Blockade of Glutathione Metabolism in IDH1-Mutated Glioma
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ABSTRACT
Mutations in genes encoding isocitrate dehydrogenases (IDH) 1 and 2 are common cancer-related genetic abnormalities. Malignancies with mutated IDHs exhibit similar pathogenesis, metabolic pattern, and resistance signature. However, an effective therapy against IDH1-mutated solid tumor remains unavailable. In this study, we showed that acquisition of IDH1 mutation results in the disruption of NADP⁺/NADPH balance and an increased demand for glutathione (GSH) metabolism. Moreover, the nuclear factor erythroid 2–related factor 2 (Nrf2) plays a key protective role in IDH1-mutated cells by prompting GSH synthesis and reactive oxygen species scavenging. Pharmacologic inhibition of the Nrf2/GSH pathway via brusatol administration exhibited a potent tumor suppressive effect on IDH1-mutated cancer in vitro and in vivo. Our findings highlight a possible therapeutic strategy that could be valuable for IDH1-mutated cancer treatment.

Introduction
Isocitrate dehydrogenases (IDH) are a family of enzymes that mediate the oxidative decarboxylation of isocitrate to a-ketoglutarate. These enzymes depend on NAD⁺ as a cofactor (1). Genetic abnormalities in IDH1/2 are common in multiple types of human tumors. For example, mutations in IDH1/2 have been found in over 80% of World Health Organization grade II/III gliomas, including astrocytoma and oligodendroglioma (3). These mutations are found in 73% of secondary glioblastomas, which are derived from lower grade gliomas, but are less frequent in primary glioblastoma multiforme (4). Furthermore, the IDH1/2 mutations are commonly identified in acute myeloid leukemia (AML), central chondrosarcoma, central/perosteal chondromas, and cholangiocarcinoma (5, 6). However, despite the widespread prevalence of these mutations, effective therapies for IDH1-mutated solid tumors remain unavailable.

The majority of cancer-associated IDH mutations are amino acid substitutions of an arginine residue in its catalytic center. For IDH1, the 132 arginine (R) residue is frequently altered to histidine (H) or cysteine (C). The R132H (73.67%) and R132C (13.35%) variant comprise over 87% of all IDH1 mutations in human. A seminal study by Dang and colleagues (7) revealed that these amino acid substitutions in IDH1 lead to a neomorphic activity of the enzyme, which is the NADP⁺-dependent consumption of a-ketoglutarate for 2-hydroxyglutarate (2-HG) production. The accumulation of 2-HG has been reported to associate with glioma oncogenesis by inhibiting a-ketoglutarate dioxygenases (8–10). Several pioneer studies also suggest that IDH mutants are associated with depletion of NADPH and glutathione (GSH), accompanied with elevated reactive oxygen species (ROS) levels (11, 12). The neomorphic catalytic function, 2-HG accumulation and occurrence of oxidative stress, suggest a distinctive oncogenesis mechanism, which could be exploited as a therapeutic vulnerability in IDH1-mutated malignancies.

In this study, we investigated the association between mutant IDH1 enzyme and ROS levels. Furthermore, we analyzed the role of nuclear factor erythroid 2–related factor 2 (Nrf2) in the regulation of GSH metabolism that maintains cellular redox homeostasis and survival. Moreover, we investigated the efficacy of Nrf2/GSH metabolism blockade as a therapeutic approach in IDH1-mutated malignancies.

Materials and Methods
Cell culture
The U251 cell was obtained from Sigma in 2015. Cells were cultured in DMEM supplemented with 10% heat inactivated FBS (Thermo Fisher Scientific) and antibiotics (Thermo Fisher Scientific) at 37°C in humidified air with 5% CO₂. Brain tumor initiating cell (BTIC) T603 (IDH1 R132H) was obtained from Dr. Timothy Chan (Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY) in 2017 (13). BTIC GSC827 (IDH1 wild-type) and GSC923 (IDH1 wild-type) were previously established in our laboratory, which are derived from patient sample following the approval of NCI Institutional Review Board (14). All BTIC lines were cultured in NBE media as described previously (15). All cell lines are PCR tested negative for Mycoplasma. For inducing gene expression, cells were treated with 100 ng/mL doxycycline (Gold Biotechnology) for at least 24 hours.

