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Reaction of platinum anticancer drugs and drug derivatives with a copper transporting protein, Atox1

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ABSTRACT

Platinum (Pt) containing anticancer drugs have been used in cancer treatment for several decades as they trigger cell death upon DNA binding. Pt-containing anticancer drugs and drug derivates with a variety of ligands around the Pt center (with Cisplatin being most well known) exist today in clinics and in clinical trials. However, a major drawback with these drugs is limited efficacy due to side reactions resulting in cell resistance. The cellular copper (Cu) transport pathway is proposed to be responsible for part of these side reactions through interactions with the Pt-containing drugs and possibly cellular export of Pt. The cytoplasmic Cu chaperone, Atox1, was recently found to bind Cisplatin in vitro and, when over-expressed in *Escherichia coli*, in vivo. Here we investigate how the chemical properties of six Pt-substances differentially affect binding, unfolding, and aggregation of Atox1 in vitro using near- and far-UV circular dichroism (CD) spectroscopy and SDS-PAGE. The results show that both ligand type and orientation dictate the interactions with Atox1. Only substances with two good leaving groups in *cis*-configuration result in near-UV CD changes that report on Cu–Pt interactions. The different substances promote Atox1 unfolding in a pattern that can be explained by ligand chemistry and geometry. Our work emphasize that ligands around the Pt-center have decisive roles in tuning protein interactions (prior to DNA binding) and therefore they also dictate the level of drug side effects and cellular resistance.

1. Introduction

1.1. Platinum anticancer drugs and resistance

Cisplatin (*cis*-diamminedichloroplatinum(II), CisPt) is a widely used anticancer drug mainly effective against testicular, ovarian, head, neck, and lung cancer tumors [1]. The mechanism of action for CisPt involves binding and cross linking of DNA through a covalent coordinate bond to the nitrogen atom on guanine, and to a lesser extent adenine. This DNA damage prevents replication and transcription, which leads to cell apoptosis [2]. Several platinum (Pt) drug analogues exist (Fig. 1) and carboplatin (*cis*-diammine(1,1-cyclobutane-dicarboxylato)platinum(II), CarboPt) and oxaliplatin (1,2-diaminocyclohexane-oxalateplatinum(II)), OxaliPt) is today also approved for use in the clinics [1–3].

The efficacy of platinum substances in cancer treatment is limited due to cell resistance. The resistance can be either intrinsic or acquired and seems to be multifactorial [3]. Several different resistance mechanisms have been proposed: for example, DNA repair, defective apoptosis, reduced uptake, and in-cell detoxification [1,3,4]. Copper (Cu) transporting proteins have been proposed to be involved in events that lead to resistance of platinum drugs. Copper transporter 1 (Ctr1), that transports copper into the cell, is also the major influx transporter for the platinum drugs. Knockout of Ctr1 renders cells resistant to the platinum drugs for both yeast and mammalian cells [5]. Copper transporting ATP:ases ATP7A and ATP7B are over expressed in CisPt resistant carcinoma cells [6,7] and have been proposed to efflux CisPt both out of the cell [6] or into intracellular vesicles and thereby lower intracellular drug concentration [8]. Human ATP7A/B has six metal binding domains in the cytoplasmic N-terminal domain. The domains each have a ferredoxin like fold and can bind one copper (I) ion each in a conserved copper-binding site, $MX_1CX_2X_3C$, in a surface exposed loop. Structurally similar to the metal binding domains is the 68-residue cytoplasmic copper chaperone Atox1 (Fig. 2) [9]. Because of the chaperone's high similarity to the ATP7A/B metal binding domains it appears likely that also Atox1 can bind CisPt, and this was recently shown when two crystal structures of Atox1 with bound CisPt were published [10]. In one of the crystal structures Atox1 is monomeric and a platinum ion is bound to the two cysteines in the copper-binding site. In the other reported crystal structure Atox1 is present as a dimer with CisPt bound to one

Abbreviations: CD, circular dichroism; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothretiol; TCEP, Tris(2-carboxyethyl)phosphine; Ctr1, copper transporter 1; NMR, nuclear magnetic resonance; OCT, organic cation transporter.

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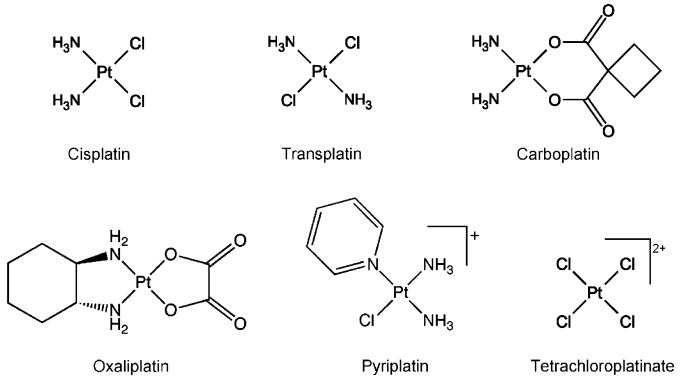

Fig. 1. Platinum substances used in this study.

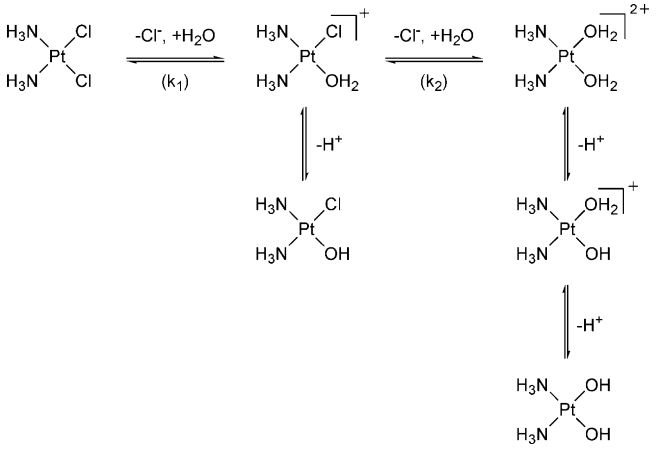

Fig. 3. Hydrolyzes scheme of CisPt. Redrawn from Berners-Price et al. [46].

