Electronic Supplementary Material (ESI) for ChemComm.

Xanthine derivatives inhibit FTO in an L-ascorbic acid-dependent manner

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SUPPORTING INFORMATION

Materials and Methods

Chemicals

Compounds used in this study are listed as bellow.

1	(±)-Lisofylline	Cayman Chemical	10010785
2	8(p-Sulfophenyl)theophylline	Santa Cruz	SC-217511
3	3-Isobutyl-1-methylxanthine	Sigma-Aldrich	I5879
4	3.7-dimethyl-1-propargyl xanthine	Santa Cruz	sc-209732
5	Pentoxifylline	TCI Chemicals	P2050
6	Pentifylline	Santa Cruz	TX75220
7	1-heptyl-3,7-dimethyl-3,7-dihydro-1H-purine-2,6-dione	Vitas M Chemical	STK394191
8	Propentofylline	Enzo Life Science	Screen-Well Neurotransmitter Library
9	Theophylline	Fujifilm Wako Pure Chemical	209-09932
10	Caffeine	Sigma-Aldrich	C0750

Purification of FTO

E. coli BL21 (DE3) transformed with the pET28b_hFTO encoding His- and Strep-tags at N- and C-terminus of human FTO, respectively, were grown at 37℃, 200 rpm to an OD_{600} of 0.8. FTO expression was induced by addition of 0.1 mM Isopropyl-β-D (−)-thiogalactopyranoside (IPTG), and cells were incubated at 18℃ 100 rpm overnight. Cells were collected by centrifugation at 4℃, 5,000 g and stored at −80℃. Cell pellets were thawed and resuspended in His A buffer (Table 1). The suspension was lysed by sonication on ice. The lysate was cleared by centrifugation and followed by filtering of the supernatant with a 0.45 μm filter. The filtered supernatant was loaded on a 1 mL HisTrap FF (Cytiva) which was equilibrated with His A buffer and FTO was eluted with His B buffer. For the second step of purification, the elution was loaded on 1 mL StrepTrapXT (Cytiva) which was equilibrated with Strep A buffer and FTO was eluted with Strep B buffer. FTO was concentrated with ultrafiltration (Amicon Ultra, 10 kDa NMWL; Merck Millipore) and buffer-exchanged into 25 mM Tris-HCl (pH 7.4), 1 mM DTT.

Table 1: Buffers used for FTO purification

Buffer	composition
His tag A buffer	20 mM $NaH_2PO_4 \cdot 2H_2O$, 500 mM NaCl, 40 mM Imidazole, 1mM DTT, pH 7.4
His tag B buffer	20 mM $NaH_2PO_4 \cdot 2H_2O$, 500 mM NaCl, 250 mM Imidazole, 1mM DTT, pH 7.4
Strep tag A buffer	100 mM Tris-HCl, 150 mM NaCl, pH 8.0
Strep tag B buffer	100 mM Tris-HCl, 150 mM NaCl, 50 mM biotin, pH 8.0

Demethylation reaction

M6A demethylase (400 nM FTO or 1.5 μM ALKBH5) was added into demethylation buffer (25 mM Tris-HCl (pH 7.4), 50 μM α-ketoglutarate, 25 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 300 μM L-ascorbic acid), followed by incubation on ice for 5 min and at room temperature for 15 min. Subsequently, synthesized ssDNA/RNA chimera probe (5′ FAM- CAT r(GGm6ACA) TATGT -3′ BHQ-1) was added and the reaction buffer was incubated for 1 hr at 25℃ and heated to stop the reaction.

FRET-based MazF cleavage assay

A part of the demethylation reaction buffer was added to MazF reaction buffer and measured the fluorescence intensity as described in a previous paper (M. Imanishi, S. Tsuji, A. Suda and S. Futaki, *Chem Commun*, 2017, **53**, 12930-12933).

Screening

Z′ factor under 400 nM FTO condition was calculated as the equation shown below.

$$Z' = 1 - {(3 \times SD_{100\%} + 3 \times SD_{0\%})}\Big/{(Mean_{100\%} - Mean_{0\%})}$$

$SD_{100\%}$: standard deviation of fluorescence intensity with 400 nM FTO, $SD_{0\%}$: standard deviation of fluorescence intensity without FTO, $Mean_{100\%}$: mean of fluorescence intensity with 400 nM FTO, $Mean_{0\%}$: mean of fluorescence intensity without FTO

Four compound libraries (Screen-Well Fatty Acid Library, Screen-Well Neurotransmitter Library, Screen-Well Endocannabinoid Library and Screen-Well Bioactive Lipid Library; Enzo Life Science) were utilized in this screening. At first, 100 μM of each compound was added into the demethylation reaction buffer and its demethylation efficiency was evaluated. Those compounds which showed >50% FTO inhibition were further evaluated for FTO inhibitory activity at 50 and 20 μM, except for compounds that inhibited MazF activity.