Reagents and treatment condition
Brusatol was purchased from Sigma and dissolved in DMSO. The final concentration used is 40 mM in vitro. Cells were treated with brusatol for 24–72 hours among different experiments in vitro. N-acetylcysteine (NAC, Sigma) was dissolved in PBS. The final concentration used is 2.5 mM/L. Catalase (Sigma) was dissolved in PBS, the final concentration used is 500 U/mL. Mannitol (Sigma) was dissolved in PBS, the final concentration used is 50 mM/L. MnTBAP
(Millipore) was dissolved in DMSO, the final concentration used is 100 μmol/L. AGI-5198 was purchased from Cellagen Technology and dissolved in DMSO. The final concentration used is 1 μmol/L. in vitro.

Plasmid, lentivirus, and stable cell line generation

The coding sequence of IDDH1 gene was inserted into pLVX-TetOne-Puro (Clontech) using restriction sites EcoRI/BamHI. The R132C/H variants were introduced by QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) and verified by Sanger sequencing. The lentivirus was packaged in HEK293T cells using pMD2.G (Addgene catalog no.12260) and psPAX2 (Addgene catalog no. 12260) system. Virus was directly added into cell culture medium. Stable expression cell lines were selected by puromycin (1–2 μg/mL).

NADP⁺/NADPH quantification

NADP⁺/NADPH was quantified by NADP⁺/NADPH-Glo Assay (Promega). Cells were seeded in 96-well plate at 4,000 cells per well. Cells were lysed in total GSH lysis reagent or oxidized GSH lysis (Promega). Cells were seeded in 96-well plate at 4,000 cells per well. Cells were probed with Image-iT Lipid Peroxidation Reagent (10 μmol/L, 1 h). Live cell imaging was performed using a Leica SP8 microscope. Stably transfected cell lines were harvested and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h. The absorbance was measured at 570 nm

ROS quantification

The quantity of ROS was measured by ROS-Glo assay (Promega). Cells were seeded in 96-well plate at 4,000 cells per well. Cells were incubated with H₂O₂ substrate for 4 hours and lysed in ROS-Glo detection solution. The ROS level was measured by luminescence signal that recorded by a Polarstar Optima Plate Reader (BMG Labtech).

GSH/glutathione disulfide quantification

Cellular level of GSH was quantified using GSH/GSSG-Glo Assay (Promega). Cells were seeded in 96-well plate at 4,000 cells per well. Cells were lysed in total GSH lysis reagent or oxidized GSH lysis reagent. The lysate was then treated with luciferin generation reagent. The luminescence signal was recorded by a Polarstar Optima Plate Reader (BMG Labtech). GSH/glutathione disulfide (GSSG) ratio was calculated through luminescence signal.

Live cell imaging

To quantify the level of oxidative damage, cells were seeded in 8-well chamber slides (Ibidi) at 10,000 cells per well. After treatment, cells were probed with Image-iT Lipid Peroxidation Reagent (10 μmol/L, 30 minutes, Thermo Fisher Scientific) or MitoSOX (5 μmol/L, 10 minutes, Thermo Fisher Scientific). Fluorescent signal was recorded using a Leica TCS SP8 STORM microscope. Oxidative stress was evaluated by measuring fluorescent intensity with ImageJ software.

Western blot analysis

Western blot was performed as described previously (16).Briefly, cells were seeded in 6-well plate at 3–5 × 10⁵ cells per well. After treatment, cells were lysed in RIPA buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The lysate was separated by NuPAGE Bis-Tris Gel (Thermo Fisher Scientific) and transferred to polyvinylidene difluoride membrane (Millipore). The membranes were blocked in Superblock Blocking Buffer (Thermo Fisher Scientific) followed by primary antibody. The quantity of target protein was revealed by horseradish peroxide–conjugated secondary antibody and Chemiluminescence Assay (Bio-Rad). The primary antibodies used in this study are listed as follow: Nrf2 (Abcam ab26352, 1:1,000), GCLC (Abcam ab41463, 1:2,000), GCLM (Proteintech 14241-1-AP, 1:2,000), SLCA7A11 (Proteintech ab37185, 1:2,000), EGFP (Thermo Fisher Scientific, A-11122, 1:2,000), β-actin (Cell Signaling Technology, 4967, 1:5,000), and HA-tag (Origene, 1:2,000).

