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Citation for the original published paper (version of record):

Espling, M., Lundin, C., Björn, E., Naredi, P., Wittung-Stafshede, P. (2013)

Interaction between anticancer drug Cisplatin and copper chaperone Atox1 in human melanoma cells.

Protein peptide letters

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Interaction Between Anticancer Drug Cisplatin and Copper Chaperone Atox1 in Human Melanoma Cells

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Cisplatin (CisPt) is one of the most common anticancer drugs used against many severe forms of cancers. However, treatment with this drug causes many side effects and often, it results in the development of cell resistance. A major part of side effects as well as cell resistance are thought to develop due to CisPt interactions with proteins prior to reaching the nucleus and the target, DNA. The copper (Cu) transport proteins Ctr1 and ATP7A/B have been implicated in cellular resistance of CisPt, possibly exporting the drug out of the cell. Recent *in vitro* work demonstrated that CisPt also interacts with the cytoplasmic Cu-chaperone Atox1, binding in or near the Cu-binding site, without expulsion of bound Cu. Whereas Ctr1 and ATP7B interactions with CisPt have been shown *in vivo* or *ex vivo*, there is no such information for Atox1-CisPt interactions. To address this, we here develop a method to probe if CisPt interacts with Atox1 in human melanoma cells. Atox1-specific antibodies were linked to magnetic beads and used to immune-precipitate Atox1 from melanoma cells that had been pre-exposed to CisPt. Analysis of extracted Atox1 with inductively coupled plasma mass spectrometry (ICP-MS) demonstrated the presence of Pt in the protein fraction. Thus, CisPt-exposed human melanoma cells contain Atox1 molecules that bind some derivative of CisPt. This study gives the first indication for the intracellular presence of Atox1-CisPt complexes *ex vivo*.

Accepted for publication in *Protein and Peptide Letters*. E-pub. ahead of print. Aug. 2013.

INTRODUCTION

Cisplatin (*cis*-(PtCl₂(NH₃)₂, CisPt) is an anticancer drug that has been used for decades to treat various malignant tumors, including melanoma, testicular, ovarian, lung, head and neck cancers. CisPt is believed to kill cancer cells via crosslinking of the DNA in the nucleus. DNA crosslinking induces apoptosis and cell death. However, the anticancer effect of CisPt is often dramatically limited by the development of cell resistance [1].

One of the major causes behind cell tolerance to CisPt appears to be CisPt interactions with extracellular or intracellular proteins, hindering CisPt from entering the nucleus and reaching the DNA. CisPt interactions with the cellular copper (Cu) transport proteins Ctr1 and ATP7A/B are implied as an important resistance mechanism. The first evidence for this correlation came from the finding that Cu metabolism was disrupted in resistant cells [2]. Further work showed that some Cu transport proteins directly interact with CisPt. Since free Cu is toxic, cells have developed a protein-based system that delivers Cu to targets (copper-dependent enzymes, e.g., ceruloplasmin) in an efficient and specific way. The general Cu transport pathway in human cells, that is conserved in all organisms, directs Cu to the secretory pathway and involves copper transporter 1 (Ctr1), Atox1, and ATP7A and ATP7B.

Ctr1 is the membrane pump that is responsible for the cellular uptake of Cu. It has been shown to play a major role in CisPt uptake and cytotoxicity [3]. The absence of Ctr1 render cells resistant to CisPt [4] whereas patients with high levels of Ctr1 in their tumors responded better to platinum drug treatment [5]. ATP7A and ATP7B are homologous Cu-transporting ATPases positioned in the *trans*-Golgi network. CisPt has been shown to trigger re-localization of these multi-domain, membrane-associated proteins towards more peripheral parts of the cell where they are thought to mediate CisPt resistance by sequestering the drug into intra-cellular vesicles [6-7]. The ATP7A/B proteins have been found to be over-expressed in CisPt resistant cancer cells [2, 8].

