**Supplementary methods**

**Chemicals**

NAD+ (Sigma N7004), folitixorin (TRC F680350), NAD(P)H-GloTM (Promega G9062), TrizmaBase (Sigma T1503), NADP+ (Sigma N5755), Bovine Carbonic Anhydrase (Sigma) and Bovine Serum Albumin (Sigma).

**Synthesis of LY345899**

See Supplementary Figure 6 for a synthesis overview.

**(4-(((2-Amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino)benzoyl)-L-glutamic acid:**([1](#_ENREF_1), [2](#_ENREF_2))Folic acid (1019 mg, 2.31 mmol) and Pb(NO3)2 (15 mg, 0.05 mmol) were weighed into a 3-necked 100 mL flask. The atmosphere was changed to N2. Degassed H2O (28 mL) was added under a stream of N2. pH was adjusted to 7-8 by addition of degassed 5 M NaOH, giving a clear orange-brown solution. Another three cycles of evacuation/N2-filling were done to ensure inert atmosphere and the temperature was adjusted to 12°C. A solution of NaBH4 (315 mg, 8.33 mmol) in degassed H2O (3 mL) was added to the cooled reaction mixture, followed by another three cycles of evacuation/N2-filling. The pH was adjusted from 9-10 to 8-9 by slow addition of 20 % degassed aq. citric acid solution over 15 minutes. Immediate analysis by LCMS showed complete consumption of starting material. pH was adjusted to 3-4 by addition of 5M HCl/5M NaOH to facilitate fine-tuning of pH. The reaction mixture was cooled on ice before quickly filtrated in air. The precipitate was washed with H2O followed by EtOH, and then dried under vacuum for four days giving the (4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino)benzoyl)-L-glutamic acid as a grey precipitate (689 mg, 80 % purity). MS (ESI+) m/z 446 [M+H]+. Due to the sensitivity towards oxygen the product was used in next step without further characterization or purification.

**(4-(3-Amino-1,9-dioxo-1,2,5,6,6a,7-hexahydroimidazo[1,5-f]pteridin-8(9H)-yl)benzoyl)-L-glutamic acid**. The cyclization with phosgene was carried out according to the method published by Tonkinson([3](#_ENREF_3)) but with modified procedures for workup and purification**.**

A slurry of the crude (4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino)benzoyl)-L-glutamic acid (634 mg, 80 % purity) in a mixture of H2O (12 mL) and toluene (10 mL) under N2 was cooled to 0°C. A solution of COCl2 (20 % in toluene, 1.5 mL, 2.8 mmol) was added dropwise, and the reaction mixture was stirred at 0-5°C. After 1.5 hours the mixture was cooled to 0°C and COCl2 (20 % in toluene, 1.5 mL, 2.8 mmol) was added dropwise. Stirring was continued at room temperature for 1hours and 15 minutes, and then a third portion of COCl2 (20 % in toluene, 0.5 mL, 0.93 mmol) was added dropwise at room temperature. After additional 35 minutes of stirring at room temperature the reaction mixture was worked up by separation of the phases (under air, i.e. not inert atmosphere). The aqueous phase was washed with toluene (3x 7 mL) to remove excess of COCl2. The combined organic phases were backextracted with H2O (1x 10 mL). The solvent was removed by blowing a stream of N2 into the flask and at the same time warming to ca 65°C for ca 4 hours, giving the crude product as a dark red semisolid.

The crude product was dissolved in 2 M NaOH (pH was checked still to be 0-1), filtered through cotton wool and purified in 5 portions by preparative LCMS on a Gilson 305 HPLC system equipped with an ACE 5 C8 (5 µm, 30 mm x 150 mm) column. 5-30 % MeCN in mixture with H2O containing 0.1 % TFA was used as gradient. The pure fractions were pooled using 3 M HCl giving (4-(3-amino-1,9-dioxo-1,2,5,6,6a,7-hexahydroimidazo[1,5-f]pteridin-8(9H)-yl)benzoyl)-L-glutamic acid as a solid after evaporation (171 mg, 16 % over 2 steps). MS (ESI+) m/z 472 [M+H]+.

141 mg of (4-(3-amino-1,9-dioxo-1,2,5,6,6a,7-hexahydroimidazo[1,5-f]pteridin-8(9H)-yl)benzoyl)-L-glutamic acid was dissolved in H2O, MeCN and excess 6 M HCl to give the HCl salt of the product as a solid (117 mg). MS (ESI+) m/z 472 [M+H]+.1H NMR (400 MHz, D2O/DCl) (7.5 mg; 0.7 mL D2O, 0.1 mL 35 wt % DCl) δ ppm 6.95 - 6.99 (m, 2 H) 6.76 - 6.81 (m, 2 H) 3.72 - 3.77 (m, 1 H) 3.35 - 3.41 (m, 1 H) 2.84 - 2.99 (m, 3 H) 2.34 - 2.41 (m, 1 H) 1.72 (m, 2 H) 1.43 - 1.50 (m, 1 H) 1.28 - 1.37 (m, 1H).

