Effects of the loss of Atox1 on the cellular pharmacology of cisplatin

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1. Introduction

The mechanism of cellular accumulation of the anticancer drug cisplatin (CDDP) is often impaired in cells with acquired resistance to CDDP. Recent data has implicated the transporters of the copper (Cu) homeostasis system in the process of uptake and efflux of CDDP. In particular, a role for the Cu importer Ctr1 has been demonstrated by the observation that the absence of Ctr1 impairs the ability of cells to accumulate CDDP and thus increases the degree of resistance of cells to the cytotoxic effects of this drug [1–4]. A large body of evidence has also documented the involvement of the two Cu exporters, Atp7a and Atp7b, in the efflux or vesicular sequestration of CDDP and shown them to be regulators of the cytotoxic effect of CDDP [5–8]. While the exact mechanism by which these Cu transporters control the intracellular levels of CDDP remains to be discovered, available data confirm a role for the cysteine, histidine and methionine rich metal binding motifs which are found in these and several other Cu homeostasis proteins such as the metallochaperone Atox1 (antioxidant protein 1) [reviewed in [9]].

The metal binding domain of Ctr1 consists of a methionine rich motif (mets motif) that binds Cu and propels it into the central pore of a homotrimeric Ctr1 complex at the plasma membrane

The current study examined the role of Atox1 in the regulation of cellular pharmacology of CDDP using a pair of fibroblast cell lines established from Atox1+/+ and Atox1−/− mice. Atox1 is a metallochaperone that is known to play a central role in distributing Cu within the cells and was recently shown to act as a Cu-dependent transcription factor. Loss of Atox1 increased Cu accumulation and reduced efflux. In contrast, loss of Atox1 reduced the influx of CDDP within the cells and was recently shown to block the CDDP-induced down regulation of Ctr1. Ctr1 was found to be polyubiquitinated in an Atox1-dependent manner during CDDP exposure. In conclusion, Atox1 is required for the polyubiquitination of Ctr1 and the Ctr1-mediated uptake of CDDP.

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2. Materials and methods

2.1. Reagents

Cell culture media and sera were purchased from HyClone (Logan, UT). Antibodies to Ctrl were from Novus Biologicals (Littletown, CO), tubulin, from Sigma Co. (St. Louis, MO) and polyubiquitin conjugates (FK1 and FK2), from BIOMOL (Exeter, UK). 64Cu was purchased from Isotrace Technologies, Inc. (O’Fallon, MO). Other chemicals were purchased from Sigma Co. (St. Louis, MO) and Fish er Scientific Co. (Tustin, CA). CDDP (PLATINOL-AQ) was received as a gift from Bristol Laboratories (Princeton, NJ).

2.2. Cell culture and assay of sensitivity

Immortalized fibroblasts from Atox1+/+ and Atox1−/− mice were generously provided by Dr. J.D. Gitlin (Washington University, St. Louis, MO) [24]. These cells were maintained and propagated in DMEM supplemented with 10% fetal bovine serum and 250 μg/mL of G418. Sensitivity to the growth inhibitory effects of CDDP and Cu was determined by measuring total cellular protein using a Bio-Rad assay dye reagent (Bio-Rad, Richmond, CA) at the end of a 5 day drug exposure. Inoculation of 6000 cells per well of a 24-well plates and a growth period of 5 days in the presence of various concentrations of Cu or CDDP was found to yield the optimal dynamic range for the Atox1+/+ and Atox1−/− cells in this assay. After 5 days of growth, cells were washed with PBS (phosphate buffered saline) and then dissolved in situ in 100 μL of lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, 10 mM Tris, pH 7.4), which was subjected to protein determination. Protein levels were measured at a wavelength of 595 nm using a Benchmark Microplate Reader (BioRad, Richmond, CA).

