**UNIVERSITY OF CALIFORNIA, SAN DIEGO**

# The Role of Copper Transporter 1 (CTR1) in the Cellular Accumulation of Platinum Drugs

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

Doctor of Philosophy

in

Biomedical Sciences

by

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2010

The Dissertation of Christopher Alan Larson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2010

# DEDICATION

To my wonderful wife, Alicia, for standing beside me and always encouraging and supporting me. Thanks for all that you do. I could never have done this without you – you truly are my better half, I love you.

To my parents, Maureen and Alan, for their undying support of me ever since I was a little kid. Thanks for always challenging me to rise up, and more importantly for always bearing with me through the hard times.

## EPIGRAPH

**All for one, one for all.**

*Alexandre Dumas père*

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# ACKNOWLEDGEMENTS

I would like to thank the following people:

Nissi Varki for her assistance with histology analysis and technical expertise.

Laarni Gapuz and the UCSD Cancer Center Histology and

Immunohistochemistry Shared Resource for their technical assistance.

Michael Petris for his assistance and expertise with relation to CTR1.

Kersi Pestonjamasp for his training and assistance with ICC imaging.

Amy Harpin and MCCT Vivarium for technical expertise and assistance with the *in vivo* studies.

Leanne Nordeman and Gina Butcher of the Biomedical Sciences Program for their never-ceasing assistance and support both administrative and non.

I would like to thank Drs. Don W. Cleveland, Daniel J. Donoghue, Seth J. Field and Nicholas J. Webster for devoting the time to serve on my dissertation committee. Your guidance, input and support has made me a better scientist and is greatly appreciated**.**

To my colleagues in the Howell lab past and present:

Alison Holzer for bringing this CTR1 project to life and for the willingness to answer all those questions, even after you left the lab. Thank you.

Goli Samimi for teaching me the ropes of the Howell Lab.

Brian Blair for being my counterpart and sounding board as well as for assistance and contributions throughout the entirety of this dissertation.

Preston Adams for indispensable assistance with these studies and for valuable discourse.

Gerald "Jack" Manorek for assistance throughout the entirety of the dissertation and for invaluable technical expertise and discourse.

Xinjian Lin for providing valuable insight and technical assistance.

Paolo Abada for valuable technical discourse.

Catherine Pesce and Danielle Jandial for help with *in vivo* work and for valuable assistance in these studies.

Angela Robles for her help with so many of the administrative aspects of all these studies.

Nicole Chung for her assistance in these studies.

Sakura Moua and Phillip Chang for assistance.

Lastly, I'd like to thank my mentor, Stephen B. Howell, for his guidance, counsel, and advice. Thanks for your commitment, encouragement and your constant challenging me to push myself harder. Most importantly, thanks for your understanding and devotion to making me a true scientist. Your constant input, and discourse is greatly appreciated. I have enjoyed my time in the lab and am grateful for all the time and effort you have put into helping me succeed in my career and life.

On a personal level I would like to acknowledge the following people for their help and support:

First and foremost my wife, Alicia, for her love, support and help. No matter the time, urgency or my unwillingness, you helped me to get things done. I love you. Thank you.

My parents, Alan and Maureen, thank you for all your support and encouragement. I will never be able to say thank you enough for all you have done for me. Thank you.

My sister, Michelle and her family- Mike, Madison and Damon. For years and years you've been there to support me and make me laugh. I wish I could express how much you've been able to help me. Thanks.

My in-laws, Don and Sally Waite. For supporting and encouraging me as a son, for understanding when we ended up so far away and the endless concern for us. Thanks.

Danny and Darcie, Tim and Jenelle and, of course, Nick. Thanks for the laughs, the late nights, way too many inside jokes, the random photos and the encouragement and support over the years.

Ed and Andrea, for years of support and concern, for always being happy to hear from us and for countless laughs.

The Three Musketeers- Shane, Wes and Mike for all the years of making sure I'd end up where I'm at.

Acknowledgements for individual chapters are as follows:

**Chapter 1**: Christopher A. Larson was the primary author of this chapter. Stephen B. Howell supervised the writing of this chapter.

**Chapter 2**: A majority of the content of Chapter 2 has been published in *Molecular Pharmacology* (Larson CA, Blair BG, Safaei R, Howell SB. The Role of the Mammalian Copper Transporter 1 in the Cellular Accumulation of Platinum-based Drugs. Mol Pharmacol. 2009 Feb;75:324, 2009.) Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Brian G. Blair assisted in the measurement of Pt

and Cu accumulation and provided helpful feedback. Roohangiz Safaei provided helpful discussion. The author would like to thank Dr. Dennis Thiele for generously providing the $CTR1^{+/+}$ and $CTR1^{-/-}$ mouse embryo fibroblasts used in these studies.

**Chapter 3**: A majority of the content of Chapter 3 has been published in *Molecular Pharmacology* (Larson CA, Blair BG, Safaei R, Howell SB. The Role of the Mammalian Copper Transporter 1 in the Cellular Accumulation of Platinum-based Drugs. Mol Pharmacol. 2009 Feb;75:324, 2009.) Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Brian G. Blair assisted in the *in vivo work* and provided helpful feedback. Roohangiz Safaei provided helpful discussion. The author would like to thank Dr. Dennis Thiele for generously providing the $CTR1^{-/-}$ mouse embryo fibroblasts used in these studies.

**Chapter 4**: A majority of the content of Chapter 4 has been accepted for publication in *Biochemical Pharmacology* (Larson CA, Adams PL, Jandial DD, Blair BG, Safaei R, Howell SB. The Role of the N-terminus of Mammalian Copper Transporter 1 in the Cellular Accumulation of Cisplatin*) Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Preston L. Adams assisted in the measurement of Pt and Cu accumulation and provided helpful feedback. Danielle D. Jandial and Brian G. Blair provided valuable assistance and feedback. Roohangiz Safaei provided helpful discussion. The author would like to thank Dr. Dennis Thiele for generously providing the $CTR1^{-/-}$ mouse embryo fibroblasts used in these studies.

**Chapter 5**: A majority of the content of Chapter 5 has been submitted for publication. Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Preston L. Adams assisted in the measurement of Pt and Cu accumulation and provided helpful feedback. Brian G. Blair provided valuable assistance and feedback. Roohangiz Safaei provided helpful discussion. The author would like to thank Dr. Dennis Thiele for generously providing the $CTR1^{-/-}$ mouse embryo fibroblasts used in these studies.

**Chapter 6**: A majority of the content of Chapter 6 has been submitted for publication. Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Preston L. Adams assisted in the measurement of Pt and Cu accumulation and provided helpful feedback. Brian G. Blair provided valuable assistance and feedback. Roohangiz Safaei provided helpful discussion. The author would like to thank Dr. Dennis Thiele for generously providing the $CTR1^{-/-}$ mouse embryo fibroblasts used in these studies.

**Chapter 7**: Christopher A. Larson was the primary author of this chapter. Stephen B. Howell supervised the writing of this chapter.

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2009 – Blair, B.G., Larson, C.A., Adams, P.L., Pesce, C.E., Safaei, R., Howell, S.B., Copper Transporter 2 (CTR2) regulates tumor growth and sensitivity to cisplatin *in vivo* (In Press)

2009 – Blair, B.G., Larson, C.A., Adams, P.L., Abada, P.B., Safaei, R., Howell, S.B., Regulation of CTR2 Expression by Copper and Cisplatin in Human Ovarian Carcinoma Cells, (In Press)

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2010 – Larson C.A., Adams P.L., Jandial D.D., Blair B.G., Safaei R., Howell S.B., The Role of the N-terminus of Mammalian Copper Transporter 1 in the Cellular Accumulation of Cisplatin. (In Press)

2010 – Larson C.A., Adams P.L., Blair B.G., Safaei R., Howell S.B., The Role of the Methionines and Histidines in the Transmembrane Domain of Mammalian Copper Transporter 1 in the Cellular Accumulation of Cisplatin, (Submitted)

2010 – Larson C.A., Adams P.L., Blair B.G., Safaei R., Howell S.B., Transport and Cytotoxicity of Cisplatin and Copper Mediated by Variant Forms of Copper Transporter 1, (Submitted)

# ABSTRACT OF THE DISSERTATION

## The Role of Copper Transporter 1 (CTR1) in the Cellular Accumulation of Platinum Drugs

by

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Doctorate of Philosophy in Biomedical Sciences

University of California, San Diego, 2010

Professor Stephen B. Howell, Chair

Platinum (Pt)-based chemotherapeutics are widely used, polar molecules that do not readily diffuse across the plasma membrane. Most patients develop resistance to Pt drugs. Pt resistance is not fully understood, however one common phenotype is decreased Pt accumulation inside the cells. As such, an understanding of how Pt molecules enter the cell is essential. The copper (Cu) pathway has been shown to be important for Pt to enter the cell.

The overall goal of the experiments described in this dissertation was understanding the role of the main copper transporter (CTR1) in the accumulation of Pt drugs inside the cell, and understanding the domains of CTR1 required for Pt transport. This was accomplished by studying cell lines that express wild type CTR1 ($CTR1^{+/+}$), cells that have a homozygous knockout of CTR1 ($CTR1^{-/-}$) and cells that have been transduced to express mutant variations of CTR1, targeted to key regions of the CTR1 molecule.

It was discovered that CTR1 is a key player in the accumulation of not only cisplatin (cDDP), but also carboplatin (CBDCA) and oxaliplatin (L-OHP); as studied by 0 second and 5 minute drug accumulation. It was also shown that the isomer transplatin is not transported by CTR1. It was demonstrated that CTR1 is a key to sensitivity to cDDP *in vitro*. Importantly, CTR1 was shown to be vital to cDDP response *in vivo*. The role of CTR1 was demonstrated by re-expressing CTR1 in the $CTR1^{-/-}$ cells and a restoration of cDDP sensitivity and accumulation was seen. Building on these observations, mutant variants of CTR1 were expressed in $CTR1^{-/-}$ cells. Analysis of the N-terminal region of CTR1 revealed a role in the accumulation of cDDP and in sensitization, but not in controlling down-regulation. Analysis of the CTR1 pore demonstrated that M150,154 residues play a role in controlling the influx of cDDP. Finally, it was demonstrated that the Y103 residue is important for controlling the influx of cDDP, sensitivity to the drug and the ability to down-regulate CTR1 in the presence of the drug. It was also discovered that C189 is important for CTR1 functionality for cDDP accumulation and sensitivity

# Chapter 1 –

# Introduction

## Platinum-based Chemotherapeutics

The discovery implicating the ability of Pt-based complexes to inhibit bacterial growth in 1965 by Rosenberg, *et al.*(Rosenberg et al., 1965), led to the development of the Pt-based group of chemotherapeutics. Numerous Pt compounds were examined for potential use as anti-cancer agents. Background information of three major Pt drugs currently approved for use in the treatment of cancer is contained in the following sections.

### Cisplatin

Cisplatin (cDDP) is a widely used chemotherapeutic agent that has a large range of application and a high level of efficacy (Chu, 1994; Fuertes et al., 2003; Jordan and Carmo-Fonseca, 2000; Rabik and Dolan, 2007). cDDP can be administered either intravenously or intraperitoneal, however immediately upon entering the body the cDDP reacts with plasma proteins and much of it becomes bound, and thus unreactive. Upon reaching the cell, the chloride side groups of the molecule (Figure 1-1 a,b) become leaving groups thus allowing the cDDP to become primed and ready to react to side groups of amino acid residues including cysteine, histidine, and methionine. While cDDP is known to bind with DNA and RNA by forming adducts of the purine bases which have been shown to inhibit transcription and synthesis

(Johnson et al., 1997; Jordan and Carmo-Fonseca, 2000; Zorbas and Keppler, 2005), the majority of these purine adducts are covalently at the $N^7$ position, generally 1,2d(GG) and 1,2d(ApG) intrastrand crosslinks; however up to 20% of the adducts may be interstrand or 1,3-intrastrand crosslinks (Johnson et al., 1997; Sedletska et al., 2005; Zorbas and Keppler, 2005). The intrastrand adducts lead to bending of the DNA which is thought to aid in the recognition of DNA damage by cellular machinery (Chu, 1994; Jordan and Carmo-Fonseca, 2000).

Adduct-damaged DNA is thought to trigger G2 cell arrest and also the activation of apoptosis. Such a response appears to be dependent on the mismatch repair system and the ability of the repair enzyme complexes to recognize the cDDP-damaged DNA(Gong et al., 1999; Lin et al., 1999). Cells that lack the mismatch repair pathway have been shown to be more resistant to cDDP damage (Gong et al., 1999; Lin and Howell, 1999; Lin et al., 1999).

**Carboplatin and Oxaliplatin**

Both carboplatin (CBDCA) and oxaliplatin (L-OHP) are later generation Pt compounds, both derived from the initial cDDP template. CBDCA, while sharing the dichloride leaving group in common with cDDP has a very distinct toxicity spectrum. The 1,1 cyclobutanedicarboxylate leaving group facilitates a much more stable compound in aqueous environments leading to a slower rate of aquation and a decreased reactivity (Jordan and Carmo-Fonseca, 2000). CBDCA has a very similar treatment profile to cDDP and also demonstrates a high level of cross-resistance with cDDP, most likely due to the similar mechanism of cytotoxicity. L-OHP is a third generation Pt compound that is very distinct from both CBDCA and cDDP, as a result

it shows very little cross-resistance with cDDP or CBDCA. L-OHP belongs to a class of Pt compounds referred to as diaminocyclohexane (DACH) compounds. L-OHP is often used to treat multiple forms of cancer and has been shown to have some cytotoxicity against cDDP-resistant tumors, both *in vitro* and *in vivo* (Raymond et al., 2002).

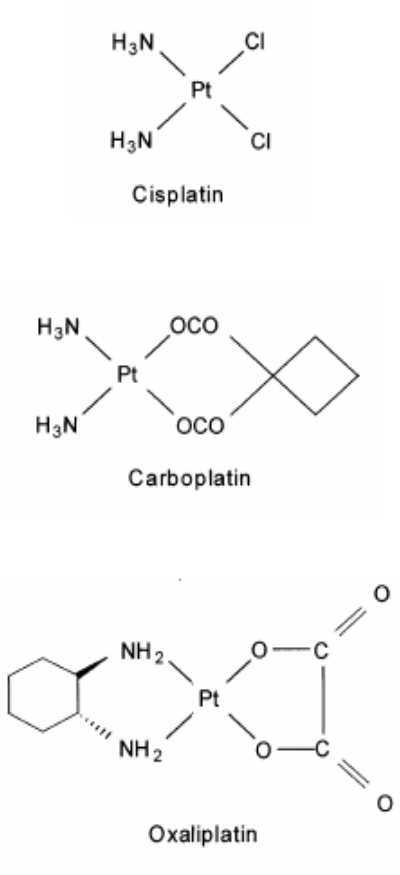


**Figure 1-1. Chemical structure of Platinum drugs.** Chemical structure of cisplatin (cDDP), carboplatin (CBDCA) and oxaliplatin (L-OHP).

## Resistance to Pt-based chemotherapeutics

The development of resistance to Pt-based drugs is a common occurrence during the course of chemotherapy (Andrews and Howell, 1990; Niedner et al., 2001; Schabel et al., 1980). Resistance to Pt drugs is thought to be a result of mutations that result in either a decrease in the total accumulation of Pt or else an increase in the ability of the cell to accommodate the mutagenic effects of the Pt drugs. Resistance to cDDP is usually of a relatively low nature, often an increase of only 1.5 – 3-fold increase in resistance is found both *in vivo* and *in vitro* (Inoue et al., 1985; Wilson et al., 1987). Previous studies have shown that such low levels of increased resistance are seen also in experimental animals (Andrews et al., 1990). Low levels of increase can still result in clinical failure of treatment (Muggia and Los, 1993).

### Rise of mutations

Due to the highly mutagenic nature of cDDP, resistance can often be acquired after a single exposure to the drug, and by extension, resistance to other drugs, including CBDCA and L-OHP (Lin et al., 1999). Development of resistance is thought to be a result of two factors: first, the genetic instability of most cancers, in conjunction with the mutagenic nature of the treatment allows for a rise of mutations in the population of the tumor. Secondly, the selective pressure of the treatment allows for an increased survival of cells that contain one or more mutations that confer some resistance to the Pt treatment. This selective pressure allows for a formation of a secondary colony of cells that are resistant to treatment, ultimately leading to the failure of treatment.

### Mechanisms of resistance

While it is known that cells can develop resistance to Pt drugs it is not known the mechanisms or systems involved in such resistance. Several mechanisms have been shown to be altered in cell lines showing resistance including changes to drug influx and efflux, down-regulation of the apoptosis and cell death pathways and deficiencies in the mismatch repair pathway (Blair et al., 2009; Chu, 1994; Crul et al., 1997; Kuo et al., 2007; Lin and Howell, 2002; Manic et al., 2003; Sedletska et al., 2005). While there appears to be no single mechanisms linked to resistance, an overall trend is that of decreased accumulation of Pt in resistant cell lines *versus* sensitive cells (Gately and Howell, 1993; Kelland et al., 1992; Oldenburg et al., 1994; Song et al., 2004; Teicher et al., 1991; Twentyman et al., 1992; Waud, 1987). The mechanisms by which cDDP may enter the cell will be discussed later in this chapter.

## Copper Transporter 1 (CTR1)

CTR1 is a unique transmembrane transporter that has been shown to be important in the regulation of not only Cu transport, but has also been implicated in the transport of cDDP, and by association has been suggested to be important for the transport of other Pt drugs. Little is known about the transporter, how it functions, how it is regulated, trafficked or even the mechanism that controls transcription of the *SLC31A1* gene (the gene associated with CTR1).

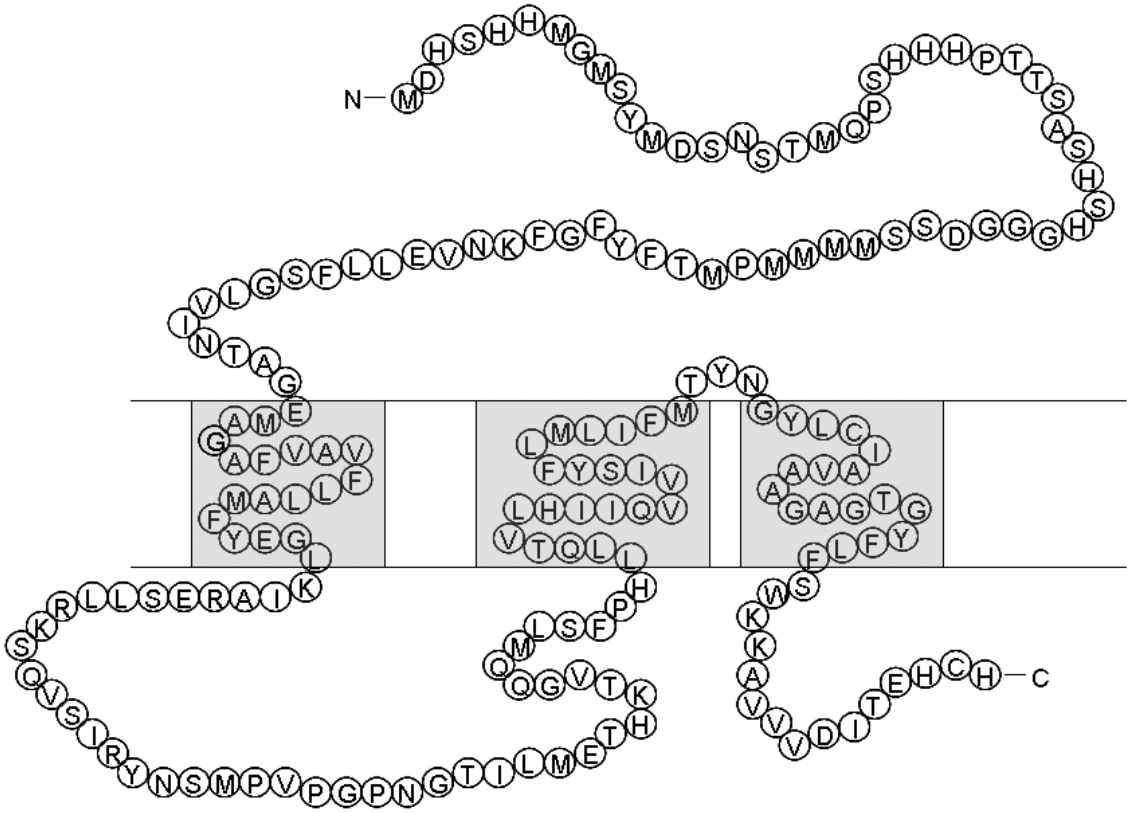

**Figure 1-2. Schematic diagram of the amino acid sequence of hCTR1.**

**Characterization of CTR1**

CTR1 was discovered while searching for complementary genes to restore the phenotype of *ctr1* deficient yeast cells (Zhou and Gitschier, 1997). Upon identifying CTR1 it was discovered that the protein CTR1 contains similar transmembrane motifs as seen in yeast, suggesting that the protein was a high-affinity Cu transporter and contained the ability to restore loss of function in *ctr1*$^{-/-}$ yeast cells (Zhou and Gitschier, 1997). Later it was shown that over expression of the hCTR1 gene had a marked influence on Cu influx in human fibroblast cells (Moller et al., 2000). CTR1 is

a highly conserved protein containing almost an identical sequence between human and mouse forms (92% sequence homology).

CTR1 is a 190 residue, 28 kDa protein that undergoes glycosylation at 2 sites on the N-terminus of the molecule resulting in a mature 35 kDa molecule (Petris, 2004). The *SLC31A1* gene consists of 573 bases across 4 exons. CTR1 has been shown to organize as a homotrimer with the N-terminus projected outside the cell and the C-terminal tail in the cytoplasm (Aller and Unger, 2006; De Feo et al., 2009; Eisses and Kaplan, 2002; Klomp et al., 2003; Lee et al., 2000; Petris et al., 2003)

The protein itself is divided into 3 distinct regions. The N-terminal extracellular hydrophobic domain of the protein is a 67 residue region of the protein that contains multiple metal-binding motifs capable of interacting with Cu. This N-terminal domain contains 4 such clusters, the first of which is the H1 region encompassing residues 3-6 and containing 3 histidines. The second is the M1 region that encompasses amino acids 7-12 and includes 3 methionines. The H2 cluster encompasses 3 histidines at positions 22-24, and the M2 region contains 5 methionines clustered together at positions 40 - 45. Prior studies have shown that, both in yeast and mammalian cells, the M2 region is required for Cu transport when environmental levels of Cu are low (Eisses and Kaplan, 2002; Guo et al., 2004; Liang et al., 2009; Puig et al., 2002), but when normal levels of Cu are available even truncation of the entire N-terminus of yCTR1 does not disable its ability to transport Cu (Puig et al., 2002).

The second region is the three transmembrane regions of the CTR1 molecule. The hydrophobic transmembrane regions create a pore when the CTR1 assembles as a

homotrimer in the plasma membrane, as seen in Figure 1-3. It is through this pore that Cu is believed to pass (De Feo et al., 2009). The inner faces of the pore contain methionines, cysteines, and histidine residues that facilitate the movement of Cu into the cell down the concentration gradient. Interactions between Cu and methionines 150 and 154, and histidine 139, are important determinants of the high affinity of CTR1 for Cu. These amino acids, along with methionines 43 and 45 and cysteine 189, are thought to form a series of stacked rings each of which can chelate $Cu^{+1}$ that is then handed down through the pore in a series of transchelation reactions (De Feo et al., 2009; Howell et al., 2010). Prior studies have established the importance of M150, M154 and H139 to the transport of Cu. These amino acids are of particular interest as structural studies suggest that they lie in the narrowest part of the pore formed by homotrimeric CTR1, and that methionines and histidines are often involved in binding Cu in other proteins. The requirement for sequential transchelation reactions appears to be the basis for the high selectivity of CTR1 which transports $Cu^{+1}$ but not $Cu^{+2}$

The third, intracellular, region consists of 2 distinct regions: the 46 residue that forms the loop between the first and second transmembrane domains, and the 15 residues that comprise the C-terminal tail of the molecule. The transmembrane loop region may be important in the regulation and trafficking of the CTR1 molecule. Contained within this region are several potential sites that may be phosphorylated, may be a direct target of PI3K or potentially a target of ubiquitin ligases such as NEDD4L, these sites include the PXPXP (107-111) domain and the tyrosine seen at position 103. The Y103 site is of particular interest because the YXXM motif within which it resides is a potential phosphorylation site and a candidate for binding to the

p85 subunit of PI3K which mediates the endocytosis of PDGF and many other surface proteins (Wu et al., 2003).

The final domain is the 15 residue C-terminal tail region of the molecule that contains two potentially key motifs, one being the lysine residues at 178 and 179 as well as the cysteine located at 189. CTR1 is known to exist as a homotrimer on the cell surface and electron crystallography indicates that it forms a pore with a narrow entrance at the extracellular end and a vestibule at the intracellular end (comprised of the C-terminal tail) (De Feo et al., 2009; De Feo et al., 2007). It has been proposed that C189 at the C-terminal end functions as a switch to open and close the pore (Wu et al., 2009a).

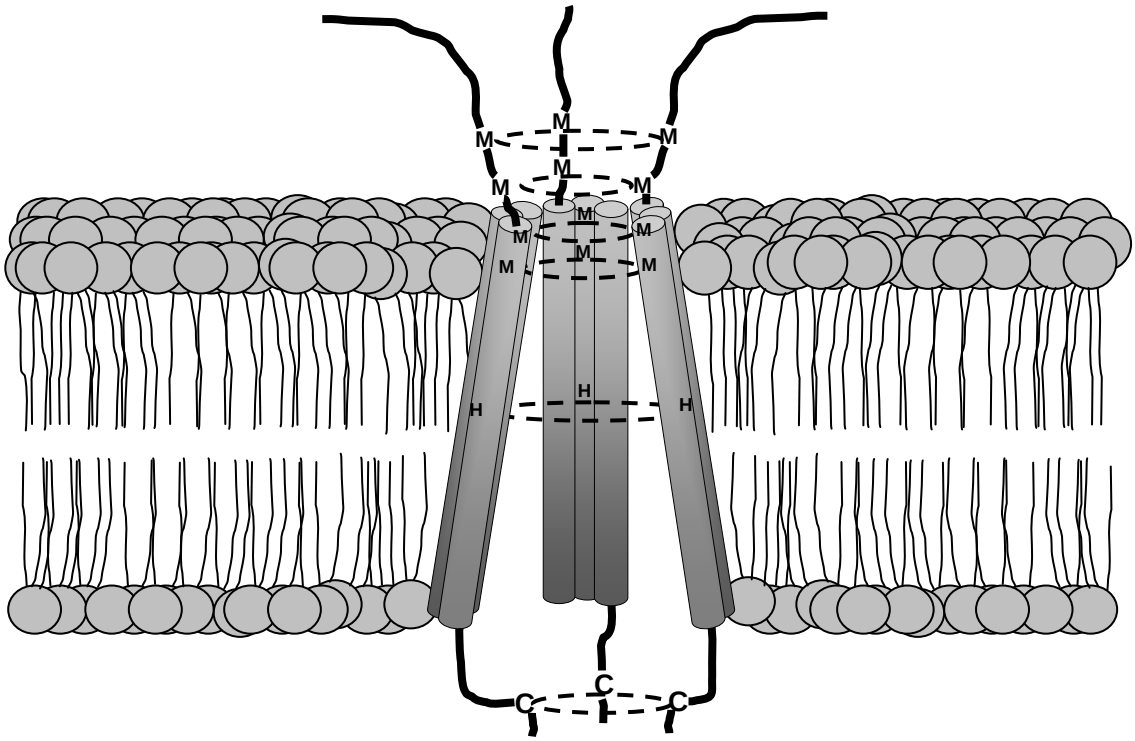


**Figure 1-3. Schematic of CTR1 homotrimer.** Schematic of CTR1 homotrimer as oriented in plasma membrane. Rings show stacked ring structure, including corresponding residues.

