Supplementary Material (ESI) for Chemical Communications

Inhibition of the Histone Lysine Demethylase JMJD2A by Ejection of Structural Zn(II)

Supporting Information

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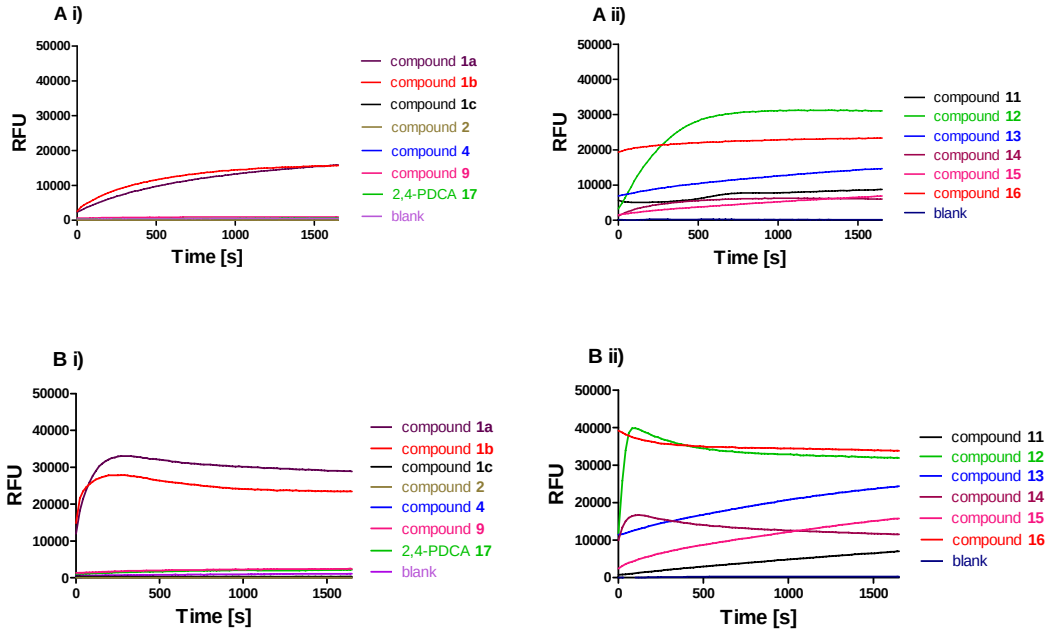
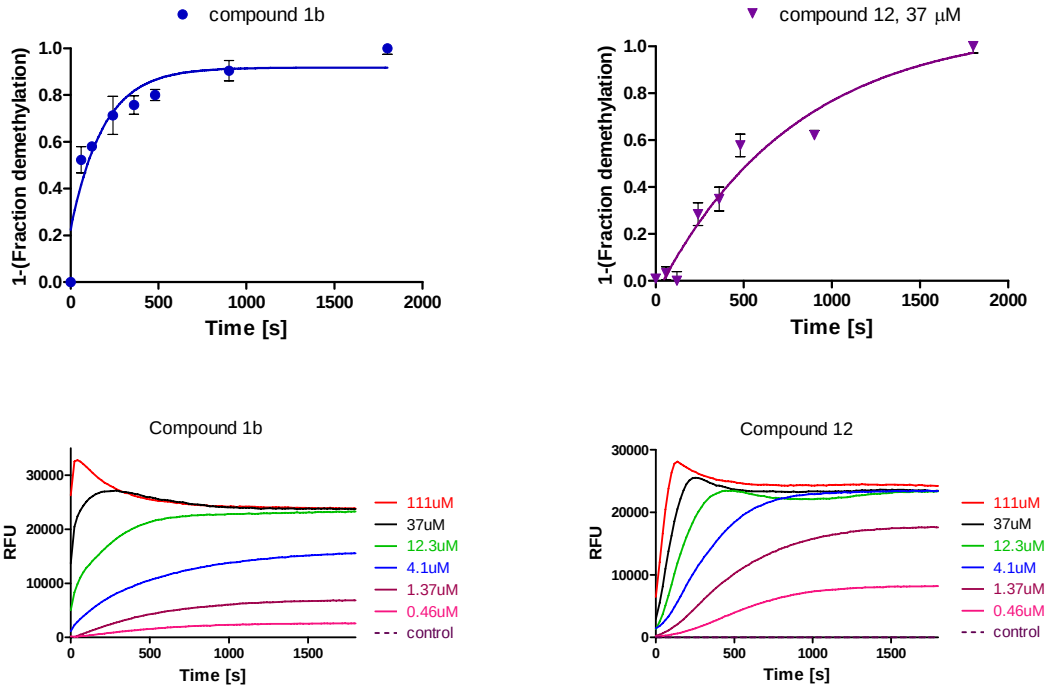
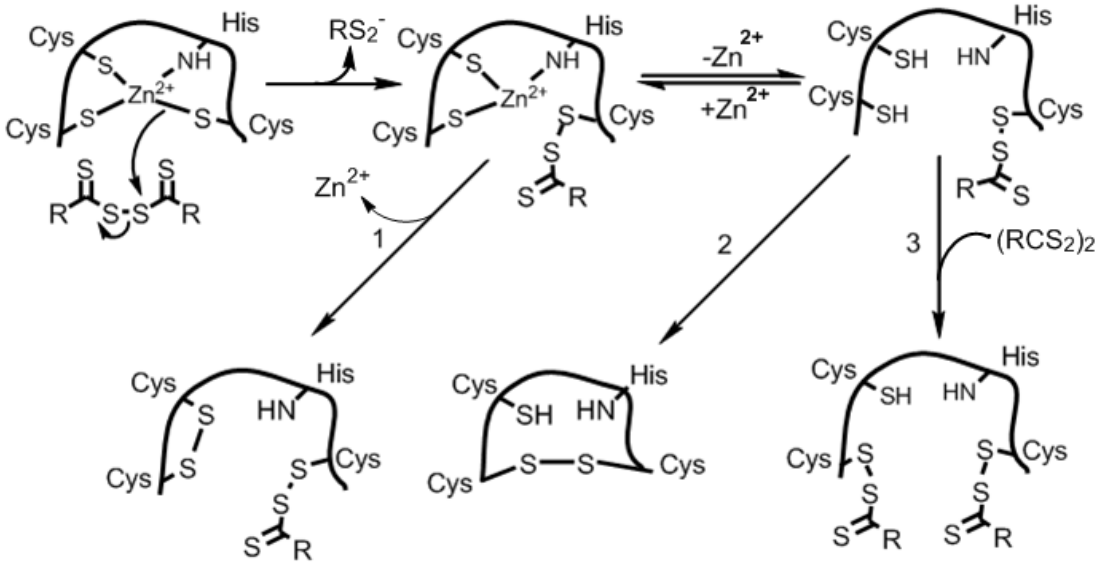
Supporting Figure 1. Kinetic traces of Zn^{2+}-release from JMJD2A upon treatment with Zn-ejectors. *S*-containing compounds (i) and *Se*-containing compounds (ii) at 2 μM (A) and 50 μM (B) dissolved in DMSO (5% v/v) were incubated with JMJD2A (2 μM) in the presence of FluoZin™-3 (1 μM) in 50 mM HEPES, pH 7.5. Linear baseline correction was applied to compensate for temperature-related signal drift.



