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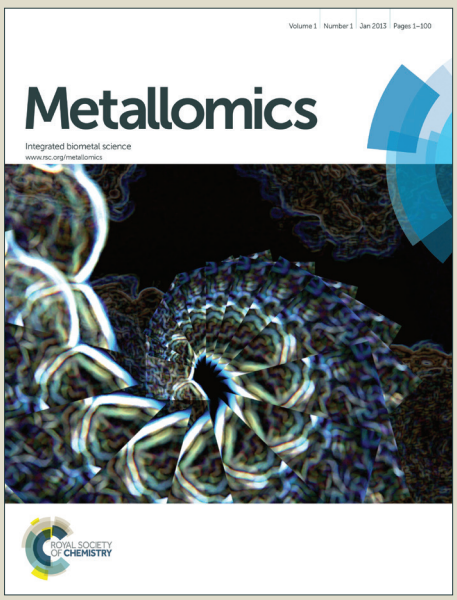

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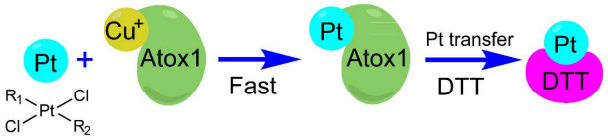
Graphic Abstract

Copper binding modulates the platination of human copper chaperone Atox1 by antitumor *trans*-platinum complexes



Cu(I) coordination enhances the reactivity of Atox1 with antitumor-active *trans*-platinum complexes and promotes platinum transfer from the protein to dithiothreitol.

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Copper binding modulates the platination of human copper chaperone Atox1 by antitumor *trans*-platinum complexes

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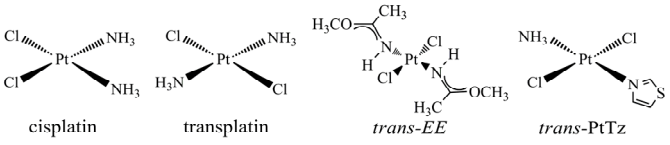
The transport system of platinum-based anticancer agents is crucial for the drug sensitivity. Increasing evidence indicates that the copper transport system is also involved in the cellular influx and efflux of platinum drugs. The copper chaperone Atox1 has been shown to bind to cisplatin *in vitro* and in cells. Previous result reveals that copper binding promotes the reaction between Atox1 and cisplatin. Here, we have performed detailed solution NMR and ESI-MS experiments to investigate the effect of Cu(I) binding on the reactions of Atox1 with two antitumor active *trans*-platinum agents, *trans*-*EE* and *trans*-PtTz. Results indicate that, similar to the reaction of cisplatin, copper coordination also enhances the platination of Atox1 by two *trans*-platinum complexes, and platinum binds to the copper coordination residues. However, copper binding promotes the *trans*-platinum transfer from Atox1 to dithiothreitol (DTT). This result is in contrast to the reaction of Atox1 with cisplatin, in which the presence of copper largely suppresses the platination of DTT. Additionally, both apo- and Cu^{I}-Atox1 react faster with *trans*-platinum complexes than with cisplatin, however, less protein aggregation is observed in the reaction of *trans*-platinum complexes. These results indicate that the roles of Atox1 in the regulation of cellular trafficking of platinum drugs are dependent on the coordination configurations.

Introduction

Cisplatin is an efficient anticancer agent in the treatment of a variety of solid tumors; the platinum binding leads to the DNA damage and induces apoptosis.[1] However, the clinical use of platinum-based drugs is seriously limited due to intrinsic and acquired resistance.[2] Since the discovery of the antitumor properties of cisplatin and its analogues, the presence of a *cis* configuration with two leaving groups has long been considered to be essential for the anticancer activity. Recent years, a number of *trans*-platinum compounds show promising antitumor activities, such as *trans*-[$PtCl_2${*E*-HN=C(OCH_3)CH_3}$_2$] (*trans*-*EE*) and *trans*-[$PtCl_2(NH_3)$(thiazole)] (*trans*-PtTz).[3, 4] In particular, *trans*-*EE* exhibits higher cytotoxicity than its *cis* congener, and also active to several cisplatin-resistant tumor cell lines.[3] These findings imply an alternative strategy for platinum drug design, especially to the cisplatin resistant cells.

