Molecular Pharmacology Fast Forward. Published on October 31, 2007 as doi:10.1124/mol.107.040980
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Transport of cisplatin by the copper efflux transporter ATP7B

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Molecular Pharmacology Fast Forward. Published on October 31, 2007 as DOI: 10.1124/mol.107.040980
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Running title: Transport of cisplatin by ATP7B

Common abbreviations: Copper (Cu)

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Number of text pages: 32

Number of Figures: 6

Number of references: 34

Number of words in the *Abstract*: 224

Number of words in the *Introduction*: 750

Number of words in the *Discussion*: 1315

Non-standard abbreviations: BCS, bathocuproinedisulfonic acid; DDP, cisplatin; ICP-MS, inductively coupled plasma mass spectrometry; PBS, phosphate buffered saline; TBS, tris buffered saline

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ABSTRACT

ATP7B is a P-type ATPase that mediates the efflux of copper (Cu). Recent studies have demonstrated that ATP7B regulates the cellular efflux of cisplatin (DDP) and controls sensitivity to the cytotoxic effects of this drug. To determine whether DDP is a substrate for ATP7B, DDP transport was assayed in vesicles isolated from Sf9 cells infected with a baculovirus that expressed either the wild type ATP7B or a mutant ATP7B that was unable to transport Cu due to conversion of the transmembrane metal binding CPC motif to CPA. Only the wild type ATP7B-expressing vesicles exhibited Cu-dependent ATPase activity, Cu-induced acyl-phosphate formation, and ATP-dependent transport of Cu. The amount of DDP that became bound was higher for vesicles expressing either type of ATP7B than for those not expressing either form of ATP7B, but only the vesicles expressing wild type ATP7B mediated ATP-dependent accumulation of the drug. At pH 4.6, the vesicles expressing the wild type ATP7B exhibited ATP-dependent accumulation of DDP with an apparent K_m of 1.2 ± 0.5 (SEM) μM and V_{max} of 0.03 ± 0.002 (SEM) nmol/mg protein/min. DDP also induced the acyl-phosphorylation of ATP7B, but at a much slower rate than Cu. Cu and DDP, each, inhibited the ATP-dependent transport of the other. These results establish that DDP is a substrate for ATP7B, but is transported at a much slower rate than Cu.

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INTRODUCTION

Cisplatin (DDP) is currently one of the most commonly used anticancer drugs. However, the efficacy of DDP decreases with repeated cycles of therapy because of the rapid development of resistance which is often due to defects in the mechanisms for drug accumulation and/or efflux (Gately and Howell, 1993). The mechanism by which cells accumulate DDP is poorly understood and at present only a small number of transporters are known to influence the uptake and efflux of this drug (reviewed by (Safaei et al., 2004)). Recent studies have linked the efflux of DDP with the expression of copper (Cu) exporters, ATP7A and ATP7B. However, it is not known whether either ATP7A or ATP7B function as direct transporters of DDP.

ATP7A and ATP7B are important constituents of the Cu homeostasis system that has evolved to deliver Cu to Cu-requiring proteins while protecting the cells from toxic effects of Cu (Culotta et al., 1999). The major Cu uptake transporter is the copper transporter 1 (CTR1), which delivers Cu to pathway-specific chaperones such as ATOX1, CCS and COX17 for delivery to the secretory compartment, cytosol and mitochondria respectively (Culotta et al., 1999). An important feature of Cu transporters and chaperones is the presence of specific histidine-, methionine-, and cysteine-rich metal binding domains that selectively bind Cu(I) and exchange it with other Cu homeostasis proteins (Huffman and O'Halloran, 2001). Recent data indicates that the Cu homeostasis system also regulates the uptake, intracellular compartmentalization and efflux of DDP (Katano et al., 2003; Safaei and Howell, 2005; Samimi et al., 2004). Available data is consistent with the concept that DDP mimics Cu in being taken up by CTR1, distributed to various intracellular compartments by the Cu chaperones, and exported from tumor

cells by ATP7A and ATP7B. However, given the exquisite selectivity of the Cu homeostasis proteins for Cu relative to other metals, and for Cu(I) rather than Cu(II), it is not known if the effects of these proteins on the transport of DDP is direct or indirect.

ATP7B is abundantly expressed in liver and brain; mutations of ATP7B are the cause of Wilson's disease and the development of liver cancer in humans and animal models (Terada et al., 1998). ATP7B is highly similar in structure and function to the other human P1-type ATPase, ATP7A, whose mutations are the cause of Menkes disease (Mercer and Camakaris, 1997). ATP7A and ATP7B are located in the *trans*-Golgi and are known to mediate the efflux of Cu (Mercer and Camakaris, 1997; Terada et al., 1998). Like other P1-type ATPases, these proteins utilize the energy of ATP hydrolysis to transport Cu across the vesicular membrane via a process that involves the formation of a transient acylphosphate intermediate (Solioz and Vulpe, 1996). Other molecular features of ATP7A and ATP7B include the presence of six N-terminal metal binding domains with the core motif of CxxC, a conserved CPC metal binding motif in the sixth transmembrane domain and the ATP-binding and hydrolyzing domain (Solioz and Vulpe, 1996). While most of the N-terminal metal binding domains of ATP7A and ATP7B are not absolutely required for Cu transport, both, the catalytic unit and the CPC motif are essential as demonstrated both by *in vitro* studies (Lowe et al., 2004) and the fact that mutations in these domains cause Menkes and Wilson's diseases (Tumer et al., 1999).

The role of ATP7A and ATP7B in the regulation of cytotoxicity of DDP, carboplatin (CBDCA) and oxaliplatin (L-OHP) are well established. Over-expression of these transporters is associated with increased resistance of cells to DDP, CBDCA and in some cases L-OHP as the study of cultured cells and tumors have demonstrated

(reviewed by (Safaei and Howell, 2005)). Current data suggest that ATP7A and ATP7B are directly involved in the vesicular sequestration (Samimi et al., 2004) and efflux of Pt drugs (Katano et al., 2002).

In this study we sought to determine whether ATP7B can actually transport DDP across a lipid bilayer membrane or whether it operates to export DDP by simply binding DDP to the surface of secretory vesicles. Using vesicles from Sf9 cells that expressed either the wild type ATP7B or a form in which its transmembrane transport function had been disabled by mutating the CPC motif to CPA, we demonstrated that, like Cu, DDP binds to ATP7B, stimulates the formation of an acyl-phosphate intermediate, induces the hydrolysis of ATP and is transported into the vesicles in an ATP-dependent manner. We report here that DDP serves as a substrate for ATP7B, but is transported at a much slower rate than Cu.

