Supporting Information

m6A-RNA Demethylase FTO Inhibitors Impair Self-Renewal in Glioblastoma Stem Cells

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Experimental Methods

Molecular Modeling with Schrödinger

*In silico* modeling of FTO inhibitors was performed using the Glide docking module of the Schrödinger 11.5 modeling software suite. A crystal structure of FTO bound to meclofenamic acid (MA) (PDBID: 4QKN) was first refined using Prime. Missing side chains and hydrogen atoms were resolved before docking and the Optimized Potentials for Liquid Simulations All-Atom (OPLS) force field and the Surface generalized Born (SGB) continuum solution model was used to optimize and minimize the crystal structures. The docking grid was generated as a 5x5x5 Å cube centered on MA. Glycerol and α-ketoglutarate were removed from the docking site prior to grid generation. Ligprep was used to generate a minimized 3D structure for all prospective FTO inhibitors using the OPLS 2001 force field. Docking was performed with Glide XP. QikProp was used to predict physicochemical properties such as clogP and membrane permeability in Caco-2 and MDCK cell lines for the 20 most promising compounds.

Protein expression and purification

The protein expression and purification protocol was adapted from Svensen and Jaffrey, 2016. *E. Coli* BL21 competent cells (New England Biolabs) were transformed with pET28-SUMO-His10-FTO plasmid (a generous gift from the Jaffrey lab) by heat shock and spread on a LB Kanamycin agar plate, then incubated overnight at 37 °C. 2-3 colonies were picked and transferred to 5 mL of LB media treated with kanamycin (0.5 mg mL⁻¹ final concentration), then grown overnight shaking at 37 °C. The overnight culture was then transferred to 2 L of LB kanamycin media and incubated at 37 °C until OD 0.8. The culture was cooled at 4 °C for 20 mins and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), then grown
shaken at 16 °C. Cell pellets were collected by centrifugation (5,000 g for 10 min at 4 °C) and
the supernatant was discarded. The pellets were resuspended in B-PER Bacterial Protein
Extraction Reagent (6 mL per gram) with DNase 1 (5U per mL, RNase-free) and incubated at 4
°C for 1 hour. The suspension was centrifuged at 10,000 g for 20 min and the supernatant was
transferred to a Talon Metal Affinity Resin column that had been pre-equilibrated with binding
buffer (50 mM NaH₂PO₄ pH 7.2, 300 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol in
RNase-free water). The supernatant was incubated with the affinity resin column at 4 °C for 1
hour with end-over-end rotation. After incubation, the column was washed with 5 bed volumes
of binding buffer, then incubated with 1 bed volume of elution buffer (50 mM NaH₂PO₄ pH 7.2,
300 mM NaCl, 500 mM imidazole, 5 mM β-mercaptoethanol in RNase-free water) for 20 mins.
After incubation, the eluant was collected and the column was incubated again with 1 bed
volume of elution buffer; the elution process was repeated until no further protein was collected
(3-5 bed volumes total). The eluant was combined and transferred to a Slyde-A-Lyzer Dialysis
Cassette (20,000 MWCO, Thermo Scientific) and dialyzed overnight at 4 °C against dialysis
buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM B-mercaptoethanol, 5% (v/v) glycerol in
RNase-free water). Protein concentration was measured by absorbance at 280 nm and calculated
by Beer-Lambert’s Law (A = ε/C, ε₅₈₀ = 95,340). ALKBH5 was expressed and purified from
pET28-SUMO-His10-ALKBH5 plasmid by the same procedure described above.

In Vitro Inhibition Assay Method

The in vitro inhibition assay method was adapted from Svenson and Jaffrey, 2016. All reactions
were performed in a 96-well plate with 200 µL assay buffer (50 mM HEPES pH 6, 300 µM 2-
oxoglutarate, 300 µM (NH₄)₂Fe(SO₄)₂·6H₂O, 2 mM ascorbic acid in RNase-free water) with 7.5
µM m⁶A⁷-Broccoli RNA and 0.250 µM FTO. Inhibitors were added in concentrations ranging
from 0.008 - 40 µM; all inhibitors were dissolved in DMSO and added to a final concentration of 0.2% DMSO. Prior to incubation, 40 µL read buffer (250 mM HEPES pH 9.0, 1 M KCl, 40 mM MgCl₂, 2.2 µM DFHBI-1T in RNase-free water) was added to bring the final well volume to 200 µL. After incubation at room temperature for 2 hours, the plates were left at 4 °C overnight (16 hours) to allow DFHBI-1T to bind to A7-Broccoli RNA. Specificity assays were performed by the same method with 0.250 µM ALKBH5. Fluorescence intensity was measured with a BioTek Synergy plate reader with FITC filters (excitation 485 nm, emission 510 nm). Sigmoidal dose-response curves were fitted in GraphPad Prism 6. All assays were performed in triplicate, with additional repetitions added as necessary.

As a negative control, inhibitors were screened at concentrations ranging from 0-40 µM as described above with 7.5 µM demethylated Broccoli instead of m⁶A7-Broccoli. No compounds were observed to significantly alter fluorescent signal of the A7-Broccoli-DHBI-1T complex at these concentrations (Figure S22).

Michealis-Menton kinetics was performed using the inhibition assay procedure described above; the activity of FTO concentrations of 0, 0.250, 0.385, 0.500, 0.625, 0.750, 1.25, 2.5, 5, and 10 µM m⁶A Broccoli were recorded for the following concentrations of FTO-02 N: 0, 0.5, 1, 10, and 40 µM and FTO-04: 0, 1, 10, 20, and 40 µM. The data were fitted in GraphPad Prism 6.

