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Mechanistic and structural basis for inhibition of copper trafficking by platinum anticancer drugs

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Abstract

Copper (Cu) is required for maturation of cuproenzymes, cell proliferation, and angiogenesis and its transport entails highly specific protein-protein interactions. In humans, the Cu chaperone Atox1 mediates Cu(I) delivery to P-type ATPases Atp7a and Atp7b (the Menkes and Wilson disease proteins, respectively), which are responsible for Cu release to the secretory pathway and excess Cu efflux. Cu(I) handover is believed to occur through the formation of three-coordinate intermediates where the metal ion is simultaneously linked to Atox1 and to a soluble domain of Cu-ATPases, both sharing a CxxC dithiol motif. The ultra-high thermodynamic stability of chelating S-donor ligands secures the redox-active and potentially toxic Cu(I) ion, while their kinetic lability allows facile metal transfer. The same CxxC motifs can interact with and mediate the biological response to antitumor platinum drugs, which are among the most used chemotherapeutics. We show that cisplatin and an oxaliplatin analogue can specifically bind to the heterodimeric complex Atox1-Cu(I)-Mnk1 (Mnk1 is the first soluble domain of Atp7a), thus leading to a kinetically stable adduct that has been structurally characterized by solution NMR and X-ray crystallography. Of the two possible binding configurations of the Cu(I) ion in the cage made by the CxxC motifs of the two proteins, one (bidentate Atox1 and monodentate Mnk1) is less stable and more reactive toward *cis*-Pt(II) compounds, as shown by using mutated proteins. A Cu(I) ion can be retained at the Pt(II) coordination site, but can be released to glutathione (a physiological thiol) or to other complexing agents. The Pt(II)-supported heterodimeric complex does not form if Zn(II) is used in place of Cu(I) and transplatin instead of cisplatin. The results indicate that Pt(II) drugs can specifically affect Cu(I) homeostasis by interfering with the rapid exchange of Cu(I) between Atox1 and Cu-ATPases, with consequences on cancer cell viability and migration.

Introduction

Copper trafficking pathways require high protein-protein specificity, and generally involve a Cu chaperone that binds and delivers Cu to specific targets and organelles. In humans, Atox1 delivers Cu(I) to the metal-binding domains (MBDs) of Atp7a and Atp7b (the Menkes and Wilson disease proteins, respectively), two Cu(I)-transporting ATPases located in the Golgi [1]; each ATPase has six soluble MBDs, which have been shown to interact differently with Atox1 in a Cu-dependent manner [2–4]. Both Atox1 and the MBDs bind one Cu(I) through two cysteine (Cys) residues located in a conserved CxxC motif [5], and Cu(I) transfer is believed to occur via an Atox1-Cu(I)-MBD complex [6]. In cells exposed to high Cu levels, Cu(I) exchange between Atox1 and MBDs promotes Atp7a translocation from the trans-Golgi network (TGN) to the plasma membrane or cytosolic vesicles [7,8].

Enhanced Cu trafficking has been associated with various types of cancer, where Cu is required for angiogenesis, cell proliferation and metastasis [9,10]. This has stimulated numerous approaches aiming at designing compounds able to interfere with Cu transport [11]. A small molecule, selected among a library of compounds, was shown to target the Cu delivery pathway to the secretory compartment, by destabilizing the interface between the Cu(I) chaperone Atox1 and the acceptor domains of Cu(I)-ATPases, thus selectively reducing cancer cell proliferation and significantly attenuating tumor growth in mouse models [12].

Pt-based anticancer drugs used in the clinics, cisplatin, oxaliplatin and carboplatin (Fig. 1a), have the ability to target sulfur-containing metal-binding sites in Cu transporters [13–17] and such behavior was shown to influence the cellular pharmacology of Pt drugs, either in terms of sensitivity or of resistance [18–20]. Thus, Cu transporters have been proposed to be involved in Pt cellular uptake and subcellular distribution to different compartments and organelles.

Although nuclear DNA is the ultimate target of cisplatin, only a few percent (1-4%) of intracellular cisplatin forms adducts with nuclear DNA [21–26]. The remaining part readily reacts with thiol-containing molecules inside the cytoplasm, including glutathione (GSH) and metallothioneins (MTs) [27,28].

As a matter of fact, cisplatin can exert prominent cytotoxicity also in enucleated cells (cytoplasts) [29–31]. Therefore, the cytostatic/cytotoxic effects do not depend solely on the genotoxic activity of cisplatin, but can originate from both nuclear and cytoplasmic signaling pathways [32–34] .

The molecular mechanisms that underlie the cytotoxic potential of cytoplasmic drugs are still poorly understood, but may involve the accumulation of reactive oxygen species (ROS) [30,33].

It was found that cells treated with cisplatin for prolonged periods often develop acquired resistance [35–37] and that such a resistance is in part associated to higher expression levels of Atox1 [38,39] and Cu(I)-ATPases [40,41]. In particular, it has been reported that overexpression of Atp7a in human carcinoma cells could result in the development of resistance [42], which is not necessarily associated to enhanced efflux of cisplatin, but may be due to altered Pt intracellular distribution (overexpressed transporters favoring drug accumulation in peripheral subcellular compartments) [40,43].

We have shown that, following an ATP concentration jump, Atp7a or Atp7b embedded in microsomes adsorbed on a gold electrode-supported membrane, can translocate Pt(II) cations, thus inducing a current transient [44]. Moreover, Pt(II) drugs can inhibit the active transport of Cu(I) by the ATPases and, viceversa, Cu(I) can inhibit the translocation of Pt(II) cations. However, we failed to observe Pt transfer between Atox1 and the first soluble Cu(I)-binding domain of Menkes ATPase under close to physiological conditions, i.e. in the presence of GSH as reducing agent [45].

We and others observed that preloading with Cu(I) can foster the interaction with Pt drugs of the dithiol motifs of Cu(I) transporters [44–47], thus we deemed of crucial importance to characterize the interaction of cisplatin and oxaliplatin with the Cu(I)-mediated complex of Atox1 and the first soluble domain of Atp7a (Mnk1 hereafter).

The present work focuses on the reactivity of Cu(I) and Pt(II) (in the form of cisplatin and oxaliplatin) with the proteins Atox1 and Mnk1. The formation of adducts has been studied by NMR spectroscopy, HPLC/ESI-MS and UV-vis spectrophotometry. The results support the idea that cisplatin can interfere with copper trafficking by forming stable mono- and hetero-dimeric adducts with the proteins in the presence of Cu(I). Site-directed mutagenesis studies on Atox1 and Mnk1 were also performed to investigate the role of individual cysteines in the formation of adducts with cisplatin, confirming that at least one Cys from each partner is necessary to form the heterodimeric adduct. We also demonstrate that the Pt-bridged heterodimeric species does not form if transplatin is used in place of cisplatin or Zn(II) is used in place of Cu(I). The kinetically stable adduct with Pt(II) bridging the two proteins in the heterodimeric complex has also been structurally characterized by X-ray crystallography. Together with the Pt(II)-bridged species (72% occupancy) there is cocrystallization of a second species (28% occupancy) in which a Cu(I) is bound to Atox1 in a monodentate manner and to Mnk1 in a bidentate manner, this corresponds to one of the two hypothesized Cu(I)-bridged forms of the functional heterodimer not yet characterized by X-ray crystallography.

