Electronic Supporting Information

for

Transporting Platinum Drugs from Copper Chaperone to ATPase: the Mechanistic Implication of Drug Efflux Mediated Cisplatin Resistance

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Contents

Experimental Details

Reagents and methods: PCR kits, Genetailor mutagenesis kit, and media were obtained from Invitrogen. Cisplatin was bought from Sigma-Aldrich. Ni-NTA resins were received from Qiagen and the chitin resins were got from New England Biolabs. Other materials were purchased from Sangon Biotech (Shanghai, China).

ESI-MS spectra were recorded on Thermo LTQ linear Orbitrap XL mass spectrometry (Thermo Fisher, San Jose, CA, USA) and data were analyzed using XCalibur software (version 2.0, Thermo Finnigan). Fast protein liquid column (FPLC) was performed on AKTA purifier (GE Healthcare) with UV detector at 280 nm. The Hitrap^{TM} Desalting column, HiLoad Superdex 75 pg 16/60 size-exclusion chromatography and the Souce15Q anion exchange column were equipped in the experiments. ICP-MS measurements were carried out on a Perkin-Elmer 560 instrument. All samples for ICP-MS were digested in nitric acid before the measurements.

Protein Expression and Purification: The gene sequence for the expression of Atox1 was amplified via PCR from a human cDNA library. The gene was inserted into pST-SG1 vector using the ligation-independent cloning (LIC) method. The plasmid was introduced into *E.* coli BL21(DE3) Gold. Cells were grown at 37 °C in LB medium until the OD_{600} reached 0.8, the protein expression was induced by 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 10 h at 25 ºC. Cells were harvested by centrifugation at 4000 rpm at 4 ºC for 20 min. The proteins were purified by a column with Ni-NTA resin (Qiagen). The His_6-tag was cleaved by TEV (tobacco etch virus) protease at 16 ºC overnight with 2 mM DTT and 0.5 mM EDTA. Proteins were purified with size-exclusion chromatography using HiLoad Superdex 75 pg 16/60 (GE Healthcare) in 20 mM potassium phosphate buffer, pH 7.4. The purity was verified by Tricine-SDS-PAGE and ESI-MS. The same procedure was used for the expression and purification of MNK4.

The plasmid for expression of CBD-Atox1 fusion protein was constructed as described previously.[1] The mutation of Cys12 and Cys15 to Ser (CBD-Atox1S) was performed on CBD-Atox1 plasmid with site-directed mutagenesis. The expression of fusion protein was conducted in *E. coli* BL21(DE).

ESI-MS analysis of the reaction of MNK4 with cisplatin: 0.5 mM apo-MNK4 was incubated with 1.3 molar equivalents of cisplatin in 20 mM potassium phosphate buffer (pH 7.4) in the presence of 2 mM DTT at 25 ºC. After different reaction time (8, 24, 36 h), the salts and unreacted cisplatin were removed through Hitrap Desalting column against pure water. Samples were diluted with a solution of water/methanol (1:1) containing 0.1% formic acid, and were directly infused to the mass spectrometer with nanospray source option. Data were collected in the positive mode in the range of 200 to 2000 m/z.

SDS-PAGE analysis of the multimerization of MNK4: 0.45 mM MNK4 was incubated with 1.3 molar equivalents of cisplatin in the presence of 1 mM DTT at 25 °C for different time. The products were analyzed with 15% Tricine-SDS-PAGE. The protein incubated with buffer in the absence of cisplatin was used as control.

Preparation of the Pt-Atox1 complex: Apo-Atox1 was incubated with equimolar cisplatin and 4 molar equivalent DTT for 3 h at 25 °C. The sample was buffer exchanged on FPLC with a Hitrap desalting column to remove unreacted cisplatin. Pt-Atox1 was concentrated by centrifugal ultrafiltration through filter membranes and the concentration of platinum was determined by ICP-MS.

Anion exchange chromatography analyzes of the reaction of MNK4 with Pt-Atox1: Pt-Atox1 was incubated with MNK4 in 20 mM potassium phosphate buffer at 25 °C for 10 h. The samples were analyzed on anion exchange column (eluents: A: 20 mM Tris-HCl, pH 8.0; B: eluent A with 1 M NaCl). The column was pre-equilibrated with 12% B. The linear gradient (0-5 min: 12% B, 5-15 min: 12-45% B) was used with 1 ml/min flow rate. The profiles were recorded by UV detector at 280 nm.

