SUPPLEMENTARY MATERIAL

Inhibiting phosphoglycerate dehydrogenase counteracts chemotherapeutic efficacy against *MYCN*-amplified neuroblastoma

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SUPPLEMENTARY MATERIALS AND METHODS

Generation of CRISPR/Cas9-mediated PHGDH knockout clones

Single CRISPR/Cas9-mediated PHGDH knockout clones were generated by transfecting the plasmids, px459_PH_ex4_80 or px459_PH_ex7_96, into BE(2)-C cells using the Effectene (Qiagen) method according to the manufacturer's directions. These plasmids encode for SpCas9 and a gRNA respectively targeting exon 4 in *PHGDH* (5'-TGCCGGAAGATCTTGCAAGA-3') or exon 7 in *PHGDH* (5'-CTTCAT CGAAGCCGTCGCCT-3'). Control cells were transfected with the px459 vector, which expresses SpCas9 (pSpCas9(BB)-2A-Puro; #48139, Addgene). After 24 h, 2 µg/ml puromycin (Thermo Fisher Scientific) was added to culture medium for 72 h to enrich for transfected cells, before performing limited dilution by seeding 0.5 to 1 cell per well into 96-well plates. All clones derived from single cells that could be expanded were subjected to western blotting to identify PHGDH knockouts.

Sample preparation for LC-MS-based shotgun proteomics

Disulfide bonds in proteins were reduced in 2 mM DTT for 30 min at 25 °C, and resulting free cysteines were alkylated in 11 mM iodoacetamide for 15 min at room temperature in the dark. Proteins were digested to peptides for LC-MS as previously described[1] using the endoproteinase, Lys-C, added (at a w/w ratio of 1:40) to samples and incubated overnight at 30 °C with gentle shaking. Immobilized trypsin beads (Applied Biosystems) and 50 nM ammonium bicarbonate were added to samples, then incubated another 4 h at 30 °C under rotation. Protein digestion was stopped by acidification (pH 2) through addition of trifluoracectic acid. A total of 18 µg peptide digest was desalted as previously described[1] for each LC run.

LC-MS-based settings for shotgun proteomics

The spray voltage of the nanospray source was set to 2.4 kV, and the ion transfer tube temperature to 260 °C. Samples were loaded onto the column with a flow rate of 450 nl/min. Elution was performed using a 240 min gradient ranging from 5 to 40 % of solvent B (80 % acetonitrile, 0.1 % formic acid) in solvent A (5 % acetonitrile, 0.1 % formic acid) with a flow rate of 400 nl/min. A 200 cm long MonoCap C18 HighResolution 2000 column (GL Sciences) was used for chromatographic separation.

Metabolite extraction for GC-MS analysis

As previously published[2], polar metabolites were extracted by methanol/chloroform/water extraction, centrifuged at 4000 x g for 10 min at 4 °C and split into two aliquots destined for metabolite and nucleotide pool quantification for the same cell samples, then dried in a vacuum concentrator overnight. For metabolite analysis using GC-MS, extracts were dissolved in methoxyamine hydrochloride solution (40 mg/mL MeOX in pyrimidine) at 30 °C for 90 min with constant shaking followed by an incubation at 37 °C for 60 min with N-methyl-N-[trimethylsilyl] trifluoroacetamide containing an alkane mixture. Extracts were centrifuged at maximum speed for 10 min, and aliquots were transferred into glass vials.

Sample preparation for direct-infusion MS

Dried extracts from 300,000 cells were resuspended in 5 mM buffer A (5 mM hexylamine, pH 6.3, adjusted with acetic acid). Samples were centrifuged at 20,000 x g for 5 min at 4 °C, then desalted and the nucleotide fraction enriched using ZipTips (Merck Millipore). Eluted samples were mixed 1:2 with methanol for direct-infusion MS.