We propose that Pt drugs, apart from targeting DNA, can also impair Cu homeostasis with relevant detrimental effect on the cellular viability and migration.

Fig. 1

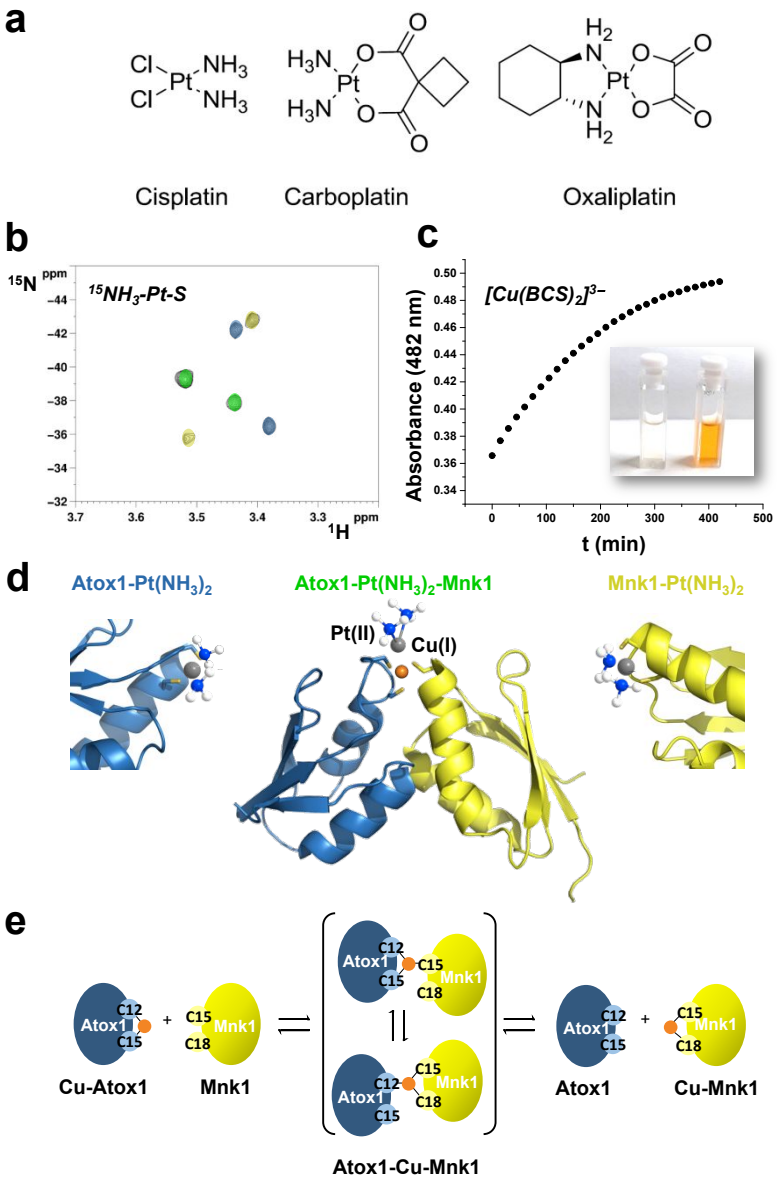

Figure 1. Cisplatin interferes with the rapid exchange of Cu(I) between the metallochaperone Atox1 and the first domain of Menkes ATPase (Mnk1). a, Chemical structures of Pt(II) anticancer drugs used in the clinics. **b,** Two-dimensional ^{1}H, ^{15}N-HSQC spectra of ^{15}N cisplatin incubated with Atox1-Cu(I)-Mnk1 complex for 4 h. Green cross-peaks belong to $^{15}NH_3$ of a platinated heterodimeric species, blue and yellow cross-peaks belong to $^{15}NH_3$ of monomeric bifunctional adducts of Atox1 and Mnk1, respectively. **c,** Time-dependent changes in absorbance at 482 nm ($[Cu^{I}(BCS)_2]^{3-}$) following addition of ^{15}N cisplatin to the Atox1-Cu(I)-Mnk1 complex, with BCS present from the beginning of the incubation,. The inset shows the color change of the solution

before addition of cisplatin and after 12 h incubation. **d,** Structural models of Atox1-Pt(NH_3)$_2$ (blue), Mnk1-Pt(NH_3)$_2$ (yellow) and heterodimeric Atox1-Pt(NH_3)$_2$-Mnk1 complex . Color codes: gray = Pt, orange = Cu, blue = N, white = H. **e,** The three-coordinate mechanism of Cu(I) exchange between Atox1 and Mnk1.

Results

Reaction of Atox1-Cu(I)-Mnk1 with cisplatin

In the presence of Cu(I), Atox1 and Mnk1 form a heterodimeric complex in solution which is in fast exchange with monomeric proteins [48]; therefore, when ^{15}N Cu(I)-Atox1 is titrated with increasing amounts of unlabeled apoMnk1 (up to one equivalent), in two-dimensional ^{1}H,^{15}N Heteronuclear Single Quantum Coherence (HSQC) spectra only one set of signals is observed throughout the titration (Supporting Fig. 1). A similar behavior is observed when an analogous titration is performed starting from ^{15}N Cu(I)-Mnk1 and adding aliquots of apoAtox1 (Supporting Fig. 2).

The weighted average chemical shift differences indicate that the most perturbed Atox1 residues belong to a positively charged patch that can interact with negative charges present on the surface of Mnk1 (Supporting Fig. 3); however, despite their surface complementarity, apoAtox1 and apoMnk1 do not form a complex in solution when mixed, indicating that the driving force to complex formation is provided by Cu(I) coordination to the CxxC consensus motifs of the two proteins [49]. Notwithstanding the attomolar affinity for Cu(I) [50,51] the complex is characterized by high kinetic lability [52], which allows rapid (faster than milliseconds) exchange between complexed and free protein. The equilibrium is insensitive to Cu scavengers present in excess in the intracellular milieu [53] and, within the complex, the metal ion undergoes a facile switch between two coordination modes (two sulfur donor atoms from one protein and one sulfur from the other) [6,54]; such a three-coordinate mechanism is also supported by NMR studies performed on Cu(I) complexes with GSH [52].

To investigate the effect of cisplatin, two equivalents of ^{15}N-labeled drug were added to the Atox1-Cu(I)-Mnk1 complex, under strict anaerobic conditions. The $^{15}NH_3$ chemical shifts of cisplatin are sensitive to the nature of donor atoms in *trans* position, therefore they can report changes in Pt coordination (the ^{15}N nuclei of ammine ligands resonate approximately between -30 and -50 ppm when trans to S, between -50 and -75 ppm when trans to Cl or N, and between -70 and -90 ppm when trans to O) [55].

