Supplementary methods for "Pharmacological targeting of MTHFD2 suppresses acute myeloid leukemia by inducing thymidine depletion and replication stress"

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Abstract

Supplementary methods for the article "Pharmacological targeting of MTHFD2 suppresses acute myeloid leukemia by inducing thymidine depletion and replication stress".

Procedure

Modified comet assay with uracil-DNA glycosylase (UNG). Briefly, THP-1 cells were treated with DMSO, MTHFD2i (TH7299 3 μM, TH9028 50 nM, TH9619 50 nM) or reference compounds (all-*trans* retinoic acid, ATRA 1 μM, methotrexate, MTX 50 nM) for 16 h, harvested and washed in PBS, then mixed with 1.2% low-melting-point agarose (Sigma Aldrich) at 37 °C and embedded on 1% agarose-coated Superfrost slides (ThermoFisher Scientific). Subsequent steps were carried out away from light. Slides were submerged in lysis buffer (2.25 M NaCl, 115 mM EDTA, 9 mM Tris, 10% DMSO, 1% Triton X-100, NaOH to pH 10) for 2 h at room temperature, washed 3x with enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 200 mg/L BSA, KOH to pH 8), then treated with uracil-DNA glycosylase (New England Biolabs, UNG 0.1 unit/slide diluted in enzyme buffer) or enzyme buffer only for 1 h at 37 °C. Slides were denatured in electrophoresis buffer (1 mM EDTA, 300 mM NaOH) for 30 min at room temperature, then subjected to 25 V and 300 mA for 30 min at 4 °C in a comet assay tank (Thistle Scientific), and followed by incubation in neutralization buffer (400 mM Tris, HCl to pH 7.5) for 45 min at room temperature. For image acquisition, slides were stained with SYBR® Gold Nucleic Acid Gel Stain (ThermoFisher) and visualized at 488 nm and 10x magnification using a Zeiss Axiovert 35 inverted fluorescence microscope. Comet tail moment for 200 cells per condition was measured live using Comet Assay IV software (Instem).

Recombinant proteins. Cloning, expression, and purification of human MTHFD2 and MTHFD1 dehydrogenase/cyclohydrolase (DC) proteins were performed as described in Gustafsson, R. *et al.* Cancer Res. 2017 Feb 15;77(4):937-948 (doi: 10.1158/0008-5472.CAN-16-1476). Mouse MTHFD2 was produced as described for human MTHFD2. MTHFD2L cDNA encoding protein lacking mitochondrial signal peptide (AA 50-347) was cloned into pET22b vector and the construct was transformed into *E. coli* Arctic Express (DE3) competent cells. The protein expression was induced by 0.5 mM IPTG at 13° C for 20 hours. Collected cells were dissolved in BugBuster protein extraction reagent (Merck/Novagen) supplemented with complete protease inhibitor cocktail (Roche), Benzonase® Nuclease (Novagen) and 0.35 mg/ml lysozyme (Sigma-Aldrich). After rotation for 20 min at RT the suspension was centrifuged, and the supernatant was applied on a Talon column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol buffer. The bound proteins were eluted with linear 0 mM-500 mM imidazole gradient. The fractions containing MTHFD2L were dialyzed against 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 0.5 mM TCEP buffer. His-tag was cleaved off and removed by passing the protein over a HisTrap column. MTHFD2L was dialyzed against 20 mM Tris-HCl, pH 8.2, 20 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM TCEP buffer and loaded onto an anion-exchange monoQ-HP column (GE Healthcare) equilibrated with the same buffer. The bound proteins were eluted with linear 20

mM - 800 mM KCl gradient. Fractions with purified MTHFD2L were combined, protein concentration was determined by Bradford assay with Coomassie Plus Protein Kit (Thermo Scientific) and BSA as protein standard. MTHFD2L protein was stored at -20 °C in 20 mM Tris-HCl, pH 8.2, 100 mM KCl, 5 mM $MgCl_2$, 1 mM TCEP, 40% glycerol buffer. SHMT1, SHMT2, and TYMS proteins were purified by the Protein Science Facility at the Karolinska Institutet. DHFR was purchased from Sigma Aldrich. Uracil DNA glycosylase was purchased from New England Biolabs (Cat. # M0280).

