Role of human copper transporter Ctr1 in the transport of platinum-based antitumor agents in cisplatin-sensitive and cisplatin-resistant cells

Im-Sook Song,[1] Niramol Savaraj,[3] Zahid H. Siddik,[2] Peiman Liu,[1] Yingjie Wei,[1] Chun Jing Wu,[3] and Macus Tien Kuo[1]

Departments of [1]Molecular Pathology and [2]Experimental Therapeutics, University of Texas M.D. Anderson Cancer Center, Houston, Texas and [3]Department of Medical Oncology, Virginia Hospital, Miami, Florida

Abstract

Recent studies have shown that the mammalian high-affinity copper transporter encoded by *Ctr1* is involved in the uptake of cisplatin. However, the roles of *hCtr1* in cisplatin-sensitive and cisplatin-resistant mammalian cells have not been investigated. Here, we show that, of five cisplatin-resistant cell lines, only one (SR2) exhibited substantial reduction in hCtr1 expression as compared with that in its sensitive line small cell lung cancers (SCLC), whereas copper efflux transporters ATP7A and ATP7B were not significantly altered. SR2 exhibited cross-resistance to carboplatin but not to oxaliplatin. Transfection of expression hemagglutinin-tagged *hCtr1* cDNA into SCLC and SR2 cells enhanced the uptake of copper, cisplatin, carboplatin, and oxaliplatin, suggesting that hCtr1 transporter can transport these platinum-based drugs. Whereas increased sensitivities to all these platinum drugs were observed in *hCtr1*-transfected SCLC cells, increased sensitivities to cisplatin and carboplatin but not to oxaliplatin were observed in *hCtr1*-transfected SR2 cells. These results suggest that SR2 acquired an additional unique intracellular resistance mechanism to oxaliplatin. Finally, using hCtr1 deletion mutants, we showed that the NH_2-terminal domain of hCtr1 was involved in transporting all these platinum-based antitumor agents. These results collectively show the importance of hCtr1 in the transport of platinum-based antitumor agents in cisplatin-sensitive and cisplatin-resistant variants. [Mol Cancer Ther 2004;3(12):1543–9]

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Requests for reprints: Macus Tien Kuo, Department of Molecular Pathology, University of Texas M.D. Anderson Cancer Center, Box 89, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-792-3256; Fax: 713-792-8424. E-mail: tkuo@mdanderson.org

Introduction

Cisplatin chemotherapy is effective in the treatment of advanced testicular cancer with a cure rate of >90%. It is also effective in treating other types of malignancies including ovarian, bladder, cervical, head and neck, and small cell lung cancers (SCLC; ref. 1). Many patients eventually relapse and become refractory to this drug (2). Two major strategies have been undertaken to improve the efficacy of cisplatin-based therapies: One is the development of platinum analogues with better therapeutic indices. Of these, carboplatin, which displays comparable therapeutic effectiveness with cisplatin, generally exhibits more favorable toxicity profiles to certain types of solid tumors than the first-generation cisplatin (3). Another platinum compound oxaliplatin (Eloxatine) exhibits activities against a wide range of tumors with a better safety profile and lacks cross-resistance with cisplatin and carboplatin (4). These developments have made platinum-based chemotherapy one of the most important treatment options for human cancers.

The other strategy is through better understanding the resistance mechanisms of cisplatin-based chemotherapy so that methods for circumventing drug resistance can be developed. Elucidating mechanisms of cell killing elicited by the platinum-based antitumor agents is a prerequisite for this strategy. Whereas it has been well recognized that cisplatin acts on multiple cellular targets representing diverse signal transduction pathways leading to cell death, DNA remains the primary target of interest (5, 6). Once inside the cells, cisplatin forms intrastrand cross-link adducts on DNA and activates DNA repair pathways (6). Thus, reduction in adduct formation (7) and enhanced repair of and/or tolerance to adducts (8) have been suggested as important mechanisms of resistance to platinum-mediated cell death.

An important issue relevant to the toxicities and mechanisms of resistance of cisplatin that has not been well addressed is the transport system of cisplatin and its related compounds. In fact, defective uptake of cisplatin has been one of the most consistently identified characteristics of cells selected for cisplatin resistance both *in vivo* and *in vitro* (9, 10). Many reports have pointed to reduced drug accumulation as a significant mechanism of cisplatin resistance. However, the platinum transporter was not identified until recent demonstration that deletion of the yeast *Ctr1* gene, which encodes the high-affinity copper

transporter, resulted in increased cisplatin resistance and reduced accumulation of cisplatin (11). Parallel studies showed that cells selected for resistance to cisplatin are cross-resistant to copper and vice versa (12). The mouse *Ctr1* homologue was identified by complementation study in yeast cells. Mouse cells lacking *Ctr1* display decreased cisplatin accumulation and increased cisplatin resistance. These observations suggested that cisplatin uptake is mediated by the copper transporter Ctr1 in yeast and mammalian cells (11).

The *Ctr1* family represents evolutionarily conserved transporters that are present in yeast, plants, and mammals. All the Ctr1 transporters contain three transmembrane domains, a NH_2-terminal methionine-rich motif consisting of three to five methionines in MxM and/or MxxxM arrangements, and a COOH-terminal cysteine or histidine motif (13). It is considered that these NH_2-terminal methionine residues are involved in the functional transporter of copper (14).