CTR1 is a highly specific molecule that is known to transport Cu(I) but not Cu(II), it is also very dependent on time, extracellular Cu concentration, extracellular K+ levels as well as extracellular pH (Lee et al., 2002a). Heavy metals such as zinc, iron, magnesium, cadmium and silver have been shown to serve as competition to $^{64}$Cu in transport; however the results are indicated only by decrease in Cu accumulation, not in actual accumulation of other compounds (Lee et al., 2002a).

There are two major mechanisms by which CTR1 may bring substrates in to the cell. The first is *via* the pore, as described above. The second is through down-regulation and endocytosis of the CTR1 molecule previously (Holzer and Howell,

2006). While it is not known to what extent each mechanism plays in accumulating Cu and cDDP in the cell. Understanding how these mechanisms both contribute to the accumulation of Cu and cDDP is important for a complete picture of cDDP trafficking, and also for understanding Cu homeostasis.

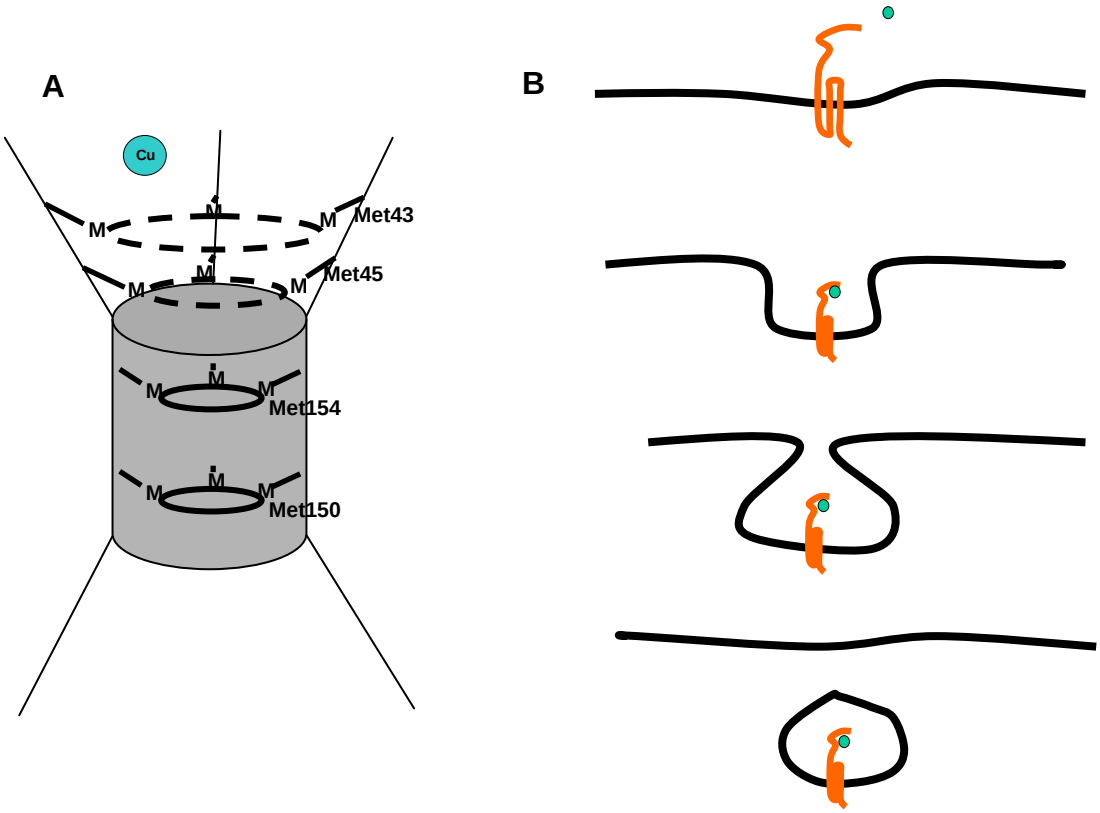


**Figure 1-4. Mechanisms of CTR1 import.** A, Copper molecule positioned next to CTR1 pore. Methionines 43, 45, 154 and 150 stacked rings shown. B, Endocytosis of CTR1 molecule upon interacting with substrate (Note while CTR1 exists as trimer, monomer is shown for simplicity.

### Cellular organization and localization

CTR1 is expressed in all mammalian tissues, with levels being highest in the liver, heart and kidney, while the lowest were found in the brain and muscle tissues

(Holzer et al., 2006b; Zhou and Gitschier, 1997). CTR1 is one of several transports that mediate the movement of Cu across the plasma membrane; it is essential for early embryonic development as knockout of both CTR1 alleles causes *in utero* lethality (Kuo et al., 2001; Lee et al., 2002b).

While the majority of the CTR1 is seen on the cell surface CTR1 is seen perinuclear and in Golgi regions of the cell (Klomp et al., 2002; Lee et al., 2002a; Petris et al., 2003). In many types of cells high concentrations of Cu trigger the endocytosis of CTR1, and in some types of cells this is accompanied by degradation (Petris et al., 2003) whereas in others it is not (Liang et al., 2009; Molloy and Kaplan, 2009). It has been hypothesized that degradation may serve to limit accumulation of toxic levels of the metal (Guo et al., 2004; Holzer et al., 2004a; Liu et al., 2007; Molloy and Kaplan, 2009). In yeast the degradation of CTR1 requires the E3 ubiquitin ligase Rsp5 (Liu et al., 2007). Similarly, in some types of cells cDDP triggers the endocytosis and degradation of CTR1 (Holzer and Howell, 2006; Jandial et al., 2009) whereas in others it does not (Liang et al., 2009). In cells in which cDDP induces degradation, CTR1 becomes ubiquitinated (Safaei et al., 2009) and inhibition of proteosome function can block the degradation (Holzer and Howell, 2006) and enhance the uptake and cytotoxicity of the drug (Jandial et al., 2009). The intracellular loop between trans-membrane domains one and two contains a tyrosine at position 103, and tyrosines in the cytosolic domains of proteins are often involved in endocytosis (Esposito et al., 2001).

## Copper transport

The initial finding that cells resistant to Cu toxicity displayed resistance to Pt drug toxicity and *vice versa* lead to discovery of an intimate linkage between the Cu homeostasis pathway and the Pt drug toxicity pathway (Fukuda et al., 1995; Naredi et al., 1994; Rixe et al., 1996). More recently evidence showing that cDDP resistance can come as a result of up-regulation of Cu export transporters further strengthened the connection between Cu transport and Pt accumulation (Higashimoto et al., 2003; Kanzaki et al., 2002; Katano et al., 2002a; Katano et al., 2003; Katano et al., 2002b; Ohbu et al., 2003). The following sections provide background information of Cu function in a cell, cellular homeostasis and transport, and the role that Cu transporters may function in mediating Pt drug transport.

### Background

Cu is an essential trace element required for the activity of multiple critical proteins, including p53, superoxide dismutase I, cytochrome *c* oxidase, lysyl oxidase, tyrosinase, ceruloplasmin and dopamine ß-hydrolase (reviewed in (Madsen and Gitlin, 2007)). The ability of Cu to undergo oxidation to Cu(II) and the reversal reduction to Cu(I) allows Cu to serve as the redox cofactor for multiple processes including detoxification of reactive oxygen species and electron transport (Linder and Hazegh-Azam, 1996). Due to the redox potential of Cu, Cu levels are highly controlled as high levels can become toxic to the cell. Excess Cu can displace other metal cofactors and cause dysfunction of normal cellular processes, as such no free Cu is usually present in a cell (reviewed in (Balamurugan and Schaffner, 2006; Bertinato and L'Abbe, 2004; Kim et al., 2008)).

**Copper Transport**

Due to the need to tightly regulate Cu levels in the cell eukaryotes have developed a complex system to control the influx, cellular trafficking, storage and efflux of Cu. To avoid toxicity Cu(I) is handed through the cell via a group of transporters and chaperones that share common metal binding motifs (MBS) usually rich in cysteine, methionine or histidine residues. As Cu enters the cell it is handed from protein to protein via MBS-containing proteins creating an environment with virtually zero (less than $10^{-18}$ M) free Cu(I) in the cell (Hamza et al., 1999; Lippard, 1999; Pena et al., 1999; Pufahl et al., 1997).

Figure 1-5 shows a schematic of the current understanding of the Cu homeostasis and metabolism pathway in mammalian cells. Cu(II) bound to ceruloplasmin reaches the cell surface where it is reduced by the FRE1 and FRE2 reductases (Georgatsou and Alexandraki, 1999; Hassett and Kosman, 1995). After being reduced to Cu(I), the Cu binds to the CTR1 molecule located at the cell surface where the CTR1 transports the Cu across the cell surface (reviewed in (Howell et al., 2010)). Upon entering the cell it is transferred to the chaperones Atox1, CCS and Cox17. Atox1 delivers Cu to the Golgi-associated transporters ATP7A and ATP7B (Huffman and O'Halloran, 2000; Klomp et al., 1997). CCS transports the Cu to the superoxide dismutase (SOD1) (Culotta et al., 1997). Cox17 is responsible for transporting Cu to the cytochrome *c* oxidase and the mitochondria (Amaravadi et al., 1997). ATP7A and 7B are thought to sequester the Cu in the *trans*-Golgi allowing the Cu to be delivered to ceruloplasmin and other Cu-dependent proteins. These two transporters also allow for the efflux of excess Cu by facilitating transport of Cu to the

plasma membrane, although it is not known whether the 7A and 7B handle this directly or merely facilitate another protein in the process, once at the plasma membrane Cu can be effluxed out of the cell (Camakaris et al., 1995; Petris et al., 1996; Petris and Mercer, 1999; Roelofsen et al., 2000).

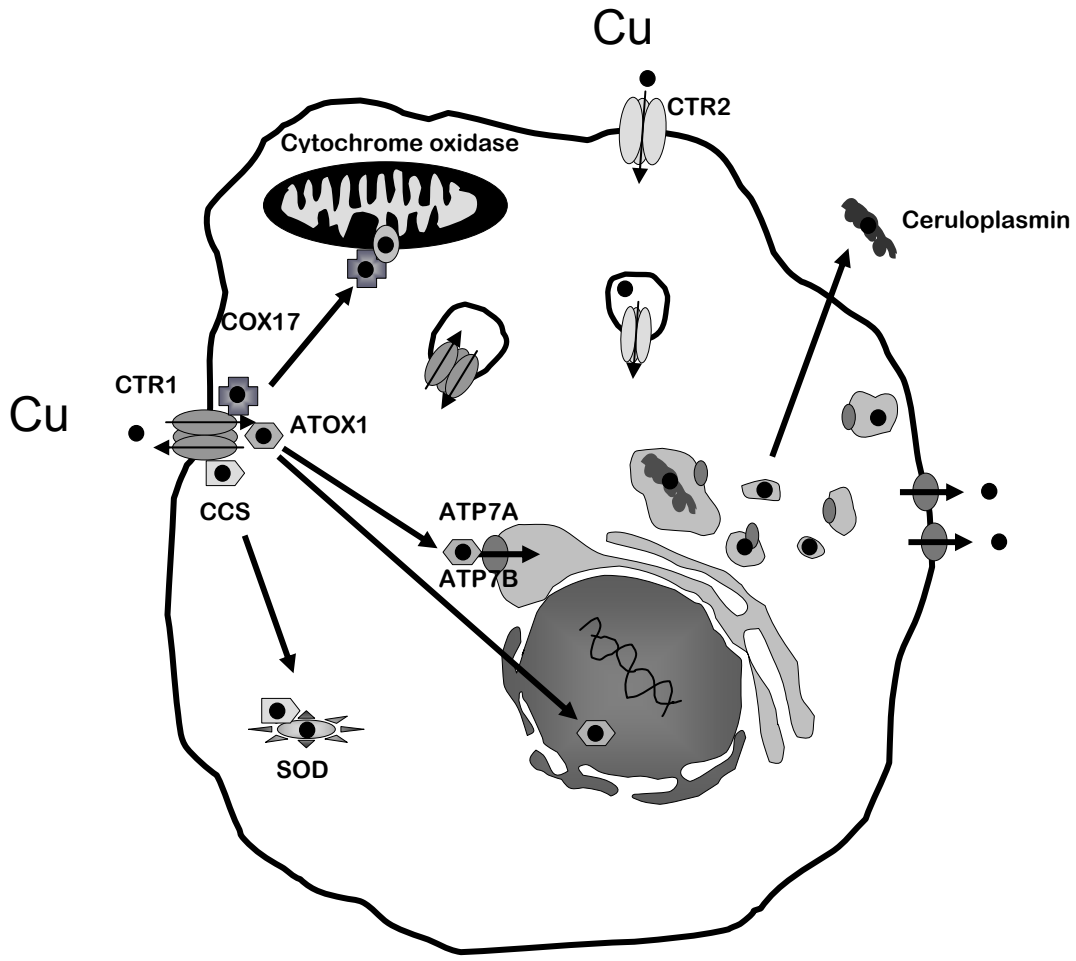


**Figure 1-5. Copper transport pathway.** Schematic showing Cu import and trafficking, including major players.

**Copper transport proteins and platinum drugs**

Due to the polar nature of Pt drugs it is not believed that they can readily diffuse across cell membranes. As a result the drugs must enter the cell via transporters such as channels, pores and pumps (Andrews and Albright, 1991; Andrews et al., 1988; Mann et al., 1991). Similar to Cu transport, cDDP transport has been shown to be modulated by pH, $K^+$ ion concentrations, as well as the presence of reducing agents (Amtmann et al., 2001; Atema et al., 1993; Blasiak et al., 2002; Chen et al., 2005; Marklund et al., 2004; Sarna and Bhola, 1993; Zhang et al., 1994). More convincing evidence of the strong link between Cu transport and Pt drugs was demonstrated when Komatsu *et al.* reported that an increase in ATP7B had been seen in cDDP resistant cells (Komatsu et al., 2000). This report and others have shown that over-expressing ATP7B results in less cDDP accumulating in the cell as well as an increase in efflux of cDDP, indicating that the transport may be directly tied to the export of cDDP from the cell (Komatsu et al., 2000; Safaei and Howell, 2005). More recently it has been shown that both ATP7A and ATP7B are over-expressed in some Pt drug resistant ovarian carcinoma cell lines, as expected these cell lines show a decreased accumulation of Cu and Pt (Katano et al., 2002a; Katano et al., 2003; La Fontaine et al., 1998). Samimi *et al.* demonstrated that alteration of ATP7A expression modulated sensitivity to Pt drugs and demonstrated a correlation between sensitivity and total Pt accumulation in the same cell lines (Samimi et al., 2004b). Direct correlation between CTR1 and Pt drug accumulation was shown by Holzer *et al.*, as well as several other groups (Holzer et al., 2004b; Ishida et al., 2002; Lin et al., 2002). While CTR1 seems to be the major player in the accumulation of Pt drugs it has been

shown repeatedly that there is a smaller amount of Pt drug that enters the cells, regardless of the presence or absence of CTR1 (Holzer et al., 2004b). Such a finding suggests that other players may exist to aid in the movement of Pt across the plasma membrane. One major candidate that appears to play a role in the movement of both Cu and Pt is CTR2 (Blair et al., 2010; Blair et al., 2009). It is easy to envision a system that utilizes both systems to control the movement of Pt and Cu into the cell.

## Summary

The Pt-containing drugs, including cDDP and CBDCA, are widely used chemotherapeutics that, while highly effective, are limited in effectiveness due to the frequent development of resistance during treatment. There is an established correlation between Cu resistance and Pt resistance. Furthermore there is a connection between the Cu pathway and Pt transport. Specifically, Copper Transporter 1 (CTR1) has been shown to play a role in the transport of Pt into cells, once inside other Cu chaperones and transporters have been shown to regulate the level of Pt in the cell. The information and previous work suggests that CTR1 functions not only as the main Pt drug transporter but that alterations to the molecule may alter the function of the transport in the movement of Pt.

## Hypothesis

The ability of CTR1 to regulate the accumulation of Pt drugs is an established phenomenon. The objective of this dissertation research was to assess the structure and

motifs of the CTR1 molecule with respect to their function in controlling the accumulation of cDDP. The hypothesis tested was that mutations to certain regions and/or key residues of hCTR1 would alter the ability of the cell to transport cDDP across the cell membrane, and furthermore would alter the cytotoxicity of the cDDP.

# Chapter 2 –

# Role of Mammalian CTR1 in the Accumulation of Platinum Drugs

## Introduction

The goal of the experiments described in this chapter was to determine the role of CTR1 in accumulating cDDP, CBDCA, L-OHP and transplatin, especially with respect to the initial uptake of drug. The role of CTR1 was studied by utilizing an isogenic pair of mouse embryonic fibroblasts, a wild type cell line ($CTR1^{+/+}$) and a line in which both alleles of CTR1 were knocked out ($CTR1^{-/-}$). Additionally a subline in which expression of wild type CTR1 was restored was created using lentiviral induction. Such re-expression facilitates the verification that the observed phenotype of the $CTR1^{-/-}$ cells is reversible, upon re-expression of CTR1. Studies of drug accumulation were utilized, as well as cytotoxicity assays, as measured using sulforhodamine B staining following a short exposure to cDDP.

## Results

### Effect of CTR1 on initial influx of Pt-containing drugs.

The effect of CTR1 on the cellular accumulation of cDDP was examined using a pair of mouse embryonic fibroblast lines, one containing wild type alleles of CTR1 ($CTR1^{+/+}$) and another in which both copies of the *CTR1* gene had been somatically knocked out ($CTR1^{-/-}$). Prior studies with this pair of cell lines had indicated that loss

of CTR1 reduced the accumulation of cDDP to 36% of control when uptake was measured following a 1 h exposure to either 2 or 10 μM cDDP. However, as shown in Figure 2-1A, even brief exposure of the $CTR1^{+/+}$ cells to 30 μM cDDP triggered rapid degradation and disappearance of CTR1 staining when examined by western blot. The reduction in CTR1 was functionally significant. As shown in Figure 2-1B, when cells were exposed to cDDP for 15 min prior to being exposed to Cu for 1 h the accumulation of Cu was reduced by 68% ($p<0.0006$). The rapid degradation of CTR1 suggested that the major contribution of this transporter to cDDP uptake may occur during the initial phase of cDDP influx. To examine this in more detail, $CTR1^{+/+}$ and $CTR1^{-/-}$ cells were exposed to 10, 30 or 100 μM cDDP and the drug was either removed immediately or after just a 5 min exposure. The cells were then thoroughly washed and the amount of cell-associated Pt was measured by ICP-MS. The range of concentrations was selected to encompass the range of concentrations anticipated in the peritoneal cavity following intraperitoneal instillation of cDDP during treatment for ovarian cancer. Figure 2-2A shows that, even when the drug was removed as quickly as possible and cells washed immediately with ice-cold saline, there was a clear difference in the amount of cDDP that became associated with the $CTR1^{+/+}$ *versus* $CTR1^{-/-}$ cells at all 3 concentrations tested. The Pt that became immediately bound to the $CTR1^{-/-}$ cells was only 33 ± 10 (SEM) % of that associated with the $CTR1^{+/+}$ cells when exposed to 10 μM cDDP. When exposed to 30 or 100 μM cDDP, the $CTR1^{-/-}$ cells bound 48 ± 20 (SEM) % and 29 ± 5 (SEM) % of the amount bound by the $CTR1^{+/+}$ cells. Since the period of exposure was <15 sec, this suggests that most of the difference in cell-associated Pt was the result of rapid binding of cDDP to

the extracellular domain of CTR1 rather than a difference in transport across the plasma membrane. Of interest, as shown in Figure 2B, there was no significant difference in the immediate binding of either CBDCA, L-OHP or transplatin between the $CTR1^{+/+}$ and $CTR1^{-/-}$ cells, a finding consistent with the generally slower rate of association of these drugs with nucleophilic targets. There was also no significant difference between the 4 drugs with respect to the amount of Pt that became immediately bound to the $CTR1^{+/+}$ cells.

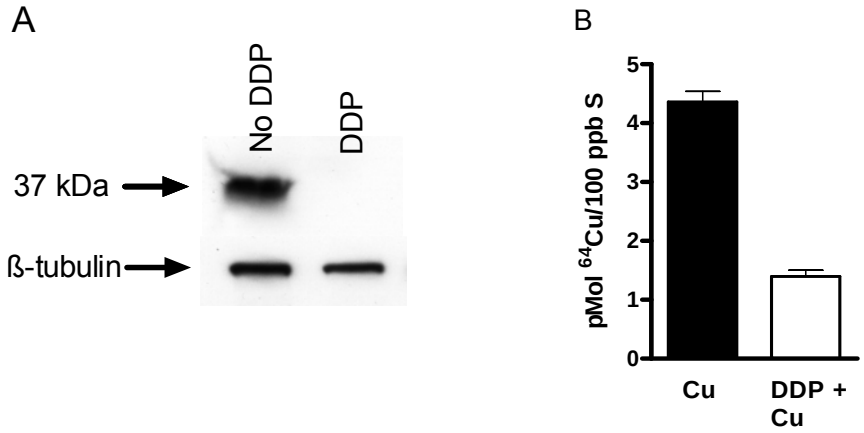


**Figure 2-1**. **Degradation of CTR1 and inhibition of Cu uptake induced by cDDP**. Panel A, western blot analysis of lysates from $CTR1^{+/+}$ cells treated with or without 30 µM cDDP for 15 min. Panel B, $^{64}Cu$ accumulation during a 1 h exposure to 2 µM Cu in $CTR1^{+/+}$ cells previously treated without (closed bar) or with (open bar) 30 µM cDDP for 15 min. Each bar represents the mean of at least 3 independent experiments each performed with 6 separate cultures. Vertical bars, SEM.

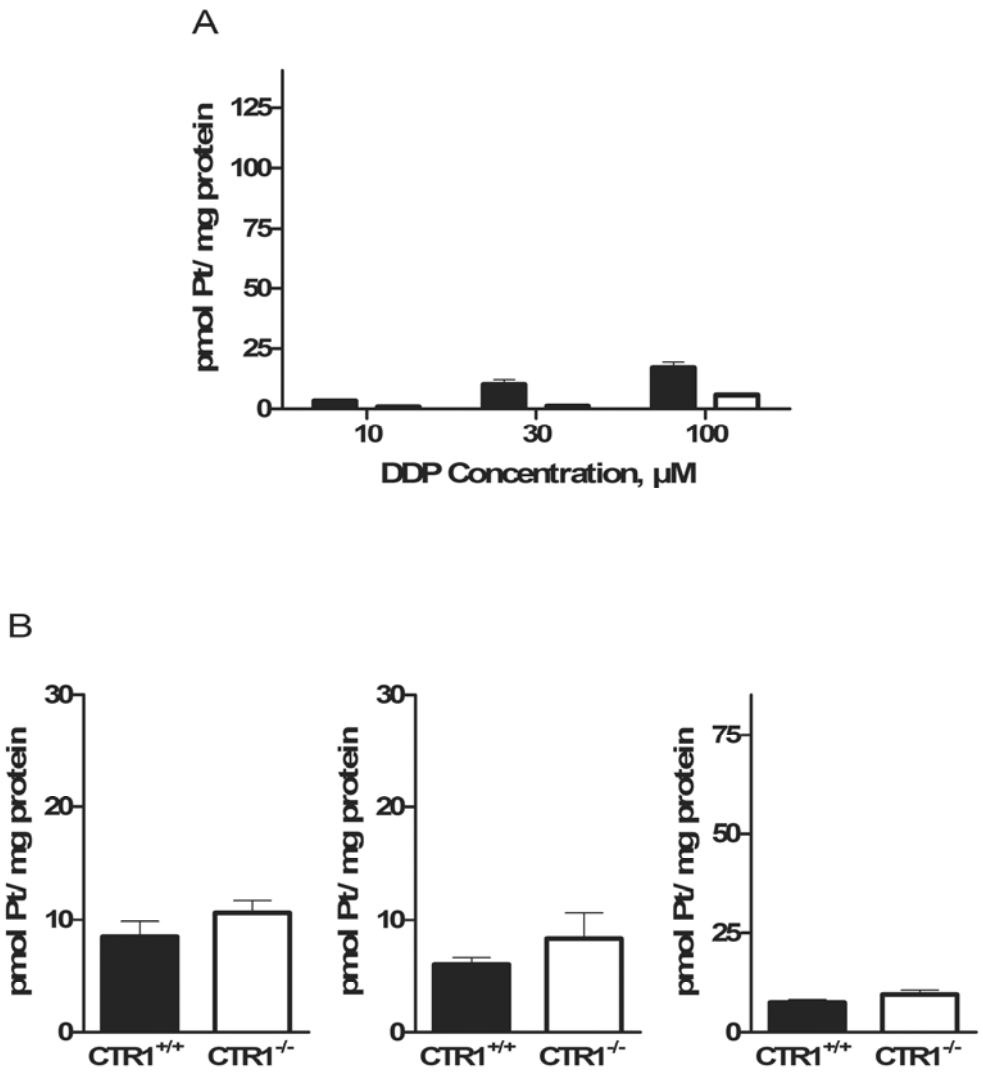


**Figure 2-2. Pt associated with CTR1$^{+/+}$ and CTR1$^{-/-}$ cells following exposure to and immediate removal of cDDP, CBDCA and L-OHP**. Panel A, cell-associated Pt following addition and immediate removal of 10, 30 or 100 µM cDDP (exposure <15 sec); panel B, cell-associated Pt following addition and immediate removal of 30 µM CBDCA and L-OHP. Closed bars, CTR1$^{+/+}$ cells; open bars, CTR1$^{-/-}$ cells. Each bar represents the mean of at least 3 independent experiments each performed with 6 separate cultures. Vertical bars, SEM.

