Supporting Information

for

Cisplatin Binds to MDM2 RING Finger Domain and Inhibits the Ubiquitination Activity

Experimental details

Materials

Cisplatin was purchased from Sigma-Aldrich (St. Louis, MO). 4-(2-Pyridylazo) resorcinol (PAR) and dithiothreitol were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Ubiquitin activating enzyme E1 and conjugating enzyme E2 were obtained from MerckMillipor. Anti-Ub antibody was obtained from Abcam (Cambridge, US). Western blot detection kit was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Ultra-purified water was prepared using a Milli-Q Synthesis System (Millipore, Bedford, MA). All other solvents and reagents were used as received.

Protein Expression and Purification

The cDNA fragment encoding the MDM2 ring-finger domain was obtained by polymerase chain reaction (PCR) amplification. The pST-GB1-MDM2-RF recombinant plasmid was constructed by inserting the PCR products into the pST-GB1 vector using the ligation-independent cloning (LIC) method. The pST-GB1 vectors encode an N-terminal poly-histidine (His_6) tag followed by a solubility enhancement tag (SET tag) of GB1. A TEV cleavage site was inserted between the SET tag and the target protein. The recombinant plasmid was transformed into BL21 (DE3) competent cells for the overexpression of fusion protein. The protein was first purified using Ni^{2+} affinity chromatography. The tag was removed by tobacco etch virus protease. The protein was further purified through gel filtration followed by high-performance liquid chromatography. The protein concentration was determined through UV absorption.

Electrospray Ionization Mass Spectrometry

All mass spectrometry experiments were conducted on an Exactive Plus (Thermo Fisher Scientific, CA, U.S.A.) mass spectrometer. For the cisplatin reactions, 50 μM MDM2-RF, were incubated with different amount of cisplatin in 50 mM ammonium acetate buffer at 37 °C for 8 hours. All samples were diluted to a final solution with 10 μM protein before injection. The positive ion mode was used in the ESI-MS experiments. Data were processed using XCalibur software (version 2.0, Thermo Finnigan).

Fluorescence Measurements

The fluorescence measurements were performed on a RF-5301PC spectrofluorometer (Shimadzu) using a quartz cuvette with a path length of 5 mm. The excitation wavelength was set at 280 nm, and the emission fluorescence spectra were recorded from 290 to 450 nm. The relative intensity of the fluorescence was calculated using the formula $(F - F_S)/ (F_0 - F_S)$, where F_0 is the initial fluorescence, F_S is the final fluorescence of in titration, and F is the fluorescence at the given concentration of metal ions.[1]

Zinc release assay

The Zn^{2+} ions released from MDM2-RF in the reactions of cisplatin were measured by UV-vis in the presence of a zinc dye 4-(2-pyridylazo) resorcinol (PAR). The reactions were performed on 30 μM MDM2-RF with different molar ratios of cisplatin for 8 h at 37 °C in 50 mM phosphate buffer, 50 μM PAR. The spectra were recorded on an Agilent 8453 spectrophotometer equipped with Peltier temperature controller.

Circular Dichroism

Circular dichroism (CD) measurements were performed on a Jasco J-810 CD spectrometer from 280 to 190 nm, flashed with high purity nitrogen. A 1.0 mm path length quartz cuvette (cleaned by distilled water and dried by nitrogen gas) was used in the measurements. All spectra were recorded in a scan speed of 100 $nm \cdot min^{-1}$ with a data pitch of 1 nm. A band width of 1 nm was used with a detector response time of 1 s. Protein samples were prepared to a final concentration of 0.15 mg/ml in 10 mM phosphate buffer. Spectra of buffer were also recorded for the baseline corrections. All measurements were repeated three times.

In Vitro Ubiquitylation Assay

The ubiquitylation reaction was performed in a volume of 20 μL in a buffer of 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, and 1 mM DTT. The reaction mixture typically contained E1 (50 ng), UBE2D2 (100 ng), ubiquitin (5 μg), and 0.5 μM of MDM2-RF for detection of MDM2-RF ubiquitylation. After incubation at 30 °C for 60 min, the reactions were stopped by the addition of SDS-PAGE sample buffer and resolved on 10% SDS-polyacrylamide gels. Ubiquitylation proteins were visualized and evaluated by Western blot using an antibody against ubiquitin for MDM2 auto-ubiquitylation.

References

1. A. Urvoas, M. Moutiez, C. Estienne, J. Couprie, E. Mintz and L. Le Clainche, *Eur. J. Biochem.*, 2004, **271**, 993-1003.

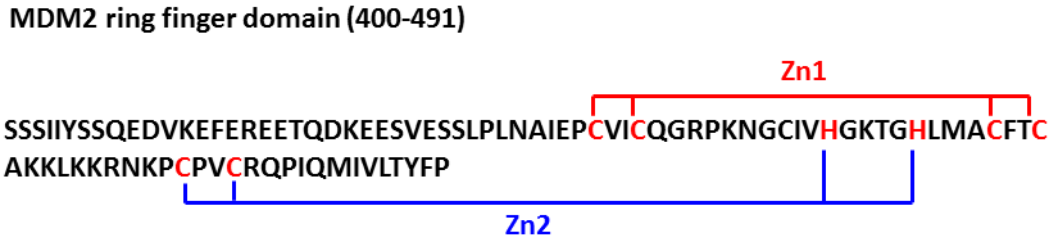

Figure S1. Protein sequences of ring finger domain of MDM2 protein (aa 400-491). The coordination residues to two zinc atoms are highlighted in red.