Calculation of IC_{50}

FTO inhibitors were serially diluted and added into demethylation buffer as referred above. The demethylation reaction buffer was incubated for 5 min on ice and for 15 min at room temperature. Subsequently, the demethylation reaction was conducted and stopped as referred above. The results were analyzed by Kaleidagraph (Synergy software, ver.4.5.2) and IC_{50} value was calculated as shown.

$$A = A_0 + \left\{ \frac{A_\infty - A_0}{1 + (\frac{[C]}{IC_{50}})^n} \right\}$$

[C]: compound concentration, A: fluorescence intensity, A_0: fluorescence intensity without FTO, A_∞: fluorescence intensity without FTO inhibitors, n: Hill coefficient

To estimate competitiveness of FTO inhibitors against cofactors, the concentration of cofactors was changed in the range under which FTO showed a similar activity.

Preparation of methylated DNA

1.2 μg plasmid DNA (pCI: Promega) was methylated by 12 U CpG methyltransferase (NEB) in methylation buffer containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, 100 μg/mL BSA and 160 μM S-adenosylmethionine at 37°C for 1 hr. The reaction was stopped by treating at 65°C for 20 minutes. The methylated plasmid was collected with a column (Wizard SV minicolumn: Promega).

Evaluation of demethylation activity of TET1

100 μM of **1**, **5**, or **6** was added into TET1 demethylation buffer (50 mM Tris-HCl(pH 7.5), 50 μM α-ketoglutarate, 25 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 300 μM L-ascorbic acid and 5 ng TET1 (Epigentek)) and the buffer was pre-incubated for 5 min on ice and for15 min at room temperature. After addition of 1.2 μg methylated plasmid DNA, samples were incubated at 37°C for 1 hr for demethylase reaction and then incubated at 65°C for 20 minutes to stop the reaction. The quantification of 5-methylcytosine was performed using MethylFlash Methylated DNA 5-mC Quantification Kit (Epigentek) according to the protocol provided with the kit.

Fluorescence Polarization assay

20 nM fluorescently tagged oligo RNA (FAM-AUUGUAUAU(m6A)CAUUUA), 1 μM FTO and serially diluted **5** or Meclofenamic acid were mixed in 25 mM Tris-HCl (pH 7.5). 30 μL of the mixture was spotted to 96-well plate (96 Well half Area, Flat Bottom, Non-Binding Surface, Black Polystyrene: CORNING). After incubating at 25°C for 1 hr, fluorescence polarization was measured using 485 nm excitation filter and 535 nm fluorescence filter, then analyzed with KaleidaGraph.

Tryptophan fluorescence quenching

Serially diluted L-ascorbic acid and 250 nM FTO were mixed in 50 mM Tris-HCl (pH 7.5). After 1 hr incubation at room temperature, fluorescence intensity of Trp was measured in the wavelength range

from 300 to 500 nm exciting with 280 nm wavelength. Affinity between L-ascorbic acid and FTO was calculated using modified Stern-Volmer equation as described below.

$$I_0/I = 1 + K_{SV}[Q]$$

[I_0]: fluorescence intensity of Trp without compounds, [I]: fluorescence intensity of Trp with compounds, K_{SV}: Stern-Volmer constant, [Q]: compounds concentration

