**Supplementary MaterialsSupplementary Materials and Methods**

**Ligand-fishing and sample preparation for mass spectrometry (MS)**

For the measurement of hit compound-binding proteins, cells were lysed with lysis buffer (20 mM Hepes-NaOH, pH 7.5 containing 1 % Triton X100, 150 mM NaCl, 50 mM NaF, 1 mM Na3VO4, cOmplete EDTA-free protease inhibitor cocktail (Roche)) and centrifuged to remove insoluble materials. The bait compounds (negative- or positive-probe) were added into cell lysate at a final concentration of 400 nM and immunoprecipitated with anti-FLAG antibody conjugated magnetic beads (Sigma-Aldrich) to capture the interacting protein.

For the competition assay, we prepared cell lysate pre-incubated with 100-fold concentration of the original FLAG-linker free hit compound against positive-probe. The hit compound interacting proteins were eluted with FLAG peptide and subjected to trichloroacetic acid precipitation. The resulting pellets were dissolved in 0.1 M ammonium bicarbonate (pH 8.8) containing 7 M guanidine hydrochloride, reduced using 5 mM TCEP (Tris (2-carboxyethyl) phosphine), and subsequently alkylated using 10 mM iodoacetamide. After alkylation, samples were digested with lysyl-endopeptidase (Wako Pure Chemical) for 3 h at 37°C and then further digested with trypsin (Thermo Fisher Scientific) for 14 h at 37°C.

**Liquid chromatography-MS/MS (LC-MS/MS) analysis**

The peptide mixture was applied to a Mightysil-PR-18 (Kanto Chemical) frit-less column (45 × 0.150 mm ID) and separated using a 0–40% gradient of acetonitrile containing 0.1% formic acid for 80 min at a flow rate of 100 nl/min. Eluted peptides were sprayed directly into a mass spectrometer (Triple TOF 5600+; AB Sciex). MS and MS/MS spectra were obtained using the information-dependent mode. Up to 25 precursor ions above an intensity threshold of 50 counts/s were selected for MS/MS analyses from each survey scan. All MS/MS spectra were searched against protein sequences of NCBI nonredundant human protein data set (NCBInrRefSeq Release 71, containing 179460 entries) using the Protein Pilot software package (AB Sciex).

**DHODH Enzyme assay**

Purified recombinant human DHODH (DHODH, 31-395aa, Human, His tag, E. coli) enzyme was purchased from ATGen (Catalog No. ATGP1615). The assay was conducted in aqueous buffer containing 100 mM Hepes, 400 mM NaCl, 10% Glycerol, 0.05% TritonX-100, 0.175 ug/mL DHODH, 0.2 mM CoQ, 0.1 mM DHO, 0.12 mM DCIP and 0.5% DMSO (pH 8.0) at room temperature, in a 384-well plate format. Compounds were added via BiomekNX, and the reaction was initiated by addition of substrates. Enzyme activity was monitored kinetically by the reduction in DCIP absorbance at 600 nm over the course of 50 min. Chemicals were purchased from Sigma-Aldrich and WAKO Pure Chemical. Absorbance measurements were obtained using a Envision plate-reading spectrophotometer.

**Antibodies**

The following antibodies were used for immunostaining: mouse monoclonal anti-Nestin (1:200, BD), biotinylated goat polyclonal anti-human Sox2 (1:200, R&D), mouse monoclonal anti-Glial fibrillary acidic protein (GFAP, 1:400, Sigma), mouse monoclonal anti-βIII tubulin (1:400, Sigma), mouse monoclonal anti-galactocerebroside (GC, 1:10, culture sup), rat monoclonal anti-GFP (1:500, Nacalai Tesque), mouse monoclonal anti-human Mitochondria (1:100, Merck Millipore), and rabbit polyclonal anti-active Caspase 3 (1:1000, Cell Signaling Technology). Antibodies were detected with Alexa568-conjugated goat anti-rabbit IgG (1:500, Life Technologies), Alexa488-conjugated goat anti-mouse, -rabbit, or -rat IgG (1:500, Life Technologies), Streptoavidin-Cy3 (1:200, Thermo Fisher) and goat anti-mouse IgG-Cy3 (1:500, Jackson ImmunoResearch).

The following antibodies were used for immunoprecipitation and western blotting: mouse anti-FLAG M2 (10 μ/ml, SIGMA), mouse anti-O-GlcNAc (1:100, Abcam) and GAPDH (1:5000, Proteintech). Antibodies were detected with horseradish-peroxidase-conjugated anti-mouse IgG (1:5000, Santa Cruz).