Atox1 is a small soluble Cu-chaperone that transports Cu between Ctr1 (i.e., cell membrane) and ATP7A/B (i.e., trans-Golgi) in the cytoplasm of human cells. Atox1 can bind one Cu^I-ion to the cysteines in a conserved surface-exposed metal binding motif, **MXCXXC**, where X is any residue [9]. We recently demonstrated that CisPt can bind to Atox1 *in vitro*, both in the absence and the presence of Cu [10]. When Cu was pre-bound to Atox1, CisPt binding resulted in the appearance of circular dichroism (CD) signals above 300 nm, likely originating from metal-metal interactions [11-13]. Thus, CisPt binding to Cu-Atox1 resulted in a ternary CisPt-Cu-Atox1 complex. Further

KEYWORDS: Copper chaperone, Cisplatin, Atox1, anticancer, immunoprecipitation, resistance

ABBREVIATIONS: CD, circular dichroism; CisPt, Cisplatin; Ctr1, Copper transporter 1; ICP-MS, Inductively coupled plasma mass spectrometry; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

studies of this complex *in vitro* showed that the two metals are positioned in or near the Cu-binding site and no other residues, except the two Cu-binding site cysteines, are involved [14]. In accord with our mutational work, a crystal structure of Atox1 in complex with CisPt demonstrated binding to the cysteines in the Cu-binding site [15]. Our *in vitro* experiments in solution also revealed that upon incubation (hours to days), binding of CisPt to Atox1 (with or without Cu) triggered protein unfolding and aggregation [10]. This result suggests that CisPt interactions with Atox1 *in vivo* may be a dead-end pathway; cell tolerance will occur due to incorporation of CisPt in unfolded protein aggregates in the cytoplasm that eventually will be degraded. However, it was shown *in vitro* by others that Atox1 can transfer CisPt to the second metal binding domain of ATP7B [16] and we recently showed that Atox1 can transfer CisPt to the fourth metal-binding domain of ATP7B prior to Pt-mediated Atox1 unfolding [14]. There are no *in vivo* or *ex vivo* studies of possible Atox1-CisPt interactions, except for an *in-cell* NMR study of over-expressed Atox1 in *E. coli* cells. Upon treatment of such cells with CisPt, the over-expressed Atox1 was found to interact with internalized Pt. With time, a Pt-bridged Atox1 homo-dimer was observed by NMR [17]. This dimer may be the initial event in the process towards Pt-mediated Atox1 unfolding and aggregation, as we found to occur with time *in vitro*. Based on the reported *in vitro* studies, one may propose that Atox1 interacts with CisPt also *in vivo* - either acting as a CisPt transporter between Ctr1 and ATP7A/B in the cytoplasm, or as a dead-end sink (if the Atox1-CisPt complexes start to unfold).

Here we have developed a method to study if Atox1 molecules in human melanoma cancer cells interact with internalized CisPt upon treatment of the cells externally with CisPt. The method is based on antibody-coupled magnetic beads followed by ICP-MS detection of metal content in the extracted Atox1. Our study provides the first evidence of Atox1-CisPt complexes *ex vivo*.

MATERIALS AND METHOD

Materials

CisPt for *in vitro* experiments was purchased from Sigma-Aldrich (Sweden) and CisPt for cell experiments from Hospira Nordic (Stockholm, Sweden). Magnetic beads M-270 Epoxy were purchased as a complete kit from Invitrogen (Dynabeads antibody coupling kit). Atox1 antibody 3B7 was purchased from Novus Biologicals. EDTA-free protease inhibitor cocktail was purchased from Roche. T-75 flasks were purchased from Sarstedt (Nümbrecht, Germany) and RPMI 1640 medium from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was purchased from BioWest (Nuaille, France). Precellys 24 glass beads for cell lysis from Bertin Technologies (Montigny-le Bretonneux, France). Hydrocortisone, insulin, human transferrin, estradiol and selenium were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of magnetic beads

Following the instructions from the manufacturer, a monoclonal IgG1 Atox1 antibody was coupled covalently to the magnetic beads. The beads were prepared in smaller batches, each one containing 5 mg beads. The capacity of the beads to bind Atox1 was tested with an *in vitro* sample of Atox1, and a clear Atox1-band was visible on SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, 16 %). The beads were also tested with a cell lysate sample (from two T-75 flasks, 5 mg antibody coupled beads) and Atox1 was the only protein-band visible on the gel, assuring the selectivity of the method. This experiment also demonstrated that microgram amounts of Atox1 were extracted from two flasks of cells when using 5 mg of antibody-covered beads.

Cell culture and cell lysate preparation

Cells of the human malignant melanoma T289 cell line [18] was incubated in nine replicated T-75 flasks, $6 - 9 \times 10^6$ cells/flask, at 37°C under a gas phase of 95% air/5% CO₂. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 nM hydrocortisone, insulin 5 µg/ml, 5 µg/ml human transferrin, 10 nM estradiol and 5 ng/ml selenium. The cells were grown to approximately 95% confluence and then exposed for 1 hour in growth medium containing 100 µM CisPt [19]. No dead cells were observed for this exposing condition.

