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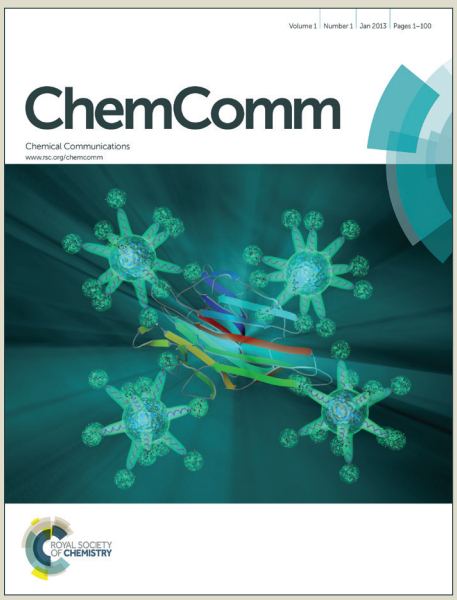

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ARTICLE TYPE

Cisplatin Binds to Human Copper Chaperone Cox17: the Mechanistic Implication of Drug Delivery to Mitochondria

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Cox17 facilitates the platinum accumulation in mitochondria, which contributes to the overall cytotoxicity of cisplatin.

Cisplatin is a widely used anticancer drug for the treatment of a large variety of solid malignancies. It is generally accepted that cisplatin targets DNA and the DNA damage triggers cell apoptosis.[1] However, only small portion (~ 1%) of the intracellular cisplatin forms DNA crosslinks.[2] On the other hand, the cytotoxicity of cisplatin to enucleated cells raises the question of non-DNA targets of platinum drugs.[2] Recent years, proteins have been recognized to be important in the mechanism of platinum drugs.[3] Protein interactions affect the drug sensitivity and resistance by regulating the drug uptake and efflux, drug detoxification, DNA repair and signaling pathways.[4] Moreover, cisplatin is also proposed to exert cytotoxicity by targeting diverse subcellular structures, including mitochondria.[5]

Mitochondria play essential roles in regulating cell apoptosis via various signaling pathways, including the change of mitochondrial transmembrane potential, the release of pro-apoptotic proteins, changes in electron transport and the alteration of cellular redox potential.[6] It has been proposed that mitochondria are also associated with the mechanism of cisplatin, and the direct interaction of cisplatin with mitochondria induces apoptosis.[7] A recent study shows that the delivery of platinum drug to mitochondria promotes the apoptosis of cancer cells.[8] Indeed, platinum has been found highly accumulated in mitochondria proteins; the apoptosis associated protein VDAC shows over 200-fold higher affinity to cisplatin than whole cellular proteins.[9] However, it is unknown how cisplatin was delivered to mitochondria.

The copper transport system has been proved to be involved in the cisplatin delivery. Copper transport protein Ctr1, which is essential for copper transport, also facilitates the uptake of cisplatin.[10] The P-type ATPases, which maintain cellular copper homeostasis, also mediates cisplatin efflux.[11] Recent studies show that the copper chaperone Atox1 can react with cisplatin and is involved in cisplatin resistance.[12] It is intelligible that cisplatin tends to bind to copper proteins since Pt(II) and Cu(I) have similar binding affinity. Thus, the copper chaperone Cox17, which is associated with copper transfer to mitochondrial proteins, could also be involved in cisplatin transfer to mitochondria.

Cox17 has three oxidation states, the oxidized $Cox17_{3S\text{-}S}$ with three disulfide bonds, the reduced $Cox17_{0S\text{-}S}$ with no disulfide bonds, and the intermediate state $Cox17_{2S\text{-}S}$ with two disulfide bonds. $Cox17_{2S\text{-}S}$ transfers Cu(I) to cytochrome C oxidase (CCO) via Sco1 and Cox11.[13] The mutation of Cox17 results in the dysfunction of CCO, leading to the respiratory defect.[14] Moreover, the over-expression of Cox17 has been found in some cancer cells, so that Cox17 has been proposed as a therapeutic target for the cancer treatment.[15] $Cox17_{2S\text{-}S}$ contains two free cysteine residues (Cys23 and Cys24) for the Cu(I) coordination; these residues are the potential binding sites of cisplatin. Cox17 can transfer from cytoplasm to mitochondria through a C-terminal mitochondrial inter-membrane space targeting signal (ITS).[16] Thus, we speculate that the binding of cisplatin to Cox17 is also correlated to the drug transport to mitochondria.