**Vectors**

Full-length human *SOX2* cDNA was amplified from E6 cDNA using KOD Plus-Ver.2 polymerase (Toyobo) according to the manufacturer’s instructions. Mutant forms of *SOX2* (K75A and S248A) were made using KOD plus-Ver.2 and sets of oligonucleotides containing mutant sequence. These cDNAs were inserted into p3xFLAG CMV10 vector to produce p3xFLAG CMV10-hSOX2wt, -hSOX2 K75A, and -hSOX2 S248A. We synthesized the following oligonucleotide DNA primers: for the full-length human *SOX2*, the 5' primer was 5’-AGAATTCAATGTACAACATGATGGAGACG-3’ and the 3' primer was 5’-AGGATCCTCACATGTGTGAGAGGGGCA-3’; for a K75A mutant, the 5' primer was 5’-GAGATCAGCGCGCGCCTGGG-3’ and the 3' primer was 5’-CCCAGGCGCGCGCTGATCTC-3’; for a S248A mutant, the 5' primer was 5’-TGGTCAAGGCCGAGGCCAG-3’ and the 3' primer was 5’-CTGGCCTCGGCCTTGACCA-3’’.

 Full-length human *DHODH* cDNA was amplified from E6 cDNA using KOD Plus-Ver.2 polymerase according to the manufacturer’s instructions. The cDNA was inserted into the pcDNA3-2xFLAG-c vector to produce pcDNA3-hDHODH 2xFLAG-c. We synthesized the following oligonucleotide DNA primers: full-length human *DHODH*, forward 5’-AGAATTCGCCACCATGGCGTGGAGACACCTGAAA-3’ and reverse 5’-TCTCGAGCCTCCGATGATCTGCTCCAAT-3’.

To knock down human *DHODH*, four short hairpin (sh) sequences were generated using InvivoGen’s siRNA Wizard (http://www.sirnawizard.com/). These sh sequences were inserted into a psiRNA-h7SKhygro G1 expression vector (InvivoGen) to produce psiRNA-h7SKhygro-hDHODHsh1-4. The knockdown efficiency of these vectors was analyzed by Western blotting (Supplementary Figure 10). We used most efficient *DHODHsh3* for the knockdown experiment. The sh3 target sequence for human *DHODH* was 5’-CCGGGATTTATCAACTCAAAC-3’. The control sh target (*egfp*) sequence was 5’-GCAAGCTGACCCTGAAGTTCA-3’.

The nucleotide sequences of cloned cDNA were verified using the BigDye Terminator Kit version 3.1 (Applied Biosystems) and ABI sequencer model 3130xl (Applied Biosystems). We transfected cells with the vectors using either the Nucleofector device according to the supplier’s instructions (Lonza) or Polyethylenimine (PEI), as previously described11,16.

**Pharmacokinetics study**

The pharmacokinetics (PK) study was performed as follows. 10580 (3 or 30 mg/kg) was orally administered to BALB/c mice (n=5). Blood sampling was performed at 0.25, 0.5, 2, 4, 8 and 24 hr after drug administration. Collected blood samples were centrifuged at 1,500 *g* for 10 min at 4˚C to obtain plasma. Concentrations of 10580 in plasma were determined by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) using an ACQUITY UPLC (Waters) and Triple Quad 5500 system (AB SCIEX). Liquid chromatography was performed using an ACQUITY UPLC BEH C18 Column (1.7 µm, 2.1 mm × 50 mm, Waters) at a flow rate of 0.6 mL/min. The mobile phases were distilled water containing 5 mM ammonium acetate, 5% acetonitrile, and 0.2% formic acid (mobile phase A) and acetonitrile containing 5 mM ammonium acetate, 5% acetonitrile and 0.2% formic acid (mobile phase B). Analytes were ionized by electrospray ionization in positive mode, and the monitoring ion was 370.1> 350.1 for 10580.