Protein interactions have been proved to play important roles in the drug resistance.[2, 5] Increasing evidence indicates that the copper transport proteins, Ctr1 and ATPases mediate the influx and efflux of platinum drugs, respectively.[6, 7] Human copper chaperone Atox1, which delivers Cu(I) ions to ATPase, is believed to be involved in cisplatin resistance. X-ray crystallography and NMR measurements demonstrated that cisplatin binds to Atox1 at the copper coordination residues.[8, 9] It has been also reported that Atox1 could regulate the cellular accumulation of cisplatin and transfer cisplatin to the metal binding domains of ATP7A and ATP7B; and high level of Atox1 was found in cisplatin-resistant cells.[10-14] Recently, we found that the copper coordination enhances the reactivity of Atox1 to cisplatin, which could affect the copper protein associated drug resistance.[15]

Due to the different coordination chemistry, *trans*-platinum complexes exhibit significantly different kinetics and binding modes from cisplatin in DNA binding.[4, 16] These divergences are considered as the origins for the different drug activities of various platinum complexes. Due to the significance and the diversity of protein reactions in drug resistance, the direct interactions of proteins with platinum complexes could also contribute to the different antitumor activities. To understand the effect of the configurations of platinum complexes on the reactions of Atox1, we performed the reactions on two antitumor active *trans*-platinum complexes (*trans*-*EE* and *trans*-PtTz, see Scheme 1). Because of the cross resistance between copper and cisplatin,[17, 18] we also studied the effect of copper binding on the platination of Atox1. The reaction rates were measured through the 2D ^{1}H-^{15}N HSQC spectroscopy with the ^{15}N isotope labeled *trans*-platinum complexes. The products were characterized using tandem mass spectroscopy. Results showed the different roles of Atox1 and copper coordination in the reactions of *trans*-platinum complexes in comparison with cisplatin.



Scheme 1. Structures of platinum complexes

Experimental

Synthesis of platinum drugs

The ^{15}N-labeled platinum complexes, *trans*-[$PtCl_2${*E*-$H^{15}N=C(OCH_3)(CH_3)$}$_2$] and *trans*-[$PtCl_2(^{15}NH_3)$(thiazole)] were synthesized according to literature methods.[19, 20]

Protein expression and purification

The gene sequence for the Atox1 expression was amplified via PCR from a human cDNA library. Atox1 in pST-SG1 vector was transformed into BL21(DE3) Gold cells. The cells were grown in LB medium at 37 °C. When OD_{600} reached 0.8, the culture was induced with 0.8 mM IPTG for 4 h. Cells were harvested by centrifugation at 4000 rpm at 18 °C for 20 min. The protein was purified using Ni^{2+} affinity chromatography. After TEV protease digestion to remove the $(His)_6$ tag, the protein was further purified through size-exclusion chromatography using Superdex 75 16/60 column (GE Healthcare).

NMR spectroscopy

Protein samples were prepared in a buffer of 50 mM sodium phosphate and 5-fold DTT at pH 7.0. For the preparation of Cu^I-Atox1, equimolar amounts of Cu(I) complex $[Cu(CH_3CN)_4]^+$ was added to apo-Atox1 in the buffer containing DTT. The NMR samples were prepared by mixing protein solutions with equimolar lyophilized platinum complexes to a final concentration of 0.8 mM. ^{1}H-^{15}N HSQC spectra were recorded immediately to monitor the reactions. All NMR experiments were carried out on a 700 MHz Varian Inova spectrometer at 25 °C. The spectral widths were set as 10 ppm (^{1}H) and 20 ppm (^{15}N, centered at 92 ppm) for the *trans-EE* reactions, and 8 ppm (^{1}H) and 20 ppm (^{15}N, centered at -65 ppm) for the *trans*-PtTz reactions. The pulse sequence was optimized with a delay $1/(4J_{NH})$ of 3.42 ms (*trans*-PtTz) or 3.21 ms (*trans-EE*). ^{15}N chemical shifts of platinum complexes were referred to $^{15}NH_4Cl$.

HPLC-ESI-MS

For time-dependent measurements, samples of 0.2 mM Atox1 were incubated with equimolar platinum complexes at 25 °C for different time. The resulting mixtures were directly analyzed by HPLC-ESI-MS. For trypsin digestion, Atox1 samples were treated with equimolar platinum complexes at 25 °C for 4 h. The unbound platinum complexes were removed on HPLC with a Zorbax eclipse XDB-C8 column. Then, trypsin (from Promega) was added to the purified protein solution in a molar ratio of 1:40 (trypsin:Atox1). The digestion mixture was incubated for 6 h at 37 °C prior to HPLC-ESI-MS analysis.

ESI-MS spectra were recorded on a Micromass Q-TOF mass spectrometer (Waters) coupled to a Waters CapLC HPLC system. Pt-Atox1 adducts were separated on a Symmetry-C8 column (Waters), while the tryptic digests of Pt-Atox1 adducts were separated on a Symmetry-C18 column (Waters). Linear gradients were used (mobile phases: A: $H_2O/CH_3CN/HCOOH$ (95:4.9:0.1); B: $H_2O/CH_3CN/HCOOH$ (4.9:95:0.1)).The eluents were directly delivered into the mass spectrometer through the ESI probe. The MS spectra were obtained in the range of 500-1800 *m/z* at a capillary temperature of 140 °C with the spray voltage 3.30 kV and cone voltage 35 V. The collision energy was set to 10 V. Collision-induced dissociation MS/MS spectra were acquired in the range of 100–2000 *m/z*, and the relative collision energy was set to 15-20 eV.

