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Supplementary Information

A Photo-Responsive Chemical Modulation of m^{6}A RNA demethylase FTO

Ling Lan,[†] Ying-Jie Sun,[†] Li Liu and Liang Cheng*

Beijing National Laboratory for Molecular Sciences (BNLMS), CAS Key Laboratory of Molecular Recognition and Function, CAS Research/Education Center for Excellence in Molecular Sciences, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China and University of Chinese Academy of Sciences, Beijing 100049, China

[†] The first two authors contributed equally to the manuscript.

chengl@iccas.ac.cn

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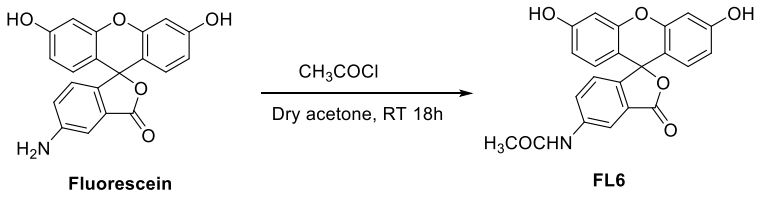
1. Procedures for the synthesis of FL6 and FL6-1/2

All reagents were commercially available. NMR spectra were recorded on Bruker AVANCE 300 MHz and 500 MHz. All chemical shifts (δ) are quoted in ppm relative to residual solvent for ^{1}H-NMR and relative to internal resonance for ^{13}C NMR. ESI-MS were recorded on Thermo Fisher Scientific (Exactive).

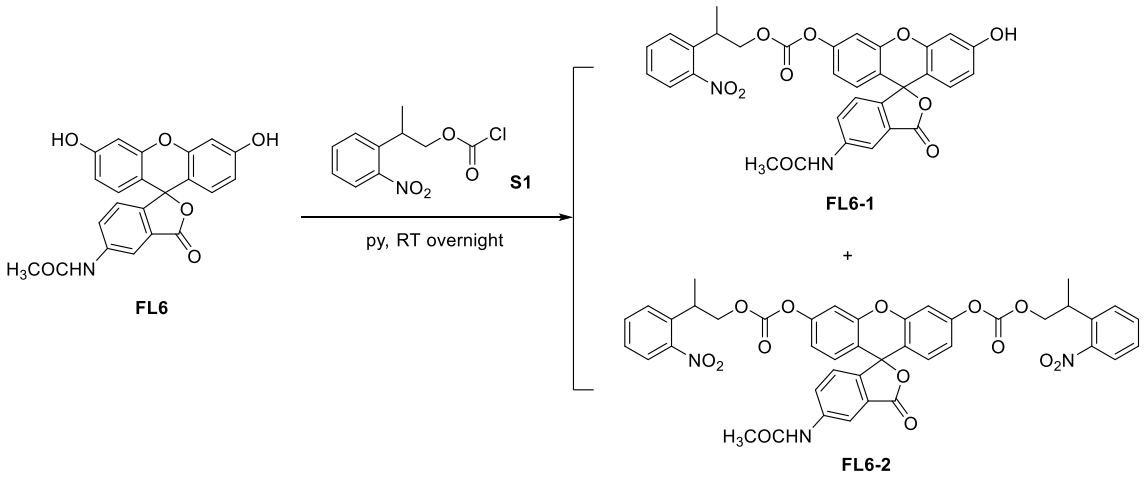
1.1 Compound FL6



Fluorescein **FL6**

To a solution of fluorescein (347 mg, 1 mmol) in anhydrous acetone (5 mL) , then acetyl chloride (290 µL, 4 mmol) was added under nitrogen protection, following an orange solid precipitated out gradually. After stirring for 18 h at room temperature, the solid was filtered out and spin-dried to obtain the product **FL6** (339 mg, 87.5%). **FL6**: ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.57 (s, 1H), 8.34 (s, 1H), 7.85 (d, J = 8.4 Hz, 1H), 7.21 (d, J = 8.4 Hz, 1H), 6.73 (s, 2H), 6.68-6.55 (m, 4H), 2.13 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.5, 169.1, 160.4, 152.6, 141.4, 129.7, 127.5, 126.5, 125.1, 114.0, 113.4, 110.4, 102.7, 24.5. HRMS calculated for $C_{22}H_{15}NO_6$ $[M+H]^+$: 390.0969, found: 390.0972.

1.2 Compound FL6-1 and FL6-2



FL6 (194.5 mg, 0.5 mmol) and **S1** (182 mg, 0.75 mmol) were dissolved in dry pyridine (5 mL). The solution was

then stirred overnight at room temperature. After stopping the stirring, spined the pyridine to dryness. The residue was separated by column chromatography (dichloromethane: ethyl acetate=20:1-10:1) to obtain a light yellow solid **FL6-1** (120 mg, 40.3%) and white solid **FL6-2** (80 mg, 19.9 %).