Once copper is transported by hCtr1, specific chaperones, cyclooxygenase-17, CCS, and HAH1 deliver copper to mitochondria, cytoplasmic superoxide dismutase, and trans-Golgi compartments, respectively (15). Two P-type ATPase family of cation transporters ATP7A and ATP7B shuttle between the trans-Golgi and the plasma membrane that mediate eliminating intracellular copper. There are reports that overexpression of ATP7B by transfection conferred cisplatin resistance associated with decreased accumulation of cisplatin and carboplatin (16, 17). These results suggest that the transporters that carry copper in and out of cells may also carry platinum compounds.

Whereas reduced cisplatin transport have been frequently observed in cisplatin-resistant variants, whether Ctr1 plays a definitive role in these cisplatin-resistant cells has not been investigated. Moreover, the roles of Ctr1, ATP7A, and ATP7B in regulating the homeostasis of platinum-based antitumor drugs in cisplatin-resistant variants remain to be investigated. We show in this communication that enhanced expression of the human *Ctr1* gene resulted in accumulation of cisplatin, carboplatin, and oxaliplatin, suggesting that hCtr1 can transport not only cisplatin but also carboplatin and oxaliplatin, albeit at the reduced rates. We also show that, although oxaliplatin transport was increased in cisplatin-resistant cells, the enhanced oxaliplatin accumulation failed to sensitize cisplatin-resistant cell lines to oxaliplatin. Thus, there must be additional intracellular roles of hCtr1 in cisplatin-sensitive and cisplatin-resistant cells.

Materials and Methods

Reagents and Cell Lines

Reagents were purchased from the following commercial sources: cisplatin, doxorubicin, vinblastine, synthetic oligonucleotides, $CuSO_4$, and antibodies against Na^+/K^+-ATPase (Sigma, St. Louis, MO); $^{64}CuCl_2$ (Washington University Medical School, St. Louis, MO); anti-tubulin and anti–lamin B antibodies (Calbiochem, Darmstadt, Germany); anti-hemagglutinin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); LipofectAMINE (Invitrogen, Carlsbad, CA); geneticin (Life Technologies, Inc., Carlsbad, CA); RNA isolation kit STAT-60 (Tel-test, Inc., Friendswood, TX); and enhanced chemiluminescence plus Western blotting detection system (Amersham Bioscience, Piscataway, NJ).

The SCLC and its cisplatin-resistant variant SR2 were described previously (18). The CP2 cisplatin-resistant cell line, originally developed from LoVo human colon carcinoma cell line (19), was obtained from Dr. Li-Ying Yang (M.D. Anderson Cancer Center, Houston, TX). The human glioblastoma cell lines T430 and A172 (20) and their respective cisplatin-resistant variants T430CR and A172CR were obtained from Dr. Akira Gomi (Jichi Medical School, Tochigi, Japan). The SCLCB line and its cisplatin-resistant variant have been described previously (21). All the cells were grown in DMEM containing 10% fetal calf serum at 37°C in 5% CO_2 atmosphere unless otherwise noted.

Construction of Wild-type Human hCtr1 and Deletion Mutants in the Expression Vectors: Transfection of Plasmid DNA

Wild-type (WT) human *hCtr1* cDNA and its NH_2- and COOH-terminal deleted mutants were synthesized by reverse transcription-PCR using appropriate primer sets containing a *Not*I recognition sequence (sequences available on request) and polyadenylated RNA from SCLC cells. The resulting PCR products covering nucleotides 156 to 745 (WT, using Genbank accession no. U83460 as a reference), 288 to 745 (NH_2-terminal deletion), and 156 to 713 (COOH-terminal deletion mutant) were digested by *Not*I, and each was cloned into TA vector (Stratagene, La Jolla, CA). The *Not*I fragments containing the respective cDNA were then transferred into the *Not*I site of CIN-HA-pcDNA3 vector, which contains a hemagglutinin tag, enhancer CIN sequences for expression, and a neomycin resistance marker for transfection selection. All the plasmids were confirmed by sequencing.

Recombinant plasmid DNA was transfected into SCLC and SR2 cells using LipofectAMINE according to the procedure provided by the vendor. Stable transfectants were selected using 400 μg/mL geneticin.

RNase Protection Assay and Northern Blot Hybridization

RNA probes for *Ctr1*, *ATP7A*, and *ATP7B* sequences were synthesized by reverse transcription-PCR. The resulting PCR products were cloned into pCRII-TOPO vector (Invitrogen). All the plasmids were confirmed by sequencing. Plasmids containing *Ctr1*, *ATP7A*, and *ATP7B* fragments were linearized, and radioactively labeled probes were synthesized using T7 or SP6 RNA polymerase. 18S RNA probe (80 nucleotides) was used as positive control. Extraction of total RNA and RNase protection were carried out according to the procedures described previously (18), generating protection signals corresponding to 120, 167, and 253 nucleotides, respectively, for hCtr1, ATP7A, and ATP7B mRNA. Northern blot hybridizations also followed the procedure described previously (18).