**ELISA Assay Methods**

The IC₅₀s of FTO-02 and FTO-04 against FTO were determined by ELISA as an orthogonal assay control. 3’-biotinylated m⁶A-RNA (5’-CCGG(m6A)CUU-3’, 0.200 µM) was incubated with 0.250 µM FTO for 2 hours at room temperature in reaction buffer (50 mM NaHEPES pH 6, 300 µM 2-oxoglutarate, 300 µM (NH₄)₂Fe(SO₄)₂·6H₂O, and 2 mM L-ascorbate) with 0-40 µM
FTO-02 or FTO-04. The reaction mixture was then incubated with neutravidin coated 96-well plates (Pierce) overnight at 4 °C, washed and blocked, incubated with m^6A^-specific primary antibody (Abcam ab151230, 1:400 dilution) for 1 hour at room temperature, washed and blocked (phosphate buffer saline with 0.1% tween-20 (PBST); blocked in 5% of non-fat milk in PBST buffer), and incubated with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, A6154, 1:5000 dilution) for 1 hour at room temperature. After extensive washing, the wells were treated with 3,3',5,5'-tetramethylbenzidine (TMB, BM Blue POD substrate by Roche Diagnostics GmbH) for 30 minutes at room temperature and the absorbance was measured at 390 nm. Absorbance was normalized to control wells for each concentration of inhibitor without cofactor 2-oxoglutarate, and the data were fit to a sigmoidal dose-response curve in GraphPad Prism 6.

**Synthetic Methods**

**General experimental procedures**

All reagents were performed under nitrogen atmosphere. Air sensitive liquids were transferred by syringe through rubber septa. Dry THF was prepared by distillation over calcium hydride. All other reagents and solvents were purchased from commercial sources and used without further purification. All solvents used for column chromatography were reagent grade. Reaction progress was monitored by analytical thin layer chromatography (TLC, silica gel 60, F254, EMD Chemicals) and visualized by UV illumination (254 nm). Compounds were purified by flash column chromatography on silica gel 60 Å (200-400 mesh, 40-63 µm) at medium pressure (20 psi). All compounds were purified to > 95% purity. NMR spectra were recorded at ambient temperature on a Brucker 600 MHz spectrophotometer (^1H NMR: 600 MHz and ^13C NMR: 150 MHz). Chemical shift values are reported in parts per million (ppm) relative to the residual
solvent peak (CDCl\textsubscript{3} or (CD\textsubscript{3})\textsubscript{2}OS). Coupling constants for \textsuperscript{1}H NMR are reported in Hz. High Resolution Mass Spectrometry (HRMS) data were acquired on an Agilent 6230 High Resolution time-of-flight mass spectrometer and reported as m/z for the molecular ion [M+H]\textsuperscript{+}.

General procedure A for Suzuki-Miyaura cross-coupling reactions

\[ \text{HO-Br} + \text{Br-OH} \xrightarrow{5 \text{ mol\% Pd(PPh\textsubscript{3})}_4, 2 \text{ equiv. } K_2CO_3} \text{THF:EtOH 5:1 reflux, 6-8 hours} \]

6-bromo-2-naphthol (0.900 g, 4.0 mmol), palladium tetrakistriphenylphosphine (0.231 g, 0.02 mmol), and potassium carbonate (1.115 g, 8.0 mmol) were placed under nitrogen atmosphere, and dissolved in dry THF (20 mL) to obtain a dark red solution. A syringe was used to transfer pyrimidine-5-boronic acid (0.500 g, 4.0 mmol) in 5 mL dry THF to the stirring solution. The reaction was heated under reflux for 6 hours. The reaction mixture was filtered over Celite and the filter cake was washed with ethyl acetate. The filtrate was concentrated under reduced pressure to obtain the crude product as a yellow solid. The crude product was purified by silica gel column chromatography (Ethyl acetate: Hexanes 2:3, Rf = 0.48). Following this procedure, twenty potential FTO inhibitors were obtained with an average yield of 54%.

Procedure B for synthesis of tert-butyl (6-bromobenzo[d]thiazol-2-yl)carbamate

6-bromobenzo[d]thiazol-2-amine (0.458 g, 2 mmol) and BOC\textsubscript{2}O (1.2 eq, 2.4 mmol) were dissolved in THF (30 mL). 4-dimethylaminopyridine (DMAP, 0.1 equivalent) was added to the solution and the reaction was stirred for 3.5 hours at room temperature. The reaction mixture was diluted in ethyl acetate (100 mL) and washed with 0.25 M HCl (50 mL), 2 M NaHCO\textsubscript{3} (100
mL), and brine. The organic layers were dried by Na$_2$SO$_4$, filtered, then concentrated to obtain the crude product. The crude product was used for Suzuki coupling via general method A without further purification.

**Procedure C for Boc deprotection of tert-butyl (6-(2-methoxypyrimidin-5-yl)benzo[d]thiazol-2-yl)carbamate**

A solution of tert-butyl (6-(2-methoxypyrimidin-5-yl)benzo[d]thiazol-2-yl)carbamate (0.720 g, 2 mmol) in dioxane (40 mL) was treated with 4M HCl in dioxane and stirred at room temperature for 1 hour. The reaction mixture was concentrated, then dissolved in ethyl acetate (100 mL) and extracted with 10% Na$_2$CO$_3$ (50 mL) and brine (2 x 50 mL). The organic layers were dried with Na$_2$SO$_4$, filtered, and concentrated to obtain the crude product as a yellow solid. The crude product was purified by silica gel column chromatography (Ethyl acetate: Hexanes 2:3, Rf = 0.48).

**Chemical Characterization Data**

6-(pyrimidin-5-yl)naphthalen-2-ol (FTO 1)

Prepared according to general procedure A. Yield 0.640 g, 2.88 mmol, 72%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 9.93 (s, 1H), 9.25 (s, 2H), 9.17 (s, 1H), 8.03 (d, $J = 2.0$ Hz, 1H), 7.75 (d, $J = 8.6$ Hz, 1H), 7.65 (d, $J = 8.6$ Hz, 1H), 7.47 (dd, $J = 8.8, 2.1$ Hz, 1H), 7.45 (d, $J = 8.8$ Hz, 1H), 7.13 (d, $J = 2.5$ Hz, 1H). $^{13}$C NMR (150 MHz, d-DMSO): 156.5, 155.3, 150.3, 150.3, 133.8, 132.8, 132.2, 130.0, 129.5, 129.4, 128.2, 125.2, 115.9, 109.5. HRMS (ESI, M+) m/z calculated for C$_{14}$H$_{10}$N$_2$O 222.0793, found 222.0795.