Results

NMR studies on the reaction between Atox1 and *trans-EE*

The reaction of apo-Atox1 with ^{15}N-labeled *trans-EE* was measured using 2D ^{1}H-^{15}N HSQC NMR spectroscopy. The signals of dichloro *trans-EE* (**1**, 89.2/7.43 ppm) and its hydrolysis species (**2**, 92.9/7.51 ppm) are in accordance with our previous report.[21] (Fig. 1A) The binding of *trans-EE* to Atox1 generated a new signal (peak **3)** at 97.6/7.81 ppm. In addition to the platinated Atox1 adduct, several *trans-EE*/DTT adducts were also detected based on the control experiments. While the formation of the platinated Atox1, the signals of *trans-EE* decreased quickly and disappeared completely after 33 min. This reaction is considerably faster than the reaction of cisplatin with apo-Atox1, in which the half-life time is about 170 min.[15]

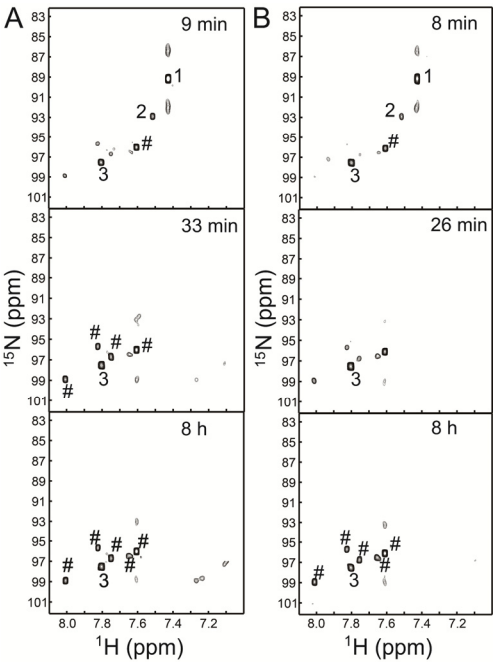


Fig. 1 ^{1}H-^{15}N HSQC spectra of ^{15}N labeled *trans-EE* at 25 °C in the reaction with Atox1 at different time. (A) apo-Atox1; (B) Cu^I-Atox1. The cross-peaks in the spectra were assigned as *trans-EE* (**1**); monochloro *trans-EE* (**2**); the adduct of *trans-EE* with Atox1 (**3**) and the adducts of *trans-EE* with DTT (#).

The reaction of Cu^I-Atox1 showed that the signals of *trans-EE* disappeared more quickly in the reaction of holo-Atox1 than that of apo-Atox1. (Fig. 1 and Fig. S1) Meanwhile, more *trans-EE*-Atox1 adducts and less *trans-EE*-DTT adducts were formed in the reaction of Cu^I-Atox1 in the early stage (~ half-hour). (Fig. 2) This result indicates that, similar to the reaction of cisplatin,[15] the copper coordination also enhances the reactivity of Atox1 with *trans-EE* and suppresses the platination of DTT. After 26 min reaction, *trans-EE*-Atox1 significantly decreased the intensity (~ 59% decreased during 8 h reaction) in the reaction of Cu^I-Atox1, while *trans-EE*-DTT increased to about twice amount. As no free *trans-EE* was present during the period, these intensity changes indicate the platinum transfer from Atox1 to DTT. (Fig. 2) On the contrary, in the reaction of apo-Atox1, the amount of platination adducts almost did not changed during this period (from 33 min to 8 h). This observation clearly indicates that the copper coordination promotes the platinum transfer from Atox1 to DTT. This result is different from the reaction of cisplatin, in which the copper coordination significantly suppresses the platination of DTT throughout the reaction.[15]

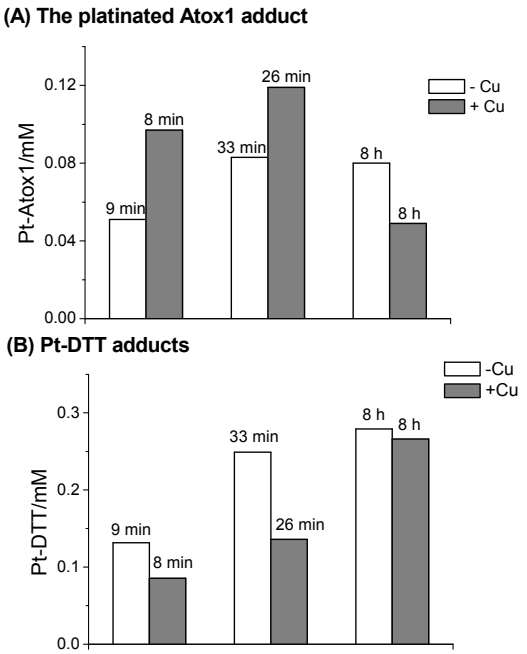


Fig. 2 The time dependent concentrations of platination adducts in the reaction of *trans-EE* with Atox1 detected in the NMR spectra. The color of columns denotes the reaction of apo-Atox1 (white) or Cu^{I}-Atox1 (gray). (A) the *trans-EE*-Atox1 adduct (the peak **3** in the ^{1}H-^{15}N HSQC spectra); (B) the *trans-EE*-DTT adducts.