After 1 h incubation of ^{15}N-labeled cisplatin with Atox1-Cu(I)-Mnk1, the ^{1}H,^{15}N HSQC NMR spectrum in the region of $^{15}NH_3$ displayed new signals, in addition to those of the dichlorido (*cis*-[$PtCl_2(^{15}NH_3)_2$], δ ^{15}N ~ -67 ppm) and monoaqua species (*cis*-[$PtCl(^{15}NH_3)_2(H_2O)]^+$, δ ^{15}N ~ -66 and -83 ppm for $^{15}NH_3$ trans to Cl and O, respectively). Two cross-peaks were characteristic of $^{15}NH_3$ trans to S (δ ^{15}N ~ -42.5 and -36.5 ppm) and were assigned to the bifunctional adduct Atox1-$Pt(^{15}NH_3)_2$ with both cysteines of CxxC bound to Pt. Other two cross-peaks were assigned to the monofunctional adduct Mnk1-$PtCl(^{15}NH_3)_2$ (δ ^{15}N ~ -66 and -38 ppm for $^{15}NH_3$ trans to Cl and S,

respectively) (Supporting Fig. 4). The above peaks are similar to those observed in the reaction of cisplatin with the free monomeric proteins [44,45,56].

After 4 h incubation, cross-peaks corresponding to the monofunctional adduct Mnk1-PtCl($^{15}NH_3$)$_2$ converted into a new pair of cross-peaks (δ ^{15}N ~ -43 and -36 ppm) assigned to the bifunctional adduct Mnk1-Pt($^{15}NH_3$)$_2$ (Supporting Fig. 4). In the same spectrum, two new cross-peaks (δ ^{15}N/^{1}H -39/3.52 and -38/3.44 ppm) appeared that were assigned to a Pt-bridged heterodimeric species with both $^{15}NH_3$ ligands trans to S atoms (Fig. 1b). We will refer to the latter species as "upfield-shifted Pt-bridged heterodimer". Notably, the latter cross-peaks were never observed in the reactions of cisplatin with the single proteins

After 12 h incubation, the $^{15}NH_3$ signals of the Pt derivatives with monomeric proteins vanished. Also the cross-peaks of the upfield-shifted Pt-bridged heterodimer faded but were replaced by two new signals, downfield-shifted along the proton dimension (δ ^{15}N/^{1}H -48.5/3.90 and -45.5/4.07 ppm, Supporting Fig. 4), which persisted even after 24 h of incubation. We will refer to the latter species as "downfield-shifted Pt-bridged heterodimer". The disappearance of the $^{15}NH_3$ signals of the Pt derivatives with monomeric proteins could be due to protein unfolding, fostered by the simultaneous binding of Cu and Pt to Atox1 or Mnk1 [39,44], as witnessed by the coalescence of some amide signals in the ^{1}H,^{15}N HSQC spectrum (Supporting Fig. 5a,b).

Addition of bathocuproine disulfonate (BCS, a ligand which binds specifically Cu(I) to yield the 1:2 chromophoric complex $[Cu^{I}(BCS)_2]^{3-}$ with high affinity ($\beta_2 = 10^{19.8}$ M^{-2}) [50]) at the end of incubation with cisplatin caused immediate disappearance of the downfield-shifted $^{15}NH_3$ signals and reappearance of those of the initial upfield-shifted Pt-bridged heterodimer (Fig. 2a). Notably, a similar behavior was observed when the physiological reducing agent GSH was added instead of BCS (Fig. 2b). It can be concluded that the cross-peaks with deshielded ammine ligands (δ ^{15}N/^{1}H -48.5/3.90 and -45.5/4.07 ppm) belong to a heterodimeric species containing a rather labile Cu localized close to Pt.

When BCS was added at the onset of incubation, all $^{15}NH_3$ signals of monomeric Atox1 or Mnk1 bifunctional adducts were still detectable after 24 h, together with the signals of the upfield-shifted Pt-bridged heterodimer, indicating that Cu(I) sequestration by BCS prevents both the formation of the downfield-shifted Pt-bridged heterodimer and the degradation of the monomeric bifunctional adducts (as also witnessed by the lack of signal coalescence in the protein HSQC spectrum, Supporting Fig. 5c,d). In the latter experiment, UV-Vis absorption spectra showed a time-dependent increase of absorbance at 482 nm due to formation of $[Cu^{I}(BCS)_2]^{3-}$ (Fig. 1c), indicating a slow release of Cu from the proteins to BCS (Fig. 1d).

The same reaction was further checked by HPLC/ESI-MS analysis. Thus, a mixture of holo ^{15}N Atox1 and apoMnk1 was treated with two equivalents of cisplatin under strictly anaerobic conditions at 25 °C and pH 7 (25 mM phosphate buffer). After 4 h incubation, the chromatogram (UV detector) showed three main peaks with retention times (t_R) of 18.5, 19.2, and 19.6 min (Fig. 3a) whose mass spectra (Supporting Fig. 6) were in accord, in the order, with the following compositions: ^{15}N Atox1-Pt$(NH_3)_2$ (7579 Da and 7710 Da), ^{15}N Atox1-Pt$(NH_3)_2$-Mnk1 (15947 Da and 16078 Da), and ^{15}N apoAtox1 (7350 Da and 7481 Da) plus apoMnk1 (8368 Da). The first peak in each deconvoluted spectrum corresponds to the expected total mass after cleavage of the N-terminal methionine of Atox1 (131 Da).

The HPLC/ESI-MS spectrum of the peak at t_R = 19.2 min was further analyzed (Fig. 3b). Each multi-charged state showed two main peaks, corresponding to ^{15}N Atox1-Pt$(NH_3)_2$-Mnk1 with or without the N-terminal methionine of Atox1, and a series of low intensity signals corresponding to ^{15}N Atox1-Pt$(NH_3)_2$-Mnk1 + 2Cu (16204 Da) and ^{15}N Atox1-Pt-Mnk1 + Cu (16107 Da, a heterodimeric species in which Pt(II) has lost the ammines). Thus, the HPLC/ESI-MS analysis supports the co-presence of Cu(I) and Pt(II) also in the first formed heterodimeric complex with upfield-shifted ammine protons.

According to the three-coordinate model of Cu(I) binding, only three cysteines of the two CxxC motifs are needed for the formation of the Atox1-Cu(I)-Mnk1 complex (Fig. 1e) [6,49]. While the two cysteines that are more N-terminal in sequence (Cys12 of Atox1 and Cys15 of Mnk1) are strictly required for the interaction, the third cysteine residue can be either Cys15 of Atox1 or Cys18 of Mnk1 [48].