6-(2-methoxypyrimidin-5-yl)naphthalen-2-ol (FTO 2)
Prepared according to general procedure A. Yield 0.525 g, 2.8 mmol, 52%. Orange solid, mp 230 °C. \(^1\)H NMR (600 MHz, d-DMSO): 9.89 (s, 1H), 9.02 (s, 2H), 8.16 (d, \(J = 2.0\) Hz, 1H), 7.82 (d, \(J = 8.7\) Hz, 1H), 7.80 (d, \(J = 8.7\) Hz, 1H), 7.76 (d, \(J = 2.5\) Hz, 1H), 7.75 (d, \(J = 2.5\) Hz, 1H), 7.15 (d, \(J = 2.6\) Hz, 1H), 3.97 (s, 3H). \(^{13}\)C NMR (150 MHz, d-DMSO): 157.8, 155.4, 155.4, 154.7, 133.9, 130.6, 129.3, 128.5, 128.2, 126.7, 125.5. 120.1, 115.9, 106.5, 56.0. HRMS (ESI, M+) \(m/z\) calculated for C\(_{15}\)H\(_{12}\)N\(_2\)O\(_2\) 252.0899, found 252.0900.

5-(3-(benzyloxy)phenyl)-2-methoxypyrimidine (FTO 3)

Prepared according to general procedure A. Yield 0.588 g, 2.01 mmol, 51%. Yellow solid, mp 230 °C. \(^1\)H NMR (600 MHz, CDCl\(_3\)): 8.71 (s, 2H), 7.47 (d, \(J = 7.3\) Hz, 2H), 7.42 (t, \(J = 7.4\) Hz, 1H), 7.41 (d, \(J = 6.1\) Hz, 2H), 7.40 (d, \(J = 2.7\) Hz, 1H), 7.36 (t, \(J = 7.3\) Hz, 1H), 7.13 (d, \(J = 1.4\) Hz, 2H), 7.04 (d, \(J = 1.6\) Hz, 1H), 5.14 (s, 2H), 4.07 (s, 3H). \(^{13}\)C NMR (150 MHz, d-DMSO): 163.4, 159.7, 156.7, 156.7, 139.7, 136.6, 130.8, 130.8, 128.9, 128.9, 128.4, 127.8, 124.2, 118.4, 114.0, 113.2, 70.4, 55.0. HRMS (ESI, M+) \(m/z\) calculated for C\(_{18}\)H\(_{16}\)N\(_2\)O\(_2\) 292.1212, found 292.1216.

6-(2-methoxypyrimidin-5-yl)benzo[d]thiazol-2-amine (FTO 4)

Prepared according to general procedure A from tert-butyl (6-bromobenzo[d]thiazol-2-yl)carbamate and (2-methoxypyrimidin-5-yl)boronic acid. FTO-04 was purified after Boc deprotection as described in procedure C. Yield 0.723 g, 2.80 mmol, 70%. Yellow solid, mp 230 °C. \(^1\)H NMR (600 MHz, d-DMSO): 8.82 (s, 2H), 7.71 (s, 2H), 7.60 (d, \(J = 8.3\) Hz, 1H), 7.47 (d, \(J = 2.0\) Hz, 1H), 7.14 (dd, \(J = 8.3, 2.0\) Hz, 1H), 3.87 (s, 3H). \(^{13}\)C NMR (150 MHz, d-DMSO): 168.7, 157.8, 155.2, 155.0, 155.0, 130.5, 123.8, 123.4, 120.6, 118.9, 55.1. HRMS (ESI, M+) \(m/z\) calculated for C\(_{12}\)H\(_{10}\)N\(_4\)OS 258.0575, found 258.0580.
5-(6-methoxynaphthalen-2-yl)pyrimidine (FTO 5)

Prepared according to general procedure A. Yield 0.595 g, 2.52 mmol, 63%. White solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 9.26 (s, 2H), 9.19 (s, 1H), 8.35 (d, $J = 1.1$ Hz, 1H), 7.98 (d, $J = 8.6$ Hz, 1H), 7.92 (dd, $J = 8.5, 2.1$ Hz, 2H), 7.40 (d, $J = 2.5$ Hz, 1H), 7.24 (dd, $J = 8.9, 2.6$ Hz, 1H), 3.90 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 157.7, 155.3, 150.3, 150.3, 135.0, 134.2, 133.9, 130.6, 129.3, 128.5, 126.7, 125.4, 120.1, 106.5, 56.0. HRMS (ESI, M+) $m/z$ calculated for C$_{15}$H$_{12}$N$_2$O 236.0950, found 236.0593.

(2-methoxy-4-(2-methoxypyrimidin-5-yl)phenyl)methanol (FTO 6)

Prepared according to general procedure A. Yield 0.374 g, 1.52 mmol, 38%. White solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.60 (s, 2H), 7.29 (d, $J = 7.9$ Hz, 1H), 7.13 (d, $J = 1.6$ Hz, 1H), 7.11 (t, $J = 2.7$ Hz, 1H), 5.10 (t, $J = 5.6$ Hz, 2H), 3.78 (s, 6H). $^{13}$C NMR (150 MHz, d-DMSO): 163.4, 157.1, 148.9, 148.9, 136.2, 131.0, 129.1, 123.5, 113.9, 61.1, 58.1, 56.0. HRMS (ESI, M+) $m/z$ calculated for C$_{13}$H$_{14}$N$_2$O$_3$ 246.1004, found 246.1009.