FL6-1: ^{1}H NMR (500 MHz, DMSO-*d*$_6$): δ 10.44 (s, 1H), 10.20 (s, 1H), 8.35 (s, 1H), 7.86-7.80 (m, 2H), 7.76-7.70 (m, 2H), 7.51 (t, *J* = 7.2 Hz, 1H), 7.26 (d, *J* = 8.5 Hz, 2H), 6.93-6.85 (m, 2H), 6.72 (d, *J* = 1.9 Hz, 1H), 6.66-6.58 (m, 2H), 4.48-4.39 (m, 2H), 3.60-3.56 (m, 2H), 2.13 (s, 3H). 1.33 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-*d*$_6$): δ 169.5, 169.0, 160.2, 152.7, 152.2, 152.0, 151.6, 150.6, 146.8, 141.6, 136.5, 133.5, 129.8, 129.7, 129.0, 128.5, 127.0, 126.8, 124.9, 124.3, 117.8, 117.6, 113.8, 113.6, 110.2, 109.7, 102.7, 82.3, 72.6, 33.2, 24.6, 18.0. HRMS calculated for $C_{32}H_{24}N_2O_{10}$ $[M+H]^+$: 597.1504, found: 597.1500.

FL6-2: ^{1}H NMR (500 MHz, DMSO-*d*$_6$): δ 10.47 (s, 1H), 8.41(s, 1H), 7.87-7.70 (m, 7H), 7.51 (t, *J* = 7.8 Hz, 2H), 7.33-7.31 (m, 3H), 7.00-6.93 (m, 4H), 4.50-4.40 (m, 4H), 3.59 (q, *J* = 6.5 Hz, 2H), 2.14 (s, 3H), 1.33 (d, *J* = 6.9 Hz, 6H). ^{13}C NMR (125 MHz, DMSO-*d*$_6$): δ 169.5, 168.8, 152.64, 152.61, 152.4, 151.2, 150.6, 146.7, 141.8, 136.5, 133.5, 129.9, 129.0, 128.5, 126.9, 126.7, 125.0, 124.3, 118.3, 117.1, 114.0, 110.3, 81.1, 72.6, 55.4, 33.2, 24.6, 18.0. HRMS calculated for $C_{42}H_{33}N_3O_{14}$ $[M+H]^+$: 804.2035, found: 804.2027.

2. FTO protein expression and purification

The expression and purification of $FTO_{\Delta N31}$ (encoding a His-tag fusion human FTO protein with *N*-terminal 31 residues truncated) was modified from previously reported methods[1]. *E.coli* BL21 (DE3) cells transformed with the pET28a-$FTO_{\Delta N31}$ plasmids (gifted from Jia Guifang's research group at Peking University) were grown at 37 °C to OD_{600} = ~1.0 and induced by 0.5mM Isopropyl β-D-thiogalactopyranoside at 18 °C for 20 hours. The cell pellets were harvested and stored at −80 °C. The cells were resuspended and sonicated in Ni-IDA binding buffer (20 mM Tris-HCl, pH = 8.0, 500 mM NaCl, 5 mM imidazole). The lysate was centrifuged and the supernatant was loaded onto a 5 mL Ni-IDA-Sepharose CL-6B affinity chromatography column at a flow rate of 0.5 $mL \cdot min^{-1}$ using Biologic LP protein purification system. The column was allowed to reach equilibrium with Ni-IDA binding buffer and eluted with Ni-IDA washing buffer (20 mM Tris-HCl, pH = 8.0, 500 mM NaCl, 10-50 mM imidazole). The fractions were diluted and eluted with Ni-IDA washing buffer (20 mM Tris-HCl, pH = 8.0, 500 mM NaCl, 250 mM imidazole) at a flow rate of 1 $mL \cdot min^{-1}$. The combined protein fractions were loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for purity analysis. Finally, were high purity of FTO protein was obtained and concentrated to 1 $mg \cdot mL^{-1}$ for further bioassays.

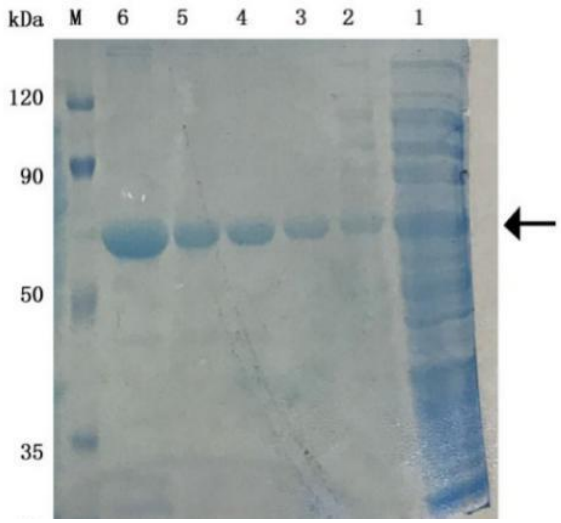


Figure S1. Gel electrophoresis of purified recombinant FTO protein. Lanes 2-6 represented 5 mM, 10 mM, 20 mM, 50 mM and 250 mM imidazole Ni-IDA elution buffer, respectively.

3. Determining the photolysis of photocaged FL6-2

Analysis of samples after 365 nm irradiation was performed with Waters ACQUITY Arc HPLC instrument (2998 PDA Detector). The standard samples of **FL6**, **FL6-1** and **FL6-2** were resolved in methanol. The experiment was conducted by periodically irradiating the **FL6-2** (30 µM) in methanol at λ = 365 nm using a LED UV lamp (180 $mW \cdot cm^{-2}$) positioned 3 cm from the sample tubes, prior to HPLC analysis. The injection volume was 40 µL. Chromatographic separation of **FL6-2** samples irradiated by UV was done with ACQUITY 5 TC-C_{18} column (5-µm particle size, 4.6 mm × 250 mm). The mobile phases (delivered at 1.0 $mL \cdot min^{-1}$) consisted of H_2O for A and methanol for B. Isocratic elution for 10 minutes at 20% A. Retention times of monitored standard **FL6**, **FL6-1** and **FL6-2** were 2.5, 3.8 and 8.8 minutes respectively.