Cells were washed three times with 7 ml of cold phosphate-buffered saline (PBS) solution (136 mM NaCl, 2.68 mM KCl, 10 mM KH₂PO₄, and 1.76 mM Na₂HPO₄) and loosened by the use of a cell scraper. The cells from two flasks were pooled and centrifuged at 1000 rpm for 5 min. The pellet was re-suspended in 100 µl of pure ice cold water and transferred to a tube containing 52 mg of glass beads with 0.5 mm diameter. Homogenization was performed using a high throughput homogenizer, Precellys 24 run at 2 cycles of 15 sec with 5000 rpm, 5 sec break. Cell debris was pelleted by centrifugation at 14000 rpm for 15 min, +4°C and the supernatant was collected for analysis.

Non-exposed control sample cells were incubated in CisPt free medium for 1 hour after being grown to 95% confluence.

Immunoprecipitation from cell lysate

To the cell lysates 1 % protease inhibitor cocktail was added. 5 mg antibody-coupled magnetic beads were added to each of the three lysate samples and the control sample. Volume of cell lysate loaded to the beads was constant for all samples, 150 µl, originating from two flasks of cells. Beads and lysate were incubated for 90 minutes on a roller in 6° C. The beads were isolated and washed thoroughly according to the manual and protein was eluted with 50 µl 0.1 M Citric acid, pH = 3.1. The samples were measured with ICP-MS for elemental analysis without further additives. The beads were regenerated quickly with PBS to be reused for new experiments.

ICP-MS

Signals for Cu (65 Da), Pt (195 Da) and S (48 Da, to quantify protein content via Met and Cys) were monitored by ICP-MS. A PerkinElmer/Sciex Elan DRC-e ICPMS instrument with O₂ as reaction gas was used for the measurements. Quantification was performed by matrix matched standards and the isotopes ⁶³Cu⁺, ⁶⁵Cu⁺, ¹⁹⁴Pt⁺ and ¹⁹⁵Pt⁺ as well as ³²S¹⁶O⁺ were monitored. Standard deviation (square root of variance among the three samples in each experiment) and single-value Anova analysis (all nine Pt-containing samples compared to the three control samples without added CisPt) were calculated in Excel.

RESULTS AND DISCUSSION

Guided by the *in vitro* findings of CisPt interactions with Atox1 [10, 14-15, 17], we decided to search for CisPt-Atox1 complexes in human cancer cells. To reach this goal, we developed a method based on Atox1-specific antibodies covalently attached to magnetic beads and ICP-MS detection. The idea was to selectively bind Atox1 present in human cancer cell lysate to the antibody-bead construct and then remove the beads (and cargo) with a magnet, followed by elution of the protein from the beads and metal detection in the isolated protein fraction by ICP-MS. In Figure 1, we show a schematic diagram of our experimental approach to extract Atox1 from human cancer cells and evaluate the presence of CisPt.

We choose a melanoma cell line for this study since CisPt is one of the most commonly used chemotherapeutic drugs to treat patients with advanced cutaneous malignant melanoma. We first evaluated several polyclonal antibodies for bead attachment before selecting the current monoclonal antibody. Next, we tested if enough Atox1 could be detected using our experimental set up (see Materials and Methods section). Atox1-specific antibodies linked to magnetic beads (5 mg antibody covered beads) were mixed with melanoma cell lysate (from two T-75 flasks) and then the beads with content were removed from the lysate with a magnet. Antibody-bound protein was eluted from the bead-antibody construct by citric acid. The eluted protein was analyzed by SDS-PAGE gel electrophoresis (Figure 2). Compared to the positive control of pure Atox1, the antibody construct in our set up binds enough Atox1 molecules (and only Atox1 molecules) to allow Coomassie stained detection of the protein on an SDS-PAGE gel. Upon visual comparison of the bands, the bead-antibody extracted Atox1 from two flasks of cells (see Materials and Methods section), corresponds to a few µg of protein at the most. A rough calculation using the assumed number of cells per flask indicates that this corresponds to extraction of about 10⁶ Atox1 molecules per cell.