Determination of platinum in MNK4 by ICP-MS: MNK4 was separated from Pt-Atox1 with anion exchange chromatography. The elution was performed in 12% B for 8 min and then turned to 100% B with 1 ml/min flow rate. The fraction of MNK4 and Pt-MNK4 was collected together. (**Fig. S3**) The protein concentration was measured using BCA assay and the platinum content was determined using ICP-MS.

Alkylation of MNK4 by N-ethylmaleimide: 0.6 mM apo-MNK4 with four molar equivalents of DTT was incubated with 20 molar folds NEM for 2 h at 25 °C in 20 mM potassium phosphate

buffer, pH 7.4. The product was buffer exchanged with 20 mM potassium phosphate buffer to remove excess NEM and DTT.

Determination of the protein concentrations on chitin column: The cell pellets from the expression of CBD-Atox1 or CBD-Atox1S were resuspended with PBS (20 mM potassium phosphate buffer, pH 7.4) and lysed by sonication for 10 min. The lysate was centrifuged at 16000 rpm for 30 min at 4 °C. The supernatant was loaded onto the chitin column. The column was rotated on a rotatory shaker at 4 °C for 20 min for the protein binding. The column was washed with PBS containing 2 mM DTT to remove impurities. The beads were shaken for 30 min with 0.3 M NaOH to elute CBD-Atox1 or CBD-Atox1S. The concentrations of CBD-Atox1 and CBD-Atox1S were measured through Bradford assays.

Reaction of Pt-Atox1 on chitin column: Chitin columns containing the same amount of CBD-Atox1 or CBD-Atox1S were used in the reaction. Cisplatin was added to the column containing 2 molar equivalent DTT, and the columns were rotated at 25 °C for 3 h in the dark for the platinum binding to CBD-Atox1 or CBD-Atox1S. The columns were washed with PBS to remove unreacted cisplatin. Then MNK4 in PBS was added to the columns for the further reaction. After 10 h rotation at 25 °C, the supernatants were collected, and the platinum in the solutions were determined by ICP-MS after the nitric acid digestion.

Reference

1. L. Zhang, Y. Zheng, Z. Xi, Z. Luo, X. Xu, C. Wang and Y. Liu, *Molecular BioSystems*, 2009, **5**, 644-650.

Table S1 The mass spectra analyses of the products in the reaction of MNK4 with cisplatin.

Composition	Molecular Formula	m/z	
		obsd. (6+)	cald. (6+)
$[MNK4+6H]^{6+}$	$C_{335}H_{559}N_{91}O_{120}S_4$	1318.326	1318.319
$[MNK4+Na+5H]^{6+}$	$C_{335}H_{558}N_{91}O_{120}S_4Na$	1321.986	1321.983
$[MNK4+Pt+4H]^{6+}$	$C_{335}H_{557}N_{91}O_{120}S_4Pt$	1350.479	1350.496
$[MNK4+Pt(NH_3)+4H]^{6+}$	$C_{335}H_{560}N_{92}O_{120}S_4Pt$	1353.324	1353.334
$[MNK4+Pt(NH_3)_2+4H]^{6+}$	$C_{335}H_{563}N_{93}O_{120}S_4Pt$	1356.159	1356.173
$[MNK4+Pt(NH_3)_2+Na+3H]^{6+}$	$C_{335}H_{562}N_{93}O_{120}S_4PtNa$	1359.826	1359.836
$[MNK4+Pt(NH_3)_2Cl+5H]^{6+}$	$C_{335}H_{564}N_{93}O_{120}S_4PtCl$	1362.320	1362.249
$[MNK4+Pt(NH_3)_2Cl+Na+4H]^{6+}$	$C_{335}H_{563}N_{93}O_{120}S_4PtClNa$	1365.812	1365.912

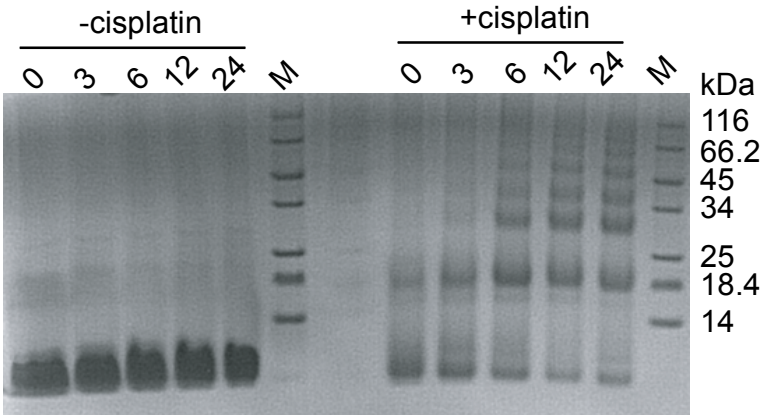

Fig. S1 Tricine-SDS-PAGE gel of MNK4 samples. MNK4 was incubated at 25 ºC without or with 1.3 molar equivalents cisplatin for different time intervals (0, 3, 6, 12, 24 h).