MTHFD2 routine inhibition assay. To determine IC_{50} values of a compound, an 11-point dose-response curve with 3-fold difference in concentration between points was generated by using an acoustic dispenser from Labcyte (Echo 550 Liquid handler). Each assay point was run in duplicate and the assay was performed in a white ProxiPlate low-volume 384-well plates (Perklin Elmer, Cat # 6008280). DMSO was used as negative control. The serial dilution in DMSO, from compound DMSO stock solution, was created by dispensing from a 384-well low dead volume microplate (Labcyte, Cat # LP-0200,) and a 384-well polypropylene microplate 2.0 (Labcyte, Cat # PP-0200,). The assay buffer consisted of 50 mM Tris (pH 8.0), 100 mM NaCl, 25 mM NaPi, 5 mM $MgCl_2$, 2 mM 2-Mercaptoethanol, 250 µM NAD^+ and 0.005% Tween 20. NAD(P)H-Glo™ detection reagent was purchased from Promega (Cat # G9061), NAD^+ from Sigma (Cat # N7004) and folitixorin from Toronto Research Chemicals (Cat # F680350). Working solution of folitixorin (0.2% DMSO) contained 10 µM folitixorin in assay buffer. MTHFD2 protein was diluted to 6.84 nM (0.24 µg/mL) in assay buffer. A total of 2.5 µL 6.84 nM enzyme was preincubated with compound or DMSO for 10 minutes. The enzymatic reaction was initiated by adding 2.5 µL 10 µM folitixorin. For background control, 5 µL 250 µM NAD^+ buffer was added to the wells in column 24. After 15 minutes reaction, 5 µL NAD(P)H-Glo detection reagent (Cat # G9061) was dispensed in all wells and the plate was incubated for 60 minutes. Luminescence was measured in a Hidex Sense plate reader for 1 sec / well. The light signal produced is proportional to the amount of NAD(P)H in the sample. IC_{50} values were calculated using a four Parameter Logistic Model or Sigmoidal Dose-Response Model in XLfit 5 (IDBS Software).

MTHFD2L inhibition assay. The assay buffer consisted of 50 mM Tris (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 2 mM 2-Mercaptoethanol, 5% Glycerol, 100 µM $NADP^+$ and 0.005% Tween 20. $NADP^+$ was purchased from Sigma (Cat # N5755). Working solution of folitixorin (2% DMSO) contained 80 µM folitixorin in assay buffer. MTHFD2L protein was diluted to 200 nM (6.5 µg/mL) in assay buffer. The assay was performed as for MTHFD2 but in white non-binding surface 384-well plates (Corning, Cat # 3824). For background control, 5 µL 100 µM $NADP^+$ and 40 µM folitixorin in assay buffer were added to control wells.

MTHFD1 inhibition assay. The assay buffer consisted of 25 mM MOPS (pH 7.3), 0.5 mM TCEP, 400 µM $NADP^+$ and 0.005% TritonX100. $NADP^+$ was purchased from Sigma (Cat # N5755). Working solution of folitixorin (1.2% DMSO) contained 60 µM folitixorin in assay buffer. MTHFD2L protein was diluted to 0.5 nM (0.017 µg/mL) in assay buffer. The assay was performed as for MTHFD2 but in white non-binding surface 384-well plates (Corning, Cat # 3824) and with the addition of 1 µL menadione 2.75 mM (Sigma, Cat # M5625) in 20% DMSO 10 min before reading. For background control, 5 µL 400 µM $NADP^+$ and 30 µM folitixorin in assay buffer were added to control wells.

DHFR inhibition assay. Inhibition of DHFR was measured using the Dihydrofolate Reductase Assay Kit purchased from Sigma Aldrich (Cat # CS0340) following manufacturer's instructions. Compounds were dissolved to 10 mM stock solutions in 10x assay buffer provided in the kit and screened at final concentrations of 100 µM. Methotrexate 1 µM was used as positive control, which gave 100% inhibition.

TYMS inhibition assay. Inhibition of TYMS was measured as previously described[76]. Enzymatic activity was measured spectrophotometrically at 340 nm by monitoring the absorbance change during the conversion of 5,10-methylenetetrahydrofolate to dihydrofolate using a Hidex Sense plate reader. Measurements were carried out at room temperature in a buffer of 50 mM Tris at pH 7.5 and 150 mM NaCl. Initial reaction rates were measured with 50 nM of purified protein, 250 µM 5,10-methylenetetrahydrofolate and 100 µM dUMP in the presence of compounds (100 µM) using the same amount of DMSO as in control. Raltitrexed (100 µM) was used as positive control, giving 100% inhibition. Initial rates and activity were analyzed using Prism (GraphPad Software).

SHMT1/2 inhibition assays. Inhibition of SHMT1 and SHMT2 was measured as previously described[77]. Compounds were tested at 100 µM and lometrexol (100 µM) was used as positive control, yielding 63% and 50% inhibition of SHMT1 and SHMT2 respectively.