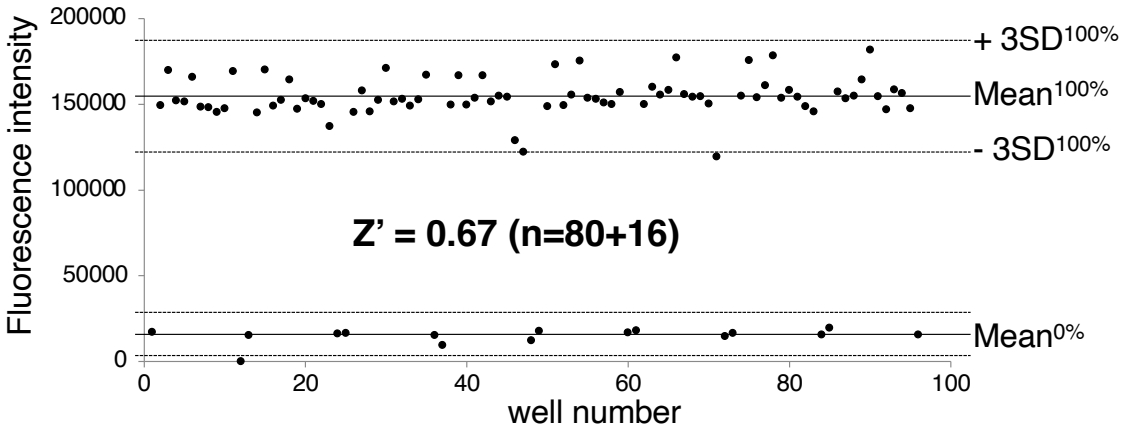

Figure S1: Verification of the screening system of FTO inhibitors using a 96-well plate. $Mean^{100\%}$ and $mean^{0\%}$ indicate the means of the fluorescence intensity of the MazF-treated samples containing the ssDNA/RNA chimera probe (5′ FAM- CAT r(GGm6ACA) TATGT -3′ BHQ-1) treated with FTO (80 samples) and without FTO (16 samples), respectively.

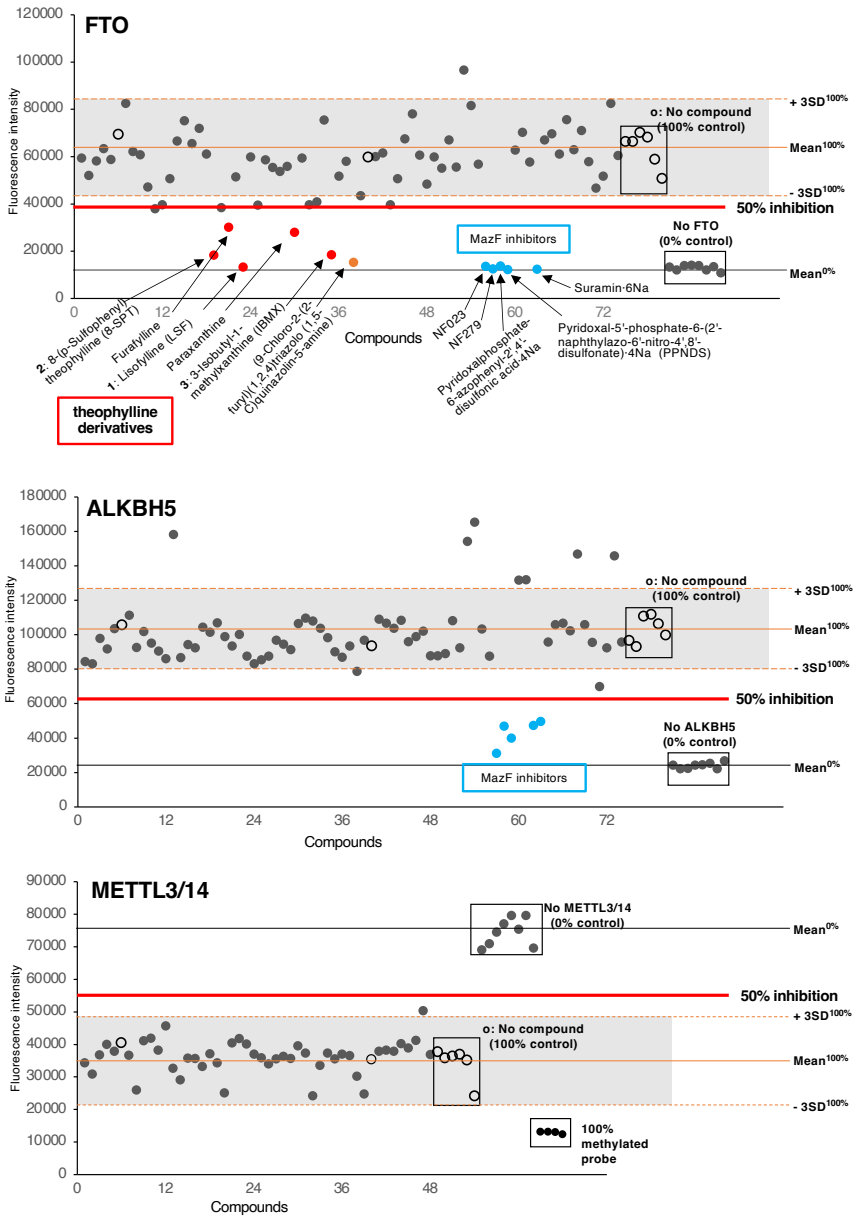

Figure S2: The results of screening of inhibitors of m6A demethylases FTO (A) and ALKBH5(B), and methyltransferase complex METTL3/14 (C) from Screen-Well Neurotransmitter Library (Purinergic and Adenosine Ligands). The compounds shown in blue inhibited RNA cleavage by MazF (data not shown) and were eliminated from the examination for METTL3/14 activity.