2.3. Accumulation and efflux of 64Cu

Whole cell accumulation of Cu was measured by incubating 5 × 10^5 of Atox1+/+ or 7.5 × 10^5 Atox1−/− cells/well in 35 mm plates using basal medium containing 2 μM Cu traced with 1–8 × 10^6 cpm 64Cu. After a 24 h exposure to 64Cu, cells were washed three times with ice-cold PBS, lysed with PBS containing 1% SDS and analyzed for 64Cu accumulation by using a Beckman 5500B Gamma Counter. For efflux, cells were loaded with 2 μM Cu traced with 1–8 × 10^6 cpm of 64Cu for 24 h and then medium was removed and cells were allowed to efflux for 0, 5, 10, 15, 30 and 60 min after which the levels of radioactivity were determined as described above. The Cu levels were normalized to the protein levels in each lysate sample.

2.4. Accumulation of CDDP in whole cell, subcellular fractions and assay of efflux of CDDP

For whole cell accumulation of CDDP 5 × 10^5 of Atox1+/+ and 7.5 × 10^5 Atox1−/− cells were inoculated l in 35 mm plates. CDDP was added to the culture medium to the final concentration of 2 μM. At different time points following CDDP exposure cells were rinsed quickly with three changes of ice-cold PBS and then lysed with 250 μL of lysis buffer (0.25% Nonidet P-40 in 100 mM Tris HCl, pH 8.0). An aliquot was set aside for protein determination and another 200 μL aliquot was mixed with 125 μL of 70% analytical grade nitric acid, heated to 65 °C overnight, diluted with 3 mL of distilled water containing 1 part per million indium (Acros Organics, Tustin, CA) and subjected to Pt measurements using a Thermo Finnigan ICP-MS (inductively coupled plasma mass spectrometry, Element 2 at the Analytical Facility at the Scripps Insti-
Biotechnology, Inc. (Rockford, IL) and then probed with a monoclonal anti-tubulin antibody from Sigma (St. Louis, MO) according to the protocol provided by the vendor. A ChemilumagerTM 4400 instrument (Alpha Innotech, San Leandro, CA, USA) was used for determining the density of protein bands.

2.7. Confocal microscopy

Cells were cultured on coverslips and allowed to attach overnight. The cells were then exposed to either 10 μM CDDP or 300 μM Cu for 15 min after which they were quickly rinsed with cold PBS and fixed with 3.7% formaldehyde in PBS for 10 min. The cells were permeabilized with 0.3% Triton-X 100 in PBS for another 10 min, blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 1 h and then incubated with a polyclonal antibody against Ctr1 or monoclonal antibodies against polyubiquitin for 12 h. Following three 15 min rinses with PBS, cells were stained with an Alexa Fluor 488 conjugated anti-rabbit secondary antibody (Molecular Probes, Seattle, WA) or a Texas red conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) along with the nuclear marker Hoechst 33342 (Molecular Probes, Seattle, WA). Cells were then washed for 15 min three more times and then mounted on slides with Gelvatol and processed for microscopy with a DeltaVision deconvolution microscope system (Applied Precision, Inc., Issaquah, WA) at the UCSD Cancer Center’s Digital Imaging Shared Resource Facility as previously described [28].

2.8. Flow cytometry

Atox1+/+ and Atox1−/− cells were cultured on 150 mm flasks until 70% confluent and then exposed to 2 μM CDDP for 15 min. The cells were quickly rinsed with cold PBS before harvesting them with trypsin and centrifuging at 1000g for 10 min. The cells were resuspended in cold PBS, fixed for 10 min with 3.7% formaldehyde in PBS, permeabilized for 10 min with 0.3% Triton-X 100 in PBS and then treated for 1 h with 1% BSA in PBS prior to incubation for 12 h with antibodies against Ctr1. After three 15 min washes with PBS, cells were incubated for 1 h with an Alexa Fluor 488-tagged secondary antibody (Molecular Probes, Seattle, WA). After washing the secondary antibody three times, 15 min each, with PBS, the cells were assayed for fluorescence using a BD FACScalibur instrument at the Flow Cytometry Shared Resource of the UCSD Cancer Center.