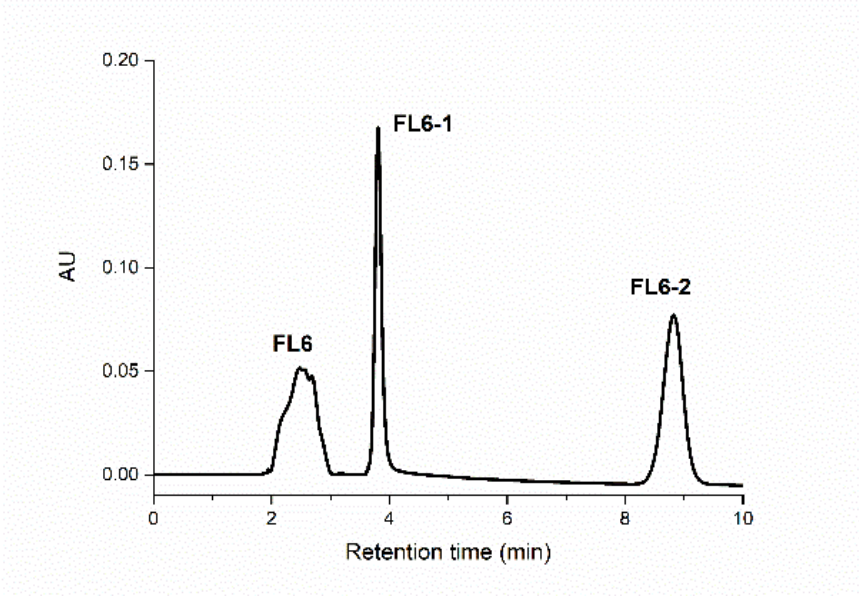
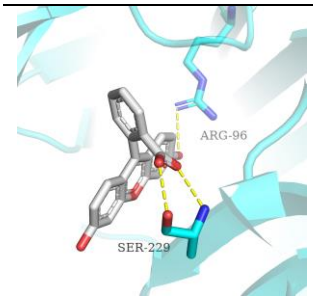
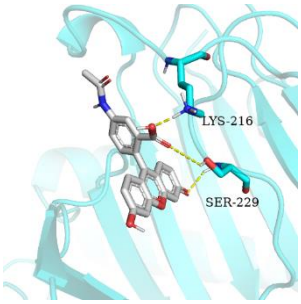


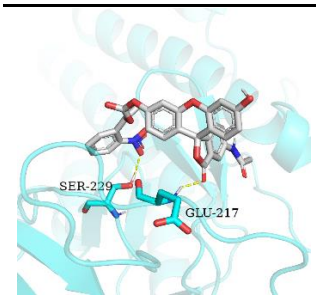
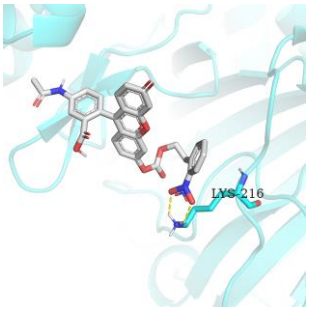
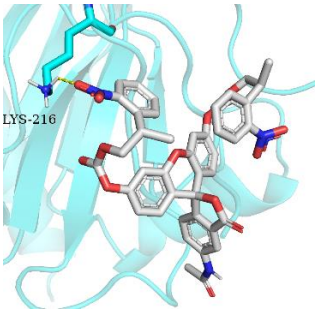
Figure S2. HPLC analysis of **FL6**, **FL6-1** and **FL6-2** standards, the concentration of all compounds is 30 µM in methanol.

4. Molecular docking for compounds binding at the FTO Active Site

Docking studies was performed to check the binding ability of photocaged compounds **FL6-1**/**FL6-2** and the inhibitor **FL6** in FTO protein. AutoDock 4.2 (The Scripps Research Institute, Molecular Graphics Laboratory)[2] was used to find out binding sites and binding energies of ligands to the receptor. In order to clarify the specific binding site of inhibitor **FL6** in FTO binding pocket, we also selected the crystal structure of FTO/fluorescein **FL4** complex (PDB: 4ZS2) to extract the protein as the receptor. The receptor (FTO) and the ligands (compounds **FL6**, **FL6-1** and **FL6-2**) PDBQT files were prepared using AutoDock Tools 1.5.6 (The Scripps Research Institute, Molecular Graphics Laboratory)[2]. The solvent water molecules of receptor were deleted and polar hydrogen were added. All other bonds were allowed to be rotatable. The binding site was surrounded with a grid-box sized 126 × 126 × 126 points with a grid spacing of 0.375 Å. The AutoDock4.2 force field was used in all molecular docking simulations and the Lamarckian Genetic Algorithm (LGA) was chose. All calculations were performed on an Intel Core™ 2 based machine running Windows® XP as the operating system. The model and the diagram were generated with PyMOL (The PyMOL Molecular Graphics System, Version 2.3.2, Schrödinger, LLC) molecular graphics software.

Table S1. Closed view of the FTO-fluorescein crystal complex and different docking modes of compounds **FL6**/**FL6-1**/**FL6-2** with FTO.