We used site-directed mutagenesis of the latter two residues to investigate the role of individual cysteines in the formation of adducts with cisplatin. Upon addition of two equivalents of ^{15}N-labeled cisplatin to a 1:1 mixture containing the WT form of one protein and the mutant of the other protein (lacking the cysteine less N-terminal in sequence), the NMR spectra showed that in both cases (Atox1-Cu(I)-C18A Mnk1 and C15A Atox1-Cu(I)-Mnk1) only a platinated heterodimeric species was formed, while no peaks related to monomers could be detected (Supporting Figs. 7 and 8). The $^{15}NH_3$ signals were sensitive to the addition of BCS, giving rise, in both cases, to a new pair of upfield-shifted signals (Fig 2a). Thus, unlike the case of wild-type proteins, in the case of mutants the formation of the heterodimeric species with downfield shifted ammine signals is observed from the beginning. Interestingly, the reaction with cisplatin was faster in the case of Atox1-Cu(I)-C18A Mnk1 than in the case of C15A Atox1-Cu(I)-Mnk1. Finally, when both mutants

were mixed together, no adducts were formed, consistent with the fact that one cysteine from each protein is not sufficient to stabilize the Atox1-Cu(I)-Mnk1 heterodimer.

SDS-PAGE, performed at the end of incubation with cisplatin, showed that in all cases (WT proteins and mutants), a dimeric species resistant to the denaturing conditions of the gel was present (Fig. 2c), which is most likely supported by a Pt bridge as witnessed by the characterization of Pt(II)-supported heterodimeric species by NMR and X-ray crystallography and the absence of heterodimeric complex resistant to denaturing gel in the absence of added Pt drug.

Fig. 2

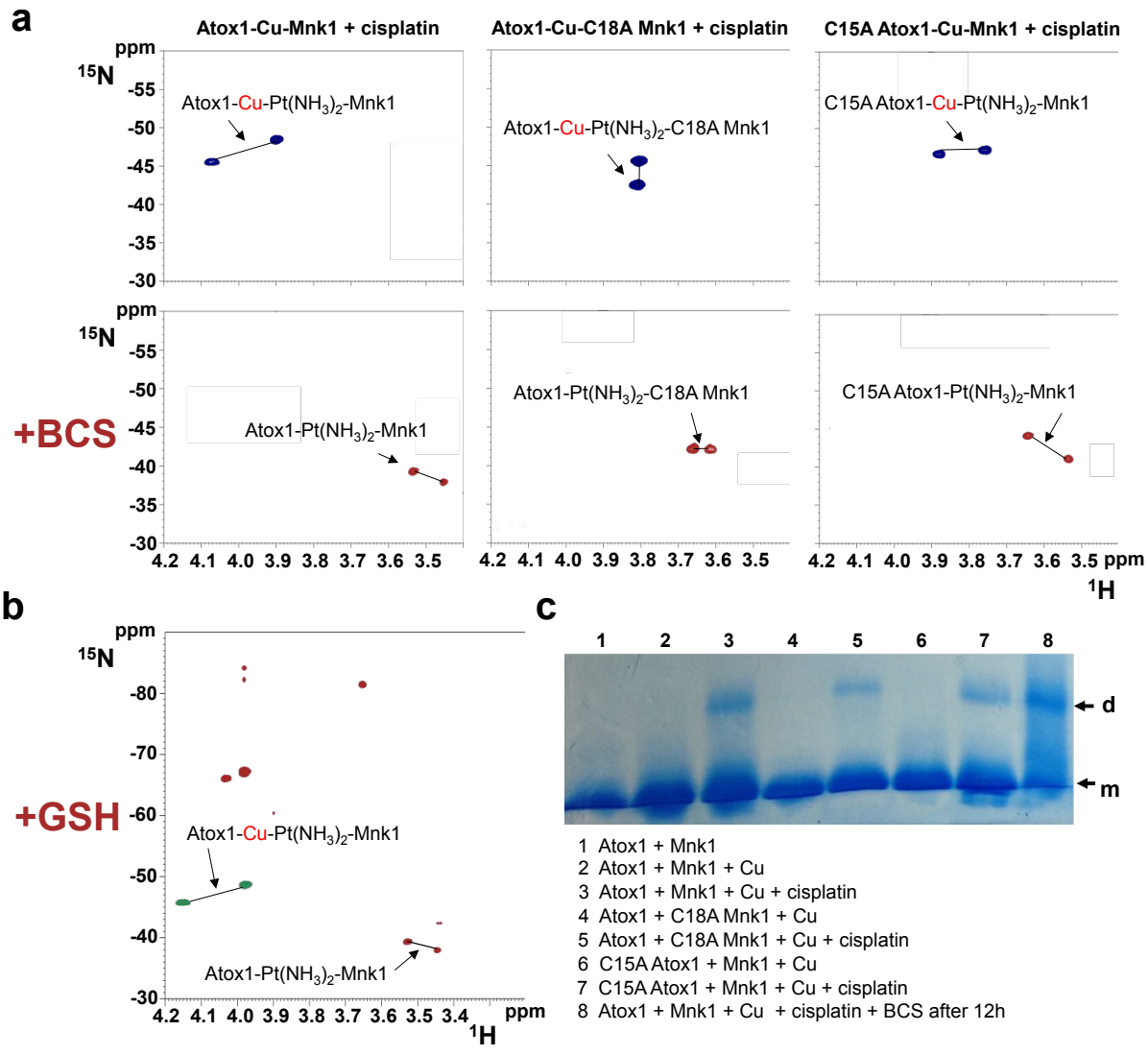

Figure 2. Binding of cisplatin stabilizes heterodimers of Atox1 and Mnk1. a, Two-dimensional ^{1}H, ^{15}N-HSQC spectra of ^{15}N cisplatin incubated for 24 h with Atox1-Cu(I)-Mnk1, Atox1-Cu(I)-C18A Mnk1 and C15A Atox1-Cu(I)-Mnk1 complexes, before and after addition of bathocuproine

disulfonate (BCS); the formulae include only the labile Cu which is shown in red. **b,** Overlay of two-dimensional ^{1}H, ^{15}N-HSQC spectra of ^{15}N cisplatin incubated for 24 h with Atox1-Cu(I)-Mnk1 complex, before (green contours) and after (brown contours) addition of glutathione (GSH); the formulae include only the labile Cu which is shown in red. **c,** SDS-PAGE of various samples incubated in the absence or in the presence of cisplatin, as indicated in the legend for lanes 1-8. The positions of protein monomers (m) and heterodimers (d) resistant to denaturing gel conditions are indicated by arrows.

The Pt-bridged heterodimeric species does not form if transplatin is used in place of cisplatin or Zn(II) is used in place of Cu(I)

For an unspecific bridging interaction, transplatin is expected to behave similarly to cisplatin, therefore an experiment was performed in which transplatin was added to a solution of the preformed Atox1-Cu(I)-Mnk1 complex. Although Atox1 exhibited some reactivity toward transplatin (a Pt-bound Atox1 was detected by HPLC/ESI-MS analysis), no peaks assignable to Pt-bridged dimeric species were detected (Supporting Fig. 9). Therefore, the *cis* orientation of the leaving ligands in the Pt substrate is essential for cross-linking Atox1 and Mnk1.

The results so far obtained also indicate that a preformed Atox1-Cu(I)-Mnk1 heterodimer is required for the formation of a Pt-containing heterodimeric species. To check whether the preformed Atox1-Mnk1 heterodimer must have a specific conformation, we performed an experiment in which cisplatin was added to a solution in which the two proteins had been incubated with Zn(II) (known to be able to bridge the two proteins) [51].