MTHFD2 high-throughput screening (HTS)

The assay buffer consisted of 50 mM Tris (pH 8.0), 100 mM NaCl, 25 mM NaPi, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.005% Tween 20 and 0.03% BSA. NAD(P)H-Glo™ detection reagent was purchased from Promega (Cat # G9062), NAD^+ from Sigma (Cat # N7004) and folitixorin from Toronto Research Chemicals (Cat # F680350). NAD^+ buffer was prepared by dissolving 250 µM NAD^+ in assay buffer. DMSO stock of folitixorin comprised 0.5 mM folitixorin in DMSO. Working solution of folitixorin (2% DMSO) contained 10 µM folitixorin in assay buffer. MTHFD2 protein was diluted to 0.2 µg/ml in NAD^+

buffer. The assay was then run according to the following consecutive steps: (1) 5 nL 10 mM compounds were transferred using the Echo550 acoustic dispenser into 384-ProxiPlates in columns 1-22. In column 23, 5 nL and 50 nL of 10 mM LY345899 in DMSO were transferred to 3 wells each to give final concentrations of 10 µM and 100 µM in triplicate. DMSO 5 nL was transferred to the remaining wells in column 23 (DMSO wells) and to column 24 (background wells); (2) A 0.5 mM folitixorin solution was then prepared in DMSO; (3) 250 µM NAD^+ solution was prepared in the assay buffer described above; (4) The 0.5 mM folitixorin DMSO solution was diluted 50 times in NAD^+ buffer to a final concentration of 10 µM; (5) the MTHFD2 enzyme was diluted to 0.2 µg/ml in the NAD^+ buffer and kept cold on ice; (6) 5 µl NAD^+ buffer was dispensed in the background wells of column 24, and the plates were kept on ice, until the assay was run at room temperature; (7) 2.5 µl 0.2 µg/ml enzyme in NAD^+ buffer was dispensed with a Multidrop dispenser in columns 1-23. The plates were centrifuged at 1000 rpm 1 min, and the mixture was allowed to pre-incubate for 10 min; (8) 2.5 µl of 10 µM folitixorin was then added with the Multidrop dispenser in columns 1-23, and the mixture was incubated for 15 min; (10) 5 µl of Promega NAD(P)H-Glo™ detection reagent (mixed according to manufacturer) was then dispensed in all wells using a Multidrop dispenser; (11) the detection mixtures was incubated for 1 h; (12) luminescence was read using a Hidex Sense plate reader.

Synthesis of TH9028

Methyl (2S)-2-[(5-aminopyridin-2-yl)formamido]-4-[1-(2-cyanoethyl)-1H-1,2,3,4-tetrazol-5-yl]butanoate (3)

Et_3N (1.0 mL, 7.3 mmol) was added to a stirred mixture of methyl (2S)-2-amino-4-[1-(2-cyanoethyl)-1H-1,2,3,4-tetrazol-5-yl]butanoate hydrochloride (0.94 g, 3.3 mmol) (synthesis has previously been described in *Chem. Pharm. Bull. 43(2) 230-235 (1995)*), 5-aminopyridine-2-carboxylic acid (0.34 g, 2.4 mmol), EDC-HCl (0.94 g, 4.9 mmol), HOBt (0.75 g, 4.9 mmol) and acetonitrile (50 mL). The reaction was stirred at r.t. over night and the solvent was removed under reduced pressure. The material was portioned between EtOAc (200 mL) and sat. $NaHCO_3$ (100 mL). The organic phase was washed with water (25 mL) and concentrated. The product was purified by flash chromatography (50 g silica, 5% MeOH in DCM as eluent). This gave methyl (2S)-2-[(5-aminopyridin-2-yl)formamido]-4-[1-(2-cyanoethyl)-1H-1,2,3,4-tetrazol-5-yl]butanoate (0.62 g, 53%). LCMS $[M+H]^+$ *m/z* 359; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 2.25 - 2.43 (m, 2 H), 2.92 - 3.00 (m, 2 H), 3.11 - 3.17 (m, 2 H), 3.64 (s, 3 H), 4.58 - 4.66 (m, 3 H), 6.02 (s, 2 H), 6.97 (dd, *J*=8.5, 2.8 Hz, 1 H), 7.69 (dd, *J*=8.5, 0.4 Hz, 1 H), 7.95 (dd, *J*=2.7, 0.6 Hz, 1 H), 8.66 (d, *J*=8.4 Hz, 1 H)