Modes	Residues	ΔG (kcal/mol)
 (FTO-**FL4**, PDB: 4ZS2)	ARG96 SER229	—
 (FTO-**FL6**)	LYS216 SER229	-5.45

 (FTO-**FL6-1**)	GLU217 SER229	-5.68
 (FTO-**FL6-1**)	LYS88 LYS216	-5.88
 (FTO-**FL6-2**)	LYS216	-2.45

5. HPLC-based assay of the inhibition of m^6A demethylation in RNA

Based on the published protocol[1, 3], reactions were typically were set up to quantitatively verify compounds inhibition of FTO demethylation activity. The solution of reactions (100 μL) contained 10 μM ssRNA (5'-CUGG(m^6A)CUGG-3') and 2 μM FTO protein in 50 mM HEPES, pH=8.0, 300 μM 2OG, 280 μM $(NH_4)_2Fe(SO_4)_2$, 2 mM L-ascorbic acid, 0.05 mg/mL BSA and serial concentrations of compounds (DMSO: water=1:1000). The samples treated by **FL6-2** were immediately irradiated at 365 nm UV light (180 mW•cm^{-2}) for 10 min after mixing, while the others were kept in dark. After incubation at 25°C for 3 h, the reaction was quenched by adding 0.5 μL EDTA (0.5 M) and heating for 5 min at 95°C.

The sample is divided into two parts, one part is directly used to HPLC analysis of RNA, and the other part is subjected to nucleoside analysis after digesting. The RNA strands were analyzed on an Agilent HPLC system equipped with Waters Sunfire® C18 column (5-μm particle size, 4.6 mm × 150 mm). The mobile phase consisted of acetic acid-triethylamine buffer (100 mM, pH=7.0~7.2) for A and Acetonitrile for B. The detection wavelength was set at 260 nm. Isocratic elution for 15 minutes at 92% A at a flow rate was 1 mL/min.

Another of reaction mixtures was digested by nuclease P1 and alkaline phosphatase. The digestion nucleosides were analyzed on an HPLC system equipped with an ACQUITY 5 TC-C18 column (5-μm particle size, 4.6 mm × 250 mm) setting mobile phase of buffer A (water contained 0.01% formic acid) and buffer B (methanol contained 0.01% formic acid) at a flow rate of 1 mL/min at room temperature. The detection wavelength was set at 266 nm. Gradient elution for 30 min. IC_{50} value for FTO inhibition by **FL6** was also calculated by using this assay in triplicate. The data was processed by Origin9.1.

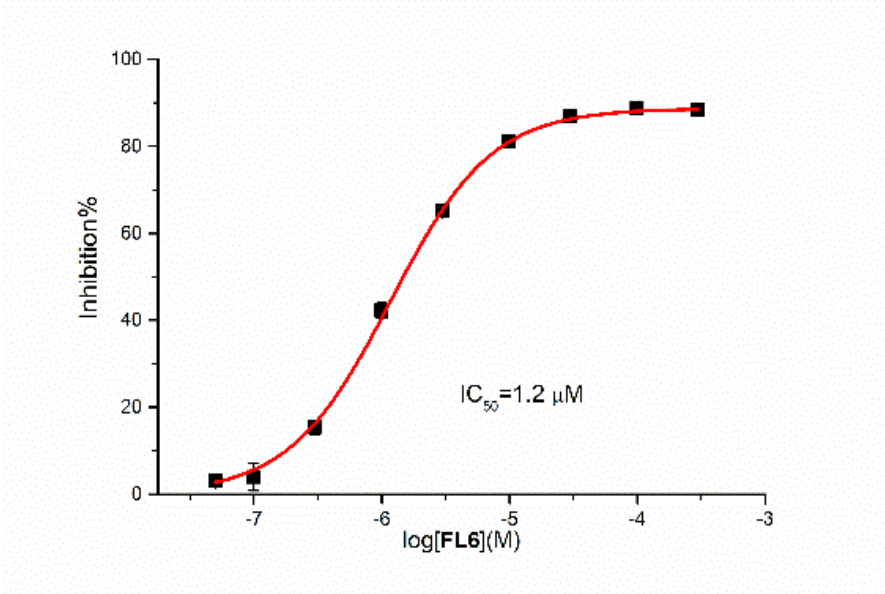


Figure S3. The IC_{50} between inhibitor **FL6** (0.1% DMSO) and recombinant FTO protein based on the substrate of m^6A-ssRNA. m^6A-ssRNA: 5′-CUGG(m^6A)-CUGG-3′. [FTO] = 2 μM, [m^6A-ssRNA] =10 μM, [**FL6**] =0, 0.3, 1, 3, 10, 30, 100, 300, 1000 μM.

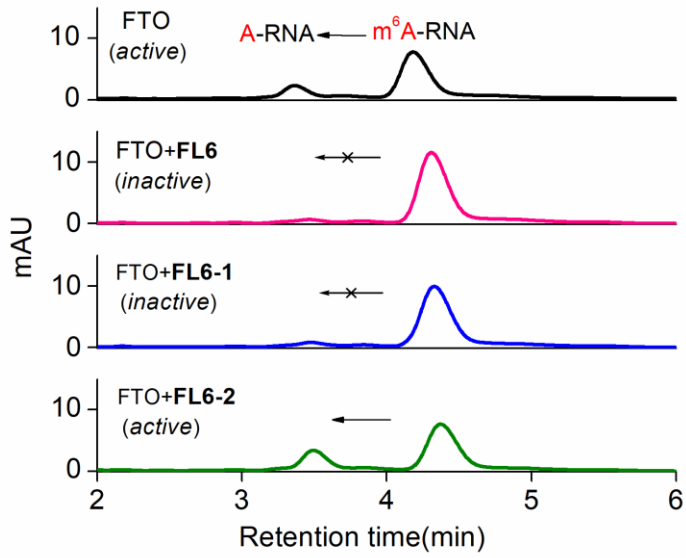


Figure S4. Analysis of the inhibitory activity of modified or unmodified **FL6** in the process of FTO demethylation, respectively. [**FL6** or **FL6-1** or **FL6-2**] = 30 μM, [FTO] = 2 μM, [m^6A-ssRNA] =10 μM.

