Copper-Transporting P-Type ATPase, ATP7A, Confers Multidrug Resistance and Its Expression Is Related to Resistance to SN-38 in Clinical Colon Cancer

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Abstract

We and others have shown that the copper transporters ATP7A and ATP7B play a role in cellular resistance to cis-diaminedichloroplatinum (II) (CDDP). In this study, we found that ATP7A transfection of Chinese hamster ovary cells (CHO-K1) and fibroblasts isolated from Menkes disease patients enhanced resistance not only to CDDP but also to various anticancer drugs, such as vincristine, paclitaxel, 7-ethyl-10-hydroxy-camptothecin (SN-38), etoposide, doxorubicin, mitoxantron, and 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11). ATP7A preferentially localized doxorubicin fluorescence to the Golgi apparatus in contrast to the more intense nuclear staining of doxorubicin in the parental cells. Brefeldin A partially and monensin completely altered the distribution of doxorubicin to the nuclei in the ATP7A-expressing cells. ATP7A expression also enhanced the efflux rates of doxorubicin and SN-38 from cells and increased the uptake of SN-38 in membrane vesicles. These findings strongly suggested that ATP7A confers multidrug resistance to the cells by compartmentalizing drugs in the Golgi apparatus and by enhancing efflux of these drugs, and the trans-Golgi network has an important role in ATP7A-related drug resistance. ATP7A was expressed in 8 of 34 (23.5%) clinical colon cancer specimens but not in the adjacent normal epithelium. Using the histoculture drug response assay that is useful for the prediction of drug sensitivity of clinical cancers, ATP7A-expressing colon cancer cells were significantly more resistant to SN-38 than ATP7A-negative cells. Thus, ATP7A confers resistance to various anticancer agents on cancer cells and might be a good index of drug resistance in clinical colon cancers. [Cancer Res 2007;67(10):4860–8]

Introduction

Drug resistance in cancer cells is a major obstacle for cancer chemotherapy. We have previously shown that cells expressing the ATP7B copper transporter are resistant to cis-diaminedichloroplatinum (II) (CDDP) and in several heavy metals (1). ATP7A, a second copper transporter, has also been implicated in CDDP resistance (2, 3). ATP7B is a P-type ATPase copper transporter, and a functional deficit of this transporter causes Wilson disease (4, 5). ATP7A has 67% amino acid identity to the ATP7B protein (6) and sequence similarity to the bacterial proteins ZntA and CopB, which confer resistance to heavy metals, including lead, zinc, cadmium, and silver (7). Copper-resistant Chinese hamster ovary (CHO) variant cells displayed a higher expression of ATP7A, amplification of the ATP7A gene, and a higher efflux rate of copper than the parental CHO cells (8). A role for ATP7A in copper metabolism in humans has been suggested by the discovery that ATP7A is the causative gene of Menkes disease (4–6).

The major biochemical defect in Menkes disease is caused by copper deficiency and results in death with progressive neurodegeneration and connective tissue disturbance in early childhood. ATP7A seems to play an important role in the transport of copper from the intestine to serum. Copper is an essential heavy metal required for several enzymes, including cytchrome c oxidase, superoxide dismutase, and lysyl oxidase (4, 5).

In this study, we provide evidence that ATP7A is involved in resistance to various anticancer agents. Furthermore, we explored mechanisms by which ATP7A might confer multidrug resistance (MDR) and determined the correlation between ATP7A expression and SN-38 resistance in clinical colon cancer cells with an ex vivo drug resistance assay, histoculture drug response assay (HDRA).

Materials and Methods

Chemicals. Commercially obtained reagents were [14C]doxorubicin (1.85 GBq/mmol; Amersham Biosciences); paclitaxel (Taxol), vincristine, doxorubicin, etoposide (VP-16), mitoxantron, CDDP, brefeldin A, and monensin (Sigma Chemical Co.; CuCl2, Wako Pure Chemical Industries Ltd.); and N-[6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]propyl]sphingosine (C2r-NBD-ceramide; Invitrogen), SN-38 and CPT-11 (Daichi Pharmaceutical Co. Ltd. and Yakult Pharmaceutical Co., respectively) were kind donations.

Cells and antibodies. ATP7A cDNA-transfected CHO-K1 cells were CHO/pCMB117, previously described as clone 600-5#3 (9), and CHO/615D.7

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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MC32a-T22/L is an immortalized fibroblast from a Menkes disease patient and MC32a/pCMB117 is an ATP7A-transfected MC32a-T22/L clone, previously described as clone A12-H9 (10). All cells were cultured in DMEM (Nissui Sefyaku Co.) containing 20 mmol/L 1-proline, 50 μmol/L uridine, 1 mmol/L sodium pyruvate, 20 mmol/L HEPES (pH 7.5), and 10% FCS. KB-C2 and KB/MRP cells were cultured as described previously (11, 12). MCF-7/AdVp3000 cells were kindly presented from Dr. Susan Bates and cultured in Iscove’s modified Eagle medium containing 10% FCS with 3,000 ng/mL doxorubicin and 5 μg/mL verapamil (13).

