Copper Transport Systems are Involved in Multidrug Resistance and Drug Transport

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Abstract: Copper is an essential trace element and several copper containing proteins are indispensable for such processes as oxidative respiration, neural development and collagen remodeling. Copper metabolism is precisely regulated by several transporters and chaperone proteins. Copper Transport Protein 1 (CTR1) selectively uptakes copper into cells. Subsequently three chaperone proteins, HAH1 (human atx1 homologue 1), Cox17p and CCS (copper chaperone for superoxide dismutase) transport copper to the Golgi apparatus, mitochondria and copper/zinc superoxide dismutase respectively. Defects in the copper transporters ATP7A and ATP7B are responsible for Menkes disease and Wilson's disease respectively. These proteins transport copper *via* HAH1 to the Golgi apparatus to deliver copper to cuproenzymes. They also prevent cellular damage from an excess accumulation of copper by mediating the efflux of copper from the cell.

There is increasing evidence that copper transport mechanisms may play a role in drug resistance. We, and others, found that ATP7A and ATP7B are involved in drug resistance against the anti-tumor drug cis-diamminedichloroplatinum (II) (CDDP). A relationship between the expression of ATP7A or ATP7B in tumors and CDDP resistance is supported by clinical studies. In addition, the copper uptake transporter CTR1 has also been reported to play a role in CDDP sensitivity. Furthermore, we have recently found that the effect of ATP7A on drug resistance is not limited to CDDP. Using an *ex vivo* drug sensitivity assay, the histoculture drug response assay (HDRA), the expression of ATP7A in human surgically resected colon cancer cells correlated with sensitivity to 7-ethyl-10-hydroxy-camptothecin (SN-38). ATP7A-overexpressing cells are resistant to many anticancer drugs including SN-38, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11), vincristine, paclitaxel, etoposide, doxorubicin (Dox), and mitoxantron.

The mechanism by which ATP7A and copper metabolism modulate drug transport appears to involve modulation of drug cellular localization *via* modulation of the vesicle transport system. In ATP7A overexpressing cells, Dox accumulates in the Golgi apparatus. In contrast, in the parental cells, Dox is localized in the nuclei, where the target molecules of Dox, topoisomerase II and DNA, are found. Disruption of the intracellular vesicle transport system with monensin, a Na^+/H^+ ionophore, induced the relocalization of Dox from the Golgi apparatus to the nuclei in the ATP7A overexpressing cells. These data suggested that ATP7A-related drug transport is dependent on the vesicle transport system. Thus copper transport systems play important roles in drug transport as well as in copper metabolism. Components of copper metabolism are therefore likely to include target molecules for the modulation of drug potency of not only anti-cancer agents but also of other drugs.

Keywords: Copper transport, CTR1, ATP7A, ATP7B, CDDP, multidrug resistance, vesicle transport.

INTRODUCTION

The importance of drug transport systems in the human body has been recognized since the discovery of the ABCB1/P-glycoprotein (P-gp) which confers multidrug resistance (MDR) to cancer cells by mediating the efflux of anticancer agents. In humans about 450 influx and efflux transporter proteins have now been identified. Since it would be difficult to predict all of the substrates of each transporter protein and, furthermore, whether a transporter can transport specific chemical agents has to be determined experimentally, the effects of most of the known transporters on the distribution and dynamics of chemical agents remain to be clarified.

We found that a copper transporter on the Golgi membrane, ATP7B, plays a role in resistance to the anticancer drug cis-diaminedichloroplatinum (II) (CDDP). This was the first report that indicated a relationship between copper metabolism and drug resistance [1]. Since then other transporters, including the related ATP7A and a copper uptake transporter CTR1, have been reported to play a role in CDDP sensitivity or resistance [2-6].

Recently we reported that the expression of ATP7A is involved not only in cellular resistance to CDDP but also in resistance to several other anticancer drugs *in vitro*. Furthermore the clinical relevance of these findings was suggested by the fact that ATP7A expressing human colon cancer cells, isolated from surgically resected tumors, are more resistant to 7-ethyl-10-hydroxy-camptothecin (SN-38) than ATP7A negative cells in an *ex vivo* histoculture drug response assay (HDRA) [7]. The discovery of a link between copper transport, and resistance to a variety of anti-cancer drugs, was a breakthrough in understanding the mechanism of transport of a wide variety of chemical agents, indicating that these agents are transported within a copper vesicle transport system. Below we describe the new paradigm of copper transport as a mechanism for drug transport in human cells.

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1. COPPER METABOLISM

Copper is an essential trace element and copper is required for the function of specific proteins involved in processes such as oxidative respiration (cytochrome *c* oxidase), neural development and function (dopamine β-monooxygenase), collagen remodeling (lysyl oxidase (LOX)) and free radical defense (superoxide dismutase). However an excess amount of copper is highly toxic. The cell has developed a number of mechanisms to ensure that there is no accumulation of excess copper and copper metabolism is regulated by several transporters and chaperone proteins [8, 9]. The membrane protein, copper transport protein 1 (CTR1) selectively uptakes copper into cells following which three chaperone proteins, namely human atx1 homologue 1 (HAH1), Cox17p and copper chaperone for superoxide dismutase (CCS) transport copper to the Golgi apparatus, mitochondria and superoxide dismutase respectively (Fig. **1**). HAH1 has a single copper-binding motif (MT/HCXXC) (the so-called metal binding site (MBS)) and transfers copper to ATP7A and/or ATP7B through the MBS [10]. ATP7A and ATP7B both supply copper to the Golgi apparatus for the synthesis of copper-containing enzymes, as well as secrete excess copper out of the cells.

2. CTR1 AND CDDP RESISTANCE

Human CTR1 (hCTR1) is a 27 kDa protein that has 3 transmembrane domains and exists at the plasma membrane as a homomultimer. The human CTR1 protein forms a symmetric homotrimer with a putative pore whose diameter was tentatively estimated to be about 9 Å [11]. CTR family members have a number of methionine rich domains in the extracellular N-terminal region as well as within the second transmembrane domain, with the exception of *Saccharomyces cerevisiae* CTR-3 (ScCTR-3) that has methionine rich domains only within the second transmembrane region but not in the N-terminal region. The methionine-rich domains contain 2-3 methionine residues arranged in the format MXM or MXXXM where X is usually a hydrophobic amino acid. Copper binding induces hCTR1 endocytosis and degradation and the extracellular methionine-rich domain ^{40}MMMMPM^{45} that is close to the transmembrane region, functions as a high affinity copper sensing site (Fig. **2**). This site is required for endocytosis of hCTR1 in response to low copper concentrations. Site-directed mutagenesis of this methionine rich sequence demonstrated that Met43 and Met45 are critical for both copper sensing and endocytosis of hCTR1 [12] . These two methionine residues are also conserved in the corresponding region of ScCTR1 in which they are located about 20 amino acids upstream from the first transmembrane spanning domain. Mutation of Met127 in ScCTR1, which corresponds to Met45 in hCTR1, also abolishes the function of ScCTR1 as a copper transporter [12]. A second methionine cluster (MXXXM) in the second transmembrane domain is also conserved between hCTR1 (^{150}MLIFM^{154}) and ScCTR1 (^{256}MLATM^{260}) (Fig. **2**). This motif is essential for copper-stimulated endocytosis and degradation of CTR1 [13-15].