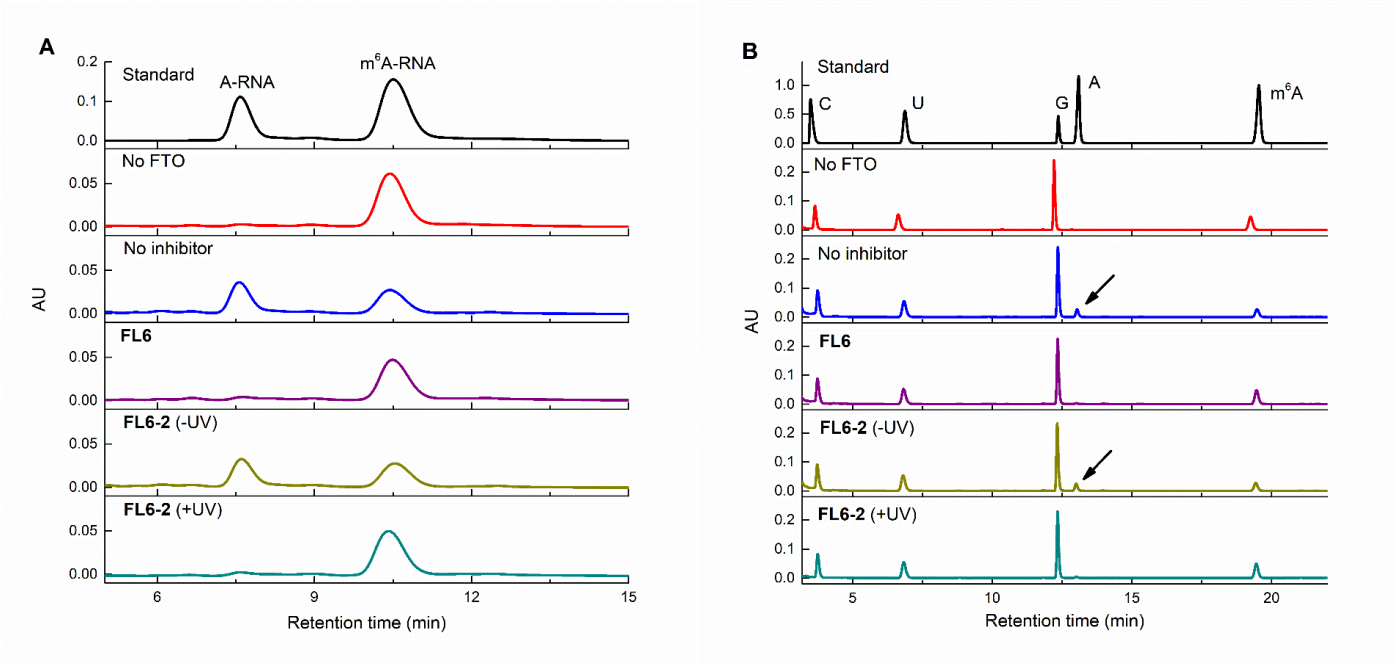


Figure S5. HPLC analysis of (A) FTO demethylation on m^6A in ssRNA and (B) digested mixtures corresponding to ssRNA in different reaction conditions, respectively. [**FL6** or **FL6-2**] = 30 μM, [FTO] = 2 μM, [m^6A-ssRNA] =10 μM.

6. Inhibition of m^6A demethylation in Cells

Human HeLa was routinely grown in a humidified incubator at 37 ^oC with 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco, C11995500BT) supplemented with 10% Fetal Bovine Serum (Gibco, 10099-141C) and 1% penicillin/streptomycin (Solarbio). Cells were seeded at a density of 7×10^6 cells into 10 cm cell culture dishes. 24 hours after plating, cells were treated in fresh medium with the photocaged **FL6-2** or uncaged **FL6** at 30 μM. Cells treated by 0.1% DMSO as a negative control. After incubation for 4 hours, following the addition of fresh medium and treatment, one set of **FL6-2** plates were irradiated for 10 minutes using 365 nm UV light (25 $mW \bullet cm^{-2}$) at 25°C, then incubated for additional 12 hours at 37°C for dot blot assay. The remaining set of plates served as control and were kept in the dark. Total RNA was separated with TRIzol Reagent (Invitrogen, 15596026) in accordance with the manufacturer's instructions. Then, mRNA was extracted from total RNA using PolyATtract® mRNA Isolation System (Promega, Z5310), followed by further removing of contaminated rRNA using NEBNext Globin & rRNA removal Kit (NEB, E7755L). The concentration and purity of mRNA were measured with Nanodrop (Thermo scientific).