2-methyl-6-(pyrimidin-5-yl)quinoline (FTO-07)

Prepared according to general procedure A. Yield 0.520 g, 2.35 mmol, 59%. White solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 9.26 (s, 1H), 8.68 (s, 2H), 8.24 (d, $J = 8.4$ Hz, 1H), 8.23 (d, $J = 2.2$ Hz, 1H), 7.89 (d, $J = 8.9$ Hz, 1H), 7.83 (dd, $J = 8.9, 2.2$ Hz, 1H), 7.48 (d, $J = 8.4$ Hz, 1H), 2.73 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 155.0, 154.8, 154.8, 150.5, 150.1, 141.9, 136.8, 130.7, 130.2, 128.7, 128.3, 125.9, 123.1, 24.0. HRMS (ESI, M+) $m/z$ calculated for C$_{14}$H$_{11}$N$_3$ 221.0953, found 221.0958.

2-methoxy-5-(6-methoxynaphthalen-2-yl)pyrimidine (FTO 8)
Prepared according to general procedure A. Yield 0.266 g, 1.00 mmol, 25%. White solid, mp 230 °C. \(^1\)H NMR (600 MHz, d-DMSO): 9.05 (s, 2H), 8.23 (d, \(J = 1.1\) Hz, 1H), 7.95 (d, \(J = 8.6\) Hz, 1H), 7.93 (d, \(J = 2.1\) Hz, 1H), 7.89 (d, \(J = 2.1\) Hz 1H), 7.37 (d, \(J = 2.5\) Hz, 1H), 7.21 (dd, \(J = 8.9, 2.6\) Hz, 1H), 3.97 (s, 3H), 3.89 (s, 3H). \(^13\)C NMR (150 MHz, d-DMSO): 163.5, 157.5, 150.3, 150.3, 133.9, 130.8, 130.3, 129.5, 128.5, 128.3, 124.1, 120.4, 120.1, 106.7, 56.5, 56.0. HRMS (ESI, M+) \(m/z\) calculated for C\(_{16}\)H\(_{14}\)N\(_2\)O\(_2\) 266.1055, found 266.1058.

5-(3-(phenylamino)phenyl)pyrimidin-2-amine (FTO-09)

Prepared according to general procedure A. Yield 0.441 g, 1.68 mmol, 42%. Yellow solid, mp 230 °C. \(^1\)H NMR (600 MHz, d-DMSO): 8.37 (s, 2H), 7.27 (t, \(J = 7.9\) Hz, 2H), 7.15 (t, \(J = 8.6\) Hz, 2H), 7.08 (d, \(J = 7.6\) Hz, 2H), 7.02 (dd, \(J = 8.2, 1.7\) Hz, 1H), 6.92 (d, \(J = 8.9\) Hz, 1H), 6.90 (t, \(J = 7.3\) Hz, 1H). \(^13\)C NMR (150 MHz, d-DMSO): 161.5, 150.2, 150.2, 140.1, 139.3, 137.2, 130.5, 129.9, 129.9, 121.4, 120.6, 120.6, 120.6, 120.4, 117.6, 117.6. HRMS (ESI, M+) \(m/z\) calculated for C\(_{16}\)H\(_{14}\)N\(_4\)O 262.1218, found 262.1225.

6-(2-aminopyrimidin-5-yl)naphthalen-2-ol (FTO 10)

Prepared according to general procedure A. Yield 0.690 g, 2.91 mmol, 73%. Yellow solid, mp 230 °C. \(^1\)H NMR (600 MHz, d-DMSO): 8.66 (s, 2H), 8.20 (d, \(J = 6\) Hz, 1H), 8.01 (s, 1H), 7.78 (d, \(J = 8.8\) Hz, 1H), 7.73 (d, \(J = 8.6\) Hz, 1H), 7.12 (d, \(J = 6\) Hz, 1H), 7.09 (dd, \(J = 8.9, 2.4\) Hz, 1H), 6.79 (s, 2H), 6.57 (s, 1H). \(^13\)C NMR (150 MHz, d-DMSO): 158.8, 158.6, 156.5, 156.5, 134.1, 132.6, 130.1, 130.2, 127.6, 127.6, 124.7, 124.0, 122.1, 110.8. HRMS (ESI, M+) \(m/z\) calculated for C\(_{16}\)H\(_{11}\)N\(_3\)O 237.0902, found 237.0900.

6-(2-methoxypyrimidin-5-yl)-2-methylquinoline (FTO 11)
Prepared according to general procedure A. Yield 0.302 g, 1.20 mmol, 30%. White solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.53 (s, 2H), 7.96 (d, $J = 8.4$ Hz, 1H), 7.93 (d, $J = 2.2$ Hz, 1H), 7.89 (d, $J = 8.9$ Hz, 1H), 7.74 (dd, $J = 8.9$, 2.2 Hz, 1H), 7.31 (d, $J = 8.4$ Hz, 1H), 4.02 (s, 3H), 2.73 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 163.4, 155.0, 154.8, 154.8, 150.5, 141.9, 136.8, 130.7, 128.7, 128.3, 125.9, 123.1, 118.4, 50.3, 21.0. HRMS (ESI, M+) m/z calculated for C$_{15}$H$_{13}$N$_3$O 251.1059, found 251.1061.

5-(6-methoxynaphthalen-2-yl)pyrimidin-2-amine (FTO 12)

Prepared according to general procedure A. Yield 0.543 g, 2.16 mmol, 54%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.68 (s, 2H), 8.09 (d, $J = 2.5$ Hz, 1H), 7.87 (d, $J = 8.8$ Hz, 1H), 7.84 (d, $J = 8.8$ Hz, 1H), 7.75 (dd, $J = 8.5$, 1.9 Hz, 1H), 7.33 (d, $J = 2.5$ Hz, 1H), 7.18 (dd, $J = 8.9$, 2.5 Hz, 1H), 6.79 (s, 2H), 3.88 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 163.4, 157.9, 156.6, 156.6, 133.9, 130.9, 130.4, 129.5, 128.5, 126.7, 123.8, 122.8, 106.5, 56.0, 25.8. HRMS (ESI, M+) m/z calculated for C$_{15}$H$_{13}$N$_3$O 251.1059, found 251.1066.