MATERIALS AND METHODS

Cell culture and viral infection. The baculoviral constructs encoding the wild type ATP7B and the CPA mutant were developed as previously described by Dr. S. Lutsenko's laboratory (Tsivkovskii et al., 2002). Adherent Sf9 cells were grown in 2% fetal calf serum (Invitrogen, Carlsbad, CA) in HYQ® SFX-INSECT™ medium (Hyclone, South Logan, UT); suspension cultures were grown in serum-free medium. Sf9 cells were infected at an MOI of 1-2 for 72 h in all experiments.

Preparation and characterization of vesicles. Vesicles were prepared from infected Sf9 cells as previously described (Samimi et al., 2004). Briefly, cells were harvested by centrifugation at 1,000 x g for 10 min, resuspended in PBS and recentrifuged at 1,000 x g and then incubated for 10 min in 1 mM sodium bicarbonate at room temperature. Cells were homogenized with 100 strokes of a manual glass homogenizer and then centrifuged again at 1,000 x g for 10 min. Sucrose was added to the supernatant at final concentration of 1.2 M, which was then placed at the bottom of a 12 ml ultracentrifuge tube (Beckman, Fullerton, CA) and layered with 250 mM sucrose in 1 mM sodium bicarbonate. The tubes were centrifuged for 1 h at 140,000 x g in a SW40 or SW41 rotor in a Beckman ultracentrifuge. All solutions contained protease inhibitors (Complete tablets, Roche Pharmaceuticals, Nutley, NJ) at concentrations recommended by the manufacturer. The band of vesicles at the interface of 1.2 M and 250 mM sucrose was isolated and then subjected to another round of purification as described above. Vesicles were pooled and centrifuged at 140,000 x g for 1 h at 4 ^{o}C in histidine storage buffer (40 mM histidine, 100 mM $MgCl_2$, 250 mM sucrose, 5 mM KCl and 1 mM DDT). The vesicles were then resuspended in the histidine buffer containing

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50 µM DTT and 200 µM bathocuproinedisulfonic acid (BSC) and placed on ice for 1 h and then pelleted by another round of centrifugation for 1 h as above. The pellet was rinsed once with the histidine buffer and recentrifuged and then resuspended in the histidine storage buffer and kept at -80 ^{o}C until use. All vesicle preparations were analyzed by SDS gel electrophoresis and Coomassie Blue staining or Western blotting to quantify the expression of ATP7B. ATPase activity was assayed as reported by Takeda et al. (Takeda et al., 1999); inorganic phosphate was measured by using the ENZCheck kit (Invitrogen, Carlsbad, CA).

Western blotting. Vesicle and whole cell lysates were dissolved in lysis buffer (150 mM NaCl, 5 mM EDTA, 1 % Triton-X 100, 10 mM Tris, pH 7.4) and, following determination of their protein levels by Bradford reagents (Bio-Rad, Richmond, CA), were subjected to electrophoresis on 4-15% gels using 1-25 µg protein /lane. A Bio-Rad trans-blot system was used to transfer the proteins to Immobilin-P membranes (Millipore, Billerica, MA). Blots were incubated overnight at 4 ^{o}C in 4% dry non-fat milk in TBST buffer (tris buffered saline, 150 mM NaCl, 300 mM KCl, 10 mM Tris, pH 7.4 and 0.01% Tween 20) and with the polyclonal antibody against ATP7B (Novus Biologicals, Littleton, CO) for 1 h at room temperature. A horseradish peroxidase conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) was dissolved in 4% milk in TBST buffer and incubated with the blot for 1 h at room temperature. After three 15 min washes, blots were exposed to the ECL chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ) and detected on x-ray films (HyBlot CL, Denville Scientific, Inc. Metuchen, NJ).

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Transport of ^{64}Cu into vesicles. The transport assay was adopted from previous studies (Lowe et al., 2004; Voskoboinik et al., 1998). The assay buffer consisted of 40 mM histidine, 100 mM KCl, 10 mM $MgCl_2$ and 50 µM dithiothreitol (DTT). Vesicles (25-50 µg protein) were first equilibrated with the buffer for 5 min at 37 ^{o}C and then ATP was added to a final concentration of 5 mM following which the incubation was continued for 5-10 min before the addition of ^{64}Cu. $CuSO_4$ was prepared as 1 M stock solution in 100 mM HCl and stored for several weeks before being traced with ^{64}Cu. ^{64}Cu, at specific activity of ~50 µCi, was obtained from the Mallinckrodt Institute of Radiology (Washington University Medical School, St. Louis, MO). Reactions were performed in sextuplicates with and without ATP. The reactions were stopped by adding 10 volumes of ice-cold histidine buffer containing 50 mM EDTA. Free copper was removed by filtration through spin columns (Genesee Scientific, San Diego, CA) and centrifugation for 1 min at 5,000 x g. Following 4 washes, each with 400 µl of the assay buffer, the spin baskets were placed in scintillation tubes and the radioactivity determined by a Beckman gamma counter (model 5500 B).

Transport of DDP into vesicles. Transport of DDP was measured using the same protocol as for ^{64}Cu with the following exceptions. Vesicles corresponding to 100-300 µg of protein were used for each sample and the free DDP was separated from the vesicles at the end of each assay by the addition of 10 ml of ice-cold histidine buffer and centrifugation of the samples at 140,000 x g in a SW40 Beckman rotor for 30 min. The membrane pellets were resuspended in 10 ml histidine buffer and centrifuged again. After the second wash, the pellets were dissolved in lysis buffer (see above), an aliquot was taken for protein assay (Bradford method) and a fixed amount of the sample containing

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100-250 µg of protein was dissolved in nitric acid and processed for ICP-MS determination of Pt levels as previously described (Safaei et al., 2005). DDP was a gift from Bristol-Myers Squibb (Princeton, NJ).