Figure 2-3A shows the net uptake of Pt when the CTR1$^{+/+}$ and CTR1$^{-/-}$ cells were exposed to 10, 30 or 100 µM cDDP for 5 min; the values shown are the total Pt associated with the cells at 5 min less that bound to the cells at time zero. At all 3

concentrations tested the net accumulation of cDDP was reduced in the $CTR1^{-/-}$ cells; this reduction was significant for the 30 ($p<0.05$) and 100 μM ($p<0.0005$) concentrations. There was a trend toward a greater effect at higher cDDP concentrations; as the concentration was increased from 10 to 100 μM the uptake in the $CTR1^{-/-}$ cells diminished from 19 to 8% of that in the $CTR1^{+/+}$ cells. Thus, loss of CTR1 had a large effect on the initial uptake of cDDP, and by comparison to prior studies (Holzer et al., 2006a), the effect was substantially larger with a 5 min relative to a 1 h period of drug exposure.

To determine whether the loss of CTR1 affected uptake of the other Pt-containing drugs, the $CTR1^{+/+}$ and $CTR1^{-/-}$ cells were exposed to CBDCA, L-OHP or transplatin at a concentration of 30 μM for 5 min. As shown in Figure 2-3B, the magnitude of the effect for CBDCA was even greater than for cDDP; the $CTR1^{-/-}$ cells accumulated only 0.4% as much CBDCA as the $CTR1^{+/+}$ cells ($p<0.03$). In contrast, while the $CTR1^{-/-}$ cells also took up less L-OHP, the uptake was fully 32% of that in the $CTR1^{+/+}$ cells ($p<0.05$) and there was no effect of the loss of CTR1 on the uptake of transplatin at all. Thus, CTR1 regulates the initial influx of the 3 clinically effective drugs, although the magnitude of the effect was less for L-OHP than for cDDP and CBDCA as had previously been reported for 1 h drug exposures (Holzer et al., 2006a). However, it has no effect on the accumulation of transplatin, a molecule having the same composition as cDDP, but with the chlorides in a trans- rather than cis-configuration around the central Pt atom, and that is very much less cytotoxic than cDDP.

A comparison of data presented in Figures 2-3A and B indicates that the initial influx of transplatin in the $CTR1^{+/+}$ cells was 1.3-fold higher than that of cDDP, whereas the influx of CBDCA and L-OHP was 39% and 42% of that for cDDP, respectively. The fact that the accumulation of transplatin was even greater than that of cDDP and that the loss of CTR1 had no effect on the accumulation of transplatin suggests that transplatin enters cells by quite a different mechanism than cDDP.

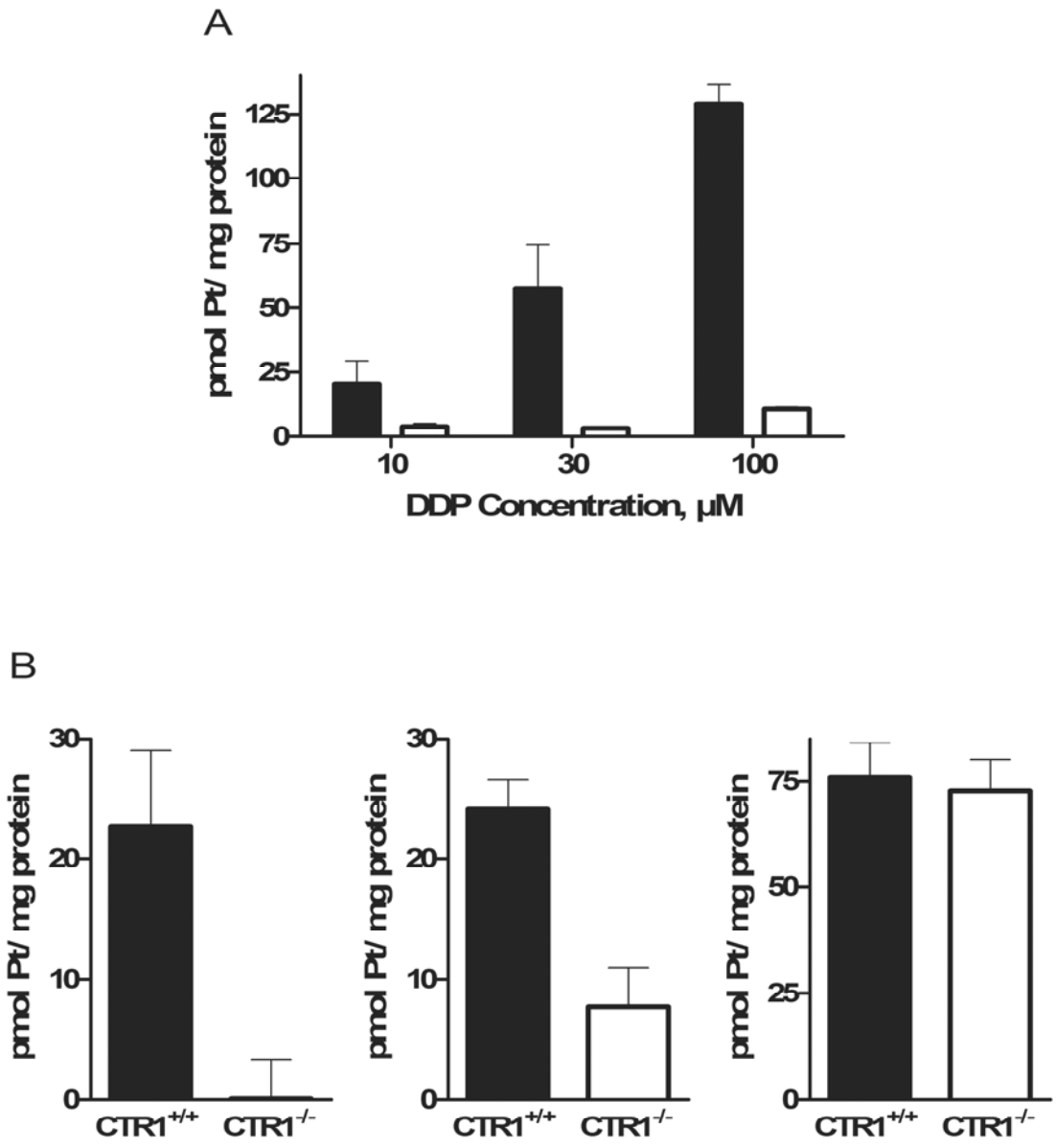


**Figure 2-3. Net Pt accumulation in CTR1$^{+/+}$ and CTR1$^{-/-}$ cells following a 5 min exposure to 10, 30 or 100 µM cDDP, or 30 µM CBCDA, L-OHP or transplatin**. Each bar represents the mean of at least 3 independent experiments each performed with 6 separate cultures. Vertical bars, SEM.

**Effect of CTR1 on the cytotoxicity of cDDP.**

CTR1 is not the only route by which cDDP can enter cells as shown by the fact that loss of CTR1 did not block uptake completely. If the cDDP entering the cell via CTR1 is an important component of the pool of intracellular drug that contributes to cell killing, then the reduction in cDDP uptake that accompanies the loss of CTR1 function should be matched by a reduction in cytotoxicity. $CTR1^{+/+}$ and $CTR1^{-/-}$ cells were treated for 5 min with increasing concentrations of cDDP, ranging from 0-400 μM, after which they were allowed to grow for 5 days before being stained with sulforhodamine B. Figure 2-4 shows that for $CTR1^{+/+}$ cells there was a concentration-dependent inhibition of growth. However, even a concentration of 400 μM cDDP produced no inhibition of the growth of $CTR1^{-/-}$ cells. Thus, the cDDP that enters the cell via a CTR1-mediated process contributes directly to the cytotoxic pool of intracellular drug rather than being effectively detoxified by sequestration into endosomes or other subcellular compartments from which it has no access to critical cytotoxic targets as might be expected if cDDP were simply chelated to CTR1, endocytosed into the cell and retained in a subcellular compartment.

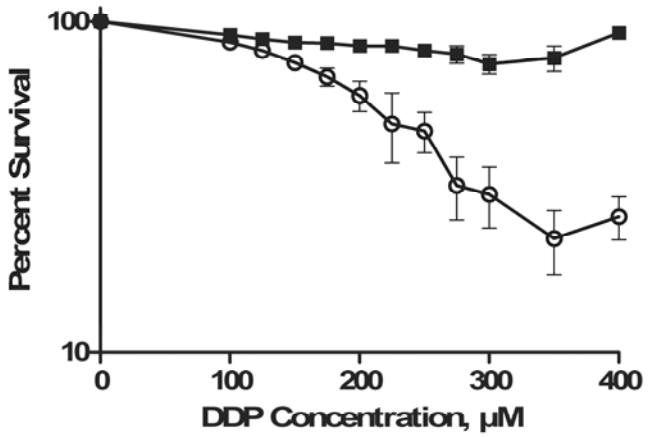


**Figure 2-4. Inhibition of CTR1$^{+/+}$ ( ○ ) and CTR1$^{-/-}$ ( ■ ) growth rate as a function of concentration following a 5 min exposure to cDDP.** Each data point presents the mean of 3 independent experiments each performed with triplicate cultures for each drug concentration. Vertical bars, SEM.

**Effect of re-expression of CTR1.**

In order to further demonstrate the importance of CTR1 to initial cDDP influx, and to demonstrate that hCTR1 as well as mCTR1 could mediate cDDP transport, the CTR1$^{-/-}$ cells were infected with a lentiviral vector expressing an hCTR1 cDNA and a clone of cells in which CTR1 expression had been restored was isolated and designed as CTR1$^{-/-/R}$. Using primers specific for mCTR1 and hCTR1 mRNA, the CTR1$^{-/-/R}$ cells were found to express hCTR1 mRNA at a level ~50% of the mCTR1 mRNA expression in the CTR1 $^{+/+}$ cells, as normalized to ß-actin expression. As shown in Figure 2-5A, re-expression of hCTR1 restored the initial influx of cDDP over the first 5 min to a level similar to that observed in the CTR1$^{+/+}$ cells. Re-expression of hCTR1 also rendered the CTR1$^{-/-}$ cells sensitive to the growth inhibitory effects of cDDP (Figure 2-5B). Thus, the reduced cDDP influx and cytotoxicity in the CTR1$^{-/-}$

cells was due specifically to the loss of CTR1 and hCTR1 was as effective as mCTR1 in mediating the initial influx and cytotoxicity of cDDP.

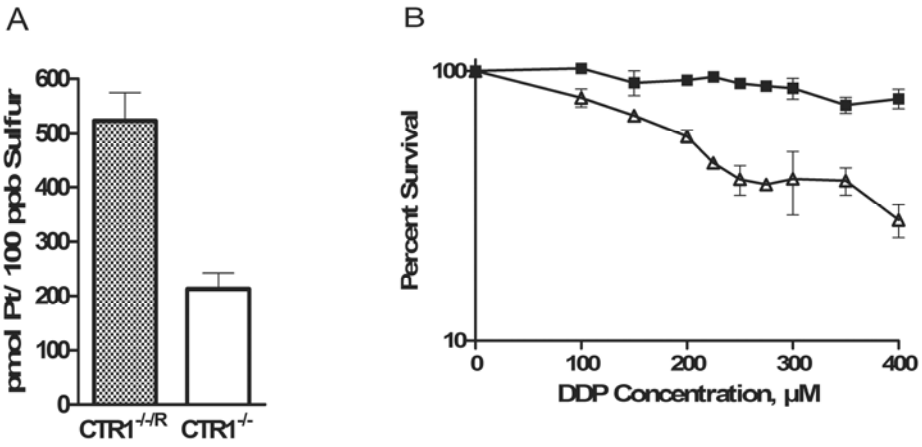


**Figure 2-5. Effect of re-expression of CTR1 in $CTR1^{-/-}$ cells on drug uptake and *in vitro* cytotoxicity.** Panel A, Pt accumulation following a 5 min exposure to 30 µM cDDP. Panel B, concentration-survival curves for $CTR1^{-/-}$ ( ■ ) and $CTR1^{-/-/R}$ ( Δ ) cells following a 5 min exposure to cDDP

## Discussion

CTR1 plays a critical role in the regulation of Cu uptake into the cell during normal development, and previous studies from this and other laboratories documented that it is also involved in the uptake of all 3 of the clinically used Pt-containing drugs. Loss of CTR1 was found to reduce the uptake of cDDP, CBDCA and L-OHP when measured at 1 h (Holzer et al., 2006a). Enhanced expression of CTR1 was also shown to increase cDDP uptake in several different cell systems (Holzer et al., 2004b; Song et al., 2004). The results of the current study provide further detail about the role and substrate specificity of CTR1 as a Pt drug transporter.

As was previously observed in human ovarian cancer cells expressing hCTR1 (Holzer and Howell, 2006), exposure to cDDP triggered rapid degradation of mCTR1 in the murine $CTR1^{+/+}$ cells suggesting that the major contribution of CTR1 to cDDP influx was likely to be during the first few minutes of drug exposure. The initial interaction of cDDP with CTR1 appears to be very rapid as demonstrated by the fact that measurable levels of Pt became associated with the cells in a CTR1-dependent manner within 15 sec. The extracellular domain of CTR1 is rich in both methionine and histidine motifs potentially capable of chelating cDDP but attempts to produce the extracellular domain in *E. coli* as a recombinant protein with which to further analyze binding have been unsuccessful due to its insolubility. It is noteworthy that the amount of Pt that became associated with the $CTR1^{+/+}$ cells when they were exposed briefly to CBDCA and L-OHP was less than that following exposure to cDDP, and that the initial binding of these two drugs did not differ significantly between the $CTR1^{+/+}$ and $CTR1^{-/-}$ cells indicating that the initial binding of these 2 drugs was not as dependent on CTR1 expression.

The availability of an isogenic pair of $CTR1^{+/+}$ and $CTR1^{-/-}$ cells allowed unequivocal demonstration that CTR1 plays a major role in the initial influx of cDDP. Over a wide concentration range, loss of CTR1 markedly reduced the initial influx of cDDP; when exposed to 10 μM cDDP for 5 min initial influx was reduced by 81%. There was a trend toward a greater magnitude of the effect at higher cDDP concentrations suggesting that other mechanisms that contribute to initial influx become saturated such that initial influx is even more dependent on CTR1. The dependence of initial cDDP influx on CTR1 was further demonstrated by restoring

CTR1 expression in the CTR1$^{-/-}$ cells. The re-expression of human rather than mouse CTR1 served to both document that restoration of CTR1 increased cDDP uptake and that human as well as mouse CTR1 was capable of mediating this effect.

Loss of CTR1 had an even greater effect on reducing the initial influx of CBDCA than that of cDDP, but, as observed when uptake was measured at 1 h in a previous study (Holzer et al., 2006a), there was a smaller effect on the initial influx of L-OHP whose uptake was reduced to only 32% of control. In addition, loss of CTR1 had no effect on the initial influx of transplatin. The fact that loss of CTR1 had a large effect on the initial influx of cDDP but no effect on that of transplatin clearly indicates that the cis configuration is essential to the participation of CTR1. It is surmised that the cis configuration is required to allow the drug to bind to the methionine and histidine-rich motifs of the extracellular domain of CTR1. Whether or not the differences in the magnitude of the effect of the loss of CTR1 on the initial influx of cDDP versus CBDCA and L-OHP is related to their ability to interact with the extracellular domain of CTR1 remains to be determined. The fact that loss of CTR1 did not alter the initial binding of CBDCA and L-OHP detectable with exposures of <15 sec is consistent with their generally slower rate of reaction with nucleophilic targets but inconsistent with the observation that loss of CTR1 had as large an affect on the initial uptake of CBDCA as it did for cDDP.

Previous studies in which over-expression of CTR1 was shown to increase whole cell Pt levels following exposure to cDDP, but not to increase the extent of DNA adduct formation or cytotoxicity (Holzer et al., 2004b), raised the question of whether the cDDP transferred into the cell in a CTR1-dependent manner was really

available to attack critical targets or was just being trafficked to sites where it was detoxified by sequestration into subcellular organelles. The results reported here clearly demonstrate that the diminished influx of cDDP associated with the loss of CTR1 was accompanied by reduced cDDP cytotoxicity, and that the cytotoxicity of cDDP could be restored by re-expression of CTR1 in the $CTR1^{-/-}$ cells. Loss of CTR1 rendered cells completely resistant to even very high concentrations of cDDP when they were exposed for 5 min *in vitro*. A previous study demonstrated a 3.2-fold loss of sensitivity when cells were exposed for 1 h (Holzer et al., 2006a). Importantly, the results of the current study showed that, despite the differences between the *in vitro* and *in vivo* exposures, this impairment of influx translated to loss of sensitivity *in vivo* as well. The $CTR1^{-/-/R}$ tumor xenografts responded well to a single maximum tolerated dose of cDDP whereas the $CTR1^{-/-}$ tumors demonstrated no detectable response at all. This suggests that CTR1 expression in tumors is likely to be an important determinant of tumor responsiveness to cDDP. A broad survey of CTR1 expression in a variety of types of cancer demonstrated marked variability in the level of expression both within and between histologically defined tumor types (Holzer et al., 2006b). It remains to be determined whether CTR1 can serve as a clinically useful biomarker of responsiveness of human cancers to cDDP, CBDCA or L-OHP.

How CTR1 mediates the uptake of cDDP, CBDCA and L-OHP remains an enigma. The pore created by the trimeric CTR1 complex through which $Cu^{+1}$ is believed to enter cells is too small to accommodate the Pt-containing drugs (Aller and Unger, 2006; De Feo et al., 2007), and recent studies with yeast CTR1 indicate that Cu and cDDP produce different changes in the function of CTR1 (Sinani et al., 2007).

Prior studies indicated that cDDP uptake by CTR1 requires micropinocytosis (Holzer and Howell, 2006) suggesting a model in which cDDP binds to the extracellular domain of CTR1 and is rapidly internalized into endosomes. A similar phenomenon has been suggested by researchers studying the yeast variant of CTR1 (Sinani et al., 2007). How cDDP escapes from such endosomes and finds its way to DNA in the nucleus remains one of the key questions about the cellular pharmacology of this drug.

## Materials and Methods

### Drugs and reagents.

cDDP was a gift from Bristol-Myers Squibb (Princeton, NJ); it contains cDDP at a concentration of 3.33 mM in 0.9% NaCl. L-OHP was a gift from Sanofi-Aventis (Malvern, PA); the powder was dissolved in $ddH_2O$ at a concentration of 10 mM. CBDCA was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in $ddH_2O$ at a concentration of 10 mM. Transplatin was obtained from Sigma-Aldrich (St. Louis, MO) and was resuspended in 0.9% NaCl at a concentration of 3.33 mM. The drugs were diluted into OptiMEM Reduced Serum Media (Invitrogen, Carlsbad, CA) to produce final concentrations of 10, 30 and 100 μM. Bradford reagent was purchased from BioRad Laboratories, Inc. (Hercules, CA) and sulforhodamine B was obtained from Sigma-Aldrich (St. Louis, MO) and 0.4% SRB (w/v) was solubilized in 1% (v/v) acetic acid solution.

### Cell types, culture and engineering.

Parental mouse embryonic fibroblasts containing wild type alleles of CTR1 ($CTR1^{+/+}$) and a subline in which both copies of CTR1 had been somatically knocked

out ($CTR1^{-/-}$) were a gift from Dr. Dennis Thiele (Lee et al., 2002b). The $CTR1^{-/-/R}$ subline was constructed by infecting the $CTR1^{-/-}$ cells with a lentivirus expressing a wild type human CTR1 cDNA using the ViraPower Lentiviral Induction kit (Invitrogen, Carlsbad, CA). Cell survival following exposure to increasing concentrations of drugs was assayed using the sulforhodamine B assay system (Monks et al., 1991). Five thousand cells were seeded into each well of a 96-well tissue culture plate. Cells were incubated overnight at 37°C, 5% $CO_2$ and then exposed for 5 min by the addition of 100 µl Pt drug-containing OptiMEM medium. After 5 min the drug-containing media was aspirated off, cells were washed once with 37°C PBS, PBS was aspirated off and cells were covered in 200 µl complete medium. Cells were allowed to grow for 5 d after which the media was removed, the protein was precipitated with 50% trichloroacetic acid and stained using 100 µl of 0.4% sulforhodamine B in 1% acetic acid at room temperature for 15 minutes. Following washing the absorbance of each well at 515 nm was recorded using a Versamax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). All experiments were repeated at least 3 times using 3 cultures for each drug concentration.

**Measurement of cellular drug accumulation.**

$CTR1^{+/+}$, $CTR1^{-/-/R}$ and $CTR^{-/-}$ cells were grown to 90% confluence in T-150 tissue culture flasks. Cells were then harvested using trypsin and $7.5 \times 10^5$ cells were placed into each well of 6-well tissue culture plates and allowed to grow overnight in 2.5 ml of media at 37°C in 5% $CO_2$. All data presented are the means of at least 3 independent experiments each performed with 6 wells per concentration tested. The next day medium was removed by aspiration and the cells were exposed to 500 µl of

drug-containing OptiMEM medium (Invitrogen, Carlsbad, CA) at 37°C for either 0 or 5 minutes after which the drug-containing medium was removed, the plates were washed 3 times with ice-cold PBS and then placed on ice. In the case of the time zero samples, the drug-containing medium was aspirated within 15 sec of the start of drug exposure. Two hundred and fifteen µl of concentrated (50-70%) nitric acid was added to each well and the plate was incubated overnight at room temperature. The following day the acid was moved into Omni-vials (Wheaton, Millville, NJ) and incubated at 65°C overnight to thoroughly dissolve all cellular debris. The following day the nitric acid was diluted with 3 ml of buffer (0.1% Triton X-100, 1.4% nitric acid, 1 ppb In in dd$H_2O$) and incubated again at 65°C overnight. Pt concentration was measured using a Perkin-Elmer Element 2 ICP-MS located at the Analytical Facility at Scripps Institute of Oceanography at the University of California, San Diego. Values were normalized to either protein concentration as determined using the Bradford reagent or total sulfur content, as measured by ICP-OES, on the same samples.

**$^{64}$Cu influx.**

Cu uptake was determined as previously described (Holzer et al., 2006a) with the following changes. Plates were incubated for 1 h rather than 30 min in the presence of Cu, plates that were pretreated with cDDP were exposed to 30 µM cDDP for 15 min at 37°C followed by 3 washings with room temperature PBS, followed by exposure to $^{64}$Cu.

**qRT-PCR.**

CTR1 mRNA levels were measured by qRT-PCR. cDNA was generated from mRNA isolated using Trizol (Invitrogen, Carlsbad, CA). Purified mRNA was

converted to cDNA using Oligo$(dT)_{20}$ priming and the SuperScript III First-Strand Kit (Invitrogen, Carlsbad, CA). qRT-PCR was performed on a Bio-Rad MyIQ qPCR machine (Hercules, CA). The forward and reverse primers for hCTR1, mCTR1 and mouse ß-actin were, respectively: gatgatgatgcctatgacct, tcttgagtccttcatagaac, actgttgggcaacagatgct, ctgctgctactgcaatgcag, gatgatgatgcctatgacct, ctctcgggctatcttgagtc, tccaggtagtcatcagct, tggcagtgctctgtgatgtc, aggtgacagcattgcttctg, gctgcctcaacacctcaac. Reactions were prepared using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), according to manufacturer's recommendations. Samples were prepared in quadruplicate and 3 independent RNA isolates were used in 3 independent experiment. Analysis was done using the Bio-Rad iQ5 system software (Hercules, CA).

**Chemiluminescent Immunoblotting.**

Western blots were performed as previously described (Holzer et al., 2004b), with the following changes: PVDF membrane (Millipore, Billerica, MA) was used in place of nitrocellulose and Pierce SuperSignal chemiluminescence detection was used (Thermo Scientific, Rockford, IL).

**Statistical Analysis.**

All 2-group comparisons were done using Student's *t*-test, where two-tailed $p \leq 0.05$ was determined statistically significant.

## Acknowledgements

The author wishes to thank Dr. Dennis Thiele for kindly providing the isogenic pair of $CTR1^{+/+}$ and $CTR1^{-/-}$ cells used in this study. The author would also like to

thank Dr. Danielle Jandial, Gerald Manorek and Nicole Chung for their technical expertise and invaluable discussion. A majority of the content of Chapter 2 has been published in *Molecular Pharmacology* (Larson CA, Blair BG, Safaei R, Howell SB. The Role of the Mammalian Copper Transporter 1 in the Cellular Accumulation of Platinum-based Drugs. Mol Pharmacol. 2009 Feb;75:324, 2009.). Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Brian G. Blair assisted in the measurement of Pt and Cu accumulation and provided helpful feedback. Roohangiz Safaei provided helpful discussion.

# Chapter 3 –

# Role of CTR1 in *in vivo* Cisplatin Cytotoxicity

## Introduction

The goal of the experiments described in this chapter was to establish the role of CTR1 *in vivo*, with respect to sensitivity to single dose, intraperitoneal cDDP. The system utilized in these studies was that of subcutaneous xenografts of mouse embryonic fibroblast onto athymic nude mice. The cell lines used were $CTR1^{-/-}$ cells and the re-expressing cell line $CTR1^{-/-/R}$ as described in the previous chapter.