**Quantitative reverse-transcription PCR**

Total RNA was extracted from cells using an RNeasy kit (Qiagen) and reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) according to the supplier’s instructions. Real-time PCR was performed on the StepOnePlus (Thermo Fisher Scientific) using THUNDERBIRD SYBR qPCR Mix (TOYOBO), according to the manufacturer’s protocol. Cycle parameters were 15 s at 95˚C and 30 s at 60˚C for 40 cycles. All gene expression data were normalized to the housekeeping gene 18S ribosomal RNA according to the ∆∆Ct method. The oligonucleotide primer sequences are as follows: human *DHODH*, forward 5’-GCCATAAATTCCGAAATCCAG-3’ and reverse 5’-ACAGCTTGGTCCTCAGGGAG-3’; *UCK1*, forward 5’-TGTCTCGAAGAGTTCTCCGG-3’ and reverse 5’-TGCACGATCAGGTTGATGGC-3’; *UCK2,* forward 5’-GACCTGTTCCAGATGAAGCTT-3’ and reverse 5’-GAATTCCTCAAAGGCAGGCTT-3’; and *18S*, forward 5’-CGGACAGGATTGACAGATTG-3’ and reverse 5’-CAAATCGCTCCACCAACTAA-3’.

**TCGA analysis**Data from 27 microarrays of human GBM and LGG were obtained from the TCGA and analysed for the expression levels of *DHODH*, *UCK1* and *UCK2*.

**Metabolite quantification**

For quantification of nucleotides and UDP-GlcNAc, 10 ml of internal standard mix (10 mM [15N5,13C10]ATP, 10 mM [15N5,13C10]GTP, 10 mM [15N3,13C9]CTP, 10 mM [15N2,13C9]UTP, 10 mM [15N5,13C10]dATP, 10 mM [15N5,13C10]dGTP, 10 mM [15N3,13C9]dCTP, 10 mM [15N2,13C10]dTTP, and 10 mM [13C9,15N2]UMP) was added to the samples, and each sample was split into two equal portions. After 10 ml of the standard mix (500 mM ATP, 500 mM GTP, 500 mM CTP, 500 mM UTP, 1 mM dATP, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 100 mM CMP, 100 mM UMP, 100 mM CDP, 100 mM UDP, and 500 mM UDP-GlcNAc) was added to one portion for quantification using a standard addition method, phosphorylated metabolites in all split samples were purified using MonoSpin TiO columns (GL Sciences) according to a manufacturer’s protocol. The samples were then reconstituted with 10 mM ammonium bicarbonate (ABC). Mass spectrometric analysis was performed using a Xevo TQ-S micro mass spectrometer (Waters) with an H-classBio system (Waters). LC-MS/MS analysis was performed according to a previous report35 with slight modification. Each sample was separated on a SeQuant ZIC-pHILIC HPLC column (5 mm, 4.6 × 150 mm; Merck Millipore) at a flow rate of 0.5 ml/min, and subsequently eluted as follows: solvent A, 10 mM ABC and 0.05% ammonium hydroxide; solvent B, acetonitrile: 0–8 min, linear gradient from 85% B to 55% B; 8–10 min, linear gradient to 0% B; 10–12 min, isocratic with 0% B; 12–12.1 min, linear gradient to 85% B; 12.1–18 min, isocratic with 85% B. Multiple reaction monitoring (MRM) was performed in positive-ion mode using nitrogen as the nebulizing gas. Experimental conditions were set as follows: ion source temperature, 150°C; desolvation temperature, 550°C; desolvation gas flow rate, 1200 l/h; capillary voltage, 2.0 kV; cone gas flow rate, 110 l/h; collision gas, argon. The conditions of the MRM transitions were as follows [cone voltage (V), collision energy (eV)]: ATP, 508.1 > 136.0 (20, 35); GTP, 524.1 > 152.0 (20, 25); CTP, 484.1 > 112.0 (20, 20); UTP, 485.0 > 96.9 (20, 25); dATP, 492.1 > 136.0 (20, 20); dGTP, 508.1 > 152.0 (20, 20); dCTP, 468.0 > 112.0 (20, 15); dTTP, 483.0 > 81.0 (50, 15); CMP, 324.0 > 112.0 (20, 10); UMP, 325.1 > 97.0 (20, 15); CDP, 404.2 > 112.0 (20, 15); UDP, 405.1 > 97.0 (20, 15); UDP-GlcNAc, 608.2 > 204.1 (20, 15); [15N5,13C10]ATP, 523.1 > 146.0 (20, 35); [15N5,13C10]GTP, 539.1 > 162.1 (20, 25); [15N3,13C9]CTP, 496.1 > 119.0 (20, 20); [15N2,13C9]UTP, 496.0 > 101.9 (20, 25); [15N5,13C10]dATP, 507.1 > 146.0 (20, 20); [15N5,13C10]dGTP, 523.1 > 162.1 (20, 20); [15N3,13C9]dCTP, 480.1 > 119.0 (20, 15); [15N2,13C10]dTTP, 495.1 > 86.0 (50, 15); [13C9,15N2]UMP, 336.1 > 102.0 (20, 15). The amount of each metabolite was quantified by calculating the peak area ratio of the target metabolite and its isotope-labeled internal standard using a standard addition method. [13C9,15N2]UMP was used for quantification of CMP and UDP-ClcNAc as their surrogate internal standards, and [15N5,13C10]ATP and [15N2,13C10]dTTP were used for quantification of CDP and UDP, respectively.