(2S)-2-[(5-{[(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)carbamoyl]amino}pyridin-2-yl)formamido]-4-(1H-1,2,3,4-tetrazol-5-yl)butanoic acid (TH9028)

methyl (2S)-2-[(5-aminopyridin-2-yl)formamido]-4-[1-(2-cyanoethyl)-1H-1,2,3,4-tetrazol-5-yl]butanoate (0.56 g, 1.6 mmol) was dissolved in dry MeCN (2 mL) and added to a solution of 4-nitrophenyl chloroformate (0.31 g, 1.6 mmol) in dry MeCN (1 mL). The reaction was heated in a sealed tube for 1 hour at 50°C and for 90 min at 80°C. A second solution was made by adding 5M NaOH (1.9 mL, 9.4 mmol) to a stirred mixture of 2,5,6-triamino-3,4-dihydropyrimidin-4-one; sulfuric acid (0.75 g, 3.1 mmol) and water (3 mL). The warm organic mixture was added to the stirred water solution at r.t. over a period of 30 seconds. The mixture was stirred in an ice-bath for 30 min and the product was collected by filtration. The material was washed with water (2x1 mL), dried and dissolved in DMSO. The intermediate ester (4) was purified by acidic prep-HPLC. The pure fractions were combined, and the solvents were removed under reduced pressure. Gave methyl (2S)-4-[1-(2-cyanoethyl)-1H-1,2,3,4-tetrazol-5-yl]-2-[(5-{[(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)carbamoyl]amino}pyridin-2-yl)formamido]butanoate (0.63 g). The material was dissolved in a mixture of water (4 mL) and 5M NaOH (1.3 mL). The reaction was stirred at r.t. for 90 min. The mixture was cooled in an ice-bath and 12M HCl was added until pH ~2 and the product was collected by filtration. The product was washed with cooled water (2x2 mL) and dried in a vacuum oven (40°C) over night. This gave (2S)-2-[(5-{[(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)carbamoyl]amino}pyridin-2-yl)formamido]-4-(1H-1,2,3,4-tetrazol-5-yl)butanoic acid (210 mg, 29%). LCMS $[M+H]^+$ *m/z* 459; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.20 - 2.32 (m, 1 H), 2.32 - 2.44 (m, 1 H), 2.87 - 3.01 (m, 2 H), 4.50 (td, *J*=8.6, 4.8 Hz, 1 H), 6.00 (br. s., 2 H), 6.25 (br. s., 2 H), 6.87 (br. s., 1 H), 7.92 (d, *J*=8.5 Hz, 1 H), 8.07 (d, *J*=7.9 Hz, 1 H), 8.69 - 8.79 (m, 2 H), 9.17 (br. s., 1 H), 10.06 (br. s., 1 H), 12.94 (br. s., 1 H), 16.00 (br. s., 1 H)

Synthesis of TH9619

Methyl 5-{[(tert-butoxy)carbonyl]amino}-3-fluoropyridine-2-carboxylate (6)

A mixture of methyl 5-bromo-3-fluoropyridine-2-carboxylate (2.4 g, 10 mmol), tert-butyl carbamate (1.4 g, 12 mmol), Cs_2CO_3 (3.9 g, 12 mmol) and dry dioxane (50 mL) was flashed six times with nitrogen. $Pd(OAc)_2$ (110 mg, 0.50 mmol) and X-Phos (480 mg, 1.0 mmol) were added and the mixture was again flashed six times with nitrogen. The sealed reaction was heated at 90°C for 21 hours. Brine (50 mL) and water (50 mL) were added and the mixture was extracted with EtOAc. The organic solvent was removed under reduced pressure and the product was purified by flash chromatography (EtOAc/iso-hexane 25/75 to 50/50 as eluent). This gave methyl 5-{[(tert-butoxy)carbonyl]amino}-3-fluoropyridine-2-carboxylate (2.1 g, 76%). LCMS $[M+H]^+$ *m/z* 271; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.49 (s, 9H), 3.85 (s, 3H), 7.92 (dd, 1H), 8.50 (t, 1H), 10.28 (br s, 1H).

5-{[(tert-Butoxy)carbonyl]amino}-3-fluoropyridine-2-carboxylic acid (7)

LiOH (1.9 g, 79 mmol) was added to a stirred mixture of methyl 5-{[(tert-butoxy)carbonyl]amino}-3-fluoropyridine-2-carboxylate (3.6 g, 13 mmol), THF (37 mL) and water (13 mL). The reaction was refluxed for 30 min. and then allowed to cool to rt. The mixture was cooled in an ice-bath and the pH was adjusted to ~2 with 2M HCl. The water mixture was extracted with EtOAc. The combined organic solvents were dried over Na_2SO_4 and removed in a rotavapor. This gave 5-{[(tert-butoxy)carbonyl]amino}-3-fluoropyridine-2-carboxylic acid (2.9 g, 85%). LCMS $[M+H]^+$ *m/z* 257; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.49 (s, 9H), 7.88 (dd, 1H), 8.48 (d, 1H), 10.22 (br s, 1H), 13.17 (br s, 1H).