5-(3-(benzyloxy)phenyl)pyrimidin-2-amine (FTO-13)

Prepared according to general procedure A. Yield 0.566 g, 2.04 mmol, 51%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.70 (s, 2H), 7.43 (d, $J = 7.3$ Hz, 2H), 7.42 (t, $J = 7.4$ Hz, 1 H), 7.40 (d, $J = 6.1$ Hz, 2H), 7.39 (d, $J = 2.7$ Hz, 1H), 7.36 (t, $J = 7.3$ Hz, 1H), 7.14 (d, $J = 1.4$ Hz, 2H), 7.04 (d, $J = 1.6$ Hz, 1 H), 6.79 (s, 2H), 5.05 (s, 2H). $^{13}$C NMR (150 MHz, d-DMSO): 161.7, 159.7, 150.7, 150.7, 137.0, 136.6, 130.8, 128.9, 128.9, 128.4, 127.8, 127.8, 120.2, 118.4, 114.0, 113.2, 70.4. HRMS (ESI, M+) m/z calculated for C$_{17}$H$_{15}$N$_3$O 277.1215, found 277.1223.

5-(2-methylquinolin-6-yl)pyrimidin-2-amine (FTO 14)
Prepared according to general procedure A. Yield 0.784 g, 3.32 mmol, 83%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.73 (s, 2H), 8.23 (d, $J = 8.3$ Hz, 1H), 8.18 (d, $J = 8.8$ Hz, 1H), 8.00 (d, $J = 1.7$ Hz, 1H), 7.94 (d, $J = 8.7$ Hz, 1H), 7.43 (d, $J = 8.5$ Hz, 1H), 6.87 (s, 2H), 2.65(s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 163.7, 157.9, 156.9, 156.9, 141.9, 138.6, 136.8, 130.7, 128.7, 128.3, 125.9, 123.1, 118.4, 25.5. HRMS (ESI, M$^+$) $m/z$ calculated for C$_{14}$H$_{12}$N$_4$ 236.1062, found 236.1070.

$N$-(2-methoxyethyl)-5-(6-methoxynaphthalen-2-yl)pyrimidin-2-amine (FTO-15)

Prepared according to general procedure A. Yield 0.744 g, 2.52 mmol, 63%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.41 (s, 2H), 8.00 (s, 1H), 7.83 (m, 2H), 7.71 (dd, $J = 8.5$, 1.7 Hz, 1H), 7.30 (d, $J = 2.3$ Hz, 1H), 7.15 (dd, $J = 8.9$, 2.5 Hz, 1H), 6.73 (s, 1H), 3.87 (s, 3H), 3.48 (m, 2H), 3.27 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 159.5, 156.7, 150.8, 150.8, 136.1, 134.1, 132.9, 129.7, 128.8, 127.9, 124.2, 120.3, 119.1, 109.7, 72.0, 58.7, 56.3, 43.5. HRMS (ESI, M$^+$) $m/z$ calculated for C$_{18}$H$_{19}$N$_3$O$_2$ 309.1477, found 309.1472.

6-(2-((2-methoxyethyl)amino)pyrimidin-5-yl)naphthalen-2-ol (FTO-16)

Prepared according to general procedure A. Yield 0.378 g, 1.28 mmol, 32%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.29 (s, 2H), 7.93 (s, 1H), 7.68 (dd, $J = 8.7$, 2.5 Hz, 2H), 7.46 (d, $J = 7.3$ Hz, 1H), 7.39 (t, $J = 7.7$ Hz, 1H), 7.32 (t, $J = 8.0$, 1H), 6.90 (dd, $J = 8.0$, 2.0 Hz, 1H), 3.95 (s, 2H), 3.46 (s, 2H), 3.25 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 159.9, 156.6, 150.3, 150.3, 134.1, 132.2, 130.3, 130.0, 129.0, 128.7, 125.7, 120.5, 116.4, 109.5, 71.8, 43.3, 56.9. HRMS (ESI, M$^+$) $m/z$ calculated for C$_{17}$H$_{17}$N$_3$O$_2$ 295.1321, found 295.1316.

7-(2-((2-methoxyethyl)amino)pyrimidin-5-yl)naphthalen-2-ol (FTO-17)
Prepared according to general procedure A. Yield 0.484 g, 1.68 mmol, 42%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.41 (s, 2H), 7.90 (s, 1H), 7.84 (d, $J = 8.4$ Hz, 1H), 7.73 (d, $J = 8.3$ Hz, 1H), 7.64 (dd $J = 8.0$, 2.0 Hz, 1H), 7.63 (d, $J = 2.5$ Hz, 1H), 7.39 (t, $J = 7.7$ Hz, 1H), 7.13 (d, $J = 7.3$ 1H), 3.94 (s, 2H), 3.47 (s, 2H), 3.28 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 160.2, 156.1, 150.1, 150.1, 135.7, 134.9, 130.0, 129.2, 127.5, 125.5, 124.1, 120.6, 118.8, 109.7, 71.6, 56.5, 43.1. HRMS (ESI, M+) m/z calculated for C$_{17}$H$_{17}$N$_{3}$O$_{2}$ 295.1321, found 295.1314.

$5$-($4$-(benzyloxy)phenyl)-$N$-(2-methoxyethyl)pyrimidin-2-amine (FTO-18)

Prepared according to general procedure A. Yield 0.698 g, 2.08 mmol, 52%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.29 (s, 2H), 7.93 (s, 1H), 7.68 (dd, $J = 8.7$, 2.5 Hz, 2 H), 7.46 (d, $J = 7.3$ Hz, 2H), 7.39 (t, $J = 7.7$ Hz, 2H), 7.32 (t, $J = 8.0$, 1H), 6.90 (dd, $J = 8.0$, 2.0 Hz, 2H), 5.16 (s, 2H), 3.95 (s, 2H), 3.46 (s, 2H), 3.25 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 159.5, 158.8, 150.1, 150.1, 137.9, 136.6, 130.6, 128.9, 128.9, 128.4, 127.8, 127.8, 120.2, 118.4, 114.0, 113.2, 71.6, 70.7, 58.7, 43.1. HRMS (ESI, M+) m/z calculated for C$_{20}$H$_{21}$N$_{3}$O$_{2}$ 335.1634, found 334.1630.