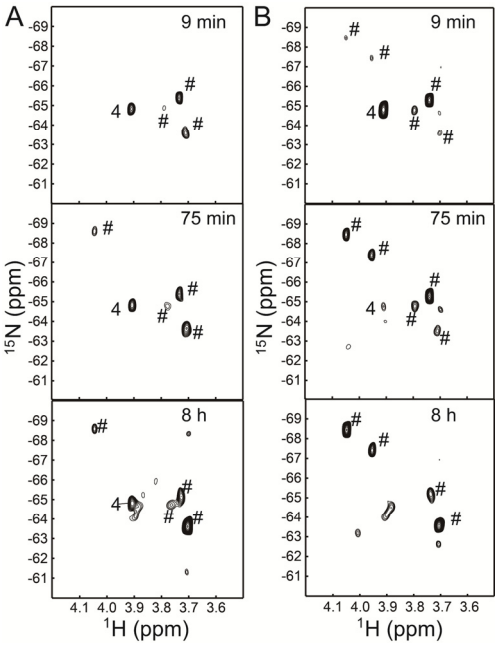


Fig. 3 ^{1}H-^{15}N HSQC spectra of ^{15}N labeled *trans*-PtTz at 25 °C in the reaction with Atox1 at different time. (A) apo-Atox1; (B) Cu^{I}-Atox1. The cross-peaks in the spectra were assigned as the adduct of *trans*-PtTz with Atox1 (**4**) and the adducts of *trans*-PtTz with DTT (#).

NMR studies on the reaction of Atox1 with *trans*-PtTz

To confirm the different effects of copper binding on the platinum transfer are dependent on the configuration of platinum complexes, the reaction of Atox1 was also performed on another antitumor active *trans*-platinum complex, *trans*-PtTz. Even no free *trans*-PtTz or its hydrolysate signals were observed in the first ^{1}H-^{15}N HSQC spectrum at 9 min of the reaction with apo-Atox1 (Fig. 3A), indicating that *trans*-PtTz is much more reactive than cisplatin or *trans-EE*. Meanwhile, three major cross-peaks appeared, including an intense cross-peak at -64.8/3.91 (**4**) ppm from *trans*-PtTz-Atox1 adduct and the other two peaks from DTT adducts. With the reaction progress, the peak **4** decreased its intensity slowly, while the amount of DTT adducts increased. This observation indicates the platinum transfer from Atox1 to DTT since no free *trans*-PtTz existed during this process.

In the reaction of Cu^{I}-Atox1, the same resonance of *trans*-PtTz-Atox1 adduct (**4**) was observed after 9 min incubation, however, with a much higher intensity. (Fig. 3B) This result confirmed that copper coordination promotes the platination of Atox1 by *trans*-PtTz as well as cisplatin and *trans-EE*. Surprisingly, this signal decreased rapidly and became undetectable within 1.5 h. Meanwhile, *trans*-PtTz/DTT adducts were observed with increasing intensities. These results clearly show that the copper coordination promotes the transfer of *trans*-PtTz from Atox1 to DTT. Therefore, copper coordination has the same effect on the platinum transfer of two *trans*-platinum complexes, but in contrast to that of cisplatin.

