

Featured Article

Increased Expression of the Copper Efflux Transporter ATP7A Mediates Resistance to Cisplatin, Carboplatin, and Oxaliplatin in Ovarian Cancer Cells

Goli Samimi,¹ Roohangiz Safaei,¹
Kuniyuki Katano,¹ Alison K. Holzer,¹
Myriam Rochdi,³ Mika Tomioka,²
Murray Goodman,² and Stephen B. Howell¹

¹Department of Medicine, the Rebecca and John Moores University of California San Diego Cancer Center and the Biomedical Sciences Graduate Program, ²Department of Chemistry, University of California San Diego, La Jolla, California, and ³Globomax Service Group, Hanover, Maryland

ABSTRACT

Purpose: The goal of this study was to determine the effect of small changes in ATP7A expression on the pharmacodynamics of cisplatin, carboplatin, and oxaliplatin in human ovarian carcinoma cells.

Experimental Design: Drug sensitivity and cellular pharmacology parameters were determined in human 2008 ovarian carcinoma cells and a subline transfected with an ATP7A-expression vector ATP7A (2008/MNK). Drug sensitivity was determined by clonogenic assay, platinum (Pt) levels were measured by inductively coupled plasma mass spectroscopy, copper (Cu) accumulation was quantified with ⁶⁴Cu, and the subcellular distribution of ATP7A was assessed by confocal digital microscopy.

Results: The 1.5-fold higher expression of ATP7A in the 2008/MNK cells was sufficient to alter Cu cellular pharmacokinetics but not confer Cu resistance. In contrast, it was sufficient to render the 2008/MNK cells resistant to cisplatin, carboplatin, and oxaliplatin. Resistance was associated with increased rather than decreased whole-cell Pt drug accumulation and increased sequestration of Pt into the vesicular fraction. Cu triggered relocalization of ATP7A away from the perinuclear region, whereas at equitoxic concentrations the Pt drugs did not.

Conclusions: A small increase in ATP7A expression produced resistance to all three of the clinically available Pt drugs. Whereas increased expression of ATP7A reduced Cu accumulation, it did not reduce accumulation of the Pt drugs. Under conditions where Cu triggered ATP7A relocalization, the Pt drugs did not. Thus, although ATP7A is an important determinant of sensitivity to the Pt drugs, there are substantial differences between Cu and the Pt drugs with respect to how they interact with ATP7A and the mechanism by which ATP7A protects the cell.

INTRODUCTION

Cisplatin (DDP), carboplatin (CBDCA), and oxaliplatin (L-OHP) are widely used to treat human cancers; however, the emergence of resistance limits their therapeutic potential. Although reduced cellular drug accumulation, increased cytoplasmic detoxification, and increased DNA repair have been implicated in resistance to DDP (reviewed in Ref. 1), the mechanisms most responsible for clinical resistance to any of the three drugs have not been well defined. Furthermore, although evidence suggesting that one or more transporters are involved in DDP uptake was published over a decade ago (2), proteins that can mediate the influx and efflux of the Pt-containing drugs in tumor cells have only been identified recently. The major copper (Cu) influx transporter CTR1 mediates the influx of these drugs (3, 4), and the Cu efflux transporter ATP7B regulates the efflux of DDP (5) and CBDCA (6).

ATP7A is involved in Cu transport from the cytoplasm into the trans-Golgi network where it serves to load Cu onto Cu-requiring enzymes, such as ceruloplasmin, and to export it from the cell via the vesicular secretory pathway. ATP7A is expressed in most tissues other than the liver; in the liver a similar function is performed by the ATP7B. The essential role of these Cu transporters was established by the fact that loss of ATP7A function causes Menkes disease and loss of ATP7B function causes Wilson's disease. Patients with either of these conditions have altered accumulation and tissue distribution of Cu (reviewed in Ref. 7). Under basal conditions, when the extracellular Cu concentration is low, ATP7A and ATP7B are localized to the trans-Golgi network where they incorporate the metal into Cu-dependent enzymes. Upon exposure to increased Cu levels, the proteins relocalize to other sites in the cell, a response that is thought to be important to their ability to limit Cu toxicity. ATP7A relocalizes in part from the trans-Golgi network to the plasma membrane (8). In contrast, ATP7B relocates primarily to intracellular vesicular compartments, presumably involved in the secretory export pathway (reviewed in Ref. 9).

The idea that Cu transporters might mediate DDP resistance was introduced by Komatsu *et al.* (5) who reported that DDP resistance was associated with increased ATP7B expression in prostate carcinoma cells. Our studies have confirmed and

Received 1/22/04; revised 4/15/04; accepted 4/22/04.

Grant support: CA95298 from NIH and DAMD17-03-0158 from the Department of Defense. This work was conducted in part by the Clayton Foundation for Research—California Division. R. Safaei and S. Howell are Clayton Foundation Investigators. The production of ⁶⁴Cu at Washington University School of Medicine is supported by the National Cancer Institute Grant R24 CA86307.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Stephen B. Howell, Department of Medicine 0058, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0058. Phone: (858) 822-1110; Fax: (858) 822-1111; E-mail: showell@ucsd.edu.

extended this concept by demonstrating that human ovarian carcinoma cells selected for DDP resistance *in vitro* are cross-resistant to Cu and *vice versa* and that ATP7A or ATP7B is overexpressed at the protein level in some DDP-resistant ovarian carcinoma cell lines (10, 11). Furthermore, overexpression of ATP7B has been reported in a wide variety of DDP-resistant tumors (12–17). Finally, studies from this laboratory have shown that forced expression of ATP7A or ATP7B in fibroblasts derived from an ATP7A-deficient Menkes patient (18) rendered these cells resistant to DDP and CBDCA (19).

To determine whether ATP7A modulates the pharmacodynamics of the platinum (Pt) drugs in fully malignant epithelial ovarian carcinoma cells and to assess the significance of small changes in ATP7A expression similar in magnitude to those observed in tumor samples (20), in the current study we examined the effect of increasing the level of ATP7A expression in human ovarian carcinoma 2008 cells on sensitivity to DDP, CBDCA, and L-OHP and on the cellular pharmacology of these agents. We report here that even a small change in ATP7A level modulates sensitivity to all three of the clinically used Pt drugs but that there are substantial differences in the way in which increased ATP7A expression affects the cellular pharmacology of Cu as opposed to the Pt drugs.