2.9. Immunoprecipitation with antibodies to Ctr1 or polyubiquitin conjugates

Whole cell extracts were prepared using lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, 10 mM Tris, pH 7.4) containing protease inhibitor tablets (Sigma, St. Louis, MO). The cell lysates were clarified by centrifugation at 10,000 g for 10 min at 4 °C and then were diluted to the final concentration of 100 μg/mL with binding buffer (50 mM Tris, 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) [29] to which protein A-Sepharose (10 μL/mL) was added and incubation continued for 1 h at room temperature. The samples were centrifuged at 1000g for 10 min at 4 °C and then 2 μg/mL of the primary antibody (anti-Ctr1, PK1 or PK2) was added and incubation continued overnight at 4 °C. The immune complexes were captured by adding 10 μL/mL of protein A-Sepharose (Thermo Scientific, Waltham, MA) for 1 h at 4 °C followed by washing five times in binding buffer. Proteins were eluted with elution buffer provided by Thermo Scientific and the samples were separated on a 4–15% polyacrylamide gels and electroblotted as described above.

2.10. Assay of 20S proteasome

The chymotrypsin-like proteasome activity was measured using the 20S Proteasome Assay Kit from Calbiochem (Gibbstown, NJ). Briefly, triplicate assays were performed in a reaction mixture that contained 178 μL of reaction buffer (25 mM HEPES and 0.5 mM EDTA, pH 7.6), 10 μL of substrate (10 μM Suc-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC)), 2 μL of SDS (0.03%) and 10 μL of cell lysate (25 μg of protein). After incubation for 30 min at 37 °C the fluorescence of liberated AMC was measured every 5 min for 30 min using excitation and emission wavelengths at 340 and 450 nm, respectively by a TECAN Infini M200 plate reader (Durham, NC). Estimates of the slope and curve fitting were made using Prism software (Prism Inc. Irvine, CA).

2.11. Statistical analysis

Groups were compared using the Student t-test assuming unequal variance.

3. Results

3.3. Effects of the loss of Atox1 on the toxicity and cellular pharmacology of Cu

These studies utilized a pair of isogenic mouse embryo fibroblasts established from either wild type mice (Atox1+/+) or mice in which both alleles of Atox1 had been deleted (Atox1−/−). Sensitivity to the cytotoxic effect of Cu was assessed by examining the effect of increasing concentrations of Cu on the growth rate of the Atox1+/+ and Atox1−/− fibroblasts over a period of 5 days. The data presented in Fig. 1A was obtained from five independent assays, each performed with triplicate cultures for each Cu concentration. The IC50 values were 337 ± 22 μM for the Atox1+/+ cells and 276 ± 9 μM for the Atox1−/− cells (p < 0.03) indicating that the loss of Atox1 was accompanied by a small increase in the sensitivity of cells to Cu.

In order to investigate the effect of the loss of Atox1 on the accumulation of Cu, Atox1+/+ and Atox1−/− cells were exposed to 2 μM 64Cu for 24 h, by which time uptake had reached steady-state; the amount of intracellular 64Cu was quantified by scintillation counting. As shown in Fig. 1B, the 64Cu level in the Atox1−/− cells was 1.8-fold higher (324 ± 16 pmol/mg protein) than that in the Atox1+/+ cells (174 ± 4 pmol/mg protein) (p < 0.0001). This difference was similar to that previously reported [25]. Results of the study of the efflux of Cu in the Atox1+/+ and Atox1−/− cells were also consistent with previously reported data [25]; Atox1+/+ cells effluxed Cu at a significantly faster rate than the Atox1−/− cells (Fig. 1C). At 30 min, the Atox1+/+ cells retained 72 ± 5% of their initial load of Cu, while Atox1−/− cells retained 97 ± 3%; at 60 min, Atox1+/+ cells only 33 ± 8% of their initial load, while the Atox1−/− cells still retained 53 ± 8% (p < 0.03). Thus, loss of Atox1 increased the steady-state level of Cu and this was associated with reduced efflux.