## Results

### Effect of CTR1 on sensitivity to cDDP *in vivo*.

$CTR1^{-/-}$ and $CTR1^{-/-/R}$ cells were inoculated subcutaneously into nu/nu mice and both types of cells formed tumors with equal frequency. Immunohistochemical analysis of sections from these tumors demonstrated robust expression of CTR1 in the $CTR1^{-/-/R}$ cells but none in the $CTR1^{-/-}$ cells (Figure 3-1). As shown in Figure 3-2, the $CTR1^{-/-/R}$ tumors grew more rapidly than the $CTR1^{-/-}$ tumors. Following a single intraperitoneal injection of a maximum tolerated dose of cDDP (10 mg/kg), there was a clear reduction in the growth of the $CTR1^{-/-/R}$ tumors but no effect on the growth of the $CTR1^{-/-}$ tumors. Thus, consistent with the impairment of influx, loss of CTR1

rendered the tumor cells completely resistant to a maximum tolerated dose of cDDP *in vivo.*

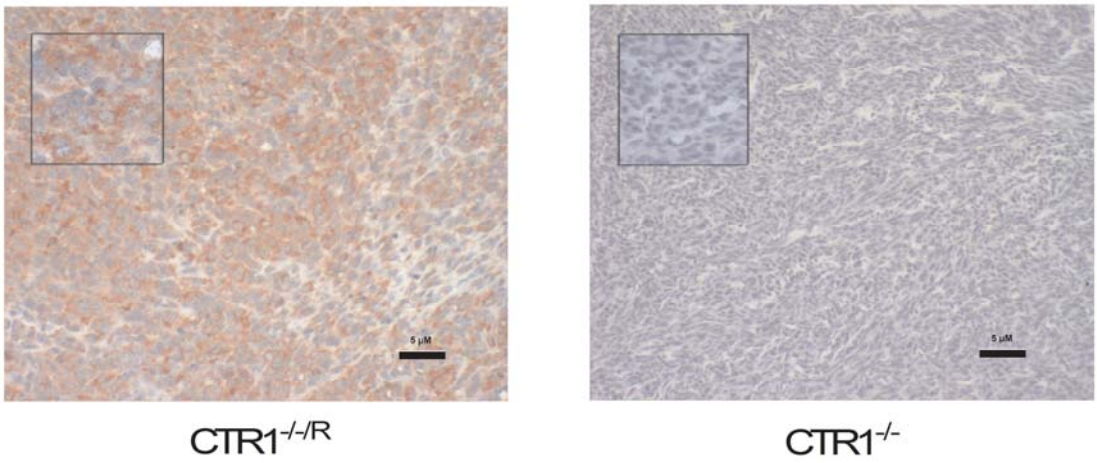


**Figure 3-1. CTR1 expression in CTR1$^{-/-/R}$ and CTR1$^{-/-}$ xenografts as determined by immunohisto chemical analysis.** Main image is at 200x, inset is at 400x magnification. Vertical bars, SEM.

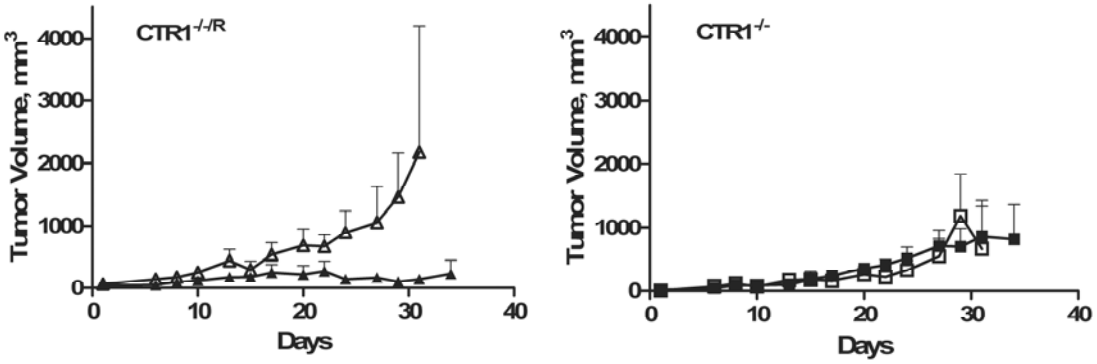


**Figure 3-2. Sensitivity of CTR1$^{-/-/R}$ and CTR1$^{-/-}$ xenografts to treatment with cDDP.** Open symbols, treatment with vehicle only; closed symbols, mice treated with a single dose of cDDP 10 mg/kg. Vertical bars, SEM.

## Discussion

Importantly, the results of the current study showed that, despite the differences between the *in vitro* and *in vivo* exposures, this impairment of influx translated to loss of sensitivity *in vivo*. The $CTR1^{-/-/R}$ tumor xenografts responded well to a single maximum tolerated dose of cDDP whereas the $CTR1^{-/-}$ tumors demonstrated no detectable response at all. This suggests that CTR1 expression in tumors is likely to be an important determinant of tumor responsiveness to cDDP. A broad survey of CTR1 expression in a variety of types of cancer demonstrated marked variability in the level of expression both within and between histologically defined tumor types (Holzer et al., 2006b). It remains to be determined whether CTR1 can serve as a clinically useful biomarker of responsiveness of human cancers to cDDP, CBDCA or L-OHP.

## Materials and Methods

### Drugs and reagents.

cDDP was a gift from Bristol-Myers Squibb (Princeton, NJ); it contains cDDP at a concentration of 3.33 mM in 0.9% NaCl.

### Cell types, culture and engineering.

Parental mouse embryonic fibroblasts containing wild type alleles of CTR1 ($CTR1^{+/+}$) and a subline in which both copies of CTR1 had been somatically knocked

out ($CTR1^{-/-}$) were a gift from Dr. Dennis Thiele (Lee et al., 2002b). The $CTR1^{-/-/R}$ subline was constructed by infecting the $CTR1^{-/-}$ cells with a lentivirus expressing a wild type human CTR1 cDNA using the ViraPower Lentiviral Induction kit (Invitrogen, Carlsbad, CA).

**Determination of drug sensitivity *in vivo*.**

In order to grow the various types of cells as xenografts $3x10^6$ cells in 100 µl were inoculated at 4 subcutaneous sites into 20 g female nu/nu mice. Cell types were randomized between shoulder and hip, left and right, assuring that there were always tumors of the same type on left and right. Tumors were allowed to grow until they were ~ 2 mm in diameter at which point each mouse received a single dose of cDDP 10 mg/kg by intraperitoneal injection. Tumor size was monitored 3 times per week for 5 weeks. Tumor volume was estimated using (length x $width^2$)/2.

**Immunohistochemistry.**

Immunohistochemical analysis was performed as previously described (Holzer et al., 2006b).

**Statistical Analysis.**

All 2-group comparisons were done using Student's *t*-test, where two-tailed $p \leq 0.05$ was determined statistically significant.

## Acknowledgements

The author would like to thank Dr. Dennis Thiele for generously providing the $CTR1^{-/-}$ mouse embryo fibroblasts used in these studies..A majority of the content of Chapter 3 has been published in *Molecular Pharmacology* (Larson CA, Blair BG,

Safaei R, Howell SB. The Role of the Mammalian Copper Transporter 1 in the Cellular Accumulation of Platinum-based Drugs. Mol Pharmacol. 2009 Feb;75:324, 2009.). Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Brian G. Blair assisted in the *in vivo work* and provided helpful feedback. Roohangiz Safaei provided helpful discussion.

# Chapter 4 –

# Role of the N-terminus of CTR1 in the Cellular Accumulation of Cisplatin

## Introduction

As discussed in chapter 1 ionic interactions between $Cu^{+1}$ and methionines, histidines and cysteines in the pore appear to determine both the selectivity of the pore for $Cu^{+1}$ and the rate of transport (Howell et al., 2010). As shown in Figure 4-1, additional clusters of methionines and histidines capable of interacting with Cu are found in the 67 amino acid extracellular N-terminal domain of CTR1. As discussed previously, the N-terminus domain contains 4 such clusters H1, H2, M1 and M2.

The goal of the experiments described in this chapter was to determine the role of the N-terminus of hCTR1, including the M2 region, with respect to the ability of CTR1 to control the cellular accumulation and cytotoxicity of cDDP. The approaches used in these studies started with the re-expression of both wild type and 2 N-terminal variant forms of hCTR1 in $CTR1^{-/-}$ mouse embryonic fibroblasts. After verifying the re-expression using qRT-PCR, western immunoblotting and immunocyto chemistry, Cu and cDDP uptake and cytotoxicity was measured.

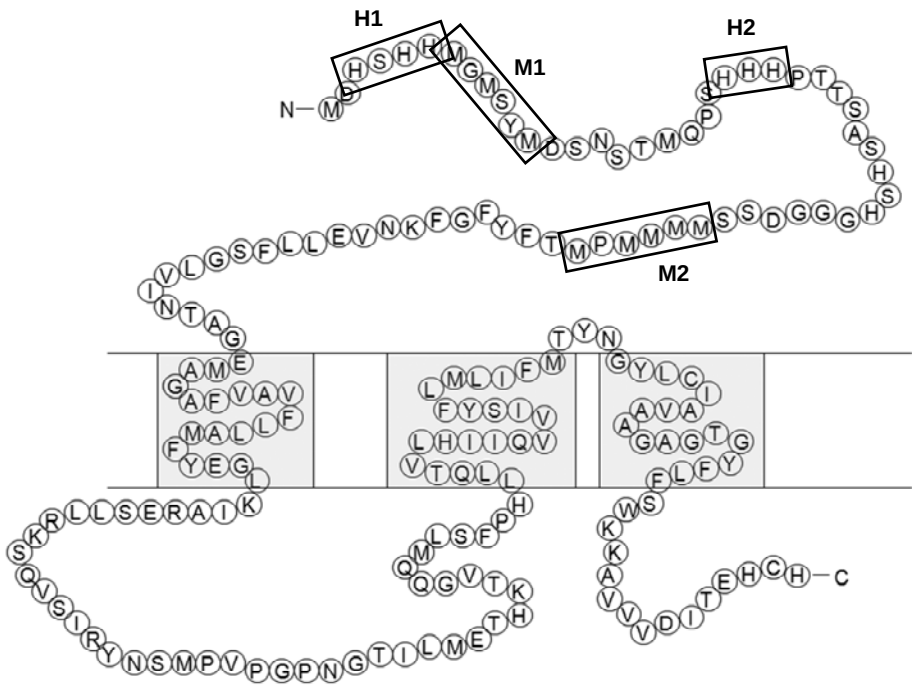


**Figure 4-1. Schematic diagram of the amino acid sequence of hCTR1**. Boxes highlight the H1, M1, H2 and M2 motifs.

## Results

### Expression of hCTR1 in CTR1$^{-/-}$ mouse embryo fibroblasts.

Prior studies of the importance of the histidine and methionine motifs in the N-terminal end of hCTR1for the transport of Cu and cDDP have been confounded by the presence of endogenous CTR1. To avoid this problem, wild type and variant forms of hCTR1 were re-expressed in mouse embryo fibroblasts in which both alleles of mCTR1 had been knocked out (CTR1$^{-/-}$ cells). qRT-PCR verified complete absence of the expression of mCTR1 in the CTR1$^{-/-}$ cell line. Lentiviral vectors containing a blastocidin resistance marker were constructed to express either wild type hCTR1 (CTR1$^{-/-/WT}$), a variant in which the $^{40}$MXXXM$^{45}$ motif was deleted (CTR1$^{-/-/M2}$), or a

variant in which the first 45 amino acids were deleted ($CTR1^{-/-/Truncated}$). Deletion of the first 45 amino acids removes all of the histidine and methionine clusters in the N-terminal domain. All 3 forms of hCTR1 contained a myc tag at the N-terminal end. Cells were infected, selected with blastocidin and then characterized with respect to the expression of each form of exogenous CTR1. Figure 4-2A shows that the wild type hCTR1 was re-expressed at a 30% higher level than either of the two variants at the mRNA level as determined by qRT-PCR. To assess the level of CTR1 protein expression at the plasma membrane, the cell surface proteins were biotinylated by exposure to sulfo-NHS-SS-biotin before lysis, then recovered on streptavadin-coated beads, and subjected to western blot analysis using an antibody to the myc tag. Figure 4-2B shows that the wild type and both variant forms of hCTR1 were correctly localized to the plasma membrane. The $CTR1^{-/-/M2}$ cells expressed plasma membrane CTR1 at a mean of 94 ± 3% of than in the $CTR1^{-/-/WT}$ cells and the $CTR1^{-/-/Truncated}$ cells expressed it at mean of 92 ± 5% of than in the $CTR1^{-/-/WT}$ cells. Wild type CTR1 was detected as a band migrating at 37 kDa corresponding to the glycosylated form of the myc-tagged monomer that has been observed in prior studies (Eisses and Kaplan, 2002; Guo et al., 2004). The CTR1 in which the $^{40}MXXXM^{45}$ was deleted, referred to as the M2 variant, also migrated at ~37 kDa and the truncated protein migrated somewhat more rapidly consistent with its smaller size.

To further assess the subcellular distribution of the CTR1 variants, the $CTR1^{-/-/WT}$, $CTR1^{-/-/M2}$ and $CTR1^{-/-/Truncated}$ cells were examined by deconvoluting microscopy using an antibody to the myc tag and a secondary fluorochrome-labeled anti-mouse antibody. Figure 4-2C shows that the distribution of CTR1 was similar for all 3 forms

with only a minority of the protein found at the plasma membrane. Thus, the N-terminal domain of CTR1 does not contain key signals required for trafficking from the ER to the Golgi and then to the plasma membrane and internal membranous compartments.

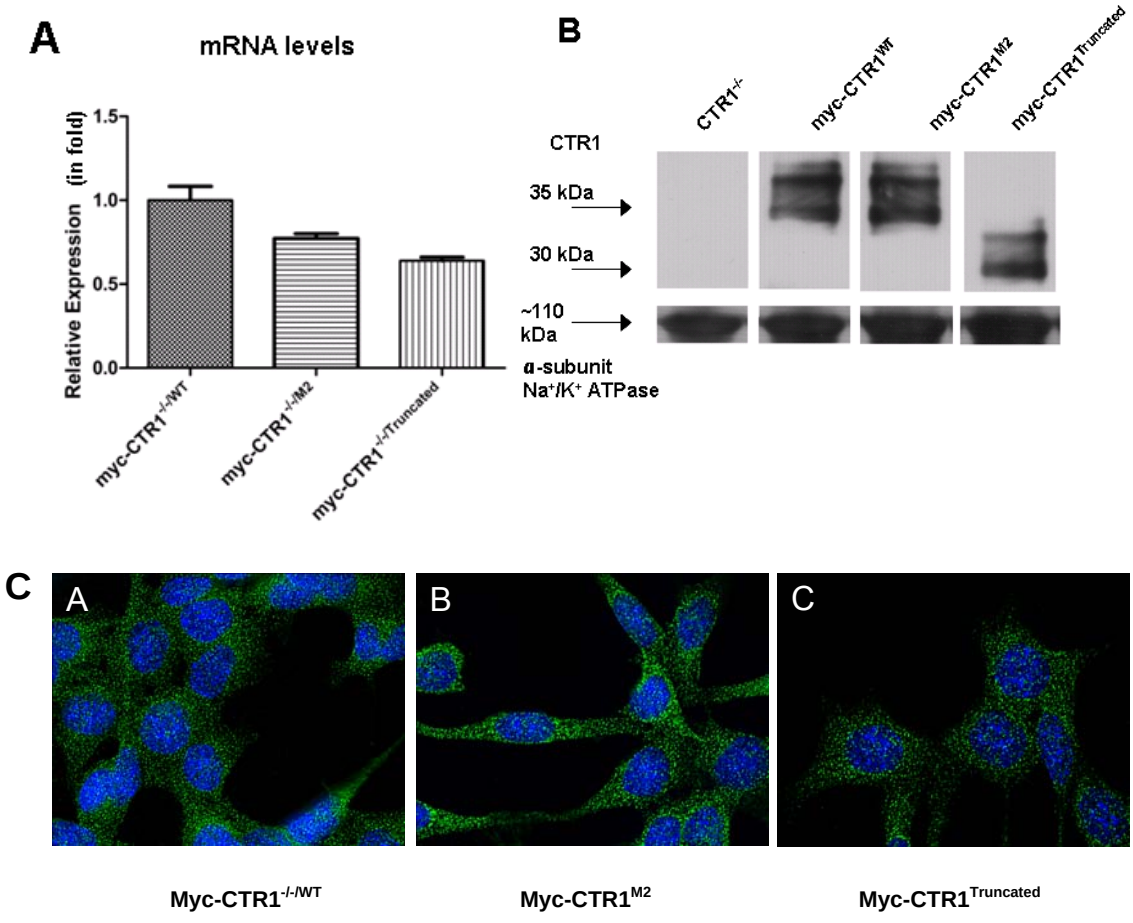


**Figure 4-2. Re-expression of wild type and variants forms of myc-hCTR1 in CTR1$^{-/-}$ cells.** A. Relative mRNA levels measured by qRT-PCR. B. Representative western blot showing the expression of myc-tagged hCTR1. C. Micrograph demonstrating the uniform expression of the myc-tagged hCTR1 protein (60x magnification). Panel A, CTR1$^{-/-/wt}$ cells; panel B, CTR1$^{-/-/M2}$ cells; panel C, CTR1$^{-/-/Truncated}$ cells. Vertical bars, ± SEM.

**CTR1 regulation of Cu uptake and cytotoxicity.**

The basal Cu content of the $CTR^{-/-}$, $CTR1^{-/-/WT}$, $CTR1^{-/-/M2}$ and $CTR1^{-/-/Truncated}$ cells was measured by ICP-MS while the cells were growing in complete DMEM medium that contains ~0.3 μM Cu. Figure 4-3A shows that re-expression of the wild type hCTR1 increased basal Cu content by 1.3-fold, whereas expression of the M2 variant increased it by 1.1-fold. Of interest was the fact that expression of the truncated variant actually decreased the basal copper level to 43% of that in the $CTR1^{-/-}$ cells. This suggests that the truncated form of CTR1 interferes with the uptake or enhances the efflux of Cu mediated by other transporters. The rate of Cu uptake was determined by measuring the Cu content following exposure of the cells to 100 μM Cu for 1 hour. As shown in Figure 4-3B, re-expression of wild type hCTR1 increased Cu uptake by 2-fold ($p < 0.001$) while neither the M2 deletion variant nor the N-terminal truncation variant enhanced Cu accumulation at all. These results indicate that, even under Cu replete conditions, the M2 component of the N-terminal domain is required for optimal Cu accumulation. The fact that deletion just the M2 motif disabled Cu transport to the same extent as deletion of the whole N-terminal domain identifies this motif as essential to optimal function of the N-terminal domain but does not exclude the possibility that individual deletion of the M1, H1 or H2 motifs might not have some effect.

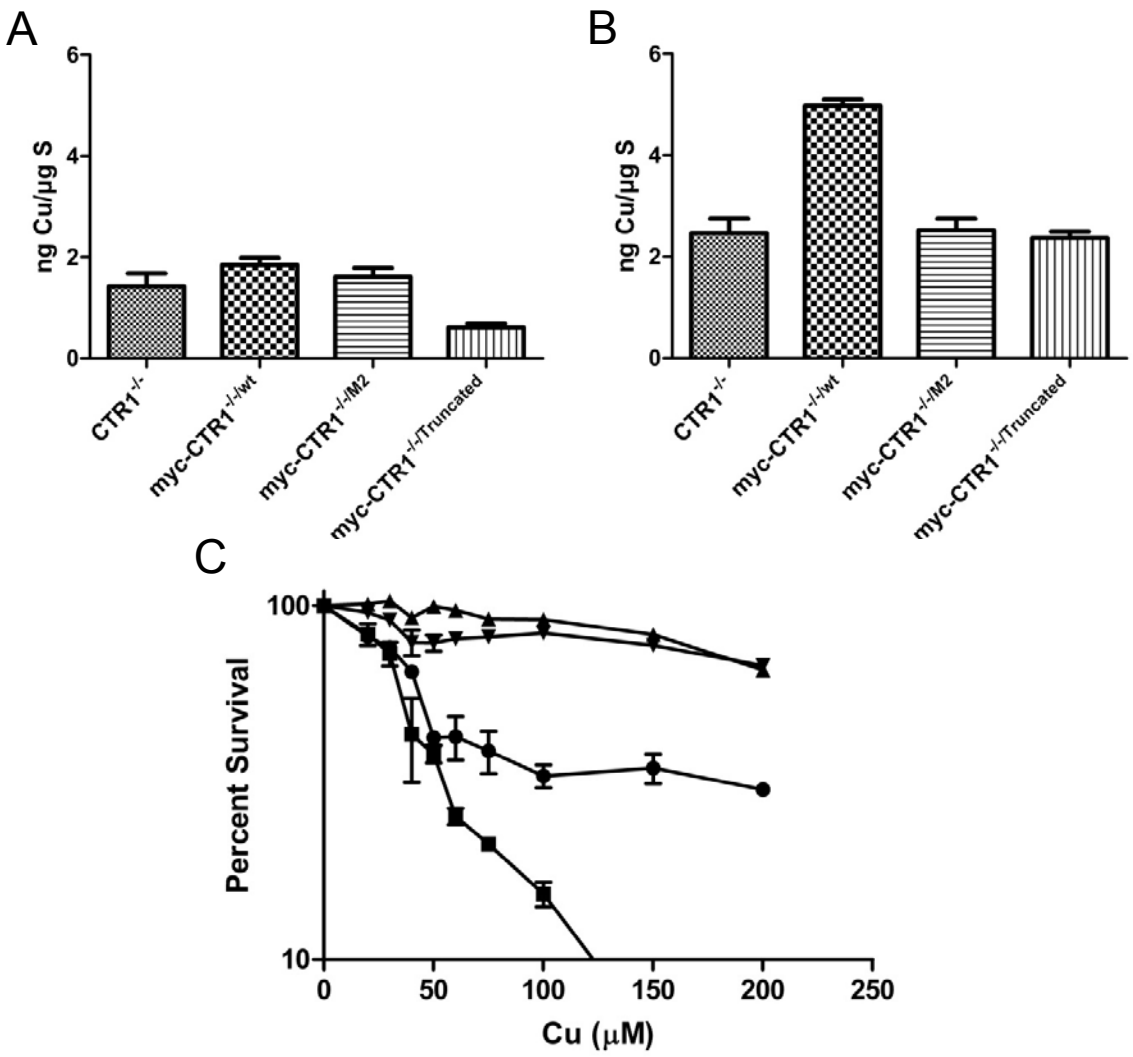


**Figure 4-3. Cu accumulation and cytotoxicity.** A, total basal Cu; B, total Cu following 1 h exposure to 100 µM Cu; C, inhibition of growth of MEF cells during 96 h continuous exposure to varying concentrations of Cu. (●), $CTR1^{-/-}$; (□), myc-$CTR1^{-/-/wt}$; (▲), myc-$CTR1^{-/-/M2}$; (▼), myc-$CTR1^{-/-/Truncated}$. Each value represents the mean of no less than 3 independent experiments each performed with 3 separate cultures. Vertical bars, ± SEM.

To determine whether the differences in Cu accumulation translated into different tolerances for the cytotoxic effect of Cu, the growth rate of the $CTR^{-/-}$, $CTR1^{-}$

$^{-/WT}$, CTR1$^{-/-/M2}$ and CTR1$^{-/-/Truncated}$ cells was measured during a 96 h exposure to increasing concentrations of Cu. As shown in Figure 4-3C, the CTR1$^{-/-/WT}$ cells were 1.2-fold more sensitive to Cu than the CTR1$^{-/-}$ cells ($IC_{50}$ 37 ± 1 *versus* 46 ± 2 μM; $p < 0.05$). In contrast, the M2 and N-terminal truncation variants were less sensitive. The $IC_{50}$ for the CTR1$^{-/-/M2}$ cells was 5.2-fold higher (238 ± 9 μM; $p < 0.01$), and that for the CTR1$^{-/-/Truncated}$ cells was 6.5-fold higher (301 ± 14 μM; $p< 0.001$), than that of the CTR1$^{-/-/WT}$ cells. Thus, consistent with the failure of the two variants to mediate as much Cu uptake as the wild type form, the cells expressing these variants were quite resistant to the cytotoxic effects of Cu. However, the finding that both variants rendered cells even more resistant than the CTR1$^{-/-}$ cells suggests that the variants are either disabling other Cu influx transporters or otherwise limiting the access of Cu to key targets capable of triggering impaired proliferation.

**CTR1 regulation of cDDP uptake and cytotoxicity.**

To assess the effect of structurally modifying the N-terminal end of hCTR1on the initial transport of cDDP, the 4 cell lines were exposed to 30 μM cDDP for 5 min, washed thoroughly and the Pt content measured by ICP-MS. Drug accumulation was measured at 5 min because the greatest effect of hCTR1 appears to be on the initial phase of uptake; the concentration of 30 μM was selected as being clinically relevant to intraperitoneal therapy. As shown in Figure 4-4A, expression of wild type hCTR1 in the CTR1$^{-/-}$ cells increased the Pt content by 4.2-fold ($p < 0.001$). Expression of the M2 variant increased the Pt content by 2.5-fold or 60% of the incremental increase in Pt content produced by the wild type hCTR1 ($p < 0.001$). Expression of the truncated variant produced a very similar enhancement. The Pt content was increased 2.3-fold or

55% of the incremental increase produced by wild type CTR1 ($p < 0.001$). Thus, both variant forms of hCTR1 retained substantial, although muted, ability to enhance cDDP uptake, despite their inability to enhance the uptake of Cu.

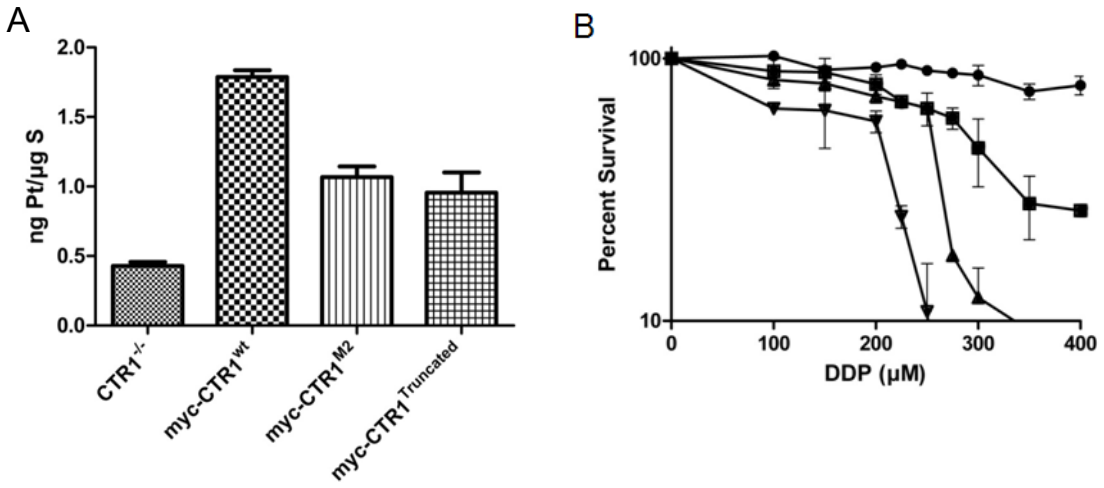


**Figure 4-4. cDDP accumulation and cytotoxicity.** A, Net accumulation of Pt in MEF cells following 5 min exposure to 30 µM cDDP. B, Inhibition of growth of MEF cells following 5 minute exposure to varying concentrations of cDDP. (●), $CTR1^{-/-}$; (□), myc-$CTR1^{-/-/wt}$; (▲), myc-$CTR1^{-/-/M2}$; (▼), myc-$CTR1^{-/-/Truncated}$. All values represent means of 6 independent experiments each containing triplicate cultures. Vertical lines, ± SEM

Figure 4-4B shows the effect of a 5 minute exposure to increasing concentrations of cDDP on the growth rate of the $CTR^{-/-}$, $CTR1^{-/-/WT}$, $CTR1^{-/-/M2}$ and $CTR1^{-/-/Truncated}$ cells during a subsequent period of 96 h as measured by sulforhodamine B staining. As anticipated, expression of wild type hCTR1 increased the sensitivity of the $CTR1^{-/-}$ cells by a factor of 2.2-fold, reducing the $IC_{50}$ from 650 ± 40 to 291 ± 7 µM ($p < 0.001$). Interestingly, expression of either variant form of

hCTR1 increased sensitivity by an even larger degree. Expression of the M2 variant increased sensitivity by 2.5-fold ($IC_{50}$ 255 ± 10 μM; $p < 0.001$), and expression of the truncated variant by 3.2-fold ($IC_{50}$ 204 ± 8 μM; $p < 0.001$). Because of the discordance between the effect of re-expressing the variant forms of CTR1 on the cellular accumulation versus the cytotoxicity of cDDP, these experiments were repeated a total of 6 times and were found to be consistent across the entire set of experiments. Thus, despite the fact that neither variant mediated cDDP accumulation as well as the wild type CTR1, the $CTR1^{-/-/M2}$ and $CTR1^{-/-/Truncated}$ cells were less able to withstand attack by cDDP.