Real-time PCR

qRT-PCR was performed as described previously (17). Total RNA was extracted from formaldehyde extracts by PureLink RNA Mini Kit (Thermo Fisher Scientific), and reverse transcript to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). Genes related to oxidative stress were analyzed by Power SYBR Green Master Mix. Primers used in this study include NQO1 (QT0005281), HMOX1 (QT00092645), NFE2L2 (QT00027384), and ACTB (QT00095431).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (18). In brief, cells were seeded in 150-mm plate at 1.2 × 10⁵ cells per plate. After treatment, cells were fixed with 1% formaldehyde for 10 minutes. Cells were collected and incubated with 1.2 M NaOH with 1% dodecyltrimethylammonium bromide. The lysate was separated into two vials for NADP⁺oxidation quantification or NADPH quantification. The luminescence signal was recorded by a Polarstar Optima Plate Reader (BMG Labtech). NADP⁺/NADPH ratio was calculated through luminescence signal.

siRNA

siRNA was designed and synthesized by Integrated DNA Technologies. Cells were seeded in 6-well plate at 5 × 10⁵ cells per well. Fifty picomole of siRNA was transfected into cells by using Lipofectamine RNAiMAX (Thermo Fisher Scientific) based on the manufacturer's protocol. The suppression of gene expression was validated by qRT-PCR. siRNAs used in this study are listed as follow: siGCLC.F: 5'- CGG AGT TTG TTG GAT GCA GCC CAC -3', GCLC.R: 5'- GGA CTG AGA CTT TGC CCT AAG AA -3', GCLM.F: 5'- ATT CCA AAC TGA GGG AGG AGC TTG TT-3', GCLM.R: 5'- ATG AGT AAC GGT TAC GAA GCA GC -3', NQO1.F: 5'- GTG GTA CAG AGG CCT CAA AA AA -3', NQO1.R: 5'- TGC TCC -3;
siGCLC.1.F: 5'- GAC TCT CCT GTG GAG -3', and SLC7A11.R: 5'- ACA TTC CTG CTT GTC TTG GT -3'.

sRNA

sRNAi was designed and synthesized by Integrated DNA Technologies. Cells were seeded in 6-well plate at 5 × 10⁵ cells per well. Fifty picomole of sRNAi was transfected into cells by using Lipofectamine RNAiMAX (Thermo Fisher Scientific) based on the manufacturer's protocol. The suppression of gene expression was validated by qRT-PCR. siRNAs used in this study are listed as follow: siGCLC.F: 5'- ACA AUA GGA CAG AUA GUU GCC AAG UGA -3', siGCLC.R: 5'- AGU UGG CUA CUA UCU UGU GCC CAA UAG T -3'; siGCLM.F: 5'- UUA AUU UUG UGA CAU UGA UGA CAA CUC -3', siGCLM.R: 5'- GGU UGC AUC AUA GUU CCA AUA UUT A -3'; siGCLM.1.F: 5'- GAA GGU UUU UGG AUA CAA UCA UGA AGC AG -3', siGCLM.1.R: 5'- UUC AUG AUA GUU UCC AAA AAA CAA T -3'; and siGCLM.2.F: 5'- CCC UCU UUU AGC UUG UAA AUA GUU GCA GCC -3', siGCLM.2.R: 5'- CUA CAU UUU ACA AGC UAA AAG AGG G -3'.

Cell apoptosis analysis

Cell apoptosis level was analyzed by Annexin V/propidium iodide (PI) Apoptosis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were seeded in 6-well plate at 3–5 × 10⁵ cells per well. After treatment, cells were harvested and incubated with FITC-conjugated Annexin V and PI for 20 minutes on ice. Cell samples were analyzed by FACS Canto II (BD Biosciences) flow cytometer.