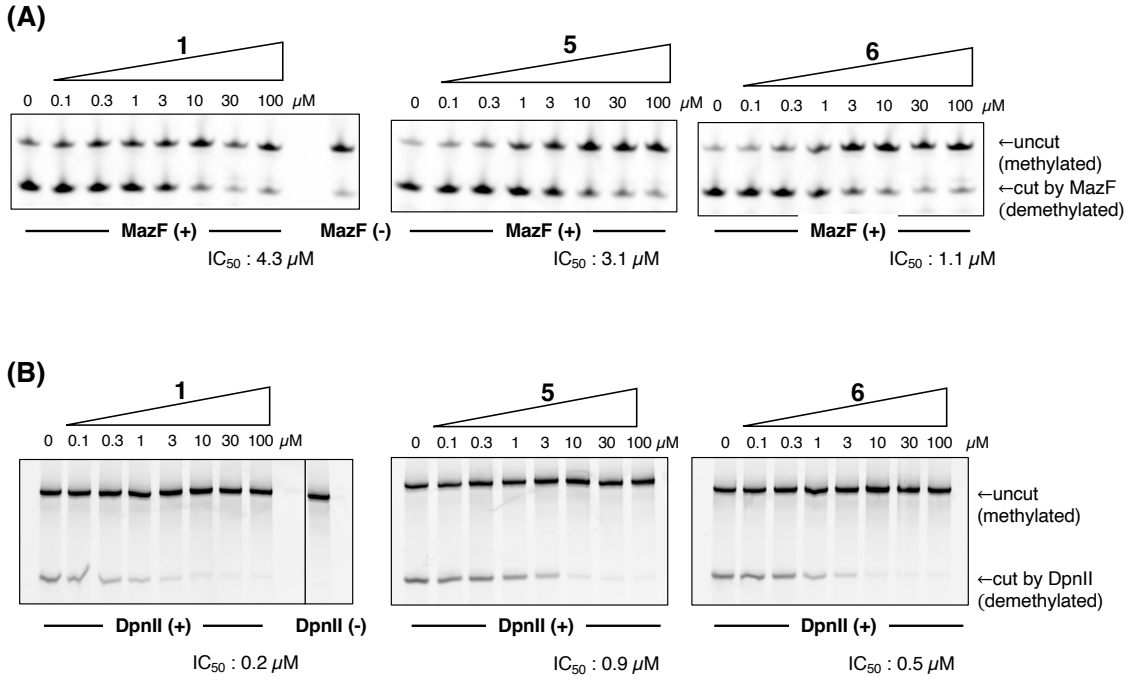

Figure S3: Inhibition of FTO by the candidate compounds, **1**, **5**, and **6**, assessed by gel electrophoresis of MazF-treated RNA (A) and DpnII-treated DNA (B). **1**; lisofylline (LSF), **5**; pentoxifylline (PTX), and **6**; pentifylline (PTF). (A) As substrates for FTO, the RNA oligonucleotide; 5′FAM-AUCAUCCAUAUAUAAG(m6A)CAUUUUA-3′ was used. After demethylation reaction in the presence of **1**, **5**, or **6**, the RNA samples were treated with MazF and electrophoresed on denaturing gels. (B) As substrates for FTO, the DNA oligonucleotide containing G(m6dA)TC sequence, 5′ FAM-TGGGAATCAAAACAC<u>AG(m6dA)TC</u>AAATGGACAGGCTCCAAGAGCTGCCATCG-3′ was used. After incubation of the substrate with FTO and inhibitors, the DNA was annealed with its complement DNA oligonucleotide and cleaved by a methylation-sensitive restriction enzyme, DpnII. The concentration of FTO inhibitors is 0, 0.1, 0.3, 1, 3, 10, 30, and 100 μM (left to right).

(A)

Fe(II) conc.	IC_{50}
0.5 μM	6.6 $\pm$ 0.6 μM
5 μM	4.2 $\pm$ 0.6 μM
50 μM	5.9 $\pm$ 2.7 μM

(B)

α-KG conc.	IC_{50}
50 μM	4.7 $\pm$ 0.5 μM
150 μM	1.9 $\pm$ 0.4 μM
500 μM	1.0 $\pm$ 0.2 μM

Figure S4: IC_{50} values of **5** in the presence of different concentration of Fe(II) and α-KG.