1,5-Diethyl (2S)-2-[(5-{[(tert-butoxy)carbonyl]amino}-3-fluoropyridin-2-yl)formamido]pentanedioate (8)

HATU (3.7 g, 9.8 mmol) was added to a stirred mixture of 5-{[(tert-butoxy)carbonyl]amino}-3-fluoropyridine-2-carboxylic acid (2.1 g, 8.2 mmol), diethyl (2S)-2-aminopentanedioate hydrochloride (3.0 g, 13 mmol), Et_3N (5.7 mL, 41 mmol) and dry THF (100 mL). The reaction was stirred for 3 hours at r.t. EtOAc (150 mL) was added and the mixture was washed with 0.5M HCl (100 mL) and sat. $NaHCO_3$ (100 mL). The organic phase was dried over Na_2SO_4 and the solvents were removed under reduced pressure. The material was purified by flash chromatography (100g silica, 0-50% EtOAc in iso-hexane). This gave 1,5-diethyl (2S)-2-[(5-{[(tert-butoxy)carbonyl]amino}-3-fluoropyridin-2-yl)formamido]pentanedioate (2.0 g, 56%). LCMS $[M+H]^+$ *m/z* 442; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.11 - 1.23 (m, 6 H), 1.49 (s, 9 H), 1.97 - 2.19 (m, 2 H), 2.33 - 2.44 (m, 2 H), 4.02 (q, *J*=7.1 Hz, 2 H), 4.07 - 4.16 (m, 2 H), 4.42 - 4.51 (m, 1 H), 7.91 (dd, *J*=13.4, 1.7 Hz, 1 H), 8.49 (dd, *J*=1.9, 1.1 Hz, 1 H), 8.72 (d, *J*=7.9 Hz, 1 H), 10.18 (s, 1 H).

1,5-Diethyl (2S)-2-[(5-amino-3-fluoropyridin-2-yl)formamido]pentanedioate (9)

TFA (10 mL) was added to a solution of 1,5-diethyl (2S)-2-[(5-{[(tert-butoxy)carbonyl]amino}-3-fluoropyridin-2-yl)formamido]pentanedioate (1.8 g, 4.1 mmol) in DCM (6 mL). The reaction was stirred at r.t. for 2 hours. The solvents were removed in a rotavapor and the material was dissolved in EtOAc (100 mL). The solvent was washed with sat. Na_2CO_3, dried over Na_2SO_4 and removed under reduced pressure to afford 1,5-diethyl (2S)-2-[(5-amino-3-fluoropyridin-2-yl)formamido]pentanedioate (1.32 g, 95%). LCMS $[M+H]^+$ *m/z* 342; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.11 - 1.21 (m, 6 H), 1.95 - 2.16 (m, 2 H), 2.30 - 2.41 (m, 2 H), 4.02 (q, *J*=7.1 Hz, 2 H), 4.06 - 4.15 (m, 2 H), 4.38 - 4.48 (m, 1 H), 6.36 (s, 2 H), 6.71 (dd, *J*=13.9, 2.2 Hz, 1 H), 7.78 - 7.84 (m, 1 H), 8.37 (d, *J*=8.1 Hz, 1 H).

1,5-Diethyl (2S)-2-{[5-(2-chloroacetamido)-3-fluoropyridin-2-yl]formamido}pentanedioate (10)

2-Chloroacetyl chloride (3.2 mL, 40 mmol) was added to a stirred solution of 1,5-diethyl (2S)-2-[(5-amino-3-fluoropyridin-2-yl)formamido]pentanedioate (12 g, 36 mmol) and Et_3N (6.1 mL, 44 mmol) in DCM (100 mL) at r.t. The reaction was stirred for 30 min and the mixture was washed with diluted Na_2CO_3. The organic phase was dried over Na_2SO_4 and removed under reduced pressure. The product was purified by flash chromatography (30-50% EtOAc in iso-hexane as eluent). This gave 1,5-diethyl (2S)-2-{[5-(2-chloroacetamido)-3-fluoropyridin-2-yl]formamido}pentanedioate (11 g, 74%). LCMS $[M+H]^+$ *m/z* 418; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 1.13 - 1.22 (m, 6 H), 1.97 - 2.19 (m, 2 H), 2.34 - 2.44 (m, 2 H), 4.03 (q, *J*=7.2 Hz, 2 H), 4.08 - 4.17 (m, 2 H), 4.37 (s, 2 H), 4.41 - 4.51 (m, 1 H), 8.12 (dd, *J*=13.0, 1.9 Hz, 1 H), 8.60 (dd, *J*=1.9, 1.1 Hz, 1 H), 8.83 (d, *J*=7.9 Hz, 1 H), 11.03 (s, 1 H).