[^{32}P]ATP Acyl-phosphate formation. The [γ-^{32}P] ATP phosphorylation of ATP7B was assayed according to the method described by Hung et al. (Hung et al., 2007). Reactions were carried out in 50 µl total volume on ice and with 50 µg of vesicle protein equilibrated with the phosphorylation buffer containing 20 mM MOPS (pH 6.8 or 4.6), 150 mM NaCl, 5 mM $MgCl_2$ and 50 µM dithiothreitol and various concentrations of $CuCl_2$, or DDP. The reaction was initiated by the addition of 1 µM [γ-^{32}P] ATP (10 Ci/mmol; Amersham Biosciences, Piscataway, NJ) and stopped at various time points by adding 50 µl of ice-cold 50% (w/v) trichloroacetic acid dissolved in 1 mM NaH_2PO_4. After incubation on ice for 15 min the samples were centrifuged at 4 ^{o}C at 20,000 x *g* for 10 min. The pellets were rinsed once with 50% trichloroacetic acid in 1 mM NaH_2PO_4 and then with 1 ml of distilled water and then dissolved in 1 ml of immunoprecipitation buffer (50 mM MOPS, pH 7.5; 150 mM NaCl; 10% glycerol; 2 mM β-mercaptoethanol, 0.1 % Triton X-100 and Roche Complete EDTA-free protease inhibitor tablet). Two µg of a polyclonal ATP7B antibody (Novus Biologicals, Littleton, CO) was added to each sample and incubation was continued overnight at 4 ^{o}C while rocking. 200 µl of immobilized protein A (Seize® Classic (A) immunoprecipitation kit, Pierce Scientific, Rockford, IL) was added to each sample and incubation was continued for 2 h at room temperature. Samples were washed with the immunoprecipitation buffer until all of the unbound protein was removed as documented by spectrophotometry (Beckman model Du 530). The immunoprecipitated phosphorylated ATP7B was then eluted in 150 µl of

elution buffer provided by the kit and aliquots of 25 µl were analyzed by SDS-PAGE; following fixation for 1 h in 10% acetic acid the gels were autoradiographed for 24-72 h at -70 °C. Some vesicle samples were treated with 200 µM BCS for 10 min prior to being incubated with [γ-^{32}P] ATP to chelate any residual Cu. In other reactions the specificity of the phosphorylation was tested by treating the samples for 10 min at room temperature with 250 µM hydroxylamine at the end of the reaction.

Statistics. Groups were compared using the Student t test assuming unequal variance. Estimates of K_m and V_{max} and curve fitting were made using Prism software (Prism Inc. Irvine, CA).

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RESULTS

Optimization of the Sf9 vesicle system for the transport of Cu. Vesicles were isolated from Sf9 cells that had been infected for 72 h with baculovirus, expressing either wild type ATP7B or a transport-defective mutant in which the CPC motif had been changed to CPA (referred to herein as the CPA mutant). As shown in Figure 1A, the level of expression of the wild type ATP7B or the CPA mutant was documented for each vesicle preparation using both Coomassie blue staining of gels and Western blotting. The different preparations were normalized based on their content of ATP7B as determined by densitometry of Western blots.

Cu-dependent ATPase activity. The vesicles expressing the wild type and the CPA mutant were assayed for Cu-dependent ATPase activity by measuring the difference in levels of inorganic phosphate released from ATP in the presence or absence of 2 μM Cu in samples that were previously treated with 250 μM of the Cu chelator BSC. As shown in Figure 1B, the Cu-dependent ATPase activity, measured at neutral pH, was present only in vesicles that expressed the wild type ATP7B. Vesicles expressing the CPA mutant had very little Cu-dependent ATPase activity; the level in these vesicles was similar to that in vesicles from un-infected Sf9 cells. The Cu-dependent ATPase activities, expressed in nmol/mg protein/min, were 9.9 ± 2.3 (SEM), 14.2 ± 2.2 (SEM), and 31.5 ± 2.1 (SEM) in vesicles from Sf9 cells, those that expressed the CPA mutant ATP7B or the wild type ATP7B, respectively. Thus, the wild type ATP7B exhibited significant levels of ($p < 0.02$) Cu-dependent ATPase activity whereas the CPA mutant form did not.

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ATP-dependent uptake of ^{64}Cu. To optimize the assay conditions for the transport of ^{64}Cu, 50 µg of the vesicles expressing wild type ATP7B were incubated for 5 or 10 min with 1-5 µM ^{64}Cu in the presence or absence of 5 mM ATP. The ATP-dependent uptake of ^{64}Cu into these vesicles was linear over the first 10 min and as shown in Figure 1C, at neutral pH, it showed saturation Michaelis-Menten kinetics. The estimated K_m was 3.4 ± 0.4 (SEM) µM and the V_{max}, 0.8 ± 0.5 (SEM) nmol Cu/mg protein/min. These values are similar to those previously reported for the vesicles expressing ATP7A (Voskoboinik et al., 1998). Figure 1D shows that, when exposed to 2 µM ^{64}Cu for 10 min, the ATP-dependent ^{64}Cu transport into vesicles that expressed wild type ATP7B was 2.7 ± 0.2 (SEM) -fold higher than in those from the control Sf9 cells ($p < 0.0007$). The transport into vesicles expressing the wild type ATP7B was 3.4 ± 0.1 (SEM) -fold higher than into those that expressed the CPA mutant ($p < 0.0005$). Thus, wild type ATP7B was capable of transporting ^{64}Cu while the CPA mutant was not, qualifying the assay system for the investigation of the role of ATP7B in the transport of DDP.

Effect of pH on the uptake of ^{64}Cu. To further validate the Sf9 vesicle assay system, we investigated the effect of pH on ^{64}Cu transport using the vesicles that expressed wild type ATP7B. As shown in Figure 2A, the rate of ^{64}Cu accumulation increased as the pH was reduced to 4.6. Figure 2B shows that, at pH 4.6, the ATP-dependent accumulation of Cu was 3.3 ± 0.1 (SEM)-fold higher in the vesicles expressing wild type ATP7B than in vesicles from uninfected Sf9 cells ($p < 0.007$), and 3.6 ± 0.5 (SEM)-fold higher than in those that expressed the CPA mutant ($p < 0.0002$). Thus, the ability of wild type ATP7B to transport Cu when exposed to 2 µM Cu was

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greater at pH 4.6 than 6.9, but the magnitude of the difference in the ^{64}Cu uptake by vesicles expressing wild type ATP7B or the CPA mutant did not vary with pH across this range. The rate of ATP-dependent uptake at pH 4.6 was measured over a range of Cu concentrations and was shown to follow Michaelis-Menten kinetics as shown in Figure 2 C. The K_m and V_{max} values for ATP-dependent ^{64}Cu transport at pH 4.6 were 6.3 ± 1.4 (SEM) μM and 1.0 ± 0.1 (SEM) nmol Cu/mg protein/min, respectively, which are both higher than those measured at pH 6.9 (see above, Figure 2 D). While the velocity of the enzyme was increased in lower pH, the affinity of the enzyme for Cu was reduced to some extent.