SDS-PAGE and HPLC/ESI-MS analysis revealed that, under the above conditions, only Pt-containing monomeric species were formed, while there was no indication, whatsoever, of formation of Pt-containing heterodimeric species (Supporting Fig. 10). The NMR investigation also showed that the reaction of cisplatin with the Atox1-Zn(II)-Mnk1 complex is very slow and leads to two sets of signals (δ ^{15}N ~ -84/-83 and -41 ppm for $^{15}NH_3$ trans to O and S, respectively), identical to those obtained by reaction of cisplatin with Zn(II)-Atox1 in the absence of Mnk1 (Supporting Figs. 11 and 12), thus indicating that, in the presence of Zn(II), cisplatin selectively binds to the Atox1 monomer. It is concluded that the use of Cu(I) is a harbinger of the Atox1-Pt$(NH_3)_2$-Mnk1 complex formation.

Crystal structure of the Pt-bridged heterodimeric species with trapped Cu(I) ion

To gain an atomistic view of the Pt-bridged heterodimeric species, a crystallographic investigation was undertaken. Since the long-time (some days) required for crystallization experiments can foster

the loss of the ammine ligands, we used an aquated form of oxaliplatin (*cis*-[Pt(SO_4)(H_2O)(1,2-*R,R*-DACH)]) (DACH = diaminocyclohexane) containing a chelated, and intrinsically less labile [57], diamine ligand (see Supporting *Materials and Methods*). First, it was proven that aquated oxaliplatin behaves as cisplatin, affording the analogous Pt-bridged heterodimeric species in which Pt retains the DACH ligand (Supporting Fig. 13).

After incubation of the Atox1-Cu(I)-Mnk1 complex with two equivalents of *cis*-[Pt(SO_4)(H_2O)(1,2-*R,R*-DACH)] and detection of the Atox1-Pt(1,2-*R,R*-DACH) -Mnk1 heterodimeric species by ESI-MS, the hanging drop technique was used to produce rod crystals having P $2_12_12_1$ symmetry. The structure (PDB entry 5T7L) was solved to a resolution of 2.8 Å (unit cell parameters and diffraction data statistics are shown in Supporting Table 1).

The asymmetric unit contains the two protein molecules (Atox1 is chain A, Mnk1 is chain B) arranged around a non-crystallographic 2-fold axis (Fig. 3c). The overall arrangement is similar to that of the heterodimer crystallized in the presence of Cd(II) (PDB entry 3CJK, 1.8 Å resolution) [48], with an overall Cα root-mean-square deviation (RMSD) of only 0.73 Å (RMSD of 0.37 Å for chain A and 0.84 Å for chain B).

Platinum binding sites

The Pt atoms have been clearly identified by using the anomalous signal (data collection was performed at 11700 eV, just above the Pt L3 edge), while the presence of Cu atoms was assessed based on the electron density map height (very weak Cu anomalous signal is expected at the above energy). Two Pt-binding sites are present, the 2Fo-Fc electron density map (in grey) and the anomalous map (in magenta) around the two Pt ions are shown in Fig. 3d.

In the first site the Pt ion (Pt1 in Fig. 3d) is bound to two cysteine residues: Cys12 of chain A and Cys15 of chain B. The two S atoms are, respectively, at a distance of 2.8 and 2.3 Å from Pt1 and form an S-Pt-S angle of 86.0° (details on bond distances and angles are given in Supporting Tables 2 and 3). Apart from the two cysteine residues, no other molecule, protein residue, or ion was found close to the Pt center, notwithstanding the starting Pt substrate contained a chelating DACH ligand. The high atomic displacement factor of Pt1 (B = 93.1 $Å^2$) may indicate a static disorder of the site, which hinders the detection of the Pt ligands in the solvent exposed region opposite the two bound cysteines; however the S-Pt-S angle of 86.0° clearly supports a square-planar coordination for the Pt center. Second shell reconstructed atoms are: W7 (4.6 Å); N of Cys12 (4.3 Å) and Cys15 (4.6 Å) and OG1 of Thr14 (4.8 Å) for chain B; N of Gly13 (4.7 Å) and O of Thr11 (4.9 Å) for chain A. The second Pt-binding site (Fig. 3d), located close to Cys41 of chain A (Atox1), will be discussed at the end of this section.

In the region of the two CxxC motifs, besides the Pt1 ion, there are also two Cu ions at 6.0 Å (Cu1) and 3.9 Å (Cu2) distance from Pt1 (Fig. 3d). Cu1 is in approximate linear coordination with the sulfur atoms of Cys15 of chain A (2.2 Å) and Cys18 of chain B (2.3 Å) forming a S-Cu-S angle of 166.7°. Unlike Cu1, Cu2 is very close to the position of the Cd(II) ion in the Atox1-Cd(II)-Mnk1 complex (3CJK model) [48]. This latter finding, coupled with the consideration that a distance of only 3.9 Å between Cu2 and Pt1 would lead to local electrostatic instability, lead us to hypothesize two alternate configurations for this heterodimer: one configuration comprising Pt1 and Cu1 and the other configuration comprising Cu2, this latter having a coordination very similar to that of Cd(II) in 3CJK. As a consequence, we split Cys12 and Cys15 of chain A and Cys15 of chain B in two conformations, one conformation corresponding to that refined from our data, the other conformation corresponding to that present in 3CJK. It is worth noting that for Cys18 of chain B the conformation refined from our data coincides with that present in 3CJK. Thus, Cu2 has a distorted tetrahedral coordination which involves Sγ of Cys12 (2.2 Å) for chain A and Sγ of Cys15 (2.6 Å) and Cys18 (2.9 Å) and Oγ1 of Thr14 (2.8 Å) for chain B. The crystallographic occupancies of the metal ions and corresponding donor atoms resulted to be 0.72 for the Pt1-Cu1 configuration, and 0.28 for the Cu2 one.

The second Pt-binding site (Fig. 3d) is located close to Cys41 of chain A (Atox1). This Pt ion (crystallographic occupancy of 0.36) is coordinated by the S atom of Cys41 (2.7 Å) and the Nζ atom of Lys3 (2.7 Å) of chain A. Two water molecules complete a distorted square-planar coordination geometry (average Pt-O distance of 2.7 Å). This site has a similarity with that recently found for the Atox1 homodimer crystallized in analogous conditions (PDB entry 4QOT) [58]. However, the Lys3 side chain assumes a different conformation in the heterodimer as compared to the Atox1 homodimer so that, after superposition of the Atox1 chains, the Pt2 atoms are 3.5 Å far apart.

Noncovalent interactions

Two salt bridges (Asp68B-Arg21A and Glu11B-Lys42B) are in common with the 3CJK model (Cd(II) heterodimer), while the two additional Asp9A-Lys60A and Asp63B-Lys25A salt bridges (the latter also forming an H-bond) replace the salt bridge Glu43A-Lys3A present in 3CJK, which has been disrupted by the reorientation of the Pt2-bound Lys3A side chain.

Although the overall number of H-bonds was greater in the 3CJK model, the present structure, 5T7L, exhibits two additional intra-dimer interactions with new Cys12A-Thr14B and Gly14A-Thr14B H-bonds fostered by the reorientation of the metal-bound residues Cys12A and Thr14B.