(2S)-2-({5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridin-2-yl}formamido)pentanedioic acid (TH9619)

2,4-Diamino-1H-pyrimidin-6-one (3.3 g, 26 mmol), $NaHCO_3$ (2.2 g, 26 mmol) and NaI (14 g, 96 mmol) were added to a stirred solution of 1,5-diethyl (2S)-2-{[5-(2-chloroacetamido)-3-fluoropyridin-2-yl]formamido}pentanedioate (10 g, 24 mmol) in DMF (75 mL). The reaction was stirred in a sealed flask at r.t. for 2 days. Brine (225 mL) was added to the reaction and the intermediate ester was collected by filtration and washed with water and MeCN. LCMS $[M+H]^+$ *m/z* 508. The material was suspended in water (200 mL) and 5M NaOH (29 mL, 140 mmol) was added. The reaction was stirred at r.t. for 30 min. The pH was adjusted to ~3 with 1M HCl and the product was collected by filtration and washed with water and MeCN. The material was dried under vacuum (40°C) over night. This gave (2S)-2-({5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridin-2-yl}formamido)pentanedioic acid (6.5 g, 60%). LCMS $[M+H]^+$ *m/z* 452; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 1.91 - 2.03 (m, 1 H), 2.03 - 2.15 (m, 1 H), 2.25 - 2.34 (m, 2 H), 3.33 (s, 2 H), 4.39 (td, *J*=8.5, 5.1 Hz, 1 H), 5.97 (s, 2 H), 6.15 (br. s., 2 H), 8.12 (dd, *J*=13.5, 2.0 Hz, 1 H), 8.57 - 8.61 (m, 1 H), 8.64 (d, *J*=7.9 Hz, 1 H), 10.05 (br. s., 1 H), 10.65 (s, 1 H), 12.44 (br. s., 2 H). $[\alpha]^{25}_D$ +10.9° (c = 0.13, 0.2M Na_2CO_3 aq).

Synthesis of TH11737

Methyl 5-amino-3-fluoropyridine-2-carboxylate (12)

TFA (2 mL) was added to a solution of 5-{[(tert-butoxy)carbonyl]amino}-3-fluoropyridine-2-carboxylic acid (190 mg, 0.70 mmol) in DCM (2 mL). The reaction was stirred at r.t. for 2 hours. The solvents were removed under reduced pressure and the material was added EtOAc (10 mL). The solvent was washed with sat. Na_2CO_3, dried over Na_2SO_4 and removed under reduced pressure to afford methyl 5-amino-3-fluoropyridine-2-carboxylate (110 mg, 92%). LCMS $[M+H]^+$ *m/z* 171; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 3.76 (s, 3 H), 6.54 (s, 2 H), 6.65 - 6.73 (m, 1 H), 7.81 - 7.86 (m, 1 H).

Methyl 5-(2-chloroacetamido)-3-fluoropyridine-2-carboxylate (13)

2-Chloroacetylchloride (0.57 mL, 7.1 mmol) was added to a stirred solution of methyl 5-amino-3-fluoropyridine-2-carboxylate (0.93 g, 5.5 mmol) and Et_3N (2.3 mL, 16 mmol) in dry THF (50 mL) at r.t. The reaction was stirred at r.t. for 30 min and sat. Na_2CO_3 (20 mL) was added. The mixture was extracted with EtOAc (100 mL). The organic phase was dried over Na_2SO_4 and removed under reduced pressure. The crude material was suspended in MeOH (5 mL) and product with improved purity was collected by filtration. Gave methyl 5-(2-chloroacetamido)-3-fluoropyridine-2-carboxylate (0.69 g, 51%). LCMS $[M+H]^+$ *m/z* 247; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 3.86 (s, 3 H), 4.37 (s, 2 H), 8.18 (dd, *J*=12.9, 2.0 Hz, 1 H), 8.59 (dd, *J*=2.0, 1.2 Hz, 1 H), 11.10 (s, 1 H).

Methyl 5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridine-2-carboxylate (14)

2,4-Diamino-1H-pyrimidin-6-one (250 mg, 2.0 mmol), $NaHCO_3$ (170 mg, 2.0 mmol) and NaI (820 mg, 5.5 mmol) were added to a stirred solution of methyl 5-(2-chloroacetamido)-3-fluoropyridine-2-carboxylate (450 mg, 1.8 mmol) in DMF (1 mL). The reaction was stirred in a sealed flask at r.t. over night. DMF (1 mL) and water (2 mL) were added to the reaction mixture and the product was collected by filtration and washed with water, 1:1 DMF/water and MeCN. The material was dried under vacuum (40°C) for 30 min. Gave methyl 5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridine-2-carboxylate (500 mg, 82%). LCMS $[M+H]^+$ *m/z* 337; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 3.32 (s, 2 H), 3.84 (s, 3 H), 5.84 (br. s., 2 H), 6.37 (br. s., 2 H), 8.16 (dd, *J*=13.3, 2.0 Hz, 1 H), 8.53 - 8.56 (m, 1 H), 10.90 (br. s., 2 H).