MATERIALS AND METHODS

Drugs. DDP and CBDCA were generously provided by Bristol-Myers Squibb (Princeton, NJ), and L-OHP was provided by Sanofi Research (New York, NY). DDP was stored as a 3.3 mM stock solution in 0.9% NaCl in the dark at room temperature, CBDCA was stored as a 27 mM stock solution in water at 4°, and L-OHP was stored as a 12.6 mM stock solution in water at –20°. Cupric sulfate was obtained from Fisher Scientific Co. (Tustin, CA).

Cell Lines. All of the cell lines were maintained at 37°C in a humidified incubator containing 5% CO₂. Ovarian carcinoma cell lines 2008/EV and 2008/MNK were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Transfections were performed with 5 µg of plasmid (MNKMYC; Ref. 21) or empty pCEP4 as a control plus 20 µl of Lipofectamine (Invitrogen) in a total of 200 µl OPTI-MEM (Invitrogen) per well. Each well was washed with OPTI-MEM, and 800 µl OPTI-MEM was then added to each well followed by 200 µl DNA/Lipofectamine mixture. Cells were incubated at 37°C for 5 h followed by the addition of 2 ml of fresh OPTI-MEM. The following day, the media was changed, and fresh RPMI 1640 was added to each well. After a subsequent 48 h, increasing concentrations of hygromycin B (Invitrogen) were added to each well, and those cells that survived were pooled together into a population identified as either 2008/EV (empty vector control cells) or 2008/MNK (ATP7A transfected cells). 2008/EV and 2008/MNK cells were subsequently maintained in the presence of 20 µg/ml hygromycin B (Invitrogen).

Western Blots. Cells were grown to ~80% confluency in T75 tissue culture flasks. The cells were trypsinized and pelleted and the pellets washed once with PBS. The cell pellets were resuspended in 50 µl radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1%

SDS, and 50 mM Tris (pH 7.5)] with added Complete Mini, EDTA-free protease inhibitor mixture tablets (Roche Diagnostics, Mannheim, Germany), and kept on ice for 30 min. The lysate was then centrifuged at 14,000 × g for 12 min at 4°C, and the supernatant was recovered. Western blots were performed with 50 µg of protein with anti-MNK (Clone 34; BD Biosciences, La Jolla, CA) using antitubulin (clone B-5-1-2; Sigma, St. Louis, MO) as a lane loading control. Antibodies were added to the membrane at dilutions of 1:2,000 and 1:20,000, respectively, overnight at 4°C. Horseradish peroxidase-conjugated antimouse (Amersham Pharmacia Biotech, Piscataway, NJ) was added at a 1:2,000 dilution for 1 h at room temp. Protein bands were visualized by standard enhanced chemiluminescence procedure (Amersham Pharmacia Biotech).

Measurement of Drug Sensitivity by Colony Formation Assay. Colony assays were performed using triplicate cultures of 200 cells/35 mm plate grown in 5 ml of medium. Cells were removed from selective media 1 day before plating. The cells were allowed to attach overnight; exposed continuously to increasing concentrations of CuSO₄ or for 1 h to increasing concentrations of DDP, CBDCA, or L-OHP; and then incubated in fresh medium until visible colonies had formed (10–14 days). The dishes were then rinsed with PBS, fixed with 100% methanol, and stained with a 1% crystal violet solution. A Chemi-Imager 400 instrument (Alpha Innotech, San Leandro, CA) was used for counting colonies containing >50 cells.

Cu Accumulation. Cells were grown until ~80% confluent in either 100-mm dishes [for inductively coupled plasma optical emission spectroscopy (ICP-OES) measurements] or plates containing six 25-mm wells (for ⁶⁴Cu measurements). For ICP-OES measurements of basal Cu content, 10 plates were used for each data point, and the cells were incubated in fresh medium containing no additional Cu. The cells were then washed three times with cold PBS, scraped from the dish, and pelleted. The pellets were dried, resuspended in 70% nitric acid, and dissolved at 65°C for 2 h, after which the samples were diluted with water to a final concentration of 5% acid. Measurements were made using a Perkin-Elmer ICP-OES (model 3000DV) located at the Analytical Facility of the Scripps Institute of Oceanography and normalized to protein levels for each cell line. For ⁶⁴Cu measurements, the six wells of a single six-well plate were used for each data point, and the cells were incubated in fresh OPTI-MEM (Invitrogen) containing 2 µM ⁶⁴Cu (Mallinckrodt Institute of Radiology, Washington University Medical School, St. Louis, MO). For efflux studies, after exposure to ⁶⁴Cu cells were washed three times in warm OPTI-MEM using a rapid sampling technique, and fresh, nonradioactive media was added for various time periods. The wells were then washed three times with cold PBS containing 2 µM non-radioactive CuSO₄ and 10 mM EDTA, lysed with a PBS solution containing 0.1% SDS and 1.0% Triton X-100, and scraped into 3 ml of scintillation fluid (National Diagnostics, Atlanta, GA). ⁶⁴Cu was quantified by scintillation counting and normalized to protein levels for each cell line.

DDP Whole-Cell Uptake and Accumulation in DNA. Cells were grown to 80% confluency in either six-well plates (whole-cell uptake) or 145-mm plates (DNA accumulation). For whole-cell uptake studies, three wells were used for each data point, and the cells were incubated in fresh medium containing

DDP for 24 h. The cells were then washed with PBS and were lysed directly by addition of 215 μ l 70% nitric acid into each well. The cells were then collected and dissolved at 65°C for 2 h, after which the samples were diluted with water to a final concentration of 5% acid. For DNA Pt accumulation studies, three plates were used for each data point, and the cells were incubated in fresh medium containing DDP for 24 h. The cells were then washed with PBS, scraped, and pelleted before isolation of genomic DNA using a Wizard[®] Genomic DNA Purification kit (Promega, Madison, WI). Harvested DNA was originally resuspended in 70 μ l of water for quantification. After quantification, 215 μ l 70% nitric acid was added to the DNA, and the samples were dissolved at 65°C for 2 h after which the samples were diluted with 3 ml of water to a final concentration of 5% acid. Measurements were made using a Thermo Finnigan inductively coupled plasma mass spectroscopy (ICP-MS; model Element2) from the Analytical Facility at the Scripps Institute of Oceanography and normalized to protein levels or DNA amounts for each cell line. Indium is added to each sample at 1 ppb as a control for flow variation.