Supporting Figure 2. Preincubation of JMJD2A with compounds **1b** and **12**. JMJD2A (2 μM) was preincubated with compounds **1b** and **12** (37 μM) for different time intervals (0-1800 s) before the demethylation reaction was initiated by addition of the peptide substrate (10 μM). Reaction was quenched with MeOH after 30 minutes and demethylation analyzed by MALDI-TOF-MS. Experiments were conducted in triplicate. The minimum and the maximum JMJD2A activities observed were normalized to 0 and 1, respectively. Plot of (1 – fraction activity) against time was generated to show dependence of JMJD2A inhibition on the duration of preincubation. Also shown are kinetic traces showing Zn-ejection (measured by FZ-3 assay) by compounds **1b** and **12**; in both the preincubation activity assay and the Zn-ejection assay, compound **1b** has faster kinetics of Zn-ejection/deactivation of JMJD2A.



Supporting Figure 3. Proposed mechanism of Zn-ejection (adapted from [1]). After the initial nucleophilic attack on a sulfur electrophile by a cysteine residue, an internal disulfide bond formation can take place with retention of covalent modification and a loss of Zn^{2+} (1); internal displacement of the covalent modification leading to internal disulfide bond formation (2); after initial covalent modification of one Cys residue, another modification can take place, leading to a doubly modified Zn-binding site (3).



Synthesis of compounds **1b**, **1c**, **2** and **7**

Synthesis of bis(pyrrolidine-thiocarbamoyl) disulfide (compound 1b)

Prepared according to a reported procedure.[2]

m.p. 119-124 °C; IR ν_{max} (KBr): 2870, 1432, 1151, 954 cm^{-1}, ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.02 (app. quin, *J*=7.0 Hz, 2H), 2.16 (app. quin, *J*=7.0 Hz, 2H), 3.97 (app. q, *J*=7.0 Hz, 4H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.2 (CH_2), 26.5 (CH_2), 50.9 (CH_2), 56.9 (CH_2), 189.1 (CS) ppm; HRMS (ESI^+): calcd for $C_{10}H_{16}N_2NaS_4$ ($M+Na^+$), 315.0089; found, 315.0085; Elemental analysis: Calcd. for $C_{10}H_{16}N_2S_4$: C 41.06, H 5.51, N 9.58; found, C 41.11, H 5.63, N 9.64.