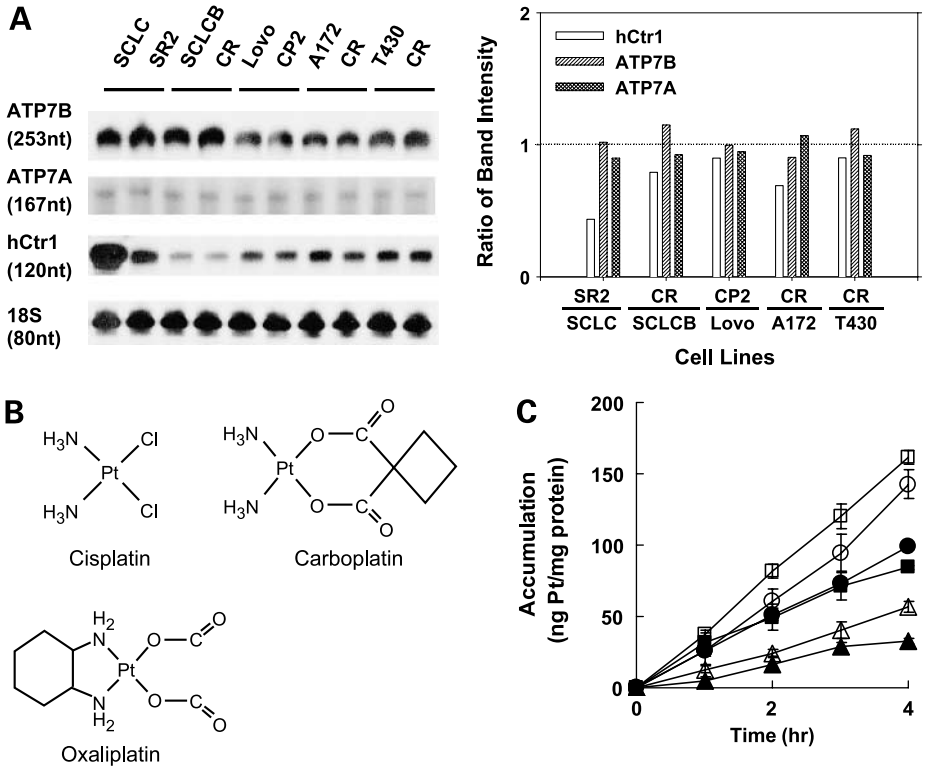

Figure 1. A, *left,* autoradiograph showing the expression of ATP7B, ATP7A, and hCtr1 mRNA in five pairs of cisplatin-sensitive and cisplatin-resistant cell lines as measured by the RNase protection assay. Expression levels of 18S RNA were used to assure equal loading. Signals were densitometrically scanned; *right,* ratios of ATP7B, ATP7A, and hCtr1 mRNA levels between the resistant cells and their corresponding sensitive cells. **B,** chemical structure of cisplatin, carboplatin, and oxaliplatin. **C,** cellular accumulation of cisplatin (○, ●), carboplatin (△, ▲), and oxaliplatin (□, ■) in parental (SCLC, ○, △, □) and cisplatin-resistant (SR2, ●, ▲, ■) cells. *Points,* mean of triplicate measurements from three independent experiments; *bars,* SD.

Fractionation of Cellular Proteins and Western Blot Analyses

To prepare total cell lysates, cells were harvested, washed twice with PBS, and suspended in 200 μL PBS buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and proteinase inhibitors. To fractionate cell lysates into cytosol, plasma membrane, and nucleus fractions, the harvested cells were washed with PBS and precipitated by low-speed centrifugation. The pellets were suspended in PBS containing 0.1 mmol/L EDTA and 0.4 mg/mL phenylmethylsulfonyl fluoride and incubated 30 minutes on an ice bath. The cell suspensions were homogenized 20 strokes with a loose fitting homogenizer (Wheaton, Millville, NJ) and centrifuged at 35,000 rpm for 30 minutes (60 Ti rotor, Beckman L8 ultracentrifuge (Beckman Coulter, Fullerton, CA). The pellet was resuspended in a buffer containing 250 mmol/L sucrose and 10 mmol/L Tris-HCl (pH 7.8), loaded onto a sucrose gradient cushion, and centrifuged at 35,000 rpm for 1 hour (SW50 rotor, Beckman L8 ultracentrifuge). The proteins over a 20% sucrose gradient were collected as a cytosolic fraction, those between 30% and 50% of sucrose were collected as a plasma membrane fraction, and pellet below 50% of sucrose gradient was collected as a nucleic fraction. Protein concentrations were determined with protein assay kit (Bio-Rad Laboratories, Hercules, CA). Total cell lysates or fractionated cell extracts (30 μg each) were separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), and probed with antibodies using the enhanced chemiluminescence plus Western blotting detection system.

Measurements of Copper Uptake

Copper uptake was measured the procedure of Holzer et al. (29). Briefly, 1×10^6 cells were plated in a 12-well plate. After 8 hours, cells were treated with 2 μmol/L $^{64}CuSO_4$. After incubation for various time intervals, cells were rinsed thrice with 3 mL each ice-cold PBS. Cell lysis buffer (0.1% Triton X-100 and 1% SDS in PBS) in a volume of 800 μL was added to the wells, and the radioactivity of the whole cell lysate was determined by gamma counter (LS 3801, Beckman Coulter). Aliquots of cell lysates (20 μL) were used to determine the protein concentration using Bio-Rad detergent-compatible protein assay kit (Bio-Rad Laboratories). The rate of copper uptake was determined from the slope of uptake-time profile.