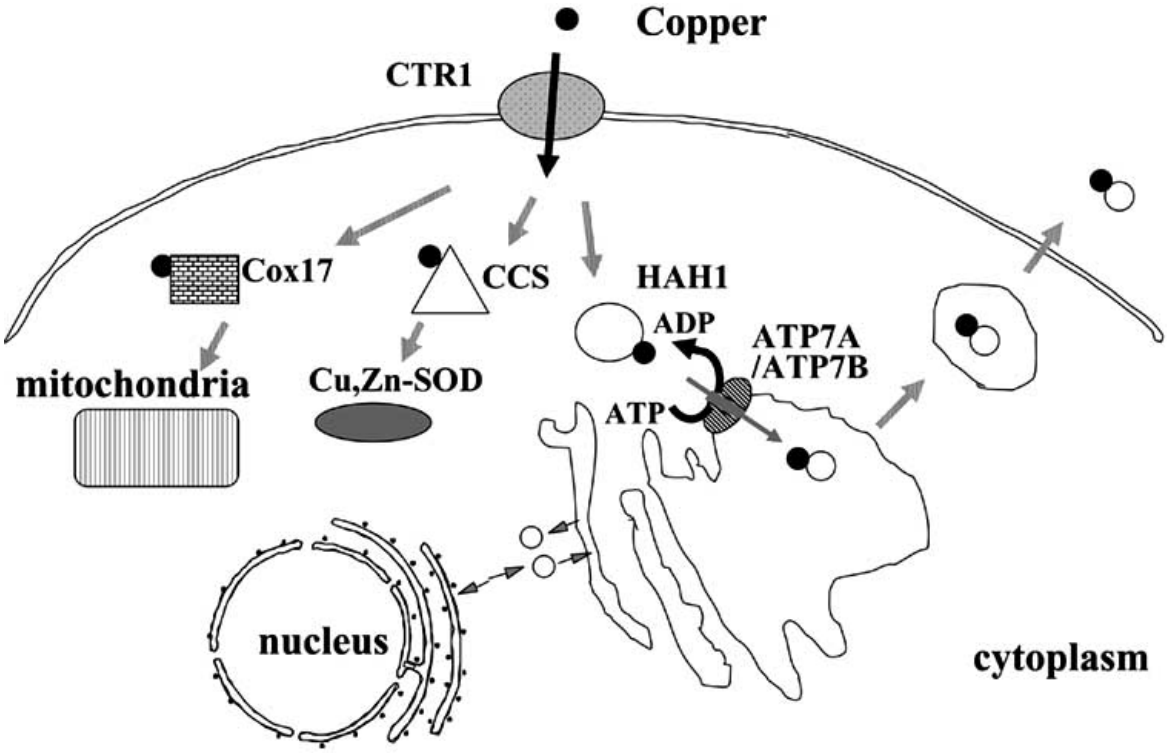


Fig. (1). Model of copper metabolism.

CTR1 selectively uptakes copper into cells. Three chaperone proteins, Cox17, CCS and HAH1 carry copper to the Golgi apparatus, mitochondria and superoxide dismutase respectively. HAH1 transfers copper to ATP7A and/or ATP7B. ATP7A and ATP7B supply copper to the Golgi apparatus for the synthesis of copper-containing enzymes and also secrete excess copper out of the cells. ATP7A is normally expressed in endometrium, prostate, testis, kidney and intestine. ATP7B is expressed primarily in the liver but also in the kidney and placenta.

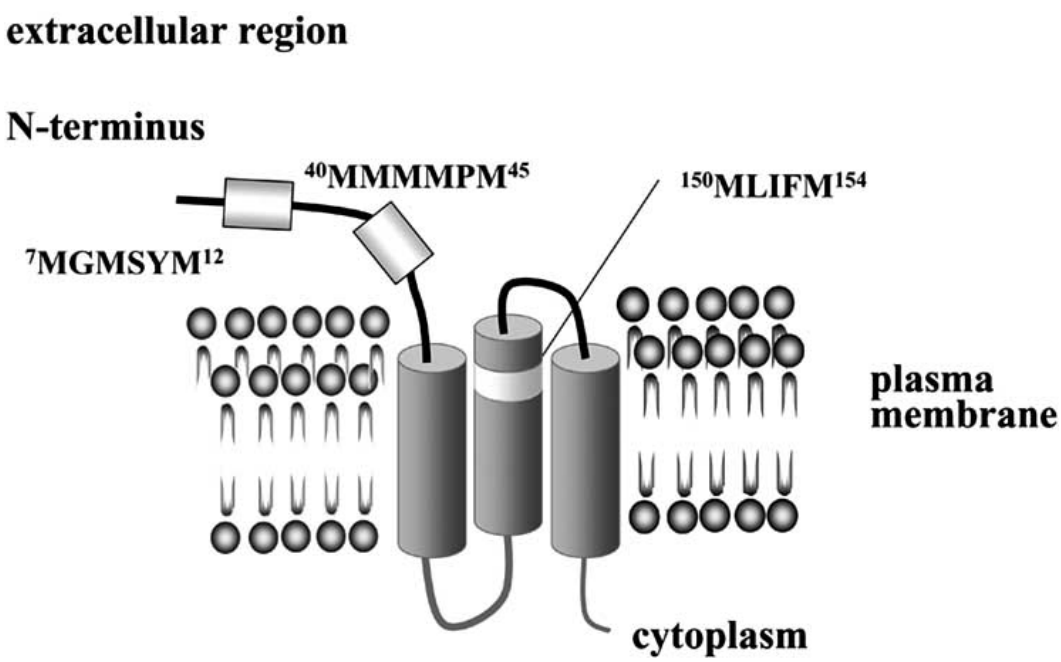


Fig. (2). Structure of human CTR1.

Human CTR1 (hCTR1) is a 27 kDa protein with 3 transmembrane domains and exists at the plasma membrane as a symmetric homotrimer with a putative pore whose diameter was tentatively estimated to be about 9 Å. Human CTR1 has three methionine rich domains, two of which are ^{7}MGMSYM^{12} and ^{40}MMMMPM^{45}in the extracellular N-terminal region and the third one is ^{150}MLIFM^{154} within the second transmembrane region. The second methionine-rich domain ^{40}MMMMPM^{45} functions as a high affinity copper sensing site. The Methionine 43 and Met45 are critical for both copper sensing and endocytosis of hCTR1. The third methionine cluster (MXXXM) in the second transmembrane domain is well conserved among CTR family proteins. This motif is also essential for copper-stimulated endocytosis and degradation of hCTR1.

CDDP is an anticancer drug composed of a neutral, square planar platinum (II) complex containing two chloride ligands oriented in a *cis* configuration. CDDP is one of the most effective chemotherapeutic agents for treating many malignancies, particularly for head and neck, testicular, ovarian, bladder, esophageal, and small cell lung cancers. However, intrinsic or acquired resistance to CDDP reduces its efficacy and this is a major obstacle in its use for chemotherapy. Multiple mechanisms of resistance against CDDP have been reported and include inactivation of the drug by thiol compounds, accelerated DNA repair and an increase in cellular metallothionein [16-19]. Proteins believed to modulate CDDP resistance include ATP binding cassette transmembrane transporters such as ABCC2/multidrug resistance protein 2 (ABCC2/MRP2), pH regulatory ATPases, vacuolar H^+-ATPase (V-ATPase) and Na^+/K^+ ATPase [20-23].

It has been reported that CDDP induces the internalization and degradation of CTR1. Indeed, CTR1 is a major pathway for the uptake of CDDP into cells and plays a role in CDDP cytotoxicity [24-28]. However, the mechanisms by which CDDP or copper induce CTR1 internalization, and by which CTR1 uptakes CDDP and copper into cells, is still controversial. It has been proposed that CDDP induces the formation of a hCTR1 multimer by binding to the hCTR1-methionine rich domains and that hCTR1 is subsequently internalized by micropinocytosis and degraded in the proteasome [29, 30].

Recently, fluorescence resonance energy transfer (FRET) analysis of labeled ScCTR1 showed that copper, but not CDDP could enhance the FRET signal from ScCTR1 and induce multimerization of ScCTR1. Mutation of the conserved methionine 127 of ScCTR1 to alanine abolished both copper-induced FRET as well as copper uptake but not the uptake of CDDP [31]. These data indicated that, although ScCTR1 can uptake both copper and CDDP, the mechanism of uptake for copper and CDDP is distinct. Thus it was suggested that, while copper can induce a conformational change in ScCTR1 leading to copper internalization, CDDP binding does not induce a conformational change but is internalized by other mechanisms, perhaps simply by co-internalization of CDDP bound to ScCTR1.

To date two different mechanisms of copper uptake by CTR1 have been proposed. One group have suggested that copper passes through a pore-like channel formed by CTR1 multimerization [11], whereas a second group suggests that copper induces a structural change in CTR1 leading to copper transport by a mechanism similar to that of drug transporters [31]. It is possible that both studies are correct and that copper might pass through a hCTR1 pore together with the induction of some structural change. Alternatively the different mechanisms may reflect species-specific differences between human and yeast CTR1 function.

Whatever the mechanism by which CDDP is transported, the *in vitro* studies described above support the view that CTR1 mediates the uptake of CDDP into cells and that the expression of CTR1 plays a role in cellular sensitivity to CDDP. These data suggest that the expression of CTR1 might be a major factor modulating CDDP sensitivity in

clinical cancers. However, no clinical evidence implying a relationship between CTR1 expression and CDDP sensitivity in human cancer samples has been reported. If the major mechanism of CDDP resistance is transient internalization and degradation of CTR1, rather than regulation of the expression of the CTR1 protein, it might be technically difficult to detect a difference in CTR1 expression in clinical samples.