Accumulation of Fluorescein-Conjugated DDP (fDDP) in Whole Cells by Flow Cytometry. 2008/EV and 2008/MNK cells were plated in T-75 flasks and grown until ~80% confluent. Cells were then treated with media alone or 2 μ M fDDP for 2 h. Treated and untreated cells were harvested in trypsin, and the cell pellets were resuspended in 0.5 ml of 5% BSA in PBS. FACSCalibur with CellQuest acquisition software (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used to collect and analyze data on at least 50,000 events per sample. Events were gated by forward and side scatter to exclude aggregates and debris, and the fluorescence intensity excited by a 488 nm laser was measured in the FL1 channel (530 \pm 15 nm).

Vesicular Preparations. Cells were plated in 145-mm plates and grown to ~80% confluency. All of the subsequent steps were performed with ice-cold solutions, and all of the spins were performed at 4°C. The cells were rinsed once with PBS and then rinsed twice with 1 mM sodium bicarbonate solution. Each plate was then scraped with 1 mM sodium bicarbonate solution with added Complete Mini, EDTA-free protease inhibitor mixture tablets (Roche Diagnostics) into a 15-ml conical tube (three plates were combined for each data point), and the cells were homogenized for 5 min. The lysate was spun at 3,000 \times g for 5 min, and the postnuclear fraction recovered and added to a 2-ml cushion of 38% sucrose in 100 mM sodium carbonate. The samples were spun in an ultracentrifuge at 25,000 rpm for 35 min, and the vesicular band was carefully recovered and transferred to a new ultracentrifuge tube. The tubes were filled with a 50 mM Tris solution containing 250 mM sucrose with added Complete Mini, EDTA-free protease inhibitor mixture tablets. The samples were again spun in an ultracentrifuge at 26,000 rpm for 45 min to pellet the vesicles. Each pellet was resuspended in 100 μ l of standard radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris (pH 7.5)], and then prepared for ICP-MS analysis as described above. For quantitative purposes, each sample was also analyzed for sulfur content using ICP-OES as described above, and sulfur levels were used to normalize total Pt accumulation.

Confocal Microscopy. Cells were cultured for 3–4 days in nonselective media on round coverslips (Fisherbrand 12–545–81, 12CIR.-1.5) in 24-well plates and treated for 1 h at 37°C with drug in OPTI-MEM (Invitrogen). All of the subsequent steps were performed at room temperature unless otherwise indicated. After three rinses in PBS, the cells were fixed for 30 min in 3.7% formaldehyde in PBS, rinsed twice with PBS, permeabilized for 15 min in 0.3% Triton X in PBS, and rinsed twice with 0.1% Tween in PBS. Cells were then blocked for 1 h in PBS containing 1% BSA and 0.1% Tween, followed by incubation with a 1:50 dilution of anti-MNK (Clone 34; BD Biosciences) for 2 h at 37°C. Cells were then rinsed three times with PBS containing 0.1% Tween and incubated with 1:2000 dilution of Hoechst 33342 (Molecular Probes) and Alexa 594 antimouse (Molecular Probes #A21125) at 1:1000 dilution for 2 h at 37°C. Cells were then rinsed three times with PBS and twice with water before mounting on glass slides with gelvatol. Slides were kept in the dark at room temperature. Confocal microscopic examination was performed at University of California San Diego Cancer Center Digital Imaging Shared Resource using a DeltaVision deconvoluting microscope system (Applied Precision, Inc., Issaquah, WA). Images were captured by \times 100, \times 60, and \times 40 lenses using SoftWorx software (Applied Precision, Inc.) on a Silicon Graphics Octane workstation.

Statistics. All of the comparisons were made using the Student *t* test with the assumption of unequal variance.

RESULTS

Cell Line Model. The human ovarian carcinoma cell line 2008 was transfected with a plasmid expressing the human MNK protein from a pCMV promoter to create the 2008/MNK subline. The 2008 cells were also transfected with the same vector containing no insert to create the 2008/EV control subline. Because the magnitude of the increase in ATP7A expression observed in cell lines selected *in vitro* for DDP resistance and in patients receiving Pt drug-based therapy is only quite modest (10, 20), and it was a goal of this study to assess the significance of such small changes in expression, a subline was identified that had only a 1.5-fold increase in ATP7A level as determined by Western blot analysis as shown in Fig. 1.

Basal Cu Content and Cu Accumulation. To determine whether the modest overexpression of ATP7A in 2008/MNK cells was sufficient to alter the pharmacodynamics of Cu, basal Cu levels were measured in cells grown in standard medium to

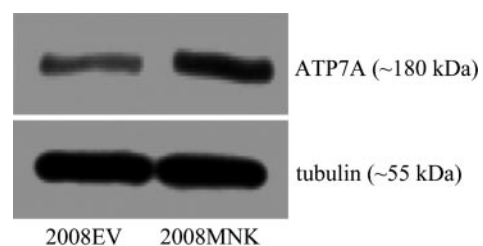


Fig. 1 Western blot analysis of ATP7A expression in 2008/MNK versus 2008/EV cells. ATP7A was detected as a single band of ~180 kDa. The blots were probed with an antitubulin antibody to serve as a lane loading control. Tubulin was detected as a single band of ~55 kDa.

which no additional Cu had been added. Under these conditions the ATP7A-transfected 2008/MNK cells contained only $45.1 \pm 13.9\%$ (SE) as much Cu/mg protein as the empty vector control 2008/EV cells. To additionally define the effect on Cu homeostasis, Cu uptake was measured after a 24-h exposure to $2 \mu\text{M}$ ^{64}Cu . The 2008/MNK cells accumulated only $69.9 \pm 2.1\%$ (SE) as much Cu as the control 2008/EV cells ($P < 0.005$). Thus, the 1.5-fold increase in ATP7A protein level detected by Western blot analysis was sufficient to reduce steady-state Cu accumulation.

Cu Uptake and Efflux Kinetics. To additionally define the effect of the increased expression of ATP7A on the cellular pharmacology of Cu, kinetic studies were performed to document the time course of Cu uptake over 1 h as well as Cu efflux over a 512-min period after a 1 h Cu exposure. Cells were exposed to $2 \mu\text{M}$ ^{64}Cu , and Cu levels were determined at various time points using a rapid harvesting and washing technique. As shown in Fig. 2A, the cellular accumulation of Cu was time-dependent for up to 1 h, and 2008/MNK cells accumulated substantially less Cu after just 20 min compared with control 2008/EV cells. Fig. 2, B and C, show the Cu efflux curves. Cu efflux from the 2008/EV and 2008/MNK cells was characterized by a very rapid initial phase from 0 to 1 min, a rapid second phase from 1 to 128 min, and a much slower terminal phase from 128 to 512 min. The efflux data were subjected to a compartmental analysis as performed in prior Cu and Pt efflux kinetic studies of DDP-resistant cell lines (10); the data were sufficient to estimate the first and second half-lives, and these are presented in Table 1. Cu efflux from the 2008/MNK cells was 3.1-fold faster for the initial phase and 1.4-fold faster for the second phase compared with that from the control 2008/EV cells. Determination of Cu efflux kinetics for the terminal phase was not possible, because the elimination of Cu during this phase was extremely slow. These results suggest that, although the magnitude of the increase in ATP7A expression in the 2008/MNK cells was modest, it was nevertheless sufficient to yield a clearly detectable phenotype with respect to the pharmacodynamics of Cu.