A sheep antibody against human ATP7A was generated using a protocol described for generation of the rabbit anti-ATP7A and was partially purified by ammonium sulfate precipitation (9). Commercially obtained antibodies were mouse monoclonal anti-P-glycoprotein (P-gp; C219; Zymed Laboratories, Inc.), anti-MDR protein 1 (MRP1; MRPm6; Progen Biotechnik GmbH), anti-MRP4 (M14; Alexis Biochemicals), horseradish peroxidase (HRP)-conjugated anti-sheep antibody (ICN), and HRP-conjugated anti-mouse and anti-rabbit antibodies (Amersham Biosciences). Rabbit anti-breast cancer resistance protein (BCRP) antibody was kindly donated by Dr. Yoshikazu Sugimoto (Kyoritsu University of Pharmaceutical, Tokyo, Japan; ref. 14).

Patient samples. This study was undertaken with the informed consent of the patients in accordance with the regulations of the Ethics Review Board of Kagoshima University (Kagoshima, Japan). The study involved 50 patients with advanced colon cancer who underwent surgical therapy without chemotherapy at Kagoshima University Hospital between July 2002 and April 2004. The specimens contained moderate to well-differentiated adenocarcinomas and primary or metastatic tumors. Thirty-four of 50 specimens were suitable for evaluation of the sensitivity of the cells to adenocarcinomas and primary or metastatic tumors. Thirty-four of 50 specimens were suitable for evaluation of the sensitivity of the cells to adenocarcinomas and primary or metastatic tumors.

Chemosensitivity assay. Chemosensitivity was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using 96-well plates and 2 × 104 cells. Cells were incubated with 20 μM doxorubicin in DMEM/10% FCS for 30 min and 2 μg/mL Hoechst 33342 was added to the medium and cells were incubated for 30 min more. After washing with PBS, the cells were observed with confocal laser microscopy (FV5000, Olympus Corp.).

Cell survival and chemosensitivity assay. Chemosensitivity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using 96-well plates and 2 × 104 CHO-K1, 3 × 105 Me32a/pCMB117, and 5 × 105 each CHO/pCMB117, CHO/615D, and Me32a-T22/2L cells per well as described previously (1).

Preparation of membrane vesicles. Membrane vesicles were prepared as described previously and stored at −80°C (1, 15). Protein concentration was determined by the Bio-Rad protein assay kit according to the manufacturer’s protocol (Bio-Rad Laboratories).

Immunoblot analyses. Membrane vesicles from the indicated cells (100 μg) or from positive control cells (20 μg) were subjected to 7.5% SDS-PAGE under reducing conditions. Immunoblotting was carried out as described previously (1). All first antibodies were used at a thousand-fold dilution, and the appropriate HRP-conjugated species-specific antibody was diluted, and the appropriate HRP-conjugated species-specific antibody was used as the second antibody. Immunoreactive bands were visualized with the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences). The intensities of fluorescent bands of ATP7A or MRP4 were quantified using ChemiDoc XRS System and Quality One 1D Analysis software (Bio-Rad Laboratories).

Semiquantitative reverse transcription-PCR and PCR of genomic DNA. RNA was isolated with Trizol (Invitrogen), first-strand cDNA was synthesized from 1 μg of total RNA using the Rever Tra Ace-α kit (Toyobo), and genomic DNA was isolated with a Genomic DNA Extraction Kit (Stratagene) according to the manufacturers’ instructions. The following PCR primers were used. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the primers were supplied with the Rever Tra Ace-α kit. For MRP1, a forward primer was designated as 5′-CACAGTGGG-GATGCTGGCCAGCAACAC-3′ and a reverse primer as 5′-TATGGCCATT-CACAGTGGG-GATGCTGGCCAGCAACAC-3′. For GAPDH, the primers were supplied with the Rever Tra Ace-α kit. For MRP1, a forward primer was designated as 5′-CACAGTGGG-GATGCTGGCCAGCAACAC-3′ and a reverse primer as 5′-TATGGCCATT-CACAGTGGG-GATGCTGGCCAGCAACAC-3′. Based on mouse genomic DNA. PCR primers were designed based on mouse BCRP mRNA and genomic DNA sequences because no sequence was available for hamster. A forward primer was designated in exon 4 as 5′-TATGGCCATT-CACAGTGGG-GATGCTGGCCAGCAACAC-3′ and a reverse primer in exon 5 as 5′-TATCCAAAGGTATGGTGGCTGGAAAGAAG-3′. All PCRs were carried out in a reaction volume of 20 μL. Individual PCR conditions were the following: PCR of GAPDH was according to the manufacturer’s protocol. PCR of cDNA used one fifth of the reverse transcriptase product, Premix ExTaq (TaKaRa Bio, Inc.), and denaturation at 97°C for 1 min followed by 25 cycles of 95°C/30 s, 65°C/1 min, and 72°C/1 min. PCR of 50 ng genomic DNA was carried out using KOD polymerase (Toyobo) with denaturation at 95°C for 5 min followed by 30 cycles of 95°C/30 s, 65°C/10 s, and 74°C/10 s. PCR of BCRP used one fifth of the reverse transcriptase cDNA, KOD polymerase, and initial reaction conditions of 30 cycles of 95°C/20 s, 55°C/10 s, and 74°C/1 s. Tests were then carried out in which the annealing temperature was varied between 50°C and 65°C. As a positive control, PCR of 50 ng genomic DNA was carried out with KOD polymerase and PCR conditions of denaturation at 95°C for 5 min followed by 30 cycles of 95°C/30 s, 65°C/19 s, and 74°C/5 s. PCR fragments were sequenced using the appropriate primer set to confirm that the band was the target DNA. The corresponding mouse genomic DNA contains a rather short intron, 627 bp in MRP1 and 545 bp in BCRP, between each set of primers.