**Details regarding crystallographic data processing, refinement and model building for MTHFD2 crystal structure**

Data processing was carried out using XDS ([4](#_ENREF_4), [5](#_ENREF_5)) and the CCP4 suite ([6](#_ENREF_6)). The structure was solved via molecular replacement using Phaser ([7](#_ENREF_7)) with a preliminary model from an initial dataset, which was solved with Molrep ([8](#_ENREF_8)) and ARP/wARP ([9](#_ENREF_9)) using pdb 1B0A as search model. Refinement in Refmac5 ([10](#_ENREF_10), [11](#_ENREF_11)), interspersed with manual building in Coot ([12](#_ENREF_12), [13](#_ENREF_13)) was needed to complete the model. Water molecules were automatically placed in the maps, using a FO-FC Fourier difference map cutoff of 3 σ, and subsequently validated to ensure correct positioning. The final protein model contains one molecule of MTHFD2, residues 36-332. Ramachandran statistics were generated using MolProbity ([14](#_ENREF_14), [15](#_ENREF_15)). All structure figures were prepared using PyMOL (http://www.pymol.org).

**Dimer analysis using PISA**

Dimer interface calculations for MTHFD2 (5TC4.pdb) was generated using the 'Protein interfaces, surfaces and assemblies' service PISA at the European Bioinformatics Institute. (<http://www.ebi.ac.uk/pdbe/prot_int/pistart.html>) ([16](#_ENREF_16)).

**Calculation of assay quality and dose-response curves**

Z´ is suitable for assessment of assay quality ([17](#_ENREF_17)) and was calculated for the MTHFD2 assay (Supplementary Table 2). The normalized signals for the dose-response curve were plotted vs log[LY345899]/M and IC50 values were determined by fitting the equation log[inhibitor] vs. response-Variable slope in the GraphPad Prism software to the data.

**Size Exclusion Chromatography**

A 10/300 GL S75 column was equilibrated on Äkta Prime with 20 mM Tris-HCl pH 8.2, 150 mM NaCl, 15 % glycerol, 1 mM TCEP. Loaded protein samples were diluted to 1 mL each within the same buffer. Three separate samples were run over the column: 200 μg each of MTHFD2, Bovine Carbonic Anhydrase and Bovine Serum Albumin.

**Schematic representation of binding sites**

Plots for the binding sites of NAD+, inorganic phosphate and LY345899 were generated using LIGPLOT+ ([18](#_ENREF_18)) using the MTHFD2 structure (5TC4.pdb).

**Sequence alignment**

Sequence alignment of MTHFD1 (Uniprot ID P11586), MTHFD2 (Uniprot ID P13995) and MTHFD2L (Uniprot ID Q9H903) was preformed using Clustal Omega v 1.2.1. Graphic representation was generated using ESpript 3.0([19](#_ENREF_19)).

**Binding site analysis**

Binding of NAD+, Mg2+ and inorganic phosphate was manually inspected using criteria from literature. ([20-25](#_ENREF_20))

**Cellular thermal shift assay (CETSA) on intact cells**

MRC-5, U-2OS and Hs-578T cells were seeded into T175 flasks at a density of 2x106 cells per mL and left to adhere overnight. Cells were washed once in PBS and treated with either 10 µM LY345899 or vehicle (DMSO) for 6h in an incubator at 37°C and 5% CO2. The cells were then harvested using trypsin, followed by trypsin inactivation with FBS-containing cell media, and centrifugation at 1500 rpm, 10 minutes, 4°C. The pellets were resuspended in TBS supplemented with complete protease inhibitor cocktail, then counted and recalculated to a density of 2x106 cells per mL. The cell suspensions were divided into aliquots, heated to indicated temperatures for 3 minutes, then cooled at room temperature for 3 minutes. Heated cells were freeze-thawed at -80°C for 3 minutes followed by 3 minutes at 37°C three times. Lysates were cleared of insoluble aggregates by centrifugation at 13,000 rpm at 4°C for 30 minutes, supernatant was transferred to new tubes and protein concentration was determined using a BCA assay (Pierce). Western blot was performed according to standard procedures. The antibodies used were mouse anti-MTHFD2 (Abcam, ab56772), mouse anti-Actin (Abcam, ab6276) and IRDye 800 CW donkey anti-mouse (LI-COR, 926-32212). Images were taken with Odyssey Fc imager (LI-COR) and analyzed with ImageJ software.

**Resazurin cell viability assay**

MRC-5, U-2OS and Hs-578T cells were seeded in a 96-well plate at a density of 3,000 cells per well in 200 µL media and treated with vehicle (DMSO) or increasing concentrations of LY345899 (2.5 µM – 50 µM). The cells were left to proliferate over a period of 72 hours in an incubator at 37°C and 5% CO2. Cells were washed once in PBS, followed by addition of 10 µg/mL resazurin sodium salt (Sigma) dissolved in fresh media. After 2-4 hours of incubation at 37°C, the fluorescent signal was measured using an excitation wavelength of 545 nm and an emission wavelength of 590 nm in a Hidex Sense plate reader.

## Supplementary References

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