**Effect on cDDP-induced down-regulation of CTR1.**

One of the most striking features of the interaction between cDDP and CTR1 in many cell types is the ability of cDDP to trigger rapid and extensive degradation of this transporter. This has been documented by western blotting and flow cytometric analysis, but the most dramatic effects are observed by immunohistochemical staining (Holzer and Howell, 2006; Holzer et al., 2004a; Jandial et al., 2009). To determine whether this reaction was mediated by interaction of cDDP with either the M2 domain or some other component of the first 45 amino acids of the N-terminal end of hCTR1, the $CTR^{-/-}$, $CTR1^{-/-/WT}$, $CTR1^{-/-/M2}$ and $CTR1^{-/-/Truncated}$ cells were exposed to cDDP for 15 min, stained with an antibody to the myc tag and examined by quantitative deconvoluting microscopy. Although degradation can be detected after 5 min of exposure, maximum differences were observed at 15 min. As shown in panels A and D of Figure 4-5, cDDP produced a marked decrease in immunocytochemically detectable hCTR1 in the $CTR1^{-/-/WT}$ cells. As shown in panels B and E the M2 variant

of hCTR1 also showed a near complete disappearance of detectable hCTR1. Similar results were shown in panels C and F with regard to the truncation variant of hCTR1. These results indicate that the methionine and histidine motifs located in the first 45 amino acids of hCTR1 are not required for the ability of cDDP to down-regulate hCTR1. Thus, although cDDP may interact with motifs in the N-terminal domain, it must also bind elsewhere in the protein at a site that triggers the degradative process.

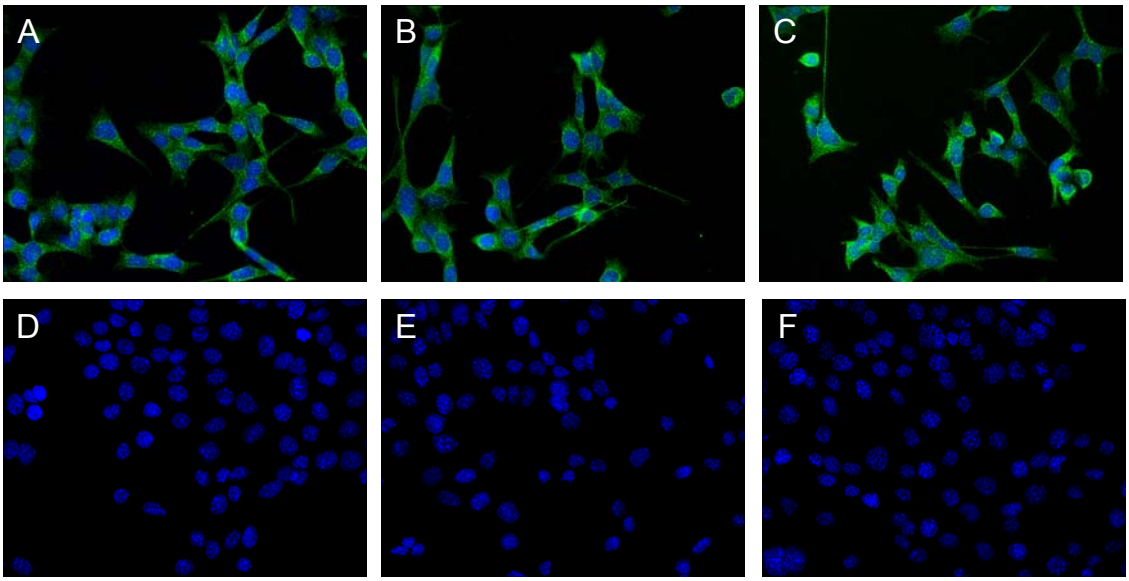


**Figure 4-5. Micrographs of MEF cells expressing hCTR1**. All panels are 20x. Panels A-C, no treatment; panels D – F, cells exposed to 30 μM CDDP treatment for 15 minutes prior to fixing. Panels A and D, $CTR1^{-/-/wt}$ cells; panels B and E, $CTR1^{-/-/M2}$ cells; panels C and F, $CTR1^{-/-/Truncated}$ cells.

## Discussion

Crystallographic analysis of hCTR1 did not identify structure for the N-terminal domain of hCTR1, but this region contains multiple methionine and histidine

motifs similar to those found in the interior of the pore formed by trimeric hCTR1 that have been proposed to mediate and regulate Cu influx by virtue of their ability to chelate Cu and cDDP (De Feo et al., 2009; De Feo et al., 2007; Howell et al., 2010). The results of the current study provide new details regarding the role of the N-terminal domain in both Cu and cDDP accumulation. Neither the M2 motif nor the entire first 45 amino acids appear to be important to the intracellular distribution of hCTR1. Consistent with most prior studies (Eisses and Kaplan, 2005; Guo et al., 2004; Puig et al., 2002), when expressed at equivalent levels in the $CTR1^{-/-}$ cells, there were no discernable differences in the subcellular distribution as detected by confocal microscopy. In addition, nearly equal amounts of the wild type and variant proteins were found on the cell surface. Thus, differences in the ability of the alternate forms of the protein to mediate Cu and cDDP uptake and cytotoxicity can be confidently attributed to the difference structure of these proteins rather than to differential levels of expression or subcellular distribution. Despite a prior report to the contrary (Liang et al., 2009), the results of the current study do not provide support for the presence of a signal essential to plasma membrane localization in the first 45 amino acids of hCTR1.

The first finding of interest is that re-expression of the N-terminal truncated variant further reduced the steady-state cellular Cu level below that found in the $CTR1^{-/-}$ cells when they were grown in standard tissue culture medium containing ~0.3 μM Cu, an effect not observed with the variant missing just the M2 motif. Cells that lack CTR1 clearly acquire Cu from their environment via other transporters, likely including the second mammalian Cu transporter CTR2 (Bertinato et al., 2007). One

possibility is that the function of these other transporters is impaired by interaction with truncated CTR1. Alternatively, the N-terminal domain of CTR1 may be required to prevent rapid efflux of Cu, a possibility given support by the previously reported finding that knockout of mCTR1 results in a large increase in the initial efflux of cDDP (Blair et al., 2009).

Prior studies performed with mutant forms of CTR1 expressed in yeast and insect cells suggested that the M2 motif in the N-terminal domain was required for uptake of Cu when environmental Cu was low, but that the M2 motif, and indeed the entire N-terminal domain, was dispensable when Cu was abundant (Eisses and Kaplan, 2005; Guo et al., 2004; Puig et al., 2002). However, it was found that, whereas re-expression of wild type hCTR1 in the $CTR1^{-/-}$ cells enhanced Cu uptake, hCTR1 containing a deletion of either the M2 motif or the first 45 amino acids N-terminal was ineffective. In addition, whereas re-expression of wild type hCTR1 enhanced the cytotoxicity of Cu, the M2 and N-terminal deletion variants actually rendered the $CTR1^{-/-}$ cells significantly more resistant to Cu. Liang *et al.* (Liang et al., 2009) also found that deletion of the first 45 amino acids obviated the ability of hCTR1 to enhance Cu uptake and rendered the cells resistant the cytotoxic effect of Cu and ascribed this to a transdominant effect of the exogenously expressed mutant hCTR1 on endogenous hCTR1. However, since the $CTR1^{-/-}$ cells contain no endogenous CTR1 another explanation must be sought. Although CTR1 has been characterized as a Cu influx transporter, these data are actually more consistent with the concept that hCTR1 functions to retain Cu in the cell, and that the M2 motif and N-terminal domain is important to this function. Both the inability of the M2 and N-

terminal truncation variants to enhance Cu accumulation, and the enhanced resistance to Cu, may be a result of enhanced efflux. The finding that deletion of just the M2 motif produced the same impairment of Cu uptake as deletion of the entire N-terminal domain is consistent with prior studies indicating the much greater importance of the M2 motif than the other histidine and methionine-rich motifs in the N-terminal domain with respect to Cu uptake (Eisses and Kaplan, 2005; Guo et al., 2004; Puig et al., 2002).

The results of the current study establish that the N-terminal domain is important but not essential to the ability of hCTR1 to mediate cDDP uptake. A recent study by Liang *et al.* (Liang et al., 2009) also showed that the M2 motif is important for cDDP uptake in small cell lung cancer cells. Truncation of entire N-terminal domain removes N15 glycosylation site whereas deletion of just the M2 motif does not; glycosylation at this site is not a determinant of Cu uptake (Eisses and Kaplan, 2002; Liang et al., 2009) and the similar effect of these changes on cDDP uptake indicates that it is also not a key determinant of cDDP uptake by hCTR1.

While there are some parallels between the effect of deleting the M2 motif and the entire N-terminal domain on Cu and cDDP uptake, there are also some clear differences. As for Cu, re-expression of wild type hCTR1 enhanced the initial uptake of cDDP; in fact the magnitude of the effect was substantially larger for cDDP than for Cu. This interesting in light of prior studies showing that small changes in the sensitivity of cells to Cu are associated with substantially larger changes in sensitivity to cDDP (Safaei et al., 2004). However, whereas the variant forms of hCTR1 failed to enhance the accumulation of Cu and actually rendered the $CTR1^{-/-}$ cells resistant to

Cu, both variants were still partially effective in enhancing cDDP uptake and rendered the cells more rather than less sensitive to the cytotoxic effect of cDDP. The former observation is consistent with the concept that, as for Cu, the M2 motif is involved in chelating cDDP during the transport process. Conversion in yCTR1 of the methionine corresponding to M45 in hCTR1 to alanine has previously been reported to block cDDP uptake in yeast (Sinani et al., 2007), and conversion of either M43 or M45 to glutamine in hCTR1 has the same effect (Liang et al., 2009). A recently proposed model suggests that, when assembled as a trimer, these motifs form a set of stacked rings of methionines and histidines that loosely chelate cDDP and pass it sequentially through the pore (De Feo et al., 2009; De Feo et al., 2007; Howell et al., 2010). Such a set of transchelation reactions has the potential to provide a path through the plasma membrane that is highly selective for metals that form weak bonds with methionine. There is now evidence supporting the concept that cDDP does in fact bind to hCTR1. Incubation of cDDP with cells increases the fraction of hCTR1 that is found in the trimeric rather than monomeric state (Guo et al., 2004; Liang et al., 2009). In addition, cDDP binds to a peptide containing the first 20 amino acids of hCTR1 that includes the H1 and M1 motifs (Wu et al., 2009b), and to an peptides with similar methionine motifs (Arnesano et al., 2007; Sze et al., 2009) as well as to the CXXC motif in ATOX1 (Boal and Rosenzweig, 2009), although it remains unclear whether the kinetics of binding are consistent with a role for the interaction in the relatively rapid process of cDDP uptake. Both the binding of cDDP to the N-terminal domain and the multimerization of CTR1 occur only after prolonged periods of incubation at concentrations of cDDP that are far higher than those found in patient plasma and the

relevance of these reactions to the regulation of cDDP transport is not currently apparent.

The results of the current study provide clear evidence that none of the motifs in the first 45 amino acids of hCTR1 are required for cDDP-induced degradation of hCTR1. Thus, in addition to interacting with the N-terminal domain, cDDP must be binding at another site in hCTR1. Chelation by the rings of methionines putatively contributed by M40 and M45 is a strong candidate for the mechanism by which conformational changes that trigger endocytosis and degradation are initiated (De Feo et al., 2009; De Feo et al., 2007; Howell et al., 2010).

The M2 and N-terminal deletion variants were less effective than the wild type hCTR1 at enhancing the initial influx of cDDP, but curiously produced a greater enhancement of the cytotoxic effect of cDDP. Thus, per unit of cDDP take up by the $CTR1^{-/-/M2}$ and $CTR1^{-/-/Truncated}$ cells there was a greater degree of cell kill. While case has been argued for movement of cDDP through the plasma membrane pore created by trimeric hCTR1 (Howell et al., 2010), there remains the possibility that hCTR1 also delivers cDDP bound to the N-terminal domain into cells by endocytosis and that deletion of M2 motif or the N-terminal domain alters the ratio of drug entering by the two routes. Loss of the histidine and methionine-rich N-terminal domain might be expected to favor entry of cDDP via the pore. Since such entry would deliver cDDP directly into the cytoplasm, whereas entry by endocytosis leaves cDDP still inside a vesicular structure, it is speculated that drug entering via the pore might be more potent. It is of interest that it has been noted previously that there are quite large differences among cell lines in the fraction of the amount of cellular oxaliplatin that

actually reaches critical targets such as DNA (Mishima et al., 2002), a feature that may reflect differences in the route of entry.

## Materials and Methods

### Drugs and reagents.

The commercial formulation of cDDP was purchased from the Moores Cancer Center pharmacy; it contains cDDP at a concentration of 3.33 mM in 0.9% NaCl. The cDDP was diluted into DMEM-RS Reduced Serum Media (HyClone, Logan, UT) to produce a final concentration of 30 μM. Bradford reagent was purchased from BioRad Laboratories, Inc. (Hercules, CA) and sulforhodamine B was obtained from Sigma-Aldrich (St. Louis, MO) and 0.4% SRB (w/v) was solubilized in 1% (v/v) acetic acid solution. Anti-myc primary antibody 9B11 was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Secondary anti-mouse, HRP-conjugated antibody was obtained from GE Healthcare (Piscataway, NJ). Hoechst 33342 nuclear stain and secondary AlexaFluor 488-conjugated anti-mouse antibody were obtained from Invitrogen (Carlsbad, CA).

### Cell types, culture and engineering.

Mouse embryonic fibroblast cell line in which both copies of CTR1 had been somatically knocked out ($CTR1^{-/-}$) was kindly provided by Dr. Dennis Thiele (Lee et al., 2002b). The myc-$CTR1^{-/-/wt}$ subline was constructed by infecting the $CTR1^{-/-}$ cells with a lentivirus expressing a wild type human CTR1 cDNA, tagged with the myc-epitope on the N-terminus of the protein, using the ViraPower Lentiviral Induction kit (Invitrogen, Carlsbad, CA). Mutations to the hCTR1 molecule were created using the

GeneTailor Site-Directed Mutagenesis Kit (Invitrogen, Carlsbad, CA) using the following primers: for the myc-CTR1$^{-/-/M2}$ deletion (residues 40-45) (cccatggtggaggagacagcagcaccttctactttggctttaagaa, ttcttaaagccaaagtagaaggtgctgctgtctcctccaccatggg), for the myc-CTR1$^{-/-/Truncated}$ (deletion of residues 1-45) mutation (caccatgggcggcagggaacaaaaacttatttctgaagaagatctgg, cttatttctgaagaagatctgggcggcaccttctactttgg, tcaatggcaatgctctgtg).

**Cell Survival Assay.**

Cell survival following exposure to increasing concentrations of drugs was assayed using the sulforhodamine B assay system (Monks et al., 1991). The optimal number of cells seeded per well was determined to be 5,000 cells in preliminary experiments. Five thousand cells were seeded into each well of a 96-well tissue culture plate. Cells were incubated overnight at 37°C, 5% $CO_2$ and then exposed for 5 min by the addition of 200 µl Pt drug-containing DMEM-RS medium. After 5 min the drug-containing media was aspirated off, cells were washed once with 37°C PBS, PBS was aspirated off and cells were covered in 200 µl complete medium. Cells were allowed to grow for 5 d after which the media was removed, the protein was precipitated with 50% trichloroacetic acid and stained using 100 µl of 0.4% sulforhodamine B in 1% acetic acid at room temperature for 15 minutes. Following washing the absorbance of each well at 515 nm was recorded using a Versamax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). All experiments were repeated at least 3 times using 3 cultures for each drug concentration.

**Immunocyto Chemistry.**

All images were analyzed using a Delta Vision Deconvolution Microscope System utilizing a Nikon TE-200 Microscope (Applied Precision, Inc., Issaquah, WA). Deconvolution and analysis was done using the softWoRx software suite (Applied Precision, Inc., Issaquah, WA).

**Measurement of cellular drug accumulation.**

Cells were grown to 90% confluence in T-150 tissue culture flasks. Cells were then harvested using trypsin and 7.5 x $10^5$ cells were placed into each well of 6-well tissue culture plates and allowed to grow overnight in 2.5 ml of media at 37°C in 5% $CO_2$. All data presented are the means of at least 3 independent experiments each performed with 6 wells per concentration tested. The next day medium was removed by aspiration and the cells were exposed to 1 ml of drug-containing DMEM-RS reduced serum medium (HyClone, Logan, UT) at 37°C for either 0 or 5 minutes after which the drug-containing medium was removed, the plates were washed 3 times with ice-cold PBS and then placed on ice. In the case of the time zero samples, the drug-containing medium was aspirated within 15 sec of the start of drug exposure. Two hundred and fifteen µl of concentrated (50-70%) nitric acid was added to each well and the plate was incubated overnight at room temperature. The following day the acid was moved into Omni-vials (Wheaton, Millville, NJ) and the wells were washed 3 times with 1 ml (3 ml total volume) of buffer (0.1% Triton X-100, 1.4% nitric acid, 1 ppb In in dd$H_2O$). Pt concentration was measured using a Perkin-Elmer Element 2 ICP-MS located at the Analytical Facility at Scripps Institute of Oceanography at the University of California, San Diego. Values were normalized to either protein

concentration as determined using the Bradford reagent or total sulfur content, as measured by ICP-OES, on the same samples.

**qRT-PCR.**

CTR1 mRNA levels were measured by qRT-PCR. cDNA was generated from mRNA isolated using Trizol (Invitrogen, Carlsbad, CA). Purified mRNA was converted to cDNA using Oligo$(dT)_{20}$ priming and the SuperScript III First-Strand Kit (Invitrogen, Carlsbad, CA). qRT-PCR was performed on a Bio-Rad MyIQ qPCR machine (Hercules, CA). The forward and reverse primers for hCTR1, mCTR1 and GAPDH were, respectively: gatgatgatgcctatgacct, tcttgagtccttcatagaac, actgttgggcaacagatgct, ctgctgctactgcaatgcag, tcaccaccatggagaaggc, gctaagcagttggtggtgca. Reactions were prepared using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), according to manufacturer's recommendations. Samples were prepared in quadruplicate and at least 3 independent RNA isolates were used in independent experiments. Analysis was done using the Bio-Rad iQ5 system software (Hercules, CA).

**Statistical Analysis.**

All multi-group analyses were done utilizing one way analysis of variance testing with Tukey's test post hoc analysis, where $p \leq 0.05$ was determined to be statistically significant. All data is expressed as mean ± SEM values.

## Acknowledgements

The author wishes to thank Dr. Dennis Thiele for kindly providing the $CTR1^{-/-}$ cells used in this study. The author would also like to thank Gerald Manorek, Dr.

Paolo Abada, Dr. Xinjian Lin, and Dr. Xiaoqin Yuan for assistance, technical expertise and valuable discussion. A majority of the content of Chapter 4 has been accepted for publication in *Biochemical Pharmacology* (Larson CA, Adams PL, Jandial DD, Blair BG, Safaei R, Howell SB. The Role of the N-terminus of Mammalian Copper Transporter 1 in the Cellular Accumulation of Cisplatin*) Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Preston L. Adams assisted in the measurement of Pt and Cu accumulation and provided helpful feedback. Danielle D. Jandial and Brian G. Blair provided valuable assistance and feedback. Roohangiz Safaei provided helpful discussion.

# Chapter 5 –

# Role of the Methionines and Histidines in the Transmembrane Domain of CTR1 in the Cellular Accumulation of Cisplatin

## Introduction

As discussed in chapter 1 the inner faces of the CTR1 pore contain methionines, cysteines, and histidine residues that facilitate the movement of Cu into the cell along the concentration gradient. The goal of the described experiments in this chapter was to assess the role of the CTR1 pore in accumulating and cytotoxicity of Cu and involved converting methionines to isoleucines, and the histidine was converted to alanine, and then re-expressed in $CTR1^{-/-}$ cells. cDDP and Cu accumulation and cytotoxicity was measured in the same manner as described in previous chapters.

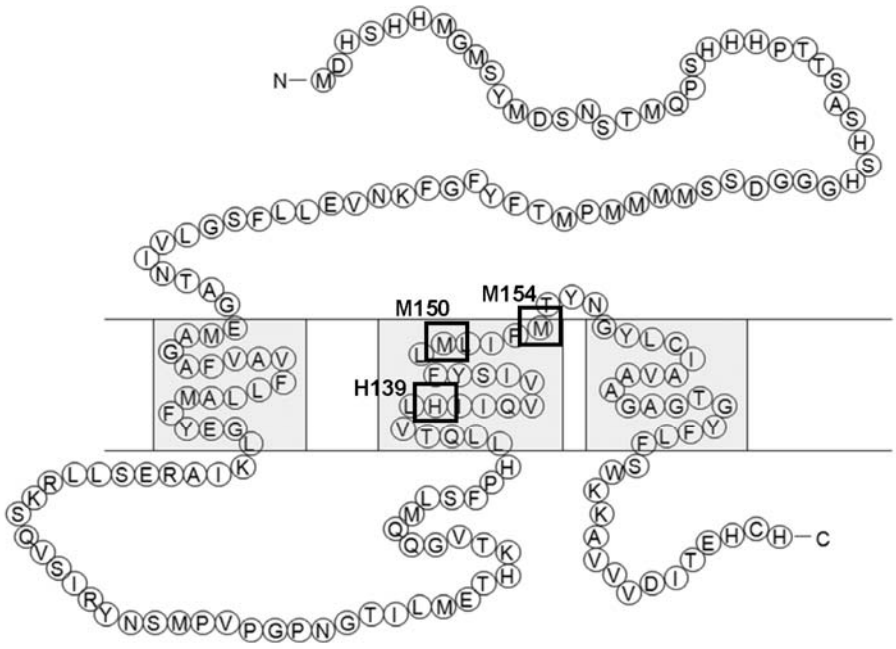


**Figure 5-1. Schematic diagram of the amino acid sequence of hCTR1**. Boxes highlight the H139 and M150, and 154 amino acids.

## Results

### Expression of CTR1 in CTR1$^{-/-}$ mouse embryo fibroblasts.

The histidines and methionines in the transmembrane section of the pore formed by homotrimeric hCTR1 potentially chelate both Cu and cDDP. The importance of $M^{150}$ and $M^{154}$ for the transport of Cu has been well established; however, prior studies of the requirement for these amino acids in the transport of cDDP have been confounded by the presence of endogenous CTR1. To avoid this problem, wild type and variant forms of hCTR1 were constitutively re-expressed in mouse embryo fibroblasts in which both alleles of CTR1 had been knocked out

($CTR1^{-/-}$ cells). Lentiviral vectors containing a blasticidin resistance marker were constructed to express either wild type hCTR1, a variant in which $M^{150}$ and $M^{154}$ were converted to isoleucines, or a variant in which the $H^{139}$ was converted to alanine, and these were used to generate $CTR1^{-/-/WT}$ , $CTR1^{-/-/M150,154I}$ , and $CTR1^{-/-/H139A}$ cells. All 3 forms of hCTR1 contained a myc tag at the N-terminal end. Cells were infected, selected with blasticidin and the resulting population characterized with respect to the expression of each form of exogenous CTR1 by qRT-PCR. Figure 5-2A shows the relative levels of CTR1 mRNA expressed in the cell lines. The two variant forms of hCTR1 were expressed at 70% of the level of the wild type hCTR1. To further validate the system, the amount of hCTR1 in the plasma membrane was determined by exposing the cells to sulfo-NHS-SS-biotin which labels cell surface proteins. The proteins were recovered onto streptavadin beads and subjected to western blot analysis using antibodies that reacted with either the myc tag or α-subunit of $Na^+/K^+$ ATPase. As shown in Figure 5-2B the CTR1 monomer was detected at 37 kDa in all 3 transduced cell lines and the α-subunit at ~110 kDa. When normalized to the level of the α-subunit, the level of expression of CTR1 in the myc-$CTR1^{-/-/H139A}$ cells was 94 ± 5% of that in the $CTR1^{-/-/WT}$ cells while expression in the myc-$CTR1^{-/-/M150,154I}$ cells was 93 ± 4%. As shown in Figure 5-2C, immunohistochemical analysis established that there were no appreciable differences in the subcellular distribution of hCTR1 in the 3 cell types. Thus, neither conversion of $M^{150}$ and $M^{154}$ to isoleucines, nor conversion of $H^{139}$ to alanine altered the trafficking of CTR1 from ER and Golgi to the cell surface or other vesicular structures.

