Supplementary Information for

Triptolide suppresses IDH1-mutated malignancy via Nrf2-driven glutathione metabolism

Di Yu[1,2,3,#], Yang Liu[1,#], Yiqiang Zhou[1], Victor Ruiz-Rodado[1], Mioara Larion[1], Guowang Xu[2,#], Chunzhang Yang[1,#]

[#]Equal Contribution

[1]Neuro-Oncology Branch, Center for Cancer Research, National Cancer Institute, MD, 20892, USA

[2]CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116023, China

[3]University of Chinese Academy of Sciences, Beijing, 100049, China

Correspondence: Chunzhang Yang, Ph.D. Investigator, Neuro-Oncology Branch Center for Cancer Research, NCI, NIH. Building 37, Room 1142E Bethesda, MD 20892**.** Phone: +1-240-760-7083**.** Email: yangc2@mail.nih.gov

Guowang Xu, Ph.D. Professor, CAS Key Laboratory of Separation Science for Analytical Chemistry, DICP, CAS. Building 18, Room 314, Dalian 116023. Phone: 0086-411-84379530. Email: xugw@dicp.ac.cn

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Supplementary Methods

Genetic silencing

Small interference RNA oligos targeting SLC7A11 were synthesized by Integrated DNA Technologies (Coralville, IA). Negative control RNA was purchased from Qiagen (Hilden, Germany). Oligos were transfected into GSC827 and TS603 cell lines using Lipofectamine RNAiMax transfection reagent (Invitrogen) according to the manufacturer's protocol. The siRNA sequences are shown as follows. siSLC7A11-1. F: 5'-AUG ACU GUG CUU CCA AGU -3'; siSLC7A11-1. R: 5'-GAU GCA UAC UUG GAA GCA -3'; siSLC7A11-2. F: 5'-CUG UAA UGA GCU UGA UCG -3'; siSLC7A11-2. R: 5'-GAA CUU GCG AUC AAG CUC -3'; siSLC7A11-3. F: 5'-UUC UUU AUA GUU GUU CCC -3'; siSLC7A11-3. R: 5'-CUG AAU UGG GAA CAA CUA -3'.

Western blotting

Total protein was extracted using RIPA lysis buffer (Thermo Fisher) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher). Protein quantification was performed with the BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were separated on NuPAGE 4-12% Bis-Tris mini gels (Life Technologies) and transferred to the membrane using the Power Blotter System (Invitrogen). The membrane was incubated with antibodies and visualized with an HRP/chemiluminescence kit (Thermo Fisher Scientific) on the ChemiDoc Imaging System (Bio-Rad). The primary antibodies used included GCLC (Abcam, ab4163, 1:1000), GCLM (Proteintech, 14241-1-AP, 1:1000), SLC7A11 (Abcam, ab37185, 1:1000), PARP (CST, 9532S, 1:1000), γH2A.X(CST, 7631P, 1:1000) and β-Actin (CST, 4970S,1:5000).

RNA extraction and quantitative real-time PCR (RT-PCR)

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) and reverse transcribed to cDNA using Superscript IV VILO Master Mix (Thermo Fisher). Gene expression was analyzed with the Human Oxidative stress Plus PCR Array (PAHS-065YA, Qiagen) or with the SYBR Green Power Master Mix Kit (Applied Biosystems). Primers used include NFE2L2 (QT00027384), HMOX1 (QT00092645), NQO1 (QT00050281), SLC7A11 (QT00002674) and ACTB (QT00095431), purchased from QIAGEN.