RNA extraction and quantitative real-time RT-PCR

Total RNA was isolated from cell lines using the RNeasy Mini Kit (Qiagen). The Thermo Fisher Scientific First-Strand cDNA Synthesis Kit was used to transcribe cDNAs for qRT-PCR analysis. Relative *PHGDH* expression was measured using SYBR Green Dye (Roche) on a StepOnePlus Real-time PCR system (Applied Biosystems), and normalized to the averaged expression of *HPRT* and *SDHA*, a gene pair that is consistently expressed in INSS stage 4 and 4S neuroblastomas[3]. The following specific primer pairs were used: *PHDGH* (forward: 5'-TTT GGG ATG AAG ACT ATA GGG TAT GA-3'; reverse: 5'-CAG CTG CTG AAC ACC AAA GG-3'), *MYCN* (forward: 5'- CCA CGT CCG CTC AAG AGT GT-3'; reverse: 5'- CCC TGA GCG TGA GAA AGC TG-3'), *HPRT* (forward: 5'- TGA CAC TGG CAA AAC AAT GCA -3'; reverse: 5'- GGT CCT TTT CAC CAG CAA GCT-3') and *SDHA* (forward: 5'- TGG GAA CAA GAG GGC ATC TG-3'; reverse: 5'- CCA CCA CTG CAT CAA ATT CAT G-3'). Data were analyzed using StepOne™/StepOnePlus™ Software version 2.3, and changes in expression were calculated using the ΔΔCt method.

Chromatin immunoprecipitation and quantitative real time PCR

Cells were lysed for ChIP in buffer containing 50 mM Tris-HCl (pH 8.1), 1 % SDS, 10 mM EDTA and Complete® protease inhibitor cocktail (Roche, Mannheim, Germany), then sonicated to obtain chromatin fragments of 200 to 1000 base pairs. ChIP was performed according to the ChIP Assay Kit protocol (Merck Millipore). The following antibodies were used: mouse monoclonal anti-MYCN (clone B8.4.B; Santa Cruz Biotechnology) and normal mouse IgG (Santa Cruz Biotechnology) as negative control. The *PHGDH* promoter region corresponding to the 3783 peak was amplified using qPCR and the primer set: 5'- GGT GAC TTT AAT CCC AAA TTG AA -3' (forward; region 120,253,334-120,253,470 [4]), 5'- GAC CAA GGA GGC ACT TCT CA -3' (reverse; region 120,253,334-120,253,470). The *PHGDH* promoter region corresponding to the 3784 peak was amplified using qPCR and the primer

set: 5'- CAG GTT ACC TCT CAA AGC CTC T -3' (forward; region 120,261,235-120,261,331 [4]), 5' - TTA TTC TCT TCA CAA CTC CAA GG-3' (reverse; region 120,261,235-120,261,331).

Western blotting and immunohistochemistry

Cells were lysed for western blotting in buffer containing 20 mM Tris-HCl, 7 M urea, 0.01 % Triton X-100, 100 mM DTT, 40 mM $MgCl_2$ and Complete® protease inhibitor cocktail. The following antibodies were used: mouse monoclonal anti-β-actin (clone AC-15, Sigma-Aldrich), mouse monoclonal anti-GAPDH (clone 6C5, Merck), mouse monoclonal anti-MYCN (clone B8.4.B, Santa Cruz Biotechnology) and rabbit polyclonal anti-PHGDH (#13428, Cell Signaling). Band density in western blots was analyzed using VisionCapt software, version 16.11a (Vilber Lourmat), and results were normalized to the respective loading controls.

A rabbit polyclonal antibody against PHGDH (#HPA021241, Atlas Antibodies) was used for immunohistochemistry on 80 formalin-fixed, paraffin-embedded tumor sections. Two pathologists independently evaluated immunostaining on an AxioImager Z1 microscope (Carl Zeiss MicroImaging, Inc.). PHGDH protein expression in tumor samples was semi-quantitatively scored from immunostaining intensity by counting the percentage of PHGDH-positive tumor cells as previously described[5].