Table 1. Species observed in the ESI-MS spectra

Pt agents	Common species	Formula	Observed *m/z*	Calculated *m/z*
trans-EE	**a1**: $[Atox1+Pt(Ime)_2+6H]^{8+}$	$C_{338}H_{567}N_{91}O_{108}S_6Pt$	1002.51	1002.62
	a2: $[Atox1+Pt(Ime)_2+DTT+6H]^{8+}$	$C_{342}H_{577}N_{91}O_{110}S_8Pt$	1021.75	1021.87
	a3: $[Atox1+2Pt(Ime)_2+4H]^{8+}$	$C_{344}H_{579}N_{93}O_{110}S_6Pt_2$	1045.02	1045.00
	a4: $[Atox1+2Pt(Ime)_2+DTT+4H]^{8+}$	$C_{348}H_{589}N_{93}O_{112}S_8Pt_2$	1064.35	1064.26
	a5: $[Atox1+2Pt(Ime)_2+2DTT+4H]^{8+}$	$C_{352}H_{599}N_{93}O_{114}S_{10}Pt_2$	1083.67	1083.51
trans-PtTz	**b1**: $[Atox1+Pt(NH_3)(Tz)+6H]^{8+}$	$C_{335}H_{559}N_{91}O_{106}S_7Pt$	997.10	997.11
	b2: $[Atox1+2Pt(NH_3)(Tz)+4H]^{8+}$	$C_{338}H_{563}N_{93}O_{106}S_8Pt_2$	1033.94	1033.98
	b3: $[Atox1+2Pt(NH_3)(Tz)+DTT+4H]^{8+}$	$C_{342}H_{573}N_{93}O_{108}S_{10}Pt_2$	1053.34	1053.23
	b4: $[Atox1+3Pt(NH_3)(Tz)+DTT+2H]^{8+}$	$C_{345}H_{577}N_{95}O_{108}S_{11}Pt_3$	1090.19	1090.11
	b5: $[Atox1+Pt(NH_3)(Tz)+DTT+6H]^{8+}$	$C_{339}H_{569}N_{91}O_{108}S_9Pt$	1016.22	1016.36

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ESI-MS studies on the species of Atox1 with *trans-EE* and *trans*-PtTz

The platinated products of Atox1 were characterized with time-dependent ESI-MS. (Fig. 4 and Fig. 5) Different from the reaction of cisplatin, the carrier ligands (iminoether, Ime) remained coordinated to platinum throughout the reaction of *trans-EE*. (Fig. 4A) The platinated Atox1 adducts were clearly observed after 10 min reaction, including [Atox1+Pt(Ime)$_2$+6H]$^{8+}$ (**a1**, m/z 1002.51) and [Atox1+Pt(Ime)$_2$+DTT+6H]$^{8+}$ (**a2**, m/z 1021.75). (see Table 1 for the peak assignments) Bis-platinated adducts (**a3**, **a4** and **a5**) were observed after 1 h reaction. As the **a1** is always the dominant peak in MS spectra, it can be concluded that the peak **3** in the ^{1}H-^{15}N HSQC spectra can be assigned to [Atox1-Pt(Ime)$_2$]. The same adducts were formed in the reaction of *trans-EE* with Cu^{I}-Atox1, however, the intensity of **a1** decreased after 1 h reaction and **a2** became dominant after 12 h reaction. (Fig. 4B) This result indicates that copper binding promotes the conversion of the Atox1 adduct (**a1**) to the Atox1/*trans-EE*/DTT ternary complex (**a2**). The formation of this ternary complex could be the preceding step of the release of *trans-EE* from Atox1 to DTT observed on the real-time NMR study.

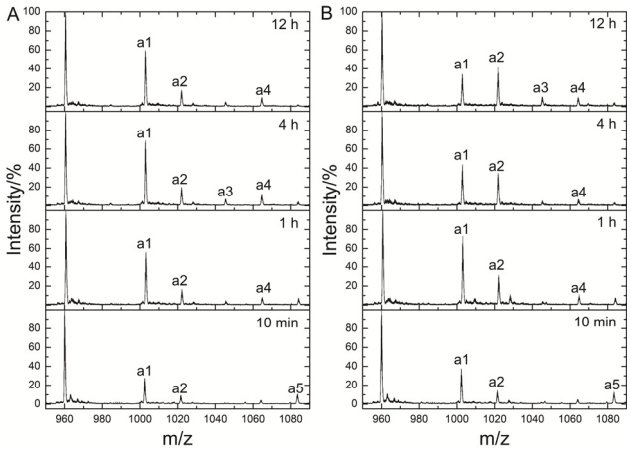

Fig. 4 Time-dependent ESI-MS spectra of Atox1 in the reaction with *trans-EE*. (A) apo-Atox1; (B) Cu^{I}-Atox1. Peaks were assigned to [Atox1+Pt(Ime)$_2$+6H]$^{8+}$ (**a1**, *m/z* 1002.51); [Atox1+Pt(Ime)$_2$+DTT+6H]$^{8+}$ (**a2**, *m/z* 1021.75); [Atox1+2Pt(Ime)$_2$+4H]$^{8+}$ (**a3**, *m/z* 1045.02); [Atox1+2Pt(Ime)$_2$+DTT+4H]$^{8+}$ (**a4**, *m/z* 1064.35); [Atox1+2Pt(Ime)$_2$+2DTT+4H]$^{8+}$ (**a5**, *m/z* 1083.67).