Measurements of Platinum Uptake

Measurement of platinum uptake was done according to the procedure described previously (22). Briefly, 10^6 cells were plated in a 60-mm Petri dish. After 24 hours, cells were treated with 40 μmol/L cisplatin, carboplatin, or oxaliplatin. After incubation for various time intervals, cells were harvested and lysed overnight in 50 μL of benzethonium hydroxide at 50°C. The lysates were acidified with 200 μL of 0.3 N HCl. Platinum concentrations were determined in an atomic absorption spectrometer (SpectrAA300, Varian, Palo Alto, CA). The rate of platinum uptake was determined from the slope of the uptake-time profile.

Determination of Drug Sensitivity

Cells grown in 96-well plates (10^4 cells per well) were continuously exposed for 72 hours to various concentrations of anticancer drugs at the following ranges: cisplatin

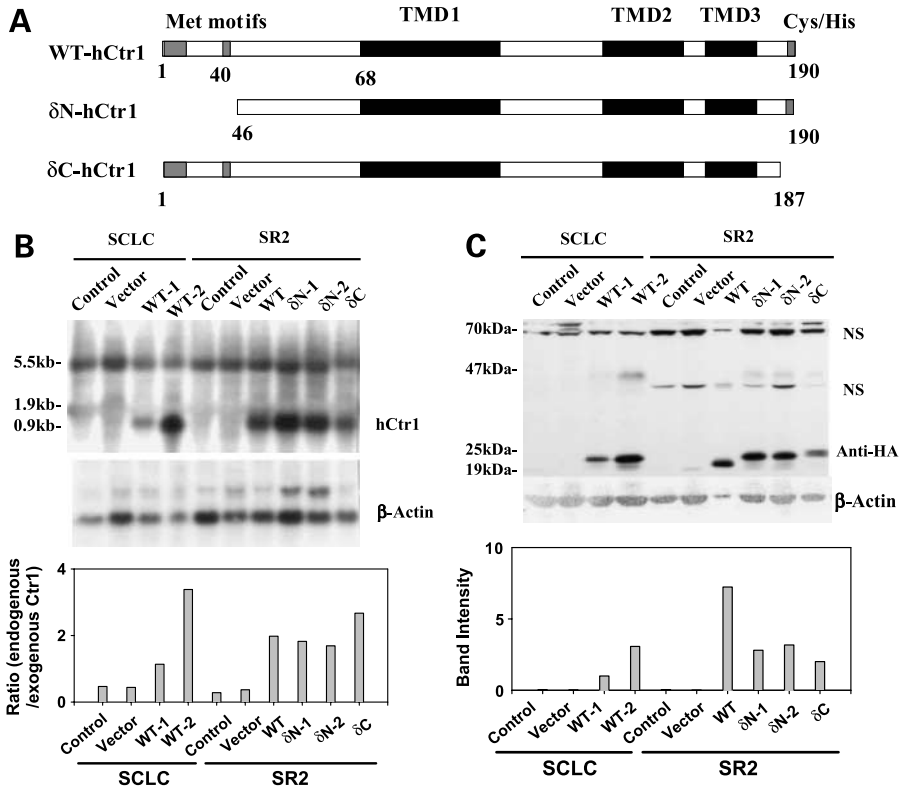

Figure 2. Expression of hCtr1 and its deleted mutants in the transfected cells. **A,** structure of WT hCtr1 and its δN and δC mutants. **B,** Northern blot analyses of expression of WT, δN, and δC hCtr1 mRNA were confirmed by measuring mRNA gene. *5.5 kb,* transcript of a pseudogene; *1.9 kb,* endogenous hCtr1 mRNA; *0.9 kb,* transcript from the transfected cDNA. Signals were densitometrically scanned; *bottom,* ratios of exogenous hCtr1 mRNA signal to the endogenous one. **C,** Western blot expression analyses of stable transfection of WT, NH_2-terminal deletion, and COOH-terminal deletion of hCtr1 transporter. Signals were densitometrically scanned; *bottom,* ratios of transfected hCtr1 protein to WT hCtr1 transfected in SCLC cell line (SCLC-WT1). SCLC and SR2 are cisplatin-sensitive and cisplatin-resistant cell lines of SCLC, respectively. SCLC vector and SR2 vector were transfected by vector only into the SCLC and SR2 cell lines; SCLC-WT1, SCLC-WT12, and SR2-WT were transfected by recombinant plasmid DNA containing WT *hCtr1* gene. SR2-δN1 and SR2-δN2 and SR2-δC were transfected with NH_2- and COOH-terminal deletion mutant, respectively.

(1–200 μmol/L), carboplatin (1–200 μmol/L), oxaliplatin (1–100 μmol/L), doxorubicin (0.01–10 μmol/L), and vinblastine (0.5–50 μmol/L). After 72 hours of incubation, 200 μL of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/mL, Sigma) was added to each well, and the plate was incubated for 4 hours. The medium was removed, and the formazan products were solubilized with 120 μL DMSO. The cell contents were measured by absorbance at 570 nm.

