

Contribution of the Major Copper Influx Transporter CTR1 to the Cellular Accumulation of Cisplatin, Carboplatin, and Oxaliplatin

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ABSTRACT

The goal of this study was to determine the ability of the major copper influx transporter CTR1 to mediate the cellular accumulation of cisplatin (DDP), carboplatin (CBDCA), and oxaliplatin (L-OHP). Wild-type murine embryonic fibroblasts (CTR1^{+/+}) and a subline in which both alleles of CTR1 were deleted (CTR1^{-/-}) were tested for their ability to accumulate platinum when exposed to increasing concentrations of DDP, CBDCA, or L-OHP for 1 h. They were also tested for their sensitivity to the growth-inhibitory effect of each drug. Platinum content was measured by ion-coupled plasmon mass spectroscopy. The experimental model was validated by measuring copper accumulation and cytotoxicity. CTR1^{-/-} cells accumulated only 5.7% as much copper as CTR1^{+/+} cells during a 1-h exposure to 2 μ M copper. When exposed to DDP, CBDCA, or L-OHP at

2 μ M, accumulation in the CTR1^{-/-} cells was only 35 to 36% of that in the CTR1^{+/+} cells. When tested at a 5-fold higher concentration, this deficit remained for DDP and CBDCA, but accumulation of L-OHP was no longer CTR1-dependent. There was an association between the effect of loss of CTR1 function on uptake of the platinum drugs and their cytotoxicity. The CTR1^{-/-} cells were 3.2-fold resistant to DDP, 2.0-fold resistant to CBDCA, but only 1.7-fold resistant to L-OHP. Thus, whereas CTR1 controls the cellular accumulation of all three drugs at low concentrations, accumulation of L-OHP is not dependent on CTR1 at higher concentrations. We conclude that L-OHP is a substrate for some other cellular entry mechanism, a feature consistent with its different clinical spectrum of activity.

Cisplatin (DDP), carboplatin (CBDCA), and oxaliplatin (L-OHP) are important chemotherapeutic agents, but the development of resistance during therapy is a common occurrence for all three. Several mechanisms that can contribute to resistance have been identified previously (Siddik, 2003); however, the molecular mechanisms are not well defined. Whereas the development of resistance is believed to be multifactorial, the most commonly identified defect is decreased drug accumulation (Andrews and Howell, 1990; Andrews et al., 1990; Gately and Howell, 1993). The reasons for decreased accumulation are unknown in part due to the fact that the pathways by which these platinum-containing drugs enter and exit from cells are defined poorly. The platinum drugs enter cells much more slowly than most other classes of small-molecule anticancer agents; current evidence suggests

that one component of their uptake is mediated by a transporter or channel (Gately and Howell, 1993). Many other metal ions enter cells on specific transporters, and acquisition of resistance to DDP is often accompanied by resistance to other metalloids (Tobey and Tesmer, 1985; Naredi et al., 1995; Romach et al., 2000). In particular, cells selected for resistance to DDP are often cross-resistant to copper and vice versa (Katano et al., 2002; Safaei et al., 2004b). Recent studies from this and other laboratories have demonstrated that both copper efflux transporters ATP7A and ATP7B modulate the export of DDP (Safaei et al., 2004a; Samimi et al., 2004a).

Copper transporter 1 (CTR1) is the major copper influx transporter. Deletion of the yCTR1 gene in *Saccharomyces cerevisiae* markedly reduces the accumulation of all three clinically available platinum-containing chemotherapeutic agents (Ishida et al., 2002; Lin et al., 2002). A preliminary study suggested that the cellular accumulation of DDP was also impaired in embryonic fibroblasts established from mice in which both mCTR1 alleles had been disrupted (Ishida et al., 2002). Forced overexpression of hCTR1 in human ovarian carcinoma cells enhanced DDP uptake (Holzer et al., 2003).

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ABBREVIATIONS: DDP, cisplatin; L-OHP, oxaliplatin; CBDCA, carboplatin; CTR1, copper transporter 1.

Increased hCTR1 expression in human small-cell lung cancer cells (Song et al., 2004) was reported to enhance the uptake of DDP, CBDCA, and L-OHP but overexpression of hCTR1 in human squamous carcinoma cells was reported to have no effect on DDP uptake (Beretta et al., 2004). Such forced overexpression of hCTR1 is toxic to human cells, and it is not clear that CTR1 functions normally at such high intracellular levels, as evidenced by the finding that increased CTR1 expression and DDP uptake into the whole cell is accompanied by minimal change in sensitivity to the cytotoxic effect of the drug and DNA adduct formation (Holzer et al., 2004).