Sensitivity to Cu, DDP, CBDCA, and L-OHP. Colony formation assays were performed to determine relative sensitivities of 2008/EV and 2008/MNK cells to increasing concentrations of Cu and the three clinically available Pt drugs, DDP, CBDCA, and L-OHP. Table 2 presents data on their relative sensitivity expressed as the ratio of the slope of the concentration-survival curve of the 2008/MNK cells relative to that for the 2008/EV cells. Although the modest overexpression of ATP7A was not sufficient to confer resistance to Cu, it was sufficient to render the cells significantly resistant to a 1 h exposure to DDP, CBDCA, and L-OHP. These results are consistent with our previous observations to the effect that even very small changes in Cu resistance are accompanied by much larger differences in DDP resistance (6, 10).

Whole Cell and DNA Pt Accumulation. To determine whether the ATP7A-mediated differences in sensitivity to the Pt-containing drugs were reflected in altered Pt whole cell accumulation or a difference in the extent of DNA adduct formation, 2008/EV and 2008/MNK cells were exposed to the equitoxic concentrations of $2 \mu\text{M}$ DDP, $50 \mu\text{M}$ CBDCA, or $6 \mu\text{M}$ L-OHP for 24 h after which the cells were washed extensively

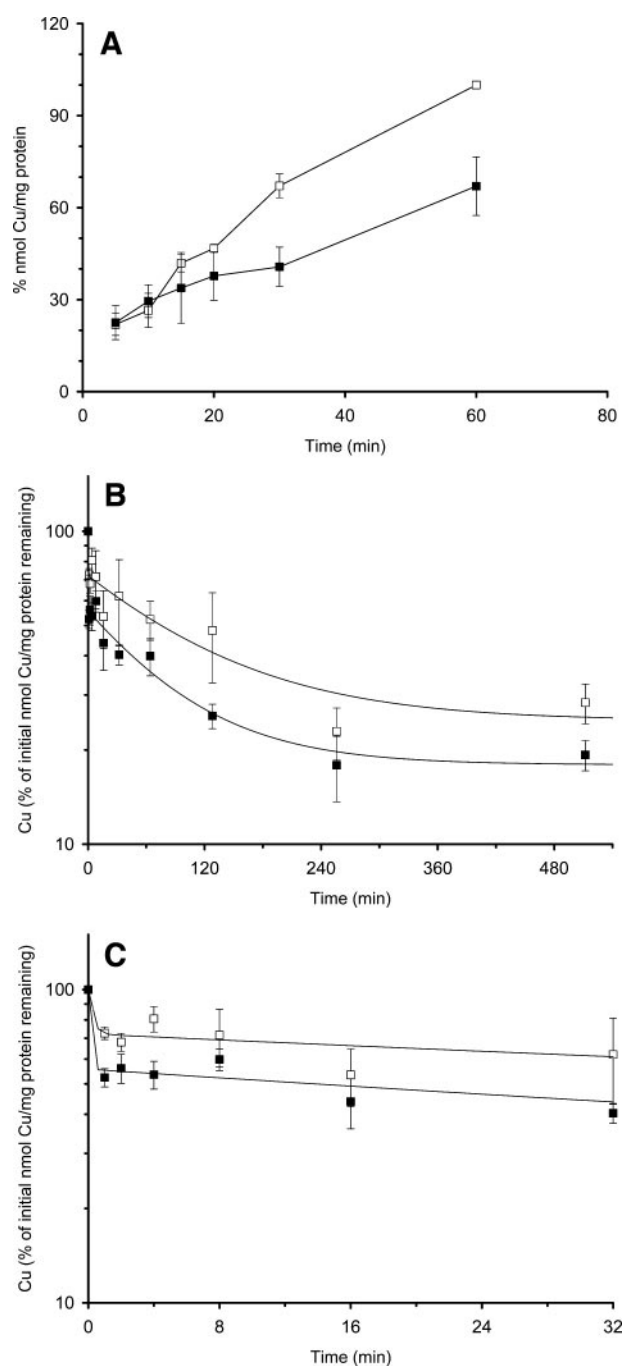


Fig. 2 Time course of copper (Cu) uptake and efflux in 2008/MNK versus 2008/EV cells. A, Cu accumulation during exposure to $2 \mu\text{M}$ CuSO_4 . B and C, Cu efflux after exposure to $2 \mu\text{M}$ CuSO_4 for 1 h. (■), 2008/MNK cells; (□), 2008/EV cells. Each data point represents the mean of three independent experiments each performed with six separate cultures; bars, \pm SE.

and cellular and DNA Pt levels quantified by ICP-MS. Neither whole cell Pt accumulation nor DNA adduct formation differed significantly between the 2008/MNK and 2008/EV cells for any of these drugs (all ratios for 2008MKNK/2008ev ranged between

Table 1 Estimated Cu^a efflux half-lives

Parameter	2008/EV	2008/MNK	Ratio ^b
T _{1/2α} (CV)	11.2 s (421%)	3.6 s (112,856%)	3.1
T _{1/2β} (CV)	82.8 min (163%)	58.5 min (70%)	1.4

^a Cu, copper; T_{1/2α}, half-life of initial rapid phase of efflux; T_{1/2β}, half-life of second phase of efflux; CV, coefficient of variation.

^b Ratio calculated as t_{1/2}[2008EV]:t_{1/2} [2008MNK].

Table 2 Relative sensitivity of 2008/MNK and 2008/EV cells to Cu,^a DDP, CBDCA, and L-OHP as determined by the slope of the concentration-survival curve determined from clonogenic assays

Drug	Slope ratio mean ± SD ^b	No. of independent experiments	95% confidence interval
Cu	0.9 ± 0.2	3	0.7–1.1
DDP	0.5 ± 0.2	4	0.3–0.7
CBDCA	0.2 ± 0.1	4	0.1–0.3
L-OHP	0.6 ± 0.2	3	0.4–0.9

^a Cu, copper; DDP, cisplatin; CBDCA, carboplatin; L-OHP, oxaliplatin.