$N$-(2-methoxyethyl)-$5$-(2-methylquinolin-6-yl)pyrimidin-2-amine (FTO-19)

Prepared according to general procedure A. Yield 0.503 g, 1.71 mmol, 43%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.70 (s, 2H), 8.24 (d, $J = 8.3$ Hz, 1H), 8.10 (d, $J = 8.8$ Hz, 1H), 8.01 (d, $J = 1.7$ Hz, 1H), 7.93 (s, 1H), 7.89 (d, $J = 8.7$ Hz, 1H), 7.44 (d, $J = 8.5$ Hz, 1H), 3.94 (s, 2H), 3.45 (s, 2H), 3.26 (s, 3H), 2.71 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 159.8, 158.1, 151.2, 151.2, 150.1, 141.9, 135.6, 133.1, 128.7, 128.3, 125.9, 123.1, 120.2, 71.5, 58.7, 43.1, 25.5. HRMS (ESI, M+) m/z calculated for C$_{17}$H$_{18}$N$_{4}$O$_{2}$ 294.1481, found 294.1485.
(2-methoxy-4-(2-((2-methoxyethyl)amino)pyrimidin-5-yl)phenyl)methanol (FTO-20)

Prepared according to general procedure A. Yield 0.584 g, 2.02 mmol, 51%. Yellow solid, mp 230 °C. $^1$H NMR (600 MHz, d-DMSO): 8.68 (s, 2H), 7.93 (s, 1H), 7.30 (d, $J = 7.9$ Hz, 1H), 7.13 (d, $J = 1.6$ Hz, 1H), 7.11 (t, $J = 2.7$ Hz, 1H), 5.10 (t, $J = 5.6$ Hz, 2H), 3.94 (s, 2H), 3.77 (s, 3H), 3.45 (s, 2H), 3.26 (s, 3H). $^{13}$C NMR (150 MHz, d-DMSO): 159.9, 157.1, 148.9, 148.9, 136.2, 131.0, 129.1, 123.5, 119.1, 113.9, 71.5, 61.1, 58.6, 58.1, 43.0. HRMS (ESI, M+) $m/z$ calculated for $C_{15}H_{19}N_3O_3$ 289.1426, found 289.1430.

**Glioblastoma cancer stem cells (GSCs) cultures**

All studies were conducted in accordance with approved IRB protocols by the University of California, San Diego. Patient derived glioblastoma stem cells such as GBM-6, GBM-GSC-23, and TS576 cell line were obtained from Frank Furnari Lab at UCSD were cultured in DMEM/F12 medium supplemented with 1:100 B27 without vitamin A, EGF (20ng/ml), FGF (10ng/ml) and penicillin-streptomycin (100IU/ml) as described earlier $^8,^9$. Neurospheres formed by generating single cell suspensions using StemPro-accutase and seeding in uncoated plates were used for screening ALK-04.

**Neurosphere formation assay**

Early passaged GSCs were used to understand the efficacy of ALK-04 on the self-renewal capacity of GSCs by neurosphere-formation assay as described earlier $^{10,11}$. In brief, GSCs were seeded at $4 \times 10^4$ cells in 24 well plate and cultured for 3 days followed by treatment with ALK-04 inhibitors at 20 μM daily for 3 days. After 3 days of treatment the images of the neurospheres were imaged with phase contrast microscope and size was measured with Image J, to understand
the effects of drugs on the self-renewal of GSCs on sphere formation. This process was also repeated for healthy neural stem cells (hNSCs) treated daily with 20 μM ALK-04 for three days to assess the therapeutic ratio.

**m^6A dot blot assay**

Polyadenylated mRNA were isolated from TS576 cells treated with either DMSO, FTO-04 (30μM), and control (shControl) or FTO lentivirus (shFTO) knockdown samples by using Magnetic mRNA Isolation Kit (New England Biolabs, S1550S). Isolated mRNA was quantified, serially diluted and denatured at 95 °C for 3 min, then chilled on ice to prevent reformation of secondary structure of mRNA. Denatured mRNA samples were spotted on an Amersham Hybond-N+ membrane (GE Healthcare, RPN3050B) and cross-linked to the membrane with UV radiation. After crosslinking the membrane was washed with phosphate buffer saline with 0.1% tween-20 (PBST) and blocked in 5% of non-fat milk in PBST buffer, and then incubated with anti-m^6A antibody (1: 1000; abcam) overnight at 4° C. The membrane was then washed as before and incubated in HRP-conjugated secondary antibodies for 1h at room temperature. The membrane was then developed with Thermo ECL SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

**Lentiviral generation and infection**

Lentiviral particles for shControl, shFTO1 and shFTO2 were prepared by co-transfection of these shRNA plasmids with psPAX.2 (1.2 μg) and pMD2.G (0.6 μg) vectors in 293FT cells using Opti-MEM and Lipofectamine 2K transfection Reagent (Invitrogen). After overnight tranfection the supernatant was removed and DMEM/F12 medium with B27 and growth factor containing medium was added to the cells. Virus containing supernatants were collected 24–48 h
after transfection and filtered at 0.22 μm and stored at -80 °C. Generated shControl and shFTO lentivirus particles were used to infect TS576 cells in the presence of Polybrene (8 μg/ml) (Millipore). After 12h lentivirus containing medium was replaced with fresh medium and samples were collected after 72h of infection.