3.2. Effects of the loss of Atox1 on the cellular pharmacology of CDDP

As shown in Fig. 2A, the Atox1+/+ cells were slightly more sensitive to the cytotoxic effects of CDDP than the Atox1−/− cells. The IC50 values for Atox1+/+ and Atox1−/− cells were, respectively, 1.0 ± 0.1 and 1.5 ± 0.1 μM (p < 0.02). Thus, although the difference in sensitivity was small, the loss of Atox1 had opposite effects on sensitivity to the growth inhibitory effects of Cu and CDDP, rendering cells more sensitive to Cu but more resistant to CDDP.

To determine whether the increased resistance of the Atox1−/− cells to CDDP was due to altered intracellular accumulation of the
drug, the uptake of CDDP in the Atox1+/+ and Atox1−/− cells was measured after exposure of the cells to 2 μM CDDP for 1 and 5 min, and 1 and 24 h. As shown in Fig. 2B, the level of Pt accumulation in the Atox1+/+ cells was significantly higher than in the Atox1−/− cells. The difference was already apparent at 1 min at which point the Atox1+/+ cells had accumulated 3.4 ± 0.1 pmol Pt/mg protein, whereas the Atox1−/− cells had accumulated 2.6 ± 0.3 pmol Pt/mg protein (p < 0.01). At 5 min the levels were 4.2 ± 0.1 and 2.4 ± 0.5 pmol Pt/mg protein for the Atox1+/+ and Atox1−/− cells, respectively (p < 0.004). The difference in accumulation persisted at 1 and 24 h. At 1 h the levels were 72.9 ± 12.8 and 45.1 ± 5.3 pmol/mg protein (p < 0.01), and at 24 h 167.0 ± 18.2 and 103.3 ± 23.4 pmol/mg protein (p = 0.03) for the Atox1+/+ and Atox1−/− cells, respectively. At 24 h the Atox1−/− cells had accumulated only 62% as much CDDP as the Atox1+/+ cells. Thus, as in the case of sensitivity to the cytotoxic effects of Cu and CDDP, the loss of Atox1 had a different effect on the uptake of CDDP in comparison to that of Cu.

To determine whether the lower level of CDDP accumulation in the Atox1−/− cells was due to a change in the ability of cells to efflux CDDP, we measured the efflux of CDDP from Atox1+/+ and Atox1−/− cells over a period of 1 h after the cells had been loaded with 2 μM CDDP for 24 h and then washed and re-incubated in drug-free medium. As shown in Fig. 2C, there was no clear difference in the ability of the Atox1+/+ and Atox1−/− cells to efflux CDDP. After 60 min of efflux the Atox1−/− cells retained 85.2 ± 0.9%,
and the Atox1+/− cells retained 80.8 ± 2.4%, of their respective initial loads of CDDP. Thus, the Atox1 protein appears to function primarily in the influx rather than the efflux of CDDP.

To assess the effect of the loss of Atox1 on the intracellular distribution of CDDP Atox1+/+ and Atox1+/− cells were exposed to 2 μM CDDP for 24 h and then subjected to subcellular fractionation using differential centrifugation. The cytosolic, microsomal and nuclear fractions were isolated and the percentage of the whole cell Pt found in each fraction was determined. As seen in Table 1, there were clear differences between the Atox1+/+ and Atox1+/− cells in the intracellular distribution of Pt. When calculated as pmol Pt/mg protein of each fraction, the cytosolic fractions of the two cell types had similar levels of Pt but the microsomal and nuclear fractions of the Atox1+/+ cells had significantly higher levels of Pt than those of the Atox1+/− cells. However, when the data was calculated as a percentage of the whole cell Pt content, no significant differences between the Atox1+/+ and Atox1+/− cells could be detected. Thus, the loss of Atox1 reduced the amount of Pt in the microsomal and nuclear compartment relative to their protein content but not the fractional distribution suggesting that loss of Atox1 had primarily affected the influx of CDDP.

