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Probing the Interaction of Cisplatin with the Human Copper Chaperone Atox1 by Solution and In-Cell NMR Spectroscopy

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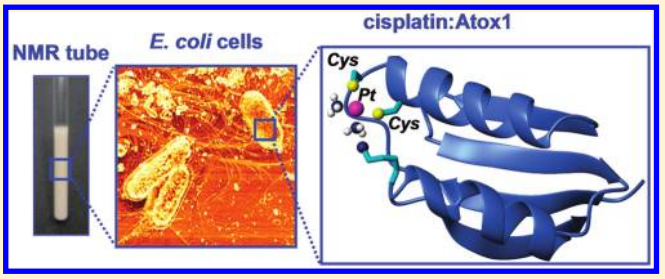
Supporting Information

ABSTRACT: Among anticancer therapeutics, platinum-based drugs have a prominent role. They carry out their antitumor activity by forming stable adducts with DNA, thus interfering with replication and transcription processes. Cellular uptake of these drugs is tightly connected to copper transport. The major Cu(I) influx transporter Ctr1 has been found to mediate transport of cisplatin and its analogues. Evidence also suggests that ATP7A and ATP7B mediate cisplatin sequestration and efflux from cells, thus influencing drug resistance. The copper-chaperone Atox1, which normally binds Cu(I) via two cysteines and delivers the metal to ATP7A/B, has also been reported to interact with cisplatin in in vitro experiments. In the present investigation we apply a combined approach, using solution and in-cell NMR spectroscopy methods, to probe intracellular drug delivery and interaction of cisplatin with Atox1. The intracellular environment provides itself the suitable conditions for the preservation of the protein in its active form. Initially a $\{Pt(NH_3)_2\}$-Atox1 adduct is formed. At longer reaction time we observed protein dimerization and loss of the ammines. Such a process is reminiscent of the copper-promoted formation of Atox1 dimers which have been proposed to be able to cross the nuclear membrane and act as a transcription factor. We also show that overexpression of Atox1 in *E. coli* reduces the amount of DNA platination and, consequently, the degree of cell filamentation.



INTRODUCTION

Copper (Cu) is an essential element in living organisms and is required as a cofactor of a variety of enzymes that regulate key biological processes, such as cellular respiration and oxidative stress defense.[1,2] At the same time, altered states of its intracellular concentrations are extremely dangerous. Accumulating evidence also suggests its fundamental role in the regulation of cell growth, in important processes such as angiogenesis, and in several pathologies including cancer and neurodegenerative diseases.[3−8] Thus, a fine balance between Cu demand and supply is crucial for survival and is tightly controlled by several transport proteins.[9,10] The soluble cytosolic Cu chaperone Atox1 (antioxidant-1, 68 amino acids), previously known as Hah1 (human atx1 homologue 1), mediates Cu(I) delivery to the Cu-transporting P-type ATPases ATP7A and ATP7B (the Menkes and Wilson disease proteins, respectively), which are responsible for Cu release to the secretory pathway of the trans-Golgi network.[11−13] Recently, a novel role for Atox1 as a nuclear Cu-dependent transcription factor has been revealed, demonstrating its implication both in activation of cell proliferation (positive expression of cyclin D1) and moderation of oxidative stress in the cardiovascular system (positive expression of extracellular superoxide dismutase SOD3).[14,15] Atox1, which is ubiquitously expressed, has a $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ ferredoxin-like structure. The metal-binding site, CxxC, is located in the loop between secondary structure elements β_1 and α_1 and is highly conserved among metallochaperones and soluble domains of Cu-transporting ATPases.[16,17] A lysine-rich region (KKTGK), located in the loop between α_2 and β_4, may represent the nuclear localization signal (NLS) for the translocation of Atox1 to the nucleus.[14]

In light of its pleiotropic roles, the attention on Atox1 is of great relevance not only for basic research but also for potential therapeutic targeting. It has been recently shown that tetrathiomolybdate (TM), a formerly used orally active agent for regulating Cu metabolism, can bind to the yeast homologue of Atox1.[18] TM forms with the Cu chaperone a stable sulfur-bridged Cu−Mo cluster, thus inhibiting Cu delivery to secreted cuproenzymes. The potent chelating and antiangiogenic activity of this drug fostered its use in the treatment of Wilson's disease, where Cu accumulation leads to hepatic and neurological disorders, as well as in the inhibition of metastatic cancer progression in a number of clinical trials.[19]

Among anticancer therapeutics, platinum (Pt)-based drugs, such as cisplatin (*cis*-$[PtCl_2(NH_3)_2]$), have a prominent role. They carry out their antitumor activity by forming stable adducts with DNA (mainly 1,2-GG or 1,2-AG cross-links between neighboring

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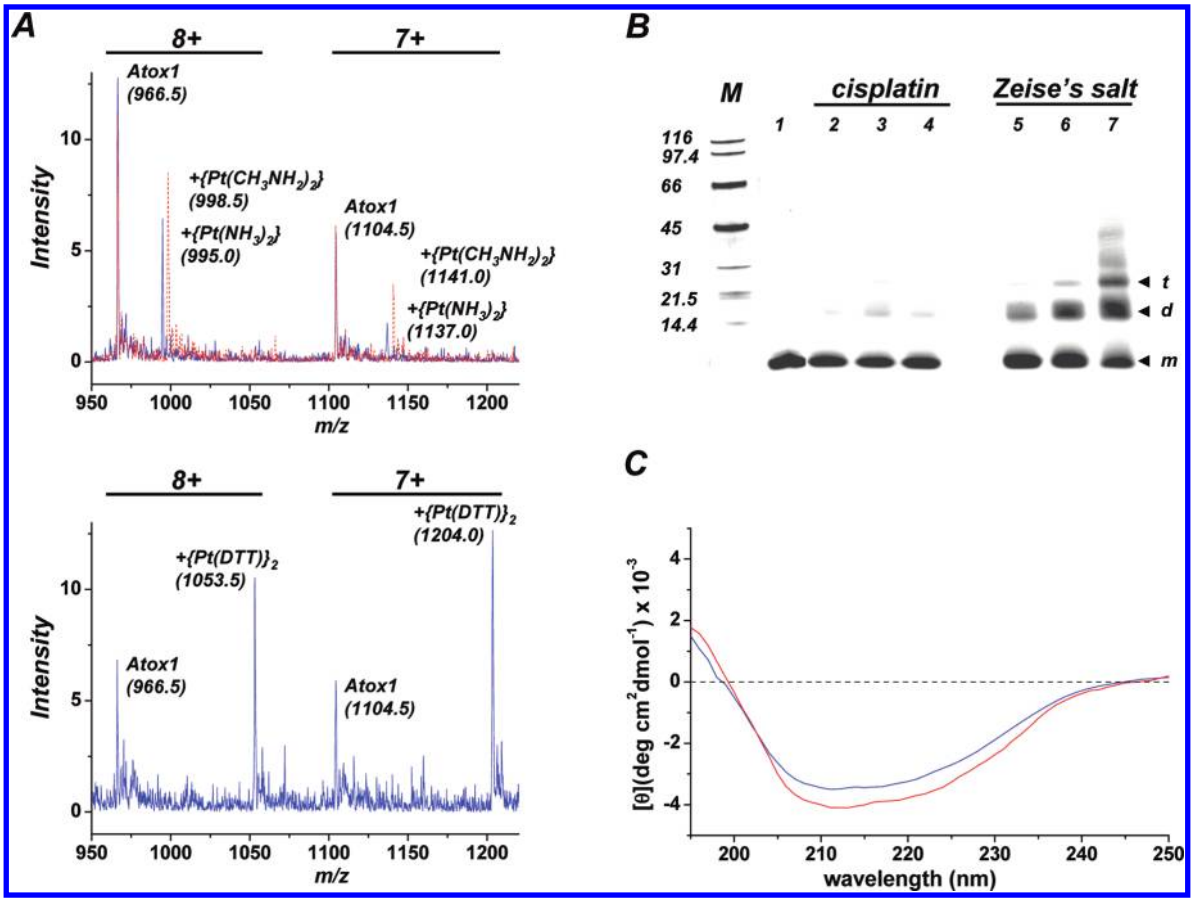