Intracellular localization of doxorubicin. MC32a-T22/L2 and MC32a/pCMB117 cells (4 × 104) were plated. Following washing with PBS, cells were incubated with 20 μM doxorubicin in DMEM/10% FCS for 30 min and 2 μg/mL Hoechst 33342 was added to the medium and cells were incubated for 30 min more. After washing with PBS, the cells were observed with confocal laser microscopy (FV5000, Olympus Corp.).

To verify the intracellular localization of doxorubicin, the cells were incubated with 30 μM doxorubicin for 1 h, washed with PBS, and incubated with 5 μM/L of the Golgi marker C2-NBD-ceramide in PBS at 4°C for 30 min (16). Following rinsing with ice-cold DMEM, the cells were incubated with 37°C for 30 min in fresh medium, washed with PBS, and observed with confocal laser microscopy (LeicaTCS4D, Leica; ref. 17). To assess the effect of brefeldin A or monensin, CHO-K1 and CHO/pCMB117 cells were incubated with 30 μM doxorubicin in DMEM/10% FCS and for 30 min at 37°C in the presence of 5 μM/L brefeldin A or 3 μM/L monensin. After washing with PBS, the cells were incubated with 5 μM/L C2-NBD-ceramide and observed with the confocal laser microscopy (LeicaTCS4D).

Quantification of doxorubicin and SN-38 accumulation in parental and ATP7A-expressing cells. To measure doxorubicin accumulation, subconfluent cells in 12-well plates were incubated with 30 μM/L cold and 0.1 μM/L [14C]doxorubicin for 1 h at 37°C. Following washing with ice-cold PBS, the cells were solubilized in 10 mmol/L phosphate buffer (pH 7.4) containing 1% Triton X-100 and 0.2% SDS and the incorporated radioactivity was determined with a liquid scintillation counter (15). To assess the effect of ATP deficient on doxorubicin accumulation, the cells were preincubated with 1 μM/L 2,4-dinitrophenol (DNP) in PBS for 30 min and then incubated with the doxorubicin-containing medium in the continued presence of 1 μM/L DNP for 1 h. The incorporated radioactivity was then determined as described above.

To measure intracellular SN-38 accumulation, 2.0 × 105 CHO-K1 cells and 3.0 × 105 CHO/pCMB117 cells were plated on a 60-mm dish. In the next day, the cells were incubated with 1 μM/L SN-38 in fresh medium for 1 h. Following washing with ice-cold PBS, the cells were harvested with a rubber scraper, washed with cold PBS, and counted with a hemocytometer. Methanol (1 mL) was added before homogenization with five strokes of a syringe with a 27-gauge needle. Insoluble debris was removed by centrifugation. The supernatant was evaporated with a concentrator. The dried pellets were resolved with 75 μL distilled water and 150 μL acetonitrile and analyzed by high-pressure liquid chromatography (HPLC) using a modification of a previously described procedure (18).

Measurement of efflux of doxorubicin and SN-38 in the parental and ATP7A-expressing cells. To estimate the efflux rate of doxorubicin, subconfluent cells in 12-well plates were incubated in fresh DMEM containing 10% FCS for 1 h and then with 30 μM/L cold plus 0.1 μM/L [14C]doxorubicin for 1 h at 37°C. After washing with PBS, the cells were incubated with medium without drugs for the indicated times at 37°C. The radioactivity of solubilized cells was determined as described above.

To examine the efflux rate of SN-38, 3.0 × 105 CHO-K1 and CHO/pCMB117 cells in six-well plates were incubated in 5 mL medium for 24 h. After washing with PBS, 4 μM/L for CHO-K1 cells and 7 μM/L for CHO/
pCMB117 cells, SN-38 was added to the medium for 1 h followed by washing with PBS. The cells were further incubated in fresh medium without SN-38 for the indicated times at 37°C and the harvested cells were counted. The SN-38 content of the cells was determined as described above.

Vesicle uptake of SN-38. Vesicle uptake of SN-38 was measured by a rapid filtration technique as described previously (18, 19). Non-specific binding of drugs to the filters was determined in the absence of membrane vesicles. Values for membrane vesicle uptake were obtained by subtracting the solution was read at 540 nm. The inhibitory index was calculated according to the following formula: inhibitory index (%) = (1 – mean absorbance of treated tumor / g) / mean absorbance of untreated tumor / g.

**Results**

Expression of ATP7A in ATP7A-transfected and parental cells. To examine the ability of ATP7A to modulate cellular resistance to anticancer agents as well as to copper, we used CHO-K1 cells and established fibroblasts from Menkes disease patients that had been transfected with ATP7A (9, 10). We first compared the protein expression of ATP7A by immunoblotting. All of the transfected cells (i.e., CHO/615D, CHO/pCMB117, and Me32a/pCMB117) expressed ATP7A, whereas weak or no ATP7A was detectable in the corresponding parental cells. Among the transfected cells, CHO/pCMB117 cells exhibited the highest expression of ATP7A (Fig. 1A).