To verify this hypothesis, we investigated the correlation between the cellular Cox17 level and the drug accumulation in mitochondria. The over-expression of Cox17 was carried out by transfecting pcDNA3.0-hCox17 recombinant plasmid into HeLa cells. The down-regulation of Cox17 was achieved by transfection of the Cox17 siRNA. The control experiments were performed by transfection of the empty vector pcDNA3.0 or the scrambled siRNA sequence. The mRNA levels of Cox17 in cells were measured using RT-PCR (Fig. S1).

After the treatment of cisplatin, mitochondria were isolated from cells and the platinum content was quantified with inductively coupled plasma mass spectrometry (ICP-MS). Data clearly show that the over-expression of Cox17 increased the platinum accumulation in mitochondria by 28% ($P < 0.05$), while the low expression of Cox17 reduced platinum level in mitochondria by 35% ($P < 0.05$, Fig. 1A). This result indicates that Cox17 is associated with the transport of cisplatin to mitochondria in cells.

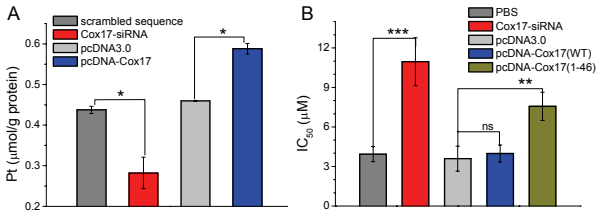

Fig. 1 Correlation of Cox17 level with the platinum accumulation in mitochondria and the cytotoxicity of cisplatin. (A) The platinum accumulation in mitochondria of the HeLa cells with different levels of Cox17 expression. Cells were incubated with 10 μM cisplatin for 10 h prior to the measurements. (B) Effect of the Cox17 level on the cytotoxicity of cisplatin. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

To verify whether the platinum accumulation in mitochondria contributes to the drug efficacy of cisplatin, we measured the inhibitory activity of cisplatin on the growth of HeLa cells with different Cox17 levels. The half inhibitive concentrations (IC_{50}) clearly showed that the down-regulation of Cox17 lowered the cytotoxicity by increasing IC_{50} from 3.6 μM to 11.1 μM (Fig. 1B, Fig. S2). This result proved that Cox17 plays important role in cisplatin cytotoxicity. It appeared that the transfection of WT Cox17 plasmid did not alter the IC_{50} value in comparison with the transfection of empty vector; however, the higher IC_{50} value was observed on the transfection of Cox17 mutant with the deletion of C-terminal mitochondrial ITS sequence ($Cox17_{1\text{-}46}$) (Fig. 1B, Fig. S2). The result indicates that the transport of Cox17 to mitochondria contributes to the cytotoxicity of cisplatin. Additionally, this observation suggested that over-expression of Cox17 could play two different roles. In addition to the delivery of cisplatin to mitochondria, the over-expressed Cox17 could also consume more cisplatin and reduce the drug interaction with the DNA target. These two opposite contributions of Cox17 caused effect of the over-expression undetectable. Nevertheless, the function of Cox17 for the delivery of cisplatin to mitochondria can be observed by comparing transfections of WT-Cox17 with the Cox17 mutant lack of C-terminal ITS. These data indicate that both the Cox17 level in the cell and its transport to mitochondria contribute to the overall drug efficacy of cisplatin.

As Cox17 is correlated with Pt accumulation in mitochondria, we further investigated the binding of cisplatin to the protein in vitro. $Cox17_{2S\text{-}S}$ was used in this work since this is the functional state of the protein for transferring Cu(I) to the target protein. $Cox17_{2S\text{-}S}$ contains two free cysteine residues (Cys23 and Cys24) for the copper binding. The redox state of $Cox17_{2S\text{-}S}$ remains stable under reaction condition (Fig. S3). $Cox17_{3S\text{-}S}$ was also used to confirm the reactivity of the two free cysteine residues.

The binding of cisplatin to apo-Cox17 was investigated using UV spectroscopy. Cox17 has very low absorbance at 280 nm as this protein has only one aromatic residue Phe61. The formation of Pt-S bonds exhibits the strong absorption at 280 nm; therefore, the increase of absorbance indicates the platinum binding to $Cox17_{2S\text{-}S}$ at the cysteine residues (Fig. 2A). Reaction on $Cox17_{3S\text{-}S}$ showed very little change of absorbance at 280 nm (Fig. 2B). This result indicates the coordination of the two free cysteines of $Cox17_{2S\text{-}S}$ to cisplatin.