5-[2-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridine-2-carboxylic acid (15)

1M NaOH (5 mL), water (5 mL) and methyl 5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridine-2-carboxylate (500 mg, 1.5 mmol) were stirred at r.t. for 30 min. The reaction mixture was filtered and the pH was adjusted to ~2 with 2M and 1M HCl. The product was collected by filtration and washed with water and MeCN. Gave 5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridine-2-carboxylic acid (240 mg, 47%). LCMS $[M+H]^+$ *m/z* 323; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 3.33 (s, 2 H), 5.95 (s, 2 H), 6.11 (s, 2 H), 8.13 (dd, *J*=13.3, 1.9 Hz, 1 H), 8.56 - 8.60 (m, 1 H), 9.99 (br. s., 1 H), 10.65 (s, 1 H), 13.16 (br. s., 1 H).

(2R)-2-({5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridin-2-yl}formamido)pentanedioic acid (TH11737)

Et_3N (0.22 mL, 1.6 mmol), HOBt (61 mg, 0.465 mmol) and EDC-HCl (89 mg, 0.465 mmol) were added to a stirred mixture of 5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridine-2-carboxylic acid (100 mg, 0.31 mmol), 1,5-dimethyl (2R)-2-aminopentanedioate hydrochloride (79 mg, 0.37 mmol) and DMSO (3 mL). The reaction was stirred in a sealed tube at r.t. over night. The intermediate ester (16) was purified by acidic prep-HPLC. The pure fractions were combined and the solvents were removed under reduced pressure. Gave 1,5-dimethyl (2R)-2-({5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridin-2-yl}formamido)pentanedioate (45 mg). LCMS $[M+H]^+$ *m/z* 480. The material was dissolved in 1M NaOH (1.5 mL). The reaction was stirred at r.t. for 10 min. 1M HCl was added until pH ~2 and the product was collected by filtration. The product was washed with water and dried in a vacuum oven (40°C) over night. Gave (2R)-2-({5-[2-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetamido]-3-fluoropyridin-2-yl}formamido)pentanedioic acid (18 mg, 13%). LCMS $[M+H]^+$ *m/z* 452; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 1.89 - 2.03 (m, 1 H), 2.05 - 2.17 (m, 1 H), 2.26 - 2.34 (m, 2 H), 3.35 (s, 2 H), 4.36 - 4.46 (m, 1 H), 6.22 (br. s., 2 H), 6.54 (br. s., 2 H), 8.11 (dd, *J*=13.3, 2.0 Hz, 1 H), 8.61 (dd, *J*=1.8, 1.2 Hz, 1 H), 8.65 (d, *J*=8.1 Hz, 1 H), 10.35 (br. s., 1 H), 10.66 (s, 1 H), 12.40 (br. s., 2 H). $[\alpha]^{25}_D$ -11.6° (c = 0.13, 0.2M Na_2CO_3 aq).

Abbreviations

aq	aqueous
Cs_2CO_3	cesium carbonate
DCM	dichloromethane
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
Et_3N	triethylamine
EtOAc	ethyl acetate
HCl	hydrochloride
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography

LCMS	liquid-chromatography electrospray mass spectroscopy
LiOH	lithium hydroxide
MeCN	acetonitrile
MeOH	methanol
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NaI	sodium iodide
Na_2CO_3	sodium carbonate
Na_2SO_4	sodium sulfate
NMR	nuclear magnetic resonance
$Pd(OAc)_2$	palladium(II) acetate
r.t.	room temperature
sat.	saturated
TFA	trifluoroacetic acid
THF	tetrahydrofuran

Stable isotope tracing and metabolite extraction. Stable isotope tracing experiments with [U-^{13}C]-serine tracer (Cambridge Isotope Laboratories) were performed in customized serine-, glycine-, and glutamine-free RPMI 1640 (Cell Culture Technologies, Switzerland). Tracer medium was supplemented with 400 µM glycine, 2 mM glutamine, 17 mM glucose, 400 µM [U-^{13}C]-serine tracer, 1% P/S, and 5 % FBS. 300,000 HL-60 cells were seeded in 12-well plates in triplicates for each experimental condition in 1 ml RPMI tracer medium, treated as indicated, and cultured for 24 h. Residual cell seeding solution was counted and cell size was determined to calculate packed cell volume (PCV) of total cells in pL at the start of each tracing experiment. After 24 h, triplicate wells per condition were counted and cell size was determined to calculate PCV at the end of the tracing experiment. All following steps for metabolite extraction were carried out on ice. First, medium of all conditions in triplicates was collected by centrifugation. To be able to determine the basal medium composition, tracer medium without cells was incubated in parallel throughout the experiment in triplicates in 12 wells. All collected media were centrifuged at 300 g for 5 min to remove residual cellular debris. Supernatant was collected and stored at -20 °C to allow for