**Effects of ATP7A on cellular resistance to anticancer drugs.** We next determined whether transfected ATP7A could confer resistance to copper or CDDP toxicity using the cell survival MTT assay. CHO/615D and Me32a/pCMB117 cells were much less resistant to copper [relative resistances (RR) of 0.95 and 1.28, respectively] than to CDDP (RRs of 2.4 and 2.4, respectively; Table 1). These data are consistent with previous reports that cells that express ATP7A at a moderate level are resistant to CDDP but not to copper (8, 22). In contrast, CHO/pCMB117, which expresses a high level of ATP7A, showed a higher resistance to copper (RR, 3.85) than to CDDP (RR, 2.5; Table 1).

To examine if ATP7A might also confer resistance to anticancer agents, we compared the sensitivity of ATP7A-expressing cells and their respective parental cells to anticancer agents using the MTT assay. Unexpectedly, all of the ATP7A-expressing cells were highly resistant to SN-38, Taxol, and vincristine. For example, CHO/pCMB117 cells showed a 147.31-, 324.95-, and 678.37-fold enhanced resistance to these agents compared with the parental cells. ATP7A-expressing cells were also moderately resistant to VP-16 and doxorubicin (20.17- and 10.30-fold enhanced resistance in CHO/pCMB117 cells) and were also resistant to CPT-11 and HDRA, ex vivo drug resistance assay. HDRA was done by SRL, Inc. as described previously (21). Briefly, four pieces of minced tumors ( 0.5 mm in diameter) were placed on a collagen sponge gel in a 24-well plate and incubated with RPMI 1640 containing 20% FCS for 7 days in the presence or absence of 20 μg/mL CDDP or 0.4 μg/mL SN-38. After adding HBSS containing collagenase and 5 mg/mL MTT in PBS to each well, the plates were incubated for 8 h. Following extraction with DMSO, the absorbance of the solution was read at 540 nm. The inhibitory index was calculated according to the following formula: inhibitory index (%) = (1 – mean absorbance of treated tumor / g) / mean absorbance of untreated tumor / g.

Statistical analysis. Differences between groups were analyzed by the Student’s t test. A P value of <0.05 was considered to be significant. Significance levels given are those for the two-tailed Student’s t test.

**Figure 1.** Expression of transporter proteins and mRNA in ATP7A-expressing cells. A, membrane vesicles (100 μg) of CHO-K1, CHO/pCMB117, CHO/615D, Me32a-T22/2L, and Me32a/pCMB117 cells and membrane vesicles (100 μg) of each control cells (right lane) were separated by 7.5% SDS-PAGE, and ATP7A, P-gp, MRP1, BCRP, and MRP4 proteins were detected with specific antibodies. Positive control cells were KB-C2, KB/MP, and MCF-7/AdVp3000 cells for P-gp, MRP1, and BCRP, respectively. Quantified indices of ATP7A and MRP4 expression level were indicated under each band. B, mRNA expression of MRP1 (top), BCRP (middle), and GAPDH used as a control (bottom) was measured by RT-PCR. Amplification of cDNA and genomic DNA was carried out as described in Materials and Methods and detected the bands of 250 bp for BCRP cDNA, 900 bp for genomic MRP1, and 700 bp for genomic BCRP. An amplified band using human BCRP cDNA as template with the same primers and the representative condition. The sequence of the 203-bp amplified cDNA and genomic DNA of MRP1 was completely identical to those in the hamster database. Although the hamster sequence of BCRP was not in the database, the sequence of the probe-encoding regions of the genomic hamster DNA was 89% identical to those of mouse BCRP (data not shown).
mitoxantron (4.69- and 10.3-fold enhanced resistance in CHO/pCMB117 cells; Table 1). For all drugs tested, the RR in CHO/pCMB117 cells was higher than that measured in CHO/615D cells. Because CHO/pCMB117 cells express the highest ATP7A protein level, this suggests a correlation between ATP7A expression and drug resistance.

**Expression of transporter proteins in CHO and Me32a cells.** To ensure that the expression of other drug transporters did not contribute to the observed resistance to drugs in these cells, we examined the expression level of P-gp, MRP1, and BCRP by immunoblotting. Neither P-gp, BCRP, nor MRP1 was detected in these cells by immunoblotting, although high levels of these proteins could be detected in control cells (Fig. 1A). Because it has been recently reported that MRP4 is related to resistance to CPT-11 (23), we also examined the expression of MRP4 and found it to be expressed at a similar level in all the cells tested.

In case that our inability to detect MRP1 or BCRP at the protein level might be due to an inability of our antibodies to cross-react with hamster proteins, we further examined the mRNA expression level of MRP1 and BCRP in CHO cells with semiquantitative reverse transcription-PCR (RT-PCR). *MRP1* mRNA expression was slightly higher in the parental cells than in the ATP7A-transfected cells (Fig. 1B). *BCRP* mRNA was not detected in any of the cells, although we were able to amplify *BCRP* genomic DNAs with these primers. Thus, the level of expression of these well-known MDR transporters in the *ATP7A*-transfected cells is unlikely to contribute to drug resistance.

