter

may inform the design of additional Pt drugs that are even better substrates for the transporter than L-OHP.

When looking at the drugs from a structural point of view, in their native form only DDP has a square planar conformation similar to that of serum protein chelated Cu. CBDCA contains a cyclobutane dicarboxylato leaving group, and L-OHP contains a bulky oxalate leaving group in addition to a diaminocyclohexane ring. These additional constituents may prevent CBDCA and L-OHP from binding to CTR1 in a manner compatible with transmembrane transport. It is important to note that, even though knockout of CTR1 reduced the uptake of CBDCA, CBDCA may not be a substrate for CTR1 at all. When CBDCA is diluted into chloride-containing tissue culture medium, the chloride displaces the cyclobutane dicarboxylato ligand generating DDP as a metabolite which can then be transported by CTR1. Although the oxalate moiety of L-OHP is also displaced by chloride, the resulting compound still carries the bulky diaminocyclohexane ring and this metabolite would not be expected to be a substrate for CTR1. The hypothesis that the CTR1-mediated uptake of CBDCA is actually secondary to generation of DDP is indirectly supported by the observation that when DDP resistance is accompanied by reduced DDP uptake it is usually accompanied by cross-resistance to CBDCA but less frequently by resistance to L-OHP (Raymond et al., 2002).

Clinical significance of hCTR1 as a DDP transporter.

Based on previous reports and data presented in this work, CTR1 appears to account for a significant fraction of all the DDP accumulated by the cell (Ishida et al.,

2002; Lin et al., 2002). 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.* (Naganuma et al., 1984) found that Cu can protect mice against DDP toxicity *in vivo*, an affect that may be explained by Cu-mediated down-regulation of hCTR1. While it is unlikely that hCTR1 is the sole influx transporter for DDP, pharmacological antagonism of its ability to reduce hCTR1 expression may be the basis for a clinically important strategy for increasing delivery of this chemotherapeutic agent to tumors.

While the studies presented here did not find evidence that hCTR1 levels at tightly linked to the responsiveness of human tumors to Pt-based therapy, it is possible that screening tumors for the level of hCTR1 expression may lend to the development of a better strategy of treatment. A biopsy of the tumor can be harvested and screened for hCTR1 protein expression levels. If the tumor does not express high levels of hCTR1, then the likelihood of getting significant amounts of DDP into the tumor is reduced. In that case, alternate drug treatments that do not utilize the Cu transport pathway, such as oxaliplatin, could be used.

Future Directions.

The data presented in the body of this work raises several additional questions that warrant investigation. First of all, conclusive evidence that DDP itself is bound by hCTR1 is still lacking. NMR using purified hCTR1 N-terminal domains or other such *ex vivo* studies could be used to determine whether DDP is directly bound by hCTR1 or if there is an accessory protein involved. To identify whether DDP binds hCTR1 in

the same manner as Cu, DDP uptake in cells transfected with hCTR1 mutants lacking

Cu binding domains could be compared to those transfected with wild-type hCTR1. If

mutations in the Cu binding motifs do not prevent DDP uptake, then mutants lacking

the N-terminal histines and cysteines could be used to determine the exact binding

region of DDP. To determine whether DDP is actually being transported by hCTR1,

DDP accumulation in cells transfected with constructs containing mutations in the

transmembrane regions of hCTR1 could be measured and compared to wild-type

constructs.

Based on the results of the MEFs lacking CTR1, approximately 60% of the

DDP accumulated by a cell in 1 h is taken up by CTR1. The mechanism of uptake for

the other 40% of cellular DDP accumulation is a clinically important question.