cysteine (Cys15) from each of the two copper-binding sites [10]. Both Drosophila Atox1$^{-/-}$ mutants [11] and mouse Atox1$^{-/-}$ fibroblast cell lines [12] are shown to have lower sensitivity to CisPt than the wild types [11,12]. It is shown that Atox1 is required for the Ctr1-mediated uptake of CisPt in to the cell [12] and that down regulation [11] or defunctionalization [12] of Atox1 can contribute to CisPt resistance [11,12]. Using in-cell NMR, CisPt was shown to bind to the cysteines in the copper-binding site when Atox1 was over expressed in procaryotic *Escherichia coli* cells [13]. We recently reported that CisPt binds to Atox1 in solution in vitro, both with and without copper [14]. When copper was present, Cu–Pt interactions were detected suggesting that both metals are in or near the copper-binding site. The platinum–Atox1 complexes were found to be unstable and unfolded and aggregated in hours to days. Moreover, we showed that Atox1 levels in tumor cells correlates with CisPt sensitivity [14]. A cell line with five-fold higher resistance to CisPt (melanoma T289 cells) [15] had considerably higher levels of Atox1 than the cell line with lower CisPt resistance (ovarian cancer 2008 cells) [16].

1.2. Platinum substances

CisPt is believed to react with DNA and proteins in its hydrolyzed form (Fig. 3). Platinum anticancer substances are administrated intravenously. Blood has a chloride concentration of ~104 mM and hydrolyzation of CisPt is suppressed but not totally prevented. Once inside the cell the chloride concentration is lower, 4–20 mM and CisPt is hydrolyzed [2,17]. The first hydrolysis step k_1 for CisPt has been reported to be $k_1 = 5.18 \times 10^{-5}\ s^{-1}$ [18] ($t_{1/2} = 3.5$ h). The rate constant for the second hydrolysis step is slower: $k_2 = 2.75 \times 10^{-5}\ s^{-1}$ [18] ($t_{1/2} = 7$ h). The geometry around the platinum ion is square planar and substitution reactions are therefore affected by the *trans effect* [19,20]. The result of this effect is that certain ligands labialize the ligands trans, or opposite, of themselves and thereby making them better leaving groups. A ligand can therefore direct substitution trans to itself in a complex [21]. The order of trans labialization for selected ligands follow: $SH_2/SR_2 > Cl^- > NH_3$/pyridine $> OH^- > H_2O$ [21]. CisPt is highly reactive toward nucleophiles, especially ones containing sulfur, and has been reported to bind to different amino acids in proteins, for example: cysteine [10,22], methionine [23–25], and histidine [25–27].

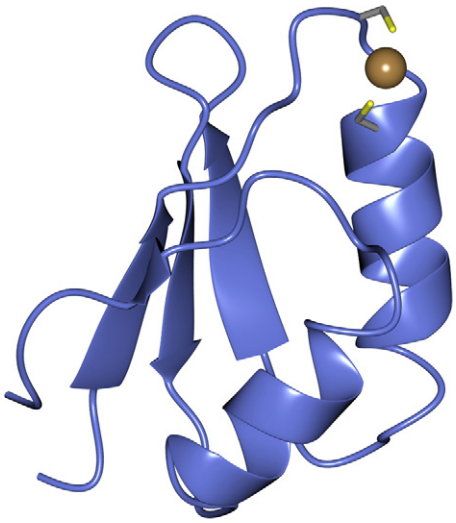

Fig. 2. The copper chaperone Atox1. Visible residues are the two cysteines (Cys12 and Cys15) in the copper-binding site.

Transplatin (trans-diamminedichloroplatinum(II), TransPt) is, even though its high similarity to CisPt, not very useful as an antitumor agent. Its cytotoxicity is significantly lower compared to CisPt [19,28]. The rate constant for the first hydrolysis step is faster for TransPt ($k_1 = 9.8 \times 10^{-5}\ s^{-1}$ [29], $t_{1/2} = 2$ h) than CisPt because of the higher trans effect of chloride over ammonia. The second hydrolysis step is for the same reasons slower for TransPt ($k_2 = <5 \times 10^{-5}\ s^{-1}$ [29], $t_{1/2} > 4$ h) compared to CisPt. The reaction rate for substitution of sulfur containing ligands is therefore higher for TransPt than for CisPt [30].

CarboPt is the second generation drug of CisPt and has a more stable leaving group which gives fewer side effects [2,31,32]. Because of the high stability of CarboPt toward an nucleophilic attack, its hydrolysis rate ($k_s = 8.14 \times 10^{-8}\ s^{-1}$ [33], $t_{1/2} = 99$ days) is much lower than that of CisPt [31]. In the presence of stronger nucleophiles like chloride or nucleophilic biomolecules a more rapid reaction can be observed for CarboPt, which is believed to be the case in vivo [31,32].

OxaliPt is active against some tumors that are resistant to CisPt and CarboPt and it is the first platinum drug to be effective against advanced colorectal cancer [2,34,35]. Most likely, the effect and tumor specificity of OxaliPt is mediated by drug uptake of organic cation transporter one and two (OCT1 and OCT2) [35]. When OxaliPt is dissolved in water it will hydrolyze and lose the oxalate group [36–38]. The hydrolysis rate of OxaliPt in vitro at 37 °C is slow: $k = 1.2 \times 10^{-6}\ s^{-1}$ ($t_{1/2} = 160$ h) [37], whereas the half-life of OxaliPt in vivo is short. The biotransformation is believed to involve chloride, bicarbonate and/or sulfur ligands [39]. When comparing CisPt and OxaliPt it was observed that OxaliPt reacts faster with sulfur containing biomolecules than CisPt, and that both drugs reacted fastest with cysteine before methionine and glutathione respectively [40]. The reaction probably occurs

through a direct attack of sulfur ligand on intact OxaliPt, prior to hydrolyzation [30,40].

Pyriplatin (*cis*-diammine(pyridine)chloroplatinum(II), cDPCP or PyriPt) is a platinum anticancer drug candidate [41]. Recent studies show that PyriPt is transported and accumulated in cells by OCT1/2 [41,42].