Proliferation assay

Cell viability and number were measured on a VI-CELL-XR Cell Viability Analyzer (BeckmanCoulter, Brea, USA) based on the trypan blue-exclusion method[6].

Colony formation assay

Cells were seeded onto 6-well plates. Colonies that grew after nine days in culture were stained with crystal violet, and the number of visualized colonies was quantified using ImageJ software, version 1.48[7].

Flow cytometry

Cells were fixated with 70% ice-cold ethanol, treated with 100 µg/mL RNase A (Thermo Fisher Scientific, Waltham, USA) and stained with 50 µg/mL propidium iodide (Thermo Fisher Scientific, Waltham, USA). Cell cycle analysis was performed on a LSR Fortessa X-20 (Beckton Dickinson, Franklin Lakes, USA) using the FlowJo-X software, version 10.0.7r2 (BD Biosciences, San Jose, USA).

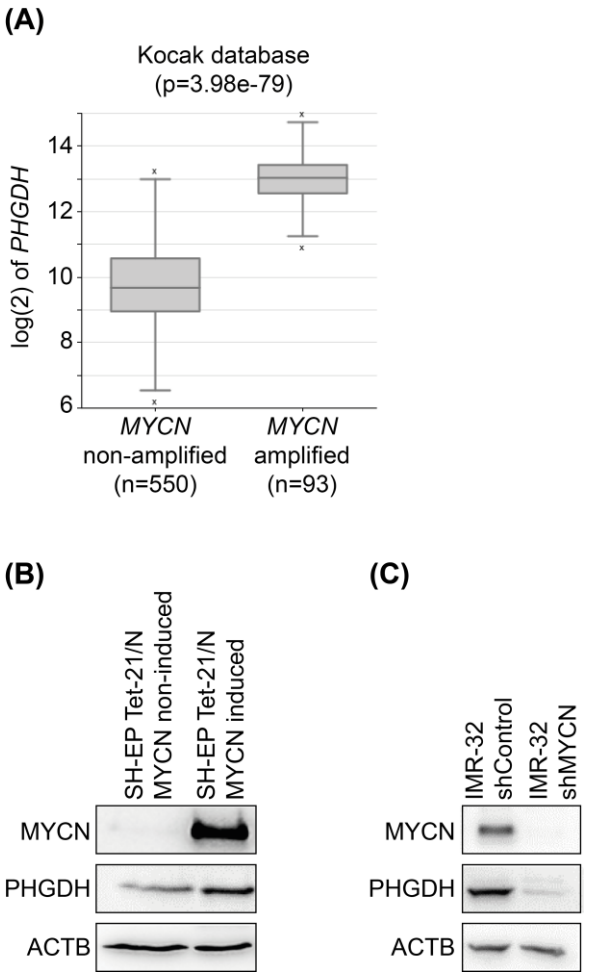
SUPPLEMENTARY TABLES

Supplementary Table S1. Characteristics of patients and tumor samples analyzed by liquid chromatography-mass spectrometry-based proteomics.

Factor	***n***
INSS stage	
I, II, III, IVS	26
IV	23
Age at diagnosis	
≤ 18 months	30
> 18 months	19
MYCN	
Diploid	38
Amplified	11

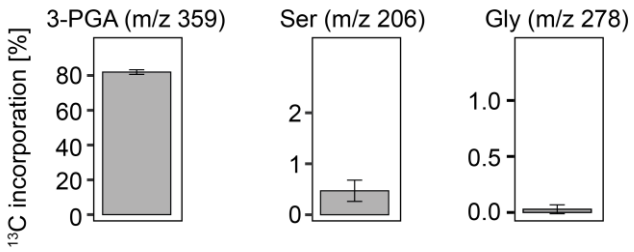
SUPPLEMENTARY FIGURES

Suppl. Fig. S1



Supplementary Fig. S1. MYCN is sufficient to control PHGDH expression. A, Correlation analysis of open-access mRNA gene expression profiles of 643 human neuroblastoma samples using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). PHGDH expression was assessed on protein level by Western blot analysis in the synthetic MYCN-inducible SH-EP-Tet21/N cell model with and without MYCN induction for 96 hours (B) and in IMR-32 cells stably transfected with a tetracycline-inducible MYCN shRNA expression system (IMR32-6TR-MYCNsh) at 72 hours of tetracycline treatment. β-actin was used as a loading control.