Figure 1. (A) (Top) ESI-MS spectra of Atox1 treated with 1 mol equiv of cisplatin, *cis*-$[PtCl_2(NH_3)_2]$ (solid blue line), or *cis*-$[PtCl_2(CH_3NH_2)_2]$ (dotted red line) and analyzed 24 h after mixing. (Bottom) ESI-MS spectra of Atox1 treated with 1 mol equiv of Zeise's salt, $K[PtCl_3(\eta^2\text{-}C_2H_4)]\cdot H_2O$, and analyzed 12 h after mixing. Peaks corresponding to two different multiply charged states (7+ and 8+) are shown, each state comprises two signals corresponding to apoAtox1 and the protein adduct with the platinum complex, respectively. In both cases the reaction between apoAtox1 (200 μM) and the platinum complex (200 μM) was conducted at 25 °C in degassed water containing 1 mM dithiothreitol (DTT). Before injection, aliquots of the reaction mixture were 10-fold diluted and 5 mM ammonium acetate buffer (pH 6) and 1% acetic acid (v/v) were added to obtain a good volatilization. (B) SDS-PAGE of apoAtox1 (lane 1) and of the reaction mixtures of Atox1 with 1 mol equiv of cisplatin (lanes 2−4) or Zeise's salt (lanes 5−7) at different time intervals after mixing: 4 h (lanes 2 and 5), 12 h (lanes 3 and 6), 24 h (lanes 4 and 7). Bands corresponding to monomeric (m), dimeric (d), and trimeric (t) protein adducts are indicated with arrowheads. (C) Far-UV CD spectra of Atox1 before (red curve) and after 24 h incubation (blue curve) with 1 mol equiv of cisplatin.

purines), thus interfering with replication and transcription processes.[20,21] Cellular uptake of these drugs occurs by a variety of mechanisms, and, interestingly, it seems to be tightly connected to Cu transport.[22,23] Ctr1, the major Cu(I) influx transporter in the cell, has been found to mediate transport of cisplatin and its analogues as well.[24] We have shown that methionine (Met)-rich motifs located in the N-terminal extracellular domain of Ctr1 are critical for the binding of both Cu(I) and Pt(II) compounds,[25,26] although the two metal ions differ in terms of chemical reactivity, distribution, and cytotoxicity. Loss of Atox1 decreases proper compartmentalization of Cu(I) and leads to diffuse distribution, highlighting the role of Atox1 in targeting.[27] In contrast, loss of Atox1 reduces the influx of cisplatin and subsequent accumulation in vesicular compartments and in DNA.[28] In particular, loss of Atox1 was found to block the cisplatin-induced down-regulation of Ctr1, but mechanistic details are still being elucidated. Evidence also suggests that ATP7A and ATP7B mediate cisplatin sequestration and efflux from cells,[29,30] thus influencing drug resistance.[31,32]

In a recent work it has been shown that cisplatin binds to the Atox1 metal-binding site regardless of the presence of Cu. Interestingly, addition of cisplatin to apoAtox1 results in no change to the circular dichroism (CD) spectrum whereas addition of cisplatin to preformed Cu(I)-Atox1 or subsequent addition of Cu to a premixed cisplatin/apoAtox1 sample results in CD changes reminiscent of CD signals of metallothioneines. Moreover, 1 equiv of cisplatin added to apoAtox1 causes little, if any, protein unfolding over two weeks. In contrast, addition of 1 equiv of cisplatin to Cu(I)-Atox1 results in unfolding with a half-life of two days at 20 °C.[33]

The X-ray structure of apoAtox1 in complex with cisplatin has been solved in two different crystalline forms, a monomeric and a dimeric adduct, depending upon the presence or absence of the tris(2-carboxyethyl)phosphine (TCEP) as cysteine (Cys)-reducing agent.[34]

In the present investigation we apply a combined approach using solution and in-cell NMR spectroscopy methods to probe intracellular drug delivery and the interaction of cisplatin with Atox1, aiming to obtain detailed molecular information in a

8362 dx.doi.org/10.1021/ja207346p |J. Am. Chem. Soc. 2011, 133, 18361–18369

physiological environment. In addition to highly cytotoxic cisplatin, Zeise's salt ($K[PtCl_3(\eta^2\text{-}C_2H_4)]\cdot H_2O$) was also chosen, owing to the strong trans-labilizing effect of the ethylene ligand which favors Pt binding to proteins and other macromolecules.