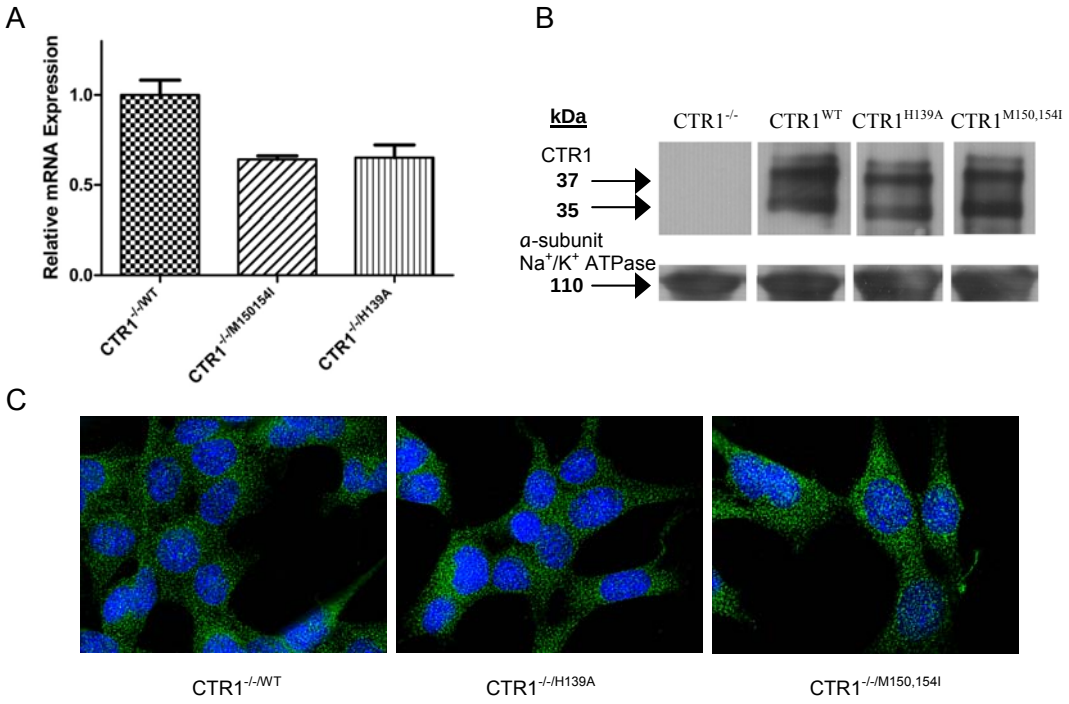


**Figure 5-2. Re-expression of myc-hCTR1 in CTR1$^{-/-}$ cells**. A, relative hCTR1 mRNA levels as determined by qRP-PR. B, representative western blot showing expression of myc-tagged CTR1. C, micrographs demonstrating uniform expression of the myc-tagged CTR1 protein (60x magnification).

**CTR1 Regulation of Cu Uptake and Cytotoxicity.**

The steady-state basal level of Cu was determined while the cells were growing in complete DMEM medium containing ~0.3 μM Cu. Figure 5-3A shows that basal Cu was 1.3-fold ($p = 0.05$) higher in the CTR1$^{-/-/WT}$ than in the CTR1$^{-/-}$ cells. Expression of the M150,154I variant reduced the basal Cu level to 89% of the found in the CTR1$^{-/-}$ cells, whereas expression of the H139A variant reduced basal Cu to just 32% ($p = 0.05$) of that in the CTR1$^{-/-}$ cells. Thus, expression of these two variant forms of CTR1 perturbed either influx, efflux or the Cu binding capacity of the cells to reduce steady-state Cu content.

Rates of Cu accumulation were analyzed by exposing the cells to media containing 100 μM Cu for 1 h. This relatively high concentration of Cu allowed measurement of cellular Cu content by by ICP-MS and has been shown previously to permit ready detection of Cu transport abnormalities (Samimi et al., 2004a). As shown in Figure 5-3B, re-expression of the wild type hCTR1 resulted in a 2-fold increase in the rate of Cu accumulation when compared to uptake in the $CTR1^{-/-}$ cells ($p = 0.002$). In contrast, neither of the hCTR1 variants was able to increase Cu uptake at all. Thus, the integrity of either or both of M150 and M154, and of H139, is essential for Cu transport to occur in this system.

To determine whether the differences in Cu accumulation translated into different tolerances to the cytotoxic effect of Cu, the growth rate of the $CTR^{-/-}$, $CTR1^{-/-/M150,154I}$, and $CTR1^{-/-/H139A}$ cells was measured during a 96 h exposure to increasing concentrations of Cu. As shown in Figure 5-3C re-expression of the wild type CTR1 resulted in a 1.2-fold increase in sensitivity relative to the $CTR1^{-/-}$ cells ($IC_{50}$ μM 37 ± 1 *versus* 46 ± 2 μM; $p = 0.003$). Neither of the CTR1 variants produced a biologically significant increase in sensitivity consistent with their failure to enhance Cu uptake.

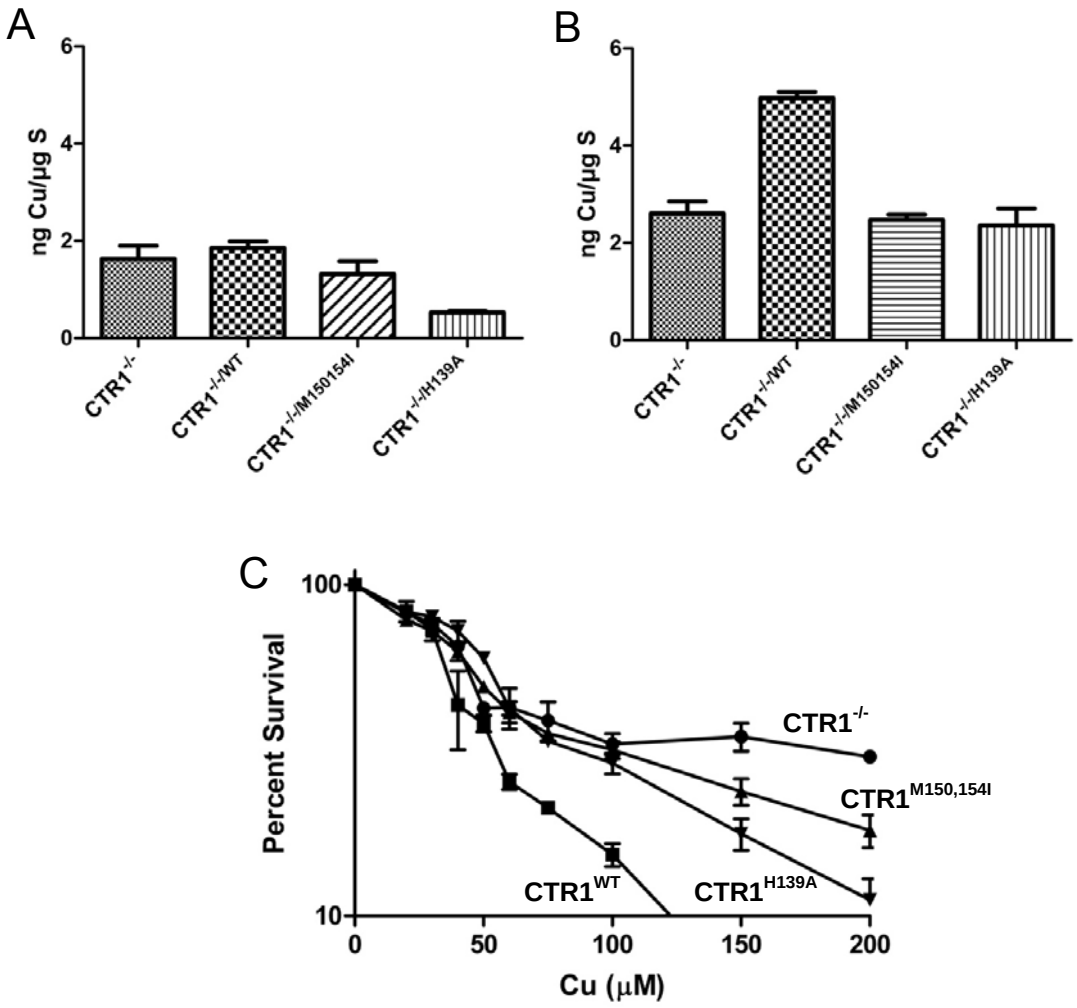


**Figure 5-3. Cu accumulation and cytotoxicity.** A, total basal Cu. B, total Cu following 1 h exposure to 100 µM Cu. C, inhibition of the growth of MEF cells during 96 h continuous exposure to varying concentrations of Cu. Each value represents the mean of no less than 3 independent experiments each performed with triplicate cultures. Vertical bars, SEM.

**CTR1 Regulation of cDDP Uptake and Cytotoxicity.**

To analyze the effect of the M150,154I and the H139A substitutions on the initial influx of cDDP, the 4 types of cells were exposed to 30 µM cDDP for 5 min, washed thoroughly and the Pt content measured by ICP-MS. As shown in Figure 5-

4A, re-expression of wild type CTR1 resulted in a 4.2-fold increase in cDDP accumulation ($p = 0.0001$). Expression of the M150,154I variant resulted in a 7.2-fold increase in cDDP accumulation relative to that in the $CTR1^{-/-}$ cells ($p = 0.0002$), and a 1.8-fold increase relative to that in the $CTR1^{-/-/WT}$ cells ($p = 0.0002$). The H139A variant also increased the initial influx of cDDP, in this case by a factor 3.5-fold over that in the $CTR1^{-/-}$ cells ($p = 0.009$) which was 85% of the increase mediated by the wild type hCTR1. Thus, rather than impairing the transport of cDDP, converting M150 and M154 to a type of amino acid not known to chelate cDDP resulted in an increase rather than a decrease in cDDP influx.

To determine whether the changes in cDDP influx translated into changes in the cytotoxicity of this drug, cells were treated with increasing concentrations of cDDP for 5 min and their growth rate assessed over the ensuing 96 h. The re-expression of wild type CTR1 resulted in a 2.2-fold increase in cytotoxicity relative to the $CTR1^{-/-}$ cells ($IC_{50}$ 291 ± 7 *versus* 650 ± 40 μM; $p = 0.002$). Consistent with its ability to increase cDDP uptake to an even greater extent, the M150,154I variant increased the cytotoxicity of cDDP by a factor of 3.1-fold ($p = 0.001$) ($IC_{50}$ of 211 ± 7 μM) or 1.6-fold more than the wild type CTR1 ($p = 0.001$). Of interest, even though the H139A variant increased cDDP uptake somewhat less than the wild type hCTR1, it actually did a better job of enhancing cDDP cytotoxicity than the wild type hCTR1. The $IC_{50}$ for the $CTR1^{-/-/H139A}$ cells was 181 ± 3 μM reflecting a 3.6-fold increase in cytotoxicity relative to the $CTR1^{-/-}$ cells ($p=0.002$), and a 1.6-fold increase over that produced by wild type CTR1 ($p = 0.0005$). Due to the discrepancy between the effect of the H139A variant on cDDP uptake versus cytotoxicity, this set of experiments was

repeated a total of 5 times, all with consistent results. Thus, whereas the M150,154I and H139A variants were unable to transport Cu, they actually mediated enhanced uptake of cDDP.

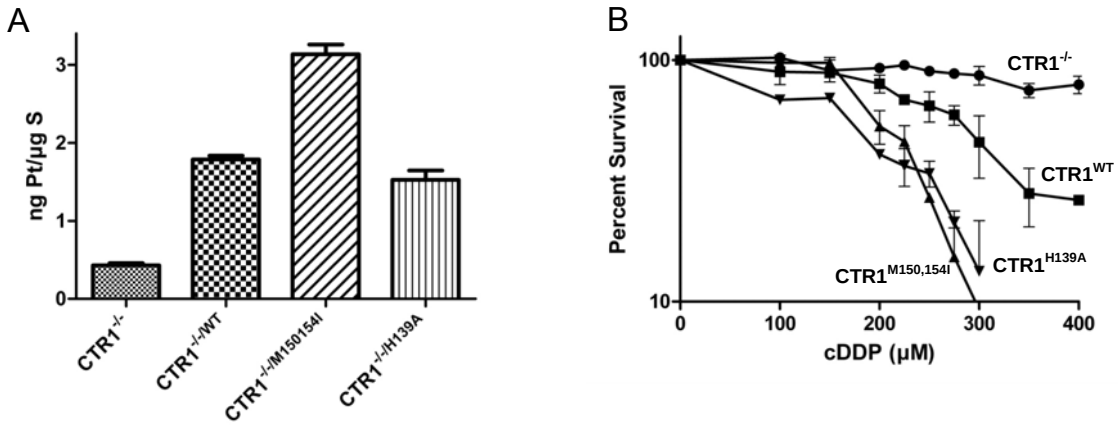


**Figure 5-4. cDDP accumulation and cytotoxicity.** A, net accumulation of Pt in MEF cells following 5 min exposure to 30 µM cDDP. B, inhibition of growth of MEF cells following 5 minute exposure to varying concentrations of cDDP. All values represent means of 6 independent experiments each performed with triplicate cultures. Vertical bars, SEM.

**Effect on cDDP-Induced Down-Regulation of CTR1.**

In mouse embryo fibroblasts cDDP triggers the rapid degradation of CTR1 via ubiquitination and subsequent degradation in the proteosome (Jandial et al., 2009). To determine whether this reaction was a result of the interaction of cDDP with M150, M154 or H139, the ability of cDDP to down-regulate the expression of CTR1 in the $CTR1^{-/-/WT}$, $CTR1^{-/-/M150,154I}$, and $CTR1^{-/-/H139A}$ cells was analyzed by immunocytochemistry. The top 3 panels of Figure 5-5 show that the distribution of hCTR1 was normal prior to cDDP exposure, and the lower 3 panels show that a 15 min exposure to 30 µM cDDP caused disappearance of nearly all the signal. Thus,

M150, M154 or H139are not essential to the down-regulation of hCTR1 indicating that cDDP must be interacting with some other part of the molecule.

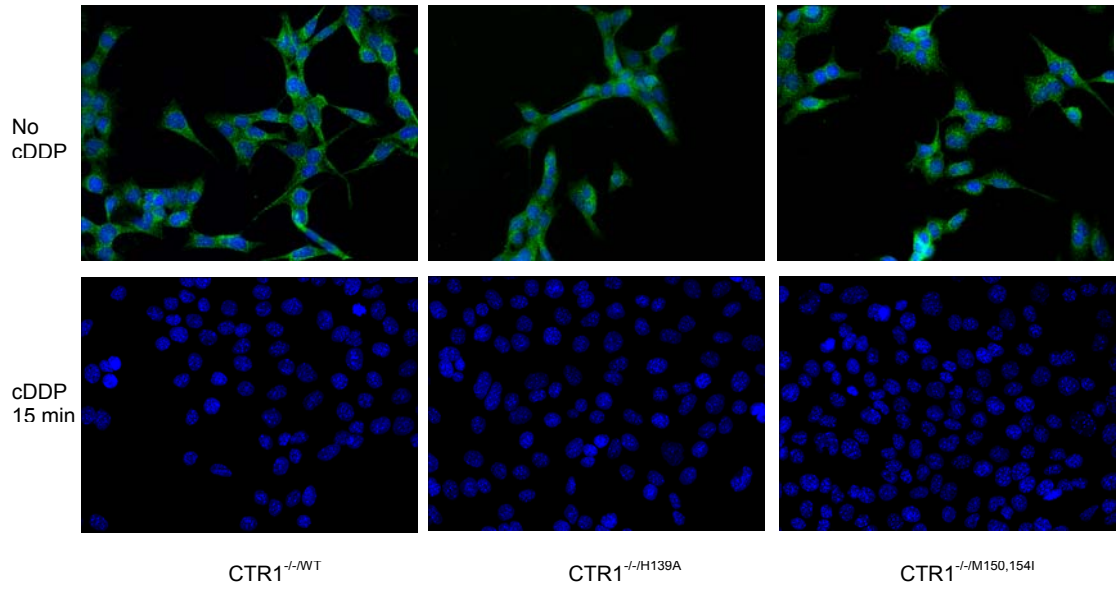


**Figure 5-5. Effect of modifying M150, 154 and H139 on ability of cDDP to trigger CTR1 degradation**. All panels are 20x. Top panels, no cDDP treatment. Bottom panels, 30 μM CDDP treatment for 15 minutes prior to fixing.

## Discussion

Electron crystallographic analysis of CTR1 suggests that when the monomers assemble into a homotrimer in the plasma membrane, M150, M154 and H139 are positioned such that they can form 3 stacked rings in the pore (De Feo et al., 2009; De Feo et al., 2007). These have been envision as mediating $Cu^{+1}$ influx through a series of transchelation reactions in which $Cu^{+1}$ is handed from one ring to the next down its concentration gradient. Based on the fact that the interaction of cDDP with methionines and histidines is very similar to that of $Cu^{+1}$, it is proposed that hCTR1 transports cDDP in a similar manner (Howell et al., 2010). However, the results

reported here indicate that, although the transchelation concept may be correct, M150, M154 and H139 play quite different roles in the transport of Cu and cDDP.

The re-expression of wild type and variant forms of hCTR1 in cells in which endogenous CTR1 is completely absent due to knockout of both alleles provides a powerful way of identifying structural components essential for function. Despite small differences in the level of expression at the mRNA level, all 3 forms of hCTR1 protein assessed in this study were expressed at equal levels at the plasma membrane. In addition, the distribution of hCTR1 in other parts of the cell appeared normal indicating that M150, M154 and H139 are not important determinants of the normal trafficking of hCTR1. This model system was further validated by the observation that re-expression of wild type hCTR1 increased Cu and cDDP uptake and enhanced sensitivity to the cytotoxic effect of both of these drugs.

The fact that the M150,154I variant of hCTR1 failed to increase the uptake of Cu, and was unable to significantly enhance the cytotoxicity of Cu, is consistent with prior studies of Cu transport in yeast, insect and mammalian cells that established the importance of these methionines under conditions of low environmental Cu (Eisses and Kaplan, 2005; Liang et al., 2009; Puig et al., 2002). Similarly, the observation that the H139A variant failed to transport Cu confirms the results of a prior study that reported that conversion of H139 to alanine reduced Cu uptake when the variant hCTR1 was expressed in insect cells (Eisses and Kaplan, 2005). The most intriguing observation to emerge from this study is that, instead of disabling cDDP transport, conversion of M150 and M154 to isoleucines significantly increased cDDP uptake well above that attained with wild type hCTR1, and that this was accompanied by a

further enhancement of the cytotoxicity of cDDP. This result indicates that the stacked rings of methionines putatively formed by M150 and M154 serve to obstruct the flow of cDDP through the pore. In the M150,154I variant the stacked rings potentially formed by H139 and C189 in the lower part of the pore remain intact and may continue to serve an essential transchelation role, but these data suggest that the interaction of cDDP with M150 and/or M154 in some way controls the rate of transport . A previous study performed in small cell lung cancer cells that contained endogenous hCTR1 found that expression of an exogenous form of hCTR1 in which either M150 or M154 were converted to glutamine reduced the uptake of cDDP and rendered the cells less sensitive to cDDP. However, this was attributed to a transdominant negative effect of the mutant hCTR1 on the wild type endogenous hCTR1 mediated by the inability to form correctly assembled trimeric complexes in the plasma membrane. The design of the prior study did not permit detection of the transport-enhancing effect of completely removing the stacked rings formed by M150 and M154.

Conversion of H139 to alanine did not significantly impair the ability of hCTR1 to mediate the uptake of cDDP, but it also did not enhance its uptake. This indicates that cDDP interaction with H139 is not essential to its transport and is consistent with the concept that the rings of methionines formed by M150 and M154 are more important determinants of flux. Of more interest is the fact that, while wild type hCTR1 and the H139A variant accumulated similar amounts of cDDP, the $CTR1^{-/-/H139A}$ cells were substantially more sensitive to the cytotoxic effect of cDDP indicating that the potency of the accumulated drug was greater. The explanation for

this is not readily apparent but may be related to alteration in the efficiency of transfer of cDDP to intracellular chaperones or a change in the ratio of cDDP that enters the cell via hCTR1 versus the other routes that must exist since $CTR1^{-/-}$ cells still accumulate measurable amounts of cDDP. The idea that the H139A variant affects other possible transport routes is bolstered by its effect on basal Cu levels. Despite the fact that conversion of H139 to alanine disabled its ability to mediate Cu uptake, it nevertheless markedly reduced the basal intracellular Cu level in the $CTR1^{-/-}$ cells.

In the mouse embryo fibroblasts cDDP triggers rapid degradation of endogenous CTR1, and the results of this study indicate that it does the same thing to exogenous hCTR1 when re-expressed in the $CTR1^{-/-}$ cells. Previous studies have documented that CTR1 becomes ubiquitinated (Safaei et al., 2009), and that inhibition of proteosome function blocks the cDDP-induced degradation of CTR1 (Holzer and Howell, 2006) and enhances the uptake and cytotoxicity of the drug (Jandial et al., 2009). The ability cDDP to trigger CTR1 degradation is compromised in cells that lack the Cu chaperone ATOX1 (Safaei et al., 2009). How cDDP initiates CTR1 ubiquitination is unknown, but the results of this study support the conclusion that it does not require interaction of cDDP with M150, M54 or H139 indicating that cDDP must bind to some other domain in hCTR1 as well.

In summary, the opposite effects of converting M150 and M154 to isoleucines on the transport of Cu and cDDP indicate that, while they play a facilitating role in moving Cu through the pore, they serve to obstruct the passage of cDDP. Thus, although both Cu and cDDP may rely on a series of transchelation reactions to pass through the hCTR1 trimeric complex, the details of the molecular interactions must be

different which provides a potential basis for selective pharmacologic modulation of Cu versus cDDP cytotoxicity

## Materials and Methods

**Drugs and Reagents**.

cDDP was acquired from the Moores Cancer Center pharmacy; it contains cDDP at a concentration of 3.33 mM in 0.9% NaCl. The cDDP was diluted into DMEM-RS Reduced Serum Media (HyClone, Logan, UT) to a final concentration of 30 μM. Bradford reagent was obtained from BioRad Laboratories, Inc. (Hercules, CA) and sulforhodamine B was purchased from Sigma-Aldrich (St. Louis, MO) and was solubilized in 1% acetic acid (v/v) at a final concentration of 0.4% SRB (w/v). Anti-myc primary antibody, clone 9B11, was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Secondary anti-mouse, HRP-conjugated antibody was purchased from GE Healthcare (Piscataway, NJ). Hoechst 33342 nuclear stain and anti-mouse AlexaFluor 488-conjugated secondary antibody were obtained from Invitrogen (Carlsbad, CA).

**Cell types, culture and engineering.**

Mouse embryonic fibroblasts containing wild type alleles of CTR1 ($CTR1^{+/+}$) and a line in which both copies of CTR1 had been somatically knocked out ($CTR1^{-/-}$) were kindly provided by Dr. Dennis Thiele (Lee et al., 2002b). The myc-$CTR1^{-/-/wt}$ subline was constructed by infecting the $CTR1^{-/-}$ cells with a lentivirus expressing wild type human CTR1 cDNA, N-terminally tagged with the myc epitope, using the

ViraPower Lentiviral Induction kit (Invitrogen, Carlsbad, CA). Point mutations were created with the GeneTailor Site-Directed Mutagenesis Kit (Invitrogen, Carlsbad, CA) using the following primers: for the myc-CTR1$^{-/-/H139A}$ mutation (tcctcacctcctgcaaacagtgctggccatcatccaggtggtcataagctac, gtagcttatgaccacctggatgatggccagcactgtttgcaggaggtgagga); for the myc-CTR1$^{-/-/M150,154I}$ mutation (aggtggtcataagctacttcctcatactcatcttcataacctacaacgggtacctctgcattg, caatgcagaggtacccgttgtaggttatgaagatgagtatgaggaagtagcttatgaccacct).

**Cell Survival Assay.**

The sulforhodamine B assay system (Monks et al., 1991) was used to determine cell survival following exposure to increasing concentrations of drug. Five thousand cells were seeded into the wells of a 96-well tissue culture plate. Cells were incubated overnight at 37°C, 5% $CO_2$ and then exposed to cDDP for 5 min, at 37°C, by the addition of 200 µl Pt drug-containing DMEM-RS medium. After 5 min the drug-containing media was removed, cells were washed once with 37°C PBS, PBS was aspirated off and cells were covered in 200 µl complete medium. Cells were allowed to grow for 5 days after which the media was removed, the plate was washed 3 times with PBS, the protein was precipitated with 50% trichloroacetic acid and stained using 100 µl of 0.4% sulforhodamine B in 1% acetic acid at room temperature for 15 minutes. Following washing of the plate, the absorbance of each well at 515 nm was recorded using a Versamax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). All experiments were repeated no less than 3 times using 3 cultures for each drug concentration.

**Immunocytochemistry.**

All images were visualized using a DeltaVision Deconvolution Microscope System utilizing a Nikon TE-200 Microscope (Applied Precision, Inc., Issaquah, WA). Deconvolution and subsequent analysis was done using the softWoRx software suite (Applied Precision, Inc., Issaquah, WA)

**Measurement of cellular drug accumulation.**

Drug accumulation was measured by inductively-coupled plasma mass spectroscopy as described previously (Larson et al., 2009); the only change made was the utilization of 1 ml of medium and DMEM-RS in place of OptiMEM.

**qRT-PCR.**

myc-CTR1 mRNA was measured via qRT-PCR. First-strand cDNA was generated from Trizol-isolated mRNA (Invitrogen, Carlsbad, CA) using Oligo$(dT)_{20}$ priming and the SuperScript III First-Strand Kit (Invitrogen, Carlsbad, CA). qRT-PCR was performed using a Bio-Rad MyIQ qPCR machine (Hercules, CA). The forward and reverse primers for hCTR1, mCTR1 and GAPDH were, respectively: gatgatgatgcctatgacct, tcttgagtccttcatagaac, actgttgggcaacagatgct, ctgctgctactgcaatgcag, tcaccaccatggagaaggc, gctaagcagttggtggtgca. Reactions utilized iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), following the manufacturer's recommendations. Samples were prepared in quadruplicate with a minimum of 3 independent RNA isolates were used in independent experiments. Analysis was done using the Bio-Rad iQ5 system software (Hercules, CA).

**Statistical Analysis.**

All 2-group comparisons utilized Student's *t*-test with the assumption of unequal variance. Data are presented as mean ± SEM.

## Acknowledgements

The author wishes to thank Dr. Dennis Thiele for kindly providing the $CTR1^{-/-}$ cells, and Dr. Jack Kaplan for providing reagents used in this study. The author would also like to thank Gerald Manorek, Dr. Paolo Abada, Dr. Xinjian Lin, and Dr. Xiaoqin Yuan for assistance, technical expertise and valuable discussion. A majority of the content of Chapter 5 has been submitted for publication. Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Preston L. Adams assisted in the measurement of Pt and Cu accumulation and provided helpful feedback. Brian G. Blair provided valuable assistance and feedback. Roohangiz Safaei provided helpful discussion.