Results

Expression of hCtr1 in Cisplatin-Resistant Variants

To determine the role of hCtr1 in cisplatin-resistant variants, we investigated the levels of hCtr1 expression in five pairs of cisplatin-resistant and cisplatin-sensitive cell lines. Levels of resistance to cisplatin ranged from 2- to 10-fold. Figure 1A shows an autoradiograph of the RNase protection results. The protection signals were analyzed by densitometry (*right*). Among the five cisplatin-resistant cell lines, SR2 exhibits >50% reduction in hCtr1 mRNA in comparison with those in the sensitive line (SCLC). The other four resistant cell line shows reduction in hCtr1 mRNA ranging from 31% to 10% with respect to their corresponding parental lines. These low levels of reduction are within the experimental variations.[4] Similar results were also obtained by Northern blot hybridization (data not shown). These observations suggest that substantially reduced *hCtr1* expression is not very frequent in the cisplatin-resistant variants. Figure 1A also shows that the mRNA levels of copper efflux pump, ATP7A and ATPB, were not significantly different between the given cisplatin-resistant and their sensitive variants.

To investigate whether expression of hCtr1 in SR2 cells correlated with rates of cisplatin uptake, we did cisplatin uptake analyses in SR2 and SCLC cells. Figure 1C showed that rate of cisplatin uptake was reduced in SR2 cells compared with that of SCLC, correlating with the reduced expression of hCtr1 mRNA in these cells. Uptakes of oxaliplatin and carboplatin were similarly reduced in the resistant line (Fig. 1C). Interestingly, the rate of oxaliplatin uptake was slightly better than, if not comparable with, cisplatin in SCLC cells, and carboplatin had lowest rates of uptake in both SCLC and SR2 cells. These results indicate that uptakes of cisplatin, carboplatin, and oxaliplatin were retarded in SR2 cells, which expressed reduced levels of hCtr1 mRNA. Lin et al. (23) showed that yeast cells deficient in Ctr1 exhibited impaired accumulation of the cisplatin analogues, carboplatin, and oxaliplatin.

Uptakes of Copper and Cisplatin Analogues in the *hCtr1*-Transfected Cells

The results shown above strongly suggested that hCtr1 transporter is capable of transporting cisplatin, carboplatin, and oxaliplatin. To substantiate these results, we transfected expression plasmids encoding WT *hCtr1* into SCLC

[4] I-S. Song, et al., unpublished data.

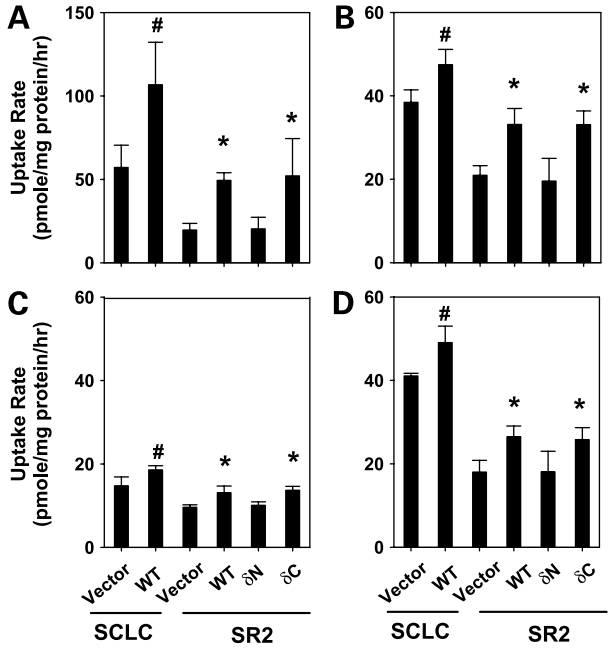

Figure 3. Uptake rates of copper (**A**), cisplatin (**B**), carboplatin (**C**), and oxaliplatin (**D**) in stable transfectant cell lines of SCLC vector, WT and SR2 vector, WT, δN, and δC. ^{64}Cu (2 μmol/L), cisplatin (40 μmol/L), carboplatin, and oxaliplatin were added to stable transfectant cell line. Cells were incubated 0–4 hours at 37°C, and uptake into the cells was measured. Rates of uptake of copper and platinum-based drugs were calculated from the slopes of the uptake-time profiles as similarly shown in Fig. 1C. Rates of ^{64}Cu uptake were similarly calculated. Representative of triplicate measurements from three independent cultured cells. *Columns,* mean; *bars,* SD. #, $P < 0.01$, significantly different from SCLC vector (Student's *t* test). *, $P < 0.01$, significantly different from SR2 vector (Student's *t* test).

and SR2 cells. For comparison, we also transfected NH_2- and COOH-terminal deleted mutants into SR2 cells. The NH_2-terminal mutant (δN) lacks the first 45 amino acids, including the two Met motifs and the flanked histidine-rich domain. The COOH-terminal mutant (δC) lacks the last three amino acid residues (Fig. 2A).

Figure 2B shows a Northern blot hybridization of hCtr1 mRNA in SCLC cells transfected with the WT construct and SR2 cells transfected with vector, WT, δN, and δC constructs. Expression of the encoded hCtr1 proteins for the corresponding transfectants was shown by the Western blot using anti-hemagglutinin tag antibody as probe (Fig. 2C). The protein levels in these transfectants were in general agreement with the mRNA levels. For unknown reasons, the δN- and δC-encoded proteins migrated more slowly than did the WT protein in the current SDS-PAGE system, such abnormal mobilities of cellular proteins, particularly membrane proteins, have been frequently reported.