Luciferase reporter assay

The Nr2-associated transcriptional activity of was determined using reporter plasmid pGL4.37[luc2P/ARE/Hygro] (Promega) containing antioxidant response element (ARE). A total of 900 ng of

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reporter plasmid and 0.1 mg of pRL-CMV were transfected into 10^5 cells in 12-well plate using Lipofectamine 3000. Luminescence was measured by Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol.

**Immunoprecipitation**

Nrf2 ubiquitination was quantified by immunoprecipitation assay as described previously (19). Cells were seeded in 6-well plate at 3–5 × 10^5 cells per well and transfected with EGFP-Nrf2 (Addgene 21549) and ubiquitin (Addgene 18712) plasmids using Lipofectamine 3000. Cells were incubated with MG-132 (MedChem Express) to suppress proteasome activity. Cells were lysed in RIPA buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) and 1% SDS. EGFP-conjugated Nrf2 were precipitated using EGFP antibody and Dynabeads Protein G Immunoprecipitation Kit (Thermo Fisher Scientific). Protein ubiquitination was measured by Western blot analysis.

**Cycloheximide pulse chase assay**

Cycloheximide pulse chase assay was performed as described previously (20). Cells were seeded in 6-well plate at 3–5 × 10^5 cells per well. Cells were transfected with EGFP-Nrf2 plasmid and exposed with 50 μg/mL cycloheximide (Sigma). Total protein was extracted and Nrf2 residue was analyzed through Western blot analysis and EGFP immunoblot.

**Xenograft**

Six- to 8-week-old NSG mice (The Jackson Laboratory) were subcutaneously injected with 5 × 10^6 TS603 cells in 100 μL PBS. Once the tumors reached over 50 mm^3, mice were randomly allocated into four groups and treated intraperitoneally with DMSO (8 μL DMSO in 100 μL PBS), brusatol (2 mg/kg, 8 μL DMSO in 100 μL PBS; refs. 21, 22), NAC (50 mg/mL, dissolved in 100 μL PBS containing 8 μL DMSO), or brusatol + NAC every other day for a total of five times. Tumor size was measured using Vernier calipers. Sixteen days after brusatol treatment, mice were sacrificed, and tumors were harvested for analysis. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals and approved by the Animal Care and Use Committee of the NIH.

**Statistical analysis**

Statistical analysis was performed with Student t test between two data groups. Differences among groups were analyzed using one-way ANOVA test followed by Student t test as the post statistical analysis. All tests were two-sided, the results were presented as mean ± SEM. **, *P < 0.05 was considered as statistically significant. All of the analysis was conducted using GraphPad Prism 7.01 (GraphPad Software).

**Results**

**Neomorphic activity in cancer-associated mutant IDH1 triggers oxidative stress**

To better understand the effect of IDH1 mutants on redox homeostasis, we established a doxycycline-induced IDH1-mutant U251 cell line (Supplementary Fig. S1A). We noticed that upon the expression of mutant IDH1 enzymes, the overall quantity of NADP+/NADPH ratio significantly