Synthesis of bis(dibenzylthiocarbamoyl) disulfide (compound 1c)

Prepared according to a reported procedure.[2]

m.p. 130-131 °C; IR ν_{max} (KBr): 2905, 1452, 1231, 921, 696 cm^{-1}, ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 5.20 (br. s., 4 H, C*H*$_2$), 5.38 (br. s., 4 H, C*H*$_2$), 6.86 - 7.73 (m, 20 H, ArC*H*) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 54.9 (*C*H$_2$), 59.0 (*C*H$_2$.), 127.5 (Ar*C*H), 127.9 (Ar*C*H), 128.3 (Ar*C*H), 128.9 (Ar*C*H), 129.1 (Ar*C*H), 134.2 (Ar*C*H), 134.9 (Ar*C*H), 196.3 (*C*=S) ppm; HRMS (ESI+): calcd for $C_{30}H_{28}N_2NaS_4$ ($M+Na^+$), 567.1028; found, 567.1028; Elemental analysis: Calcd. for $C_{30}H_{28}N_2S_4$: C 66.14, H 5.18, N 5.14; found: C 66.18, H 5.09, N 5.18.

Synthesis of sodium diethylcarbamodithioate (compound 2)

Prepared according to a reported procedure.[2]

m.p. 80-84 °C; IR ν_{max} (KBr): 2978, 2074, 1672, 1615, 1476 cm^{-1}, ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.08 (t, *J*=7.0 Hz, 6H), 3.97 (q, *J*=7.0 Hz, 4H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 12.6 (CH_3), 46.1 (CH_2), 212.0 (CS) ppm; Elemental analysis: Calcd. for $C_5H_{10}NnaS_2$: C 35.07; H 5.89; N 8.18; found, C 35.18; H 5.88; N 8.12.

Synthesis of octane-1,8-dithiol diacetate (compound 7)

Acetic anhydride (0.64 ml, 6.72 mmol) was added dropwise to a solution of 1,8-octanedithiol (0.31 ml, 1.68 mmol), triethylamine (1.17 ml 8.40 mmol) and DMAP (20 mg, 0.17 mmol) in dry DCM (10 ml) at room temperature under an atmosphere of nitrogen. The mixture was stirred for 4 h before the addition of sodium hydrogen carbonate solution (15 ml sat. aq.). The layers were separated and the aqueous phase extracted with DCM (2 × 10 ml). The combined organic extracts were washed with water (30 ml) and brine (30 ml) before being dried over $MgSO_4$ and concentrated *in*

vacuo to give an off-white solid (0.58 g). The crude product was purified by flash chromatography eluting with 1-10% Et_2O/petrol to give diacetate **7** as a white solid (0.43 g, 98%); R_f = 0.59 (4% Et_2O/petrol); mp 37-38 ^oC; ν_{max} (KBr): 2923 (CH), 2854 (CH), 1687 (C=O) cm^{-1}; δ_H (400 MHz, $CDCl_3$) 1.29-1.37 (8H, m, 3-H_2, 4-H_2, 5-H_2 and 6-H_2), 1.56 (4H, br. quint., *J* 7.5, 2-H_2 and 7-H_2), 2.33 (6H, s, 2 × -OAc), 2.86 (4H, t, *J* 7.5, 1-H_2 and 8-H_2); δ_C (100 MHz, $CDCl_3$) 28.7 (CH_2), 28.9 (CH_2), 29.1 (CH_2), 29.4 (CH_2), 30.6 (CH_3), 196.1 (C=O); *m/z* (ESI) 285 ($[MNa]^+$, 100%), 263 ($[MH]^+$, 50%).

Expression and purification of JMJD2A and PHD2 were according to reported methods.[3,4]

Methods for MALDI-TOF MS turnover assay[5]