ATP7B-mediated transport of DDP. To determine whether DDP can actually serve as a substrate for APT7B-mediated transmembrane transport, vesicles expressing wild type ATP7B were incubated with 800 nM DDP in the presence or absence of ATP. As shown in Figure 3A, while ATP-dependent transport of DDP was detectable at pH 6.9, highest levels of DDP transport occurred at pH 3.6. At all pH levels examined the uptake was linear over the first 10 min. At pH 4.6, vesicles expressing wild type ATP7B transported DDP at a rate of 5.3 ± 0.5 (SEM) pmol/mg protein/min. ATP-dependent transport of DDP demonstrated saturation as the DDP concentration approached 3 μM (Figure 3B). The estimated K_m was 1.2 ± 0.5 (SEM) μM and the V_{max} was 0.03 ± 0.002 (SEM) nmol/mg protein/min. Thus, while DDP had a somewhat greater affinity than Cu for ATP7B it was transported at a much slower rate as evidenced by the fact that the V_{max} for DDP was nearly 28-fold lower than that for Cu.

The ATP-dependent transport of DDP at pH 4.6 into vesicles from uninfected Sf9 cells, and into vesicles expressing either the wild type ATP7B or the CPA mutant, is

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shown in Figure 3C. Whereas ATP-dependent transport of DDP was measurable in the vesicles expressing wild type ATP7B, no ATP-dependent transport was detected in vesicles isolated from uninfected Sf9 cells or those expressing the CPA mutant. No differences in transport were noted when an acetate rather than histidine-containing buffer was used indicating that, although histidine may interact with both Cu and DDP, any interaction that might have occurred did not modify transport under the conditions of this assay.

Binding of DDP to wild type and mutant ATP7B. To determine whether the failure of the mutant ATP7B to transport DDP was due to lack of binding of DDP to ATP7B, the vesicles were exposed to 2 μM DDP for 10 min in the absence of ATP. As shown in Figure 4, the amount of vesicle-associated DDP was 1.8 ± 0.01 (SEM) -fold higher in vesicles expressing the CPA mutant, and 2.0 ± 0.02 (SEM) -fold higher in vesicles expressing the wild type ATP7B, than in those containing no exogenous ATP7B ($p < 0.0002$ for both). However, there was no significant difference in the amount of DDP associated with vesicles expressing either of the two forms of ATP7B. This result indicates that the wild type and mutant forms of ATP7B, in both of which the N-terminal metal binding domains are intact, bind DDP equally well. Thus, the failure of the CPA mutant to transport DDP is not due to loss of binding capacity. In this respect DDP and Cu are similar as there was no significant difference in the level of ^{64}Cu that was bound to vesicles that expressed the wild type ATP7B as opposed to the mutant form of ATP7B in the absence of ATP.

Effect of DDP on the transport of Cu and *vice versa.* If DDP is either a substrate for the transport function of ATP7B, or is able to bind to the metal binding

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domains in the N-terminus of ATP7B, then it may interfere with the ability of ATP7B to transport Cu. The data presented in Figure 5A show that, when exposed to 2 μM ^{64}Cu, even at 0.5 nM, DDP significantly reduced ^{64}Cu accumulation. This suggests that, even at concentrations well below those attained in the plasma of patients receiving standard doses of DDP, the drug significantly disables the ATP7B-mediated transport of Cu. To determine whether Cu also inhibited the transport of DDP, vesicles expressing wild type ATP7B were incubated with 800 nM DDP at pH 4.6 in the presence of increasing concentrations of Cu. The data presented in Figure 5B indicate that inhibition of DDP transport occurred even at a Cu concentration of 0.1 μM and was maximal at 2 μM. Thus, Cu and DDP are able to inhibit each other's transport by ATP7B. The effect of Cu was mainly on ATP-dependent transport of DDP and not on the binding of DDP to ATP7B as evidenced by the relatively similar levels of DDP association with vesicles in the absence of ATP over the range of Cu concentrations tested. For example, when the amount of DDP associated with the vesicles in the absence of ATP was measured following incubation for 10 min, the level of DDP bound to the wild type vesicles was 25.4 ± 2.9 pmol/mg protein in the absence of Cu and 31.9 ± 3.2 pmol/mg protein in the presence of Cu. No information is currently available on whether DDP alters the binding of Cu in the absence of ATP.

Formation of transient acyl-phosphate from [γ-^{32}P] ATP in the presence of Cu and DDP. One of the hallmarks of the transport of Cu by ATP7B is the formation of a transient acylphosphate intermediate. To validate the vesicle system with respect to this endpoint we investigated the transient phosphorylation of ATP7B in the presence of various concentrations of Cu and at different time intervals. Since the transport of DDP

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was higher at pH 4.6, we assayed the acyl-phosphorylation of ATP7B during exposure to Cu at this pH. As shown in the left panel of Figure 6A, ATP7B very rapidly formed an acyl-phosphate intermediate in the presence of 800 nM Cu with the maximum signal occurring during the first 20 seconds of the incubation with [γ-^{32}P] ATP. This reaction was inhibited when the Cu chelator BCS was present at a concentration of 200 μM, and the phosphorylation was completely reversed by treatment with 250 μM hydroxylamine (data not shown) confirming that the signal corresponded to the acyl-phosphate structure. The highest level of ATP7B phosphorylation, in a 5 min assay period, occurred at a Cu concentration at 3 μM (Figure 6B, left panel).

To determine whether DDP, like Cu, was capable of inducing the formation of a transient acylphosphate form of ATP7B, vesicles expressing wild type ATP7B were exposed to increasing concentrations of DDP for various lengths of time. As shown in right hand panel of Figure 6A, DDP stimulated acyl-phosphorylation of ATP7B but at a much slower rate and to lower levels than that produced by Cu. Whereas Cu produced maximal acyl-phosphorylation at 20 s, at a concentration of 800 nM, DDP produced maximum acyl-phosphorylation of ATP7B at ~5 min (Figure 6A). Higher concentrations of DDP were also required to produce significant levels of acyl-phosphate intermediates when compared to Cu (Figure 6B).