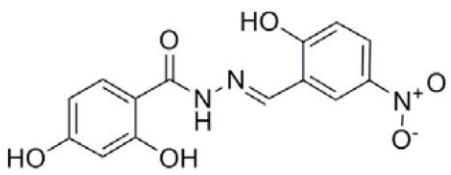
Suppl. Fig. S2



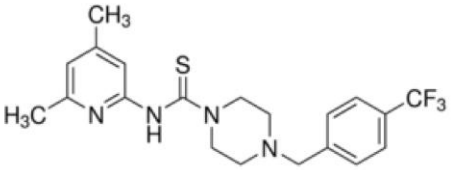
Supplementary Fig. S2. Incorporation of glucose-derived carbons into 3-PGA, serine and glycine. SH-SY5Y cells were cultured for 48 hours and pulsed with medium containing fully labeled ^{13}C-glucose for 10 min before harvest for quantitative metabolomics. Plotted bars represent percentages of ^{13}C-label incorporation into the indicated molecules ($n \geq 2$). 3-PGA, 3-phosphoglycerate; Ser, serine; Gly, glycine.

Suppl. Fig. S3

PKUMDL-WQ-2101 Ref. 34

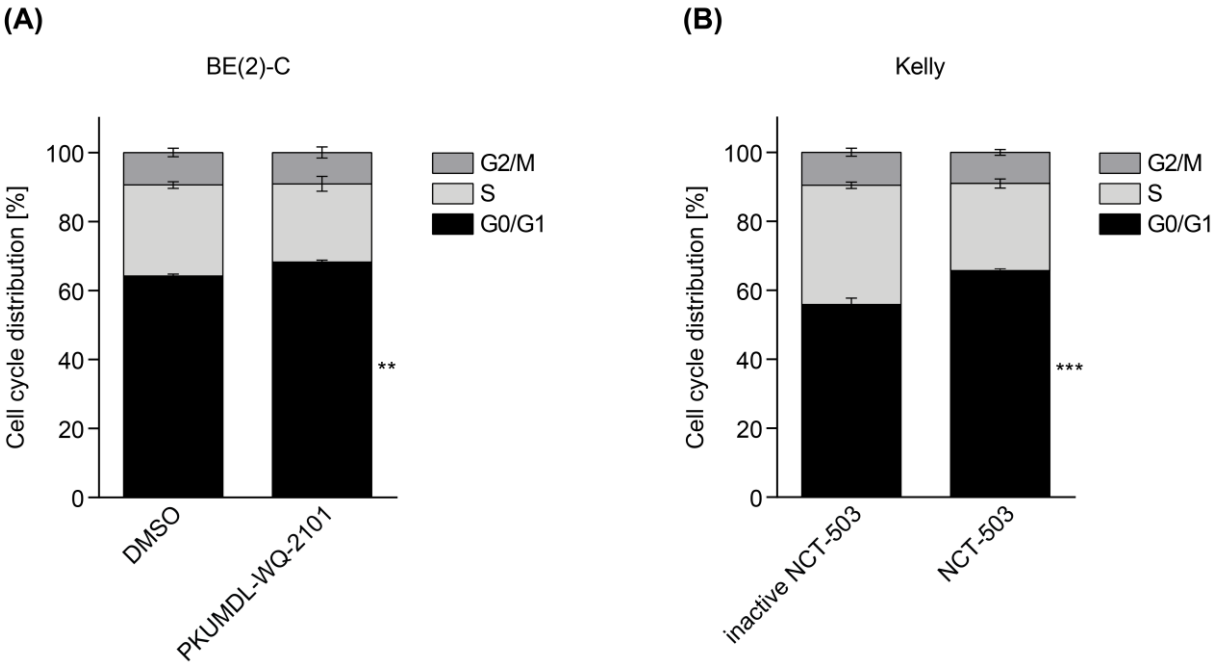


NCT-503 Ref. 35

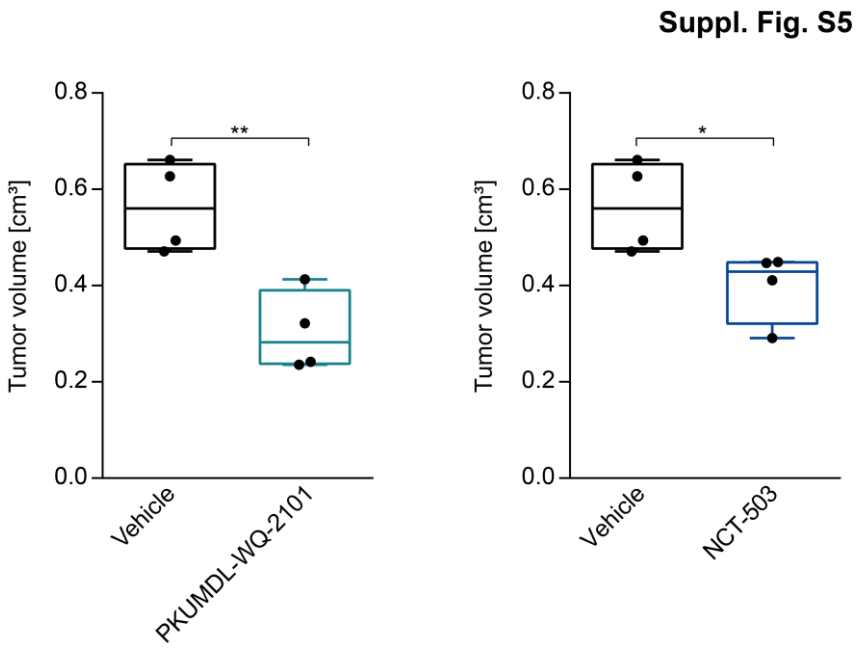


Supplementary Fig. S3. Overview of the chemical structures of PHGDH inhibitors PKUMDL-WQ-2101 and NCT-503.

Suppl. Fig. S4



Supplementary Fig. S4. PHGDH inhibitor treatment induces a G_0/G_1 cell cycle arrest in neuroblastoma cells. BE(2)-C (A) and Kelly (B) cells were cultured for 48 hours with 10 µM PKUMDL-WQ-2101, 10 µM NCT-503 or solvent control and labeled with propidium iodide before harvest for fluorescence activated cell sorting. Plotted bars represent mean ± SD (n = 3). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.



Supplementary Fig. S5. Short-term anti-tumor efficacy of PHGDH inhibitor monotherapy in mice harboring patient-derived xenograft (PDX) tumors. Box-and-whisker plots show the distribution of subcutaneous PDX 14647 tumor volumes after 10 d of treatment as indicated. NOG mice hosted PDX xenograft tumors, and 4 mice were included in each study group. Monotherapy added either 20 mg/kg/d PKUMDL-WQ-2101 ip daily or 40 mg/kg/d NCT-503 ip daily and vehicle control was 0.1 ml 0.9 % NaCl iv once weekly and 0.2 ml 10 % DMSO and 0.1 ml 0.1 % Tween-20 ip once daily. *$P < 0.05$, **$P \leq 0.01$.

SUPPLEMENTARY REFERENCES

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