^b Ratio of the slope of the concentration-survival curve for 2008/MNK to that for 2008/EV.

1.0 and 1.1). Thus, whereas 2008/MNK cells were relatively more resistant to treatment with these Pt-containing drugs, the modest overexpression of ATP7A did not affect accumulation of the Pt drugs to the same extent as it did ⁶⁴Cu in this cell system. This suggests that increased expression of ATP7A either protects these cells against the cytotoxicity of the Pt drugs by a mechanism not directly dependent on whole cell Pt accumulation or DNA adduct formation as reflected by Pt in DNA or that the magnitude of the effect was too small to be quantified by the assays used.

Accumulation of fDDP in Whole Cells. Although the increase in ATP7A expression in the 2008/MNK cells was only modest, it was sufficient to perturb Cu homeostasis and render the cells resistant to DDP, CBDCA, and L-OHP. Whereas ICP-MS measurements failed to show a difference in whole cell Pt accumulation, the possibility remained that differences in Pt accumulation might be too small to be detected by this method. Therefore, whole cell drug accumulation was measured using fDDP, shown previously to mimic the cellular pharmacology of DDP in epithelial cells (22). After a 2-h exposure to either medium alone or to 2 μM fDDP, cell-associated fluorescein levels were measured by flow cytometry. As shown in Table 3, the 2008/MNK cells unexpectedly accumulated significantly more rather than less fDDP than the 2008/EV cells ($P < 0.04$ for calculations based on differences in geometric mean fluorescence).

Pt Accumulation in Vesicular Compartments. The results obtained with fDDP suggest the possibility that ATP7A mediates sequestration of the drug into a compartment from which it is unable to reach the DNA and exert cytotoxicity. To determine whether this was the case, the Pt content of the vesicular fraction was determined in 2008/EV and 2008/MNK cells exposed to 2 μM DDP, 50 μM CBDCA, or 6 μM L-OHP for

24 h. The results, presented in Table 4, demonstrate increased vesicular accumulation in 2008/MNK cells compared with 2008/EV cells of all three of the drugs that was statistically significant for DDP ($P < 0.03$) and CBDCA ($P < 0.02$) and trended in the same direction for L-OHP. These data supports the notion that, in this cell system, ATP7A functions to sequester these drugs in intracellular vesicles thus limiting access to the nucleus and conferring resistance.

ATP7A Trafficking in 2008/MNK Cells. Increases in extracellular Cu concentration trigger the relocalization of ATP7A from the *trans*-Golgi to the plasma membrane in many cell types. To document that this was observed in the 2008/MNK cells and to determine whether the Pt drugs produced the same effect, 2008/MNK cells were exposed to 500 μM Cu, 2 μM DDP, 50 μM CBDCA, or 10 μM L-OHP for 1 h, and the location of ATP7A was determined in untreated and treated cells by confocal digital microscopy. Fig. 3, A and B, show that in untreated 2008/MNK cells ATP7A resides in the perinuclear region, verifying the localization of ATP7A in the region of the *trans*-Golgi in cells grown in the absence of added Cu (reviewed in Ref. 9). Fig. 3, C and D, show that upon treatment with 500 μM Cu, ATP7A redistributed away from the *trans*-Golgi toward the cell surface, verifying that Cu induces relocalization of ATP7A in 2008/MNK cells. However, upon treatment with 2 μM DDP (Fig. 3, E and F), 50 μM CBDCA (Fig. 3, G and H), or 10 μM L-OHP (Fig. 3, I and J), ATP7A remained localized mainly to the perinuclear region. Thus, although the level of ATP7A in the 2008/MNK cells was sufficient to perturb Cu homeostasis and ATP7A relocalized normally in response to Cu, at the concentrations tested none of the Pt drugs triggered observable ATP7A relocalization. Thus, in contrast to the situation for Cu where ATP7A-mediated resistance is associated with relocalization to the plasma membrane, ATP7A-mediated

Table 3 Whole cell accumulation of fluorescent cisplatin following a 2-h exposure to 2 μM fDDP^a

Cell line	Geometric mean of fluorescence ± SE ^b
2008/EV	73.9 ± 1.1
2008/MNK	86.5 ± 1.5 ^c

^a fDDP, fluorescein-conjugated cisplatin.

^b Values are the mean ± SE of three independent experiments.

^c $P < 0.04$ for comparison with 2008/EV cells.

Table 4 Accumulation of Pt^a in vesicles after a 24-h exposure to DDP, CBDCA, or L-OHP

Drug	ng Pt/mg sulfur mean ± SE ^b		
	2008/EV	2008/MNK	Ratio
DDP, 2 μM	0.6 ± 0.4	1.2 ± 0.7	2.1 ± 0.2 ^c
CBDCA, 50 μM	0.8 ± 0.2	2.1 ± 0.4	2.5 ± 0.0 ^d
L-OHP, 6 μM	0.9 ± 0.5	1.6 ± 0.6	1.7 ± 0.6

^a Pt, platinum; DDP, cisplatin; CBDCA, carboplatin; L-OHP, oxaliplatin.

^b Mean ± SE of at least two independent experiments.

^c $P < 0.03$.

^d $P < 0.02$.