7. m^{6}A dot blot assay

The mRNA samples were diluted in nuclease-free water, denatured at 95 °C for 3 minutes and placed on ice. Then 1 μL of the RNA samples was dotted onto a positively charged nylon membrane (Millipore, INYC00010). The RNA samples were cross-linked onto the membrane *via* UV irradiation at 254 nm (CL-1000 Ultraviolet crosslinker, 120 mJ/cm^2, 3 minutes). After UV crosslinking, the membrane was blocked with 5% nonfat dry milk for 1 hour at room temperature and incubated with rabbit-anti-m^{6}A antibody (Abcam, 151230, 1: 1000) at 4 °C overnight. Finally, the membrane was washed with 1 × PBST buffer 3×, then incubated with the HRP-conjugated goat anti-rabbit IgG (Abbkine, A21020, 1:4000) and developed with ECL Plus Western Blotting substrate (Solarbio, PE0010). The luminescence signal was detected with the CCD imaging system (Tanon-5200Multi). The membrane was stained with 0.1 % methylene blue (MB) as loading control. The image results were analysis by ImageJ [4].

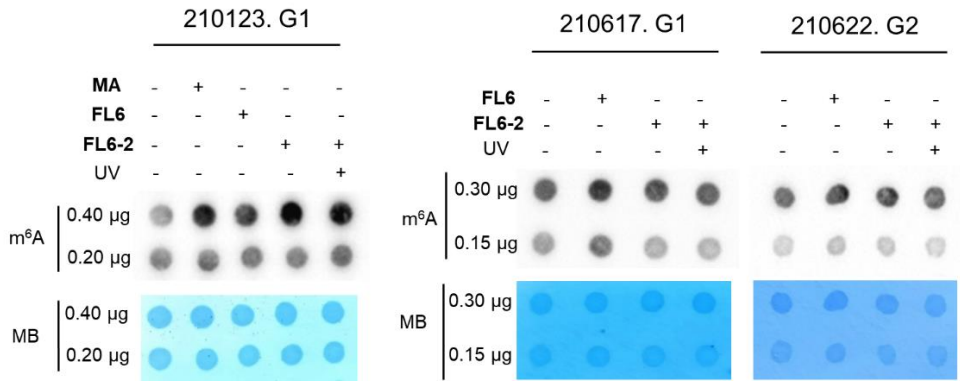


Figure S6. Determination of m^{6}A abundance in mRNA samples of HeLa cells treated with **FL6** or **FL6-2** (30 μM) before and after UV light *via* dot blot assay. Meclofenamic acid (MA, 20 μM) as a positive control was used in the 210123.G1 experiment.

8. Cell fluorescence imaging and flow fluorescence analysis

HeLa cells incubated with small molecules **FL6** (30 μM) and **FL6-2** (30 μM) for 4 hours were washed three times with PBS buffer and placed under a high-resolution fluorescence imaging system (Eclipse Ti-E) for imaging. FITC channels were used for image capture. HeLa cells treated with 0.1% DMSO for the same time served as a control. At the same time, flow cytometric fluorescence detection was also conducted on flow cytometer (CyoFlex, B2-R0-V2) to verify the results of fluorescence imaging and m^6A dot blots.