Fig. 5 shows the ESI-MS results of the reactions between Atox1 and *trans*-PtTz. Nearly identical adducts were generated in the reactions of apo- and Cu^{I}-Atox1. The adduct (**b1**) at *m/z* 997.10, which was assigned to [Atox1+Pt(NH$_3$)(Tz)+6H]$^{8+}$, is always dominant in both reactions. This composition should represent the adduct **4** in the NMR spectra. Four minor adducts (**b2**~**b5**) were also detected. In all these adducts, two carrier ligands (NH$_3$ and thiazole) remain coordinated to platinum; this result is also observed in the reaction of *trans-EE*. Consistent with the NMR results, the adduct **b1** formed faster and also decreased more rapidly in the reaction of Cu^{I}-Atox1 than that of apo-Atox1, further supporting the conclusion that the copper coordination enhances the reaction rate of *trans*-PtTz with Atox1 and promotes the *trans*-platinum transfer from Atox1 to DTT.

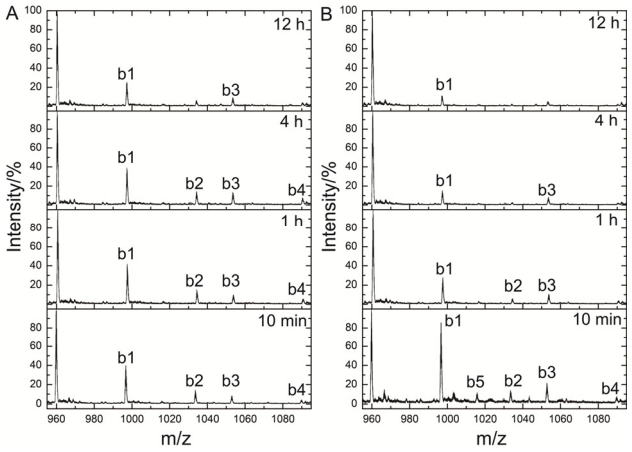

Fig. 5 Time-dependent ESI-MS spectra of Atox1 in the reaction with equimolar *trans*-PtTz. (A) apo-Atox1; (B) Cu^{I}-Atox1. Peaks were assigned to [Atox1+Pt(NH$_3$)(Tz)+6H]$^{8+}$ (**b1**, *m/z* 997.10); [Atox1+ 2Pt(NH$_3$)(Tz)+4H]$^{8+}$ (**b2**, *m/z* 1033.94); [Atox1+2Pt(NH$_3$)(Tz)+DTT+ 4H]$^{8+}$ (**b3**, *m/z* 1053.34); [Atox1+3Pt(NH$_3$)(Tz)+DTT+2H]$^{8+}$ (**b4**, *m/z* 1090.19); [Atox1+Pt(NH$_3$)(Tz)+DTT+6H]$^{8+}$ (**b5**, *m/z* 1016.22).

Platinum binding sites

Our previous result shows that cisplatin binding to the copper coordination residues (Cys12 and Cys15) of Atox1 regardless of the presence of copper or not.[15] Here, we analyzed the effect of copper coordination on the *trans*-platinum binding sites in Atox1 using tandem MS with trypsin digestion. After digestion, the same platinated peptides were detected in the reaction of apo-Atox1 as those in the reaction of Cu^{I}-Atox1 for each *trans*-platinum complex. (Fig. 6 and Fig. S2) Both *trans-EE* and *trans*-PtTz were found to bind to the same peptide fragment (H^{4}EFSVDMTC^{12}GGC^{15}AEAVS R^{21}) as cisplatin does. Additionally, *trans-EE* could also bind to the sequence of V^{40}CIESEHSMDTLLATLK^{56}, which contains a potential platinum binding site Cys41. (Fig. S2) This additional binding site may relate to the multi-platinated Atox1 adducts. No corresponding fragment was detected in the adducts of cisplatin or *trans*-PtTz, probably due to the low abundance in the MS spectra.

Tandem MS was used to identify the platinum binding sites by analyzing the fragmentation of the platinated peptide. For the reaction of *trans*-PtTz, the [P1+Pt(NH$_3$)(Tz)+H]$^{3+}$ ion (*m/z* 731.71) was selected as the precursor ion for collision-induced dissociation experiments. The resulting ESI-MS/MS spectra and corresponding fragmentation schemes are shown in Fig. 7. The fragment ions (b and y) were annotated according to the Biemann nomenclature.[22] On the other hand, the platinated fragments were identified by the characteristic isotope pattern of the platinum and annotated as b* and y*. In Fig. 7B, the largest b_8 in the b_n ions and the smallest b_9* in the b_n* ions indicate that C9 (Cys12 in Atox1) should be a platination site and the other binding site is involved in the G10-R18 sequence. There is no y* ions so that the other site could not be directly read from the fragmentation result through the comparison of the y_n ions with the y_n* ions. However, the Pt^{2+} should favor bonding with