# Chapter 6 –

# Transport and Cytotoxicity of Cisplatin and Copper Mediated by Variant Forms of Copper Transporter 1

## Introduction

As discussed in chapter 1, CTR1 contains two intracellular domains, the first being a loop connecting the first and second transmembrane domains and the second being the 15 amino acid C-terminal tail region. The goal of the experiments described in this chapter was to determine the role of the two intracellular regions in the accumulation and cytotoxicity of Cu and cDDP. The methods utilized in this chapter started with re-expression of 2 variant forms of hCTR1. One in which Y103 was converted to alanine and one in which C189 was converted to a serine in $CTR1^{-/-}$ mouse embryo fibroblasts. As in previous chapters the accumulation and cytotoxicity of both Cu and cDDP were analyzed. Furthermore, via immunocyto chemistry the ability of cDDP and Cu to trigger degradation of hCTR1 was assessed.

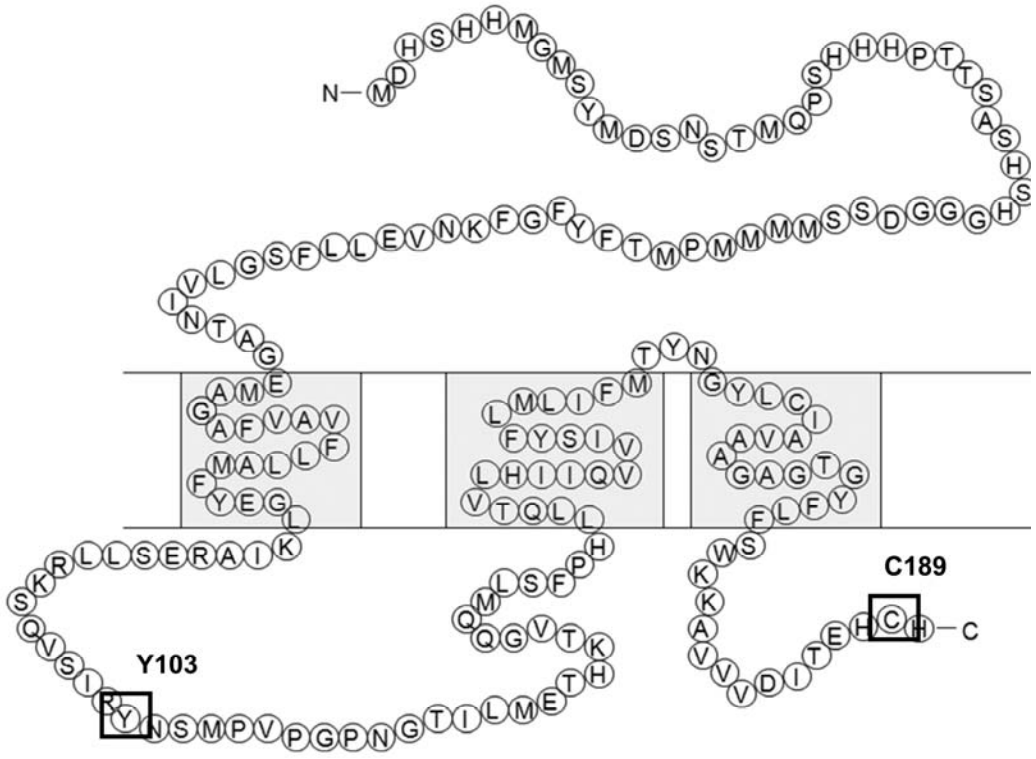


**Figure 6-1. Schematic diagram of the amino acid sequence of hCTR1**. Boxes highlight the Y103 and C189 residues.

## Results

### Expression of CTR1 in CTR1$^{-/-}$ Mouse Embryo Fibroblasts.

Prior studies of the importance of various components of hCTR1 for Cu transport have sometimes been confounded by the presence of endogenous CTR1. To avoid this problem, wild type and variant forms of hCTR1 were re-expressed in mouse embryo fibroblasts in which both alleles of CTR1 had been knocked out (CTR1$^{-/-}$ cells). Lentiviral vectors containing a blastocidin resistance marker were constructed to express either wild type hCTR1, a variant in which the Y103 was converted to

alanine, or a variant in which C189 was converted to serine. $CTR1^{-/-}$ cells were infected and selected with blastocidin to generate the $CTR1^{-/-/WT}$, $CTR1^{-/-/Y103A}$ and $CTR1^{-/-/C189S}$ sublines that were then characterized with respect to the expression of each form of exogenous CTR1. Figure 2A shows that the Y103A and C189S mRNA were expressed at 80% and 67% of that of the wild type hCTR1, respectively. To assess the level of CTR1 protein expression at the plasma membrane, the cell surface proteins were biotinylated by exposure to sulfo-NHS-SS-biotin before lysis, then recovered on streptavadin-coated beads and subjected to western blot analysis using an antibody to the myc tag. Figure 2B shows that the wild type and both variant forms of hCTR1 were correctly localized to the plasma membrane. The $CTR1^{-/-/Y103A}$ cells expressed plasma membrane CTR1 at a mean of 96 ± 4% of that in the $CTR1^{-/-/WT}$ cells and the $CTR1^{-/-/C189S}$ cells expressed it at mean of 91 ± 5% of that in the $CTR1^{-/-/WT}$ cells. Wild type CTR1 was detected as a band migrating at 37 kDa corresponding to the glycosylated form of the myc-tagged monomer that has been observed in prior studies (Eisses and Kaplan, 2002; Guo et al., 2004). As shown in Figure 2C, immunohistochemical analysis using a deconvoluting microscope established that there were no appreciable differences in the subcellular distribution of hCTR1 in the 3 cell types. Thus, neither conversion of Y103 to alanine nor C189 to serine altered the trafficking of CTR1 from ER and Golgi to the cell surface or other vesicular structures.

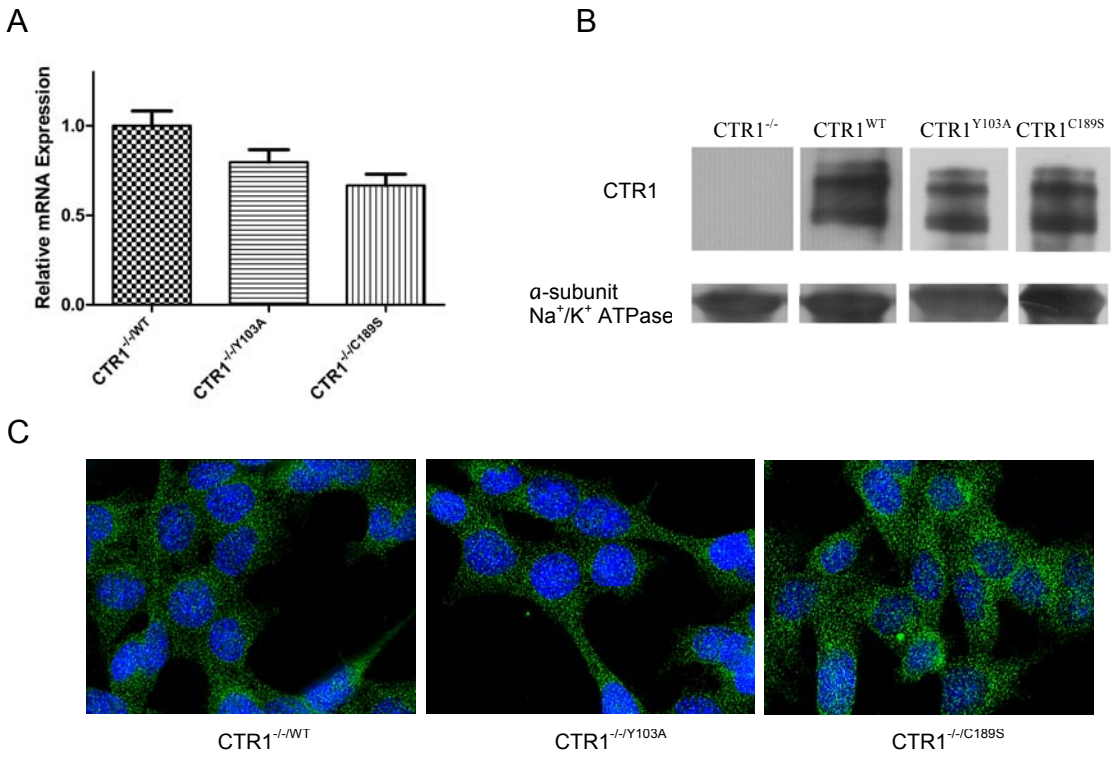


**Figure 6-2. Re-expression of hCTR1 in CTR1$^{-/-}$ cells**. A, relative hCTR1 mRNA levels as determined by qRT-PCR. B, representative western blot showing expression of myc-tagged CTR1. C, micrographs demonstrating uniform expression of the myc-tagged CTR1 protein (60x magnification).

**CTR1 Regulation of Cu Uptake and Cytotoxicity.**

The steady-state basal level of Cu was determined while the cells were growing in complete DMEM medium containing ~0.3 μM Cu. Figure 6-3A shows that basal Cu was not significantly altered by re-expression of wild type CTR1 in the CTR1$^{-/-/WT}$ cells. Expression of the Y103A variant reduced the Cu level to 55% of that in the CTR1$^{-/-}$ cells, whereas the C189S variant had no impact on basal Cu content. Thus, expression of the Y103A variant perturbed some component of the Cu homeostasis system, other than endogenous CTR1 which is missing in these cells, to

reduce steady-state Cu content. Such a reduction suggests that the non-CTR1 influx or efflux mechanisms are impacted when the Cu transport function of CTR1 is disabled.

Rates of Cu accumulation were analyzed by exposing the cells to media containing 100 μM Cu for 1 h. As shown in Figure 6-3B, re-expression of the wild type hCTR1 resulted in a 2-fold increase in the rate of Cu accumulation when compared to uptake in the $CTR1^{-/-}$ cells ($p = 0.002$). In contrast, neither of the hCTR1 variants was able to significantly increase Cu uptake. Thus, the integrity of both Y103 and C189 was required for Cu transport to occur in this system.

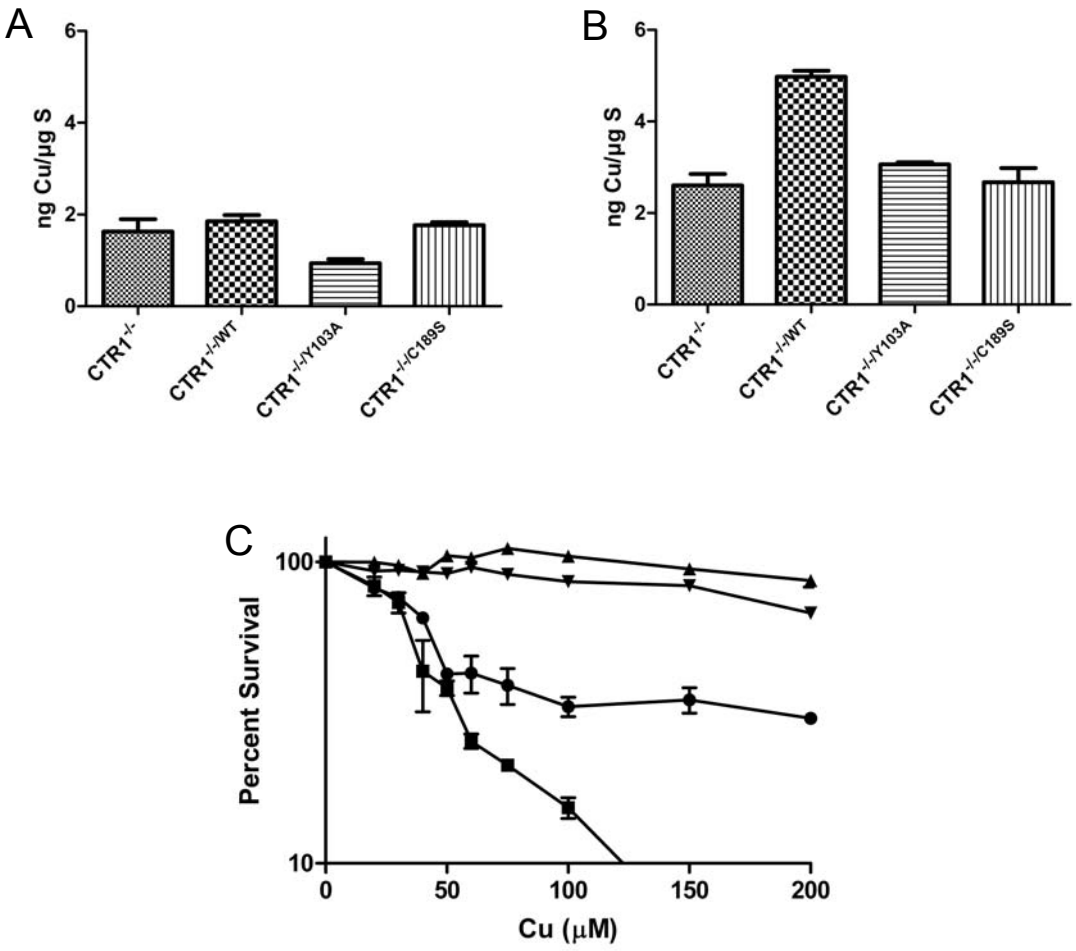


**Figure 6-3. Cu accumulation and cytotoxicity.** A, total basal Cu; B, total Cu following 1 h exposure to 100 µM Cu; C, inhibition of growth of MEF cells during 96 h continuous exposure to varying concentrations of Cu. (●), $CTR1^{-/-}$; (■), myc-$CTR1^{-/-/WT}$; (▲), myc-$CTR1^{-/-/Y103A}$; (▼), myc-$CTR1^{-/-/C189S}$. Each value represents the mean of no less than 3 independent experiments each performed with 3 separate cultures. Data for concentrations >200 µM not shown. Vertical bars, ± SEM.

To determine whether the differences in Cu accumulation translated into different tolerances to the cytotoxic effect of Cu, the growth rate of the $CTR1^{-/-/WT}$, $CTR1^{-/-/Y103A}$ and $CTR1^{-/-/C189S}$ cells was measured during a 96 h exposure to increasing concentrations of Cu. As shown in Figure 6-3C, re-expression of the wild

type CTR1 resulted in a 1.2-fold increase in sensitivity relative to the $CTR1^{-/-}$ cells ($IC_{50}$ 37 ± 1 *versus* 46 ± 2 μM ; p = 0.003). Neither of the CTR1 variants increased sensitivity to Cu consistent with their failure to enhance Cu uptake; in fact they rendered the $CTR1^{-/-}$ cells quite a bit more resistant to Cu. The $IC_{50}$ for the $CTR1^{-/-/Y103A}$ cells was 307 ± 3 μM ; this is 8.2-fold ($p = 5 \times 10^{-7}$) higher than the $IC_{50}$ of the $CTR1^{-/-/WT}$ cells and 6.7-fold ($p = 1 \times 10^{-6}$) higher than that of the $CTR1^{-/-}$ cells. Likewise, the $IC_{50}$ for the $CTR1^{-/-/C189S}$ cells was 234 ± 3 μM; this is 6.3-fold ($p = 2 \times 10^{-6}$) higher than that of the $CTR1^{-/-/WT}$ cells and 5.1-fold higher than that of the $CTR1^{-/-}$ cells ($p = 2 \times 10^{-6}$). Since these two variants were unable to enhance Cu uptake, one might have expected the $CTR1^{-/-/Y103A}$ and $CTR1^{-/-/C189S}$ cells to have the same sensitivity as the $CTR1^{-/-}$ cells. However, instead they exhibited a gain of function phenotype; they provided substantial protection against the cytotoxic effect of Cu.

**CTR1 Regulation of cDDP Uptake and Cytotoxicity.**

To analyze the effect of the Y103A and C189S substitutions on the initial influx of cDDP, the 4 types of cells were exposed to 30 μM cDDP for 5 min, washed thoroughly and the Pt content measured by ICP-MS. A short duration of drug exposure was used because prior studies have shown that the greatest effect of CTR1 is on initial cDDP influx. As shown in Figure 6-4A, re-expression of wild type CTR1 resulted in a 4.2-fold increase in cDDP accumulation ($p = 9 \times 10^{-5}$). Expression of the Y103A variant resulted in only a 1.4-fold increase in cDDP accumulation, which was only 33% of the increase produced by re-expression of wild type CTR1 (p = 0.0009). Expression of the C189S variant failed to increase cDDP uptake at all, and in fact reduced it to only 78% of that observed for the $CTR1^{-/-}$ cells. Thus, while the Y103A

variant retained some cDDP transport capability, the C189S variant appeared to be transport dead.

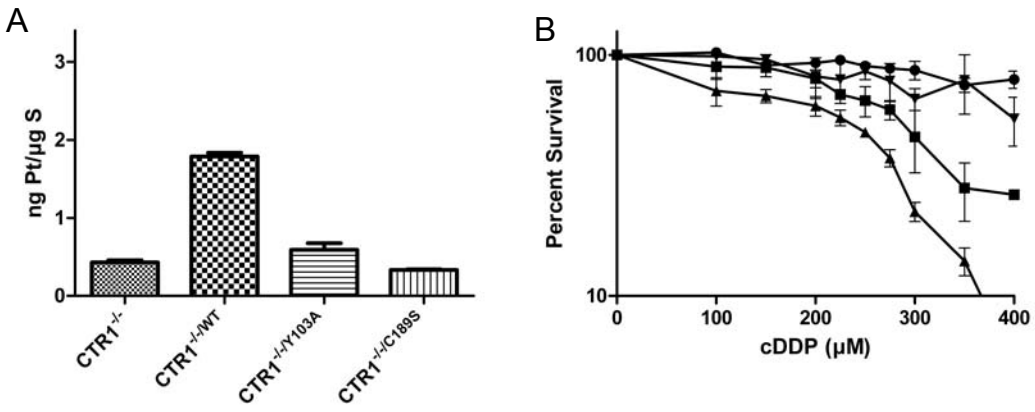


**Figure 6-4. cDDP accumulation and cytotoxicity**. A, Net accumulation of Pt in MEF cells following 5 min exposure to 30 µM cDDP. B, Inhibition of growth following 5 minute exposure to varying concentrations of cDDP. (●), $CTR1^{-/-}$; (■), $CTR1^{-/-/WT}$; (▲), $CTR1^{-/-/Y103A}$; (▼), $CTR1^{-/-/C189S}$. All values represent means of 6 independent experiments each containing triplicate cultures. Data for concentrations >400 µM not shown. Vertical lines, ± SEM.

To determine whether the changes in cDDP influx translated into changes in the cytotoxicity of this drug, cells were treated with increasing concentrations of cDDP for 5 min and their growth rate assessed over the ensuing 96 h in the absence of drug. The re-expression of wild type CTR1 resulted in a >2-fold increase in cytotoxicity relative to the $CTR1^{-/-}$ cells ($IC_{50}$ 291 ± 6 *versus* 647 ± 23 µM; p = 0.003). Despite the fact that it was much less effective at enhancing uptake, the Y103A variant enhanced the cytotoxicity of cDDP to an even greater extent than wild type CTR1 ($IC_{50}$ 242 ± 2 µM cDDP, p = 0.01). In contrast, the C189S variant had no impact on the cytotoxicity of cDDP. The fact that the Y103A variant produced a large enhancement of cDDP cytotoxicity while minimally increasing its uptake indicates

that its expression modulates other mechanisms that determine cellular tolerance to this drug.

**Effect on Cu- and cDDP-Induced Down-Regulation of CTR1.**

In mouse embryo fibroblasts cDDP triggers the rapid endocytosis and degradation of CTR1 via ubiquitination and subsequent degradation in the proteosome (Jandial et al., 2009). Y103 lies in a YXXM motif that is a potential phosphorylation site and a candidate for binding to the p85 subunit of phosphatidylinositol 3-kinase which mediates the endocytosis of many surface proteins (Wu et al., 2003). To determine whether Y103 is required for cDDP-induced degradation of hCTR1, or whether C189 is the site at which the interaction with cDDP occurs that triggers this reaction, the ability of cDDP to down-regulate the expression of CTR1 in the $CTR1^{-/-/WT}$, $CTR1^{-/-/Y103A}$ and $CTR1^{-/-/C189S}$ cells was analyzed by immunocytochemistry. Panels A-C in Figure 5 show the distribution of hCTR1 prior to cDDP exposure, and panels D-E show the cells after a 15 min exposure to 30 μM cDDP. In the absence of cDDP the distribution of hCTR1 was normal in all 3 types of cells. There was complete loss of detectable CTR1 in the $CTR1^{-/-/WT}$ and $CTR1^{-/-/C189S}$ cells in response to treatment with cDDP; however, there was clear impairment of the down-regulation of hCTR1 in the $CTR1^{-/-/Y103A}$ cells. Quantitative image analysis of total intensity demonstrated only a 50% loss of signal. This indicates that Y103 is required for the normal trafficking of hCTR1 in response to cDDP exposure. The finding that conversion of C189 to serine did not interfere with down-regulation demonstrates that this site is not essential for down-regulation under conditions where interaction with cDDP can still occur at other sites in the molecule.

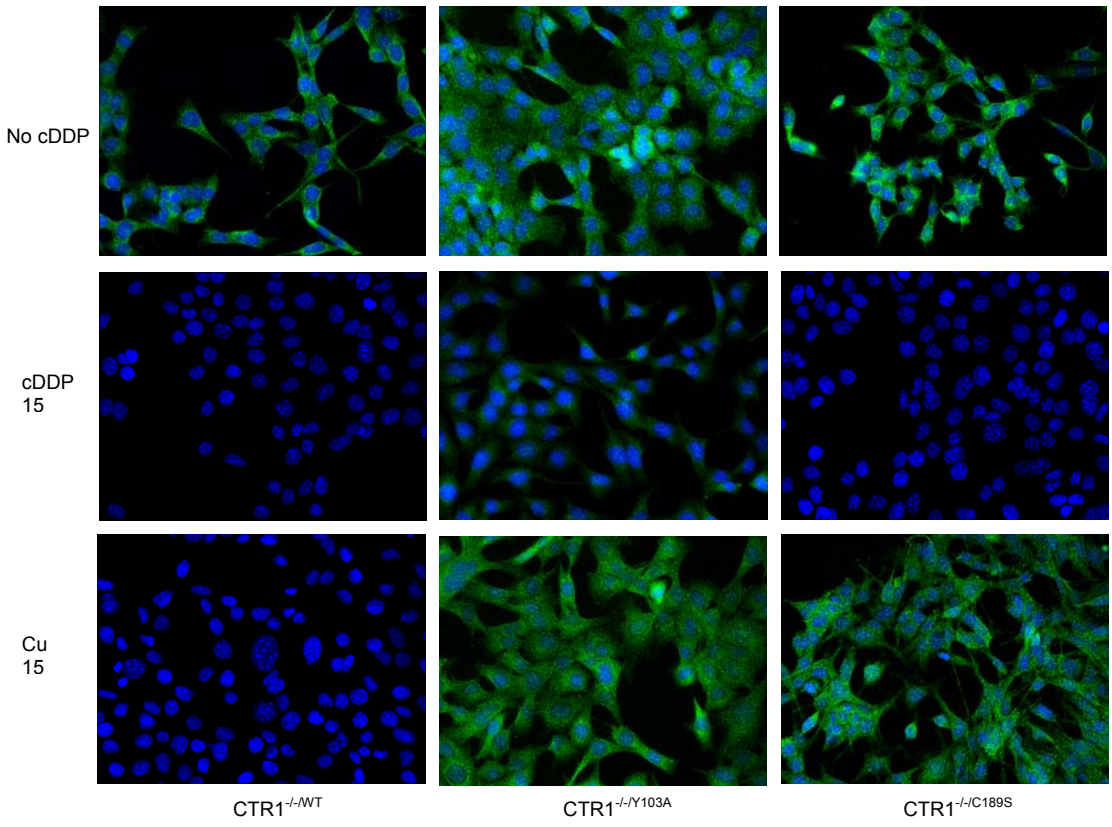


**Figure 6-5. Effect of modifying Y103 and C189 on ability of Cu and cDDP to trigger CTR1 degradation.** All panels are 20x. Top panels A-C, no treatment; middle panels, cells exposed to 30 µM cDDP for 15 minutes; bottom panels, cells exposed to 100 µM Cu for 15 minutes prior to fixing

## Discussion

The re-expression of wild type and variant forms of hCTR1 in cells in which endogenous CTR1 is completely absent due to knockout of both alleles provides a powerful way of identifying structural components essential for function. Despite small differences in the level of expression at the mRNA level, all 3 forms of hCTR1 protein assessed in this study were expressed at equal levels at the plasma membrane.

In addition, the distribution of hCTR1 in other parts of the cell appeared normal indicating that Y103 and C189 are not important determinants of the normal trafficking of hCTR1. This model system was further validated by the observation that re-expression of wild type hCTR1 increased Cu and cDDP uptake and enhanced sensitivity to the cytotoxic effect of both of these drugs.

The intracellular loop that spans transmembrane domains 1 and 2 had previously been thought to play little role in the accumulation of either Cu or cDDP. However, this domain contains several motifs that are of interest not only to the transport function of hCTR1 but also to its potential role as a scaffold that facilitates the binding of other proteins. In *Xenopus* the CTR1 molecule has been shown to be important for signaling during embryo development (Haremaki et al., 2007), and the intracellular loop is a likely site for the docking of proteins that participate in this pathway. The phsophorylation of tyrosines in the cytosolic domains of transmembrane proteins is known to be involved in both clathrin and non-clathrin mediated endocytosis (Esposito et al., 2001). The Y103 site in hCTR1 is of particular interest because of its location within a YXXM motif that is a candidate for binding to the p85 subunit of PI3K which mediates the endocytosis of PDGF and many other surface proteins (Wu et al., 2003). PI3K is involved in protein sorting in different trafficking pathways such as agonist-induced endocytosis (Martin, 1998) and multivesicular body formation (Futter et al., 2001).

It was found that converting Y103 to alanine had large effects on the function of hCTR1, and that these effects were quite different for Cu *versus* cDDP. Expression of the Y103A variant reduced basal Cu level, severely impaired Cu transport and

rendered cells highly resistant to the cytotoxic effect of Cu. In contrast, while it markedly reduced cDDP uptake, it enhanced rather than diminished the cytotoxicity of cDDP. Since loss of the Y103 did not impair Cu uptake below that observed in the $CTR1^{-/-}$ cells, but nevertheless rendered these cells highly resistant to Cu, Y103A is a gain of function mutation with respect to Cu. It is also a gain of function mutation with respect to cDDP since, despite its limited ability to mediate cDDP uptake, it rendered $CTR1^{-/-}$ cells even more sensitive to the cytotoxic effect of cDDP than did the wild type hCTR1. The effects of the Y103 mutation on cytotoxicity of either Cu or cDDP cannot easily be explained by the effect of this mutation on Cu and cDDP transport alone. However, the finding that alteration of Y103 enhances the cytotoxicity of cDDP, and the likelihood that the effect of Y103 is mediated through its phosphorylation, identifies a novel strategy for pharmacologically enhancing cDDP efficacy through the use of kinase inhibitors.