The amino acid sequence of murine CTR1 shares 92% homology with human CTR1 (Kuo et al., 2001). Whereas deletion of both mCTR1 alleles produced embryonic lethality, CTR1^{+/+} and CTR1^{-/-} embryo fibroblast cell lines were successfully established from the parental and knockout mice (Lee et al., 2001). To refine understanding of how CTR1 modulates the cellular pharmacology of the platinum-containing drugs, we carried out further investigations using this isogenic pair of mouse embryo fibroblasts. We report here quantitative studies of the effect of loss of mCTR1 function on cellular accumulation of the three clinically used platinum-containing drugs and on sensitivity to the cytotoxic effects of these agents. The results indicate that CTR1 regulates the cellular accumulation of all three drugs at concentrations attainable in humans but that at 5-fold higher concentrations, although accumulation of DDP and CBDCA is still CTR1-dependent, L-OHP accumulation becomes CTR1-independent, indicating that it is a substrate for another cell-entry mechanism.

Materials and Methods

Reagents. DDP was a gift from Bristol-Myers Squibb (Princeton, NJ). The clinical formulation (Platinol-AQ) containing 3.33 mM DDP was kept in the dark at room temperature. A 100 μ M stock was created by diluting the drug in 0.9% NaCl. CBDCA was purchased from Sigma (St. Louis, MO), and a stock solution was prepared at 10 mM in water. L-OHP was a generous gift from Sanofi Pharmaceuticals (Malvern, PA). A stock solution was prepared at 10 mM in water. Copper in the form of cupric sulfate was obtained from Fisher Scientific (Tustin, CA). Protein concentration was measured using Bradford's reagent from Bio-Rad Inc. (Hercules, CA). ⁶⁴CuSO₄ was purchased from the Mallinckrodt Institute of Radiology (Washington University Medical School, St. Louis, MO).

Cell Lines. The murine embryonic fibroblasts, originally isolated from day-7 embryos and immortalized using SV40 large T antigen, were cultured as described previously (Lee et al., 2002). Cells were grown in 20% fetal bovine serum, 2 mM glutamine, 1 $\times$ nonessential amino acids, 55 μ M 2-mercaptoethanol, 50 mg/liter uridine, and 110 mg/ml pyruvate.

Measurement of ⁶⁴Cu Accumulation. Copper uptake measurements were made using cell cultures that were 80% confluent in the 30 mm wells of a six-well plate. After the addition of prewarmed media containing 2 μ M ⁶⁴CuSO₄, the plates were incubated at 37°C in 5% CO₂ for 30 min. At the end of the incubation period, the plates were placed on ice, and the wells were rinsed three times with 3 ml of ice-cold phosphate-buffered saline. Cell lysis buffer (0.1% Triton-X and 1% SDS in phosphate-buffered saline) in a volume of 500 μ l was added to the wells, and the lysate was harvested by scraping the dish twice before it was transferred to tubes for γ counting on a Beckman Gamma 5500B (Beckman Coulter, Fullerton, CA). Six cultures were harvested for each data point. Lysates from a set of identical cultures not exposed to ⁶⁴CuSO₄ were used to measure protein concentration.

Platinum Accumulation Assays. Cells were plated in six-well plates in media without any drugs. Once the cultures were 80% confluent, 2 or 10 μ M DDP, 2, 10, or 12 μ M CBDCA, or 2, 6, or 10 μ M L-OHP was added, and plates were incubated at 37°C in 5% CO₂ for 1 h. The plates were then placed on ice, and wells were rinsed three times with 3 ml of ice-cold phosphate-buffered saline. Cells were harvested by dissolving them in 70% nitric acid at 65°C for at least 2 h. Samples were diluted to 5% nitric acid with water containing 1 ppb indium and 0.1% Triton X-100. Platinum content was determined using inductively coupled plasmon mass spectroscopy (Element2; PerkinElmer Life Sciences, Boston, MA) on an instrument available through the Analytical Facility at the Scripps Institute of Oceanography (San Diego, CA). Lysates from a set of identical cultures suspended in 0.1% Triton X-100 and 1% SDS in phosphate-buffered saline were used to measure protein concentration.