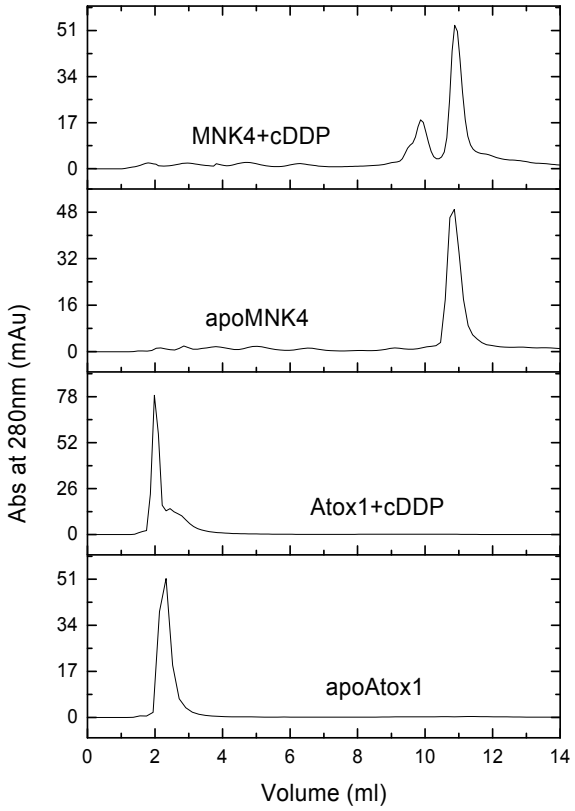

Fig. S2 Anion exchange chromatography analysis of Atox1 and MNK4. The eluents were A: 20 mM tris-HCl, pH 8.0; B: A + 1 M NaCl. The column was pre-equilibrated with 12% B. The linear gradient (0-5 min: 12% B, 5-15min: 12-45% B) was used with 1 ml/min flow rate.

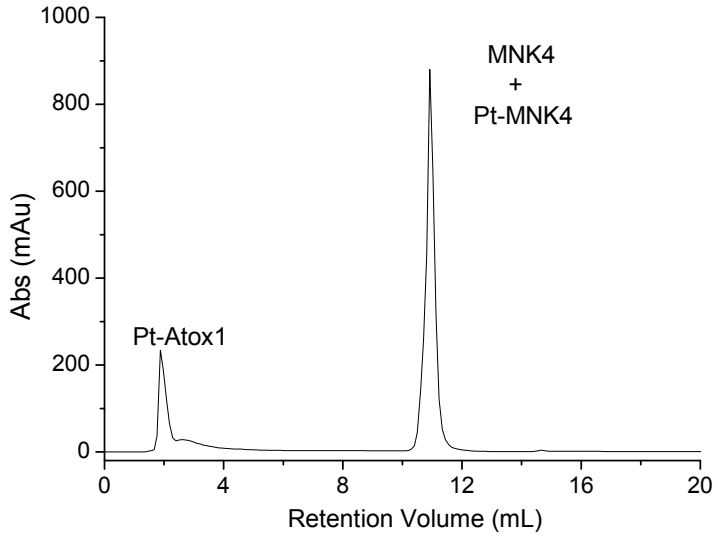

Fig. S3 Anion exchange chromatography analysis of the products of incubation of Atox1-cDDP and MNK4 for ICP-MS measurement. The column was pre-equilibrated with 12% B. The elution was performed in 12% B for 8 min and then turned to 100% B with 1 ml/min flow rate.