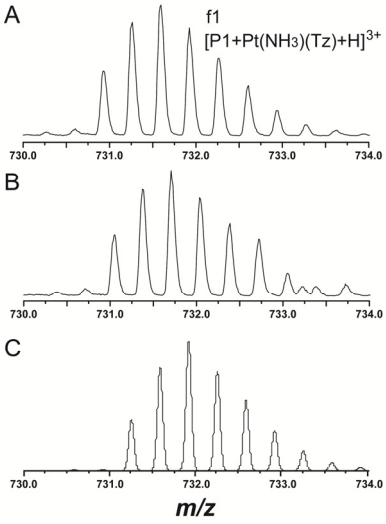

Fig. 6 Selected ESI-MS spectra of platinated peptides from trypsin digestion of (A) Atox1 and (B) Cu^{I}-Atox1 incubated with *trans*-PtTz for 4 h. The isotopic distribution of the f1 peak (the most abundant isotopomer at *m/z* 731.71, $[P1+Pt(NH_3)(Tz)+H]^{3+}$) is well consistent with the simulated pattern (C). P1 represents the peptide $H^{4}EFSVDMTC^{12}GGC^{15}AEAVSR^{21}$.

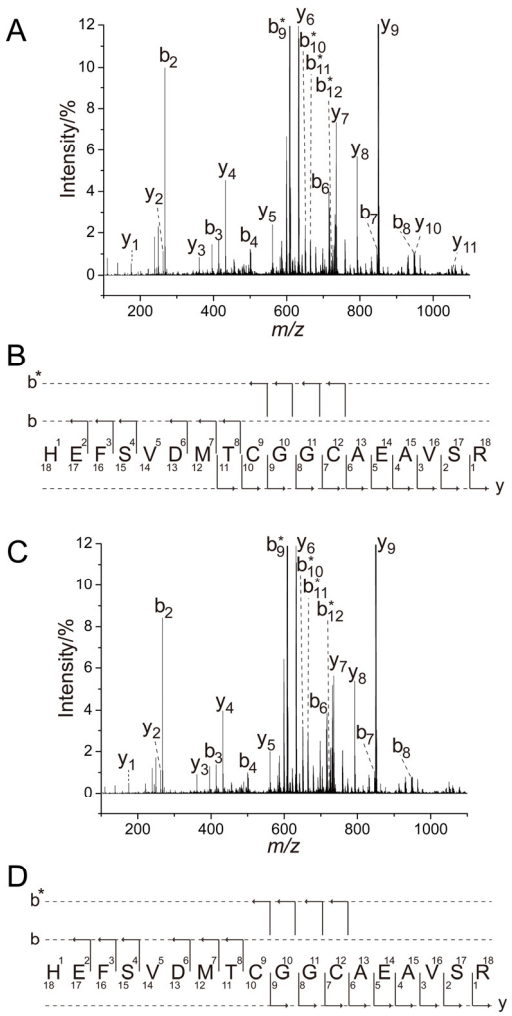

Fig. 7 ESI-MS/MS spectra of the triply charged ion f1 at *m/z* 731.71 from trypsin digestion of (A) Atox1 and (C) Cu^{I}-Atox1 treated with *trans*-PtTz for 4 h. Fragmentation schemes based on the spectra (A) and (C) are shown in (B) and (D), respectively.

sulfur ligands based on coordination chemistry. Therefore, the C12 (Cys15 in Atox1) is the most potential coordination site in the G10-R18 sequence. Similar result was obtained for the reaction of *trans*-PtTz with Cu^{I}-Atox1, indicating that *trans*-PtTz still binds to the copper coordination residues in the presence of copper. (Fig. 7D) The tandem MS experiments were also performed on the samples from the reaction of Atox1 with *trans-EE*, and results show that *trans-EE* binds to the copper binding sites in both apo-Atox1 and holo-Atox1. (Fig. S3) Thus, copper coordination does not change the platinum binding sites, although modulates the platination rate of Atox1 and the *trans*-platinum transfer from Atox1 to DTT.

Discussion

Increasing evidence indicates that copper proteins are involved in the mechanism of platinum drugs.[6, 7, 23, 24] The copper chaperone Atox1 could bind to cisplatin and has been proposed to involved in the intracellular trafficking and drug resistance of cisplatin.[6] Recently, we found that copper binding could promote the interaction of Atox1 with cisplatin.[15] In order to investigate the influence of the configurations of platinum complexes on the reactions of Atox1, we carried out detailed NMR and MS studies to probe the reactivity of Atox1 to two antitumor active *trans*-platinum complexes in the absence and presence of copper. Results reveal that *trans*-platinum complexes also preferentially bind to the copper coordination residues (Cys12 and Cys15) in both apo-Atox1 and Cu^{I}-Atox1. However, copper coordination facilitates the platination of Atox1 by the *trans*-platinum complexes.