Figure 1. Schematic drawing of the Atox1 extraction method from cancer cells exposed to CisPt. Magnetic beads covered with Atox1 antibody were incubated with cell lysate from human cancer cells exposed to CisPt. Beads were extracted with a magnet from the lysate and Atox1 was eluted from the bead-antibody construct with citric acid for subsequent measurement of Pt- (and Cu- and S-) content in the Atox1 fraction with ICP-MS.

Next, we wanted to expose the cells to CisPt, incubate them, and finally wash them thoroughly followed by analysis of extracted Atox1 for metal content. Since the procedure involved elution of protein from the magnetic bead-antibody construct by citric acid, it was feasible to directly perform analysis of the protein fraction by inductively coupled plasma mass spectrometry (ICP-MS), which is a method for elemental analysis of liquid samples [20]. The detection of metals bound to proteins is straightforward with ICP-MS since buffers and the polypeptide chemistry do not contain metal ions [21]; in case of CisPt, the Pt element is detected and no conclusion regarding its ligation can be drawn. To correlate metal content to protein content, sulfur (S) can be monitored which will report on the number of S in the protein (Met and Cys residues). The protein concentration is then estimated by correlating the intensity of the signal to a sulfur standard, and counting the amount of sulfurs in the amino acid sequence. For small volume samples the ICP-MS intensity for each element can be monitored over time. An average of the raised intensity detected when the sample passes

the detector is calculated and correlated to element standards to gain concentrations of the detected elements. Here we used time-dependent ICP-MS to simultaneously probe Pt, Cu and S content in Atox1 samples extracted from our melanoma cells, treated or not treated with CisPt.

Figure 2. SDS-PAGE Coomassie stained gel of extract from lysate of two flasks of melanoma cells upon using the antibody-bead construct as described in the text (lane E). For comparison, three positive controls of purified Atox1 (lanes 1, 2, and 3) are also shown containing 5, 10 and 24 μ g protein, respectively. The molecular weight of the protein in lane E matches that of purified Atox1 (7.5 kDa). No other protein except Atox1 is extracted from the lysate in amounts possible to detect on an SDS-gel, indicating, as expected, high selectivity for Atox1 by the antibody-bead construct.

Three separate experiments (starting with new cell cultures) were performed with three replicates (i.e., 6 flasks with cells exposed to CisPt in each experiment) in each for CisPt exposed cells. To assure that there was no source of CisPt other than CisPt accumulated intracellular in these experiments, the cells were thoroughly washed according to a previously established protocol, leaving negligible amounts of free CisPt outside the cells [19]. We also performed three control experiments (2 flasks with cells in each experiment), with the same procedure, but without CisPt addition.

As expected, ICP-MS analysis of extracted and eluted Atox1 from the control experiments revealed that there was no Pt present in these samples (Figure 3A). For these samples, we detected S and Cu signals, indicating that a minor fraction of the Atox1 molecules contained Cu. In contrast, Pt signals were detected in all nine Atox1 samples extracted from the cells exposed to CisPt (Figure 3B). An Anova single-value analysis on the pooled nine Pt-containing samples compared to the three control samples gave a p-value of 0.095. From the intensity of

the average ICP-MS signal, and normalization to standards for each element, rough estimates of amounts of protein (S), Cu and Pt were made in the extracted Atox1 samples. We determined that between 1-5 % of the extracted Atox1 molecules are bound to Cu; only about 0.5 % of the Atox1 molecules are bound to CisPt in the CisPt treated samples (Figure 3C).

The low levels of bound metals may indicate that at our cell growing/exposure conditions, most Atox1 is found in the apo-form in the cells. However, we cannot exclude that the result is due to partial metal precipitation in the acid elution step or that the monoclonal antibody we use may have higher affinity for the apo protein over Cu- or Pt-bound forms. Notably, technical issues with reducing agents (containing sulfur) necessary for *in vitro* loading of Atox1 with Cu and Pt prohibited a thorough investigation of this issue.

There appears to be increased Cu levels in Atox1 samples from CisPt treated samples (Figure 3AB) although the protein content (S signal) appears the same. This result may be expected since we have previously found *in vitro* that both metals can bind to the same Atox1 molecule simultaneously [10] and that the presence of Cu appears to enhance Pt binding [14]. Nonetheless, if Atox1 is shuttling Pt (and Cu) between Ctr1 and ATP7A/B, there may only be a low steady-state level of the metallated Atox1 form.