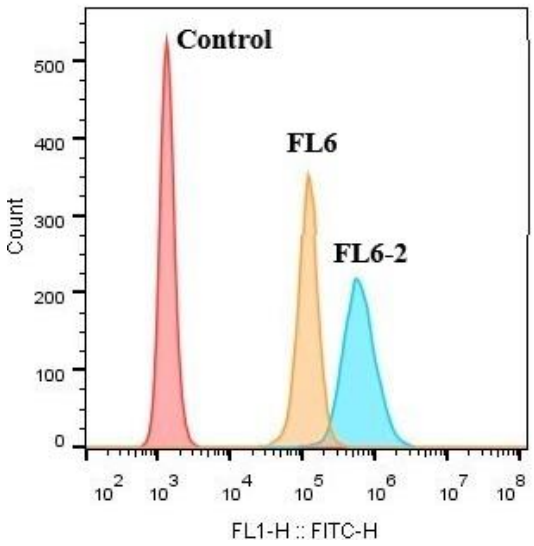
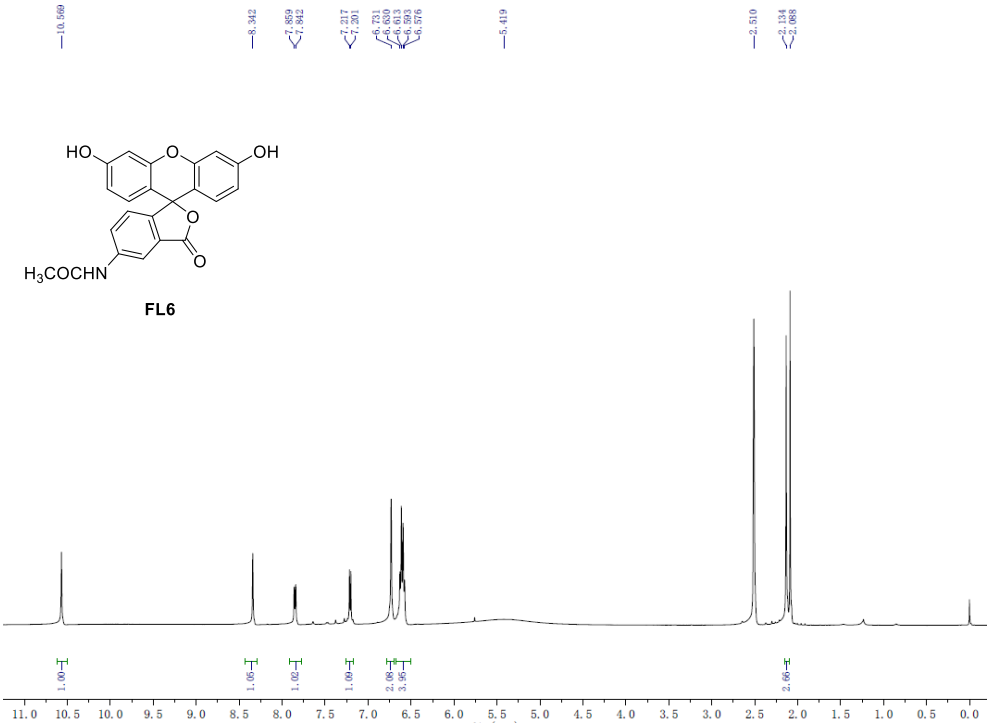
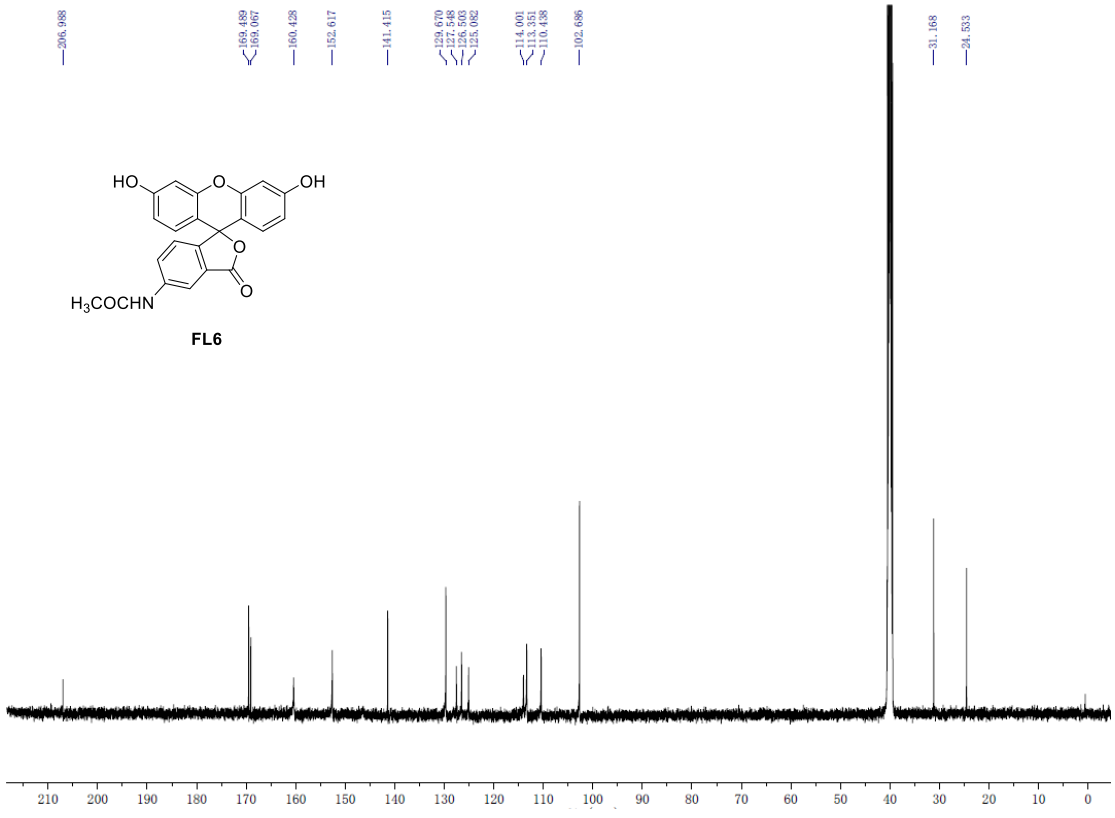


Figure S7. Flow cytometric fluorescence detection of HeLa cells incubated with small molecules for 4 hours. Control (0.1%DMSO), **FL6** (30 μM), **FL6-2** (30 μM)