Figure 3A shows that rates of ^{64}Cu uptake were 1.9- and 2.6-fold, respectively, higher in the cisplatin-sensitive (SCLC) and cisplatin-resistant (SR2) cell lines transfected with WT *hCtr1* than those transfected with empty vector. These results suggest that the recombinant hCtr1 construct was functional. Figure 3A also shows that deletion of NH_2-terminal amino acid residues but not the COOH-terminal ones abolished the enhanced ^{64}Cu uptake. Puig et al. (14) showed that NH_2-terminal sequences, particularly the Met motif ($M^{40}MMMPM^{45}$), are involved in copper transport in mammalian cells. It was also reported that hCtr1 mutants, where Cys^{161} and Cys^{189} (the last cysteine) were replaced with serine, also mediated copper uptake (24), suggesting that COOH-terminal residues are not required for copper transport. Our present results are consistent with these observations.

Elevated expression of hCtr1 in SCLC cells by transfection also enhanced cisplatin uptake. Likewise, transfecting *hCtr1* into SR2 cells enhanced the uptake of cisplatin. However, whereas transfecting the δC mutant enhanced the rate of cisplatin uptake, transfecting the δN mutant did not (Fig. 3B). These results suggest that the extracellularly located NH_2-terminal amino acid sequence is required for cisplatin transport. Similar results were found in the uptake of carboplatin (Fig. 3C) and oxaliplatin (Fig. 3D). Our results also showed that, in most cases, transfection of hCtr1 alone could not completely restore drug uptake, despite significant expression levels of the exogenous hCtr1 in these cells, suggesting other factors may be involved for maintaining cisplatin homeostasis.

To rule out the possibility that the inability of transporting cisplatin in the δN mutant was due to failure of protein targeting to the membrane, we fractionated the cell lysates of the transfected cells into cytosolic, plasma membrane, and nuclear fractions. We did Western blot analyses using antibodies to marker proteins for each fraction (i.e., tubulin for cytosol, Na^+/K^+-ATPase for plasma membrane, and lamin B for nucleus). Figure 4 shows that the fractionation scheme provided little cross-contamination among each other. These results also showed that the NH_2-terminal deleted hCtr1 protein, like WT and COOH-terminal deleted versions, was associated

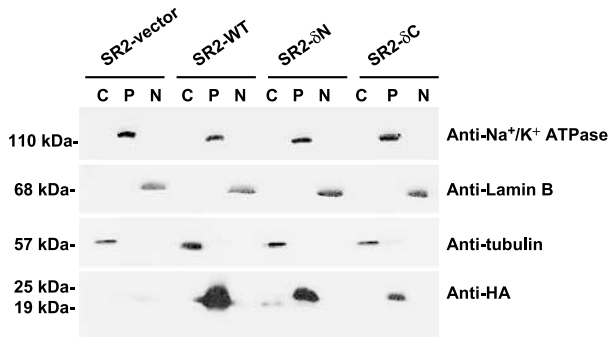

Figure 4. Western blot analyses of cellular distribution of the HA-tagged hCtr1 protein in the cytosolic (*C*), plasma membrane (*P*), and nucleic (*N*) fractions in various transfected cell lines. Cell fractions were separated by discontinuous sucrose gradient centrifugation as described in Materials and Methods. Antitubulin, anti-Na^+/K^+-ATPase, and anti–lamin B antibodies were used to probe cytoplasmic, plasma membrane, and nuclear fractions, respectively. Anti-HA antibody was used to probe hCtr1 protein encoded by the transfected cDNA.

with plasma membrane. These results strongly suggested that the functional defect of δN deletion mutant could not be attributed to mislocalization of the transporter. We note that the abnormal mobilities of hCtr1 WT and δN and δC proteins as mentioned above (Fig. 2C) were also evident in this SDS-PAGE analysis.

Sensitization of hCtr1-Transfected SCLC and SR2 Cells to Platinum Analogues

To investigate whether overexpression of hCtr1 by transfection would sensitize the transfected cells to platinum analogues, we determined the IC_{50} values of SCLC cells and their WT-transfected counterpart to various cytotoxic compounds. As shown in Table 1, the WT-transfected SCLC cells were more sensitive to cisplatin, carboplatin, and oxaliplatin. However, transfecting WT *hCtr1* into SR2 cells could only sensitize to the toxic effects of cisplatin and carboplatin but not oxaliplatin. Similar results were observed with transfecting δC mutant but not with δN. These results are consistent with the results described above that the δN mutant encodes a nonfunctional hCtr1. The inability to sensitize WT- and δC-transfected SR2 cells to oxaliplatin suggested that SR2 cells have a unique oxaliplatin-specific mechanism in addition to the reduced hCtr1 expression that contributes to the overall resistance profile to the platinum analogues. SR2 did not exhibit cross-resistance to doxorubicin and vinblastine, and transfection of functional *hCtr1* into SCLC and SR2 cells did not change the sensitivities of these cells to these agents, suggesting that the multidrug resistance gene was not involved in the sensitivities.