For quantification of orotate and DHO, the methanol extract was split into three equal portions. After equal volume of water, the standard mix-1 (200 nM orotate and 500 nM DHO), or the standard mix-2 (500 nM orotate and 50,000 nM DHO) was added to each aliquot for quantification using a standard addition method, LC-MS/MS analysis was performed. Each sample was separated on a SeQuant ZIC-pHILIC HPLC column (5 mm, 4.6 × 150 mm) at a flow rate of 0.5 ml/min, and subsequently eluted as follows: solvent A, 10 mM ABC and 0.05% ammonium hydroxide; solvent B, 10 mM ABC, 90% acetonitrile and 0.05% ammonium hydroxide: 0–1 min, isocratic with 100% B; 1–7 min, linear gradient to 50% B; 7–8 min, linear gradient to 0% B; 8–10 min, isocratic with 0% B; 10–10.1 min, linear gradient to 100% B; 10.1–16 min, isocratic with 100% B. MRM was performed in negative-ion mode. Experimental conditions were set as follows: capillary voltage, -1.5 kV; other parameters were the same as nucleotides analysis. The conditions of the MRM transitions were as follows [cone voltage (V), collision energy (eV)]: orotate, 154.9 > 110.9 (20, 10); DHO, 157.1 > 112.9 (20, 8). The amount of each metabolite was quantified by calculating the peak area ratio of the target metabolite using a standard addition method.

Simultaneous and absolute quantification of nucleoside triphosphates using liquid chromatography–triple quadrupole tandem mass spectrometry. Genes and Environment.

**Tumorigenesis**

Mice were anesthetized with 10% pentobarbital for the injection procedures. For the subcutaneous tumor model, GICs (1x106) were suspended in 50 µl of Matrigel (BD Biosciences) and injected subcutaneously into the hip of NOD/SCID mice aged 5-8 weeks. When the tumor reached 5-6 mm in length and width, the mice were daily administered 200 µl of saline, 30 mg/kg 10580 in 200 µl of 0.5% Methyl cellulose (Wako Pure Chemical) or 25 mg/kg Brequnair sodium (BRQ, TOCRIS) in 200 µl of PBS for 11 days. Dose was determined by the time-course analysis of the plasma concentration of 10580 (Supplementary Figure 1). The tumor size was measured daily, as described previously10.

**Supplementary References**

35. Matsuda S, Kasahara T. Simultaneous and absolute quantification of nucleoside triphosphates using liquid chromatography-triple quadrupole tandem mass spectrometry. *Genes Environ.* 2018;40:13.

**Supplementary Figure Legends**

**Supplementary Figure 1. PK study of 10580**

10580 (3 or 30 mg/kg) was orally administered to BALB/C mice. Blood samples were collected at 0.25, 0.5, 2, 4, 8 and 24 hr after drug administration. Concentrations of 10580 in plasma were determined by LC/MS/MS. Red dashed lines indicate GI50 (25.8 ng/ml). Error bar indicates ±SD.