3. PHYSIOLOGICAL FUNCTION OF ATP7A AND ATP7B

Defective ATP7A and ATP7B genes are the underlying cause of Menkes and Wilson's disease respectively [32-36]. These P-type ATPase Cu transporters exist at the Golgi membrane and share 67% amino acid identity. Both molecules have 6 MBSs in the N-terminal cytoplasmic region, 8 membrane spanning segments, a phosphatase domain in the 3rd cytoplasmic loop and a phosphorylation domain (DKTG), and an ATP binding domain, in the 4th cytoplasmic loop (Fig. **3**) [8]. ATP7A is expressed in the majority of tissues except for the liver, and ATP7B is expressed primarily in the liver but also in the kidney and placenta. ATP7A effluxes copper from enterocytes into the portal circulation. The liver is a center of copper homeostasis and most of the newly absorbed copper from the small intestine goes into this organ. In the liver, copper is supplied to endogenous enzymes, is incorporated into ceruloplasmin which is secreted into the blood, and excess copper is secreted into the bile [9]. It has been reported that the cellular localization of ATP7A and ATP7B proteins changes according to the copper concentration. At basal copper concentrations both ATP7A and ATP7B reside in the trans-Golgi network (TGN) whereas in the presence of elevated copper they redistribute to the plasma membrane or to a cytoplasmic vesicular compartment [37, 38]. Transient phosphorylation at an invariant aspartate of the DKTG sequence in the 4th cytoplasmic loop (Fig. **3**), that forms an acyl-phosphate intermediate, induces the trafficking of both proteins [39, 40]. Recent studies under more physiological conditions suggested that, at high concentrations of copper ATP7A mainly relocalizes to novel vesicles in the basolateral region of epithelial cells and ATP7B traffics to pericanalicular vesicles in hepatocytes. Specific peptide motifs within ATP7A and ATP7B have been suggested to play critical roles in the sorting of both proteins. A C-termilal dileucine motif (LL1487-1488) of ATP7A is important for its retrieval by the Golgi following endocytosis at the plasma membrane and for its targeting to vesicles at the basolateral membrane. ATP7B has a similar (LLL1454-1456) trileucine motif that is expected to have a role in the recycling of ATP7B to the TGN from the pericanalicular vesicles. [40-42]. A C-terminal DTAL sequence of ATP7A is a putative PDZ (PSD-95, DLG and ZO-1) motif required for its sorting and/or retention at the basolateral membrane of MDCK cells under conditions of elevated copper. No putative PDZ binding motif has been identified at the C terminus of ATP7B [41, 43]. Although the structure and cellular function of these proteins appear to be very similar, the symptoms of Menkes and Wilson's disease are quite different from each other. The basis for this distinction is the different tissues that are affected as well as differential copper responsive trafficking of the two proteins. In Menkes disease, cop-

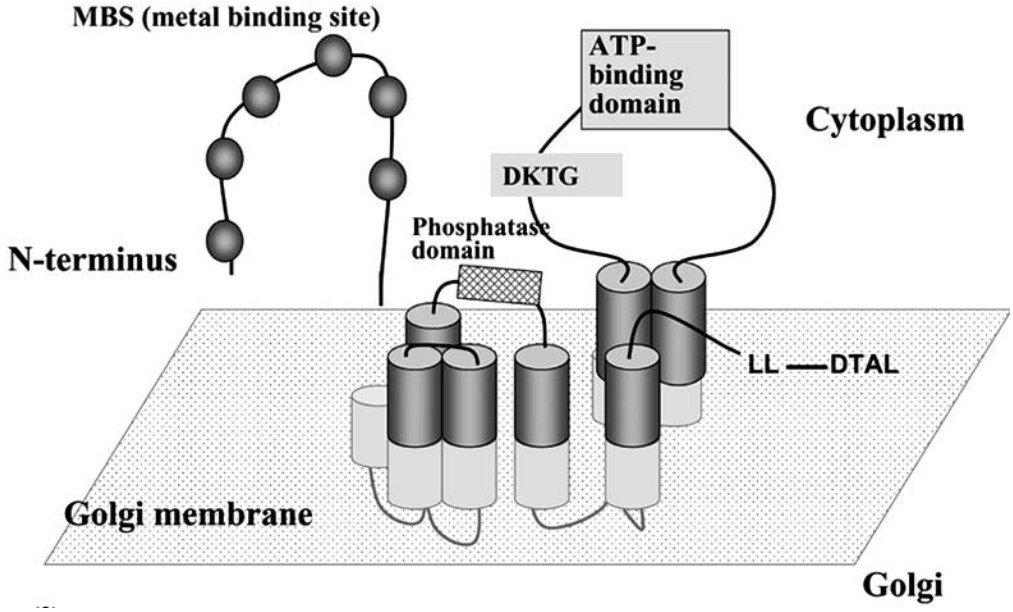


Fig. (3). Structure of ATP7A /ATP7B.

ATP7A and ATP7B are P-type ATPase Cu transporters that exist at the Golgi membrane and share 67% amino acid identity. Both molecules have 6 copies of the sequence MT/HCXXC, termed the metal binding site (MBS), in the N-terminal cytoplasmic region, 8 membrane spanning segments, a phosphatase domain in the third cytoplasmic loop and a phosophorylation site and an ATP binding domain in the fourth cytoplasmic loop. A C-terminal dileucine motif (LL1487-1488) in ATP7A is important for its endocytic retrieval by the trans-Golgi network (TGN) and for targeting to the basolateral membrane. ATP7B has a similar (LLL1454-1456) trileucine motif that is expected to have a role in recycling of ATP7B to the TGN. A C-terminal DTAL sequence is a putative PDZ motif required for sorting and/or retention of ATP7A at the basolateral membrane of MDCK cells under conditions of elevated copper. No putative PDZ binding motif has been identified at the C terminus of ATP7B.

per accumulates only in enterocytes and kidney epithelial cells, and other organs are in a copper-deficient condition. Menkes disease is usually fatal within 3 years and is characterized by symptoms such as connective tissue weakness (bone change and arterial rupture), hypopigmentation, hypothermia, and progressive cerebral degeneration. In contrast, Wilson's disease is an autosomal recessive copper toxicosis and massive amounts of copper accumulate in the liver and subsequently in other tissues. Wilson's disease patients manifest chronic liver disease and progressive neurological impairment [44].

4. ATP7A AND ATP7B CUPPER TRANSPORTERS AND CDDP RESISTANCE - *IN VITRO* STUDIES

In several CDDP resistant cells lines, not only is the accumulation of CDDP frequently decreased but the efflux of CDDP from the cells is enhanced and the cells often show cross-resistance to other metals such as antimony, arsenite or cadmium [45-48]. Cross-resistance to cadmium has also been reported for copper resistant hepatic cancer cells that upregulate ATP7B [49]. Thus these ATP7A and ATP7B overexpressing cells show some similarity to certain CDDP resistant cell lines. These observations suggested a possible link between ATP7B function and CDDP resistance. Indeed a number of studies have supported this potential link. In a previous study we showed that ATP7B-overexpressing epidermoid carcinoma KB cells (KB/WD) were 8.9-fold more resistant to CDDP than the parental cells. The accumulation of CDDP in KB/WD was also lower than that in the parental cells and the efflux of CDDP was enhanced. We have shown that ATP7B is overexpressed in CDDP-resistant prostate carcinoma PC-5 cells but not in the parental PC-3 cells nor in the revertant PC-5R cells [1]. Furthermore, it has been shown that ATP7B cDNA-transfected ovarian and squamous carcinoma cells were 1.6- or 2.6-fold resistant respectively to CDDP. These cells were also resistant to carboplatin (CBDCA) showing a reduction in its accumulation and an augmentation of its efflux rate [2, 50]. It has also been reported that the level of ATP7A and/or ATP7B was increased in three CDDP-resistant cell lines in comparison to the level in the respective parental cells. The accumulation of CDDP and copper in the three CDDP resistant cell lines was 38-67% and 27-46% of the level in the sensitive parental cells respectively. In a comparison of one pair of resistant and sensitive cell lines, the initial influx rate of both copper and CDDP was lower, whereas the terminal efflux rate was higher in the resistant cells compared to that in the sensitive cells [2].

The accumulated *in vitro* data therefore suggest a strong link between ATP7A and ATP7B expression and CDDP resistance.

5. ATP7A AND ATP7B TRANSPORTERS AND CDDP RESISTANCE IN CLINICAL CANCERS

Consistent with the observed *in-vitro* correlation between ATP7A and ATP7B function and CDDP resistance, studies also show a correlation between the aberrant expression of ATP7A or ATP7B and CDDP resistance in clinical cancers. ATP7A is normally expressed in intestine, endometrium, prostate, testis and kidney, but is not detected in the other major organs by immunohistochemistry. However, ATP7A expression has been detected in some of the most common human malignancies including prostate (7 of 7), breast (10 of 10), lung (8 of 8), colon (5 of 8), and ovary (6 of 7), as well as in a wide variety of other types of malignancy [3]. A correlation between ATP7A expression and survival has also been reported. In one study it was reported that ATP7A was expressed in 28 of 54 (51.8%) ovarian carcinomas before treatment. Patients with increased ATP7A expression after treatment (18 of 54) exhibited poorer survival [3].