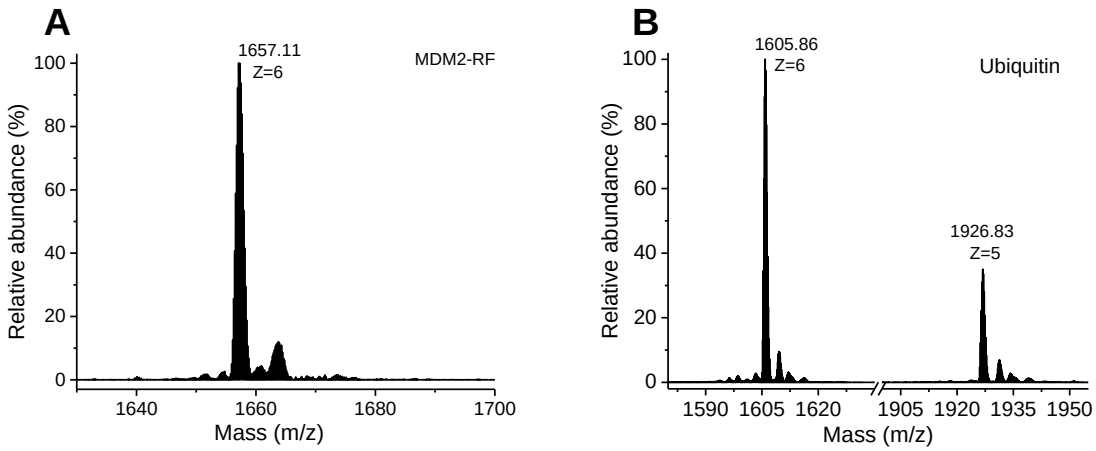

Figure S2. ESI-MS characterization of proteins. **(A)** MDM2-RF, **(B)** Ubiquitin. The m/z values and charges are labelled in spectra.

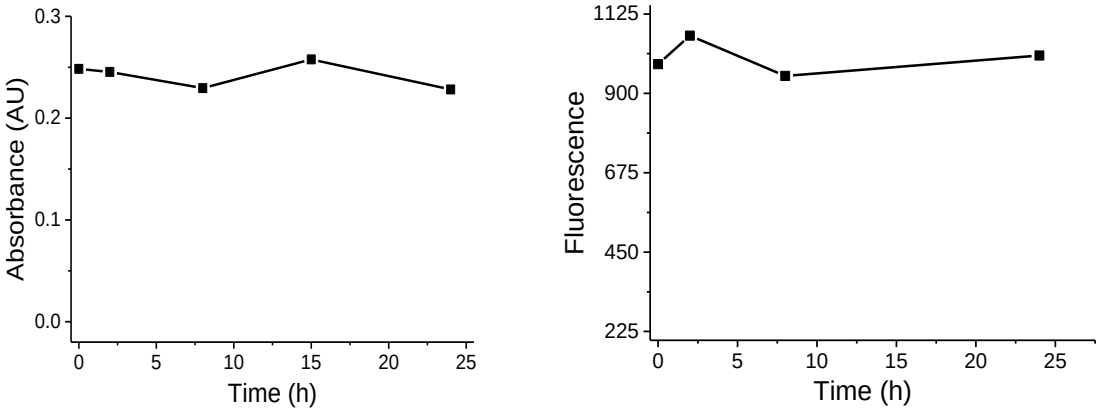

Figure S3. (**A**) Ellman's assay of the thiol content of MDM2-RF in the air. MDM2-RF (5 µM) was incubated with different time at 37 °C. UV spectra were recorded after adding 50 µM DTNB for 10 min. **(B)** Fluorescence measurement of MDM2-RF was incubated with different time in the air. The reactions were performed in 15 µM MDM2-RF, 20mM phosphate buffer.

Table S1. The analysis of ESI-MS peaks detected in Figure S2

Protein	Composition	Formula	m/z (charge)	MW: obsd./cald.	Mass error (ppm)
MDM2-RF	Zn_2- MDM2	$C_{416}H_{678}N_{122}O_{133}S_9Zn_2$	1657.11 (+6)	9936.66/9936.10	56.36
Ubiquitin	Ub	$C_{425}H_{689}N_{125}O_{128}S_1$	1605.86 (+6) 1926.83 (+5)	9629.16/9629.97	-84.11

Table S2. The analysis of the products from the reaction of cisplatin with MDM2-RF

Protein	Composition	Formula	m/z (charge)	MW: obsd./cald.	Mass error (ppm)
MDM2-RF	Zn_2-MDM2	$C_{416}H_{678}N_{122}O_{133}S_9\ Zn_2$	1657.11 (+6)	9936.66/9936.10	56.36
	MDM2+Pt	$C_{416}H_{680}N_{122}O_{133}S_9Pt$	1668.12 (+6)	10002.72/10002.40	31.99
	MDM2+Pt-NH_3	$C_{416}H_{683}N_{123}O_{133}S_9Pt$	1670.95 (+6)	10019.70/10019.43	26.95
	Zn-MDM2+Pt	$C_{416}H_{678}N_{122}O_{133}S_9\ ZnPt$	1678.77 (+6)	10066.62/10065.80	81.46
	Zn-MDM2+Pt-NH_3	$C_{416}H_{682}N_{123}O_{133}S_9\ ZnPt$	1681.75 (+6)	10084.50/10083.82	67.43
	MDM2+Pt_2	$C_{416}H_{678}N_{122}O_{133}S_9\ Pt_2$	1700.28 (+6)	10195.68/10195.50	17.65
	MDM2+Pt_2-NH_3	$C_{416}H_{681}N_{123}O_{133}S_9\ Pt_2$	1703.12(+6)	10212.72/10212.50	21.54