**Doxorubicin distribution in Me32a-T22/L2 and Me32a/pCMB117 cells.** Because ATP7A seemed to play a role in drug resistance, we next analyzed potential mechanism(s) by which ATP7A might modulate drug resistance. It has previously been shown that the cytotoxicity of anticancer agents depends on their subcellular localization. We therefore determined if the expression of ATP7A could alter the intracellular distribution of anticancer drugs using doxorubicin as a model system. Doxorubicin has an intrinsic red fluorescence that facilitates observation of its cellular localization. We therefore treated Me32a/pCMB117 or the parental Me32a-T22/L2 cells with doxorubicin and then observed its intracellular distribution with confocal laser fluorescence microscopy. In Me32a-T22/L2 cells, doxorubicin fluorescence was localized predominantly in the nuclei (Fig. 2A, top). In contrast, in the ATP7A-expressing cells, doxorubicin was detected as a punctate cytoplasmic distribution of fluorescence around the nuclei, suggesting that ATP7A could alter the intracellular localization of doxorubicin (Fig. 2A, bottom). Because ATP7A is localized mainly in the Golgi membrane, it seemed likely that the punctate distribution of doxorubicin that we observed in the ATP7A-expressing cells reflected colocalization of doxorubicin and ATP7A in the Golgi.

We tested this hypothesis by using C6-NBD-ceramide as a marker for the Golgi apparatus. In CHO/pCMB117 and Me32a/pCMB117 cells, the fluorescence of doxorubicin and C6-NBD-ceramide was clearly colocalized (Fig. 2B, *f* and *l*), whereas in CHO-K1 and Me32a-T22/L2 cells (Fig. 2B, *c* and *i*) it was not. Therefore, one mechanism by which ATP7A modulates drug resistance seems to be by relocation of the drug away from the nucleus, which would limit its cytotoxicity.

**Effect of brefeldin A and monensin on the intracellular localization of doxorubicin in CHO/pCMB117 cells.** To assess the role of trans-Golgi network of anticancer agents in ATP7A-expressing cells, we observed the effects of brefeldin A and monensin on the doxorubicin localization in CHO/pCMB117 cells. Brefeldin A partially and monensin almost completely change the doxorubicin distribution from the Golgi to the nuclei (Fig. 2B, *c* and *i*). These data strongly suggested that the function of trans-Golgi network is involved in the doxorubicin localization in the Golgi of ATP7A-expressing cells.

**Effect of ATP7A on the intracellular accumulation and efflux of doxorubicin and SN-38.** A second mechanism by which ATP7A might mediate drug resistance is by an effect on the intracellular accumulation and efflux of the drug. To explore this possibility, the effect of ATP7A on cellular accumulation of doxorubicin and SN-38 was examined. CHO-K1 and CHO/pCMB117 cells were incubated in medium containing [14C]doxorubicin or SN-38 for 60 min and the level of doxorubicin or SN-38 in cell lysates was then measured.

Intracellular amounts of doxorubicin (166.9 nmol/L/10⁴ cells) and SN-38 (79.5 ng/10⁵ cells) in CHO/pCMB117 cells were

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**Table 1. Drug and metal resistance in ATP7A-expressing cells**

<table>
<thead>
<tr>
<th>Agent</th>
<th>CHO-K1 IC₅₀</th>
<th>CHO/615D IC₅₀</th>
<th>CHO/pCMB117 IC₅₀</th>
<th>Me32a-T22/2L IC₅₀</th>
<th>Me32a/pCMB117 IC₅₀</th>
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</thead>
<tbody>
<tr>
<td>SN-38</td>
<td>0.100 ± 0.025</td>
<td>0.587 ± 0.62</td>
<td>14.78 ± 1.60</td>
<td>0.028 ± 0.003</td>
<td>1.20 ± 0.44</td>
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<tr>
<td>CPT-11</td>
<td>42.69 ± 12.47</td>
<td>108.95 ± 14.47</td>
<td>200.34 ± 10.87</td>
<td>6.166 ± 1.134</td>
<td>81.03 ± 20.03</td>
</tr>
<tr>
<td>Taxol</td>
<td>0.054 ± 0.008</td>
<td>0.80 ± 0.057</td>
<td>14.6 ± 17.83</td>
<td>0.0030 ± 0.0005</td>
<td>0.028 ± 0.0080</td>
</tr>
<tr>
<td>Vincriistine</td>
<td>0.077 ± 0.012</td>
<td>0.280 ± 0.010</td>
<td>1.08 ± 0.01</td>
<td>0.0043 ± 0.00084</td>
<td>0.30 ± 0.185</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.306 ± 0.070</td>
<td>6.74 ± 0.73</td>
<td>19.27 ± 1.72</td>
<td>1.107 ± 0.425</td>
<td>14.48 ± 7.76</td>
</tr>
<tr>
<td>VP-16</td>
<td>2.899 ± 0.389</td>
<td>23.59 ± 1.45</td>
<td>6.579 ± 0.00</td>
<td>2.095 ± 0.766</td>
<td>56.52 ± 7.51</td>
</tr>
<tr>
<td>Mitoxantron</td>
<td>1.912 ± 0.123</td>
<td>12.22 ± 0.40</td>
<td>19.68 ± 2.37</td>
<td>0.095 ± 0.0020</td>
<td>0.40 ± 0.03</td>
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<tr>
<td>CDDP</td>
<td>5.82 ± 0.26</td>
<td>13.70 ± 0.14</td>
<td>1.470 ± 0.76</td>
<td>6.56 ± 0.177</td>
<td>15.71 ± 0.71</td>
</tr>
<tr>
<td>Copper</td>
<td>0.074 ± 0.012</td>
<td>0.070 ± 0.0021</td>
<td>0.28 ± 0.06</td>
<td>0.130 ± 0.036</td>
<td>0.17 ± 0.02</td>
</tr>
</tbody>
</table>