Since the higher degree of resistance of Atox1+/− cells to treatment with CDDP was consistent with the lower accumulation of CDDP in DNA we measured the levels of Pt–DNA adducts in the two cell types following exposure to 2 μM CDDP for 24 h. As shown in Fig. 2D, the loss of Atox1 was associated with a reduced level of Pt in DNA. The Atox1+/− cells accumulated 34.4 ± 6.7 pmol Pt/mg DNA whereas the Atox1+/− cells accumulated only 18.8 ± 5.5 pmol Pt/mg DNA (p < 0.02). The magnitude of the difference in DNA adducts was in the same range as the difference in whole cell uptake of CDDP.

In summary, loss of Atox1 was associated with a small degree of resistance to CDDP, reduced influx and accumulation in the whole cell, the nuclear compartment and in the DNA but did not change the efflux of CDDP.

### 3.3. Effect of loss of Atox1 on Cu- and CDDP-induced down regulation of Ctrl

Since the primary consequence of the loss of Atox1 was a reduction in the uptake of CDDP, we examined the effect of the loss of Atox1 on the initial influx of CDDP mediated by Ctrl. Ctrl is the major Cu influx transporter and has been shown to mediate the initial phase of CDDP influx in human ovarian carcinoma cells [2]. Exposure to either CDDP or Cu has been shown to rapidly down regulate Ctrl expression in several cell types [30]. We examined the levels of Ctrl in the Atox1+/+ and Atox1+/− cells before and after exposure to CDDP. A Western analysis of the Ctrl+/+ and Atox1+/− cells is shown in Fig. 3A; in both cell types the monomorphic form of Ctrl1 was detected as a band migrating at ~28 kDa. In untreated cells, the levels of Ctrl1 were similar relative to the lane loading controls. However, when the Atox1+/+ and Atox1+/− cells were exposed for 15 min to 10 μM CDDP or 300 μM Cu, only the Atox1+/+ cells exhibited a CDDP- or Cu-induced down regulation of the Ctrl1 (Fig. 3A). Exposure of Atox1+/− cells to either compound resulted in Ctrl1 expression levels that were unchanged from those found in the untreated cells. Therefore, while both CDDP and Cu triggered the down regulation of Ctrl1 only in the Atox1+/+ cells, neither reduced Ctrl1 in the Atox1+/− cells.

To assess the subcellular localization of Ctrl1 in Atox1+/+ and Atox1+/− cells before and after exposure to CDDP and Cu, we examined the cells by confocal fluorescence microscopy after exposing them to 10 μM CDDP or 300 μM Cu for 15 min prior to staining with an antibody to Ctrl1. As shown in Fig. 3B, while the overall subcellular distribution of Ctrl1 was not visibly different in the two cell lines prior to drug treatment, a 15 min exposure to 10 μM CDDP or 300 μM Cu triggered down regulation of Ctrl1 only in the Atox1+/+ cells. This result was confirmed by a flow cytometric examination that demonstrated the reduction of Ctrl1 levels in the Atox1+/+ but not in Atox1+/− cells following exposure to 2 μM CDDP; exposure to CDDP in fact produced a small increase in Ctrl1 level in the Atox1+/− cells (Fig. 3C). This result suggests that the reduced ability of Atox1+/− cells to accumulate CDDP may be due to the inability of CDDP to trigger the endocytotic process that putatively brings the CDDP into the cell and that accompanies the down regulation of Ctrl1 during CDDP exposure.