Considering the rapid down regulation of hCTR1, it is possible that hCTR1 is

responsible for the initial uptake of DDP and a second, slower mechanism is

responsible for subsequent accumulation of DDP. One possible secondary mechanism

is through transferrin and the transferrin receptor. Iron (Fe) and copper have many

similarities, including their essential role in the respiratory pathway and other cellular

proteins and the ability to form free oxygen radicals. Similar to the proteins of the Cu

transport pathway, transferrin transports iron in the blood and is responsible for

preventing iron from forming free radicals. Like Cu, ceruloplasmin in the blood may

be necessary for loading iron onto transferrin. Additionally, transferrin has several

histidine sites available for coordination with Fe. Studies have shown that DDP binds

transferrin (Allardyce et al., 2002), and observations made in DDP uptake studies can

be explained by uptake and internalization with transferrin. For example, the use of

endocytotic inhibitors in ovarian carcinoma cells dramatically decreases DDP accumulation. Transferrin is brought into the cell through clathrin mediated endocytosis, which is prevented by most of the common endocytotic inhibitors such as nystatin and methyl-B-cyclodextran, thus explaining the lack of DDP accumulation.

Additionally, the role of the chaperone proteins in DDP transport could be a clinically relevant question. Our studies indicate that hCTR1 level is not a prime determinant of tumor resistance to DDP, but perhaps the chaperone proteins responsible for the intracellular movement of Cu and DDP do influence drug sensitivity. The cellular chaperone responsible for delivering Cu and perhaps DDP to the nucleus would be a good candidate protein in the study of DDP resistance. Mutations in said chaperone could result in altered affinities for DDP resulting in less DDP reaching nuclear DNA. Likewise, decreases in the protein levels of that chaperone could also lead to decreased DDP delivery to the nucleus. Alterations in another Cu chaperone, Cox17, could also affect DDP cytotoxicity through alterations in mitochondrial functions.

Possible mechanisms of DDP transport following internalization by hCTR1.

Perhaps the biggest question that has yet to be addressed is what happens to the DDP once it is internalized by hCTR1. The results detailed in this work suggest several possible models of DDP accumulation in human tumor cells. In all these hypothetical models, DDP is bound by hCTR1 present on the plasma membrane. hCTR1 is then rapidly internalized through the endocytotic process known as macropinocytosis. The proteasome then degrades hCTR1 within 15 min of exposure to

DDP. What happens to the DDP and how it reaches the nucleus and other organelles to trigger apoptosis is undetermined. One could hypothesize a model in which the DDP is released from hCTR1 into a vesicle, where it is acquired by some other heavy metal binding protein and is eventually transported to the DNA. It is also possible that the DDP is released from the vesicle when it merges with other cellular membranes. Based opn currently available data, it appears likely that the Cu transport pathways continue to be utilized in the intracellular transport of DDP. Work by several laboratories has shown that the Cu efflux proteins ATP7A and ATP7B play a role in effluxing DDP (reviewed in (Safaei et al., 2004)). If that is the case, then the unknown chaperone protein responsible for delivering Cu to the nucleus may be rapidly interacting with the macropinocytosed vesicles containing DDP bound hCTR1 to acquire the DDP for transport to the nucleus. It is also important to note that even though adducts in the nuclear DNA are thought to be the main mechanism of DDP cytotoxicity, there are other aspects of the cellular function that are also dramatically affected by DDP and could contribute significantly to DPP cytotoxicity. For example, the mitochondria requires significant amounts of Cu. It is possible that DDP mimics Cu and thus transported to the mitochondria by the cellular chaperone, Cox17, for use in the Cu requiring enzymes of the respiratory chain. If DDP does replace Cu in complex IV of the respiratory chain, then complex IV would not be able to undergo the redox reactions necessary for the generation of ATP in the mitochondrial matrix, resulting in cell death. Similarly, DDP could be poisoning other proteins in the cell by replacing Cu, leading to improper function resulting in cell death.

Conclusions.

In conclusion, I have established that while hCTR1 does play a role in the transport of DDP into the cell, its level of expression is not tightly linked to the DDP-resistant phenotype in human ovarian cell lines or tumors. hCTR1 is expressed at high levels in certain types of normal human tissue and the tumors that arise from these tissues. Finally, the cellular trafficking of hCTR1 most likely involves the continual cycling of the protein. On exposure to DDP hCTR1 is rapidly internalized through macropinocytosis and degraded by the proteasome. These findings provide insight into how the Cu homeostasis mechanisms modulate the cellular pharmacology of the Pt drugs that have relevance to the development of novel strategies for the therapeutic use of these important drugs.

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