DISCUSSION

Previous studies from this laboratory demonstrated that cells selected for DDP resistance often over-express ATP7B, that forced over-expression of ATP7B renders cells resistant to DDP (Katano et al., 2002; Katano et al., 2004; Komatsu et al., 2000; Samimi et al., 2004), and that fluorochrome-tagged DDP co-localizes with ATP7B in human ovarian carcinoma cells (Katano et al., 2004). Such over-expression of ATP7B is associated with increased amounts of DDP in the vesicular fraction of cells, and increased efflux of DDP (Katano et al., 2004). These prior studies suggested that, in a manner similar to its effect on Cu, ATP7B may serve to sequester DDP into the secretory export pathway either by binding the drug to the surface of ATP7B-expressing vesicles or by transporting the DDP into such vesicles. The results of the current study establish that DDP can indeed bind to ATP7B and become transported across the vesicular membrane by promoting the utilization of ATP.

The expression of either the wild type or a transport-deficient form of ATP7B at high levels in vesicles isolated from Sf9 cells provides a powerful system for studying the function of this transporter (Tsivkovskii et al., 2002). The Sf9 cells expressed substantial amounts of both forms of ATP7B and incorporated them into the vesicle membranes as had been previously documented (Tsivkovskii et al., 2002). The vesicles expressing wild type ATP7B were functional with respect to Cu transport as evidenced by Cu-dependent, BCS-inhibitable ATPase activity and their ability to accumulate ^{64}Cu in an ATP-dependent manner. In contrast, the vesicles expressing the CPA mutant lacked Cu-dependent ATPase activity and failed to transport Cu. In the presence of Cu, the wild type ATP7B was capable of forming a transient acylphosphate intermediate whereas the

mutant ATP7B was not. The results of this study confirm for ATP7B the utility of this model system that has previously been used to study the function of CCC2 (Lowe et al., 2004) and ATP7A (Hung et al., 1997).

A novel finding to emerge from this study was that the Cu transport by wild type ATP7B increased substantially as the pH was reduced from 6.9 to 4.6. The mechanism of this effect is not known, but in this respect ATP7B functions in a manner similar to other membrane ATPases such as those in the renal brush border (Eiam-Ong et al., 1993). It is likely that optimal transport of Cu into the vesicles requires the function of acid sequestering pumps such as the H^+-ATPase as previously demonstrated (Chavez-Crooker et al., 2001). It is also noteworthy that many prokaryotic and eukaryotic organisms increase the expression of ATP7B during acid exposure and as a result of acid adaptation (Reeve et al., 2002).

The vesicular uptake of Cu by wild type ATP7B was strongly inhibited by DDP even when DDP was present at a concentration 4,000 times lower than Cu; in the presence of 2 μM Cu the ATP-dependent transport of Cu was reduced to 31 ± 2 % upon addition of 0.5 nM DDP. DDP has been reported to inhibit other P-type ATPases such as the Na-K-ATPase (Sakakibara et al., 1999), the H^+-ATPase (Shiraishi et al., 2000), and the Mg^{2+}-ATPase (Bhatnagar and Ramalah, 1998). Rather than competing with the normal substrates for these transporters, it is likely that DDP disables these enzymes by binding to key thiol groups. The ability to bind DDP may underlie the increase in resistance to DDP that is observed following over-expression of some of these enzymes (for example see (Kishimoto et al., 2006). It is of substantial interest that DDP-associated nephrotoxicity is accompanied by severe Cu deficiency (DeWoskin and Riviere, 1992). It

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is also likely that Cu and DDP are uncompetitive inhibitors of each other's transport by ATP7B as is commonly found in multi-reactant systems. Further studies on the effects of DDP and Cu on each other's binding and transport are needed to elucidate the nature of this mutual inhibition.

The central observation of this study was that vesicles expressing either the wild type or the mutant ATP7B bound substantially more DDP than vesicles expressing no exogenous ATP7B in the absence of ATP; however, only the transport-proficient form of ATP7B mediated the ATP-dependent accumulation of DDP. Thus, at clinically relevant concentrations, DDP both binds to and is transported by ATP7B although the estimated V_{max} suggests that DDP is transported much more slowly than Cu. Since DDP is capable of reducing the transport of Cu at even very low concentrations, it appears likely that DDP may also interfere with its own transport by ATP7B and this may explain the discrepancy between its potency with respect to inhibition of Cu transport and the estimated K_m for its own transport. Cu was able to inhibit the ATP-dependent transport of DDP in a concentration-dependent manner with 50% inhibition being achieved at a Cu concentration of ~150 nM suggesting that Cu can modulate the transport of DDP with high efficiency. High resolution structural studies of the interaction of DDP with the metal binding and channel domains of ATP7B are now needed to determine how Cu and DDP regulate each other's transport.

Although native DDP is the dominant species in chloride-containing buffer, some DDP is found in a monoaquated form and reduction in pH favors the conversion of monoaquated DDP from the hydroxo to the aqua form (Martin, 2006). Thus, despite the short duration of this assay, it cannot be concluded with certainty that the native form of

DDP and not an aquated form of this drug is the substrate for ATP7B. As was the case for the transport of Cu, the transport of DDP was more extensive at a pH of 4.6 than at 6.9. Since DDP itself is not known to undergo significant structural changes over this pH range, it is likely that the effect of pH is primarily on the ATP7B rather than the substrate. Perhaps protonation of certain amino acid residues of ATP7B produces a conformational change in this protein that facilitates the transport of DDP. The ability of ATP7B to transport metalloids other than Cu has parallels to other Cu efflux pumps. ATP7A is known to bind other metals such as silver (Cobine et al., 2000), and the metallochaperone Atx1 has been shown to deliver cadmium and mercury to CCC2, the ATP7B equivalent in yeast (Morin et al., 2004). It has also recently been reported that ATP7A is capable of conferring resistance to Pb in astroglia in a manner that required the metal binding domains of this protein (Qian et al., 2005).

ATP7B is found in the membranes of secretory vesicles and it can clearly mediate resistance to DDP and enhance its intracellular sequestration (Safaei, 2006). In some types of cells this results in the accumulation of higher intracellular levels of DDP, presumably because the drug-loaded vesicles are not readily exported (Samimi et al., 2004). However, in other types of cells increased expression of ATP7B actually enhances the efflux of DDP (Katano et al., 2004). In principle, ATP7B could produce these effects either by simply binding DDP to the surface of vesicles destined for export, or by actually transporting DDP into the interior of such vesicles. The finding that very high levels of DDP associate with the ATP7B-expressing vesicles suggests that the ability of ATP7B to bind DDP may be an important feature of this protein. However, given the picomolar level of DDP accumulation in cells, it is evident from this data that ATP-dependent

vesicular sequestration/efflux of DDP by ATP7B is very likely to be adequate to explain the ability of ATP7B to modulate DDP cytotoxicity. Increased expression of ATP7A has also been shown to render cells resistant to DDP (Samimi et al., 2004). Although no information is currently available on the ability of ATP7A to transport DDP, given the structural similarity of ATP7A and ATP7B, and the widespread expression of ATP7A, this question is now of substantial interest.