**Quantification of m⁶A and m⁶Am by HPLC-MS/MS**

Polyadenylated RNA was analyzed by LC-MS/MS/MS as described previously.¹²
Table S1. Inhibition Data for FTO Inhibitors against FTO and ALKBH5. ClogP and permeability parameters calculated by QikProp.

| Structure | Name | clogP (octanol/water) | Permeability (nm/s) | Enzymatic IC<sub>50</sub> FTO | Enzymatic IC<sub>50</sub> ALKBH5 |
|-----------|------|-----------------------|---------------------|-----------------------------|----------------------------------|
| ![Structure](image1.png) | FTO-1 | 2.04 | 873 | 427 | 41.7 ± 1.2 | > 40 |
| ![Structure](image2.png) | FTO-2 | 3.00 | 1338 | 677 | 2.18 ± 1.3 | 85.5 ± 5.7 |
| ![Structure](image3.png) | FTO-3 | 4.69 | 4410 | 2460 | ND | ND |
| ![Structure](image4.png) | FTO-4 | 2.00 | 632 | 562 | 3.39 ± 2.5 | 39.4 ± 3.1 |
| ![Structure](image5.png) | FTO-5 | 2.67 | 2880 | 1552 | 13.38 ± 2.3 | > 40 |
| ![Structure](image6.png) | FTO-6 | 2.30 | 1335 | 665 | 13.8 ± 2.4 | 64.4 ± 6.3 |
| ![Structure](image7.png) | FTO-7 | 2.27 | 2101 | 1104 | 29.1 ± 2.4 | > 40 |
| ![Structure](image8.png) | FTO-8 | 3.75 | 4411 | 2460 | 10.0 ± 1.8 | 16.4 ± 2.1 |
| ![Structure](image9.png) | FTO-9 | 2.79 | 624 | 297 | 43.8 ± 2.4 | 5.2 ± 2.9 |
| ![Structure](image10.png) | FTO-10 | 1.60 | 255 | 113 | 48.1 ± 3.5 | 36.1 ± 3.1 |

ND = Not determined
| Structure | Name | clogP (octanol/water) | Permeability (nm/s) | Enzymatic IC$_{50}$ FTO | Enzymatic IC$_{50}$ ALKBH5 |
|-----------|------|----------------------|---------------------|--------------------------|---------------------------|
| ![Structure](image1.png) | FTO-11 | 3.35 | 3218 | 1750 | 11.3 ± 1.1 | 19.5 ± 2.7 |
| ![Structure](image2.png) | FTO-12 | 2.48 | 842 | 411 | 18.3 ± 1.7 | > 40 |
| ![Structure](image3.png) | FTO-13 | 3.37 | 842 | 411 | 36.7 ± 3.1 | 14.9 ± 1.8 |
| ![Structure](image4.png) | FTO-14 | 2.11 | 615 | 292 | 59.6 ± 4.8 | > 40 |
| ![Structure](image5.png) | FTO-15 | 3.45 | 963 | 475 | ND | ND |
| ![Structure](image6.png) | FTO-16 | 2.89 | 292 | 130 | 46.5 ± 3.1 | > 40 |
| ![Structure](image7.png) | FTO-17 | 2.89 | 292 | 130 | 51.9 ± 4.7 | > 40 |
| ![Structure](image8.png) | FTO-18 | 3.69 | 963 | 475 | > 40 | > 40 |
| ![Structure](image9.png) | FTO-19 | 2.44 | 702 | 337 | 25.2 ± 4.9 | 53.5 ± 5.2 |
| ![Structure](image10.png) | FTO-20 | 1.21 | 287 | 128 | 17.2 ± 2.9 | 90.2 ± 7.8 |

ND = Not determined
Table S2. Calculated Physicochemical Data for MA, FB23, and FB23-2. ClogP and permeability parameters calculated by QikProp. Inhibition data for MA was obtained as described in the methods. Inhibition data for FB23 and FB23-2 against FTO and ALKBH5 is reported from Huang *et. al* 2019.