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed using the ChIP-IT Express Enzymatic kit (Active Motif) as previously described (1). Control IgG and antibody targeting Nrf2 (Active Motif) were used. For PCR amplification, the following primers specific for the GCLC, GCLM and SLC7A11 promoters were used. GCLC promoter, Forward: 5'- CGC AGT TGT TGT GAT ACA GCC -3', Reverse: 5'- GGA CTG AGA CTT TGC CCT AAG AA -3'. GCLM promoter, Forward: 5'- ATT CCA AAC TGA GGG AGC TGT TT -3', Reverse: 5'- ATG AGT AAC GGT TAC GAA GCA CT -3'. SLC7A11 promoter, Forward: 5'- AGC TTC CCA CAA AGT CGA AG -3', Reverse: 5'- ACA TTC CTG CTT GTC TTG GT -3'. NQO1 promoter, Forward: 5'- TTT GCT GAG TCA CCA GTG C-3', Reverse: 5'- TTT GCT GAG TCA CCA GTG C -3'. The input DNA of each sample was used as a control.

Caspase 3/7 activity assay

Caspase 3/7 activity was measured using the Caspase-Glo 3/7 Assay (Promega).10,000 Cells were seeded in 96-well plate and treated with DMSO, 30 nM triptolide or triptolide combined with 0.1 mg/mL catalase for 24 hr. Luminescence was generated by adding caspase-3/7 substrate and measured by Epoch plate reader.

Annexin V/PI apoptosis assay

Cell apoptosis was analyzed with the Annexin-V/PI Kit (Thermo Fisher) according to the manufacturer's protocol. Cells were treated with DMSO, 30 nM triptolide, or triptolide combined with 0.1 mg/mL catalase for 96 hr. Cells were harvested and stained with FITC labeled Annexin-V and P.I. (Invitrogen) for 20 min on ice. Cell samples were analyzed by the FACSCanto II flow cytometer (B.D.).

Glutathione assay

Total glutathione and oxidized glutathione disulfide (GSSG) were measured with the GSH/GSSG-Glo Assay Kit (Promega) following the manufacturer's instructions. Total glutathione minus GSSG equals reduced glutathione (GSH). Ten thousand cells were seeded to a 96-well plate and treated with DMSO, 30 nM triptolide or triptolide combined

with 0.1mg/mL catalase for 24 hr. Luminescence was measured with a microplate reader and normalized to the protein quantification.

ROS assay

ROS levels were measured using the ROS-Glo H_2O_2 Assay kit (Promega) and CM-H_2DCFDA (Life Technologies) according to the manufacturer's instructions. For ROS-Glo H_2O_2 Assay, cells were incubated with an H_2O_2 substrate, which can generate a luciferin precursor. After adding the luciferin detection reagent, luminescence was measured using a microplate reader and normalized to the protein levels. For CM-H2DCFA, cells were resuspended in PBS containing ten μM CM-H_2DCFDA at 37 °C for 15 min. Cells were analyzed by a FACSCanto II flow cytometer (B.D.).

DNA damage assay

DNA damage level was evaluated using DNA fragmentation, 8-oxoguanine level, and comet assay. For DNA fragmentation assay, 300-500 ng of genomic DNA was resolved by electrophoresis on 4-20% TBE gel (Invitrogen). The gel was then stained with SYBR Safe and visualized on the Bio-Rad ChemiDoc Imaging System. The level of 8-oxoG was measured using the DNA/RNA Oxidative Damage ELISA Kit (Cayman) according to the manufacturer's protocol. An alkaline Comet assay was performed as previously described (2). In brief, cells were resuspended in agarose, spread on glass slides, and lysed in lysis buffer overnight at 4°C. Electrophoresis was performed at a voltage of 1 V/cm for 20 min. After electrophoresis, DNA was stained by SYBR Safe and visualized by fluorescence microscopy. The tail moment was measured and quantified by the OpenComet plugin of ImageJ software (3).

Cell proliferation

Cell proliferation was analyzed using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen), following the manufacturer's instructions. Fifteen thousand cells were seeded to μ-slide 8-well chambered coverslips and treated with DMSO or 30 nM triptolide for 24 hr after plating. After washing with PBS, cells were incubated with 10 μM EdU for 2 hr, and DNA was stained by Hoechst 33342. The cells labeled 488 azide were defined

as EdU positive cells. Images were taken by Zeiss LSM710 confocal microscope, and ten random fields were analyzed using ImageJ software.