Overall, the larger number of intra-dimer noncovalent interactions stabilizes the 5T7L heterodimer, which assumes a more closed configuration than the 3CJK heterodimer.

Fig. 3

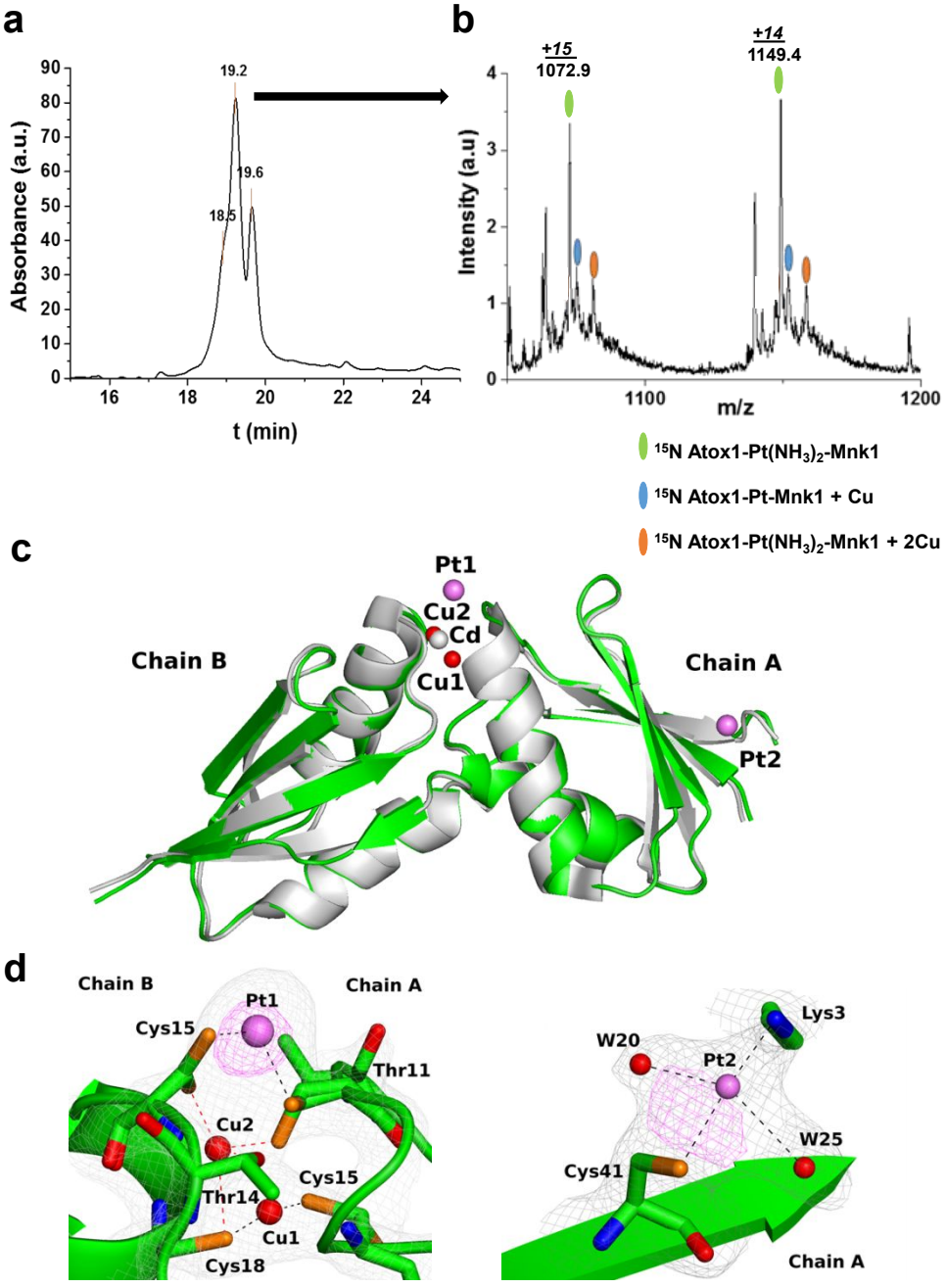

Figure 3. Stoichiometry and crystal structure of platinated heterodimers of Atox1 and Mnk1. **a,** HPLC-UV chromatogram of ^{15}N Atox1-Cu(I)-Mnk1 complex incubated for 4 h with two equivalents of cisplatin. **b,** HPLC/ESI-MS spectrum of the peak eluted at t_R= 19.2 min; only the two

most intense multi-charged states are displayed and the assigned peaks are labeled with ovals. **c,** Crystal structure of the heterodimeric complex (green) crystallized in the presence of [Pt(SO_4)(H_2O)(1,2-*R,R*-DACH)], superimposed to that of the Atox1-Cd(II)-Mnk1 complex (light gray, PDB entry 3CJK). Atox1 (chain A) and Mnk1 (chain B) molecules are shown in cartoon representation. Pt, Cu and Cd atoms are shown as magenta, red, and gray spheres, respectively. **d,** Platinum binding sites Pt1 and Pt2. Protein residues within 3 Å from Pt and Cu are shown as sticks, with C, O, N, and S atoms colored in green, red, blue, and yellow, respectively. The 2Fo-Fc electron density map (grey, 2σ for Pt1 and 1σ for Pt2 sites) and the anomalous map (magenta, 6σ for Pt1 and 3σ for Pt2 sites) are also shown. Dashed lines are drawn between the metal ions and the closest protein donor atoms. Red and black dashed lines indicate the two different metallic cores (one comprising Pt1 and Cu1 and the other Cu2) of the crystallized heterodimer.

Discussion

Previous studies have shown that Atox1 is able to bind to Pt in a bidentate manner forming the species Atox1-Pt$(NH_3)_2$ [46,56]; in contrast, Mnk1 first generates the monofunctional adduct Mnk1-PtCl$(NH_3)_2$, which subsequently evolves towards the bidentate species Mnk1-Pt$(NH_3)_2$ [44].

In the presence of GSH, Atox1 does not compete successfully with the physiological thiol for Pt binding; in contrast, Mnk1 does coordinate to Pt [45]. Remarkably, preloading with Cu enhances the reactivity of both proteins toward cisplatin: Cu(I)-Atox1 readily coordinates Pt even in the presence of GSH and Cu(I)-Mnk1 gives directly the bifunctional adduct Mnk1-Pt$(NH_3)_2$ [44,45]. Increased platination rate, as a consequence of preloading with copper, has also been demonstrated for other soluble domains of Atp7a and Atp7b [47].

In competition experiments, cisplatin reacts with Mnk1 in preference to Atox1 [45]; however, Pt-loaded Atox1 is unable to handover Pt to Mnk1.

A reactivity non ascribable to that of the single proteins is observed in the case of reaction of cisplatin with the Atox1-Cu(I)-Mnk1 heterodimeric complex.