 **Supplementary Figure 2. Metabolome analysis of 10580-treated GICs**Representative data of metabolites, pyrimidine ribonucleotides (A), purine ribonucleotides (B), deoxyribonucleotide triphosphates (C) and UDP-GlcNAc (D) in control (DMSO, white column) and 10580-treated (black column) E6 and E16 GICs. Error bar indicates ±SD. t test was used for statistical significance. \* *p<*0.05, \*\* *p<*0.01, \*\*\* *p<*0.001. **Supplementary Figure 3. Increased expression of *DHODH* in GICs/GICRs and GBM**(A) Quantitative RT-PCR analysis of the expression of *DHODH*, *UCK1* and *UCK2* in NSC, GICs (E6 and E16) and GICRs (E6R and E16R). The mRNA levels were shown as fold change over mRNA levels in NSC. (B) Expression data (from 27 microarrays) of *DHODH*, *UCK1* and *UCK2* from the TCGA database. Error bars indicate ±SD. Statistical significance was determined by the *t-*test. (C) Clinical data from the cBioPortal database suggested that increased *DHODH* mRNA (red line, Z-score>2) has correlated with a poorer prognosis, survival (left panel) and disease free (right panel), in GBM. (D) Uridine addition (circles) competitively inhibited 10580 cytotoxicity, but the addition of other ribonucleosides, such as adenosine (diamond), guanosine (square) and cytidine (triangle), did not. Error bar indicates ±SD. t test was used for statistical significance. \* *p<*0.05, \*\* *p<*0.01, \*\*\* *p<*0.001. **Supplementary Figure 4. 10580 induced cell cycle arrest and cell death in GICs**The E6 and E16 GICs were cultured in the presence of 10580 for the indicated time and then immunolabelled for BrdU (green) and CASP3 (red), a marker of cell death. All nuclei were counterstained with DAPI (blue). Scale bar, 100 μm. **Supplementary Figure 5. Neural marker expression in 10580-treated GICs**The E6 and E16 GICs were cultured in the presence of 10580 for the indicated time and then immunolabelled for the NSC markers (A) NESTIN (green) and SOX2 (red) and the differentiation markers (B) βIII tubulin (green), GFAP (red) and GC (green). All nuclei were counterstained with DAPI (blue). Scale bar, 100 μm. **Supplementary Figure 6. 10580 prevented GICR tumorigenesis with the decreased SOX2 immunoreactivity and the increased cell death**

(A) Representative images of H&E-stained E6R and E16R tumours from mice treated with saline or 10580. Lower panels show high magnification images. (B) Representative images of E6R and E16R tumours immunostained for SOX2 (red) and mitochondria (green). (C) Representative images for E6R and E16R tumours immunostained for SOX2 (red) and active CASP3 (green). Nuclei were counterstained with DAPI (blue). Scale bar: 1 mm in A and 100 µm in B and C.

**Supplementary Figure 7. LMB and UDP-GlcNAc prevented the 10580-induced nuclear export of SOX2**(A) The E6 and E16 GICs were cultured in the presence of 10580 (10 μM) with or without LMB (0.15 nM) for 1 day and then immunolabelled for SOX2 (red). Nuclear SOX2+ cells were shown as a percentage of the total cells. (B) The E6 and E16 GICs were cultured in the presence of 10580 (10 μM) with or without UDP-GlcNAc (1 mM) for 2 days and then immunolabelled for SOX2 (red) and NESTIN (green). All nuclei were counterstained with DAPI (blue). Scale bar, 100 μm.**Supplementary Figure 8. The OGT inhibitor OSMI-1 induced cell death and nuclear SOX2 export in GICs**(A) The E6 and E16 GICs were cultured in the presence of various concentrations of OSMI-1 for 3 days, and cell viability was examined using MTT assays. Error bar: ± SD. t test was used for statistical significance. \* *p<*0.05, \*\* *p<*0.01, \*\*\* *p<*0.001. (B) The E6 and E16 GICs were cultured in the presence of OSMI-1 (20 μM) for 2 days and then immunolabelled for SOX2 (red) and NESTIN (green). Nuclear SOX2+ cells were shown as a percentage of the total cells. All nuclei were counterstained with DAPI (blue). Scale bar, 100 μm.

**Supplementary Figure 9. CRM1 and O-GlcNAcylation regulate nuclear export of SOX2, positively and negatively.**

(A) 10580-treated E6 and E16 GICs expressing either wild-type SOX2 (SOX2 wt) or SOX2 mutants (SOX2 K75A and S248A) were immunostained for SOX2 (red) and NESTIN (green). (B) Dose-dependent effects of 10580 in GICs expressing control (open circle), SOX2 wt (upper panels, closed circle), SOX2 K75A (middle panels, closed circle) or SOX2 S248A (lower panels, closed circle). Error bar: ± SD. t test was used for statistical significance. \* *p<*0.05, \*\* *p<*0.01, \*\*\* *p<*0.001.

**Supplementary Figure 10. Knockdown efficiency of *DHODHsh* expression vectors**The FLAG-tagged human DHODH expression vector was transfected with a *control sh* (C) or *DHODH sh1-4* expression vectors into Cos7 cells. Cell extracts were harvested 2 days after transfection and were analysed by Western blotting using anti-FLAG and anti-GAPDH (loading control) antibodies. Molecular weight (MW) standards (Bio-Rad) are shown in kilodaltons (kDa).