subsequent analysis of formate exchange rates from the medium. Formate extraction, derivatization, and quantification was performed using GC-MS analysis as previously described[28]. For intracellular metabolite extraction after [U-^{13}C]-serine tracing, pelleted HL-60 cells were washed with 1 ml cold PBS and subsequently resuspended in 400 µl ice-cold extraction solvent (5:3:2 MeOH/ACN/H_2O). Intracellular metabolites were extracted by shaking at 4 °C for 10 min. After a final centrifugation, supernatant was collected, stored at -80 °C, and submitted to LC-MS analysis of intracellular metabolite labeling. LC-MS analysis of nucleotides was detailed in BioRxiv Preprint Kiweler *et al.* 2021 (https://doi.org/10.1101/2021.05.27.445928).

Genomic 2'-deoxyuridine (dU) measurements. Cells were lysed by passing through 21G and 23G syringe needles and subsequent incubation at 37 °C for 1 h with 1000 RPM shaking in a buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1% SDS, 100 mM DTT, 0.1 mg/mL proteinase K (Worthington Biochemical), 0.1 mg/mL RNase A (Sigma-Aldrich), 50 µM tetrahydrouridine (THU, Merck Millipore). DNA was subsequently extracted from the lysates with 25:24:1 phenol:chloroform:isoamyl alcohol, followed by two washes with 24:1 chloroform:isoamyl alcohol and isopropanol precipitation using 10 M ammonium acetate to precipitate the DNA. RNA and free nucleotides were then removed from the DNA samples by treatment with 4 µg RNase A in 10 mM ammonium bicarbonate (pH 7.0), 10 mM $MgCl_2$ for 30 min at 37 °C, followed by a subsequent isopropanol precipitation. Next, the DNA samples were hydrolyzed and dephosphorylated to single nucleosides as previously described (Galashevskaya, A. et al. DNA Repair. 2013 Sep;12(9):699-706. doi: 10.1016/j.dnarep.2013.05.002). DNA was hydrolyzed to nucleosides by treatment with 0.8 U Nuclease P1 (Sigma-Aldrich), 80 U Benzonase® Nuclease (Santa Cruz Biotechnology), and 7.5 U Antarctic Phosphatase (New England Biolabs) in 50 µl reactions containing 10 mM ammonium acetate (pH 5.5), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 240 µM THU for 60 min at 37 °C. Enzymes were then precipitated and removed from the reactions by adding three volumes of ice-cold acetonitrile to the reactions, incubating for 10 min on ice, centrifugation at 16,000 RCF for 30 min at 4 °C. The supernatants were transferred to new tubes and lyophilized until dry. To separate dU from dC, the samples were redissolved in water and fractionated on an Agilent 1200 HPLC system (with a UV detector set to 260 nm to identify the canonical nucleosides) and a mixed mode Primesep 200 column (2.1 mm x 150 mm, 5 µm, SieLC) kept at 30 °C using a flow rate of 0.4 mL/min and water and acetonitrile as mobile phase, each containing 0.1% formic acid, as the mobile phase. The 12-min-long HPLC gradient was as follows: 5% acetonitrile for 30 s, ramp to 35% acetonitrile by 1.5 min to 2.5 min, and return to 5% acetonitrile by 2.51 min. The dU-containing fractions were collected from 1.6-1.7 min and vacuum centrifuged until dry. The pellets were redissolved in water and analyzed by LC-MS/MS using a reverse phase column (2.1 mm x 150 mm, 1.8 µm, EclipsePlusC18 RRHD, Agilent Technologies) kept at 25 °C with a flow rate of 0.3 mL/min on a 1290 Infinity II HPLC coupled to a 6495 Triple Quadrupole mass spectrometer with an electrospray ion source (Agilent Technologies). Water and methanol were used as the mobile phase, each containing 0.1% formic acid. The 13-min-long HPLC gradient was as follows: 5% methanol for 3 min, ramp to 13% methanol by 3.5 min, ramp to 17% methanol by 5.5 min to 7 min and return to 5% methanol by 8 min. Analysis was performed in positive ionization multiple reaction monitoring mode, using the mass transitions 229.08 → 113.0 and 232.08 → 116.0 for 2´-deoxyuridine

(dU), $^{13}C^{15}N_2$-dU, respectively. Unmodified nucleosides were measured on an API5500 triple quadrupole mass spectrometer (Applied Biosystems) with a reverse phase column at room temperature (Zorbax SB-C18 2.1 mm × 150 mm, 3.5 µm, Agilent Technologies). The HPLC method used a flow rate of 300 µL/min with an isocratic flow of 25% B for 2 min with the column heated to 40 °C. The mass transitions used were 252.1 → 136, 228.1 → 111.9, 268.1→152, and 243.1→127 m/z for dA, dC, dG, and dT, respectively.