RESULTS

Electrospray Mass Spectrometry. The stoichiometry of adducts formed by Atox1 with cisplatin or Zeise's anion ($[PtCl_3(\eta^2\text{-}C_2H_4)]^-$) was determined by ESI-MS. The ESI-MS spectrum of apoAtox1 shows a series of multiply charged states corresponding to the expected mass of the recombinant protein (see Supporting Information for details). When cisplatin is added to Atox1 in an equimolar amount, ESI-MS spectra of the reaction mixture show a new set of signals, whose intensity increases with time, indicating the formation of an adduct between Atox1 and the Pt complex. The mass difference between corresponding charged states indicates binding of a $\{Pt(NH_3)_2\}$ moiety to Atox1 (Figure 1A, top, solid blue line). When the reaction is performed with the cisplatin analogue *cis*-$[PtCl_2(CH_3NH_2)_2]$, peaks with an additional mass corresponding to a $\{Pt(CH_3NH_2)_2\}$ moiety are observed, again indicating that the major adduct with Atox1 is formed by loss of two chlorido ligands from the Pt substrate (Figure 1A, top, dotted red line). We cannot exclude that other Pt−protein adducts are formed, but their yield must be rather small. The signals corresponding to the $\{Pt(NH_3)_2\}$−Atox1 adduct, if compared to those of apoAtox1, reach a maximum intensity about 24 h after mixing. At the same time, the absolute intensities of all peaks start to decrease, suggesting protein aggregation induced by Pt binding.

The reaction of Zeise's salt with apoAtox1 in equimolar amounts proceeds faster than that of cisplatin and leads to $\{Pt(DTT)\}_2$−Atox1 (DTT = dithiothreitol, used as Cys-reducing agent) as the prevalent species after 12 h (Figure 1A, bottom). As already observed in the case of cisplatin, with time all signals weaken and finally disappear probably from the formation of aggregates. To test the latter hypothesis, we performed gel electrophoresis on Atox1 samples incubated with cisplatin or Zeise's salt for different time intervals (4, 12, and 24 h). In the case of Zeise's salt, in addition to the monomer band of Atox1 at about 7 kDa, dimer and trimer bands of Atox1, whose intensities increase with time, are observed. In contrast, the reaction with cisplatin leads only to a weak dimer band at about 15 kDa after 24 h incubation (Figure 1B). In both cases (cisplatin and Zeise's salt) circular dichroism does not indicate significant change in protein secondary structure as inferred by a stable spectrum profile with only a time-dependent decrease of signal intensity upon addition of the Pt complex (Figure 1C).

Since Met-rich motifs located in the N-terminal extracellular domain of Ctr1 are able to bind cisplatin and replace the two chlorido and the two ammine ligands, we tested whether Atox1 is capable of removing Pt bound to methionines. For this purpose, we used an octapeptide, called Mets7, containing three Met residues and mimicking the minimal Met-rich motif of Ctr1, that we already characterized for its ability to react with Pt(II) compounds.[25] The reaction between Mets7 and cisplatin or Zeise's anion leads to an adduct containing the peptide and the Pt(II) ion, with all original Pt ligands being replaced by the three Met S atoms and a O/N donor coming from the peptide.[25] This species reacts quickly with Atox1, giving back apoMets7 as inferred by ESI-MS. Thus, Atox1 can readily remove Pt bound to Met-rich motifs. Remarkably, the newly formed adduct(s) between Pt and Atox1 is(are) extremely stable because even the classical method used to remove Pt(II) cross-linked to DNA,[35] consisting of an overnight incubation in 0.2 M NaCN (basic pH) at 45 °C followed by extensive washing, is unable to remove most of the Pt bound to Atox1, as determined by atomic absorption spectroscopy. If, however, the Pt−Atox1 adduct is treated with a low molecular weight thiol chelator, such as 3-thiapentane-1,5-dithiolate $[S(CH_2CH_2S)_2]^{2-}$ (TPDT), Pt is readily and quantitatively removed. Note that, unlike TPDT, DTT, used as Cys-reducing agent and present in excess in our reactions, gives weaker interactions and is unable to compete with Atox1 for binding to cisplatin.

Solution NMR spectroscopy. To detect Atox1 residues involved in Pt coordination we recorded $^1H,^{15}N$-HSQC NMR spectra on a uniformly ^{15}N-labeled sample of Atox1. Upon interaction with 1 equiv of cisplatin, some $^1H/^{15}N$ cross-peaks, corresponding to different amide signals of Atox1, experience a time-dependent moderate shift accompanied by a decrease in intensity (Figure 2A). The observed chemical shift changes are mainly localized in the protein region encompassing the Cu(I)-binding motif CxxC (Figure 2B). The $^1H,^{13}C$-HSQC spectra recorded on an Atox1 sample selectively labeled at the Cys residues (^{13}C), indicate that, from the early beginning, the cross-peaks of Cys12 and Cys15, unlike those of Cys41, are broadened beyond detection (Figure 2C). These observations are in accord with a chemical exchange between free and coordinated Atox1 (see Discussion).

The fate of the ammine ligands in the reaction of ^{15}N-labeled cisplatin with Atox1 was also monitored by $^1H,^{15}N$-HSQC NMR spectroscopy. The ^{15}N chemical shifts are very diagnostic because they are extremely sensitive to the nature of the donor atom that is in trans position.[36] Addition of unlabeled Atox1 to a solution of ^{15}N-labeled cisplatin causes reduction of the signals of the dichlorido (*cis*-$[PtCl_2(^{15}NH_3)_2]$, $\delta^{15}N = -67$ ppm) and monoaqua species (*cis*-$[PtCl(^{15}NH_3)_2(H_2O)]^+$, $\delta^{15}N = -66$ and -83 ppm for $^{15}NH_3$ trans to Cl and to H_2O, respectively) and the appearance of cross-peaks relative to $^{15}NH_3$ trans to Cys sulfur ($\delta^{15}N \sim -40$ ppm) (Figure 2D). The intensity of these latter peaks decreases as the reaction progresses, indicating a release of $^{15}NH_3$ from the complex owing to the trans effect of sulfur.[37] Because the solution is buffered at pH 7, the signal of free $^{15}NH_4^+$ ($\delta^{15}N = 7$ ppm) can barely be detected due to rapid exchange with the solvent.

In-Cell NMR Spectroscopy. As a first attempt to monitor the effect of Pt drug binding to Atox1 in a living cell, we applied in-cell NMR spectroscopy to *E. coli* BL21-Gold(DE3) cells expressing a uniformly ^{15}N-labeled protein. Atox1 expression was induced by addition of 1 mM isopropyl thiogalactoside (IPTG). We initially optimized cell growth conditions by removing from culture medium any paramagnetic metal ion which would otherwise prevent detection of protein signals (see Supporting Information for details). Because the spectral resolution and the short cell lifetime are limiting aspects of this technique we applied band-selective optimized flip-angle short-transient (SOFAST)-HMQC spectroscopy to obtain 2D $^1H,^{15}N$-correlation spectra of the protein in a few seconds and with a high signal-to-noise ratio. Long acquisition times may yield spectra that do not represent the cell physiological state because, after some time, oxygen starvation may change cell metabolism.