Reaction of cisplatin with wild-type proteins

In the case of wild-type proteins the heterodimeric complex Atox1-Cu(I)-Mnk1 is in equilibrium with the monomeric species [48,54]. In the first hour of incubation only Pt-adducts of monomeric proteins are formed; however, while monomeric Atox1 gives the bifunctional adduct Atox1-Pt$(NH_3)_2$ notwithstanding the presence of Mnk1, monomeric Mnk1, similarly to the case of absence of Cu, forms first the monodentate Mnk1-PtCl$(NH_3)_2$ adduct that only afterwards affords the bidentate Mnk1-Pt$(NH_3)_2$ species. The latter results can be explained by looking at the solution structure of the Cu(I)-mediated complex between the yeast homologues of Atox1 and Mnk1 (Atx1 and the first domain of Ccc2 ATPase) [49]. In the Cu(I)-mediated heterodimeric complex Atx1 was found to have a conformation more similar to its monomeric Cu(I)-loaded state (helix α1 fully formed and Cys15 in helical conformation, at variance with the *apo* state), which would favor the formation of a bifunctional adduct with cisplatin. In contrast, the ATPase domain was found to undergo a conformational rearrangement with partially unwound helix α1, which would disfavor the formation of the bifunctional adduct with cisplatin. Thus, the conformational changes observed in the yeast homologues are consistent with the observed reactivity toward cisplatin of human proteins when in a complex with a bridging Cu(I) ion, i.e. Atox1 reacts as in its Cu(I)-bound form while Mnk1 reacts as in its *apo* form.

Unlike the case of mutants (see following discussion), the initially formed Atox1-Pt$(NH_3)_2$-Mnk1 heterodimeric complex (at 4 h incubation time) has upfield-shifted $^{15}NH_3$ signals, and only at longer

reaction time (after 12 h incubation) it undergoes transition to a downfield-shifted Pt-bridged heterodimer containing a labile Cu that can be rapidly removed by BCS or GSH giving back the first formed heterodimeric complex with upfield-shifted $^{15}NH_3$ signals. The initial formation of the upfield-shifted Pt-bridged heterodimer in the case of the wild-type proteins may reflect the increased metal-loading capacity of the four thiolate ligands which, after coordination of Pt(II) to the more N-terminal cysteines of the CxxC motifs, can retain the Cu(I) ion in an internal/buried site involving the less N-terminal cysteines (Cys15 of Atox1 and Cys18 of Mnk1). This possibility is excluded in the case of one wild-type protein and one mutant, due to the lack of the second internal cysteine. With time some Cu can be taken up from the solution by the initially formed heterodimer yielding the downfield-shifted Pt-bridged heterodimer containing a labile Cu close to Pt which, if removed by BCS, gives back the initial upfield-shifted Pt-bridged heterodimer.

Most likely, the downfield-shifted Pt-bridged heterodimeric complex cannot form in physiological conditions due to the presence of GSH, which has been shown to be able to remove the labilized Cu.

Interestingly, both the nature of the pre-loaded metal ion (Cu(I)) and the *cis* configuration of the Pt substrate are required for the formation of the Pt-bridged heterodimeric complex, a clear example of metal template specificity. Indeed, by using Zn(II) in place of Cu(I) and transplatin in place of cisplatin no Pt-bridged heterodimeric species was formed. This specific recognition of the Atox1-Cu(I)-Mnk1 heterodimer by *cis*-oriented Pt(II) complexes is rather peculiar and not envisaged up to now.

Concerning the absence of interprotein cross-link when transplatin was used instead of cisplatin, we recall that both the intermolecular interactions and the metal ion bridging the two proteins contribute to the stability of heterodimeric adducts [48,49]. It is likely that the two more N-terminal cysteines are suited for a *cis*-coordination to platinum rather than for a *trans*-coordination as required by the *trans* isomer. Due to steric reasons, transplatin is also unable to form 1,2-intrastrand cross-links between two adjacent purines on a DNA strand, and this was related to its lack of pharmacological activity [21].

Concerning the different behavior of the Cu(I)- and Zn(II)-supported heterodimers, the results of the present investigation show that even in the case of Cu(I), which can assume two different configurations in the Atox1-Cu-Mnk1 heterodimer, one configuration (monofunctional Atox1 and bifunctional Mnk1) is less reactive towards Pt than the other (bifunctional Atox1 and monofunctional Mnk1). This implies the necessity of a fine matching between the preformed heterodimer (with either Cu(I) or Zn(II) bridging ion) and the attacking Pt substrate; most likely Zn(II) cannot provide such a fine matching.

Reaction of cisplatin with mutated proteins

The reaction of ^{15}N-labeled cisplatin with a 1:1 mixture containing the WT form of one protein and the mutant of the other protein (either C15A Atox1 or C18A Mnk1) leads to the direct and exclusive formation of a Pt-bridged heterodimeric complex with downfield-shifted $^{15}NH_3$ signals from which a Cu(I) ion can be rapidly removed by BCS leading to a Pt-bridged heterodimeric complex with upfield-shifted $^{15}NH_3$ signals. The absence of Pt adducts with monomeric proteins, suggests that the lack of a cysteine in one partner shifts the equilibrium completely towards the Cu(I)-bridged heterodimeric form, which has no tendency to dissociate into monomers. Moreover, the immediate formation of the Pt-heterodimeric complex with downfield-shifted $^{15}NH_3$ signals indicates that the pre-loaded Cu(I) ion cannot be retained buried inside (as in the case of wild-type proteins) due to the presence of only one internal cysteine, therefore this Cu(I) ion is immediately available for colocalization near the Pt atom. Finally, the greater reactivity toward cisplatin of Atox1-Cu(I)-C18A Mnk1 with respect to C15A Atox1-Cu(I)-Mnk1 could stem from a different solvent accessibility as well as from a different ability to reorient the two cysteines that bind to Pt (each protein contributes only one cysteine sulfur to Pt, the more N-terminal cysteine of the CxxC motifs, with the two ammines completing the square planar coordination geometry).

X-ray structure of the wild-type heterodimeric species

The crystallization of a Pt-containing heterodimeric species provided a unique opportunity to gain structural insights also into the elusive Cu-bridged forms.

The analysis of the crystalline structure pointed to the coexistence of two species. In one species a Cu atom (Cu2) is bound to Atox1 in a monodentate manner and to Mnk1 in a bidentate manner (which corresponds to one of the two hypothesized Cu(I)-bridged forms of the functional heterodimer). This Cu coordination mode, which has never been structurally characterized before, corresponds to that found in the Cd(II)-bridged heterodimer previously reported [48]. Such a Cu(I) coordination should be the only one admitted in the C15A Atox1-Cu(I)-Mnk1 mutant, which has been found to be scarcely reactive toward Pt(II). The second hypothesized Cu(I)-bridged form of the functional heterodimer has Cu(I) bound to Atox1 in a bidentate manner and to Mnk1 in a monodentate manner. This latter coordination should be the only one admitted in the Atox1-Cu(I)-C18A Mnk1 mutant, which has been found to be more reactive toward Pt(II). Indeed, looking at the arrangement of thiols in Fig. 3d, it can be guessed that Cys15 of Mnk1 and Cys12 and Cys15 of Atox1 are less suited to three-coordinate a Cu(I) ion, therefore the corresponding heterodimer is expected to be more reactive toward Pt(II). This explains why the conformation with monodentate

Atox1 and bidentate Mnk1 (being intrinsically more stable) has been observed in the solid state for both Cu(I) (Cu2 in the present investigation) and Cd(II). Importantly, apart from the structural considerations made above, the experiments performed with mutated proteins have shown that, of the two possible binding configurations of the Cu(I) ion in the cage made by the CxxC motifs of the two proteins, one (bidentate Atox1 and monodentate Mnk1) is more reactive towards cisplatin and hence less stable than the other.