Generally, metal binding could significantly lower the reactivity of metalloproteins to platinum compounds, especially when the metal shares the same coordination residues as the platinum.[25, 26] The results here indicate that the bindings of copper and platinum to Atox1 are not simply in competition. It has been proposed that the Cu-Pt interaction is present in the reaction of Cu^{I}-Atox1 only with *cis*-platinum complexes containing two exchangeable leaving groups.[13, 27] In this work, however, we found that Cu(I) binding promotes the reactions of Atox1 also with the *trans*-platinum complexes. The structural investigations on Atox1 have demonstrated that, upon Cu(I) binding, both Cys12 and Cys15 show decreased dynamics and their side-chains become closer to each other.[28, 29] In addition, the more solvent exposure of Cys15 thiol in Cu^{I}-Atox1 could make the cysteines more accessible for platinum binding.

Cisplatin has been reported to bind to Atox1 and lead to the protein unfolding and aggregation of *in vitro*.[13] Our previous result showed that in the reaction of cisplatin, the enhanced Atox1 platination by the copper coordination promotes the Atox1 aggregation.[15] Here we demonstrated that the *trans*-platinum complexes also induced the aggregation of Atox1, and this aggregation is more obvious in the reaction of Cu^{I}-Atox1. (Fig. S4) However, much less protein aggregation was observed in reactions of the *trans*-platinum complexes, even though they are more reactive to Atox1 than cisplatin. These divergences could be correlated with the different coordination chemistry of these platinum complexes. It has been observed that cisplatin could release the ammine ligands in the reaction with Atox1.[15] The two additional coordination sites generated by loss of ammine ligands from cisplatin can facilitate the formation of inter-molecular complexes. On the contrary, the nitrogen ligands are always retained in the reaction of *trans*-platinum complexes. (Fig. 4 and Fig. 5) It has been proposed that the aggregation of Atox1 could associate with the cisplatin resistance.[13] Results here highlight the different mechanisms of Atox1 in the cellular process of *trans*- and *cis*-Pt complexes; in addition, this process is also modulated by the copper binding.

Kinetic properties have been proved to be crucial in the determination of the drug activity of platinum complexes. It is well known that higher reactivity of transplatin causes its lower antitumor activity than cisplatin.[30, 31] Replacing the ammine ligands of transplatin with bulky ligands could lead to lower reactivity but higher antitumor activity.[4] For instance, *trans*-*EE* shows the similar DNA binding rate to cisplatin.[21] Nevertheless, the two *trans*-platinum complexes are much more reactive than cisplatin in the reactions with both apo- and holo-Atox1. This result could be explained by the strong *trans* effect of thiol group.[32] With the binding of first cysteine residue, the other leaving group is *trans* to thiol group in the *trans*-platinum complexes, which makes the second substitution very fast. This is not the case in the reaction of DNA, in which the coordination of guanine N7 has much lower *trans* effect. On the other hand, it is interesting to note that DTT has different effects between the reactions of *trans*- and *cis*-platinum complexes. Cu(I)-binding facilitates the DTT-induced *trans*-platinum release from Atox1 but suppresses the cisplatin release, although Cu(I)-binding promotes the platination of Atox1 by all three platinum complexes. The significantly different reactivity and binding stability of platinum complexes to proteins could correlate to the diverse mechanisms of various drugs.

As a copper chaperone protein, Atox1 executes its function by binding cuprous ions and delivering them to the target proteins (ATP7A and ATP7B) in cells.[33, 34] ATP7A and ATP7B have been proved to be involved in the resistance of platinum drugs.[35, 36] Both ATP7A and ATP7B contain six metal binding domains (MBDs) in the cytoplasmic N-terminal domain, and the overall structure and the conserved copper binding motif of each MBD are very similar to that of Atox1.[37] Therefore, it is very likely that the copper binding may also promote the platination of ATP7A and ATP7B, which consequentially influences the sequestration or efflux of platinum complexes. Moreover, more folded *trans*-platinum-Atox1 species and readily *trans*-platinum release from Atox1 suggest a possible mechanism of copper in the regulation of the trafficking of *trans*-platinum complexes: copper binding promotes the transfer of *trans*-platinum complexes from Atox1 to ATP7A and ATP7B, for the subsequent cellular export.

Conclusions

In conclusion, this work demonstrates that copper binding promotes the platination of Atox1 by two antitumor active *trans*-platinum complexes, although platinum binds to the copper coordination residues of the protein. In comparison to cisplatin, *trans*-platinum complexes are much more reactive to Atox1, however, induce less protein aggregations. In addition, cuprous ions demonstrate different effects on the platinated Atox1 adducts of *trans*-platinum relative to cisplatin. The Cu^{I}-coordination facilitates the *trans*-platinum transfer from Atox1 to DTT, whereas significantly suppresses the platination of DTT by cisplatin. As Atox1 is a copper chaperone and executes functions with copper coordination, the modulation of platination by copper indicates that the Atox1 protein plays different roles of *cis*- and *trans*-platinum agents in the intracellular trafficking, and could be correlated to cross resistance between copper and platinum drugs.

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