Lipid peroxidation assay

Lipid peroxidation was detected using the Image-iT™ Lipid Peroxidation Kit (Invitrogen). Fifteen thousand cells were seeded to μ-slide 8-well chambered coverslips and treated by DMSO, 30 nM triptolide, or triptolide combined with 0.1 mg/mL catalase for 24 hr. Live cells were stained with 10 μM dye for 30 min, and DNA was stained by Hoechst 33342 for 5 min. Images were taken using a Zeiss LSM710 confocal microscope. Five random fields were analyzed using ImageJ software.

Limited dilution assay

Cells were counted and plated at clonal density for a limited dilution assay (125, 62, …, 2 cells/well in 96-well plates). Cells were treated with DMSO or 30 nM triptolide for three weeks and analyzed under a microscope. Cell clusters larger than 100 μm in diameter were defined as positive cells. The negative rate for each sample was calculated.

Sphere formation assay

Five thousand cells were seeded in 6-well plates and cultured in single-cell suspension for the sphere formation assay. Cells were treated with DMSO, 30 nM triptolide, or 60 nM triptolide for two weeks to allow sphere formation. Phase-contrast images were taken. Spheres were counted from 10 random fields.

Cell viability assay

8,000 to 10,000 cells per well were seeded in 96-well plate. Cell viability was evaluated using Cell Counting Kit-8 (Dojindo) according to the manufacturer's instructions. Absorbance was measured at 450 nm using an Epoch plate reader (BioTek).

Immunohistochemistry and TUNEL assay

Immunohistochemistry was performed in tumor sections. The sections were deparaffinized and rehydrated, and antigen retrieval was conducted by boiling in citrate

buffer (pH 6.0) for 5 min. The sections were incubated with primary antibodies overnight at 4 °C, followed by HRP labeled secondary antibody at room temperature for 30 min. Bound antibodies were visualized using a DAB kit. The primary antibodies used include Nrf2 (CST, 1:1000), SLC7A11(Abcam, 1:500), GCLC (Abcam, 1:500), Ki67 (Abcam, 1:1,000) and PCNA (Santa Cruz, 1:1,000). TUNEL assay was performed using the DeadEnd Colorimetric TUNEL System (Promega) following the manufacturer's protocol. The slides were detected using DAB and counterstained using hematoxylin.