While several studies indicate that ATP7A and ATP7B are capable of transporting Cu only in its reduced form (Cu(I)), data from other P-type ATPases such as Znt indicate that this class of enzymes may be capable of transporting other heavy metals when the metals are present in high concentrations (Liu et al., 2006). The observation that ATP7A- and ATP7B-transfected cells accumulate high levels of DDP in their vesicular compartment following exposure to low concentrations of the drug suggests that DDP also may be a substrate for these transporters (Katano et al., 2003; Samimi et al., 2003).

Molecular Pharmacology Fast Forward. Published on October 31, 2007 as DOI: 10.1124/mol.107.040980
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ACKNOWLEDGMENTS

We thank Mrs. Claudette Zacharia for help with the preparation of this manuscript. We are also grateful to Drs. Svetlana Lutsenko and Ruslan Tsivkovskii for the gift of baculovirus constructs.

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FOOTNOTES

Grant support: This work was supported by the NIH grant CA78648-08 and a grant from the Clayton Medical Research Foundation, Inc. The production of ^{64}Cu at Washington University School of Medicine was supported by the National Cancer Institute grant R24-CA86307.

FIGURE LEGENDS

Figure 1 Characterization of vesicles from Sf9 cells. A, Coomassie blue (left) and Western blot analysis (right) of 10 μg of lysate from a representative preparation of vesicles expressing no exogenous ATP7B (lane 1), vesicles expressing the CPA mutant (lane 2) and vesicles expressing the wild type ATP7B (lane 3). B, ATPase activity expressed as nmol of inorganic phosphate released per mg protein per min in the presence (gray bar) or absence (black bar) of Cu in vesicles expressing the wild type ATP7B. C, ATP-dependent uptake of ^{64}Cu measured at Cu concentrations ranging from 1-5 μM in vesicles expressing the wild type ATP7B. Assay of ^{64}Cu uptake was performed at pH 6.9. D, ATP-dependent uptake of 2 μM ^{64}Cu by vesicles expressing no exogenous ATP7B (open bar), the CPA mutant (Mutant, light gray bar) and vesicles expressing the wild type ATP7B (WT, dark gray bar) at pH 6.9. In all panels vertical bars indicate SEM of at least 3 independent experiments, number of samples for each data point per experiment is 6.

Figure 2 Effect of pH 4.6 on the ATP-dependent uptake of ^{64}Cu by vesicles expressing no exogenous ATP7B (Sf9), vesicles that express the CPA mutant (Mutant) and the vesicles that express the wild type ATP7B (WT). A, uptake ^{64}Cu into vesicles expressing the wild type ATP7B at different pH conditions. B, relative accumulation of ^{64}Cu measured at pH 4.6 into vesicles expressing no exogenous ATP7B (Sf9, open bar), vesicles expressing the CPA mutant (Mutant, light gray bar) and vesicles

expressing wild type ATP7B (WT, dark gray bar). C, kinetics of ^{64}Cu uptake into vesicles expressing the wild type ATP7B at pH 4.6. The curve is a Michaelis-Menten fit with K_m of 6.3 ± 1.4 (SEM) μM and V_{max} of 1 ± 0.1 (SEM) nmol/mg protein/min. Inset is the Lineweaver-Burk plot of the 1/V vs. 1/S. D, kinetics of ^{64}Cu uptake into vesicles expressing the wild type ATP7B at pH 6.9. The curve is a Michaelis-Menten fit with K_m of 3.4 ± 0.4 (SEM) μM and V_{max} of 0.8 ± 0.5 (SEM) nmol/mg protein/min. Inset is the Lineweaver-Burk plot of the 1/V vs. 1/S. In all panels vertical bars indicate SEM of at least 3 independent experiments, number of samples for each data point per experiment is 6.

Figure 3 ATP-dependent accumulation of DDP into Sf9 vesicles. A, effect of pH on accumulation of DDP by vesicles expressing the wild type ATP7B. B, kinetics of DDP uptake into vesicles expressing the wild type ATP7B at pH 4.6. the curve is a Michaelis-Menten fit with K_m of 1.2 ± 0.5 (SEM) μM and V_{max} of 0.03 ± 0.002 (SEM) nmol/mg protein/min. Inset is the Lineweaver-Burk plot of the 1/V vs. 1/S. C, relative accumulation of DDP into vesicles expressing no exogenous ATP7B (Sf9), vesicles expressing the CPA mutant (Mutant) and vesicles expressing the wild type ATP7B (WT). In all panels vertical bars indicate SEM of at least 3 independent experiments, number of samples for each data point per experiment is 6.

Figure 4 Association of DDP with isolated vesicles. Binding of 2 μM DDP to vesicles expressing no exogenous ATP7B (Sf9, open bars), vesicles

expressing the CPA mutant (Mutant, light gray) and vesicles expressing the wild type ATP7B (WT, dark gray) during a 10 min assay. Vertical bars indicate SEM of at least 3 independent experiments, number of samples for each data point per experiment is 6.

Figure 5 Inhibitory effects of DDP and Cu on the vesicular uptake of ^{64}Cu and DDP. A, Effect of increasing concentrations of DDP on the uptake of 2 μM ^{64}Cu by vesicles expressing the wild type ATP7B. B, effects of different concentrations of Cu on the uptake of 800 nM DDP by vesicles expressing the wild type ATP7B. In all panels vertical bars indicate SEM of at least 3 independent experiments, number of samples for each data point per experiment is 6.

Figure 6 Cu- and DDP-induced acyl-phosphate formation by wild type ATP7B (WT). A, time course in seconds (S) of γ[^{32}P]-ATP7B formation induced by 800 nM Cu (left panel) and DDP (right panel). B, formation of γ[^{32}P]-ATP7B in the presence of various concentrations of Cu (left panel) or DDP (right panel) over a period of 3 min. Each experiment was repeated at least 3 times.