Drug Sensitivity Assay. Six-well plates were seeded with 1000 cells per well, and 24 h later, the medium was replaced with medium containing various concentrations of either DDP, CBDCA, L-OHP, or copper. After 3 days of continuous exposure, cultures were trypsinized and stained with trypan blue. The number of live cells per well was determined by counting cells that excluded trypan blue with a hemocytometer. Each drug concentration was tested in six cultures per cell line, and each experiment was repeated three times.

Statistics. Tests of significance used a two-sided paired Student's *t* test with the assumption of unequal variance; *p* values of <0.05 were considered significant. All *p* values are for the comparison of the values for the CTR1^{+/+} versus the CTR1^{-/-} cells.

Results

Effect of the Loss of CTR1 on Copper Accumulation.

Previous studies have shown that CTR1 plays a substantial role in the cellular accumulation of copper. To verify the influence of CTR1 on copper uptake and validate this cell line model for the study of the platinum-containing drugs, CTR1^{+/+} and CTR1^{-/-} cells were exposed to a 2 μ M concentration of ⁶⁴Cu for 1 h and then washed extensively before the accumulation of copper was measured by γ counting. This concentration of copper is near the *K_m* value of 1 μ M reported for murine CTR1 (Lee et al., 2002). As shown in Table 1, the cells lacking CTR1^{-/-} accumulated only 5.7% as much copper as the CTR1^{+/+} cells. Thus, as reported previously (Lee et al., 2002), loss of CTR1 markedly impaired the influx of copper in these cells.

TABLE 1

Accumulation of copper, DDP, CBDCA, and L-OHP in CTR1^{-/-} cells as a percentage of that in CTR1^{+/+} cells

Data are presented as mean $\pm$ S.E.M.

Drug & Concentration	Comparative Cell Accumulation	<i>p</i> Value
	%	
⁶⁴ Cu		
2 μ M	5.7 $\pm$ 2.0	1.36 $\times$ 10 ⁻¹⁰
DDP		
2 μ M	36.3 $\pm$ 9.7	0.007
10 μ M	30.0 $\pm$ 1.7	0.00025
CBDCA		
2 μ M	35.1 $\pm$ 5.5	0.00008
10 μ M	49.5 $\pm$ 19.3	0.017
12 μ M	45.6 $\pm$ 19.0	0.0033
L-OHP		
2 μ M	36.3 $\pm$ 9.7	0.007
6 μ M	84.9 $\pm$ 9.0	0.148
10 μ M	110.6 $\pm$ 14.6	0.427

Effect of the Loss of CTR1 on DDP, CBDCA, and L-OHP Accumulation. The accumulation of the platinum-containing drugs was quantified by measuring the platinum content of the whole cell after a 1-h exposure to either 2 or 10 μM DDP, CBDCA, or L-OHP or concentrations that were found to be equitoxic to human ovarian carcinoma cells in clonogenic assays performed in an earlier study (Samimi et al., 2004b). The equitoxic concentrations of the drugs were 2 μM for DDP, 12 μM for CBDCA, and 6 μM for L-OHP. This resulted in data on the accumulation in CTR1^{+/+} and CTR1^{-/-} cells being available for two concentrations of DDP and three concentrations for CBDCA and L-OHP. As shown in Table 1, when exposed to 2 μM DDP for 1 h, the CTR1^{-/-} cells accumulated only 36.3% as much platinum as the CTR1^{+/+} cells. The magnitude of the effect of the loss of CTR1 expression was no less when the accumulation was measured after a 1-h exposure to 10 μM DDP. A similar pattern was observed for CBDCA. When exposed to a concentration of 2 μM , the CTR1^{-/-} cells accumulated only 35.1% as much platinum as the CTR1^{+/+} cells, and the magnitude of this deficit was only slightly less at 10 or 12 μM . However, L-OHP exhibited a different pattern. When the cells were exposed to 2 μM L-OHP, the loss of CTR1 had a substantial effect, and the accumulation in the CTR1^{-/-} cells was only 36.3% of that in the CTR1^{+/+} cells. However, as the concentration of L-OHP was increased first to 6 and then to 10 μM , the magnitude of the effect of the loss of CTR1 diminished to the point at which there was no deficit at 10 μM .