By comparison with reference maps obtained on the purified protein, the in-cell NMR spectra indicate that Atox1 is in the

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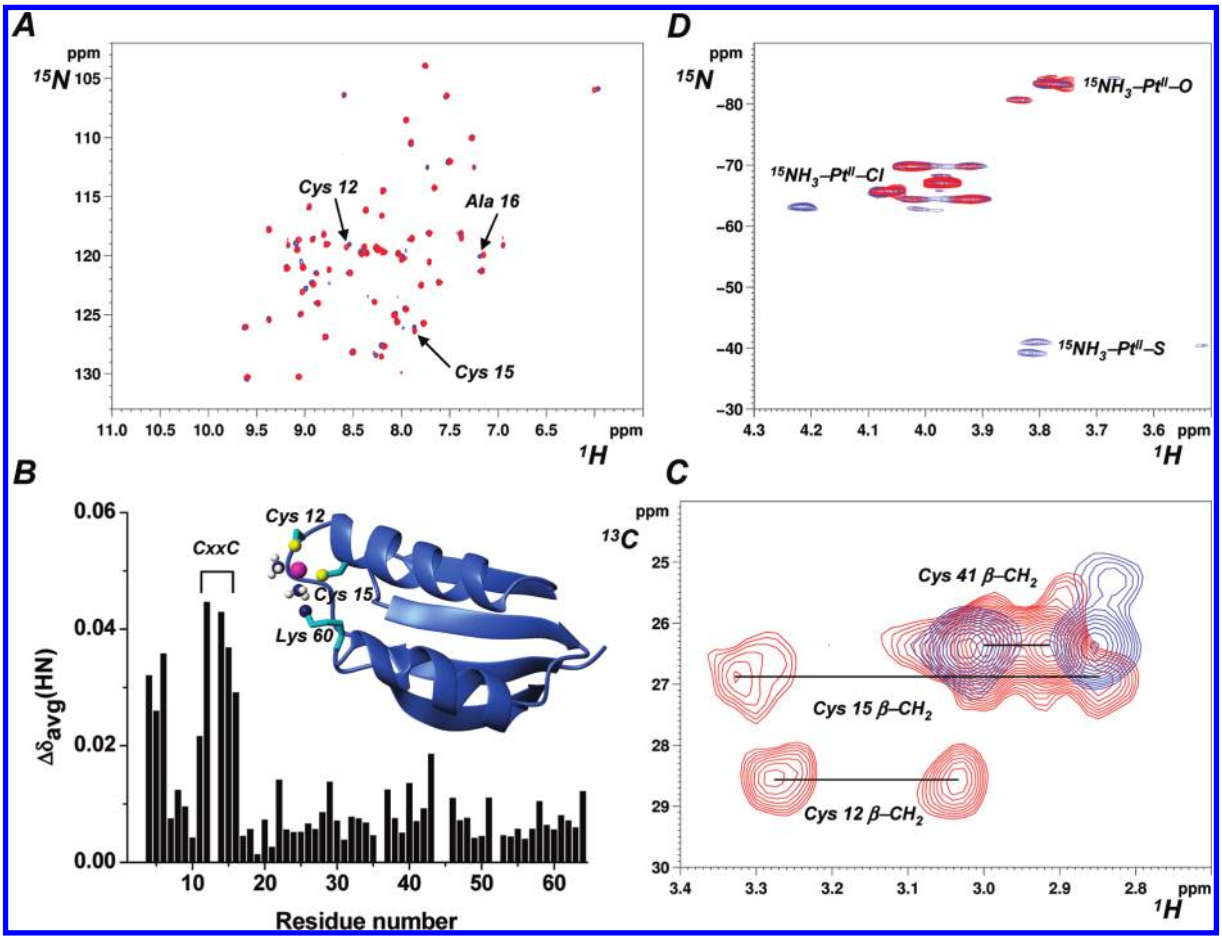

Figure 2. (A) Overlay of $^{1}H,^{15}N$-HSQC spctra of ^{15}N-labeled Atox1 in the absence (red contours) and in the presence (blue contours) of 1 mol equiv of cisplatin (4 h after mixing). Cross-peaks of residues Cys12, Cys15, and Ala16 are indicated with arrows. (B) Weighted average chemical shift differences $\Delta\delta_{avg}$(HN) of Atox1 in the presence and in the absence of 1 mol equiv of cisplatin. The inset shows a structural model of the adduct between Atox1 (PDB ID 1TL5) and the {*cis*-Pt(NH_3)$_2$} cisplatin moiety. The Pt(II) ion is shown as magenta sphere. Side chains of Cys12, Cys15, and Lys60 are shown as cyan sticks. S = yellow, N = blue, H = white. (C) Overlay of $^{1}H,^{13}C$-HSQC spectra (β-CH_2 region) of Atox1 containing ^{13}C-selectively labeled cysteines in the absence (red contours) and in the presence (blue contours) of 1 mol equiv of cisplatin (4 h after mixing). Resonance assignments are reported. (D) Overlay of $^{1}H,^{15}N$-HSQC spectra of ^{15}N-labeled cisplatin in the absence (red contours) and in the presence (blue contours) of 1 mol equiv of Atox1 (4 h after mixing). The cross-peaks are assigned to $^{15}NH_3$ trans to O, Cl, and S donor atoms, respectively. The presence of four satellite signals around the cross-peak assigned to free cisplatin (*cis*-[$PtCl_2$($^{15}NH_3$)$_2$], $\delta^{15}N = -67$ ppm) is due to *J* coupling of ^{1}H and ^{15}N nuclei with ^{195}Pt (having a natural abundance of 33.8%). The differential broadening of satellite pairs is due to cross-correlation effects between dipolar and chemical shift anisotropy relaxation.[36]

reduced apo state, the form competent to bind metal ions. Most of the signals are superimposable to those of the in vitro maps, with only little shifts and increased line widths for the in-cell protein resonances. In-cell NMR spectra show additional peaks in a narrow region of the spectrum (7.5−8.5 ppm) corresponding to ^{15}N-labeled metabolites and other molecules (Figure 3A). To verify that no protein signals from leaked protein in the liquid surrounding the cells were present, after each in-cell experiment we followed a standard protocol[38] consisting of cell recovery from the tube, sample centrifugation, and recording of an NMR spectrum of the supernatant. Negative results were always obtained.