A further assessment of the effect of the loss of Atox1 on the CDDP- and Cu-induced down regulation of Ctrl1 was made by assaying the uptake of 64Cu following a 15 min treatment of cells with 10 μM CDDP. We reasoned that since Ctrl1 is the major transporter of Cu, its down regulation by CDDP should diminish the levels of 64Cu uptake to a greater extent in the Atox1+/+ cells than the Atox1+/− cells. Cu accumulation in the CDDP-treated Atox1+/+ cells was 54.0 ± 1.3% of that in untreated Atox1+/ cells while Cu accumulation in the CDDP-treated Atox1+/− cells was 83.3 ± 2.4% of that in untreated Atox1+/− cells (p < 0.01 in both cases). Thus, the functional down regulation of Ctrl1 by CDDP was less severe in the Atox1+/− than Atox1+/+ cells.

### 3.4. Effect of the loss of Atox1 on the proteasomal degradation of Ctrl1

Current evidence suggests that Ctrl1 transports Cu and CDDP by different mechanisms. In mouse fibroblasts and human ovarian carcinoma cells CDDP causes rapid down regulation of Ctrl1 even at very low concentrations (1–2 μM) while Cu achieves this only at very high concentrations (>200 μM). Previous data indicates that the CDDP- and Cu-induced down regulation of Ctrl1 involves non-clathrin mediated endocytosis followed by degradation in the 26S proteasome. Proteasomal degradation often involves the formation of ubiquitinated intermediates that serve to direct the molecule to the proteasome. To determine whether Ctrl1 is ubiquitinated during CDDP- and Cu-induced degradation, Atox1+/+ and Atox1+/− cells were incubated for 4 h with the proteasome inhibitor bortezomib at a concentration of 40 nM and then exposed to 300 μM Cu or 10 μM CDDP for 15 min. The cells were then stained with antibodies to Ctrl1 and polyubiquitin. As shown in Fig. 4, confocal microscopic examination showed that the extent of co-localization of the Ctrl1 and polyubiquitin signal, shown by the yellow color, was much greater in the Cu- and CDDP-treated Atox1+/+ cells than in the equivalently treated Atox1+/− cells. In

### Table 1

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<tr>
<th>Cell fraction</th>
<th>Percent of total Pt in fraction, mean ± SEM</th>
<th>pmol Pt/mg protein, mean ± SEM</th>
<th>p Valuea</th>
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<tr>
<td></td>
<td>Atox1+/+ cells</td>
<td>Atox1+/− cells</td>
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<tr>
<td>Cytosol</td>
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<td>73.9 ± 6.6</td>
<td>21.4 ± 0.4</td>
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<tr>
<td>Microsomal</td>
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<td>6.1 ± 0.9</td>
<td>221.8 ± 10.9</td>
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<tr>
<td>Nuclear</td>
<td>24.0 ± 4.6</td>
<td>20.0 ± 4.8</td>
<td>318.5 ± 21.6</td>
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a For difference in Pt content.
addition, in the Atox1−/− cells CDDP altered the patter of co-localization of Ctr1 and polyubiquitin more extensively than Cu. This analysis demonstrated a role for Atox1 in this process as very little co-localization of Ctr1 and the polyubiquitin signal was observed in the Atox1−/−/− cells under any of the conditions examined.

Western blot analysis of the total lysates from Atox1+/+ and Atox1−/− cells treated with bortezomib prior to exposure to 10 μM CDDP using antibodies against polyubiquitin conjugates indicated that the levels of a wide range of polyubiquitinated proteins were much higher in the Atox1+/+ than the Atox1−/− cells following CDDP exposure (data not shown). To demonstrate that Ctr1 becomes ubiquitinated during exposure to CDDP, and provide further evidence for a difference in ubiquitination between the Atox1+/+ and Atox1−/− cells, the two cell lines were treated for 4 h with bortezomib and then with 10 μM CDDP for an additional 15 min prior to preparing lysates for immunoprecipitation with antibodies against Ctr1 or polyubiquitin conjugates. As shown in
the left panel of Fig. 5A, when equal amounts of protein (1.7 μg) immunoprecipitated by the Ctr1 antibody were resolved on SDS gels and probed with anti-polyubiquitin antibody a polyubiquitinated protein of ~130 kDa and several lower molecular weight forms were detected. The intensity of these bands was lower in the Atox1+/− cells. Their intensity increased in response to CDDP exposure in the Atox1+/+ cells but not in the Atox1−/− cells. Similar results were obtained when cell lysates from bortezomib and CDDP-treated cells were immunoprecipitated with anti-polyubiquitin antibodies and probed on Western blots with anti-Ctr1 antibodies (Fig. 5A, right panel). Thus, the level of polyubiquitinated Ctr1 increased in Atox1+/+ cells following exposure to CDDP whereas it decreased in the Atox1−/− cells under similar conditions. This result establishes that Ctr1 does become polyubiquitinated, and that this is defective when Atox1 is deficient.