Effect of the Loss of CTR1 on Sensitivity to the Growth-Inhibitory Effects of Copper, DDP, CBDCA, and L-OHP. Because the CTR1^{+/+} and CTR1^{-/-} cells do not form discrete colonies when cultured on plastic, the effect of loss of CTR1 function on sensitivity to the cytotoxic effect of each drug was determined by quantifying the inhibition of proliferation during a 72-h exposure to increasing concentrations of drug. A 1-h exposure to drug, such as was used to assess the effect of the loss of CTR1 on accumulation, produced cytotoxicity only at drug concentrations unattainable in humans. Figure 2 depicts the inhibition of growth as a function of drug concentration using continuous exposure, which allowed the assessment of the effect of lower concentrations. The CTR1^{-/-} cells were 1.4 ± 0.4 (mean $\pm$ S.E.M.)-fold more resistant to copper than their wild-type counterparts. It is interesting to note that the loss of CTR1 produced a larger degree of resistance to DDP than to copper; cells lacking CTR1 were 3.2 ± 0.4 -fold resistant to DDP (95% confidence interval, 3.1–3.6). The CTR1^{-/-} cells were only 2.0 ± 0.8 -fold resistant to CBDCA, and in a set of four experiments, this difference did not reach statistical significance. There was only a 1.7 ± 0.6 -fold difference in sensitivity to L-OHP between the two types of cells, which was not statistically significant. Thus, consistent with the effect on the loss of CTR1 on drug uptake, loss of CTR1 produced different degrees of resistance to DDP, CBDCA, and L-OHP. It is interesting to note that although there was a marked difference in the uptake of ⁶⁴Cu, the difference in copper IC₅₀ values was not great. In contrast, for cells treated with DDP, there was a closer correspondence between the reduction in uptake and the increase in the IC₅₀ value.

Discussion

The isogenic pair of CTR1^{+/+} and CTR1^{-/-} cells used in this study provides a powerful model in which to refine understanding of the role of CTR1 in the influx of the platinum-containing chemotherapeutic agents. Prior studies of the ability of CTR1 to mediate platinum drug accumulation in human cells have used forced expression of exogenous CTR1 and have been complicated by the presence of significant amounts of endogenous CTR1 (Beretta et al., 2004; Holzer et al., 2004; Song et al., 2004). As reported previously (Lee et al., 2002), the accumulation of copper in the CTR1^{-/-} cells was found to be markedly reduced in the CTR1^{-/-} cells when they were exposed to low concentrations of copper for 1 h. Accumulation in the CTR1^{-/-} cells was just 5.7% of that in the CTR1^{+/+} cells. Loss of CTR1 function had a smaller

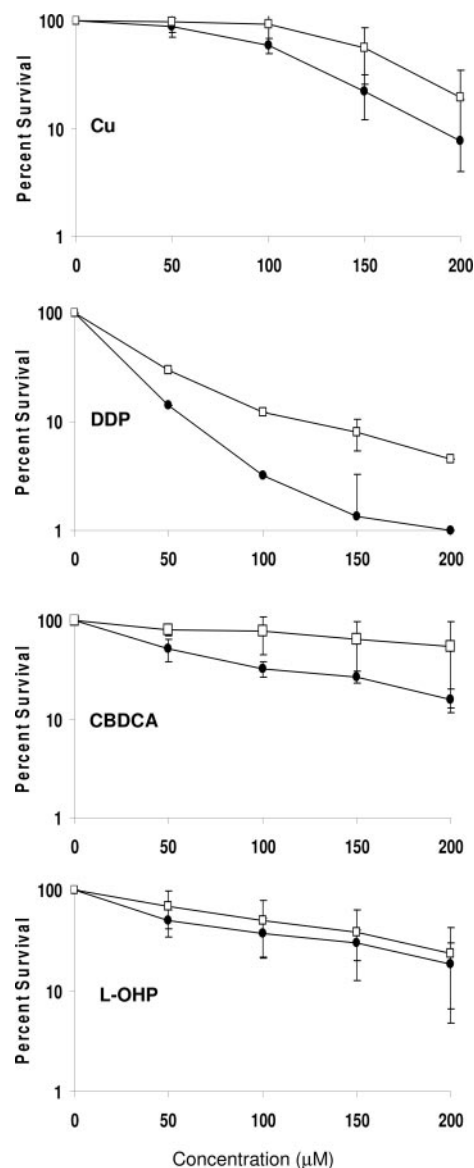


Fig. 1. Inhibition of the growth of CTR1^{+/+} and CTR1^{-/-} cells by DDP, CBDCA, and L-OHP. Each curve is a plot of the percentage of cells surviving after a 72-h continuous exposure to drug. ♦, CTR1^{+/+} cells; □, CTR1^{-/-} cells. Each point represents the mean of three independent experiments each performed with six replicate cultures. Vertical bars, S.E.M. Where S.E.M. bars are missing, they are smaller than the symbol.