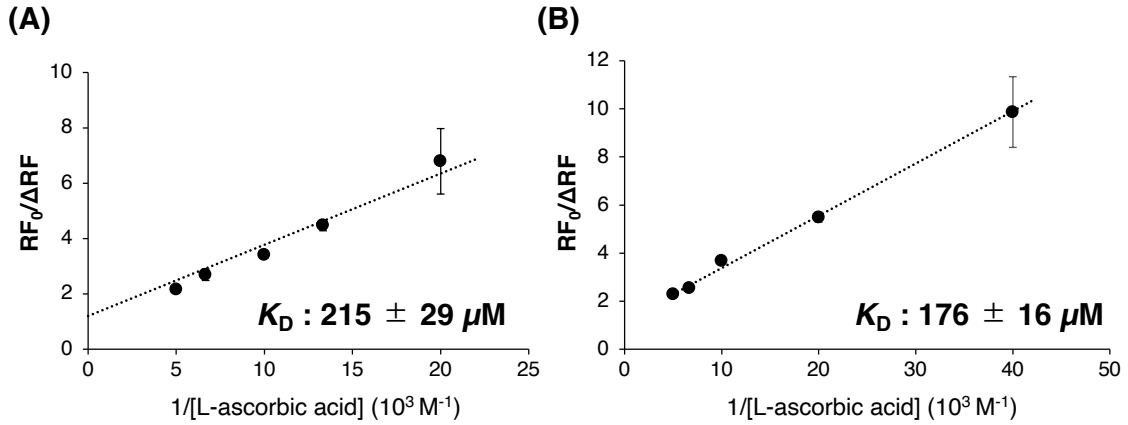

Figure S5: Tryptophan fluorescence quenching assay by titration of L-ascorbic acid to FTO. (A, B) Fitting curves of fluorescence titration without (A) and with 50 μM of the compound **5** (B) to modified Stern-Volmer equation.

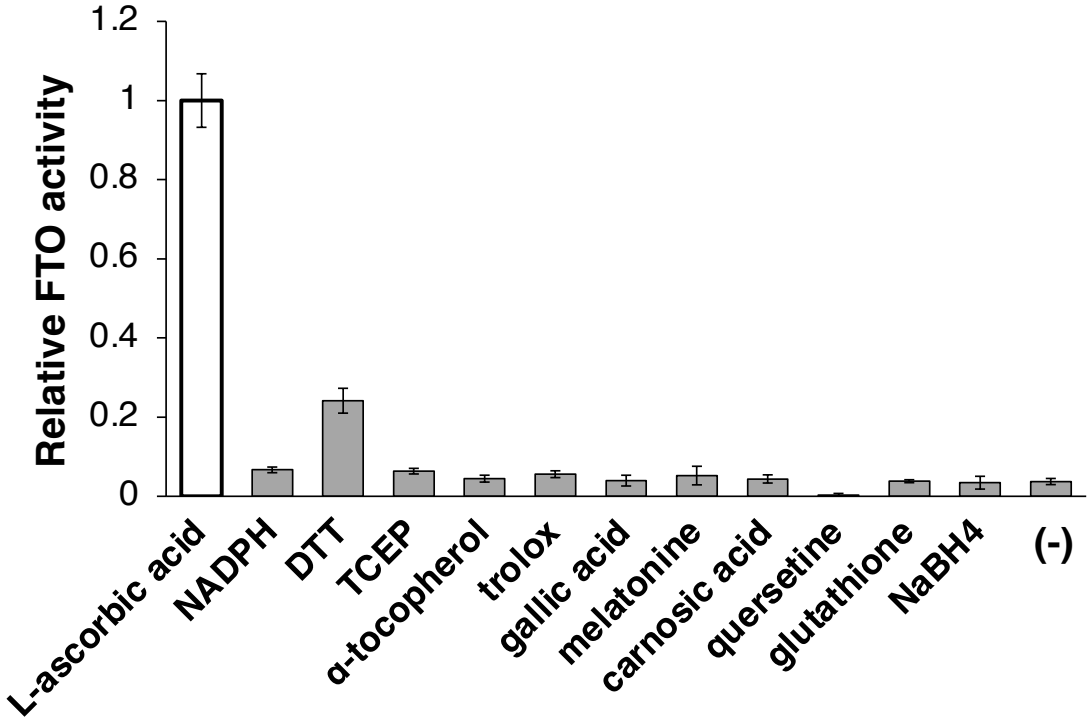

Figure S6: None of examined reducing agents but not L-ascorbic acid proceeded the demethylation reaction of FTO. The demethylation of RNA by FTO was examined by FRET-based MazF cleavage assay. In the demethylation reaction mix, 1 mM of each reducing agent was used instead of 1 mM L-ascorbic acid.