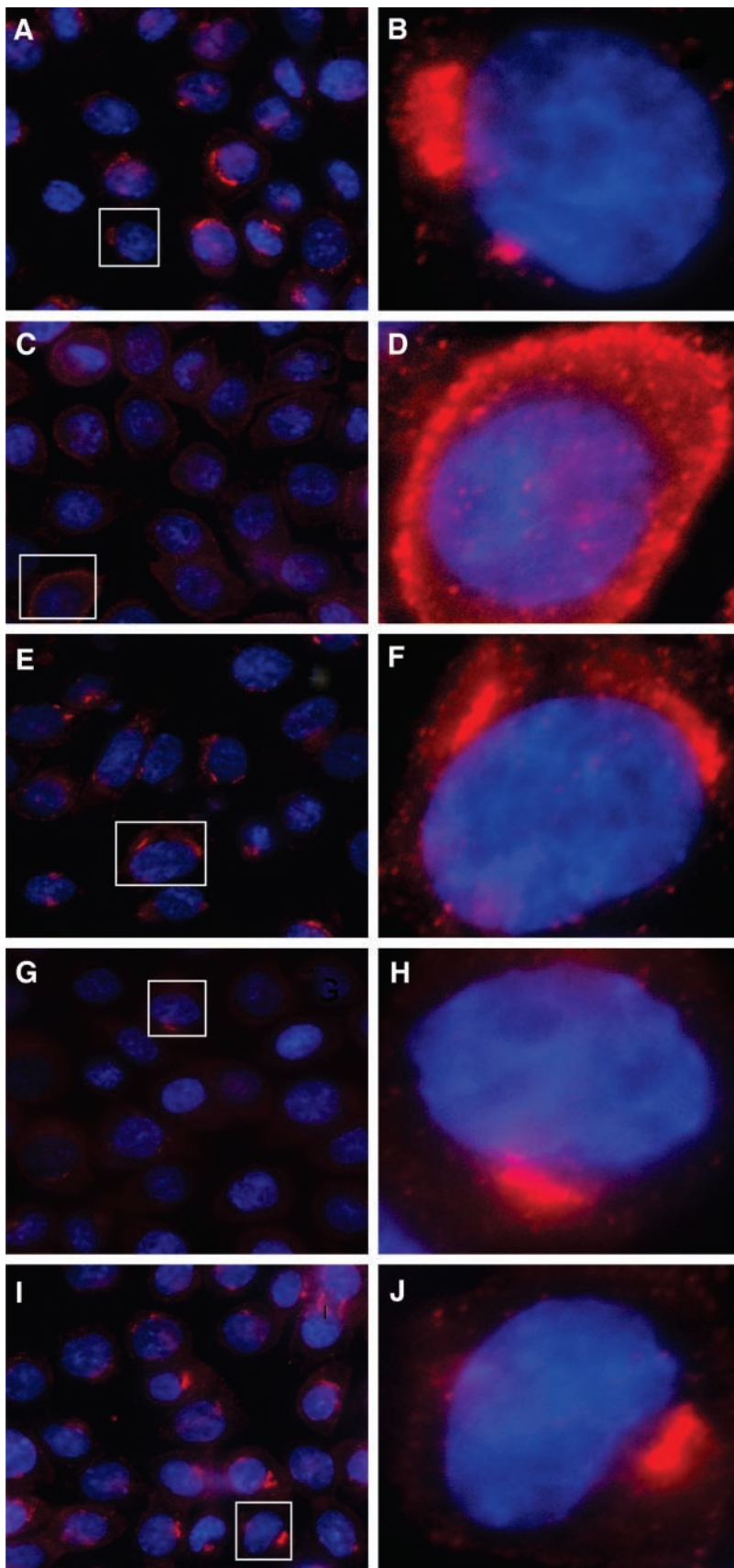


Fig. 3 Immunofluorescence localization of ATP7A in 2008/MNK cells after exposure to copper, cisplatin, carboplatin, or oxaliplatin. The nuclear marker Hoechst 33342 stains the nucleus blue. Alexa fluor 594-tagged antimouse antibody indirectly stains ATP7A red. *A* and *B*, perinuclear localization of ATP7A in untreated 2008/MNK cells; *C* and *D*, relocation of ATP7A in 2008/MNK cells after a 1-h exposure to 500 μM copper; *E* and *F*, mainly perinuclear localization of ATP7A in 2008/MNK cells after a 1-h exposure to 2 μM cisplatin; *G* and *H*, perinuclear localization of ATP7A in 2008/MNK cells after a 1-h exposure to 50 μM carboplatin; *I* and *J*, perinuclear localization of ATP7A in 2008/MNK cells after a 1-h exposure to 10 μM oxaliplatin.

resistance to DDP, CBDCA, and L-OHP occurred in the absence of an equivalent degree of drug-induced relocalization. This suggests that there are substantial differences between Cu and the Pt drugs with respect to how they interact with ATP7A and with respect to the mechanism by which ATP7A protects the cell against these compounds.

DISCUSSION

Earlier studies have indicated that sensitivity to the Pt drugs can be regulated by the Cu influx transporter CTR1 (3, 4) and the Cu exporter ATP7B (5, 10). The results reported here indicate that another Cu transporter, ATP7A, is also capable of modulating sensitivity to this class of drugs and that this effect is observable in ovarian cancer cells. Furthermore, the results document that even a quite modest change in ATP7A level is pharmacodynamically significant. These findings provide additional support for the concept that the Cu transporters are important to the pharmacodynamics of the Pt-containing drugs and specifically identify small changes in ATP7A expression as being of relevance in ovarian cancer.

Western blot analysis of ovarian carcinoma cell lines selected *in vitro* for acquired resistance to DDP (10) and immunohistochemical analysis of ovarian carcinoma samples from patients failing DDP and/or CBDCA-containing primary chemotherapy (20) have demonstrated only modest changes in ATP7A protein levels. The 2008/MNK cells exhibited a similar modest 1.5-fold increase in the expression of ATP7A relative to that in the 2008/EV cells and, thus, provide a good model in which to study the effect of clinically relevant changes in ATP7A expression on the pharmacodynamics of DDP, CBDCA, and L-OHP. The ATP7A expressed from the transfected vector was clearly functional in that it produced the expected changes in the cellular pharmacology of Cu. First, Cu content was reduced when the cells were grown under either basal or Cu-supplemented conditions. Second, detailed analysis of Cu uptake and efflux kinetics demonstrated that the rate of Cu accumulation was reduced and that Cu efflux from 2008/MNK cells was substantially more rapid than from the 2008/EV cells, at least over the time period where the kinetics could be accurately defined. Thus, this pair of cell lines constitutes a well-defined system with which to investigate the effect of increased ATP7A on the pharmacodynamics of the Pt drugs. It is of interest that despite producing readily detectable changes in the cellular pharmacology of Cu, a 1.5-fold increase in ATP7A in these ovarian carcinoma cells did not produce a measurable degree of resistance to the cytotoxic effect of Cu. The reason for this is unknown.

Increased expression of ATP7A in the 2008/MNK cells resulted in resistance to the Pt drugs that was greatest for CBDCA and least for L-OHP. Although the magnitude of the resistance was small, it is in the range sufficient to account for clinical failure of DDP therapy (23). However, analysis of whole cell Pt accumulation and DNA adduct formation did not demonstrate a decrease in drug uptake. However, a more sensitive measure of drug uptake made using fDDP showed clearly that the 2008/MNK cells accumulated more rather than less fDDP. In addition, whereas Cu triggered a readily observable relocalization of ATP7A away from the perinuclear region, this

was not observed with DDP, CBDCA, or L-OHP at the concentrations at which the 2008/MNK cells displayed resistance to the cytotoxic effect of these drugs. This indicates that Cu and the Pt drugs interact with ATP7A in different ways, and that ATP7A mediates resistance to the Pt drugs by a mechanism that is different in some way from that by which it controls sensitivity to Cu. In this cell system, ATP7A did not function to limit accumulation of the Pt drugs as it did for Cu, but rather enhanced whole cell drug accumulation as detected by fDDP. This suggests that ATP7A may function to detoxify the Pt drugs by sequestering them into vesicles that are not subsequently as readily exported as are the vesicles into which ATP7A sequesters Cu.