To study the interaction of cisplatin with Atox1, cells were grown in contact with various concentrations of Pt drug and cell viability was assessed by plating colony tests (see Supporting Information for details). Remarkable cell growth inhibition was observed for Pt drug concentrations higher than 10 μM as a consequence of strong cisplatin cytotoxicity. Therefore, 10 μM was considered an upper limit concentration to maintain good cell viability for in-cell NMR experiments with cisplatin. Unlike cisplatin, cells were still viable (although with reduced numbers of colonies) when treated with up to 100 μM concentration of Zeise's salt, thus allowing a higher amount of Pt to enter the cells. This amount was determined, by atomic absorption spectroscopy, to be about 70% of added Pt in the case of cell exposure to 100 μM Zeise's salt and about 10% of added Pt in the case of cell exposure to 10 μM cisplatin (in both cases strictly analogous experimental conditions were used). This percentage of Pt uptake is consistent with previously published data.[39]

For in-cell NMR analysis, Atox1-expressing *E. coli* cells were grown overnight at 37 °C in Luria−Bertani (LB) medium and then harvested and resuspended in ^{15}N-minimal medium (composed of 10 mL of 5X M9 salts, 1 mL of 20% glucose, 100 μL of 1 M $MgSO_4$, 50 μL of 0.1 M $CaCl_2$, 50 mg of $(^{15}NH_4)_2SO_4$, 50 μL of 1 mg/mL biotin, 50 μL of 1 mg/mL thiamin). After 4 h IPTG induction in the absence or in the

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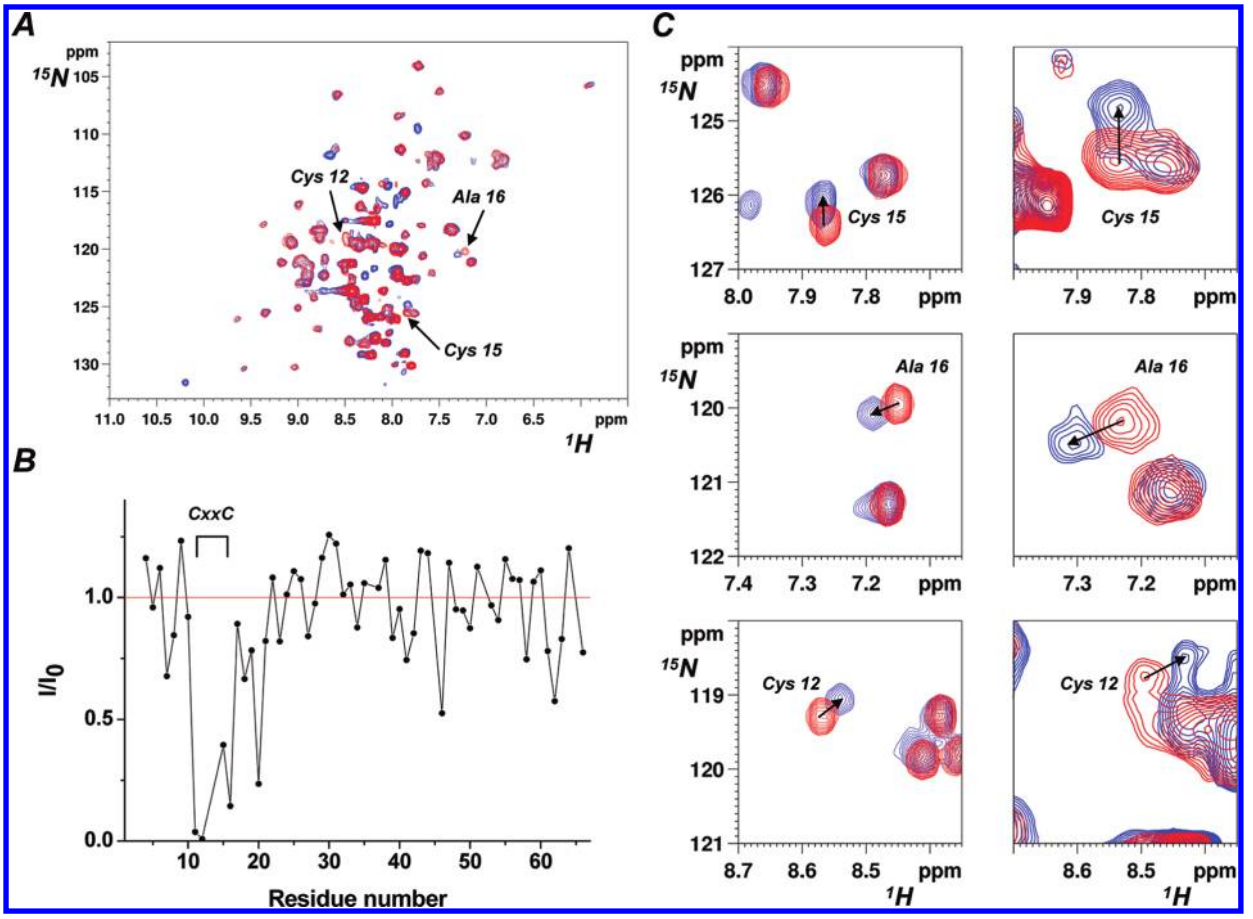

Figure 3. (A) Overlay of $^{1}H,^{15}N$-SOFAST-HMQC spectra of *E. coli* cells expressing ^{15}N-labeled Atox1 after 4 h IPTG induction in the absence (red contours) and in the presence (blue contours) of 10 μM cisplatin. Cross-peaks of residues Cys12, Cys15, and Ala16 are indicated with arrows. (B) Intensity profile obtained as the ratio of cross-peak intensities relative to ^{15}N-labeled Atox1 in $^{1}H,^{15}N$-SOFAST-HMQC spectra of *E. coli* cells after 4 h IPTG induction in the presence and in the absence of 10 μM cisplatin. (C) (Left) Overlay of three regions of in vitro $^{1}H,^{15}N$-HSQC spectra of ^{15}N-labeled Atox1 in the absence (red contours) and in the presence (blue contours) of 1 mol equiv of cisplatin (4 h after mixing). (Right) Overlay of three regions of $^{1}H,^{15}N$-SOFAST-HMQC spectra of *E. coli* cells expressing ^{15}N-labeled Atox1 after 4 h IPTG induction in the absence (red contours) and in the presence (blue contours) of 10 μM cisplatin. Chemical shift changes induced by cisplatin are indicated with arrows for residues Cys12, Cys15, and Ala16.