SI Figures

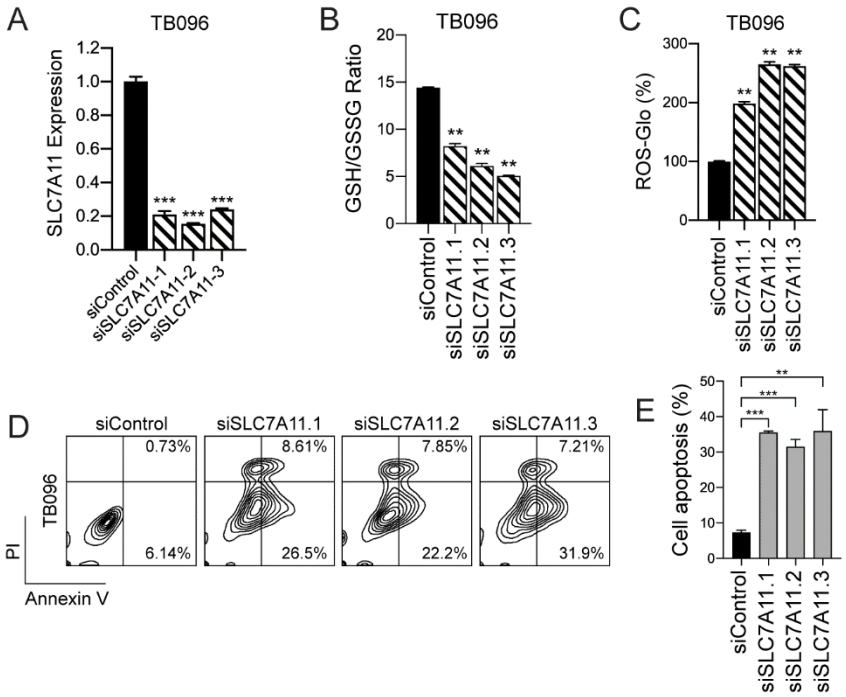

Fig. S1. (A) RT-PCR measured the siRNA knockdown efficiency of SLC7A11 in TB096 cells. ***p<0.001. **(B)** GSH/GSSG-Glo assay measured the GSH/GSSG ratio in TB096 after siRNA knockdown of SLC7A11. **p<0.01. **(C)** ROS-Glo H_2O_2 assay measured the ROS level in TB096 cells after depletion of SLC7A11. **p<0.01. **(D)** Cell apoptosis assay using Annexin V and PI staining in TB096 cells after depletion of SLC7A11. **(E)** Quantification of apoptotic cells in Fig. S1D. ***p<0.001, **p<0.01.

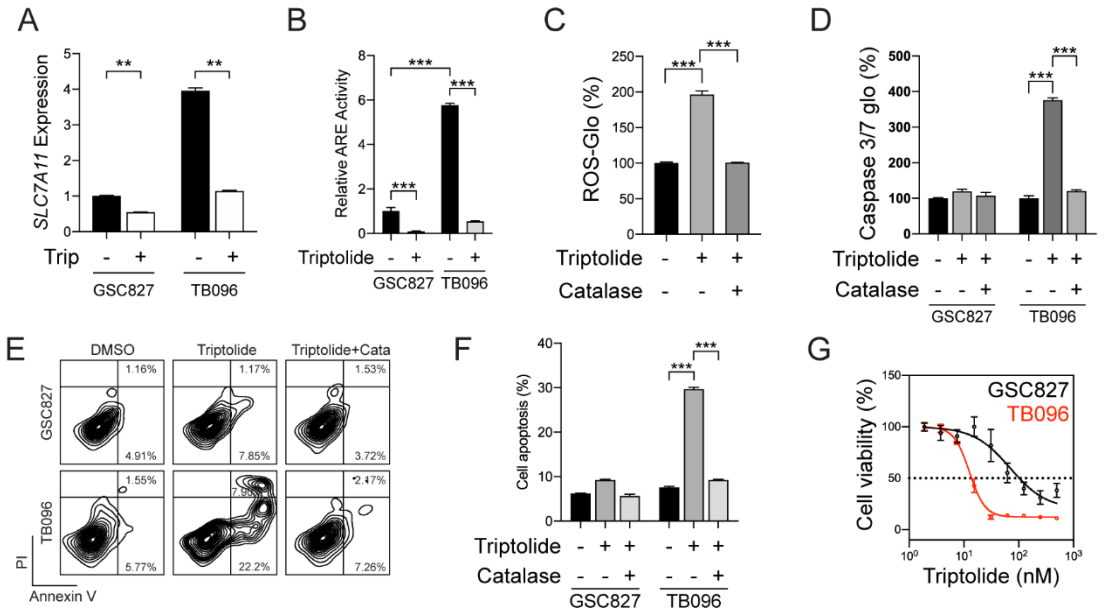

Fig. S2. (A) RT-PCR measured SLC7A11 expression in GSC827 and TB096 after treated with triptolide (30 nM, 24 hr). **p<0.01. **(B)** Luciferase reporter assay measured the ARE-driven transcriptional activity in GSC827 and TB096 cells. ***p<0.001. **(C)** ROS-Glo H_2O_2 assay measured the ROS level in TB096 cells after treated with triptolide. ***p<0.001. **(D)** Caspase 3/7-Glo assay measured the caspase 3/7 activity in GSC827 and TB096 after treated with triptolide. ***p<0.001. **(E)** Cell apoptosis assay using Annexin V and PI staining in GSC827 and TB096 after treated with triptolide. Cata, Catalase. **(F)** Quantification of apoptotic cells in Fig. S2F. ***p<0.001. **(G)** Dose response cell viability assay using CCK8 assay in GSC827 and TB096 cells after treated with different doses of triptolide.

References:

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