Previous studies of the effect of converting C189 to serine demonstrated little effect on the $K_m$ or $V_{max}$ for Cu transport when the hCTR1 variants were expressed at high levels in insect cells (Eisses and Kaplan, 2002; Eisses and Kaplan, 2005), but two prior studies have noted that C189 appears to be important for the assembly of hCTR1 multimers as fewer dimeric forms were observed on SDS PAGE analysis (Eisses and Kaplan, 2005; Lee et al., 2007). However, the results of the current study demonstrated quite large effects of the loss of C189 on the transport and cytotoxicity of both Cu and cDDP. Conversion of C189 to serine reduced transport capability for Cu and also rendered the $CTR1^{-/-/C189S}$ cells disproportionately more resistant than even the $CTR1^{-/-}$ cells. This suggests that, although no change in the distribution of the

variant was noted, variant C189S limits access of Cu to the critical targets that trigger apoptosis; this may be an effect of the intracellular component rather than the plasma membrane component of the variant hCTR1. In the case of cDDP, the C189S variant hCTR1 appears to have lost all transport function and as a result produced no change in cDDP cytotoxicity. The discrepancy between its effects on Cu and cDDP provide one line of evidence that the molecular details of how Cu and cDDP are transported are quite different.

One of the most dramatic consequences of the exposure of mouse embryo fibroblasts to cDDP is the subsequent rapid degradation of both endogenous CTR1 and exogenously re-expressed hCTR1. Previous studies have documented that CTR1 becomes ubiquitinated (Safaei et al., 2009), and that inhibition of proteosome function blocks the cDDP-induced degradation of CTR1 (Holzer and Howell, 2006) and enhances the uptake and cytotoxicity of the drug (Jandial et al., 2009). The ability cDDP to trigger CTR1 degradation is compromised in cells that lack the Cu chaperone ATOX1 (Safaei et al., 2009). How cDDP initiates CTR1 ubiquitination is unknown, but the results of this study support the conclusion that it does not require interaction of cDDP with C189. Whether there is a single unique site in hCTR1 that mediates this effect, or whether binding to any of the methionines or histidines in hCTR1 is sufficient remains to be determined.

The results of this study provide additional evidence that, despite the fact that $Cu^{+1}$ and cDDP appear to be substrates for the CTR1 transporter, the molecular details of the mechanism by which CTR1 imports cDDP and Cu are different. Other evidence comes from studies in yeast where FRET analysis of the interaction between the C-

terminal tails of yCTR1 monomers showed that Cu but not cDDP brought them closer together. This may be a reflection of the ability of $Cu^{+1}$ to bind 3 or 4 sulfur atoms, whereas cDDP and its variants are expected to only form bis-adducts with just 2 sulfur atoms. A variant of yCTR1 defective in Cu transport nevertheless still enhanced cDDP accumulation (Sinani et al., 2007). Some N-terminal methionine-rich motifs that are dispensable for Cu transport are required for cDDP uptake (Sinani et al., 2007). Mutational studies that address differences between cDDP and Cu transport in mammalian cells are limited. A recent study (Liang et al., 2009) showed that the MXXXM motifs in the N-terminal domain are required for both Cu and cDDP uptake in small cell lung cancer cells transfected with a vector that over-expressed hCTR1, but Cu and cDDP exhibited different kinetics. In addition, cDDP but not Cu increases the fraction of CTR1 that is found in the trimeric rather than the monomeric state (Guo et al., 2004; Liang et al., 2009). Refinement of the understanding of how cDDP is transported by hCTR1 is important because cellular uptake is a direct determinant of cytotoxicity, and insights into how transport can be manipulated have the potential to enhance overall efficacy of chemotherapy with this agent.

## Materials and Methods

**Drugs and Reagents**.

cDDP was purchased from the pharmacy at the Moores Cancer Center; it contains 3.33 mM cDDP in 0.9% NaCl. The cDDP was diluted into DMEM-RS Reduced Serum Media (HyClone, Logan, UT). Bradford reagent was purchased from BioRad Laboratories, Inc. (Hercules, CA) and sulforhodamine B was purchased from

Sigma-Aldrich (St. Louis, MO) and solubilized in 1% acetic acid (v/v) at a final concentration of 0.4% SRB (w/v). Anti-myc primary antibody 9B11 was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Secondary, anti-mouse, HRP-conjugated antibody was obtained from GE Healthcare (Piscataway, NJ). Hoechst 33342 nuclear stain and anti-mouse AlexaFluor 488-conjugated secondary antibody were both obtained from Invitrogen (Carlsbad, CA).

**Cell types, Culture and Engineering.**

Mouse embryonic fibroblasts containing wild type alleles of CTR1 ($CTR1^{+/+}$) and a line in which both copies of CTR1 had been somatically knocked out ($CTR1^{-/-}$) were graciously provided by Dr. Dennis Thiele (Lee et al., 2002b). The myc-$CTR1^{-/-/WT}$ subline was constructed by infecting the $CTR1^{-/-}$ cells with a lentivirus expressing wild type human CTR1 cDNA, N-terminally tagged with the myc epitope, using the ViraPower Lentiviral Induction kit (Invitrogen, Carlsbad, CA). Point mutations to the CTR1 molecule were generated using the GeneTailor Site-Directed Mutagenesis Kit (Invitrogen, Carlsbad, CA) using the following primers: for the myc-$CTR1^{-/-/Y103A}$ mutation (gcgtaagtcacaagtcagcattcgcgccaattccatgcctgtcccaggacca, tggtcctgggacaggcatggaattggcgcgaatgctgacttgtgacttacgc), for the myc-$CTR1^{-/-/C189S}$ mutation (atggaacaaaaacttatttc, tcaatggctatgctctgtgatatc).

**Cell Survival Assay.**

The sulforhodamine B cell survival assay (Monks et al., 1991) was used to determine cell survival following exposure to increasing concentrations of drug. Five thousand cells were seeded into wells of a 96-well tissue culture plate. Cells were incubated overnight at 37°C, 5% $CO_2$ followed by exposure to cDDP for 5 min, at

37°C, by the addition of 200 µl Pt drug-containing DMEM-RS medium. After 5 min the drug-containing media was aspirated off, cells were washed once with 37°C PBS, PBS was aspirated off and cells were covered in 200 µl 37°C complete medium. Cells were grown for 5 days after which the media was removed, the plate was washed 3 times with PBS, the protein was precipitated with 50% trichloroacetic acid and stained using 100 µl of 0.4% sulforhodamine B in 1% acetic acid at room temperature for 15 minutes. Following washes of the plate, the SRB was resolubilized in 100 µl of 10 mM Tris-HCl, the absorbance of each well at 515 nm was recorded using a Versamax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). All experiments were repeated no less than 3 times using 3 cultures for all drug concentrations.

**Immunocytochemistry.**

All images were visualized using a DeltaVision Deconvolution Microscope System tethered to a Nikon TE-200 Microscope (Applied Precision, Inc., Issaquah, WA). Deconvolution and subsequent analysis was performed using the softWoRx software suite (Applied Precision, Inc., Issaquah, WA)

**Measurement of Cellular Drug Accumulation.**

Drug accumulation was measured by ICP-MS as described in chapter 2, with the only modification being the use of 1 ml of medium and the use of DMEM-RS instead of OptiMEM.

**qRT-PCR.**

myc-CTR1 mRNA was quantified using qRT-PCR. First-strand cDNA was generated from mRNA isolated from Trizol (Invitrogen, Carlsbad, CA) using Oligo$(dT)_{20}$ priming and a SuperScript III First-Strand Kit (Invitrogen, Carlsbad, CA).

qRT-PCR was performed on a Bio-Rad MyIQ qPCR machine (Hercules, CA). The forward and reverse primers for hCTR1, mCTR1 and GAPDH were, respectively: gatgatgatgcctatgacct, tcttgagtccttcatagaac, actgttgggcaacagatgct, ctgctgctactgcaatgcag, tcaccaccatggagaaggc, gctaagcagttggtggtgca. All reactions utilized iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), following the manufacturer's recommendations. Samples were prepared in quadruplicate with no less than 3 independent RNA isolates used in independent experiments. Analysis was done utilizing the Bio-Rad iQ5 system software (Hercules, CA).

**Statistical Analysis.**

All 2-group comparisons utilized Student's *t*-test with the assumption of unequal variance. Data are presented as mean ± SEM.

## Acknowledgements

The authors wish to thank Dr. Dennis Thiele for kindly providing the $CTR1^{+/+}$ and $CTR1^{-/-}$ cells used in this study, and Dr. Michael Petris for valuable discussions. The authors also wish to thank Gerald Manorek, Dr. Paolo Abada, Dr. Xinjian Lin, and Dr. Xiaoqin Yuan for assistance, technical expertise and valuable discussion. A majority of the content of Chapter 6 has been submitted for publication. Christopher A. Larson was the primary researcher and author for this chapter. Stephen B. Howell supervised and directed the research in this chapter. Preston L. Adams assisted in the measurement of Pt and Cu accumulation and provided helpful feedback. Brian G. Blair provided valuable assistance and feedback. Roohangiz Safaei provided helpful discussion.

# Chapter 7 –

# Discussion

## Summary

The overall goal of the studies presented in this dissertation was to determine the role of CTR1 in Pt drug transport; and the importance of key regions of the CTR1 molecule with respect to the transport of Pt drugs, more specifically with a focus on cDDP. Furthermore, to determine what effects are seen, when CTR1 mutations are introduced, with respect to both Pt uptake and sensitivity. Additional studies were conducted on the same mutants to assess the Cu transport function of these variant forms of CTR1. The results presented here show that key regions of CTR1 are required to not only transport cDDP but also Cu, furthermore it is shown that the decreased ability to transport cDDP translated to an increased level of resistance to cDDP with respect to the wild type CTR1. The initial process required validation of the ability to re-express CTR1 in cells that are somatic knockouts for both copies of the allele. Such a system allowed for the study of the role of hCTR1 *in vivo*, which showed the great importance that hCTR1 plays in the sensitivity of tumors to a single dose of cDDP. The studies presented in this dissertation focus on the initial uptake of cDDP (specifically the first five minutes of exposure). The reason for focusing on this time frame is due to the drastic differences seen in Pt accumulation in cells that do or do not express CTR1 during the first five minutes. Mutational analysis of the N-terminus of hCTR1 showed that removal of the M2 region (the second met motif),

resulted in a decrease in both the cDDP accumulation and the sensitivity. Further truncation of the first 45 residues showed no appreciable changes when compared to removal of just the M2 region. Mutations to key residues in the intracellular region of the molecule revealed the importance of the methionines located at locations 150 and 154. Alterations showed an increase in cDDP uptake and increased sensitivity. Assessment of the histidine located at position 139 revealed no appreciable change in cDDP uptake versus wild type, however an increase in sensitivity was seen. A mutation to a key residue (tyrosine 103) between the first and second transmembrane region revealed a potential regulator for not just cDDP accumulation but also for trafficking of the molecule away from the cell surface. Finally a mutation that affected the C-terminal tail of CTR1 showed how disrupting a suspected player in the multimerization of CTR1 can affect accumulation of, and sensitivity to, cDDP.

The results of these studies has given a much greater insight into not only the role of CTR1 with respect to the initial uptake and sensitivity of cDDP, but also insight into the roles of some of the domains of the CTR1. A more complete understanding of how regions of can affect both Cu and cDDP uptake and also how these same regions play a role in controlling the down-regulation of CTR1 from the cell surface is essential to understanding how CTR1 regulation can have a major clinical impact on cDDP treatment.

## Role of CTR1 in platinum drug accumulation

There are 3 major platinum drugs utilized in the treatment of cancer: cisplatin, carboplatin and oxaliplatin. While there are differences in the leaving groups's

affinities for residues such as methionines and histidines, the final mechanism of cytotoxic action and the subsequent cross-resistance that is developed is the same in all drugs. The differences in molecular sizes and leaving group affinities resulted in differences in initial binding, showing that cDDP binds more readily to $CTR1^{+/+}$ cells than $CTR1^{-/-}$ cells. Furthermore, there appeared to be no appreciable differences in the initial binding of CBDCA or L-OHP. Also tested was the isomer of cisplatin, transplatin. There was no real difference there. While this is rather non-informative, it does suggest that the more complex leaving groups require longer to facilitate binding than the rather simplistic chlorides of cisplatin. The more interesting story manifests itself upon looking at the net accumulation of these drugs after a 5 minute exposure. All three clinically relevant drugs, cDDP, CBDCA and L-OHP, show a statistically significant increase in accumulation in the $CTR1^{+/+}$ cells *versus* the $CTR1^{-/-}$ cells. Also noteworthy was the transplatin, which showed no appreciable difference in accumulation between the two cell lines, and also showed accumulation levels higher than any of the other platinum drugs. The observations here coincide with observations seen previously with regard to 1 hour accumulation in $CTR1^{+/+}$ and $CTR1^{-/-}$ cells (Holzer et al., 2006a), and also with respect to an increase in uptake following over-expression of CTR1 (Holzer et al., 2004b; Ishida et al., 2002; Petris et al., 2003). The next step was to appreciate the role that CTR1 has in cDDP cytotoxicity. Previously it was shown that CTR1 has an impact on cDDP sensitivity following a 1 hour exposure to the drug (Holzer et al., 2006a). It was found that loss of CTR1 resulted in a statistically significant loss of sensitivity. The implications of these

findings contribute to an understanding of how CTR1 might play a role in the accumulation of cDDP in patients.

Previously it has been shown that CTR1 can be down regulated in the presence of cDDP and that down-regulation results in decreased accumulation of drug (Holzer and Howell, 2006; Jandial et al., 2009). This was validated in the MEF cell line utilized in these experiments.

To further validate the unique role of CTR1 as the major player a system was developed that allowed for re-expression of CTR1 in the $CTR1^{-/-}$ cells. A system such as this facilitates a much cleaner system than an over-expressing system. While previous groups have utilized over-expression there is some concern as to the validity when so much CTR1 is present. As such, a clean system featuring only the re-expressed CTR1 provides a unique system to study not only the wild type CTR1's ability to restore lost phenotypes, but also to look at the function of mutant versions of CTR1.

Upon developing a lentiviral system that allows for transduction of hCTR1 in the $CTR1^{-/-}$ cells, and performing the initial selection it became important to validate the system. The reintroduction of CTR1 results in a restoration of both the cDDP accumulation, but also a restoration of sensitivity. Experiments showed a statistically significant restoration of cDDP accumulation and of sensitivity to cDDP after a 5 minute exposure. This became important as it allowed for a system to analyze the role of CTR1 *in vitro,* and later *in vivo.*

The results discovered in these experiments allow for a significant increase in the understanding of what CTR1 does with respect to facilitating the uptake of cDDP,

and also with respect to the sensitivity of cells to cDDP when CTR1 is present or absent.

## Role of CTR1 *in vivo* insensitivity to platinum

The role of CTR1 *in vitro* in accumulating, and sensitivity to, cDDP is important. However, more important are the implications with respect to *in vivo*. The rationale of moving the system into *in vivo* lies in understanding how the changes in accumulation *in vitro* might translate into changes in sensitivity when presented as a xenograft model. The studies shown here utilized a subcutaneous xenograft model to mimic tumors in a patient. The finding that the cells respond very differently to a single treatment of cDDP based on the presence or absence of cDDP becomes very significant with respect to helping to understand why it is that patients in the clinic may not respond to Pt drug treatments. The decrease in sensitivity to the cDDP may be a result of accumulating less Pt inside the cell. This is an area that needs to be further evaluated to help understand the mechanism of resistance. Immediately apparent, however, is the obvious role that CTR1 expression might play in Pt resistance seen in patients. It is a real possibility that patients who possess a resistant phenotype have an altered level of expression of CTR1, or a mutant version of the molecule that renders the Pt accumulation impaired.

Of great interest was the observed difference in growth rates between the $CTR1^{-/-}$ and the $CTR1^{-/-/R}$ tumors. The presence of CTR1 resulted in a drastic increase in the tumor volume, an increase of approximately 2-fold. While there is no immediate explanation for this, it is known that Cu is an essential element in VEGF expression,

and that a deficiency may result in decreased angiogenesis, thus resulting in smaller tumors. While it may be something as indirect as impaired angiogenesis, it may also be a direct inability to grow as fast due to an impaired electron transport chain, and as a result a decreased efficiency in ATP production within the $CTR1^{-/-}$ tumors.

## Understanding cDDP and Cu with respect to CTR1

The way to understanding cDDP and Cu with respect to CTR1 is to understand that the mechanisms by which these molecules enter into the cell are most likely distinct (Guo et al., 2004; Sinani et al., 2007). Mutational analysis of the CTR1 molecule targeting 3 distinct regions, with 2 mutations per region, allows for analysis of CTR1 functionality. Re-expression utilizing the $CTR1^{-/-}$ background allows for analysis of only the variant forms of CTR1 without interference of wild type transporter.

### Understanding cDDP and CTR1

The easiest way to understand the phenotypes of the CTR1 mutants is to divide them into three distinct groups with respect to cDDP:

**Group 1**- Mutations that alter the amount of cDDP entering the cell, but the alteration in sensitivity corresponds. Two such mutations exist: M150,154I and C189S. The effect in these cells is opposite of each other. The methionines result in an increased amount of cDDP entering into the cell and as a result there is an increase in the cytotoxic effect of the cDDP exposure. The C189S mutation results in a significant decrease in the amount of cDDP entering the cell upon exposure, and as a result renders the cells more resistant to cDDP than wild type CTR1. While the phenotype

between these two mutants is converse the trend is the same. In both of these cell lines a mutation in CTR1 has been identified that plays a role in the gatekeeping effect of CTR1. With respect to the M150,154I mutation the CTR1 no longer possesses an ability to control how much cDDP is entering the cell, as a result almost 2-fold more cDDP enters the cell *versus* wild type. Such a finding shows the role the methionines play in regulating what enters the pore of the CTR1. The C189S mutation suggests one of two things, either the CTR1 molecule in general is impaired or the C189 is required for cDDP transport.

**Group 2-** Mutations that result in less cDDP entering the cell, but result in an increased sensitivity to cDDP. Three mutations exist within this group: the M2 deletion, the N-terminal truncation and the Y103A mutation. While it is easy to lump the N-terminal based mutations together it is important to note that the M2 region is only a partial player as there is a greater increase in sensitivity when the whole N-terminus is truncated. Such observations suggest that while the M2 region seems to be the main component of the N-terminus with respect to facilitating cDDP uptake, other regions including the H1, H2 and M1 may all play a role in the regulation of sensitivity of the cell to cDDP. The last mutation, Y103A, is a completely different type of mutation and the phenotype observed may suggest a role for the transmembrane loop in trafficking and directing cDDP around the cell. Disruption of the system could result in misdirection of the cDDP resulting in a heightened sensitivity to the drug.

**Group 3-** Mutations that result in cDDP accumulation comparable to wild type, but have an increased sensitivity. The only mutation seen in this category is the

H139A mutation. While disruption of the CTR1 pore seems to result in no change in the amount of cDDP getting into the cell, there appears to be a great difference in how the cell handles the cDDP entering the cell. Such a distinct phenotype may be the result of a change in trafficking patterns in the cell. Possibly the cell handles the cDDP in a distinct fashion, such a way that cause the same amount of cDDP to be significantly more toxic.

**Understanding Cu and CTR1**

With respect to understanding CTR1 and Cu there are 2 divisions that need to be made, the first is with respect to basal level of Cu accumulation. There are two groups here.

**Group 1-** Those mutations that accumulate basal levels of Cu roughly equal to wild type CTR1. The mutations in this group include M2 deletion, M150,154I and C189S. There appears to be nothing distinct about these mutations and how they handle relatively low levels of Cu.

**Group 2-** Those mutations that accumulate basal levels of Cu below that of wild type CTR1. Mutations that belong to this group are the N-terminal truncation, H139A and Y103A. These mutations have an effect on how the cell is able to handle lower Cu situations. Interestingly the N-terminal truncation seems to indicate that the other domains of the N-terminus, not the M2, play a key role in allowing Cu uptake in low Cu conditions. At this point it is unclear why these other mutations may have a significant role in the basal Cu level accumulation.

It is important to note that none of the CTR1 mutants was able to restore Cu uptake in Cu replete conditions. This suggests that all of these mutations play a role in the ability of the cell to adapt to higher Cu levels.

The other way to divide the CTR1 mutations, with respect to Cu, is to look at the sensitivity of the cells to continuous exposure to Cu. There are two groups of mutants in this classification.

**Group 1-** Mutations that have sensitivity levels approximately equal to $CTR1^{-/-}$ cells. These mutations are M150,154I and H139A. Such an observation leads to an understanding of how the pore of CTR1, while important to the accumulation of Cu, appears to play no major role in how the cells deal with the potential toxicity of Cu entering into the cell.

**Group 2-** Mutations that result in cells much less sensitive to Cu toxicity. Mutations in this group are both N-terminal mutations, M2 and the truncation, and both intracellular mutations, Y103A and C189S. These mutations all point to a role that CTR1 possesses beyond that of merely being a pore that solutes pass through. These mutations suggest that not just cDDP, but also Cu is handled in such a fashion that may be directed to more than one location upon entering a cell. This multiple trafficking idea allows for an understanding of not only how all the chaperones coexist in the cell, but also how molecules like CTR2 and ceruloplasmin may play a role in regulating how much Cu stays in the cell. An obvious next step here is to look at the efflux rates of Cu in these cells to understand if these mutations result in an altered rate of efflux, accounting for the decreased sensitivity.

## Understanding CTR1 down-regulation with respect to CTR1 mutations

The ability of CTR1 to down-regulate is a highly studied trait. As discussed in chapter 1 CTR1 has been shown to not only down-regulate in the presence of cDDP, but also in the presence of Cu. This down-regulation is a rapid loss of CTR1 from the cell surface, and results in a decreased accumulation of solute as seen in chapter 2. Analyzing the CTR1 mutants with respect to their ability to down-regulate allows for grouping of the mutants into 3 groups.

**Group 1-** Mutations that retain the ability to down-regulate CTr1 in the presence of cDDP and Cu as normal, compared to wild type. Mutations in this group are: both N-terminal mutations, M2 and the truncation, and H139A. These three mutations help elucidate the areas where down-regulation is not regulated. The N-terminus in general appears to not play a role in controlling the presence of CTR1 on the cell surface. This comes as a surprise as previous speculation had suggested that the methionine rich regions may be sensors for triggering down-regulation. The H139A results suggest that the center of the CTR1 pore is not a player in triggering down-regulation either.

**Group 2-** Mutations that can down-regulate CTR1 in the presence of cDDP, but not in the presence of Cu. This group is important as it allows for a basic understanding of which residues are key only to the Cu-facilitated down-regulation of CTR1. Furthermore, these findings reinforce the concept that CTR1 treats cDDP and Cu in very distinct fashions. The mutations in this group are M150,154I and C189S.

These mutations are both members of the proposed stack rings that seem to play a role in the movement of Cu into the cell. It is interesting to note that these two mutations represent the opening and end of the main pore of CTR1. Both have been suggested to be important as regulators of the pore, serving as gate-keepers for the CTR1 molecule. While it is currently unclear how these two mutations alter the ability of CTR1 to down-regulate in the presence of Cu, it certainly helps in understanding how Cu can trigger such a response.

**Group 3-** This group is comprised of one mutation that can neither down-regulate CTR1 in the presence of Cu nor in the presence of cDDP. This mutation is Y103A. This mutation is thought to be a player in the potential signaling cascade that might be related to CTR1. Furthermore, this residue may be a sight of phosphorylation and may serve as a docking site for both PI3K and possibly ubiquitin ligases. With this residue showing such an interesting phenotype, it raises questions of whether this residue is the main player in controlling down-regulation, and if so how? Does this involve signaling *via* mechanisms such as phosphorylation? There are many questions raised from this interesting mutation.

## Basal Cu and cDDP sensitiviy

A novel observation seen in multiple mutations is a correlation between a decreased basal content and hypersensitivity to cDDP. Mutations Y103A, H139A and the N-terminal truncation all result in a decrease in the total basal Cu present. Furthermore, all three of these mutations resulted in a dramatic increase in the sensitivity of these cells to cDDP insult. While there appears to be no known

correlation between these two observations, there is an apparent trend between these two phenotypes. Of interest is the idea that basal levels of Cu may make the cell more sensitivite to cDDP, as this phenotype may be exploitable in clinical settings. These observations may also help to shed some light on how cDDP is working to kill cells and how the Cu pathway is tied to cDDP movement and toxicity, not just with respect to transporters, but also targets and mechanisms of action of cDDP in these cells.

## Future Directions

The discoveries that are a result of the experiments in this dissertation raise many questions with regard to further understanding not just the role that CTR1 plays in sensitizing cells to cDDP and facilitating the uptake of Pt drugs, but also in understanding how cDDP interacts with CTR1. Also the ways that Cu and cDDP differ in their interactions with CTR1 suggest the need to further understand how these molecules bind and are transported by CTR1. Several of the mutations described here possess unique phenotypes that may be exploited as possible pharmacological targets in facilitating an increased accumulation of cDDP in cells that may have become resistant. The cDDP resistant phenotypes seen not just in the $CTR1^{-/-}$ cells, but also seen in some of the mutations may have very real implications in the patient and clinic setting. A study of CTR1 levels and mutations in tumor samples and cDDP-resistant cell lines is needed to help understand more of the implications observed through these experiments in this dissertation.

## Conclusions

In conclusion I have shown through these experiments that CTR1 is the major transporter of cDDP and is responsible for the sensitivity of cells both *in vitro* and *in vivo*. I have been able to show that via distinct mutations that CTR1 handles Cu and cDDP accumulation in very distinct ways, furthermore the sensitivity of the cells is not always a direct result of how much cDDP, or Cu, enters the cell. Rather sensitivity seems to be a mixture of drug entering the cell and how the CTR1 handles the drug. I have been able to identify key residues not only required for Cu influx both at basal levels and also in Cu replete conditions, but I have been able to identify residues responsible for the controlled accumulation of cDDP into cells. Lastly I have been able to identify some of the residues responsible for down-regulation of CTR1 in the presence of both Cu and cDDP.

## Acknowledgements

Christopher A. Larson was the primary author of this chapter. Stephen B. Howell supervised the writing of this chapter.

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