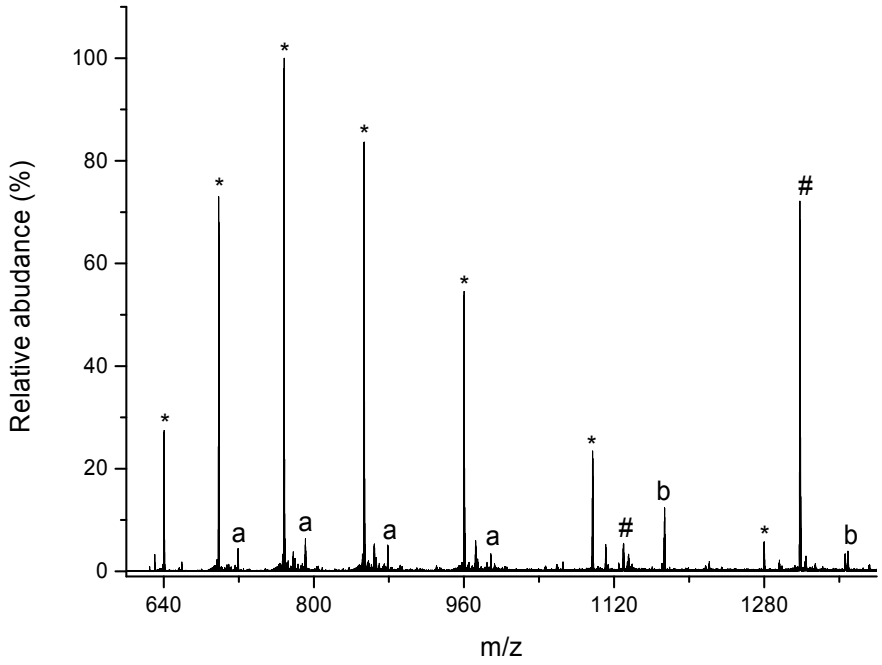

Fig. S4 ESI-MS analysis of the products of the reaction of Pt-Atox1 and MNK4. MNK4 was incubated with Pt-Atox1 in phosphate buffer at 25 ºC for 10 h. Symbols denote: Atox1 (*), MNK4 (#), [Atox1+$Pt(NH_3)_2$] (a), [MNK4+$Pt(NH_3)(PO_4)$] (b).

Table S2 The mass spectra analyses of the products of MNK4 and Pt-Atox1.

Composition	Molecular Formula	m/z obsd./cald. (charges)
[Atox1]	$C_{332}H_{547}N_{89}O_{106}S_6$	1097.276/1097.281 (7+); 960.241/960.247 (8+); 853.678/853.665 (9+); 768.392/768.399 (10+)
[Atox1+$Pt(NH_3)_2$]	$C_{332}H_{553}N_{91}O_{106}S_6Pt$	988.741/988.637 (8+); 878.878/878.900 (9+); 791.094/791.111 (10+); 719.268/719.283 (11+)
[MNK4]	$C_{335}H_{553}N_{91}O_{120}S_4$	1318.326/1318.319 (6+); 1130.137/1130.132 (7+)
[MNK4+$Pt(NH_3)(PO_4)$]	$C_{335}H_{559}N_{92}O_{124}S_4PtP$	1369.329/1369.667 (6+); 1173.860/1174.143(7+)

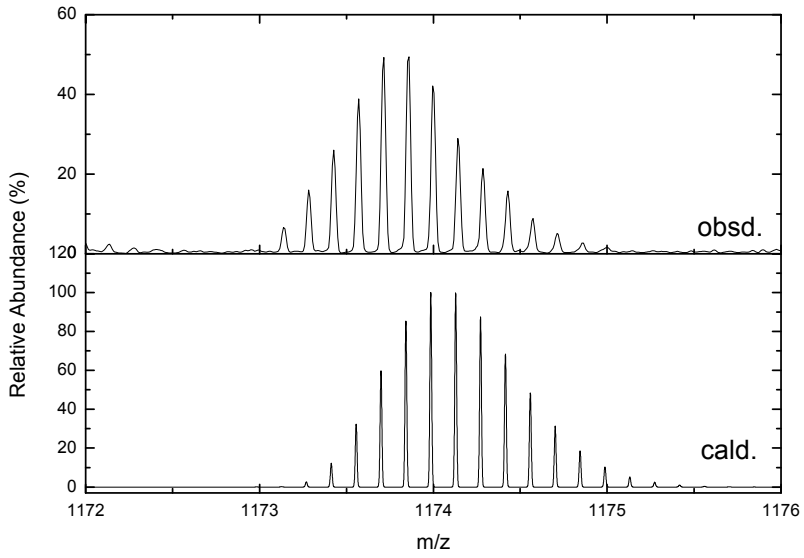

Fig. S5 The theoretical and observed ESI-MS isotope patterns of $[MNK4+Pt(NH_3)(PO_4)+8H]^{7+}$ positive charged.