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# The role of the N-terminus of mammalian copper transporter 1 in the cellular accumulation of cisplatin☆

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ABSTRACT

The mammalian copper transporter 1 (CTR1) is responsible for the uptake of copper (Cu) from the extracellular space, and has been shown to play a major role in the initial accumulation of platinum-based drugs. In this study we re-expressed wild type and structural variants of hCTR1 in mouse embryo fibroblasts in which both alleles of mCTR1 had been knocked out ($CTR1^{-/-}$) to examine the role of the N-terminal extracellular domain of hCTR1 in the accumulation of cisplatin (cDDP). Deletion of either the first 45 amino acids or just the $^{40}$MXXM$^{45}$ motif in the N-terminal domain did not alter subcellular distribution or the amount of protein in the plasma membrane but it eliminated the ability of hCTR1 to mediate the uptake of Cu. In contrast it only partially reduced cDDP transport capacity. Neither of these structural changes prevented cDDP from triggering the rapid degradation of hCTR1. However, they did alter the potency of the cDDP that achieved cell entry, possibly reflecting the fact that hCTR1 may mediate the transport of cDDP both through the pore it forms in the plasma membrane and via endocytosis. We conclude that cDDP interacts with hCTR1 both at $^{40}$MXXM$^{45}$ and at sites outside the N-terminal domain that produce the conformational changes that trigger degradation.



## 1. Introduction

Copper (Cu) is a critical element in the normal function of all cells. Cu plays a key role in controlling not only metabolism but is also instrumental in redox regulation and p53 activity, and may even be involved in cellular trafficking (reviewed in [1]). Cu homeostasis involves multiple transporters and chaperones all working to maintain adequate levels of intracellular Cu but at the same time to protect the cell from the toxicity of this metal (reviewed in [2–4]). The importance of Cu homeostasis is evidenced by the fact that mutations that disturb the distribution of Cu cause serious disorders such as Menkes and Wilson's diseases [5,6].

Copper transporter 1 (CTR1) is the major high affinity Cu influx transporter [7] and is essential for embryonic development [8]. Human and mouse CTR1 exhibit 92% sequence homology [9]. In both species CTR1 monomers assemble to form a homotrimeric structure that contains a small flexible pore that allows $Cu^{1+}$ to pass into the cell down a concentration gradient [10–14]. 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 [15]. As shown in Fig. 1, additional clusters of methionines and histidines capable of interacting with Cu are found in the 67 amino acid extracellular hydrophobic N-terminal domain of CTR1. This 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 [16–19], but

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*Abbreviations:* cDDP, cisplatin; CTR1, copper transporter 1; ICP-MS, inductively coupled plasma mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectroscopy; PBS, phosphate buffered saline; TBS, tris buffered saline.

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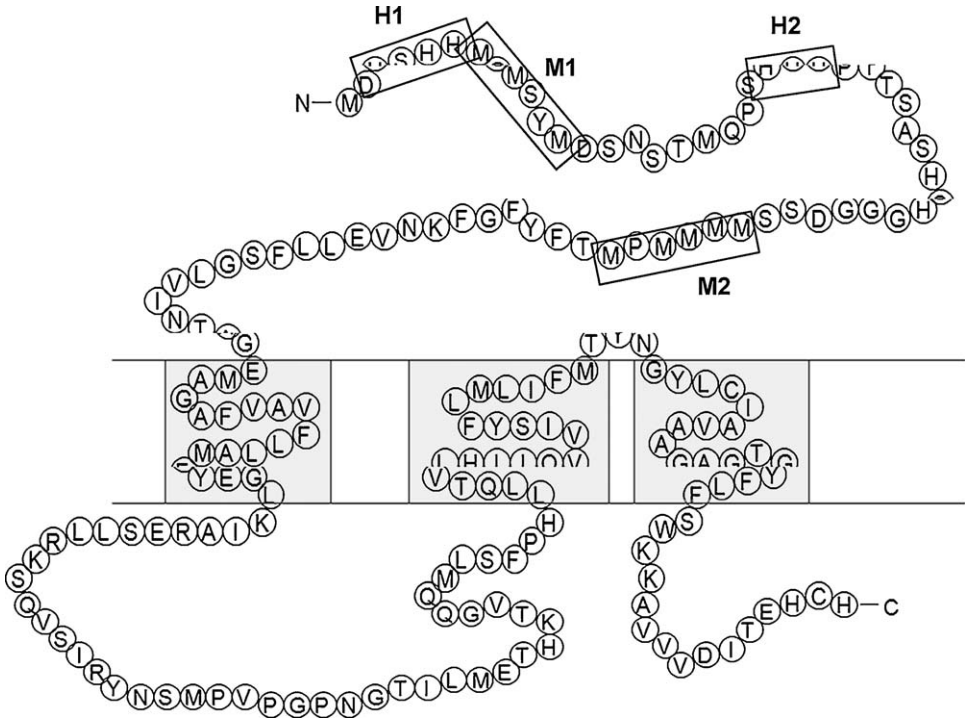


**Fig. 1.** Schematic diagram of the amino acid sequence of hCTR1. Boxes highlight the H1, M1, H2 and M2 motifs.

when normal levels of Cu are available even truncation of the entire N-terminus of yCTR1 does not disable its ability to transport Cu [19].

CTR1 is of interest with respect to cancer chemotherapy because it mediates the cellular accumulation and efficacy of cDDP and the other platinum-containing drugs carboplatin and oxaliplatin [20–22]. Knockout of both alleles of CTR1 markedly reduces the uptake of cDDP in both yeast and mammalian cells and renders them resistant to the cytotoxic effects of this drug [21,23,24]. Re-expression of wild type CTR1 in cells in which both alleles of CTR1 have been knocked out ($CTR1^{-/-}$ cells) restores cDDP accumulation and cytotoxicity both *in vitro* and *in vivo* [20]. In many types of cells high levels of Cu trigger the degradation of CTR1, an effect capable of limiting further Cu accumulation. cDDP likewise causes the down-regulation of CTR1 but does so at much lower concentrations and substantially more rapidly than Cu [25,26].

In the current study we examined 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. We used the approach of re-expressing wild type and variant forms of hCTR1 in mouse embryonic fibroblasts in which both alleles of endogenous CTR1 had been deleted. We report here that loss of the M2 cluster, either as a result of deletion of just this region or through deletion of the entire first 45 amino acids, reduced the ability of hCTR1 to transport cDDP into the cell but paradoxically increased its cytotoxicity.

## 2. Materials and methods

### 2.1. 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 Bio-Rad 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).

### 2.2. 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. Thiele [8]. 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, ttcttaaagccaaagtagaaggtgctgctgtctcctccaccatgggg), for the myc-$CTR1^{-/-/Truncated}$ (deletion of residues 1–45) mutation (caccatgggcggcagggaacaaaaacttatttctgaagaagatctgg, cttatttctgaagaagatctgggcggcaccttctactttgg, tcaatggcaatgctctgtg).

### 2.3. Cell survival assay

Cell survival following exposure to increasing concentrations of drugs was assayed using the sulforhodamine B assay system [27]. The optimal number of cells seeded per well was determined to be 5000 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