Discussion

Previous studies have shown that both mouse and human Ctr1 proteins are capable of stimulating copper accumulation in mammalian cells (25, 26). The present study further showed that transfection of the *hCtr1* gene into human cells increased uptakes of cisplatin and its analogues. These results confirm the role of this copper transporter in transporting cisplatin. The observation that hCtr1 transports not only cisplatin but also carboplatin and oxaliplatin further strengthens the importance of hCtr1 in platinum-based cancer chemotherapy.

Another important finding described in this communication is that, whereas elevated expression of hCtr1by transfection sensitized SCLC cells to cisplatin, carboplatin, and oxaliplatin, elevated expression of hCtr1 in SR2 cells can only sensitize cisplatin and carboplatin, but not oxaliplatin, despite the fact that these SR2 transfectants accumulated elevated amounts of oxaliplatin. These results suggest that SR2, which was established by continuous exposure to cisplatin in culture, has acquired a resistance mechanism unique to oxaliplatin that differs from that of cisplatin. Thus, at least two resistance mechanisms were involved during the selection of SR2 (i.e., reduction of hCtr1 expression), which contributed to the reduced transport rates of cisplatin and its analogues, and the yet-to-be-identified mechanism for neutralizing the cytotoxicity of oxaliplatin but not cisplatin and carboplatin.

Oxaliplatin is generally more potent than cisplatin when tested against a panel of human tumor cell lines (27). Our present findings are consistent with the implications that modes of cytotoxicities and mechanisms of resistance to oxaliplatin are not entirely similar to those of cisplatin and carboplatin. From the pharmacologic point of view, our results show that enhanced accumulation of certain platinum analogues by elevated expression of hCtr1 in cisplatin-resistant variants does not necessarily translate into enhanced cell killing. These observations have important clinical implications, particularly when modulation of *hCtr1* expression in cisplatin-resistant cells is considered for enhancing the efficacy of chemotherapy with platinum-based antitumor drugs.

Whereas this article was being reviewed, two-related articles were published: Beretta et al. (28) reported that, whereas transfecting expression human hCtr1 cDNA into cisplatin-resistant A431 cells conferred increased uptake of copper, no changes in cisplatin uptake and cellular

Table 1. Sensitivity (IC_{50}, μmol/L) of SCLC vector and WT, and SR2 vector, WT, δN, and δC cells to cisplatin, carboplatin, oxaliplatin, doxorubicin, and vinblastine

	Cisplatin	Carboplatin	Oxaliplatin	Doxorubicin	Vinblastine
SCLC					
Vector	21.8 ± 0.9	80.3 ± 7.6	24.5 ± 3.5	0.30 ± 0.06	11.1 ± 0.9
WT	12.3 ± 2.5*	33.6 ± 11*	15.5 ± 5.9†	0.37 ± 0.07	10.8 ± 2.0
SR2					
Vector	110.6 ± 15‡	109.7 ± 15‡	18.8 ± 5.7	0.23 ± 0.04	9.3 ± 3.2
WT	39.7 ± 3.7§	65.3 ± 12§	21.1 ± 14	0.32 ± 0.05	10.7 ± 3.3
δN	131.8 ± 11	113.3 ± 5.9	19.4 ± 5.8	0.24 ± 0.06	9.3 ± 3.9
δC	35.2 ± 13§	71.5 ± 6.6§	20.4 ± 6.5	0.23 ± 0.08	12.1 ± 1.3

NOTE: Data are expressed as means ± SD of triplicate measurements from three independent experiments.
*$P < 0.01$, significantly different from the results of SCLC vector (Student's t test).
†$P < 0.05$, significantly different from the results of SCLC vector (Student's t test).
‡$P < 0.01$, significantly different from the results of SCLC vector (Student's t test).
§$P < 0.01$, significantly different from the results of SR2 vector (Student's t test).

sensitivity to the drug was observed. Holzer et al. (29) reported that, whereas enhanced expression of hCtr1 in A2780 ovarian carcinoma cell line by transfection was associated with increases in copper and cisplatin accumulations, only marginal alternation of the survival rates in the transfected cells was observed. These results and those we describe here are not consistent with each other. Whereas the discrepancies are not apparent at the present, these results collectively suggest a complex mechanism of platinum-mediated cytotoxicity beyond the expression of hCtr1 that may depend on cell type–specific intracellular compartmentalization/targeting of the transported heavy metals. Further studies are needed to critically elucidate the effects of copper/cisplatin homeostasis and cytotoxicities in different cell contexts.

By employing deletion mutants, we found that the extracellularly located NH_2-terminal amino acid residues, but not the COOH-terminal motif, are important for transporting platinum-based drugs. Because the NH_2-terminal but not the COOH-terminal residues are also involved in the transport of copper, these results further strengthen the roles of hCtr1 in transporting platinum-based drugs in addition to copper. Although we observed that the expression levels of the copper-eliminating efflux transporters ATP7A and ATP7B were not significantly altered in five independently established cisplatin-resistant or cisplatin-sensitive pairs, their importance in clinical cisplatin resistance remains to be systematically investigated.

In conclusion, we have shown the important roles of copper transporter system in cancer pharmacology of platinum-based antitumor agents in cultured cells. Further studies of the clinical relevance of this system are needed. These investigations are currently under way in our laboratories. These studies may ultimately lead to a better utilization of platinum-based antitumor agents and thereby improve their chemotherapeutic efficacy.