In contrast to the above studies we found that there was no statistically significant correlation between CDDP resistance and ATP7A expression in human colon cancer cells using an HDRA *ex vivo* drug resistance assay although we did find that ATP7A oeverexpressing cells are resistant to CDDP *in vitro*. The discrepancy between our study and the other studies might be due to intrinsic differences between colon and ovarian cancer. I would like to emphasize that our data do not mean that ATP7A is not involved in CDDP resistance in clinical cancer. Eight ATP7A expressing tumor cells were not sensitive to CDDP, and 3 CDDP sensitive tumor cells were ATP7A negative. Our data indicated that more than 90% (31/34) of human colon cancer cells were resistant to CDDP and the high ratio of CDDP resistance in colon cancers may conceal the significance of the correlation between CDDP resistance and the ATP7A expression [7].

ATP7B expression has been examined in several different cancer tissues using immunohistochemistry and/or RT-PCR and significantly higher expression has been detected in gastric (41.2%, 21/51) [51], breast (22.0%, 9/41) [52], esophageal (76.5%, 13/17) [53], ovarian (34.6%, 36/104) [54], hepatocellular (21.1%, 4/19) [55], oral squamous (54.9%, 28/51) [56] and endometrial carcinoma cells (37.3%, 19/51) [57] when compared with adjacent non-neoplastic cells. The expression level of ATP7B correlated with a lower grade of differentiation in gastric, breast, ovarian and uterine carcinoma cells [51, 52, 54, 57]. In esophageal and oral squamous cell carcinoma (SCC) cells, patients with ATP7B positive tumors tended to show an inferior response to CDDP based chemotherapy. In oral SCC, endometrial cancer and ovarian cancer patients with ATP7B positive tumors had significantly decreased overall survival rates than those with ATP7B negative tumors [53, 56-58].

The above studies indicate that ATP7A and ATP7B show promise as indicators of response to CDDP and as potential targets for anti-tumor therapy. However, intensive study is necessary on far more clinical samples from different organs to elucidate the exact relationship between the expression of ATP7A and ATP7B and CDDP, CDBCA and L-OHP resistance in clinical cancers.

6. MECHANISMS OF ATP7A AND ATP7B RELATED DRUG RESISTANCE

6.1. Relationship of Copper Resistance and Multidrug Resistance to ATP7A Expression

The importance of ATP7A in mediating drug resistance is not limited to its effect on CDDP. We have further found that ATP7A overexpressing cells are resistant to many anti-

cancer drugs including SN-38, carbonyloxycamptothecin (CPT-11), VCR, paclitaxel, etoposide, doxorubicin (Dox) and mitoxantrone (Table **1**) [7]. The fact that the ATP7A or ATP7B over-expressing cells were not highly resistant to copper in comparison to the resistance induced by CDDP or other chemical agents is consistent with previous data although the exact cause is unknown (Table **1**) [1, 59]. One possible explanation is that, in CHO-K1 cells, metallothioneine, that is known to sequester copper, is not expressed. Thus the lack of meallothioneine may contribute to the low resistance against copper [60]. However, a number of factors may mediate copper toxicity and the mechanisms of cellular copper toxicity are still not fully clarified. Copper would be expected to participate in Fenton-like reactions and to generate hydroxyradicals that are potentially toxic. Hydroxyradicals damage not only DNA in the nuclei but also lipids and proteins in the mitochondria and lysosomes thereby damaging the membranes of these organelles. This is in contrast to the target molecules of most anticancer agents which reside in the nuclei [61-63]. It has also been recently reported that in *Escherichia coli* copper could suppress iron-mediated oxidative DNA damage through unknown mechanisms [64]. Furthermore copper may modulate cellular apoptosis through induction of the secretion of activated acid sphyngomyelinase (Asm) from leukocytes [65]. Therefore additional mechanisms of copper toxicity are possible and remain to be elucidated. Thus a number of factors may contribute to the fact that ATP7A overexpressing cells are not highly resistant to copper. However, since copper is toxic for many cellular organelles, sequestration effects in ATP7A expressing cells as is described below might not be as protective against copper toxicity as other anticancer agents. Alternatively the affinity and capacity of proteins/molecules that facilitate sequestration might be related to the difference of relative resistance of ATP7A expressing cells.

6.2. ATP7A- and ATP7B-Induced Sequestration of Anticancer Agents into Vesicles as a Potential Mechanism of Drug Resistance

Several studies have suggested that a major mechanism by which ATP7A and ATP7B modulate drug resistance is through sequestration of drugs within intracellular vesicles. Thus, confocal microscopy has shown that F-DDP, (CDDP conjugated to cyclohextlmethylamino fluorescein (CHMA-F)), co-localized with transfected ATP7A or ATP7B in vesicles that were identified as secretory export pathway vesicles and lysosomes (Fig. **4**). [66, 67]. No co-localization of the same fluorescein molecule lacking the CDDP moiety was observed. Fluorescein conjugation did not alter the properties of CDDP as F-DDP mimicked the toxicity of CDDP, and cells that were resistant to CDDP were also resistant to F-DDP [66]. These data, together with subsequent studies by the same group, suggested that ATP7A and ATP7B might function in CDDP resistance by sequestration of CDDP into vesicles of the secretory pathway and lysosomes [50, 66, 68]. In a second study, ATP7A-transfected ovarian cancer cells, that were resistant to CDDP, CBDCA and Oxaliplatin (L-OHP), also showed enhanced sequestration of platinum (Pt) into the vesicular fraction after a 24 h exposure to CDDP, CBDCA or L-OHP [59]. Furthermore, in ATP7A expressing cells Dox was detected as a punctate cytoplasmic distribution of fluorescence around the nucleus that overlapped with the Golgi apparatus marker, N-{6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]caproyl}- sphingosine (C6-NBD-Ceramide) [7]. In contrast, Dox fluorescence localized predominantly in the nuclei of the parental cells. Thus sequestration of anticancer agents within cellular vesicles appears to play a major role in the mechanism of ATP7A and ATP7B induced anticancer drug resistance.

6.3. The Potential Role of Vesicle Acidification in the Sequestration of Anticancer Agents

Although ATP7A and ATP7B clearly sequester drugs within membrane enclosed cellular vesicles the mechanism by which they do so is as yet unclear. Other drug transporters also sequester drugs within vesicles as part of their mechanism of drug resistance. For some of these transporters acidification of the vesicles has been proposed to play a major role in the mechanism by which the drugs are sequestered. Thus the acidic conditions within the vesicle are believed to help retain drugs that are basic in character.

Table 1. Drug and Metal Resistance of ATP7A Expressing Cells (from Reference [7])

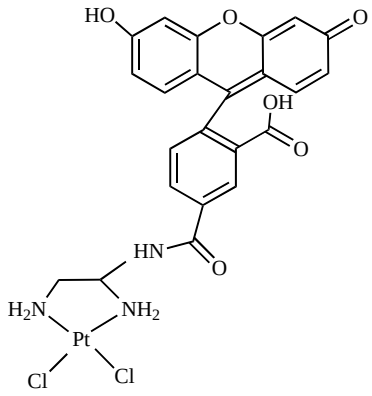
Agent/Cells	CHOK1 IC_{50} [b]	CHO/pCMB117 IC_{50}	RR	Me32a-T22/2L IC_{50}	Me32a/ pCMB117 IC_{50}	RR
SN-38	0.100 ± 0.025	14.78 ± 1.60	147.31*	0.028 ± 0.003	1.20 ± 0.44	42.94*
CPT11	42.69 ± 12.47	200.34 ± 10.87	4.69*	6.166 ± 1.134	81.03 ± 20.03	13.14*
Taxol	0.054 ± 0.008	17.83 ± 11.99	324.95*	0.00030 ± 0.0005	0.028 ± 0.0080	93.39*
VCR	0.077 ± 0.012	52.58 ± 13.95	678.37*	0.0043 ± 0.00084	0.30 ± 0.185	70.07*
Dox	1.306 ± 0.070	19.27 ± 1.72	14.75*	1.107 ± 0.425	14.48 ± 7.76	13.08*
VP-16	2.899 ± 0.389	67.95 ± 0.00	20.17*	2.095 ± 0.766	56.52 ± 7.51	26.98*
MX	1.912 ± 0.123	19.68 ± 2.37	10.30*	0.095 ± 0.0020	0.40 ± 0.03	4.16*
CDDP	5.82 ± 0.26	14.70 ± 0.76	2.5*	6.56 ± 0.177	15.71 ± 0.71	2.4*
Cu	0.074 ± 0.012	0.28 ± 0.06	3.85*	0.130 ± 0.036	0.17 ± 0.02	1.28*

CHO/pCMB117 is a human ATP7A cDNA-transfected CHO cell. Me32a-T22/2L is a fibroblast cell line derived from a Menkes disease patient. Me32a/pCMB117 is human ATP7A cDNA-transfected Me32a-T22/2L cell. CHO/pCMB117 highly express ATP7A, whereas Me32a/pCMB117 cells moderately express ATP7A. None of these cells significantly express ABCB1/P-gp, ABCC1/MRP1 nor ABCG2/BCRP by immunoblotting.