Tetrachloroplatinate (TetraclPt) is a platinum(II) dianion with four chloride ligands [43]. The rate constants for the first chloride substitution is $k_1 = 6.6 \times 10^{-5}\ s^{-1}$ [44] in basic conditions and $k_1 = 3.6 \times 10^{-5}\ s^{-1}$ [45] in acid conditions ($t_{1/2} = 3$ h and 5 h, respectively). More details of the platinum substances are described in the supplement.

Supplementary material related to this article found, in the online version, at doi:10.1016/j.bcp.2012.01.018.

Here we systematically test a range of platinum(II) substances for binding and perturbation of the sulfur containing copper transport protein, Atox1 in vitro. Our findings demonstrate a direct correlation between chemical and geometrical properties of the platinum ligands and the effects on protein properties.

2. Material and methods

2.1. Protein expression and purification

Expression and purification of Atox1 have been described previously [14]. The plasmid was kindly provided by A.C. Rosenzweig (Northwestern University, Evanston, IL).

2.2. CD spectroscopy and gel analysis

Cisplatin, transplatin, di-potassium-tetrachloroplatinate, oxaliplatin, pyriplatin, dithiothreitol (DTT), NaCl, $CuCl_2$ and 2-(N-morpholino)ethanesulfonic acid hydrate (MES) were purchased from Sigma–Aldrich (Sweden). Carboplatin was purchased from Strem Chemicals Inc. (Germany). CD spectra were recorded on a Jasco J-720 Spectropolarimeter at 20 °C. Copper was added to the samples in the form of $CuCl_2$ (20 mM in H_2O) and reduced to Cu^1 by DTT in the buffer. The platinum substances were dissolved in water (CisPt 2 mg/ml, TransPt 1 mg/ml, TetraclPt 10 mg/ml, PyriPt 10 mg/ml, CarboPt 10 mg/ml, OxaliPt 4 mg/ml). CisPt and TransPt samples were shortly heated with help of the microwave to enhance solubility, all platinum stock solutions were incubated and pre-hydrolyzed for three days. To avoid degradation products the platinum stock solutions were used within five days. Protein concentration was 50 μM in all experiments and DTT concentration in protein samples was kept five-fold higher (0.25 mM). The sample buffer was 50 mM NaCl, 20 mM MES, 0.25 mM DTT, pH 6, and all experiments were measured at 20 °C. Time dependences of Pt-induced unfolding of Atox1 were measured with far-UV CD at 220 nm in sealed cuvettes over the course of two weeks. 5 eq. Pt-substance were used in all cases except for control samples of apo- and holo-Atox1 (where 1 eq. $CuCl_2$ was added to the latter). Samples for titrations of Pt-substances probed by near-UV CD (260–400 nm) were incubated 10 min before measurements. 0–5 eq. Pt-substance was titrated to premixed holo-Atox1 in one set of titrations and 0–5 eq. $CuCl_2$ was added to premixed 1:1 Atox1–Pt-substance in another set. For titration experiments baselines of everything except protein were subtracted from each spectrum. CD signals at 220 nm as a function of time were fitted to single exponential decays (Kaleidagraph) to obtain rate constants for Pt-substance triggered Atox1 unfolding. $t_{1/2}$ is calculated as $\ln 2/k$.

For gel analysis SDS-PAGE was used. Samples with 5 eq. Pt-substance with either apo- or holo-Atox1 were mixed and incubated for different times (4 days, 2 days, 1 day, 4 h and freshly made). SDS-dye was added to the samples (no boiling) and loaded onto a 16% SDS-gel under non-reducing conditions.

3. Results

3.1. Platinum-induced Atox1 unfolding

Far-UV CD-spectroscopy probes protein secondary structure (Fig. 4A). Normally, apo Atox1 is folded in buffer during at least two weeks at 20 °C (Fig. 4C). In case of holo Atox1, unfolding starts after ~80 h, probably due to copper mediated oxidation (Fig. 4D). Adding Pt-substances to apo Atox1 induce loss of secondary structure, observed as a reduction of negative CD-signal at 220 nm with time (Fig. 4C and D). In all cases, 5:1 ratio of Pt-substance to apo Atox1 was mixed. With OxaliPt and PyriPt, the highest extent of apo Atox1 unfolding is observed. Also with TetraclPt and CisPt are the extents of Atox1 unfolding large. With TransPt, apo Atox1 unfolds to a lesser extent than with the other substances. With CarboPt the unfolding profile for Atox1 is similar to that for apo Atox1 alone and thus there may be no, or little drug binding (Fig. 4C).

Next, the same experiments were repeated with the Cu-form of Atox1. Addition of a Pt-substance with holo Atox1 accelerates unfolding to a larger extent than for the corresponding samples with apo Atox1. The order of unfolding potency for the different Pt-substances match between the holo and apo forms of Atox1. For holo Atox1 the highest extent of unfolding is observed with PyriPt and CisPt. With OxaliPt and TetraclPt, large levels of unfolding are also seen whereas with TransPt less unfolding of holo Atox1 is detected. With CarboPt, the reaction parallels that for holo Atox1 alone.

CD-data at 220 nm as a function of time was fit to single exponential decay equations (except CarboPt) to obtain rate constants for Pt-substance triggered Atox1 unfolding. In Fig. 4B the fits of the PyriPt Atox1 data are shown and the different unfolding extents for apo and holo due to 5 eq. PyriPt are emphasized. The unfolding rate constants are summarized in Table 1. TetraclPt and TransPt induce unfolding of apo Atox1 the fastest (half-life 0.9 h and 1.7 h respectively), followed by OxaliPt (10 h) and CisPt (12 h) induced Atox1 unfolding. With PyriPt, apo Atox1 unfolds slowly with a half-life of 28 h. For holo Atox1, the relative order of unfolding rates with the different substances match the behavior with apo Atox1, although the rates generally are slower. TransPt and TetraclPt induce the most rapid unfolding rate of holo Atox1 (half-life 3.0 h and 3.5 h, respectively), followed by OxaliPt (9.8 h) and CisPt (23 h) induced unfolding. With PyriPt the protein unfolds the slowest with a half-life of 48 h. OxaliPt is the only substance that induces a similar unfolding rate with both apo and holo Atox1.