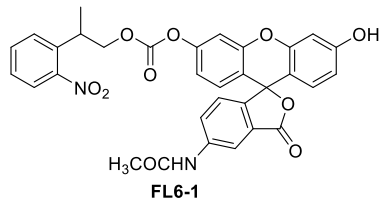
9. NMR spectra



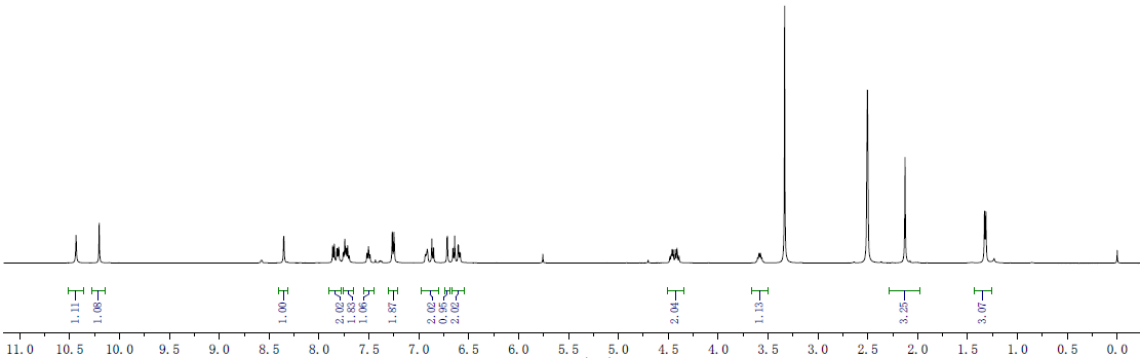
FL6

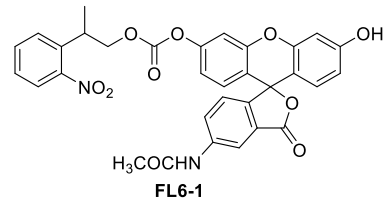


FL6

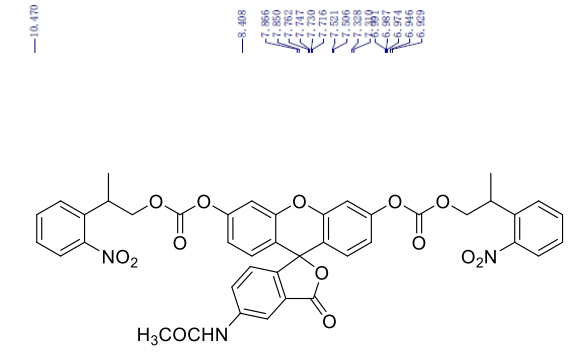


FL6-1

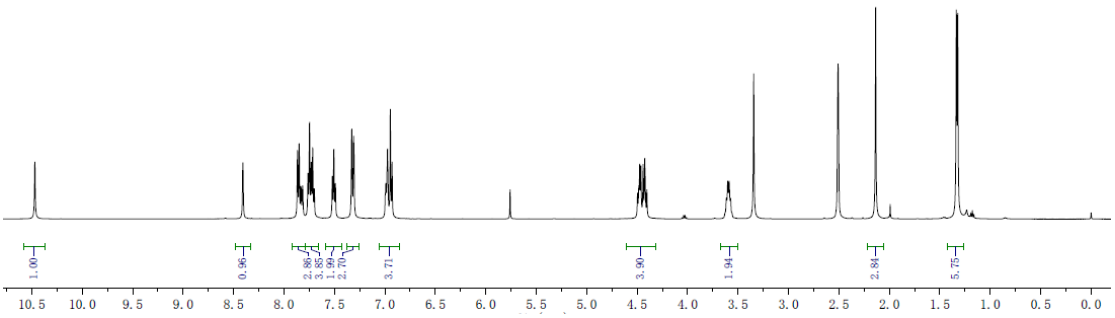


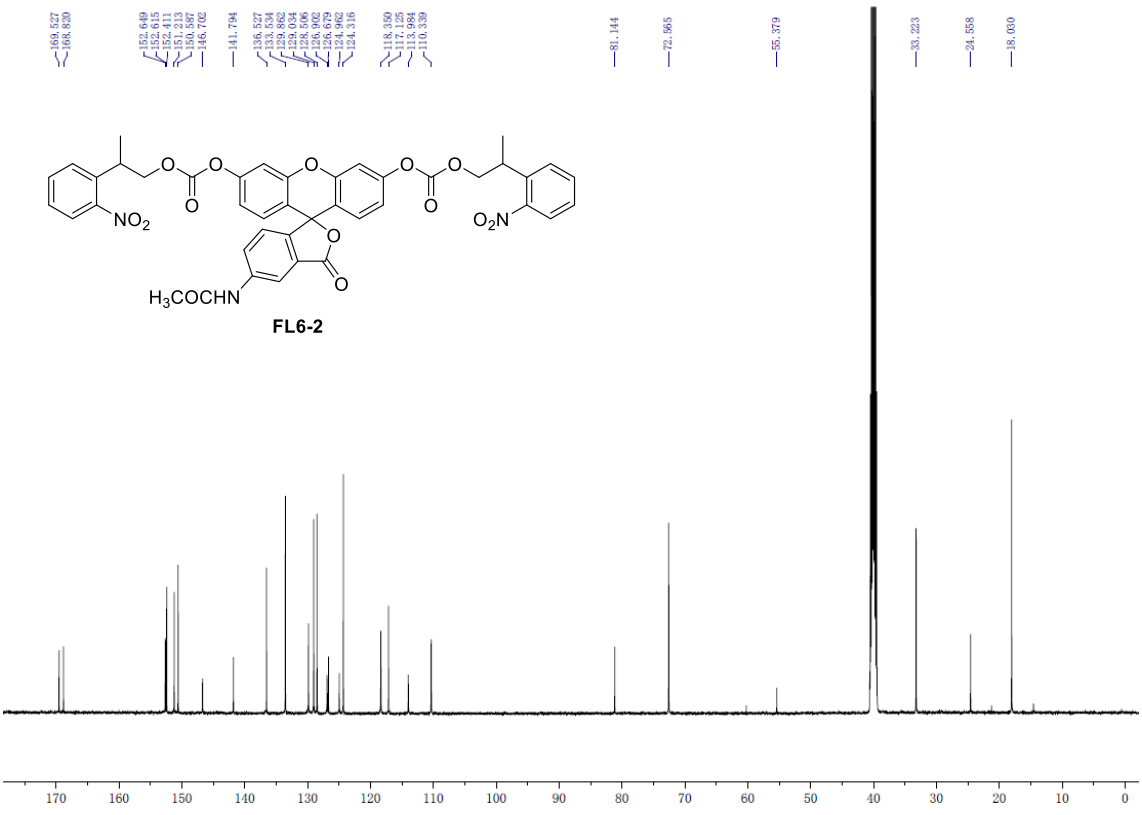


FL6-1



FL6-2





FL6-2

10. References

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