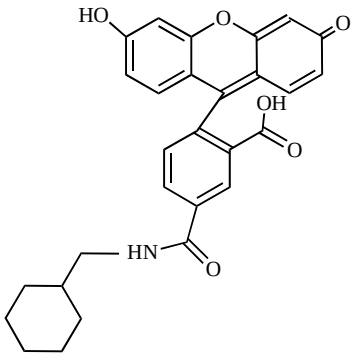
[a]RR is relative resistance, i.e. the IC_{50} value for CHO/pCMB117 or Me32a/pCMB117 divided by the IC_{50} value for CHO-K1 or Me32a-T22/2L cells respectively.

[b]IC_{50} values are the means ± SDs (μM) from triplicate determinations in the MTT assay.

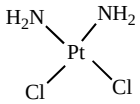
*indicates statistical significance ($P<0.05$).



F-DDP



cyclohexytlmethylamino-fluorescein (CHMA-F)



CDDP

Fig. (4). Chemical structure of F-DDP, fluorescein (CHMA-F) and CDDP.

The chemical formulae of cyclohexytlmethylamino-fluorescein (CHMA-F), fluorescein-conjugated CDDP (F-DDP) and CDDP are shown.

The role of vesicle acidification in drug sequestration has been analyzed by a number of different approaches including analysis of the expression of V-ATPase that plays a critical role in acidification of vesicles, analysis of the effect of inhibitors of V-ATPase, analysis of cells that are impaired in acidification of vesicles, and by the use of drugs such as ionophores, that directly modify acidification of intracellular vesicles. By using these approaches a number of studies suggest a positive link between vesicle acidification and drug resistance. Thus, HL-60 derived anticancer drug resistant cell lines had a higher expression of the V-ATPase subunit c than the parental cells by northern blotting and a lower accumulation of daunomycin (Dau) than parental cells [69]. The V-ATPase inhibitors Bafilomycin A1, and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD), decreased the efflux, and enhanced the accumulation of, Dau in these resistant cells [70]. Acidification impaired MCF-7 cells are drug sensitive but the drug resistant cell line MCF-7adr derived from these MCF-7 cells had normal acidic vesicles. Furthermore, following treatment of the MCF-7adr cells with the H^+/K^+ ionophore nigericine, and the Na^+/H^+ ionophore monensin, these MCF-7adr cells became sensitive to Dox [71]. In a separate study using these MCF-7 cells it was suggested that cells that were not impaired for acidification could sequester weak basic anticancer drugs such as anthracyclines and vinca alkaloids, within vesicles thereby becoming resistant to these drugs. In this study Dox distributed to the nuclei and the cytosol of the parental MCF-7 cells but localized in perinuclear vesicles of the MCF-7/ADR cells. Treatment with the V-ATPase inhibitors Bafillomycin A and concanamycin A (CCMA) changed Dox distribution in MCF-7/ADR cells from the vesicles to the cytosol and the nuclei [72]. The P-gp expressing PKSV-PRcol50 colchicine resistant cell line, derived from transformed renal epithelial PKSV-PR cells, was found to have more acidic lysosomes than the parental cells. Bafilomycin A and CCMA reversed the anthracycline drug resistance of these cells without affecting the efflux of Dau [73].

However, the role of acidification of vesicles in drug sequestration is still controversial and may not be applicable to all drugs or to all drug transporters. Previously we found that 5-*O*-Benzoylated Taxinine K (BTK), a new taxoid from the Japanese yew *Taxus Cuspidata* could render cells expressing the drug transporters ABCB1/P-gp and ABCC1/MRP1 more sensitive to Dox and VCR through different mechanisms. Following BTK treatment the relative resistance of ABCC1/MRP1 expressing cells to Dox and VCR decreased from 33.9 to 15.8 and from 21.8 to 7.17, respectively. There was no difference in the acidification of intracellular vesicles of the ABCC1/MRP1 expressing cells and its parental cells as judged by Acridine Orange staining. The effect of BTK was selective in that it inhibited the transport function of ABCB1/P-gp, but did not inhibit the transport of leukotriene C_4 by ABCC1/MRP1, nor was it able to reverse the attenuation of the accumulation of Dox in the ABCC1/MRP1 cells. However it did disturb the acidification of the vesicles in the ABCC1/MRP1 expressing cells and changed Dox distribution in ABCC1/MRP1 expressing cells [74]. Another group also reported that transiently expressed ABCC1/MRP1 co-localized with a lysozome marker and that Dox was sequestered in the vesicles of these cells. However 100 nM concanamycin A could not inhibit the sequestration of Dox following transient transfection of the cells suggesting that sequestration may occur through a mechanism other than through the action of V-ATPase [75].

In the case of ATP7A we have found that, although ATP7A overexpressing cells are more resistant to Dox and VCR they show similar acidification of intracellular vesicles to that of the parental cells by Acridine orange staining (unpublished data). However, we also found that monensin can relocate Dox from intracellular vesicles to the nuclei of ATP7A overexpressing cells suggesting that vesicle acidification may play some role in the effect of ATP7A on drug distribution [7]. These conflicting results concerning the role of acidification in ATP7A function may perhaps be due to the inability of Acridine orange to detect subtle differences in pH in the vesicles of ATP7A overexpressing cells compared to the parental cells. It must also be taken into account that the effect of monensin may not be limited to disruption of vesicle acidification by inhibition of V-ATPase. Therefore the role of vesicle acidification in the modulation of drug distribution by ATP7A remains to be clarified in future studies.

In summary, although acidification of intracellular vesicles clearly plays a role in drug sequestration within the cell, acidification *per se* cannot fully explain the mechanism by which drugs are sequestered. Although acidification of intracellular vesicles might also play a role in ATP7A related drug resistance, clearly more detailed analyses of the relationship between acidification of vesicles and drug resistance of ATP7A overexpressing cells is required.

6.4. Uptake Mechanisms of Anticancer Drugs from the Cytosol into Vesicles

In order for ATP7A to sequester drugs within intracellular vesicles the drugs must be taken up from the cytosol into the vesicle. The mechanism by which this is achieved, and the role of ATP7A, or indeed drug transporters in general, in this uptake is not very well understood.

Studies undertaken by our group, as well as by other groups, suggest that drug transporters may indeed play a role in drug uptake into intracellular vesicles. We found that in KB cells expressing transfected ABCC1/MRP1, Dox distributes in perinuclear vesicles whereas in the parental KB cells Dox distributes within the nuclei. The ABCC1/MRP1-expressing KB cells are also more resistant to Dox and VCR than the parental KB cells with no detectable difference in vesicle acidification between the ABCC1/MRP1 expressing cells and the parental cells [74]. Another group carried out crosslinking studies to determine the potential role of the ABCC/MRP1 transporter in the uptake of Dox into cellular vesicles [75]. In that study Dox distributed to the preinuclear region in transiently transfected ABCC1/MRP1 expressing HeLa cells and its cellular localization overlapped with that of CFP-tagged ABCC1/MRP1. Crosslinking with a cell membrane permeable cross-linker BMH, that can crosslink both plasma and intracellular membrane MRP1, inhibited MRP1 function and resulted in a redistribution of Dox to the nuclei. In contrast, crosslinking with a membrane impermeable protein cross-linker BM[PEO]4, that can only crosslink and inhibit MRP1 on the plasma memrane, could not inhibit sequestration of Dox in the vesicles of the MRP1-expressing cells [75]. These data suggested that MRP1 must play some role in the uptake of Dox into membrane vesicles. They also suggest that ABCB1/P-gp and ABCG2/BCRP have similar functions to ABCC1/MRP1 in drug uptake [75].