presence of 10 μM cisplatin, cells were washed and resuspended in fresh ^{15}N-minimal medium to obtain a concentrated sample (see Supporting Information for details). SOFAST-HMQC spectra were immediately recorded to show differences between the samples obtained with or without cisplatin treatment (Figure 3A). The changes in intensity, upon treatment with cisplatin, of the assigned apoAtox1 cross-peaks were measured and plotted as a function of the protein sequence (Figure 3B). The most affected signals belong to residues of the CxxC metal-binding motif. Focusing on the largest spectral changes upon interaction with cisplatin ($^{1}H/^{15}N$ cross-peaks of Cys12, Cys15, and Ala16), new cross-peaks shifted in the same direction as the corresponding cross-peaks in the analogous in vitro experiment are observed (Figure 3C). Given the similarity of spectral changes for the two sets of data, it is inferred that cisplatin has crossed the cell membrane and interacts with the CxxC metal-binding motif of Atox1.

Platinum Cellular Distribution. To have an independent measure of cisplatin binding to recombinant Atox1 in *E. coli*, 10 μM cisplatin was added to 50 mL of cell culture medium supplemented with seleno-L-methionine (SeMet). After 4 h IPTG induction, cells were washed several times to ensure that no Pt was present in the supernatant, pelleted, and sonicated. The membrane fraction was found to contain only a negligible amount of Pt whereas the cell extract (1 mL) contained about 50 μM Pt, as determined by atomic absorption spectroscopy. Therefore, about 10% of total extracellular Pt was accumulated inside the cells. The lysate was subjected to gel electrophoresis to separate SeMet-Atox1 from other cellular components. The extreme stability in 0.2 M NaCN solution of the Pt−Atox1 adduct formed in vitro (see above) made us confident that the intracellular complex did not dissociate during this step. Elemental analysis via ICP-MS on the digested gel slice corresponding to the Atox1 monomer band shows the simultaneous presence of both Se and Pt in a 1:4 ratio, corresponding to 0.75 Pt atoms per protein molecule (Atox1 contains three SeMet residues), while no trace of Pt was detected in a corresponding gel slice in the absence of cisplatin treatment or in the absence of IPTG induction (Figure 4A).

Due to the lack of compartmentalization, DNA is a readily accessible target in the bacterial cytosol. It is known that cisplatin can bind to DNA nucleobase nitrogens in competition with several kinetically favored target sites in *E. coli* cells.[40,41] To determine the effect of Atox1 expression on Pt binding to DNA, DNA was extracted from the resuspended cell pellet and its concentration determined by UV absorption. The amount of Pt

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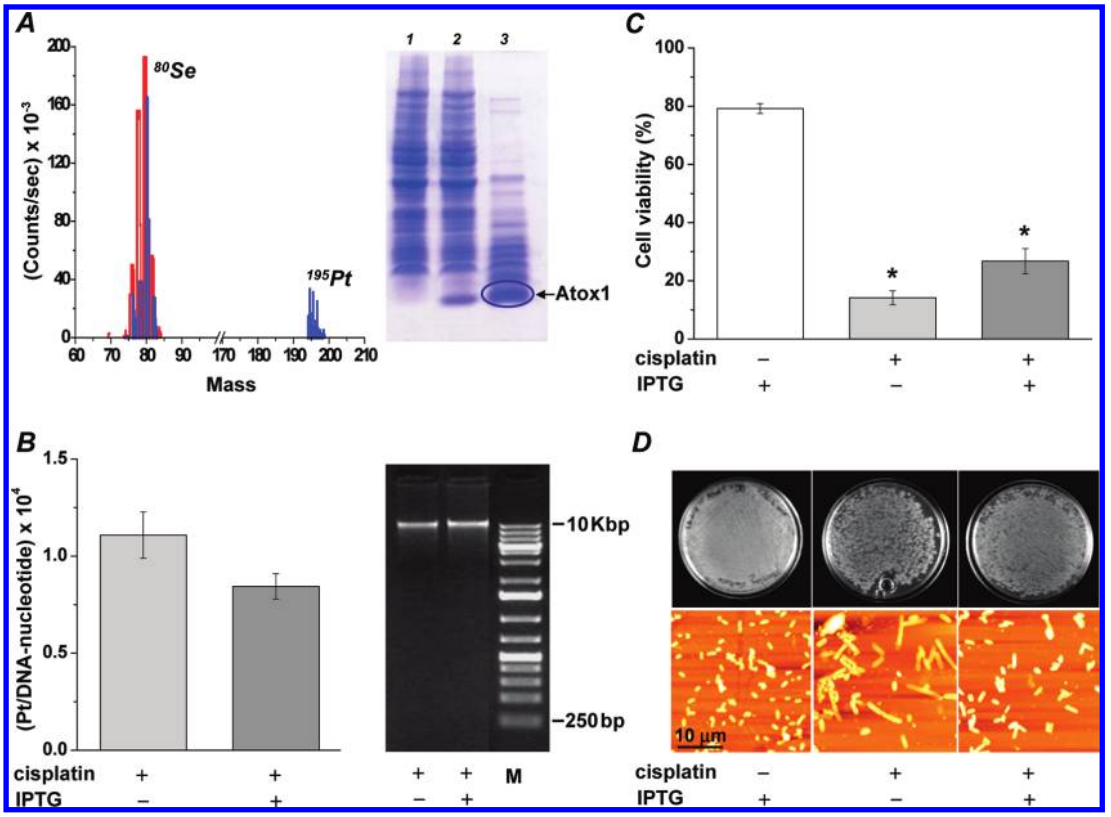