*IC₅₀ values are means ± SDs from triplicate determinations with MTT assay.
† The value that IC₅₀ for CHO/pCMB117, CHO/615D, or Me32a/pCMB117 divided with IC₅₀ for CHO-K1 or Me32a-T22/2L cells, respectively.
‡ Indicates statistically significant (*P* < 0.05).
Figure 2. Effect of ATP7A expression on intracellular localization of doxorubicin. A, Me32a-T22/L2, parental cells, and Me32a/pCMB117, ATP7A-expressing cells, were incubated with 20 μmol/L doxorubicin (Dox) in DMEM/10% FCS for 30 min and 2 μg/mL Hoechst 33342 was added and cells were incubated for 30 min more. Doxorubicin (red; middle left), Hoechst 33342 (blue; left), and phase-contrast image (middle right) of the same field were merged (right). B, CHO-K1 (a–c), CHO/pCMB117 (d–f), Me32a-T22/L2 (g–i), and Me32a/pCMB117 (j–l) cells were incubated with 20 μmol/L doxorubicin (red; a, d, g, and j) and the Golgi marker, 5 μmol/L C6-NBD-ceramide (green; b, e, h, and k). Right, c, f, i, and l, merged images. C, CHO-K1 (top) and CHO/pCMB117 (bottom) cells were incubated with doxorubicin (red) and with the Golgi marker C6-NBD-ceramide (green) in the absence (left) and presence of brefeldin A (middle) or monensin (right), and the merged images are shown.
significantly lower than those (229.7 nmol/L/10⁴ cells and 151.5 ng/10² cells, respectively) in the parental CHO-K1 cells (Fig. 3A and B). This defect of doxorubicin accumulation was attenuated with 1 mmol/L DNP, an uncoupler of oxidative phosphorylation. These data strongly suggest that the decreased doxorubicin accumulation in CHO/pCMB117 cells is due to an ATP-dependent doxorubicin transporting activity of ATP7A.

We next determined if the decreased accumulation of doxorubicin and SN-38 in ATP7A-expressing cells was due to an enhanced active efflux of doxorubicin and SN-38. We therefore compared the efflux of [¹⁴C]doxorubicin or SN-38 in parental CHO-K1 and CHO/pCMB117 cells by measurement of the cellular level of doxorubicin or SN-38 over a period of 60 min. The efflux rate of doxorubicin and SN-38 from CHO/pCMB117 cells was enhanced compared with the efflux rate from CHO-K1 cells (Fig. 4A and B). Thus, at 60 min, only 48% of doxorubicin and 52.4% of SN-38 were retained within the ATP7A-expressing cells compared with 78.5% and 72.4%, respectively, retained in the parental CHO-K1 cells, suggesting that ATP7A could enhance the cellular efflux of the drugs.

Figure 3. Effect of ATP7A expression on intracellular accumulation of doxorubicin and SN-38. A, CHO-K1 (white columns) and CHO/pCMB117 (black columns) cells were preincubated with or without DNP for 30 min and then further incubated with [¹⁴C] and cold doxorubicin for 1 h. The radioactivity of solubilized cells was determined with a liquid scintillation counter. The results indicate the intracellular accumulation of doxorubicin (nmol/L/10⁴ cells). P = 0.0013 (a) and 0.0014 (b). Bars, SD. B, CHO-K1 (white column) and CHO/pCMB117 (black column) cells were incubated with 1 μmol/L SN-38 for 30 min, and SN-38 extracted from the cells was measured by HPLC. P = 0.012, the difference is significant.

Figure 4. Effect of ATP7A expression on doxorubicin and SN-38 efflux from cells and uptake of SN-38 by membrane vesicles expressing ATP7A. A, CHO-K1 (○) and CHO/pCMB117 (●) cells were incubated with 30 μmol/L doxorubicin for 1 h and then further incubated in doxorubicin-free medium for the indicated times. After washing with ice-cold PBS, the radioactivity of solubilized cells was determined with a liquid scintillation counter. The results are expressed as the percentage of doxorubicin accumulated intracellularly. P < 0.005, for all times. B, CHO-K1 and CHO/pCMB117 cells were incubated for 1 h with SN-38. The cells were washed thrice with PBS and further incubated in fresh medium without SN-38 for the indicated times. SN-38 was extracted from the cells as described in Materials and Methods. SN-38 was quantified by HPLC. The difference at 10, 20, and 60 min is significant (P values were 0.001, 0.001, and <0.0001, respectively). ○, CHO-K1 cells; ●, CHO/pCMB117 cells. Points, mean of triplicate determinations; bars, SD. C, uptake of SN-38 by membrane vesicles from CHO-K1 (white column) or CHO/pCMB117 (black columns) cells was examined over 15 min by a rapid filtration technique. Points, average of triplicate measurements following subtracting of the data in the absence of ATP from that in the presence of ATP; bars, SD. Uptake of SN-38 by membrane vesicles from CHO/pCMB117 was significantly higher at 15 min than that by membrane vesicles from CHO-K1 cells (P = 0.017).
We also examined that the accumulation and efflux of vincristine and Taxol with $[^{3}H]$vincristine and $[^{3}H]$Taxol; however, we did not find any significant differences of accumulation and efflux of these drugs between CHO-K1 and CHO/pCMB117 cells. These data indicate that the accumulation and efflux is not important for vincristine and Taxol resistance of CHO/pCMB117 cells.