Fig. 4 presents a schematic that outlines the hypothesis that ATP7A causes resistance by sequestering the Pt drugs into vesicles and summarizes the supporting evidence. Cu, being the normal substrate, is sequestered into vesicular structures by ATP7A and triggers the relocalization required for efficient export of vesicles from the cell. Thus, an increase in ATP7A expression results in decreased whole Cu accumulation (18, 24). In contrast, whereas the Pt drugs appear to be sequestered efficiently, as evidenced by the fact that small increases in ATP7A are even better at protecting the cell against the cytotoxicity of the Pt drugs than the cytotoxicity of Cu, they fail to trigger vesicle relocalization and export. Thus, resistance is accompanied by an increase rather than a decrease in whole cell drug accumulation. Although it remains possible that the resistance is an epiphenomenon not directly linked to the transport function of ATP7A, the model is supported by the recent observation that a fluorescent form of DDP colocalizes with the

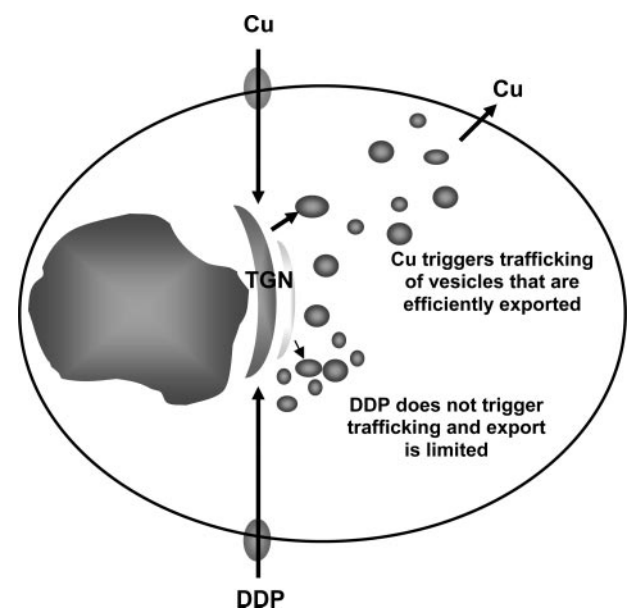


Fig. 4 Schematic diagram of hypothesized differences in copper (Cu) and platinum (Pt) drug vesicular sequestration, trafficking, and export pathways. Cu and the Pt drugs enter predominantly via hCTR1 and are sequestered by ATP7A into the *trans*-Golgi network (TGN) and vesicles of the secretory pathway. Cu triggers relocalization of vesicles that are then efficiently exported. The Pt drugs fail to trigger relocalization, and the export of vesicles containing these drugs appears to be limited.

other Cu export transporter ATP7B (25) and that cells lacking the sequestering activity of ATP7A are hypersensitive to these drugs (19). This latter observation identifies ATP7A as a potential target for therapeutic intervention.

The concept that sequestration may not be directly linked to export in the case of the Pt drugs is consistent with earlier studies of the cellular pharmacology of the Pt drugs. One might have expected that when more drug enters the cell, more will be found in the DNA. However, in ovarian carcinoma cell lines of different origin there is no correlation between the fraction of Pt in the whole cell that is found in the DNA after exposure to DDP and sensitivity to the cytotoxic effect of the drug (26). This lack of an association between the fraction of whole cell Pt that is found in the DNA and sensitivity has also been observed for L-OHP in ovarian carcinoma cells (27). This is understandable if, indeed, the extent to which the Pt drug becomes sequestered into poorly exportable vesicular structure varies between cells. For a given Pt adduct load in DNA, a cell that does a good job of sequestering the drug into vesicles would be expected to exhibit a large whole cell accumulation and *vice versa*. The results of the current study suggest that ATP7A is capable of mediating such sequestration. Another point to be considered is whether ATP7A-mediated sequestration may serve to protect a target other than DNA. Although DNA damage is thought to be the primary mechanism by which the Pt drugs exert their cytotoxicity, it is clear that a variety of other types of cellular injury occur upon exposure to DDP due to its reactivity with thiols and proteins (28). This ability to damage proteins may underlie the hypothesized difference in the extent to which vesicles can be exported after loading with Cu as opposed to the Pt drugs. The facile export of Cu-loaded vesicles involves complex protein-protein interactions that may be disrupted by the Pt drugs and in a human hepatoma cell line DDP has in fact been shown to block the export of Cu (11).

The concept that ATP7A sequesters the Pt drugs into vesicles that are then not readily exported is additionally supported by studies of the forced expression of ATP7A in a fibroblast cell line isolated from a Menkes patient (19). Restoration of the expression of ATP7A in this cell line resulted in decreased sensitivity to DDP and CBDCA. However, similar to the current study, whereas ATP7A limited the accumulation of Cu it increased rather than reduced accumulation of the Pt drugs. These results suggest that whereas ATP7A may succeed in sequestering both Cu and the Pt drugs into vesicles of the secretory pathway, only in the case of Cu is the actual export of these vesicles readily accomplished.

Cells selected for DDP resistance are cross-resistant to Cu, but the magnitude of the resistance to DDP is substantially greater than that to Cu (10). Cells selected for resistance to Cu are cross-resistant to DDP, and again the resistance to DDP exceeds that to Cu (11). This same phenomenon was observed in cells engineered to overexpress ATP7B (6). In the current study, the increase in ATP7A expression conferred a clinically significant degree of resistance to the Pt drugs in the absence of any resistance to Cu. The basis for this curious differential potency with respect to resistance is unknown but must reflect differences in the interaction of Cu and the Pt drugs with the transporters.

Immunohistochemical studies have demonstrated that

ATP7A is not detected in most normal tissues (20). In contrast, ATP7A expression is detectable in many malignant tissues, even those of which the cognate normal tissue does not express this protein (20). A previous study from this laboratory sought associations between ATP7A expression, as measured by immunohistochemistry, and various clinical parameters in paired ovarian carcinoma tumor samples from 54 patients obtained prior to and after Pt drug-based therapy (20). The results demonstrated that patients whose tumors became enriched for cells expressing ATP7A after at least three cycles of Pt-based treatment had impaired survival compared with those whose tumors did not (20). In combination with the results of the current study, this finding provides substantial evidence that ATP7A expression in ovarian cancer modulates sensitivity to the Pt drugs, and that the magnitude of the effect has clinical significance. Overall, these studies provide strong support for the notion that sensitivity to DDP and its analogs is regulated in part by the transporters and chaperones that evolved to manage Cu homeostasis.