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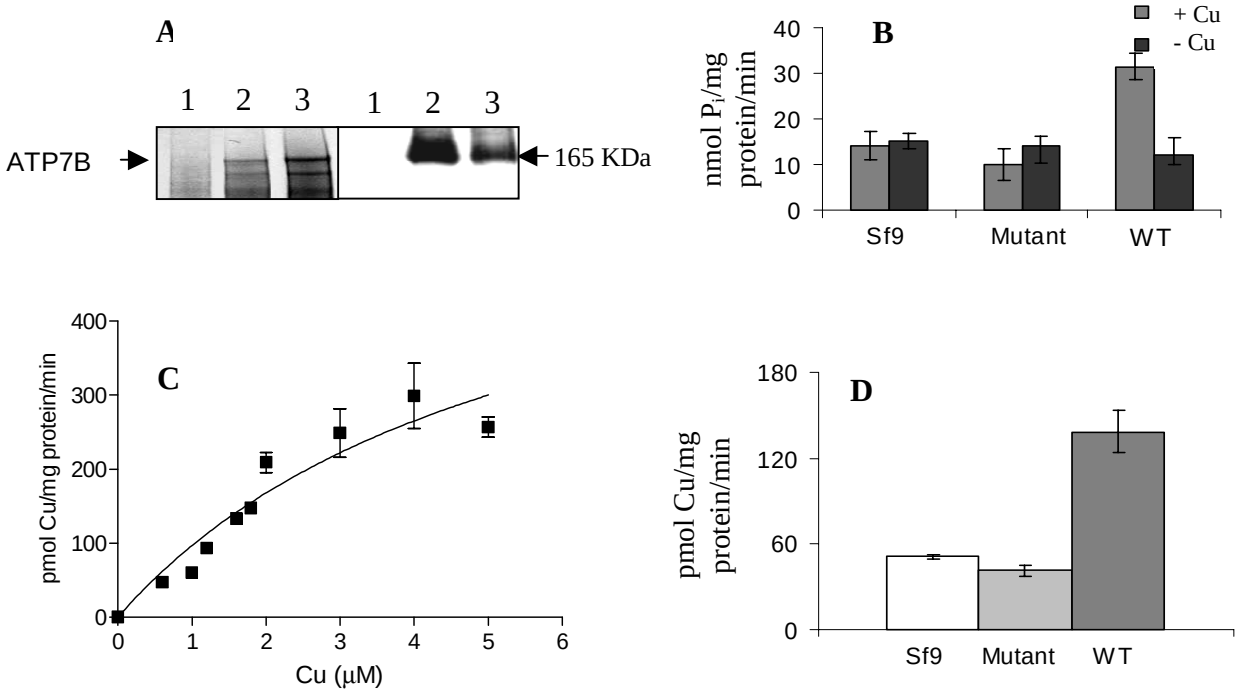

Figure 1

Figure 2

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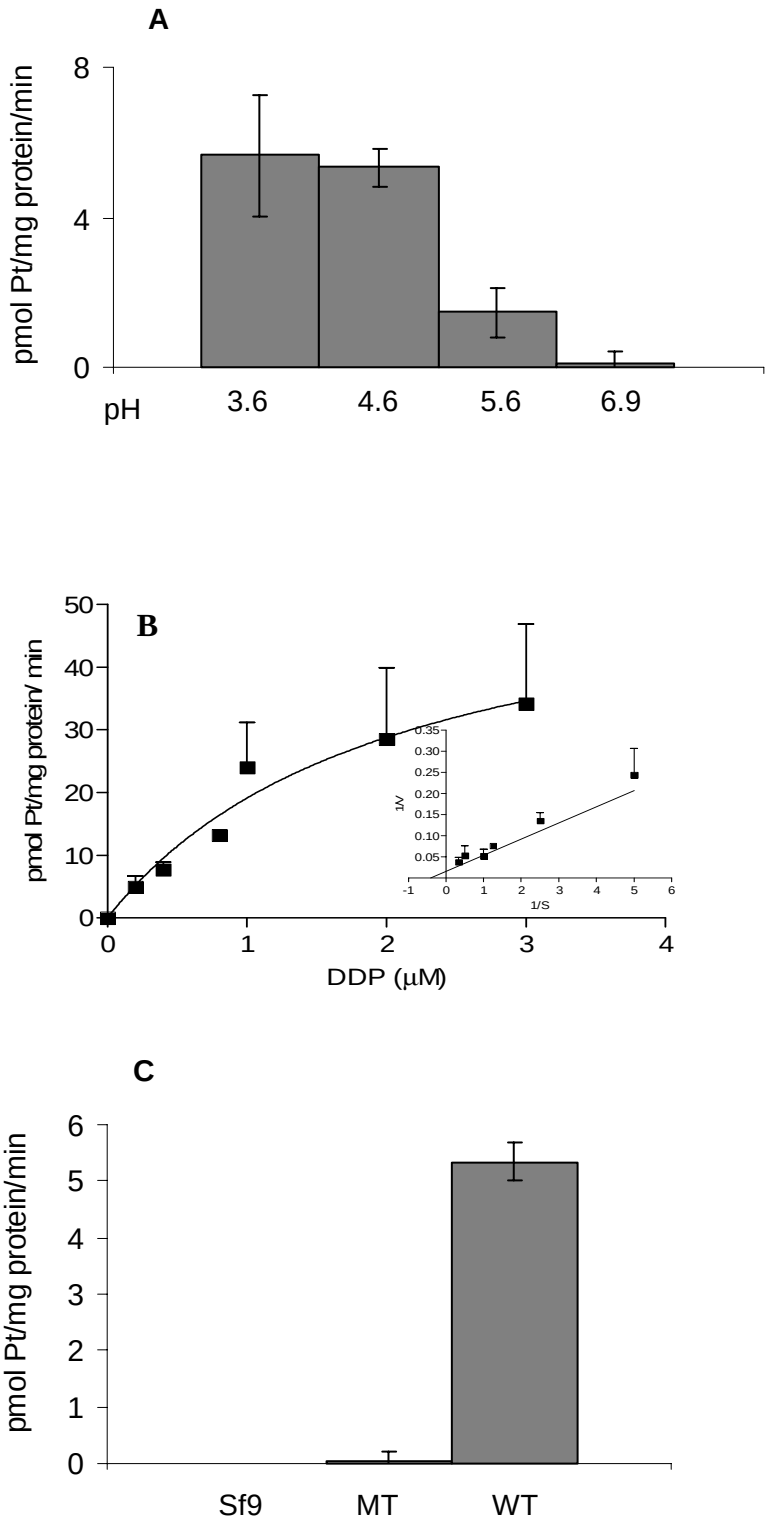