The platination of Cox17 were also monitored by HPLC combined with ICP-MS. The binding of cisplatin does not change the retention time of Cox17 on HPLC (at 14 min) (Fig. S4). Nevertheless, due to the increased absorbance of Pt-$Cox17_{2S\text{-}S}$ at 280 nm, the platinated adducts showed the increased HPLC peak with the detection at 280 nm (but not at 220 nm, see Fig. S4). Therefore, the peak intensity in HPLC profiles provides a feasible probe to characterize the platination of $Cox17_{2S\text{-}S}$. Results showed that the peak of $Cox17_{2S\text{-}S}$ increased significantly during 12 hours incubation, whereas no detectable change was observed in the reaction of $Cox17_{3S\text{-}S}$ (Fig. 2C). This result is consistent with the UV measurements. As the UV absorbance cannot detect the platinum coordination to non-sulfur ligands, we further measured the platination of Cox17 using ICP-MS. Results clearly show the binding of platinum to $Cox17_{2S\text{-}S}$ (the inset in Fig. 2C), in accordance with the absorbance increases. However, the platinum binding to $Cox17_{3S\text{-}S}$ was also detected, though in lower content (the inset in Fig. 2C, Fig. S5). This result indicates that, without free cysteine residues, $Cox17_{3S\text{-}S}$ is still reactive to cisplatin. Although cisplatin can react with sulfur atoms in disulfide bonds,[17] UV results indicate that cisplatin does not bind to disulfide cysteines in $Cox17_{3S\text{-}S}$. 2D NMR and ESI-MS results indicate that the methionine residue could be the binding site of $Cox17_{3S\text{-}S}$ (Fig. S6).

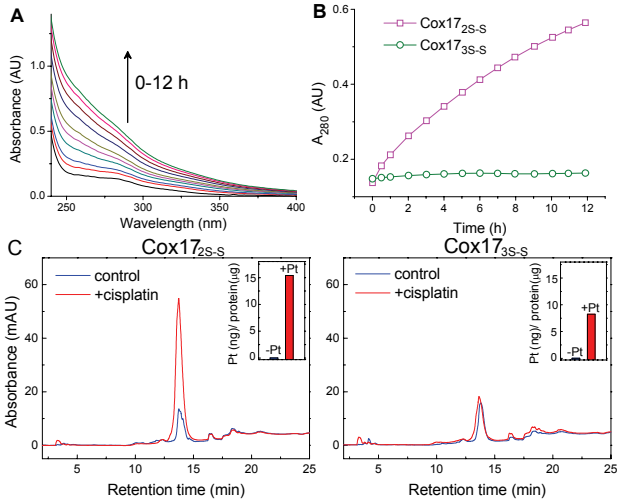

Fig. 2 Reaction of Cox17 with cisplatin. (A) Time dependent UV spectra of $Cox17_{2S\text{-}S}$ in the reaction of cisplatin. (B) The absorbance of Cox17 at 280 nm as a function of reaction time. Magenta square: $Cox17_{2S\text{-}S}$, Green circular: $Cox17_{3S\text{-}S}$. (C) HPLC profiles of $Cox17_{2S\text{-}S}$ (left) and $Cox17_{3S\text{-}S}$ (right) reacted with cisplatin for 12 h. HPLC profiles were recorded on UV detection at 280 nm. The insets show platinum content measured using ICP-MS. All reactions were performed on 100 μM Cox17 with 200 μM cisplatin in 20 mM MES (pH 6.0) at 37 °C. Samples were prepared in an anaerobic glove box.

The platination adducts of Cox17 were characterized using ESI-MS. The reference spectra on the apo-proteins confirmed the composition of apo-$Cox17_{3S\text{-}S}$ and apo-$Cox17_{2S\text{-}S}$ (MW: 7180.5 and 7182.5 Da, respectively, see Fig. S7). After the incubation with cisplatin, several new peaks were observed on the ESI-MS spectra. The mass increases indicate the binding of cisplatin in the forms of $[Pt(NH_3)_2Cl]$, $[Pt(NH_3)_2]$, $[Pt(NH_3)]$ and [Pt], suggesting the stepwise loss of ligands from cisplatin. Nearly the same products were generated in the reactions of $Cox17_{2S\text{-}S}$ and $Cox17_{3S\text{-}S}$, while product distributions are different in two reactions (Fig. 3, Fig. S8, and Table S2). The relative peak abundance indicates that the release of ammine is more pronounced in the reaction of $Cox17_{2S\text{-}S}$ than that of $Cox17_{3S\text{-}S}$. The release of ammine often occurs when cisplatin coordinates to cysteine residues due to the strong *trans* effect of the thiol ligand. This could happen either in the solution or during the ESI-MS process.[18] Therefore, the easier release of ammine ligand in the reaction of $Cox17_{2S\text{-}S}$ further supports the assumption that cysteine residues are the platinum binding sites of $Cox17_{2S\text{-}S}$.