4. Discussion

The results of this study demonstrate that, like the Cu transporters Ctr1, Atp7a and Atp7b, the metallochaperone Atox1 also regulates the cellular pharmacology of CDDP. Key findings from this study are that Atox1 regulates the intracellular accumulation, compartmentalization and cytotoxicity of CDDP by mechanisms that are distinct from those that are involved in Cu homeostasis, as is evidenced by the effects of the loss of Atox1 on the uptake rather than efflux of CDDP.

Atox1 is known to regulate Cu levels by facilitating its efflux through a process that involves receiving Cu from Ctr1 and handing it to the exporters Atp7a/b in the trans-golgi compartment via intimate protein–protein interactions. The effect of loss of Atox1 on the cellular pharmacology of Cu observed in this study is consistent with this prior understanding of the role of this protein as an acceptor and donor of Cu [25]. Thus, loss of Atox1 resulted in greater accumulation of Cu, reduced efflux and had a small increase in the sensitivity of the cells to Cu. These results provided excellent validation that the pair Atox1+/+ and Atox1−/− cells used in this study can serve as a reliable model system from which to assess the influence of Atox1 on the cellular pharmacology of CDDP.

The effect of the loss of Atox1 on the cellular pharmacology of CDDP was quite distinct from the effect on Cu suggesting that Atox1 functions differently with respect to the two metalloids. Whereas loss of Atox1 primarily affected the efflux of Cu, it significantly impaired the accumulation of CDDP. The reduced ability of the Atox1−/− cells to accumulate CDDP was reflected in the higher degree of resistance of these cells to CDDP and their lower accumulation of Pt–DNA adducts. It is surprising, however, that the degree
of resistance of Atox1/−/− cells was quite small; one reason may be that Atox1 has several and perhaps opposing regulatory functions in cells as is evidenced by the involvement of this protein in transcription [31] and oxido-reduction processes [32,33]. Loss of Atox1 is known to affect the expression of Atp7a [25] and SOD3 [34] and can be reasonably expected to affect the level of Cu-regulated proteins such as XIAP [35] and p53 [36]. In addition, Atox1 can potentially affect the cellular responses to CDDP through Cu-dependent processes; the role of Cu-containing antioxidants in conferring CDDP resistance to cells is well documented [37].

The higher levels of CDDP accumulation found in the Atox1+/+ cells, particularly in the nuclei and in the vesicular compartment which are known to associate with the Atox1, suggests that Atox1 may be a CDDP-binding protein. However, since the microsomes and nuclei of both cell types accumulated similar fractions of the total cellular CDDP, it appears that the main role of Atox1 is in the influx of CDDP rather than in subcellular compartmentalization of the drug.