Figure 4. (A) (Left) ICP-MS intensity (counts-per-second data) of Se and Pt isotopes in the SDS-PAGE slice (circled in blue on the right image) corresponding to SeMet-Atox1 monomer band from *E. coli* cell extracts after 4 h IPTG induction in the absence (red contours) and in the presence (blue contours) of 10 μM cisplatin. (Right) SDS-PAGE of uninduced *E. coli* cells (lane 1), of *E. coli* cells expressing SeMet-Atox1 after 4 h IPTG induction (lane 2) and their soluble fraction after cell lysis (lane 3). (B) Platinum binding to DNA (left) and agarose gel electrophoresis of isolated genomic DNA (right) of *E. coli* cells grown for 4 h with or without IPTG induction of Atox1 expression in the presence of 10 μM cisplatin. The range of base pairs (bp) of DNA size marker (M) is indicated. (C) Viability of *E. coli* cells grown for 4 h with or without IPTG induction of Atox1 expression in the absence or in the presence of 10 μM cisplatin, after overnight incubation at 37 °C on nutrient agar. Error bars represent the mean ± SD of five replicates. *, $p < 0.05$. (D) Colonies on nutrient agar plates after overnight incubation at 37 °C (top) and topographic AFM images (bottom) of *E. coli* cells grown for 4 h with or without IPTG induction of Atox1 expression in the absence or in the presence of 10 μM cisplatin.

associated to DNA was found to undergo 15−20% reduction upon IPTG induction of Atox1 expression, suggesting that cisplatin binding to Atox1 makes the drug less available for binding to DNA (Figure 4B).

Because unrepaired DNA lesions caused by cisplatin trigger signal pathways leading to cell death, it is expected that the amount of Pt associated to DNA inversely correlates with the number of viable cells. This was assessed by cell viability assays conducted on *E. coli* grown for 4 h, with or without IPTG induction and in the presence or absence of 10 μM cisplatin. After overnight incubation at 37 °C on nutrient agar plates, the number of colonies was measured. Atox1 expression induces 15−20% recovery of cell viability, compared to uninduced cell cultures, upon cisplatin treatment. The reduction in cell viability which can be ascribed to the heterologous expression of a human protein in *E. coli* host strain has been taken into account (Figure 4C).

The improved colony forming ability associated with Atox1 expression reflects the changes in cell morphology highlighted in atomic force microscopy images of *E. coli* cell cultures (Figure 4D). In the absence of cisplatin, *E. coli* cells grow normally with regular shape and size, but when exposed to the drug they fail to divide and appear as beaded filaments. A marked reduction of filamentation is observed upon IPTG induction, indicating that Atox1 expression partially protects *E. coli* cells from the toxic effects of cisplatin.

DISCUSSION

Chaperones involved in the transport of essential Cu ions contain surface-exposed metal-binding sites which are ideally suited to bind metallodrugs, including Pt-based anticancer drugs such as cisplatin. Cu trafficking and delivery to the correct target is a tightly controlled process driven by affinity gradients and characterized by fast ligand-exchange kinetics.[42] Unlike Cu ions, Pt has slower ligand-exchange kinetics, usually of the same order of magnitude as the rate of cell division in tumor cells, and consequently cisplatin intracellular distribution is thought to be a kinetically controlled process which may affect cell function and replication.[43]

We have applied in-cell NMR spectroscopy[44−46] to probe the interaction of cisplatin with the human Cu chaperone Atox1 in a physiological environment. We were interested to unravel the molecular details of the early steps of this process, i.e., before drug degradation and changes in cell metabolism could take place. The in-cell NMR spectroscopy has also some advantages. Atox1 is very sensitive to oxidation and must be handled with precautions. Usually it is recommended to operate in the presence of a reducing agent, such as DTT or TCEP. It occurs that these low molecular weight reductants are also good ligands for soft metal ions such as Pt(II). These problems can be overcome by performing the reaction in intact cells, where the intracellular

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environment provides itself the suitable conditions for the preservation of the protein in its active form. Solution ESI-MS and NMR experiments on the purified protein in the presence of DTT were used to complement the in-cell analysis. The two approaches indicate that cisplatin binds to the CxxC motif of Atox1 both in vitro and in *E. coli* cells (Figure 3C), without large structural rearrangements (Figure 1C).

In a recent work by Boal and Rosenzweig, two adducts of cisplatin with human Atox1 were crystallized, a monomeric and a dimeric form,[34] neither of them featuring the 1:1 adduct, with *cis*-$Pt(NH_3)_2$ bound to the two S atoms of the CxxC motif, found in our investigation (Figures 1A and 2B). The monomeric crystalline form was obtained in the presence of TCEP as reducing agent. Bound to Pt were the P atom of one TCEP molecule, a N, S-chelated Cys12 (with the amidic nitrogen of Cys12 *trans* to the phosphine), and the S atom of Cys15.[34] Therefore, the two S atoms are trans to one another, and cisplatin has completely lost its ammine ligands. It is likely that the coordination of TCEP, and subsequent trans labilization of the ammine, has favored the N-chelation of the otherwise monodentate S-bound Cys12, leaving to the S atom of Cys15 the only choice to coordinate to Pt trans to the S atom of Cys12.

The dimeric crystalline form was obtained by reacting cisplatin with Atox1 in the absence of TCEP and contained a Pt atom with its two cis ammine ligands but, instead of having an S,S-chelated Atox1, there were two protein molecules monocoordinated through the S atom of their Cys15 residues while the two Cys12 residues remained uncoordinated.[34] The failure of the Cys12 side chains to displace the ammines can appear surprising; however, note that the overall disposition of the four S atoms (two of coordinated Cys15 and two of uncoordinated Cys12) is tetrahedral with a Cys15,Cys12 S,S distance of 4.0 Å, a bit too long for cis-coordination to a square-planar Pt(II). In contrast, the disposition of the four S atoms is suitable for a tetrahedral coordination, as observed in the metal-bridged dimeric structures of Atox1 formed in the presence of Cu(I), Cd(II), and Hg(II).[47] As matter of fact, the two uncoordinated Cys12 residues shift minimally (if compared to their orientation in the Cu(I), Cd(II), and Hg(II) adducts) to accommodate the ammine ligands and each Cys12 maintains the H-bond with the Thr11 side chain of the second Atox1 molecule in the dimer.