3.2. Pt-substance binding mode in Atox1

We earlier showed that Pt–Cu interactions (in the case of CisPt) can be detected by near-UV CD [14]. When adding CisPt to holo Atox1 a new positive CD-peak arises at 360 nm (Fig. 5), in parallel with a decrease in signals at 300 and 270 nm. These CD bands likely correspond to d^8–d^{10} metal–metal interactions [47]. We find that despite that all substances (except CarboPt) interact with Atox1 (as they trigger unfolding) not all substances give rise to a change in the near-UV CD spectrum. There were no near-UV CD changes upon addition of TransPt, CarboPt and PyriPt to Cu-Atox1. However, adding 5 eq. OxaliPt to holo Atox1 changes the near-UV CD-spectrum in a similar manner as CisPt although the positive peak is shifted toward 350 nm and the decrease in signal at 300 nm and 270 nm is not as dramatic. Upon TetraclPt addition to holo Atox1, a positive peak develops at 390 nm and there appears a large negative signal at 270 nm.

Titration of Cu to apo Atox1 results in a shift in the CD-signal at 280 nm (Supplementary Fig. 1) reporting on Cu-binding to the two cysteines, as described previously [48]. Adding Cu to a premixed

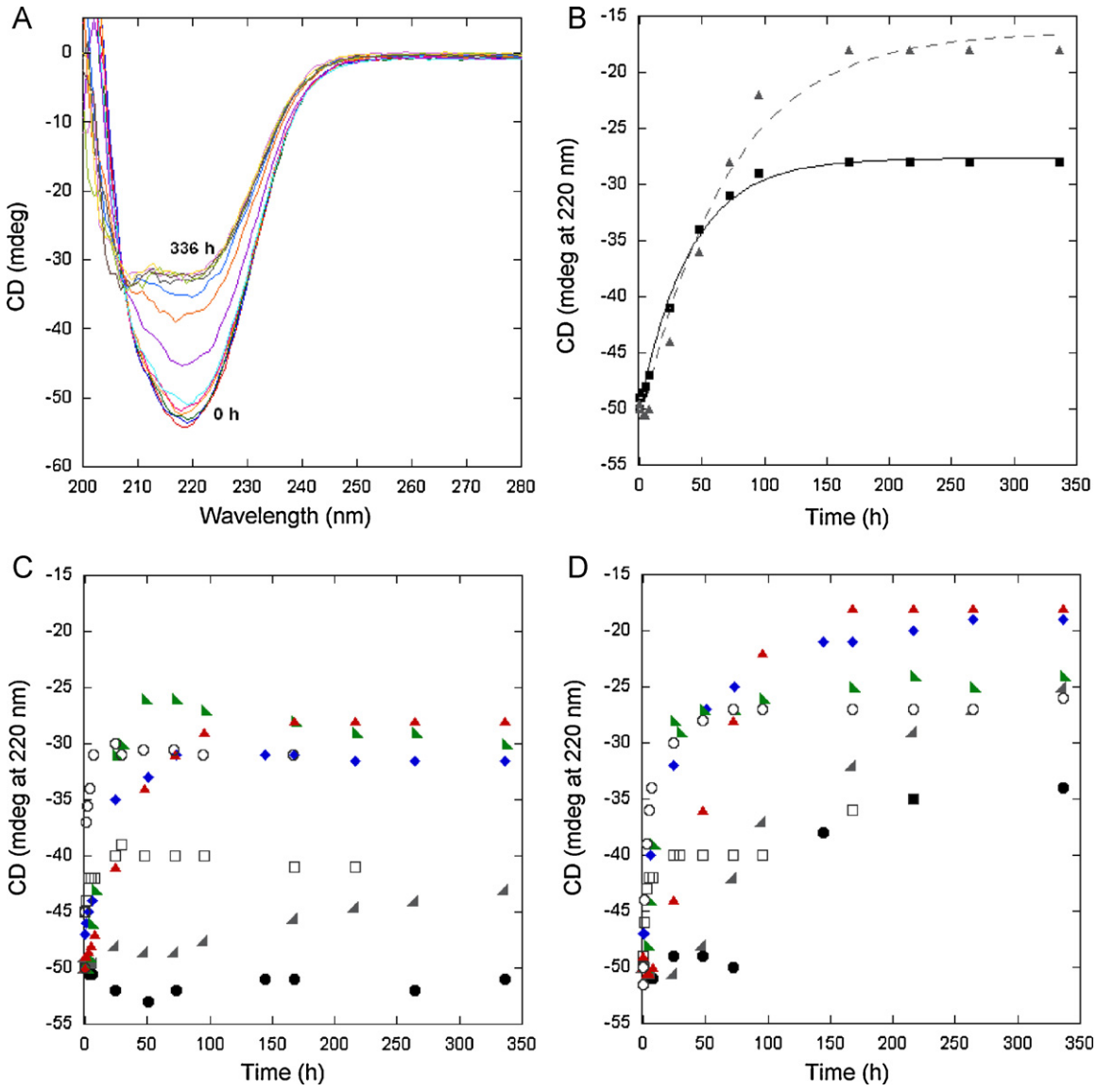

Fig. 4. Platinum-substance induced unfolding of Atox1. (A) Far-UV CD of apo Atox1 with added 5 eq. PyriPt, signal change over time. Timepoints are: before added substance, 0, 1, 3, 5, 8, 24, 48, 72, 96, 168, 216, 264, 336 h. (B) Fitting of a single exponential decay to CD signal change over time at 220 nm for apo (-■-) and holo (-▲-) Atox1 and 5 eq. PyriPt. (C) Unfolding of apo Atox1 with different Pt-substances. CD signal at 220 nm plotted against time. ● Apo Atox1, ◆ +5 eq. CisPt, □ +5 eq. TransPt, ◢ +5 eq. CarboPt, ◣ +5 eq. OxaliPt, ▲ +5 eq. PyriPt, ○ +5 eq. TetraclPt. (D) Unfolding of holo Atox1 with different Pt-substances. CD signal at 220 nm plotted against time. ● Holo Atox1, ◆ +5 eq. CisPt, □ +5 eq. TransPt, ◢ +5 eq. CarboPt, ◣ +5 eq. OxaliPt, ▲ +5 eq. PyriPt, ○ +5 eq. TetraclPt.