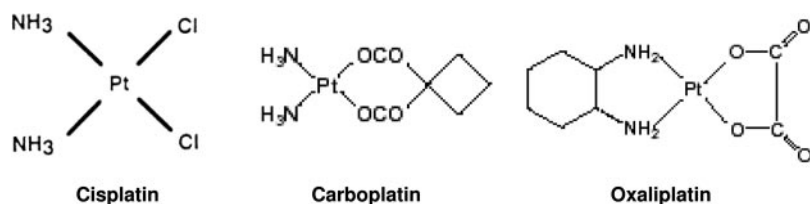


Fig. 2. Schematic drawings of the structure of DDP, CB-DCA, and L-OHP.

but still substantial effect on the uptake of all three platinum-containing drugs when cells were exposed to 2 μ M concentration of drug. At this concentration, accumulation was $\sim 36\%$ of that in the wild-type cells. In the case of DDP and CBDCA, this deficit in accumulation was still evident when cells were exposed to a 5-fold higher concentration; however, in the case of L-OHP, the deficit produced by the loss of CTR1 disappeared at the higher concentration. This suggests that CTR1 contributes importantly to the cellular uptake of all three drugs at the low concentrations typically attained in patient plasma, whereas at higher concentrations, L-OHP enters the cell predominantly via another mechanism.

S. cerevisiae express two high-affinity copper influx transporters, yCTR1 and yCTR3 (Petrus, 2004). Knockout of yCTR1 in yeast in which yCTR3 was disabled reduced the accumulation of DDP, CBDCA, and L-OHP similar to the results obtained with the CTR1^{+/+} and CTR1^{-/-} mouse embryo fibroblasts (Ishida et al., 2002; Lin et al., 2002). Thus, despite the fact that yCTR1 contains eight N-terminal copper-binding motifs while mCTR1 contains only two, both transporters seem to function similarly with respect to platinum drug accumulation.

In the mammalian cells, there was an association between the extent to which platinum drug uptake was impaired in the CTR1^{-/-} cells and the extent to which sensitivity to the growth-inhibitory effect of the drug was reduced. The loss of CTR1 had a greater effect on the potency of DDP than CBDCA, but inspection of the curves in Fig. 1 indicates that even at very high drug concentrations, CTR1 was still important to the growth-inhibitory effect of these drugs. This suggests that other routes of entry for DDP and CBDCA into the cell do not become dominant over CTR1, even at high drug concentrations. In contrast, loss of CTR1 had less effect on the growth-inhibitory potency of L-OHP, consistent with the observation that the concentration required to inhibit growth was already greater than that at which the deficit in accumulation produced by loss of CTR1 had disappeared.

The fact that deletion of CTR1 did not completely eliminate DDP and CBDCA accumulation indicates that CTR1 is not the only route by which these drugs enter mammalian cells. This mirrors the situation for copper; even though the loss of CTR1 reduced copper uptake by 94%, and this transporter is essential for embryonic viability, copper nevertheless enters the cell in amounts sufficient to sustain growth. To date, no other transporter has definitively been shown to mediate the influx of copper in mammalian cells (Lee et al., 2002). Likewise, loss of CTR1 function reduced DDP and CBDCA uptake to only 36% of control, indicating that there is yet another mechanism by which DDP and CBDCA enter cells that has not yet been characterized.

The results of this study provide strong evidence that, at low concentrations, CTR1 mediates cellular accumulation of all three platinum-containing drugs currently used in pa-

tients but that L-OHP differs from DDP and CBDCA in that its dependence on CTR1 diminishes at higher concentrations. The concept that these drugs have different influx transporters is consistent with their different spectrum of action against various types of human cancer. The structures of DDP, CBDCA, and L-OHP are shown in Fig. 2. L-OHP differs from DDP and CBDCA in that it contains a bulky diamino-cyclohexane ring, a feature that seems to make it a substrate for a non-CTR1-dependent entry mechanism at higher concentrations. Given the exquisite specificity of CTR1 for copper relative to other metal ions, it is surprising that any of these drugs is a substrate for the CTR1-mediated accumulation mechanism.

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