In our solution experiments cisplatin retains the ammine ligands and the S atoms of Cys12 and Cys15 coordinate in cis positions (Figure 2D). The 1:1 adduct appears to be stable and is the only species detected in ESI-MS. However, our NMR results were consistent with a fast-to-intermediate exchange between free and coordinated Atox1. Although fast exchange is generally considered unlikely for Pt(II) complexes, there are some exceptions. For instance fast exchange between coordinated and free L was observed in the case of $[Pt(R_5\text{-dien})L]^{2+}$ complexes (R = H or Me, L = mono or bidentate N-donor ligand, dien = diethylenetriamine).[48] A possible explanation was that a strained bis-chelated ligand (R_5-dien) can easily open up, providing a free coordination position for an extra L. The chelate ring would then reclose, expelling one of the two L's. In the present case, the *cis*-$[Pt(NH_3)_2(Atox1\text{-}S15,S12)]$ derivative could be characterized by having a slightly strained chelate ring which could open up, affording a free position for coordination of a second Atox1 molecule. Ring-closing of one of the two Atox1 molecules would afford the original adduct. In this context, the Atox1 dimer crystallized by Boal and Rosenzweig would represent the intermediate species. An indirect support to our hypothesis comes from the observation that, in both crystal structures, the S atoms of Cys12 and Cys15 are quite distant from one another.[34]

With two cis-oriented ammines and two labile chlorides, cisplatin is expected to react with biological targets, releasing first the two chlorides while keeping the two ammines. However, if the target is a soft ligand, in a subsequent reaction the ammine may become labile.[43] Indeed, at longer incubation times we observed loss of the ammines and protein dimerization. Adduct formation and protein dimerization are more rapidly observed in the reaction with Zeise's anion, owing to the strong trans labilizing effect of the ethylene ligand and to the presence of three leaving chlorido ligands (Figure 1B).

For the in-cell NMR experiments, growth conditions (4 h) in the presence of IPTG induction and cisplatin concentration of 10 μM were chosen in such a way to have good cell viability and sufficient amount of Pt entering the cells. Pt binding to Atox1 in cell was further demonstrated by ICP-MS, and a cytoprotective role of Atox1 against cisplatin toxicity was evidenced by colony forming ability (Figures 4A and 4C). We also noticed that the reaction between Atox1 and cisplatin is slower if performed in cell lysate than in intact cells. For instance, 1 mL of cell extract incubated with 50 μM cisplatin (the total amount of 50 nmol of Pt corresponds to that which enters an equivalent population of cells in the experiment performed on living cells) gives rise, after 12 h reaction, to the same spectral changes already observed in the cell after 4 h treatment. A possible explanation for the faster reaction in living cells than in the cell lysate is that intracellular macromolecular crowding increases the effective protein concentration (estimated to be in the millimolar range).[49] The increase in rate could also involve the labilization of the ammine ligands. For instance, no $^{15}NH_3$ signals were detected in $^{1}H,^{15}N$-SOFAST-HMQC spectra of *E. coli* cells after 4 h IPTG induction in the presence of ^{15}N-labeled cisplatin. In contrast, $^{15}NH_3$ signals were still observed in the cell lysate after 4 h reaction.

In principle, we cannot exclude cisplatin binding to other molecules prior to Atox1. Thioether S atoms have been shown to be kinetically favored sites for Pt coordination[50,51] although, for longer incubation times, coordination can shift to the thermodynamically preferred His and Cys residues.[40] We indeed observed that a three-Met peptide from the Cu transporter Ctr1 (Mets7) releases Pt to the dithiol motif of Atox1 to generate a highly stable adduct. Migration of Pt from S donor ligands has also been invoked to account for cisplatin binding to purine N_7 sites of DNA.[50,51] Glutathione is a putative Pt-binder present in high amount in the cytosol; however, a recent study has shown that two-thirds of the formed Pt adducts in cancer cell extracts had a molecular mass >3 kDa and that glutathione cannot account for more than 20% of the total Pt adducts.[52]

We have shown that, at least in the case of bacterial cells, where there is no compartmentalization of DNA, the overexpression of Atox1 has the effect of reducing the extent of DNA platination and ameliorates cell viability. The situation could be different in eukaryotic cells, where DNA is separated from the cytosol and confined in the nucleus. It has been proposed that in a dimeric form Atox1 can act as a Cu-dependent transcription factor, able to cross the nuclear membrane and to interact with a consensus sequence of DNA.[53] At this stage of the investigation, it cannot be excluded that dimeric Atox1 species, which we have shown to form from monomeric Atox1 upon Pt binding and which, at least in part, could still keep the two ammines coordinated to Pt as observed in the dimeric species characterized by Boal and

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Rosenzweig,[34] could also be able to cross the nuclear membrane and interact with DNA.

The route of Pt dislocation to the nucleus as outlined above has to compete with the trans labilizing effect common to S-donor ligands and consequent loss of the ammines. In the latter hypothesis S-donor bioligands and, among these, Atox1 could be responsible for cisplatin sequestration and inactivation with beneficial effect on cell viability (Figure 4D). We have shown that overexpression of Atox1 reduces the extent of cisplatin-induced filamentous growth and cell division arrest in *E. coli*, the cell morphology changes that led Rosenberg to hypothesize the anticancer properties of cisplatin.[54] Interestingly, it has been reported that some cancer cell lines with higher resistance to cisplatin have levels of Atox1 considerably higher than those of cells with lower cisplatin resistance. In addition, there is indication that exposure to cisplatin can increase cellular levels of Atox1.[33]

We also want to emphasize the strict analogy existing between the Cu(I)-binding sites of Atox1 and those of the soluble domains of the Wilson ATPase and of the active site of thioredoxin, all characterized by a CxxC motif. Recently, four Cu(I)-binding domains of the Wilson ATPase, with a fold similar to Atox1, were shown to bind and sequester Pt, thus reducing *E. coli* filamentation, suggesting a possible mechanism of resistance to cisplatin chemotherapy associated to overexpression of Cu transporters in cancer cells.[55] Also thioredoxin overexpression has been reported for several malignancies, and high levels of thioredoxin have been shown to lead to increased human cancer cell resistance to cisplatin.[56] A thiol bidentate macrochelate is formed in human cancer cells by reaction of cisplatin with the thioredoxin active site motif CxxC,[57] and, ever since, cisplatin has been evaluated as a potential thioredoxin inhibitor.

CONCLUSION

Solution and in-cell NMR experiments prove that cisplatin binds to the CxxC Cu(I)-binding motif of Atox1 and initially retains the two ammine ligands. On one hand, cisplatin-stable binding to Atox1 may interfere with the main cytotoxic activity of the drug, i.e., nucleobase binding and cross-linking. On the other hand, cisplatin bound to Atox1 may alter Cu metabolism and redox surveillance linked to cellular defense processes, thus providing a possible alternative mechanism to cytotoxicity. Last, but not least, cisplatin-induced Atox1 dimer formation could represent a vehicle for cisplatin to translocate into the nucleus and interfere with DNA transcription.

ASSOCIATED CONTENT

Supporting Information. Supporting methods; complete ref 32. This material is available free of charge via the Internet at http://pubs.acs.org.

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