sample of apo Atox1 and 1 eq. CisPt does not give the "normal" signal change indicative of regular Cu-binding to the apo protein, but instead a positive peak at 360 nm appears, and the spectrum resembles that found upon adding CisPt to holo Atox1 (Fig. 6). Based on this, it appears that regardless of order of addition, the same ternary Cu–Pt Atox1 complex form that has distinct spectral features indicating that the Cu and Pt ions are close to each other and interact. In contrast, titrating Cu to samples with apo Atox1 premixed with 1 eq. TransPt, CarboPt and PyriPt, respectively results in "normal" Cu-binding shifts in the CD-spectra. As we have concluded that these Pt-substances (except CarboPt) bind to Atox1 it appears that the binding mode here restricts Pt–Cu interactions.

Table 1
Rates (k and $t_{1/2}$) and extents (after 75 h incubation) of Pt-substance induced unfolding of apo and holo Atox1.

	k_1 apo 10^{-3} (s^{-1})	$t_{1/2}$ apo (h)	Unf. apo (mdeg)[a]	k_1 holo 10^{-3} (s^{-1})	$t_{1/2}$ holo (h)	Unf. holo (mdeg)[a]
CisPt	57.5	12	−31	30.6	23	−25
TransPt	413	1.7	−40	231	3.0	−40
CarboPt	[b]		−49	[b]		−42
OxaliPt	67.4	10	−26	70.7	9.8	−27
PyriPt	24.5	28	−31	14.3	48	−28
TetraclPt	738	0.9	−31	200	3.5	−27

[a] After 75 h incubation. Start signals are approximately −50 mdeg, and this value is retained for pure apo and holo Atox1 after 75 h.
[b] Not possible to fit an exponential decay.

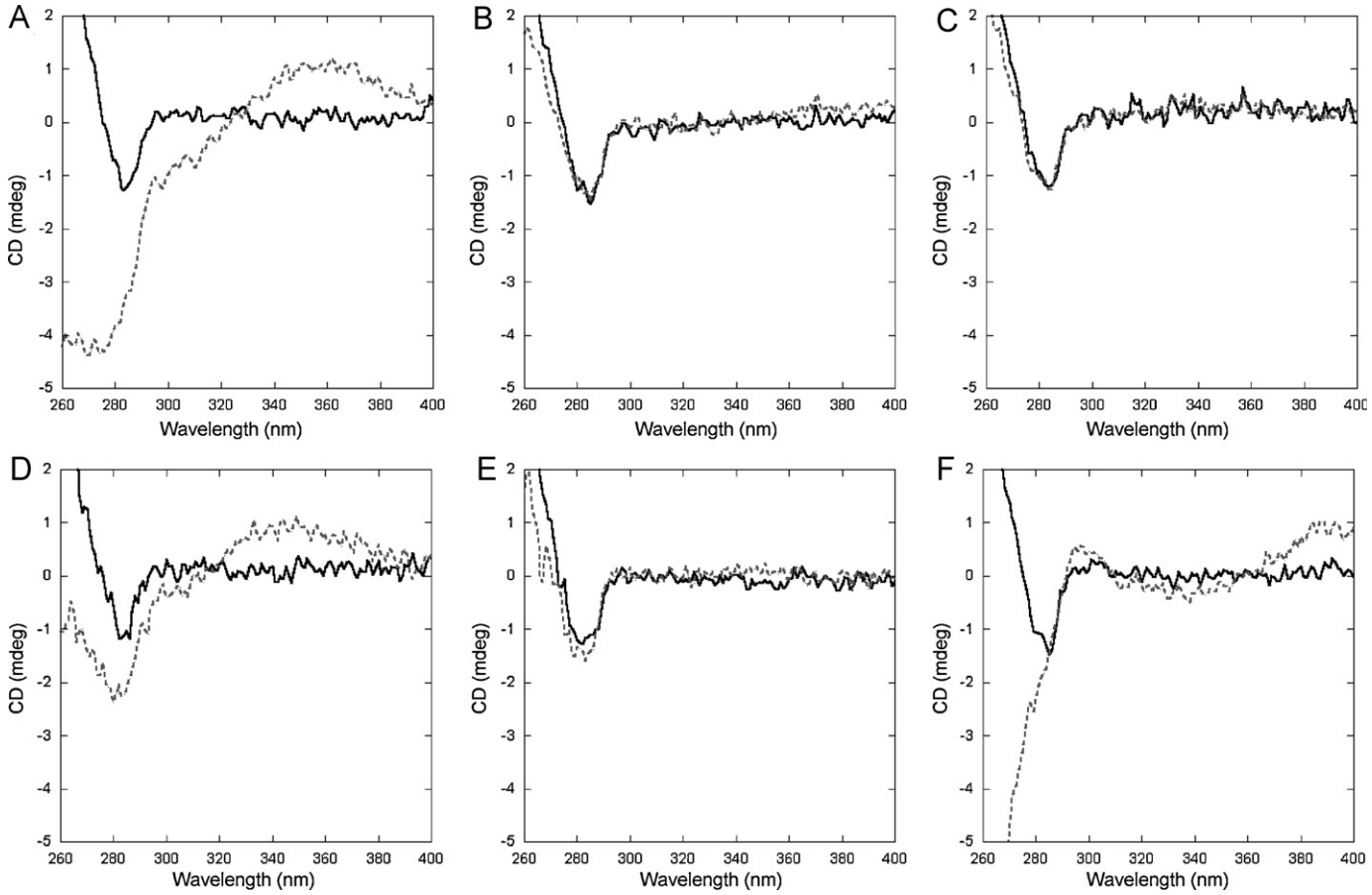

Fig. 5. Near-UV CD of holo Atox1 (solid line) and added 5 eq. platinum substance (dotted line). (A) CisPt. (B) TransPt. (C) CarboPt. (D) OxaliPt. (E) PyriPt. (F) TetraclPt.