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References

1. Loehrer PJ, Einhorn LH. Drugs five years later. Cisplatin. Ann Intern Med 1984;100:704 – 13.

2. Giaccone G. Clinical perspectives on platinum resistance. Drugs 2000; 59:9 – 17.

3. Lokich J, Anderson N. Carboplatin versus cisplatin in solid tumors: an analysis of the literature. Ann Oncol 1998;9:13 – 21.

4. Raymond E, Faivre S, Chaney S, et al. Cellular and molecular pharmacology of oxaliplatin. Mol Cancer Ther 2002;1:227 – 35.

5. Siddik ZH. Biochemical and molecular mechanisms of cisplatin resistance. Cancer Treat Res 2002;112:263 – 84.

6. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22:7265 – 79.

7. Fink D, Aebi S, Howell SB. The role of DNA mismatch repair in drug resistance. Clin Cancer Res 1998;4:1 – 6.

8. Johnson SW, Laub PB, Beesley JS, et al. Increased platinum-DNA damage tolerance is associated with cisplatin resistance and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines. Cancer Res 1997;57:850 – 6.

9. Andrews PA, Howell SB. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. Cancer Cell 1990;2:35 – 43.

10. Gately DP, Howell SB. Cellular accumulation of the anticancer agent cisplatin: a review. Br J Cancer 1993;67:1171 – 6.

11. Ishida S, Lee J, Thiele DJ, et al. Uptake of the anticancer drug cisplatin mediated by copper transporter Ctr1 in yeast and mammals. Proc Natl Acad Sci U S A 2002;99:14298 – 302.

12. Katano K, Kondo A, Safaei R, et al. Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper. Cancer Res 2002;62:6559 – 65.

13. Puig S, Thiele DJ. Molecular mechanisms of copper uptake and distribution. Curr Opin Chem Biol 2002;6:171 – 80.

14. Puig S, Lee J, Lau M, et al. Biochemical and genetic analyses of yeast and human high affinity copper transporters suggest a conserved mechanism for copper uptake. J Biol Chem 2002;277:26021 – 30.

15. Kruh GD. Lustrous insights into cisplatin accumulation: copper transporter. Clin Cancer Res 2003;9:5807 – 9.

16. Komatsu M, Sumizawa T, Mutoh M, et al. Copper-transporting P-type adenosine triphosphatase (ATP7B) is associated with cisplatin resistance. Cancer Res 2000;60:1312 – 6.

17. Katano K, Safaei R, Samimi G, et al. The copper export pump ATP7B modulates the cellular pharmacology of carboplatin in ovarian carcinoma cells. Mol Pharmacol 2003;64:466 – 73.

18. Yamane Y, Furuichi M, Song R, et al. Expression of multidrug resistance protein/GS-X pump and γ-glutamylcysteine synthetase genes is regulated by oxidative stress. J Biol Chem 1998;273:31075 – 85.

19. Yang LY, Li L, Jiang H, et al. Expression of ERCC1 antisense RNA abrogates gemcitabine-mediated cytotoxic synergism with cisplatin in human colon tumor cell defective in mismatch repair but proficient in nucleotide excision repair. Clin Cancer Res 2000;6:773 – 81.

20. Gomi A, Shinoda S, Sakai R, et al. Elevated expression of DNA polymerase β gene in glioma cell lines with acquired resistance to 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea. Biochem Biophys Res Commun 1996;227:558 – 63.

21. Xu J, Tyan T, Cedrone E, et al. Detection of 11q13 amplification as the origin of a homogeneously staining region in small cell lung cancer by chromosome microdissection. Genes Chromosomes Cancer 1996;17:172 – 8.

22. Siddik ZH, Boxall FE, Harrap KR. Flameless atomic absorption spectrophotometric determination of platinum in tissues solubilized in hyamine hydroxide. Anal Biochem 1987;163:21 – 6.

23. Lin X, Okuda T, Holzer A, et al. The copper transporter Ctr1 regulates cisplatin uptake in *Saccharomyces cerevisiae*. Mol Pharmacol 2002;62: 1154 – 9.

24. Eisses JF, Kaplan JH. Molecular characterization of hCtr1, the human copper uptake protein. J Biol Chem 2002;277:29162 – 71.

25. Moller LB, Petersen C, Lund C, et al. Characterization of the hCTR1 gene: genomic organization, functional expression, and identification of a highly homologous processed gene. Gene 2000;257:13 – 22.

26. Lee J, Prohaska JR, Thiele DJ. Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. Proc Natl Acad Sci U S A 2001;98:6842 – 7.

27. Pendyala L, Creaven PJ. *In vitro* cytotoxicity, protein binding, red blood cell partitioning, and biotransformation of oxaliplatin. Cancer Res 1993;53:5970 – 6.

28. Beretta GL, Gatti L, Tinelli S, et al. Cellular pharmacology of cisplatin in relation to the expression of human copper transporter CTR1 in different pairs of cisplatin-sensitive and -resistant cells. Biochem Pharmacol 2004; 68:283 – 91.

29. Holzer AK, Samimi G, Katano K, et al. The copper influx transporter hCTR1 regulates the uptake of cisplatin in human ovarian carcinoma cells. Mol Pharmacol 2004;66:817 – 23.

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