Effect of ATP7A on SN-38 uptake by membrane vesicles. To determine if ATP7A had a direct effect on SN-38 transport, we examined SN-38 uptake by membrane vesicles from CHO/pCMB117 cells and compared it with that of CHO-K1 cells. Uptake of SN-38 by ATP7A-expressing membrane vesicles was 1.45-fold higher than that of the parental cells at 15 min, strongly suggesting that ATP7A is directly involved in SN-38 transport (Fig. 4C).

ATP7A is overexpressed in clinical tumors and its expression is related to SN-38 resistance. Because ATP7A seems to play a role in resistance to anticancer drugs, this raised the possibility that it might be a useful target for antitumor therapy. To determine if ATP7A does play a role in drug resistance in clinical cancer, we first evaluated the expression of ATP7A in clinical colon cancer specimens with an immunohistochemical method. ATP7A was detected in 8 of 34 (23.5%) tumors but not in adjacent normal epithelial cells (Supplementary Table S1; Supplementary Fig. S1A). These data are consistent with a previous report (2) and suggest that ATP7A might be aberrantly expressed in cancer cells. We next tested if ATP7A might correlate with drug resistance in these tumors. Because CPT-11 and CDDP are frequently used for colon cancer treatment, we examined drug resistance to these anticancer agents. The average inhibitory indices of ATP7A+ tumors treated with SN-38 (10.6%) were significantly lower than those of ATP7A- tumors (8.1%); $P = 0.009478), but expression of BCRP and MRP1 of the tumors had no relation with inhibitory indices (Supplementary Fig. S1B). Twelve cases of ATP7A+ tumors showed inhibitory indices that were $>50$%. In contrast, the inhibitory indices of ATP7A- tumors were $<20$% (Supplementary Table S1). On the other hand, the average inhibitory indices obtained following treatment with CDDP were similar between ATP7A+ (20.8%) and ATP7A- (27.0%) tumors (Supplementary Fig. S1B). Thus, ATP7A expression seems to contribute to specific drug resistance of clinical cancer specimens.

Discussion

This article sheds new light on the potential cellular functions of ATP7A. The lower cellular resistance to copper than to CDDP in cells that express ATP7A at a moderate level (CHO/615D and Me32a/pCMB117 cells) is consistent with previous studies (8, 9, 22). In our study, ATP7A-expressing cells were only slightly more resistant to CDDP than the parental cells. This is in contrast to a previous study in which expression of the ATP7A protein was increased in three CDDP-resistant ovarian carcinoma cell lines that were 5.7- to 8.1-fold more resistant to CDDP than the parental cells (3). These differences might reflect the different types of cells used in each study.

Unexpectedly, the CHO cells expressing ATP7A were highly resistant to several anticancer agents, such as SN-38, vincristine, and Taxol (Table 1). This resistance could not be attributed to the presence of other well-known MDR proteins, as we did not detect the expression of BCRP and P-gp and could not detect any difference of expression of MRP1 or MRP4 between the ATP7A-expressing cells and the parental cells (Fig. 1).

That the drug resistance observed in ATP7A-expressing cells is due to ATP7A is further suggested by the fact that the drug resistance profile of ATP7A-expressing cells differs from that of other drug-resistant, protein-expressing cells. Whereas ATP7A are highly resistant to both Taxol and SN-38, P-gp-expressing cells are not resistant to SN-38, and MRP1-expressing cells are only resistant to Taxol at a low level. BCRP-expressing cells are extremely resistant to mitoxantron but are only slightly resistant to Taxol (13). However, both CHO/pCMB117 and Me32a/pCMB117 cells were highly resistant to Taxol (RRs, 324.95 and 93.39, respectively) but only moderately resistant to mitoxantron (RRs, 10.30 and 4.16, respectively). Thus, ATP7A-expressing cells were resistant to various agents with different chemical structure and molecular targets that are different from the spectra of structures and targets reported for other MDR cells. These data indicate that the spectrum of anticancer agents to which ATP7A confers resistance is unique among MDR cells.

We also investigated potential mechanisms by which ATP7A might induce drug resistance. We showed that doxorubicin was preferentially localized to the Golgi apparatus in ATP7A-expressing cells instead of to the nuclei that is its localization in the parental cells (Fig. 2B). In addition, ATP7A expression led to increased uptake of SN-38 by membrane vesicles (Fig. 4C). These data suggest that these agents are transported to the Golgi apparatus together with ATP7A. Furthermore, they are consistent with previous studies of fluorescein-labeled cisplatin (F-DDP) in suggesting that compartmentalization of doxorubicin and SN-38 within the Golgi apparatus is one mechanism whereby ATP7A modulates drug resistance (22).