With OxaliPt and TetraclPt premixed with apo Atox1 followed by Cu-addition result in similar features as found in the reverse titrations.

Supplementary material related to this article found, in the online version, at doi:10.1016/j.bcp.2012.01.018.

3.3. Aggregation of Pt-destabilized Atox1

The Pt-substances unfolded Atox1 to different degrees as measured by CD. This may be due to different levels of unfolding but most likely due to variations in unfolded state, aggregation and precipitation. Therefore, protein aggregation status was analyzed by SDS-gels. Samples of apo and holo Atox1 were incubated with 5 eq. Pt-substance for different length of time, four days to freshly prepared (Fig. 7). Control samples with apo and holo Atox1 showed no signs of aggregation for this time period (Supplementary Fig. 1). In presence of all Pt-substances the amount of aggregation in samples with holo Atox1 is higher than with apo Atox1. This correlates with the larger extent of unfolding found in the holo-Atox1 far-UV CD-experiments. For addition of CisPt mostly dimers and trimers are found, with the amount increasing with incubation time. For TransPt, four days of incubation with holo Atox1 gives oligomeric species with two, three, and four Atox1 molecules, while the other TransPt samples mostly show traces of dimers. Both CarboPt and OxaliPt give only weak oligomeric bands and mostly dimers. PyriPt gives few or no oligomeric bands on the gel. SDS-gel experiments with TetraclPt give very weak bands and bands with monomeric apo and holo Atox1 is lacking. The fresh samples show monomeric Atox1 as well as dimeric bands. This can be explained by the fast reaction of TetraclPt with Atox1: Large aggregates will form quickly and they do not enter the gel resulting in the apparent lack of bands.

4. Discussion

4.1. Discussion of results

The anticancer Pt-substances are believed to react with DNA and cross link via two *cis*-ligands. Even so their way through the body and cells seems to vary considering their preferences for different types of tumors and their various side effects. These differences are to some extent mediated by their interactions with proteins, for example in the transport into the cell and protein binding related side effects. The ligands around the Pt-center in these types of drugs result in different chemical properties and this may be linked to different reaction patterns on the way to the DNA and thereby a decisive difference in cancer treatment. We focus on one protein, the copper chaperone Atox1 that is proposed to be involved in the resistance mechanism of CisPt by direct binding of the drug [10,13]. Here, reactions of Atox1 with a selection of Pt-substances with differences in ligands and ligand orientation were studied in vitro. Our solvent conditions were chosen to mimic the in vivo scenario as much as possible. Atox1 and CisPt may both be in the μM range in the cytoplasm [49,50].

In the unfolding experiments we found distinct differences in both unfolding extent and unfolding rate with the different platinum substances. The Pt-complex triggered unfolding rate of the protein is dependent on the hydrolysis rate and extent of the platinum substance, since the hydrolyzed form is more reactive to protein binding. TransPt hydrolyzes faster than CisPt because