Figure 3

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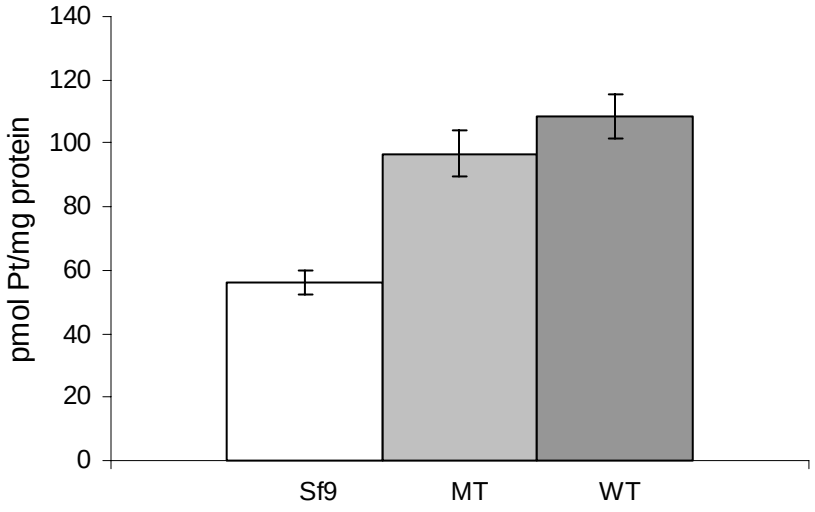

Figure 4

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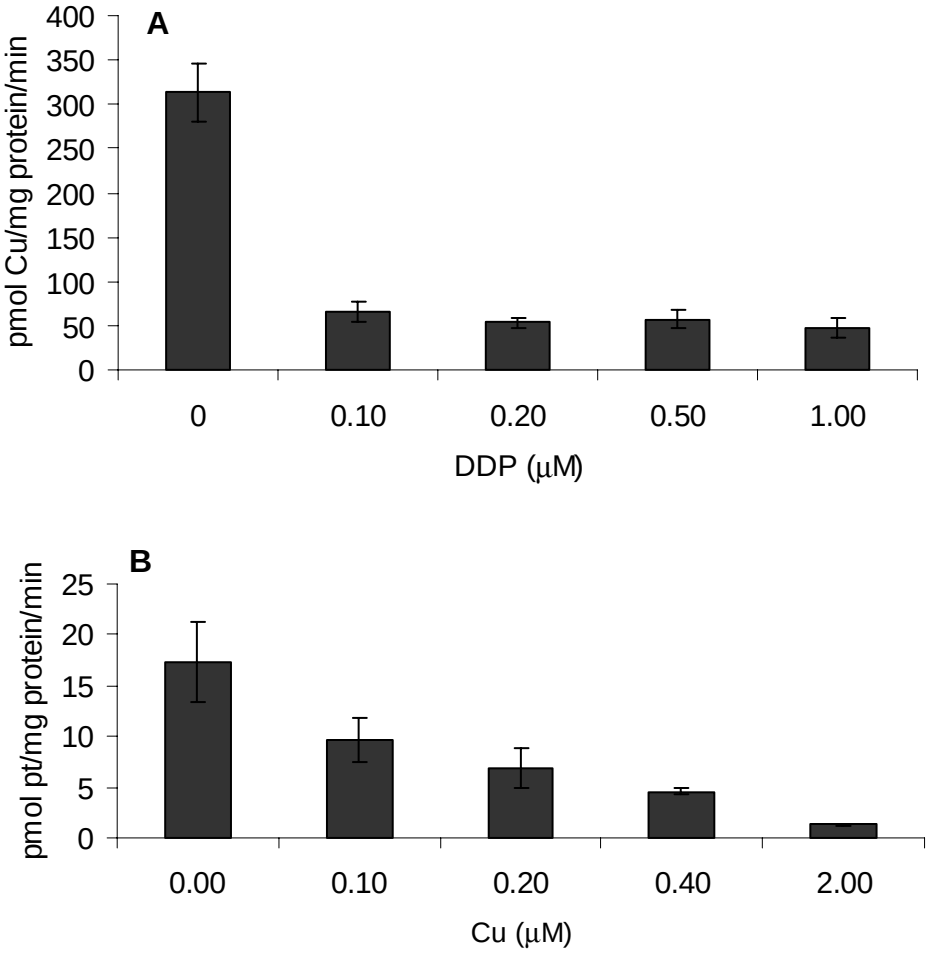

Figure 5

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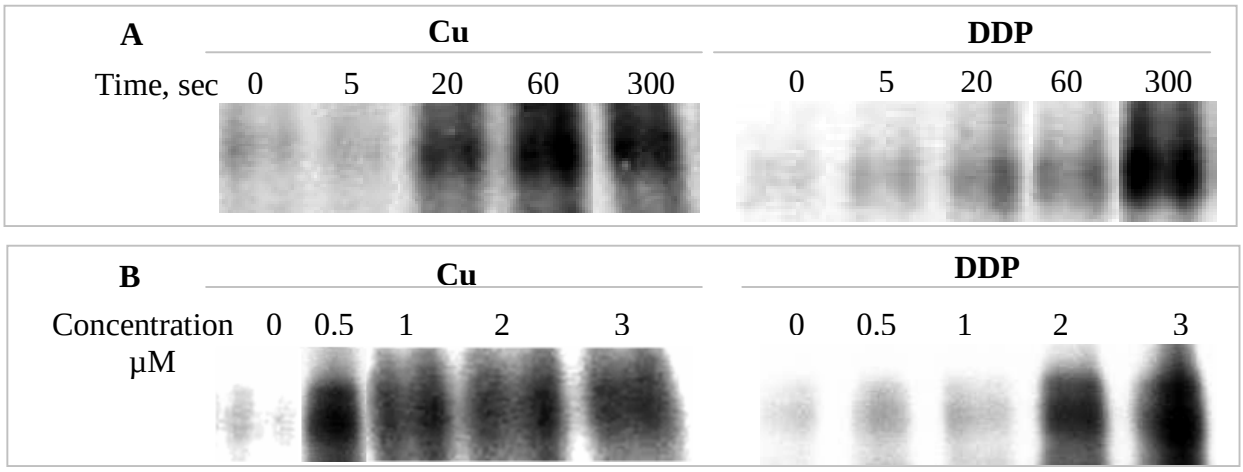

Figure 6