| Structure | clogP | Permeability (nm/s) | Enzymatic IC$_{50}$ | Enzymatic IC$_{50}$ |
|-----------|-------|---------------------|----------------------|----------------------|
| ![MA structure](image) | MA | 4.93 | 327 | 638 | 12.5 ± 1.8 | > 40 |
| ![FB23 structure](image) | FB23 | 4.96 | 97 | 210 | 0.06 | > 40 |
| ![FB23-2 structure](image) | FB23-2 | 3.46 | 240 | 428 | 2.6 | > 40 |
Figure S1. Predicted binding pose of FTO-01 at the MA binding site of FTO. A π-π stacking interaction is observed with Tyr 108.
Figure S2. Predicted binding pose of FTO-02 at the MA binding site of FTO. A water mediated hydrogen bond is expected between the pyrimidine ring of FTO-02 and the backbone of Glu 234. A $\pi-\pi$ stacking interaction is observed with His 231.
Figure S3. Predicted binding pose of FTO-03 at the MA binding site of FTO. A π–π stacking interaction is observed with His 231, and a hydrogen bonding interaction is expected between Arg 322 and the pyrimidine ring of FTO-03.
Figure S4. Predicted binding pose of FTO-04 at the MA binding site of FTO. A π-π stacking interaction is observed with His 231, and a hydrogen bonding interaction is expected between Arg 96 and the benzothiazole ring of FTO-04.
Figure S5. Predicted binding pose of FTO-05 at the MA binding site of FTO. A π-π stacking interaction is observed with Tyr 108.
Figure S6. Predicted binding pose of FTO-06 at the MA binding site of FTO. A hydrogen bond is observed between Arg 322 and the pyrimidine ring of FTO-06. A π-π stacking interaction is observed with His 231.
Figure S7. Predicted binding pose of FTO-07 at the MA binding site of FTO. A water-mediated hydrogen bond is observed between the backbone of Glu 234 and the nitrogen atom of the 2-methylquinoline ring. A π-π stacking interaction is observed with Tyr 108.
Figure S8. Predicted binding pose of FTO-08 at the MA binding site of FTO. A hydrogen bond is observed between Arg 322 and the oxygen atom of the 2-methoxypyrimidine ring.
Figure S9. Predicted binding pose of FTO-09 at the MA binding site. The pyrimidine ring is observed to form a hydrogen bond to Arg 322, and a π-π stacking interaction with His 231.
Figure S10. Predicted binding pose of FTO-10 at the MA binding site of FTO. A water-mediated hydrogen bond is observed between Glu 234 and the pyrimidine ring of FTO-10. A hydrogen bond is observed between the amino group of the 2-aminopyrimidine and Tyr 106.
Figure S11. Predicted binding pose of FTO-11 at the MA binding site of FTO. A hydrogen bond is observed between Arg 322 and the nitrogen atom of the 2-methylquinoline ring.
Figure S12. Predicted binding pose of FTO-12 at the MA binding site of FTO. A hydrogen bond is observed between the pyrimidine ring of FTO-12 and Arg 322.
Figure S13. Predicted binding pose of FTO-13 at the MA binding site of FTO. A benzene ring in FTO-13 is observed to form π-π stacking interactions with His 231 and the pyrimidine ring is predicted to form a hydrogen bond with Arg 322.
Figure S14. Predicted binding pose of FTO-14 at the MA binding site of FTO. A hydrogen bond is observed between the amino group of the 2-aminopyrimidine ring of FTO-14 and Tyr 106.
Figure S15. Predicted binding pose of FTO-15 at the MA binding site of FTO. The pyrimidine ring of FTO-15 is predicted to form a hydrogen bond to Arg 322. Tyr 295 and Arg 316 are observed to form a bifurcated hydrogen bond to the alcohol group of FTO-15.
Figure S16. Predicted binding pose of FTO-16 at the MA binding site of FTO. A π-π stacking interaction is observed between His 231 and the pyrimidine ring of FTO-16. Arg 322 is predicted to form a hydrogen bond to the alcohol group of FTO-16.
Figure S17. Predicted binding pose of FTO-17 at the MA binding site of FTO. A π-π stacking interaction is observed between His 231 and the napthol ring of FTO-17. The backbone of Met 226 is predicted to accept a hydrogen bond from the alcohol group of FTO-17.
Figure S18. Predicted binding pose of FTO-18 at the MA binding site of FTO. A benzene ring of FTO-18 is observed to form π-π stacking interactions with His 231 and Tyr 108, and the pyrimidine ring of FTO-18 is expected to form a hydrogen bond to Arg 322. Tyr 295 and Arg 316 are predicted to form a bifurcated hydrogen bond to the alcohol group of FTO-18.
Figure S19. Predicted binding pose of FTO-19 at the MA binding site of FTO. A water-mediated hydrogen bond is observed between the backbone of Glu 234 and the nitrogen atom of the 2-methylquinoline ring of FTO-19. A π-π stacking interaction is observed between the quinoline ring and Tyr 108.
Figure 20. Predicted binding pose of FTO-20 at the MA binding site of FTO. A π-π stacking interaction is observed with His 231, and hydrogen bonds are predicted with Arg 322 and Arg 316.
Figure S21. Inhibition of FTO by meclofenamic acid. The observed IC\textsubscript{50} value of 12.5 µM is comparable to literature values.

Figure S22. Demethylation Assay Negative Control. FTO-1-20 do not significantly alter fluorescent signal of the demethylated Broccoli-DHBI-1T complex.
Figure S23. DMSO Control for Demethylation Assays. DMSO does not significantly impair enzyme function or fluorescent signal until concentrations exceed >1%.

| DMSO Concentration (%) | Normalized Activity (%) | Standard Deviation |
|------------------------|-------------------------|--------------------|
| 0                      | 100                     |                    |
| 0.1                    | 101.251                 | 3.1804             |
| 0.2                    | 100.092                 | 1.933              |
| 0.5                    | 98.781                  | 1.9726             |
| 1                      | 92.3781                 | 2.525              |
| 5                      | 80.1264                 | 1.16               |
| 10                     | 58.3666                 | 5.6681             |

Figure S24. Inactive FTO controls. A. Normalized activity of wt FTO and inactive FTO in the presence of 0-40 µM FTO-02. B. Normalized activity of wt FTO and inactive FTO in the presence of 0-40 µM FTO-04.
Figure S25. IC₅₀ curves for FTO-02 and FTO-04 against FTO by ELISA Assay

Figure S26. Velocity plots for FTO-02 and FTO-04. A. FTO-02 approaches a common $v_{\text{max}}$ for all concentrations of inhibitor, consistent with a competitive mechanism of inhibition. B. FTO-04 approaches a common $v_{\text{max}}$ for all concentrations of inhibitor, indicating FTO-04 is a competitive inhibitor.
Figure S27. Effects of FTO knockdown on neurosphere size in TS576 cells. A. Representative images of TS576 cells derived neurosphere after lentivirus knocking down of FTO (shControl and shFTO) B. Neurosphere size was quantified by ImageJ and the size distribution is shown in control and FTO KD group. Box and whisker plots show 10–90 percentile. N > 50 neurospheres per group. **p < 0.01 by Student’s t test. C. qRT-PCR showing lentivirus KD efficiency of FTO in TS576.
Figure S28. m^6A mRNA dot blot assays of TS576 treated with shFTO, DMSO, or FTO-04. A. m^6A dot blot assays using poly(A)^+ mRNA of TS576 glioblastoma cells knockdown with shControl and shFTO lentivirus. B. m^6A dot blot assays using poly(A)^+ mRNA of TS576 glioblastoma cells knockdown with DMSO and FTO-04.
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NMR Spectra
600 MHz (CD$_3$)$_2$SO
FTO-02
600 MHz (CD$_3$)$_2$SO
FTO-02
600 MHz (CD$_3$)$_2$SO FTO-06
600 MHz (CD$_3$)$_2$SO
FTO-09
125 MHz (CD$_3$)$_2$SO
FTO-10
600 MHz (CD$_3$)$_2$SO
FTO-12
150 MHz (CD$_3$)$_2$SO
FTO-16
600 MHz (CD$_3$)$_2$SO
FTO-18
$600 \text{ MHz } (\text{CD}_3)_2\text{SO}$

FTO-20