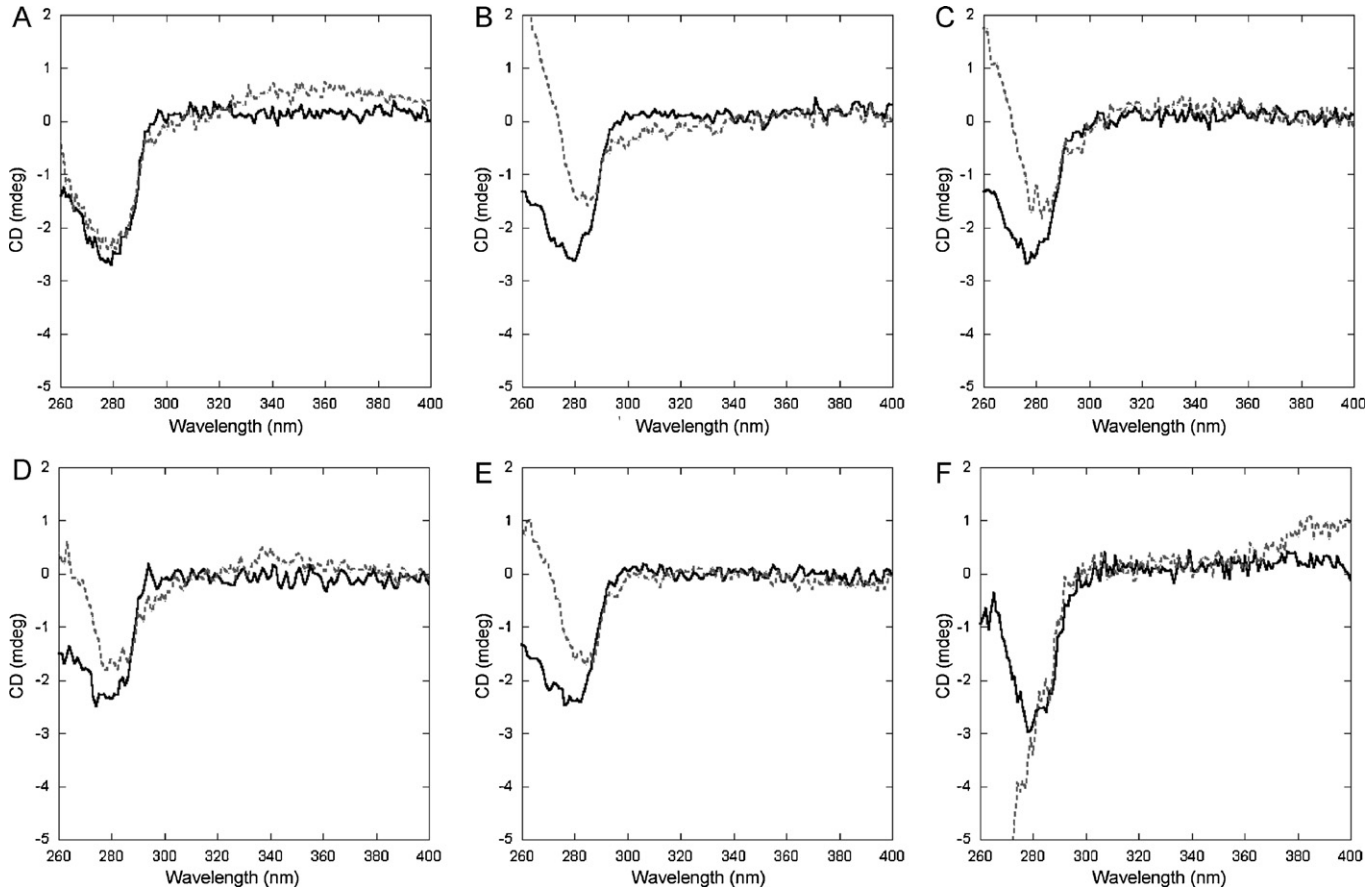

Fig. 6. Near-UV CD of apo Atox1 incubated 10 min with 1 eq. platinum substance (solid line) and added 2 eq. Cu (dotted line). (A) CisPt. (B) TransPt. (C) CarboPt. (D) OxaliPt. (E) PyriPt. (F) TetraclPt.

chloride has a larger trans effect than amine. This correlates well with our finding that TransPt unfolds Atox1 faster than CisPt. TetraclPt also has fast hydrolysis, and more positions likely to hydrolyze, which causes an even faster unfolding of Atox1. OxaliPt causes slower protein unfolding than TransPt and TetraclPt, in line with its slower hydrolysis rate, but faster than CisPt which might be surprising. However previous studies have shown that sulfur ligands like cysteine can bind to OxaliPt prior to hydrolyzation [30,40]. OxaliPt is believed to retain its 1,2-diaminocyclohexane moiety in cell like environments, thereby having only two active positions for binding the protein. The high unfolding extent by OxaliPt may be because of the hydrofobicity of the group forming interactions with hydrophobic parts of the unfolded protein. The high extent of unfolding for PyriPt, despite only one ligand likely to

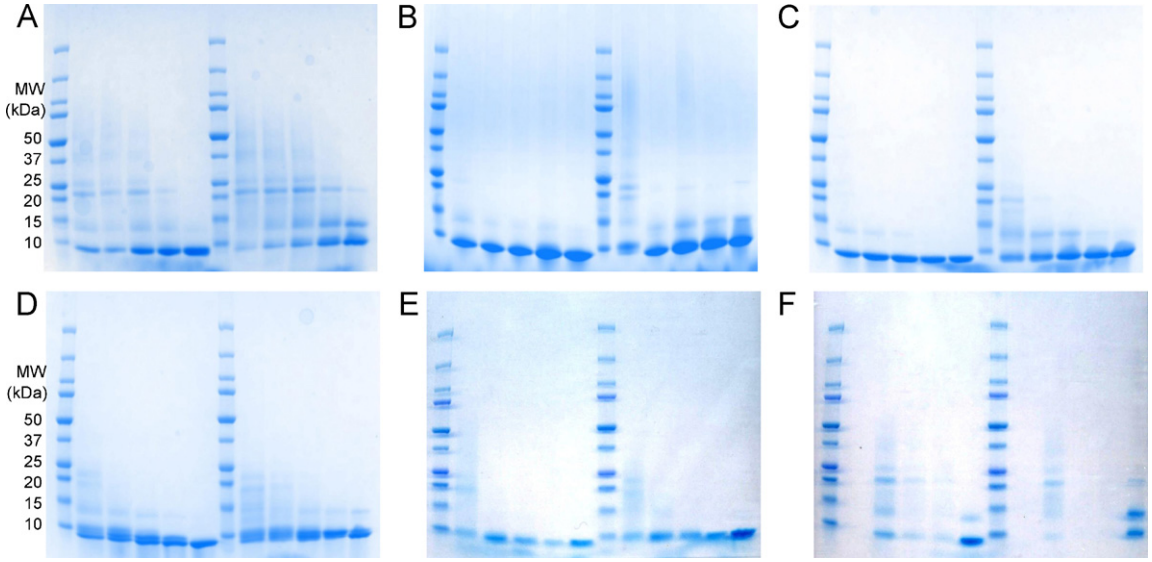

Fig. 7. Apo and holo Atox1 aggregation monitored with SDS-gel electrophoresis. Apo Atox1 is to the left and holo Atox1 to the right in all spectra. Time for incubation of samples is from left to right for both apo and holo Atox1: 4 days, 2 days, 1 day, 4 h and freshly prepared. (A) CisPt. (B) TransPt. (C) CarboPt. (D) OxaliPt. (E) PyriPt. (F) TetraclPt.

hydrolyze and react may in a similar way as for OxaliPt be explained with the pyridine ligand searching hydrophobic interactions and thereby causing protein unfolding. TransPt exhibits low extent of Atox1 unfolding despite a fast rate. When a sulfur ligand from the protein binds to a hydrolyzed position, the ligand trans in the Pt-complex will become unstable. Another protein residue might bind there and thereby destabilize the already formed protein-TransPt bond opposite. Since the amine ligands are less likely to hydrolyze and are not destabilized by the trans effect, TransPt will have only two active positions that destabilize each other in a fluctuating manner. TransPt should thereby have less driving force to form stable bonds with protein side chains than CisPt, which can bind at least two sulfur ligands simultaneously. CarboPt hydrolyzes very slowly, and hydrolysis is believed to be necessary for its interactions with thiol-ligands such as cysteine. This corresponds with the lack of distinct signals initiating Atox1 binding in our experiments. In general, all unfolding rates are slower for holo than for apo Atox1, likely because the holo configuration is more stable [48] and the cysteines are occupied, at least initially. Inspection of the extent of unfolding did show that it is more extensive for holo Atox1 in all cases. This is in agreement with the intrinsic reactivity of copper when released from the protein and when the reducing agent has oxidized.