The binding of cisplatin to the copper binding site was investigated by the quantification of free thiol of $Cox17_{2S\text{-}S}$ using the Ellman's assay. The decrease of thiol content of Cox17 during the reaction of cisplatin indicates the platinum binding at the free cysteine residues, i.e. the copper coordination sites. The

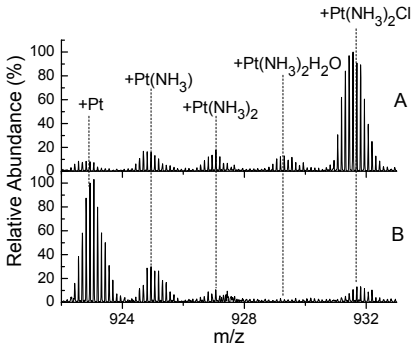

Fig. 3 ESI-MS spectra of platinated Cox17 adducts with 8+ charged peaks. (A) $Cox17_{3S\text{-}S}$; (B) $Cox17_{2S\text{-}S}$. The Cox17 proteins were incubated with 5 molar equivalent of cisplatin at 37 °C for 6 h prior to the ESI-MS measurements.

half-life time ($t_{1/2}$ = 4.2 h) was obtained from the time dependent decay of thiol content (Fig. 4A). Since Cox17 exerts its biological function as a copper chaperone, we verified whether the Cu(I) coordination affect the reaction of cisplatin. The reaction of Cu^{I}-$Cox17_{2S\text{-}S}$ with cisplatin was investigated using the copper release assay with the Cu(I) dye bicinchoninic acid (BCA). Adding cisplatin to Cu^{I}-$Cox17_{2S\text{-}S}$ in the presence of BCA led to gradually increasing of absorption at 562 nm, indicative of the release of Cu(I) from Cox17 and the formation of $Cu(BCA)_2$ (Fig. 4B). The result indicates that the reaction of cisplatin with Cu^{I}-bound $Cox17_{2S\text{-}S}$ leads to the copper release from the protein, suggesting the dysfunction of this protein. In addition, the half-life time ($t_{1/2}$ ~ 1.3 h) indicates that the reaction of Cu^{I}-bound Cox17 is about 3 times faster than that of apo-Cox17. This result is similar to the reaction of Atox1 that copper coordination promotes the platination,[19] which is probably due to the Cu-Pt interaction in the platination adducts.[20] Hence, the copper release from Cox17 could the subsequent step of the protein platination.

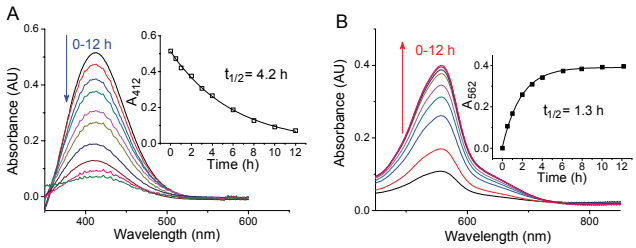

Fig. 4 (A) Time-dependent Ellman's assay on apo-$Cox17_{2S\text{-}S}$ reacted with cisplatin. The inset shows the absorbance at 412 nm (A_{412}) versus reaction time. (B) Time-dependent copper release assay on Cu^{I}-$Cox17_{2S\text{-}S}$ in reaction with cisplatin. The inset shows the absorbance at 562 nm (A_{562}) versus reaction time. Both reactions were performed on 100 μM Cox17 and 200 μM cisplatin in 20 mM MES (pH6.0) at 37 °C.

In summary, this work demonstrates that the copper chaperone Cox17 is involved in the transport of cisplatin to mitochondria and this process contributes to the overall cytotoxicity of cisplatin. *In vitro* interactions show that cisplatin binds to the Cox17 protein at the copper binding site. The binding of cisplatin results in the copper release from the protein. On the other hand, the copper binding enhances the reactivity of Cox17 to cisplatin. This finding reveals a novel role of Cox17 in the mechanism of platinum drugs and provides an insight into the transport pathway of cisplatin to mitochondria.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Experimental details, HPLC, ESI-MS, and UV-visible spectra]. See DOI: 10.1039/b000000x/

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