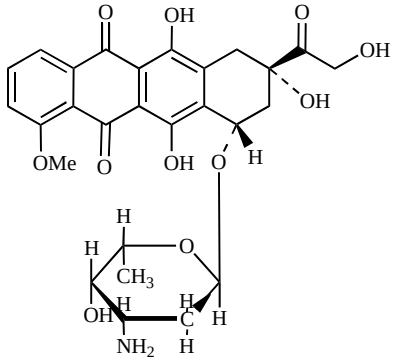
Several lines of evidence also implicate ATP7A and ATP7B directly, or indirectly, in drug uptake into vesicles. In three ATP7A over expressing cell lines Dox accumulated in the Golgi and membrane vesicles and these ATP7A overexpressing cells had higher activity in the uptake of SN-38 than the parental cells [7]. ATP7B over expressing cells are 4.1-fold more resistant to CDDP, and also show a higher accumulation of Pt into intracellular vesicles than control cells [66]. Furthermore, in CDDP resistant ovarian cancer cells, which over express ATP7A and ATP7B, sequestration of CDDP into the vesicular compartment is enhanced and F-DDP is localized to the lysosomes, Golgi and the secretory compartment [59, 68]. More recently it was shown that ATP7B directly transports CDDP and the precise kinetics of copper and CDDP transport by ATP7B was characterized. In this study CDDP uptake by ATP7B expressing membrane vesicles was inhibited by copper, and CDDP induced ATPase activity and acyl-phosphate modification of ATP7B [76].

The combined data suggest that ATP7A and ATP7B play important roles in the sequestration of anticancer agents and are candidate uptake pumps for the transport of chemical agents into intracellular vesicles. If so, then ATP7A and ATP7B would be important target molecules for cancer chemotherapy.

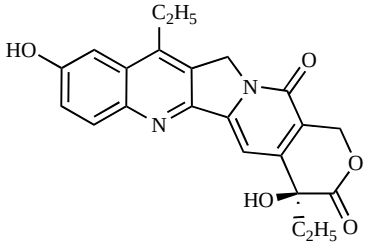
6.5. Drug Selectivity in ATP7A Related Multidrug Resistance

In ATP7A and ATP7B mediate drug resistance, the mechanism by which these transporters distinguish between different drugs is an area of active investigation. Both Dox and F-DDP accumulate in the Golgi of ATP7A overexpressing cells [7,68]. Dox and F-DDP have common structural features and both drugs are composed of multiple polycyclic aromatic hydrocarbons linked to either an anthracene backbone (Dox) or a xanthene structure (F-DDP). This structural similarity suggested that structural features of the drugs might be important for ATP7A-mediated accumulation of the drug in vesicles.

In this respect it was initially puzzling why the fluorescent CHMA-F, that is structurally almost identical with F-DDP, was localized in the cytoplasmic region rather than the Golgi, of both the parental and the drug resistant cells. However, calculation of Slog P, which is an improved method for



Doxorubicin



SN-38

Fig. (5). Chemical structure of Dox and SN-38.

the prediction of the octanol-water partition coefficient of a molecule using MOE (Molecular Operating Environment, Chemical Computing Group Inc., Montreal, Quebec, Canada), indicated that the physicochemical characteristics of CHMA-F and F-DDP are different [77]. Thus the SlogP value of FDDP (0.6126) is similar to that of other anticancer compounds such as Dox (0.0968) and SN-38 (0.6458), whereas that of CHMA-F (4.4349) is much higher than that of FDDP. Thus the chemical character of the drug appears to be an important factor that determines its recognition by ATP7A and its intracellar localization (Fig. **5**).

Although ATP7A and ATP7B modulate the cellular localization of CDDP and anticancer agents the exact mechanism by which they do so is not clear. Even though we succeeded in monitoring the increment of SN-38 uptake by membrane vesicles from ATP7A overexpressing cells, there is no concrete evidence that anticancer agents other than CDDP might be directly taken up by ATP7A or ATP7B. Therefore there are several possible mechanisms to explain the function of ATP7A in MDR: 1) ATP7A might directly uptake drugs into the Golgi apparatus. 2) ATP7A might enhance uptake of drugs into the Golgi lumen in concert with an unknown transporter(s). In this respect one possibility is that ATP7A might help to acidify vesicles. 3) ATP7A might induce the expression of an unknown transporter(s). Since these mechanisms are not mutually exclusive, some and / or all of above mechanisms may function simultaneously. The evidence to date, especially the data concerning the uptake of SN-38 by membrane vesicles from ATP7A overexpressing cells, and the transport of CDDP by ATP7B, strongly suggests that ATP7A /ATP7B directly transport a variety of drugs. Future work should therefore focus on elucidation of the interaction between ATP7A /ATP7B and anticancer agents. Our current work is directed towards understanding the induction of ATPase activity and acyl-phosphate modification of ATP7A and ATP7B by anticancer agents.

6.6. The Potential Role of Vesicle Trafficking in Drug Efflux and Drug Resistance

Theoretically, if cells entrap a drug within vesicles, then the cells become resistant to this drug as the drug is prevented from reaching its cellular targets. However, a further advantage in the entrapment of drugs within intracellular vesicles is that many intracellular vesicles undergo trafficking within the cell, and drug entrapment within secretory vesicles may thus also play a role in drug efflux from the cell. There is both direct and indirect evidence that support this latter possibility.

In CDDP resistant cells, inhibition of vesicle trafficking with brefeldin-A, Wortmannin or H89 blocked the loading of F-DDP into secretory pathway vesicles, decreased the lysosomal fraction of CDDP resistant cells to 40% of that of the parental cells, and enhanced the exosomal export of CDDP [67]. As described previously, CDDP resistant cells that overexpress ATP7A and/or ATP7B have a higher terminal efflux rate of CDDP than the parental cells [2]. In ATP7A expressing cells the accumulation of Dox and SN-38 was reduced and the efflux of both drugs was enhanced [7].

However, the ability of ATP7A to function in drug efflux is not universal for all drugs. We have found that the accumulation and efflux of VCR and Paclitaxel in ATP7A expressing cells was not any different from those of the parental cells [7]. This result is consistent with a study from a different group that reported that the overall cellular accumulation of Pt or F-DDP increased rather than decreased after exposure to CDDP, CBDCA, L-OHP or F-DDP [78]. Therefore increased drug efflux by ATP7A might show selectivity for particular chemical agents.

Clearly further study is necessary to characterize both the role of vesicle trafficking in drug efflux mediated by ATP7A and ATP7B, and the mechanism by which ATP7A or ATP7B expression might directly or indirectly affect the vesicle transport system.

7. POTENTIAL RELATIONSHIP OF ATP7A EXPRESSION WITH THE MALIGNANT PHENOTYPE

In addition to its role in MDR, recent evidence suggests the possibility that ATP7A upregulation may be related to the malignant phenotype. It has been shown that ATP7A is upregulated in pancreatic cancer compared to chronic pancreatitis [79] and that ATP7A and the Cu containing enzyme LOX are more highly expressed in invasive breast cancer cell lines than in noninvasive lines [80]. If the correlation between ATP7A expression and the malignant phenotype can be confirmed in future studies ATP7A may prove to be an important target molecule for cancer therapy.

8. CONCLUSIONS AND FUTURE PERSPECTIVES

Copper transport systems appear to play important roles in drug transport as well as in copper metabolism. Components of copper metabolism are therefore likely to include target molecules for the modulation of drug potency not only of anti-cancer agents but also of other drugs. Further study is necessary to elucidate the roles of the copper transport system in clinical cancers and the mechanism by which it mediates drug transport.

It may be difficult to specifically target the copper transport system for the modulation of drug trafficking without having an adverse effect on copper metabolism. However, if a functional screening procedure can be devised it may be possible to improve drug resistance to some chemical agents as well as to decrease malignancy related to ATP7A and ATP7B.

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REFERENCES

[1] Komatsu, M.; Sumizawa, T.; Mutoh, M.; Chen, Z. S.; Terada, K.; Furukawa, T.; Yang, X. L.; Gao, H.; Miura, N.; Sugiyama, T.; Akiyama, S. *Cancer Res.*, **2000**, *60*, 312.

[2] Katano, K.; Kondo, A.; Safaei, R.; Holzer, A.; Samimi, G.; Mishima, M.; Kuo, Y. M.; Rochdi, M.; Howell, S. B. *Cancer Res.*, **2002**, *62*, 6559.

[3] Samimi, G.; Varki, N. M.; Wilczynski, S.; Safaei, R.; Alberts, D. S.; Howell, S. B. *Clin. Cancer Res.*, **2003**, *9*, 5853.