Previous data from this laboratory have demonstrated that Ctrl regulates the initial uptake of CDDP through a mechanism that involves macropinocytosis linked to subsequent rapid degradation of Ctrl [38]. The prior studies also demonstrated that the rapid disappearance of Ctrl from the plasma membrane and its subsequent degradation following exposure to CDDP can be blocked when macropinocytosis and proteasomal activity are pharmacologically or biologically disabled [30]. The observation that loss of Atox1 impairs the ability of CDDP to down-regulate Ctrl is novel and indicates that Atox1 plays a central role in the process of CDDP-induced Ctrl internalization and degradation. The reduced uptake of CDDP in the Atox1/−/− cells is consistent with the concept that Ctrl mediates CDDP uptake by binding the drug extracellularly and internalizing it by macropinocytosis. The fact that the down regulation of Ctrl produced by pre-treatment of cells with CDDP resulted in a lower uptake of 64Cu confirms that the down regulation of Ctrl by CDDP is physiologically significant. Furthermore, the fact that pre-treatment of cells with CDDP produced a greater effect on 64Cu uptake in the Atox1+/+ than the Atox1/−/− cells confirms that Atox1 plays a role in this internalization process. How then does Atox1 regulate the influx of CDDP via Ctrl? Previous studies have demonstrated that Atox1 interacts with the C-terminal end of Ctrl [21]. It is likely that this interaction facilitates the internalization, ubiquitination and subsequent degradation of Ctrl by the proteasome. Evidence that Atox1/−/− cells are defective in the ubiquitination and degradation of Ctrl confirms this view. The interaction of Atox1 with the Ctrl may not be required for the influx of CDDP which seems to be unimpaired in Atox1/−/− cells; however, Ctrl degradation in response to Cu nevertheless occurs when the Cu concentration is very high and this suggests that, through Atox1, Ctrl serves as a sensor of toxic metals in the cellular environment. It is intriguing to speculate that the absence of Atox1 may also influence the function of the Ctrl in the FGF signaling mechanisms which were recently demonstrated in Xenopus [39].

The observation that a mammalian Ctrl is ubiquitinated is new and confirms previous data from yeast [40]. The mouse Ctrl has two lysine residues at the C-terminal end (residues 185 and 186, accession # CH466527.2) that may serve as the interaction site for ubiquitination enzymes. The observation that Atox1 is required for the polyubiquitination of Ctrl is also novel and provides evidence that Atox1 has other functions in addition to its role as a Cu chaperone. Whether the effect of Atox1 on the ubiquitination of Ctrl is due to its direct interaction with Ctrl [21] or through secondary mechanisms such as transcription remains unknown. However, the failure of the Atox1/−/− cells to down-regulate Ctrl during exposure to CDDP is not related to a reduction in overall proteasomal activity which we found to have increased in these cells. In the absence of CDDP the untreated Atox1+/+ and Atox1/−/− cells ubiquitinate Ctrl at similar levels, but in the presence of CDDP there is a failure of ubiquitination in the Atox1/−/− cells. Since CDDP has previously been shown to bind ubiquitin [41], it is possible that Atox1 competes with ubiquitin for binding to CDDP and thus protects the available ubiquitin pool from inactivation by CDDP. Another possibility is that the higher Cu levels in the Atox1/−/− cells inhibits the proteasomal activity [42] or sequesters ubiquitin or both. This later possibility, however, is unlikely as pre-treatment of cells with CDDP failed to alter the rate of Ctrl degradation or the levels of polyubiquitination of this protein.

It is interesting to note that the Atox1/−/− cells have higher levels of chymotrypsin-like proteasome activity than the Atox1+/+ cells. The increase in the level of proteasome activity may be an adaptive response to the inhibitory effects of the higher intracellular Cu found in the Atox1/−/− cells [42], or a reflection of the reduced abundance of appropriately polyubiquitinated proteins in the Atox1/−/− cells. It is also possible that Atox1 acts as a transcriptional regulator of the proteasome subunits many of which contain the GAAGA, the putative binding site for Atox1 within their proximal or distal promoter regions [31].
5. Abbreviations

Atox1  antioxidant 1
BSA  bovine serum albumin
Ctrl  copper transporter 1
CDDP  cisplatin
DMEM  Dulbecco’s modified Eagle medium
ICP-MS  inductively coupled plasma mass spectrometry
PBS  phosphate buffered saline
TBST  tris buffered saline with Tween 20

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