ACKNOWLEDGMENTS

We thank Dr. Kevin Walda (Scripps Institute of Oceanography Analytical Facility, San Diego, CA) for technical assistance, Dr. Michael J. Petris (University of Missouri-Columbia, Columbia, MO) for helpful discussions, and Dr. Anthony P. Monaco (Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom) for generously providing the MNKMYC vector for transfection studies. We also thank Dennis J. Young in the University of California San Diego Cancer Center's Flow Cytometry Shared Resource for expert advice.

REFERENCES

- Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 2003;22:7265–79.
- Andrews PA, Albright KD. Role of membrane ion transport in cisplatin accumulation. In: Howell SB, editor. Platinum and other metal coordination compounds in cancer chemotherapy. New York: Plenum Press; 1991. p. 151–9.
- Lin X, Okuda T, Holzer A, Howell SB. The copper transporter CTR1 regulates cisplatin uptake in *saccharomyces cerevisiae*. *Mol Pharmacol* 2002;62:1154–9.
- Ishida S, Lee J, Thiele DJ, Herskowitz I. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proc Natl Acad Sci USA* 2002;99:14298–302.
- 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.
- Katano K, Safaei R, Samimi G, Holzer A, Rochdi M, Howell SB. The copper export pump ATP7B modulates the cellular pharmacology of carboplatin in ovarian carcinoma cells. *Mol Pharmacol* 2003;64:466–73.
- Suzuki M, Gitlin JD. Intracellular localization of the Menkes and Wilson's disease proteins and their role in intracellular copper transport. *Pediatr Int* 1999;41:436–42.
- Petris MJ, Mercer JF, Culvenor JG, Lockhart P, Gleeson PA, Camakaris J. Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. *EMBO J* 1996;15:6084–95.
- Pena MM, Lee J, Thiele DJ. A delicate balance: homeostatic control of copper uptake and distribution. *J Nutr* 1999;129:1251–60.
- 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.

11. Safaei R, Katano K, Samimi G, et al. Cross-resistance to cisplatin in cells with acquired resistance to copper. *Cancer Chemother Pharmacol* 2004;53:239–46.
12. Nakayama K, Miyazaki K, Kanzaki A, Fukumoto M, Takebayashi Y. Expression and cisplatin sensitivity of copper-transporting P-type adenosine triphosphatase (ATP7B) in human solid carcinoma cell lines. *Oncol Rep* 2001;8:1285–7.
13. Kanzaki A, Toi M, Neamati N, et al. Copper-transporting P-Type Adenosine Triphosphatase (ATP7B) Is Expressed in Human Breast Carcinoma. *Jpn J Cancer Res* 2002;93:70–7.
14. Nakayama K, Kanzaki A, Ogawa K, Miyazaki K, Neamati N, Takebayashi Y. Copper-transporting P-type adenosine triphosphatase (ATP7B) as a cisplatin-based chemoresistance marker in ovarian carcinoma: comparative analysis with expression of MDR1, MRP, LRP and BCRP. *Int J Cancer* 2002;101:488–95.
15. Ohbu M, Ogawa K, Konno S, et al. Copper-transporting P-type adenosine triphosphatase (ATP7B) is expressed in human gastric carcinoma. *Cancer Lett* 2003;189:33–8.
16. Miyashita H, Nitta Y, Mori S, et al. Expression of copper-transporting P-type adenosine triphosphatase (ATP7B) as a chemoresistance marker in human oral squamous cell carcinoma treated with cisplatin. *Oral Oncol* 2003;39:157–62.
17. Higashimoto M, Kanzaki A, Shimakawa T, et al. Expression of copper-transporting P-type adenosine triphosphatase in human esophageal carcinoma. *Int J Mol Med* 2003;11:337–41.
18. La Fontaine SL, Firth SD, Camakaris J, et al. Correction of the copper transport defect of Menkes patient fibroblasts by expression of the Menkes and Wilson ATPases. *J Biol Chem* 1998;273:31375–80.
19. Samimi G, Katano K, Holzer AK, Safaei R, Howell SB. Modulation of the cellular pharmacology of cisplatin and its analogs by the copper exporters ATP7A and ATP7B. *Mol Pharmacol*; in press; 2004.
20. Samimi G, Varki NM, Wilczynski S, Safaei R, Alberts DS, Howell SB. Increase in expression of the copper transporter ATP7A during platinum drug-based treatment is associated with poor survival in ovarian cancer patients. *Clin Cancer Res* 2003;9:5853–9.
21. Francis MJ, Jones EE, Levy ER, Ponnambalam S, Chelly J, Monaco AP. A Golgi localization signal identified in the Menkes recombinant protein. *Hum Mol Genet* 1998;7:1245–52.
22. Molenaar C, Teuben JM, Heetebrij RJ, Tanke HJ, Reedijk J. New insights in the cellular processing of platinum antitumor compounds, using fluorophore-labeled platinum complexes and digital fluorescence microscopy. *J Biol Inorg Chem* 2000;5:655–65.
23. Andrews PA, Jones JA, Varki NM, Howell SB. Rapid emergence of acquired cis-diamminedichloroplatinum(II) resistance in an in vivo model of human ovarian carcinoma. *Cancer Commun* 1990;2:93–100.
24. Camakaris J, Petris MJ, Bailey L, et al. Gene amplification of the Menkes (MNK; ATP7A) P-type ATPase gene of CHO cells is associated with copper resistance and enhanced copper efflux. *Hum Mol Genet* 1995;4:2117–23.
25. Katano K, Safaei R, Samimi G, et al. Confocal microscopic analysis of the interaction between cisplatin and the copper transporter ATP7B in human ovarian carcinoma cells. *Clin Cancer Res*; in press; 2004.
26. Johnson SW, Laub PB, Beesley JS, Ozols RF, Hamilton TC. 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.
27. Mishima M, Samimi G, Kondo A, Lin X, Howell SB. The cellular pharmacology of oxaliplatin resistance. *Eur J Cancer* 2002;38:1405–12.
28. Wang K, Lu J, Li R. The events that occur when cisplatin encounters cells. *Coord Chem Rev* 1996;151:53–88.