Our method is not proposed to be quantitative; instead the purpose is to simply demonstrate the presence of Pt-coordinated Atox1 in a biological environment *ex vivo*.

Figure 3. Pt-, Cu- and S-content in Atox1 extract determined by ICP-MS. Sulfur (S, 48 Da) was used to monitor Atox1 concentration (via Met and Cys content). Cu (64 Da) is the natural substrate of Atox1 and Pt (195 Da) was monitored to detect CisPt (or some derivative thereof) binding. **A.** ICP-MS signal traces for an Atox1 sample extracted from cells not treated with CisPt (control). Traces are from an on-line measurement where the increase in intensity as a function of time shows how the sample passes the detector. Signals for the sample between 30s and 70s are averaged, and then compared to standards for each element to give an estimate of the concentration of that element in the actual sample. Whereas Atox1 (S) and Cu are present in this sample, no Pt can be detected in the control sample. **B.** ICP-MS signal traces for an Atox1 sample extracted from CisPt-exposed cells. Here, both metals and Atox1 are detected, demonstrating that the extracted Atox1 interacts with both Cu and Pt. **C.** ICP-MS data summarized in a bar plot with mean and standard deviation recalculated to Pt-content per Atox1 molecule. The CisPt-treatment of cells were repeated three times (1, 2, and 3), and each time three replicates were performed (in total, 18 flasks of cells grown, 2 for each individual experiment). The same amount of lysate was loaded onto the beads each time. In parallel with every set of three replicas of CisPt-treatment, one control (Ctrlis) sample were prepared in which the same procedures were performed but without the addition of CisPt. The y-axis corresponds to the fraction of detected Atox1 molecules that bind Pt (Pt per Atox1, in mol %); with this presentation, there is no contribution from possible variation of amount extracted Atox1 between samples.

An issue that should be addressed in future studies is if there are unfolded Atox1-Pt aggregates in the treated cells (as we found to develop with time *in vitro* [10]). If so, these Atox1-Pt aggregates may not be recognized by the monoclonal antibody and the level of Atox1-CisPt determined by the current method will be an under-estimate. To assess this possibility, one may try to use a polyclonal antibody in the set up instead of, as here, a monoclonal antibody. Using polyclonal antibodies may also be helpful to investigate if the metal loading of Atox1 inside the cells is truly as low as detected here, or if this is due to preferential binding of the apo-form of Atox1 to the antibody.

For the above assay, we assume that all Pt that is retained by the antibody-bead method is bound to Atox1. Thus, we neglect any non-specific binding of internalized free Pt to the bead/antibody construct. This assumption appears reasonable since it has been determined that for the same CisPt exposure levels, incubation times, and cells as here, most of the intracellular CisPt is bound to proteins and only between 10 and 100 ng Pt is free (i.e. not bound to proteins) per ml lysate (E Björn; data not shown). To address this question directly, we performed a control experiment for possible nonspecific binding of free CisPt to the magnetic beads loaded with antibodies *in vitro*. When calibrating the *in vitro* concentrations used of free CisPt in this experiment to the free Pt-concentration estimated to be present in these cells, it emerges that nonspecific binding of free CisPt to the antibody/bead construct could contribute with an ICP-MS signal one to two orders of magnitude lower, at most, than what is actually detected. Therefore, the detected Pt in the extracted Atox1 samples originates from Pt bound to Atox1.

Many recent studies have emphasized that cellular Cu transport proteins (e.g., Ctr1, ATP7A/B) play a role in CisPt trafficking/resistance inside human cells (e.g., reviewed in [22]). Our study demonstrates for the first time that CisPt interacts with the Cu chaperone Atox1 *ex vivo*, in melanoma cells. However, we want to emphasize that our study does not give any information on if the detected Pt-Atox1 interactions are linked to cell resistance to the drug, or not. Nonetheless, our observation gives support for the importance of previous *in vitro* work of Atox1 interactions with CisPt [10, 15, 17, 23] and calls for future *in vivo* studies. To investigate if CisPt interactions with Atox1 affect cell resistance to the drug, CisPt exposure to cells with over-expressed Atox1 followed by a cell proliferation assay is suggested.

ACKNOWLEDGEMENTS

Designed study: MPE, PWS
 Performed study and collected data: MPE, CL, EB
 Analyzed data: MPE, EB, PN, PWS
 Wrote paper: MPE, PWS

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