ATP7A has been implicated in the efflux of copper across the serosal membrane of gut epithelial cells to serum, and the intestinal cells of the Menkes disease patients show a decreased efflux of copper (4, 5). Our study provides further evidence for a role for ATP7A in the efflux of specific molecules from cells. Thus, the efflux rate of doxorubicin and SN-38 from the CHO/pCMB117 cells was enhanced compared with that from CHO-K1 cells (Fig. 4B), indicating that enhancement of drug efflux is one of the mechanisms whereby ATP7A modulates drug resistance. The lower rate of efflux mediated by ATP7A-expressing cells compared with that mediated by the plasma membrane transporters MRP1 and P-gp is likely due to the dependence of ATP7A-mediated transport on the vesicle transport system. It is still unclear whether all anticancer drugs are transported in a similar manner as copper by ATP7A. A second secretory pathway has been suggested to mediate the efflux of F-DDP and may also contribute to the efflux of SN-38 and doxorubicin (24).

CHO/pCMB117 cells were highly resistant to vincristine and Taxol; however, the accumulation and efflux of those agents did not increase. Because ATP7A-expressing CHO/pCMB117 cells have the well-developed Golgi apparatus as shown in Fig. 2B and Golgi apparatus is closely related to microtubules, the overexpression of ATP7A might have some effects on the microtubules, the target molecule of vincristine and Taxol, whereby ATP7A enhances drug resistance.

Brefeldin A partially and monensin almost completely change the distribution of doxorubicin in CHO/pCMB117 cells. These data
strongly suggested that the trans-Golgi network has an important role of ATP7A-related drug resistance. Brefeldin A disturbs the protein traffic from endoplasmic reticulum to the Golgi apparatus and changes the distribution of ATP7A to a juxtanuclear structure; however, its effect on the function of ATP7A is not clear (25). Monensin was reported to reorient the doxorubicin-resistant cells with disturbing of acidification of the organelles (26). Although vesicular acidification is an important factor of the drug resistance against weak base chemotherapeutic agents, the precise mechanisms to trap the drugs into the vesicles are unknown. ATP7A could be a candidate as an uptake transporter of these anticancer agents.

ATP7A is expressed in normal small intestinal cells but not in the colon. However, we found that 23.5% of clinical colon tumors aberrantly express ATP7A and that the expression of ATP7A in cancer cells is significantly correlated to the inhibitory index values obtained with HDRA. HDRA is a useful ex vivo drug sensitivity assay because of the good correlation between the inhibitory indices obtained with HDRA and the clinical outcome after chemotherapy (21, 27). Our findings strongly suggested that ATP7A expression is correlated to CPT-11 resistance, but not to CDDP resistance, in clinical cancer cells. This result partly accounts for the fact that ATP7A-expressing cells were more resistant to SN-38 than to CDDP in vitro. However, it does not necessarily mean that ATP7A is not important in the CDDP resistance of colon cancer cells because none of the ATP7A* tumors were judged to be sensitive (inhibitory index, >50%) to CDDP, whereas 3 cases of ATP7A* tumors out of 34 cases were sensitive to CDDP (Supplementary Table S1). Thus, ATP7A expression might be only one of several factors contributing to CDDP resistance in colon cancers. Evaluation of ATP7A expression in cancer cells may be a useful back-up method for the prediction of drug sensitivity to SN-38 because not all clinical samples are suitable for HDRA, which depends on the quality and quantity of the specimen. Furthermore, HDRA cannot be applied to retrospective studies. About CPT-11 resistance, we are now collecting more clinical specimens and investigating the expression of other resistance related transporters in colon cancers.

Recent evidence indicates that ATP7A is up-regulated in pancreatic cancer compared with chronic pancreatitis (28) and that ATP7A and the copper-containing enzyme lysyl oxidase are more highly expressed in invasive breast cancer cell lines than in noninvasive lines (29). Thus, ATP7A expression might be related to the malignant phenotype in addition to its role in MDR. A further follow-up study will be necessary to elucidate the answer to this question.

In summary, we found that ATP7A is important not only for copper homeostasis but also for drug resistance to anticancer agents. Because ATP7A is more broadly expressed than ATP7B, ATP7A expression could be a more common cause of drug resistance in clinical cancers than ATP7B. Furthermore, these findings suggested that ATP7A might have an important role in the pharmacokinetics of other agents beside anticancer drugs. Further study is needed to elucidate the correlation between ATP7A expression and clinical prognosis and the effects of ATP7A on the trans-Golgi network traffic and pharmacokinetics of other drugs.

**References**


ATP7A and Drug Resistance and Clinical Drug Resistance

In the article on ATP7A and drug resistance and clinical drug resistance in the May 15, 2007 issue of Cancer Research (1), there is an error in Table 1. The IC$_{50}$ value of CHO/pCMB117 against vincristine should be 52.58 ± 13.95.

Copper-Transporting P-Type ATPase, ATP7A, Confers Multidrug Resistance and Its Expression Is Related to Resistance to SN-38 in Clinical Colon Cancer

Satsuki Owatari, Satoshi Akune, Masaharu Komatsu, et al.

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