In the second species present in the crystal structure, a Pt atom (Pt1) is bound to the more N-terminal cysteines of Atox1 and Mnk1 and a Cu atom (Cu1) is bound to the internal cysteines of the same proteins. It can be hypothesized that, after coordination of Pt to the more exposed cysteines, the Cu(I) ion is pushed down reaching a new energy minimum. The observation in the ESI-MS spectra of heterodimeric fragments containing both Pt and Cu points to a good stability of the second species present in the crystal structure. In this heterodimeric complex the NMR signals of the ammines bound to Pt would not be affected by the presence of the buried Cu(I) ion, due to the large Cu-Pt distance (> 6 Å), therefore the initially formed heterodimeric complex has upfield-shifted $^{15}NH_3$ signals, and only at longer reaction time it undergoes transition to a downfield-shifted Pt-bridged heterodimer. In contrast, in the case of mutants the presence of only one internal cysteine does not allow such an internal stabilization of the Cu(I) ion, as a consequence the Pt-bridged heterodimer formed right from the beginning contains downfield-shifted $^{15}NH_3$ signals and a labile Cu that can be rapidly removed by BCS. This fully explains the different behavior of wild-type and mutated heterodimers.

Biological implications of platinum binding to dithiol motifs of copper proteins

Pull-down experiments in yeast cells have shown that cisplatin binds to CxxC motifs of Atox1 and protein disulfide isomerases (PDI), thereby inhibiting their function and ultimately resulting in the induction of endoplasmic reticulum stress [59]. Moreover, in a yeast two-hybrid screen using Atox1 as bait, many of the proteins found to be confident interaction partners contain CxxC motifs, indicating that interactions between these and Atox1 can also be mediated by Cu [60]. In the light of the present investigations, all these processes are keen to be disrupted by cisplatin.

Cisplatin treatment also causes a significant increase (doubling) of ROS levels [12]. Mechanistically, this may be a consequence of thiol blockage and metal dishomeostasis. Unlike the kinetically labile and exchangeable Cu(I) ion, Pt(II) acts as an efficient thiol scavenger as it irreversibly modifies the two-cysteine motifs and, by blocking the redox-sensitive thiol groups, contributes to oxidative stress. While an anticancer therapy that favors ROS accumulation may be more effective in inducing programmed cell death [61], a rise in intracellular Cu, consequent to a blockage of Cu efflux

machinery, can lead to increased levels of Cu-ATPases, which in turn cause enhanced drug sequestration and resistance to Pt chemotherapy. Thus, Pt drugs, by impacting Cu transport, can induce their own resistance. The arrangement of all six N-terminal MBDs of Menkes ATPase is regulated by Cu and controls the intracellular localization and catalytic activity of the pump [3,62]. The Pt ability to cross-link Atox1 and Mnk1 suggests that tethering Atox1 to Atp7a soluble domains may compromise the Cu(I)-mediated trafficking of Menkes ATPase to the plasma membrane, while Pt may become stuck between the proteins.

Such a mechanism of Pt cross-linking can be extended to other MBDs of Cu-ATPases and their Cu-dependent complexes with Atox1 and other proteins containing the dithiol motif. For instance, lysosomal exocytosis and Cu clearance are regulated by the Cu-dependent interaction between MBD6 of Wilson ATPase and the p62 subunit of dynactin [63], both containing a CxxC motif [64,65]. Binding to p62 allows Atp7b-containing membranes to anchor the dynein motor and, therefore, to be translocated to the biliary surface.

By binding to N-terminal MBDs of Cu-ATPases, Pt drugs can also alter their overall arrangement and recognition by the protein translocation machineries. Changes in the subcellular localization of Cu-ATPases can facilitate sequestration of cisplatin in vesicular structures of the endosomal pathway [40,43], which may prevent drug binding to genomic DNA, thereby contributing to cisplatin resistance and chemotherapy failure [66]. Moreover, thiol modifications have important implications also for the mechanism of extracellular vesicle release [67].

Based on these findings, cisplatin, rather than being just a cargo for Cu transporters, can interfere deeply with Cu transport and metabolism. Interestingly, the co-administration of Pt drug and a Cu chelator, such as tetrathiomolybdate (TM), can increase the nuclear availability of cisplatin and resensitize resistant cells [68]. TM was shown to inhibit the reaction of cisplatin with Cu-Atox1 [69] by forming a Mo-centered trimeric protein cluster with trapped Cu ions [70]. Similarly, the reaction of Pt drugs with MBDs of Cu-ATPases is fostered by Cu ions. Finally, the present investigation has shown that also Pt bridging of Atox1 to MBDs of Cu-ATPases requires the presence of Cu.

Conclusion

In conclusion, *cis*-Pt(II) compounds, which are among the most used chemotherapeutics, have the ability to bind CxxC motifs and form interprotein cross-links between the Cu chaperone Atox1 and the N-terminal domains of Menkes ATPase, thus hindering the release of Cu to cuproenzymes and the efflux of excess Cu from the cell. Indeed, increased copper content has been observed in human cancer cells after cisplatin treatment [71,72]. Interestingly, the Pt-bridged heterodimeric species does not form if transplatin is used in place of cisplatin or Zn(II) is used in place of Cu(I). The kinetically

stable Pt(II)-bridged adduct has also been characterized by X-ray crystallography. Together with the Pt(II) bridged species (72% occupancy) there is cocrystallization of a second species (28% occupancy) in which a Cu(I) is bound to Atox1 in a monodentate manner and to Mnk1 in a bidentate manner, this corresponds to one of the two hypothesized Cu(I)-bridged forms of the functional heterodimer not yet characterized by X-ray crystallography. The same conformer has been shown, through studies of site-directed mutagenesis, to be more stable and less reactive towards Pt(II) than the second conformer comprising bidentate Atox1 and monodentate Mnk1. After Pt(II) coordination, a Cu(I) ion can still be retained at the metal-binding site, but it is labilized and can be released to glutathione (a physiological thiol) or to other complexing agents. Conclusively, Pt drugs, apart from targeting DNA, can also impair Cu homeostasis by interfering with the rapid exchange of Cu(I) between Atox1 and Cu-ATPases with relevant detrimental effect on the cellular viability and migration.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details, additional NMR experiments, HPLC/ESI-MS spectra and crystallographic data. The structure of Pt-containing Atox1-Cu(I)-Mnk1 heterodimer has been deposited in the Protein Data Bank as entry 5T7L.

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