[4] Lin, X.; Okuda, T.; Holzer, A.; Howell, S. B. *Mol. Pharmacol.*, **2002**, *62*, 1154.

[5] Safaei, R.; Howell, S. B. *Crit. Rev. Oncol. Hematol.*, **2005**, *53*, 13-23.

[6] Kuo, M. T.; Chen, H. H.; Song, I. S.; Savaraj, N.; Ishikawa, T. *Cancer Metastasis Rev.*, **2007**, *26*, 71.

[7] Owatari, S.; Akune, S.; Komatsu, M.; Ikeda, R.; Firth, S. D.; Che, X. F.; Yamamoto, M.; Tsujikawa, K.; Kitazono, M.; Ishizawa, T.; Takeuchi, T.; Aikou, T.; Mercer, J. F.; Akiyama, S.; Furukawa, T. *Cancer Res.*, **2007**, *67*, 4860.

[8] Voskoboinik, I.; Camakaris, J.; Mercer, J. F. *Adv. Protein Chem.*, **2002**, *60*, 123.

[9] Mercer, J. F.; Llanos, R. M. *J. Nutr.*, **2003**, *133*, 1481S.

[10] Wernimont, A. K.; Huffman, D. L.; Lamb, A. L.; O'Halloran, T. V.; Rosenzweig, A. C. *Nat. Struct. Biol.*, **2000**, *7*, 766.

[11] Aller, S. G.; Unger, V. M., *Proc. Natl. Acad. Sci. USA*, **2006**, *103*, 3627.

[12] Puig, S.; Lee, J.; Lau, M.; Thiele, D. J. *J. Biol. Chem.*, **2002**, *277*, 26021.

[13] Lee, J.; Pena, M. M.; Nose, Y.; Thiele, D. J. *J. Biol. Chem.*, **2002**, *277*, 4380.

[14] Petris, M. J.; Smith, K.; Lee, J.; Thiele, D. J. *J. Biol. Chem.*, **2003**, *278*, 9639.

[15] Guo, Y.; Smith, K.; Lee, J.; Thiele, D. J.; Petris, M. J. *J. Biol. Chem.*, **2004**, *279*, 17428.

[16] Andrews, P. A. *Cancer Treat. Res.*, **1994**, *73*, 217.

[17] Akiyama, S.; Chen, Z. S.; Sumizawa, T.; Furukawa, T. *Anticancer Drug Des.*, **1999**, *14*, 143.

[18] Mukai, M.; Kanzaki, A.; Chen, Z. S.; Miyashita, H.; Sumizawa, T.; Furukawa, T.; Haraguchi, M.; Takebayashi, Y.; Takamatsu, H.; Akiyama, S. *Oncol. Rep.*, **2002**, *9*, 839.

[19] Wernyj, R. P.; Morin, P. J. *Drug Resist. Updat.*, **2004**, *7*, 227.

[20] Taniguchi, K.; Wada, M.; Kohno, K.; Nakamura, T.; Kawabe, T.; Kawakami, M.; Kagotani, K.; Okumura, K.; Akiyama, S.; Kuwano, M. *Cancer Res.*, **1996**, *56*, 4124.

[21] Koike, K.; Kawabe, T.; Tanaka, T.; Toh, S.; Uchiumi, T.; Wada, M.; Akiyama, S.; Ono, M.; Kuwano, M. *Cancer Res.*, **1997**, *57*, 5475.

[22] Andrews, P. A.; Mann, S. C.; Huynh, H. H.; Albright, K. D. *Cancer Res.*, **1991**, *51*, 3677.

[23] Murakami, T.; Shibuya, I.; Ise, T.; Chen, Z. S.; Akiyama, S.; Nakagawa, M.; Izumi, H.; Nakamura, T.; Matsuo, K.; Yamada, Y.; Kohno, K. *Int. J. Cancer*, **2001**, *93*, 869.

[24] Ishida, S.; Lee, J.; Thiele, D. J.; Herskowitz, I. *Proc. Natl. Acad. Sc. USA*, **2002**, *99*, 14298.

[25] Beretta, G. L.; Gatti, L.; Tinelli, S.; Corna, E.; Colangelo, D.; Zunino, F.; Perego, P. *Biochem. Pharmacol.*, **2004**, *68*, 283.

[26] Song, I. S.; Savaraj, N.; Siddik, Z. H.; Liu, P.; Wei, Y.; Wu, C. J.; Kuo, M. T. *Mol. Cancer Ther.*, **2004**, *3*, 1543.

[27] Zisowsky, J.; Koegel, S.; Leyers, S.; Devarakonda, K.; Kassack, M. U.; Osmak, M.; Jaehde, U. *Biochem. Pharmacol.*, **2007**, *73*, 298.

[28] Holzer, A. K.; Katano, K.; Klomp, L. W.; Howell, S. B. *Clin. Cancer Res.*, **2004**, *10*, 6744.

[29] Guo, Y.; Smith, K.; Petris, M. J. *J. Biol. Chem.*, **2004**, *279*, 46393.

[30] Holzer, A. K.; Manorek, G. H.; Howell, S. B. *Mol. Pharmacol.*, **2006**, *70*, 1390.

[31] Sinani, D.; Adle, D. J.; Kim, H.; Lee, J. *J. Biol. Chem.*, **2007**, *282*, 26775.

[32] Mercer, J. F.; Livingston, J.; Hall, B.; Paynter, J. A.; Begy, C.; Chandrasekharappa, S.; Lockhart, P.; Grimes, A.; Bhave, M.; Siemieniak, D.; Glover, T. W. *Nat. Genet.*, **1993**, *3*, 20.

[33] Chelly, J.; Tumer, Z.; Tonnesen, T.; Petterson, A.; Ishikawa-Brush, Y.; Tommerup, N.; Horn, N.; Monaco, A. P. *Nat. Genet.*, **1993**, *3*, 14.

[34] Vulpe, C.; Levinson, B.; Whitney, S.; Packman, S.; Gitschier, J. *Nat. Genet.*, **1993**, *3*, 7.

[35] Bull, P. C.; Thomas, G. R.; Rommens, J. M.; Forbes, J. R.; Cox, D. W. *Nat. Genet.*, **1993**, *5*, 327.

[36] Tanzi, R. E.; Petrukhin, K.; Chernov, I.; Pellequer, J. L.; Wasco, W.; Ross, B.; Romano, D. M.; Parano, E.; Pavone, L.; Brzustowicz, L. M.; Devoto, M. ;Peppercorn, J. ; Bush, A.I. ; Sternlieb, I.; Pirastu, M. ; Gusella, J. F. ; Evgrafov, O.; Penchaszadeh, G. K. ; Honig, B.; Edelman, I.S.; Soares, M.B. ;Scheinberg, I.H.;Gilliam, T.C. *Nat. Genet.*, **1993**, *5*, 344.

[37] Petris, M. J.; Mercer, J. F.; Culvenor, J. G.; Lockhart, P.; Gleeson, P. A.; Camakaris, J. *Embo J.*, **1996**, *15*, 6084.

[38] Payne, A. S.; Kelly, E. J.; Gitlin, J. D. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 10854.

[39] Petris, M. J.; Voskoboinik, I.; Cater, M.; Smith, K.; Kim, B. E.; Llanos, R. M.; Strausak, D.; Camakaris, J.; Mercer, J. F. *J. Biol. Chem.*, **2002**, *277*, 46736-42.

[40] Cater, M. A.; La Fontaine, S.; Mercer, J. F. *Biochem. J.*, **2007**, *401*, 143.

[41] Greenough, M.; Pase, L.; Voskoboinik, I.; Petris, M. J.; O'Brien, A. W.; Camakaris, J. *Am. J. Physiol .Cell Physiol.*, **2004**, *287*, C1463.

[42] La Fontaine, S.; Mercer, J. F. *Arch. Biochem. Biophys.*, **2007**, *463*, 149.

[43] Stephenson, S. E.; Dubach, D.; Lim, C. M.; Mercer, J. F.; La Fontaine, S., A. *J. Biol. Chem.*, **2005**, *280*, 33270.

[44] Danks, D. M., In *The Metabolic and Molecular Basis of Inherited Disease* 7th ed.; Scriver, C. R.; Beaudet, A. L.; Sly, W. S.; Valle, D., Eds. MacGraw-Hill New York, **1995**; pp 2211-2235.

[45] Nakagawa, M.; Nomura, Y.; Kohno, K.; Ono, M.; Mizoguchi, H.; Ogata, J.; Kuwano, M. *J. Urol.*, **1993**, *150*, 1970.

[46] Naredi, P.; Heath, D. D.; Enns, R. E.; Howell, S. B. *Cancer Res.*, **1994**, *54*, 6464.