JMJD2A, disodium 2-OG, ascorbic acid and [ARK(me3)STGGK-NH2] peptide solutions were made up in 50 mM HEPES buffer, pH 7.5. Fe^{2+} solution was prepared from $(NH_4)_2Fe(SO_4)_2$ dissolved in 20 mM HCl to make 400 mM stock solution, which was then diluted to the final concentration using MilliQ water. Together, these were incubated with inhibitor (small molecules were dissolved in DMSO, final in-assay volume was always 5% of total assay mix) for 30 min at 37 °C, before 1:1 quenching with methanol. When DMSO was present in the sample, methanol quenching was followed by addition of four volumes of 20 mM triammonium citrate. 1 μL of the diluted assay mixture was then mixed with 1 μL of recrystallized α-Cyano-4-hydroxycinnamic acid (CHCA, Laser BioLabs) and spotted onto the MALDI-TOF-MS plate before analysis. Pre-incubation experiments were carried out with JMJD2A, Fe^{2+}, ascorbate and inhibitor pre-incubated for 15 min at 37°C, prior to the addition of peptide and 2-OG. Relative proportions of a particular peptide species (i.e. trimethylated, dimethylated or monomethylated peptide) were calculated by taking the ratio of one methylation state's peak intensity in the mass spectrum to the sum of all three methylation states' peak intensities.

$$\%me_3 = 100 \times \frac{I_{me3}}{I_{me3} + I_{me2} + I_{me1}}$$

Percentage demethylation at varied inhibitor concentrations was then used to calculate IC_{50}s for inhibitors.

Methods for FluoZin-3™ Zn ejection assay

A Novostar spectrophotometer (BMG Labtechnologies) was used for measurements of fluorescence intensity of Zn-specific fluorophore FluoZin^{TM}-3. Before each experiment, a calibration curve (between 0-2 μM Zn^{2+}) was obtained with varied concentrations of Zn^{2+} dissolved in MilliQ water. For assays, 1mM solution of FluoZin^{TM}-3 (Invitrogen) was prepared in 50 mM HEPES buffer, pH 7.5, and then diluted in the same buffer to 10 μM stock solution. 10 μL of the stock solution was pre-mixed with 10 μL of 20 μM enzyme and 30 μL buffer to make the enzyme mix. A solution of compound in DMSO (5 μL) was mixed with 45 μL buffer. The compound solution was then mixed with the enzyme-FZ-3 solution to give a total volume of 100 μL, with final concentrations of FluoZin^{TM}-3, enzyme and DMSO of 1 μM, 2 μM and 5%, respectively. Mixing of the enzyme mix and the Zn-ejector solution took place immediately before the plate was inserted into the spectrophotometer for the first reading. The assay plate was shaken automatically for 5 seconds after each reading. Readings were taken for 90 cycles at a rate of 20 seconds per cycle.

Methods for non-denaturing ESI-MS binding assay

JMJD2A was desalted using a Bio-Spin 6 Column (Bio-Rad, Hemel Hempstead, UK) in 300 mM ammonium acetate (pH 7.5). Fe^{2+} solution was prepared as described above. The protein (final concentration 15 μM) was mixed with 1 eq of Fe^{2+} and 10 eq of inhibitor and incubated for 20 minutes at room temperature prior to ESI-MS analysis. Data were acquired on a Q-TOF mass spectrometer (Q-TOF micro, Micromass,Altrincham, UK) interfaced with a Nanomate (Advion Biosciences, Ithaca, NY, USA) with a chip voltage of 1.70 kV and a delivery pressure 0.25 psi (1 psi = 6.81 kPa). The sample cone voltage was either 80 V or 200 V with a source temperature of 40 °C and with an acquisition/scan time of 10s/1s. Calibration and sample acquisition were performed in the positive ion mode in the range of 500–5,000 *m*/*z*. The pressure at the interface between the atmospheric source and the high vacuum region was fixed at 6.60 mbar. Data were processed with MASSLYNX 4.0 (Waters).

Methods for Differential Scanning Fluorimetry[6]

DSF was used to determine T_m values for JMJD2A in the presence of small molecule Zn ejectors. It was performed using MiniOpticon™ Real-Time PCR Detection System (Bio-Rad). SYPRO orange (Invitrogen) dye was used for unspecifing binding to hydrophobic residues and its increase in fluorescence monitored as a function of time. For measurements of the effect of small molecules on the stability of proteins, compounds were dissolved to appropriate concentration in DMSO, such that the final volume of DMSO in a sample was 2.5 μL (5%, v/v); 2.5 μL compound in DMSO, 10 μL of 20 μM protein, 10 μL of 5x SYPRO orange (diluted 1:1000 in 50 mM HEPES buffer from the stock solution in DMSO supplied) and 27.5 μL buffer were mixed on ice. The chamber was cooled to 4 °C before inserting the sample plate and the readings of fluorescence were taken between 4-95 °C, increasing the temperature linearly for 30 minutes. FAM (492 nm) and ROX (610 nm) filters were used for excitation and emission, respectively. Software automatically performed global minimum subtraction. The T_m is calculated using the Boltzmann equation $y = LL + \frac{UL - LL}{1 + e^{\frac{T_m - x}{a}}}$, where LL and UL are values of minimum and maximum intensities and *a* corresponds to the slope of the curve within T_m. All measurements were performed in triplicate.

References

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