The near-UV CD titration experiments are carried out under a relatively short time frame (~2 h), but since all substances are pre-hydrolyzed in pure water a fraction of the substances will be ready to react. The changes in near-UV CD-signal when adding CisPt to holo Atox1 have previously been described by us [14]. The new spectral features that appear resemble the ones from metallothioneins, small cysteine rich peptides that bind many metals interacting in large clusters [51–53]. The CD-signals are proposed to arise from d^8–d^{10} interactions of adjacent metal ions [47]. We therefore hypothesize that the near-UV CD-signals is a result of simultaneous Cu and Pt binding to Atox1 in close proximity, likely to or near the cysteines in the copper-binding site. Although the unfolding experiments show that all Pt-substances studied (except CarboPt) interact with Atox1, not all result in near-UV CD changes. TransPt and PyriPt do not give any change in near-UV CD-signal. For TransPt this can be explained by that the orientation of the ligands matters when it comes to form an Atox1-Cu–Pt complex. PyriPt might be unable to interact with the copper-binding site because it has only one good leaving group and a bulky ligand next to it that can cause steric hindrance. In contrast, signal changes in near-UV CD for OxaliPt and TetraclPt are similar to those of CisPt. Because OxaliPt is believed to retain its 1,2-diaminocyclohexane group the two available sites for binding are in the *cis*-position. Since the three substances giving a change in CD-signal when added to holo Atox1 all have good leaving groups in *cis*-position, and the ones that lack change in CD-signal do not, this appear to be an important characteristic for the ability to bind in or near the copper-binding site in Atox1 in a way that allows for Cu–Pt interactions. Making the reverse titration, adding Cu to a pre-mixture of apo Atox1 and 1 eq. of Pt-substance, we can determine if Cu can bind as normal or if the Pt-substance hinders the copper-binding. When Cu is added to the pre-mixture of CisPt, OxaliPt and TetraclPt together with Atox1 new positive peaks appears, similar to the ones seen for Pt-complex titration to holo Atox1. Thus, in these cases, similar ternary complexes seem to form regardless of order of metal addition. TransPt and PyriPt do not interfere with the holo protein signal and in agreement presence of these two substances did not interfere with the normal spectral changes for copper-binding. Since the substances still bind to Atox1 as deducted from their effect on unfolding, they bind at a different site (not the copper-binding site; there is a third cysteine in Atox1, Cys41) or the Pt-substance is kicked out from the copper-binding site upon Cu addition. This reasoning correlates with the idea that these two substances do not have two ligands in *cis*-position.

We used non-reducing SDS-gels to investigate possible covalent aggregates during the course of the experiments. The holo Atox1 samples with added Pt-substance aggregates more than apo, due to Cu-mediated oxidations at later stages of incubation. For TetraclPt there is a lack of Atox1 monomers and only weak oligomeric bands for all but the fresh samples. Since TetraclPt has four possible binding positions, cross linking likely result in large protein aggregates that do not enter the gel. In accordance with, blue stain was visible in the loading wells, indicating large aggregates. TransPt gives less oligomerization than CisPt in agreement with the lower unfolding extent detected by CD. The limited ability to crosslink for PyriPt and OxaliPt is due to, for PyriPt fewer good leaving ligands, and for both substances steric hindrance that results in less aggregation compared to CisPt. Finally CarboPt induces few oligomeric bands, probably because of lack of interactions with Atox1 cysteine residues.

5. Summary and conclusion

CisPt that is hydrolyzed approximately to one third after the pre-hydrolyzation time [14] readily reacts with Atox1 and gives new near-UV CD-signals in the near-UV region followed by protein unfolding. Since CisPt hydrolyzes at two *cis*-positions and will bind sulfur containing ligands there, the amino groups opposite will dissociate due to the trans effect and CisPt can eventually use all four ligands for binding to Atox1. TransPt hydrolyzes faster than CisPt and is also reported to bind to sulfur ligands faster than CisPt because of the trans effect [30]. In accordance with, TransPt unfolds Atox1 faster than CisPt, however, the extent of unfolding is lower. The lower extent is explained by ligand geometry, TransPt bind with at most two bonds to Atox1, and these bonds will destabilize each other. That TransPt does not give rise to any changes in near-UV CD indicating that the two ligands able to bind Atox1 needs to be in *cis*-configuration in order to detect Pt–Cu interactions. CarboPt that probably only have traces of hydrolyzed product after the pre-hydrolyzation time, due to the slow hydrolyzation rate ($t_{1/2}$ = 99 h), does not give near-UV CD-signal changes and protein unfolding matches that of the pure protein, in accord with no or little protein binding. However, one should bear in mind that CarboPt is biotransformed in vivo [31,32], otherwise it would be too stable to react with DNA, and therefore its interactions with Atox1 in the cell may resemble more of the ones with CisPt. A hypothesis is that sulfur ligands is part of the biotransformation making CarboPt lose its chelate ring, to later be replaced by DNA ligands [54]. OxaliPt will have only a small fraction of hydrolyzed product in the start of the experiments but it still interacts readily with the protein giving Pt–Cu features and causing unfolding. This is probably because of direct attack of the sulfur ligand to the unhydrolyzed substance. The high extent of unfolding with OxaliPt is explained by hydrophobic effects. There are no studies of PyriPt hydrolysis, but one can speculate that the chloride ligand will be hydrolyzed when dissolved in water. PyriPt causes slow Atox1 unfolding probably due to the presence of only one good leaving group. Similar to OxaliPt, the hydrophobic pyridine group might favor unfolding by interacting with the hydrophobic core of Atox1, resulting in the larger extent of unfolding observed. PyriPt does not give signals for Cu–Pt interactions and as TransPt, PyriPt lacks two good leaving groups in *cis*-position. TetraclPt with its fast hydrolyzation and four good leaving groups unfolds Atox1 most rapidly of the studied substances and also gives large changes in near-UV CD.

We conclude that the ligands around the Pt-center in platinum-based anticancer drugs dictate how the substances interact with Atox1 and their hydrolysis rate defines the speed of the reactions.

The findings reported here will apply also to other cysteine-containing proteins inside and outside the cells. Since a large part of administrated Pt-drug is believed to bind proteins and thereby not reach the DNA, new designs limiting protein interactions may enhance the anticancer effect.

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