[47] Chen, Z. S.; Mutoh, M.; Sumizawa, T.; Furukawa, T.; Haraguchi, M.; Tani, A.; Saijo, N.; Kondo, T.; Akiyama, S. *Exp. Cell Res.*, **1998**, *240*, 312.

[48] Fujii, R.; Mutoh, M.; Niwa, K.; Yamada, K.; Aikou, T.; Nakagawa, M.; Kuwano, M.; Akiyama, S. *Jpn. J. Cancer Res.*, **1994**, *85*, 426.

[49] Schilsky, M. L.; Stockert, R. J.; Kesner, A.; Gorla, G. R.; Gagliardi, G. S.; Terada, K.; Miura, N.; Czaja, M. J. *Hepatology*,**1998**, *28*, 1347.

[50] Katano, K.; Safaei, R.; Samimi, G.; Holzer, A.; Rochdi, M.; Howell, S. B. *Mol. Pharmacol.*, **2003**, *64*, 466.

[51] Ohbu, M.; Ogawa, K.; Konno, S.; Kanzaki, A.; Terada, K.; Sugiyama, T.; Takebayashi, Y. *Cancer Let.*, **2003**, *189*, 33.

[52] Kanzaki, A.; Toi, M.; Neamati, N.; Miyashita, H.; Oubu, M.; Nakayama, K.; Bando, H.; Ogawa, K.; Mutoh, M.; Mori, S.; Terada, K.; Sugiyama, T.; Fukumoto, M.; Takebayashi, Y. *Jpn. J. Cancer Res.*, **2002**, *93*, 70.

[53] Higashimoto, M.; Kanzaki, A.; Shimakawa, T.; Konno, S.; Naritaka, Y.; Nitta, Y.; Mori, S.; Shirata, S.; Yoshida, A.; Terada, K.; Sugiyama, T.; Ogawa, K.; Takebayashi, Y. *Int. J. Mol. Med.*, **2003**, *11*, 337.

[54] Nakayama, K.; Kanzaki, A.; Terada, K.; Mutoh, M.; Ogawa, K.; Sugiyama, T.; Takenoshita, S.; Itoh, K.; Yaegashi, N.; Miyazaki, K.; Neamati, N.; Takebayashi, Y., *Clin. Cancer Res.*, **2004**, *10*, 2804.

[55] Sugeno, H.; Takebayashi, Y.; Higashimoto, M.; Ogura, Y.; Shibukawa, G.; Kanzaki, A.; Terada, K.; Sugiyama, T.; Watanabe, K.; Katoh, R.; Nitta, Y.; Fukushima, T.; Koyama, Y.; Inoue, N.; Sekikawa, K.; Ogawa, K.; Sato, Y.; Takenoshita, S. *Anticancer Res.*, **2004**, *24*, 1045.

[56] Miyashita, H.; Nitta, Y.; Mori, S.; Kanzaki, A.; Nakayama, K.; Terada, K.; Sugiyama, T.; Kawamura, H.; Sato, A.; Morikawa, H.; Motegi, K.; Takebayashi, Y. *Oral Oncol.*, **2003**, *39*, 157.

[57] Aida, T.; Takebayashi, Y.; Shimizu, T.; Okamura, C.; Higasimoto, M.; Kanzaki, A.; Nakayama, K.; Terada, K.; Sugiyama, T.; Miyazaki, K.; Ito, K.; Takenoshita, S.; Yaegashi, N. *Gynecol. Onco.*, **2005**, *97*, 41.

[58] Nakayama, K.; Kanzaki, A.; Ogawa, K.; Miyazaki, K.; Neamati, N.; Takebayashi, Y. *Int. J. Cancer*, **2002**, *101*, 488.
[59] Samimi, G.; Safaei, R.; Katano, K.; Holzer, A. K.; Rochdi, M.; Tomioka, M.; Goodman, M.; Howell, S. B. *Clin. Cancer Res.*, **2004**, *10*, 4661.
[60] Camakaris, J.; Petris, M. J.; Bailey, L.; Shen, P.; Lockhart, P.; Glover, T. W.; Barcroft, C.; Patton, J.; Mercer, J. F. *Hum. Mol. Genet.*, **1995**, *4*, 2117.
[61] Sokol, R. J.; Twedt, D.; McKim, J. M., Jr.; Devereaux, M. W.; Karrer, F. M.; Kam, I.; von Steigman, G.; Narkewicz, M. R.; Bacon, B. R.; Britton, R. S.; Neuschwander-Tetri, B. A. *Gastroenterology*, **1994**, *107*, 1788.
[62] Seth, R.; Yang, S.; Choi, S.; Sabean, M.; Roberts, E. A. *Toxicol. In Vitro*, **2004**, 18, 501.
[63] Arciello, M.; Rotilio, G.; Rossi, L. *Biochem. Biophys. Res. Commun.*, **2005**, *327*, 454.
[64] Macomber, L.; Rensing, C.; Imlay, J. A. *J. Bacteriol.*, **2007**, *189*, 1616.
[65] Lang, P. A.; Schenck, M.; Nicolay, J. P.; Becker, J. U.; Kempe, D. S.; Lupescu, A.; Koka, S.; Eisele, K.; Klarl, B. A.; Rubben, H.; Schmid, K. W.; Mann, K.; Hildenbrand, S.; Hefter, H.; Huber, S. M.; Wieder, T.; Erhardt, A.; Haussinger, D.; Gulbins, E.; Lang, F. *Nat. Med.*, **2007**, *13*, 164.
[66] Katano, K.; Safaei, R.; Samimi, G.; Holzer, A.; Tomioka, M.; Goodman, M.; Howell, S. B. *Clin. Cancer Res.*, **2004**, *10*, 4578.
[67] Safaei, R.; Larson, B. J.; Cheng, T. C.; Gibson, M. A.; Otani, S.; Naerdemann, W.; Howell, S. B. *Mol. Cancer Ther.*, **2005**, *4*, 1595.
[68] Safaei, R.; Katano, K.; Larson, B. J.; Samimi, G.; Holzer, A. K.; Naerdemann, W.; Tomioka, M.; Goodman, M.; Howell, S. B. *Clin. Cancer Res.*, **2005**, *11*, 756.

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[69] Ma, L.; Center, M. S. *Biochem. Biophys. Res. Commun.*, **1992**, *182*, 675.
[70] Marquardt, D.; Center, M. S. *J. Natl. Cancer Inst.*, **1991**, *83*, 1098.
[71] Schindler, M.; Grabski, S.; Hoff, E.; Simon, S. M., *Biochemistry*, **1996**, *35*, 2811.
[72] Altan, N.; Chen, Y.; Schindler, M.; Simon, S. M., *J. Exp. Med.*, **1998**, *187*, 1583.
[73] Ouar, Z.; Bens, M.; Vignes, C.; Paulais, M.; Pringel, C.; Fleury, J.; Cluzeaud, F.; Lacave, R.; Vandewalle, A. *Biochem. J.*, **2003**, *370*, 185.
[74] Okumura, H.; Chen, Z. S.; Sakou, M.; Sumizawa, T.; Furukawa, T.; Komatsu, M.; Ikeda, R.; Suzuki, H.; Hirota, K.; Aikou, T.; Akiyama, S. I. *Mol. Pharmacol.*, **2000**, *58*, 1563.
[75] Rajagopal, A.; Simon, S. M., *Mol. Biol. Cell*, **2003**, *14*, 3389.
[76] Safaei, R.; Otani, S.; Larson, B. J.; Rasmussen, M. L.; Howell, S. B. *Mol. Pharmacol.*, **2008**, *73*, 461.
[77] Wildman, S. A.; Crippen, G. M., *J. Chem. Inform. Comp. Sci.*, **1999**, *39*, 868.
[78] Samimi, G.; Katano, K.; Holzer, A. K.; Safaei, R.; Howell, S. B. *Mol. Pharmacol.*, **2004**, *66*, 25.
[79] Crnogorac-Jurcevic, T.; Gangeswaran, R.; Bhakta, V.; Capurso, G.; Lattimore, S.; Akada, M.; Sunamura, M.; Prime, W.; Campbell, F.; Brentnall, T. A.; Costello, E.; Neoptolemos, J.; Lemoine, N. R. *Gastroenterology*, **2005**, *129*, 1454.
[80] Nagaraja, G. M.; Othman, M.; Fox, B. P.; Alsaber, R.; Pellegrino, C. M.; Zeng, Y.; Khanna, R.; Tamburini, P.; Swaroop, A.; Kandpal, R. P. *Oncogene*, **2006**, *25*, 2328.