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**Figure 1.**

Cancer-associated IDH1 mutants trigger oxidative stress. A, NADP⁺ level was measured in U251 cells with doxycycline (Dox)-induced expression of IDH1-mutant enzymes (R132C and R132H). **, *P < 0.01. B, Measurement of NADP⁺/NADPH ratio in U251 cells with expression of IDH1-mutant enzymes. **, *P < 0.01. C, ROS-Glo measurement in U251 cells with expression of IDH1-mutant enzymes. **, *P < 0.01. D, ROS-Glo measurement in IDH1-R132H U251 cells with AGI-5198 (50 μM) treatment (1 μmol/L, 24 hours; **, *P < 0.01). E, Lipid peroxidation staining measures membrane oxidative damage in U251 cells with expression of IDH1-mutant enzymes. Scale bar, 10 μm. F, Quantification of lipid peroxidation in E. **, *P < 0.01. G, MitoSOX staining measures mitochondrial ROS in IDH1-mutated U251 cells. Scale bar, 10 μm. H, Quantification of MitoSOX signal in G. **, *P < 0.01.
increased (Fig. 1B), suggesting that the neomorphic enzyme activity of mutant IDH1 exhausted the cellular pool of NADPH. The balance between NADP⁺ and NADPH is a critical factor to maintain cellular redox homeostasis, as NADPH is a general cofactor in reductive biosynthetic reactions, and to provide electrons for metabolic pathways, such as the reduction of GSSG back to GSH and ROS neutralization (23). Furthermore, by quantification of H₂O₂, we showed that mutant IDH1 enzymes led to severe oxidative stress in both U251 cells and BTIC TS603 (Fig. 1C, Supplementary Fig. S2A). Such an increase in ROS levels depended on the presence of mutant enzymes. The treatment with AGI-5198, a specific inhibitor of mutant IDH1 (13), reduced ROS accumulation in cells expressing the IDH1 R132H variant (Fig. 1D). Furthermore, the elevated ROS levels led to oxidative stress to macromolecules and subcellular organelles, evidenced by increased lipid peroxidation (Fig. 1E and F) and mitochondrial ROS level (Fig. 1G and H). The ROS scavenger catalase and MnTBAP, but not mannitol, abrogated the accumulation of ROS in IDH1-mutated cells, indicating the majority form of oxidative stress is derived from hydrogen peroxide and superoxide anion (Supplementary Fig. S1B).

**GSH metabolism supports redox balance and survival in IDH1-mutated cells**

Considering the remarkable ROS accumulation in IDH1-mutated cells, we speculate that antioxidant pathways, such as GSH-dependent ROS scavenging systems, may be triggered to maintain redox homeostasis. To test this hypothesis, we first measured the protein levels of GSH synthesis enzymes by Western blotting. We found that upon introduction of pathogenic mutant IDH1 enzymes, the levels of key enzymes in GSH biosynthesis, such as glutamate-cysteine ligase (GCLC, catalytic subunit; GCLM, modifier subunit), and cystine/glutamate transporter (SLC7A11, xCT transporter), increased (Fig. 2A). We also noticed that the levels of Nrf2 protein (NFE2L2), the major transcriptional factor responsible for ROS sensing, increased in IDH1-mutated cells. The enhancement of Nrf2 and GSH-dependent ROS scavenging pathways in IDH1-mutated U251 cells, as well as IDH1-mutated BTIC TS603, was also confirmed by qPCR (Fig. 2B, Supplementary Fig. S2B). To further understand the role of GSH in IDH1-mutated cells, we quantified GSH/GSSG levels in U251 cells expressing R132C/H IDH1 mutants. We recorded a substantial decrease in the GSH/GSSG ratio compared with that in cells expressing

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**Figure 2.**

GSH de novo synthesis support cellular physiology in IDH1-mutated cells. A, Western blot measures the expression of GSH synthesis enzymes in U251 cells with IDH1-mutant expression. β-Actin was used as internal control. B, qRT-PCR analysis measures mRNA level of GSH synthesis enzymes. *P < 0.05; **P < 0.01. C, GSH and GSSG level was measured in IDH1-mutated U251 cells. Catalase (Cata) was used as exogenous ROS scavenger (500 U/mL, 24 hours; **P < 0.01). D, Annexin V/PI apoptotic analysis in IDH1-mutated U251 cells with genetic silencing of GCLC and GCLM. E, Quantification of apoptotic cells in D, F, ROS-Glo assay in IDH1-mutated U251 cells with genetic silencing of GCLC and GCLM.
wild-type IDH1, suggesting that there is an elevated demand from GSH-dependent ROS scavenging (Fig. 2C). We also measured GSH/GSSG levels in BTICs, the result consistently showed that IDH1-mutated BTIC TS603 has lower GSH/GSSG ratio compared with IDH1 wild-type BTIC GSC287 and GSC 923 (Supplementary Fig. S2C). Moreover, the addition of an exogenous antioxidant enzyme, catalase, partially restored the GSH/GSSG ratio, indicating that the GSH/GSSG imbalance could be a result of GSH oxidation by hydrogen peroxide decomposition pathways (e.g., GSH peroxidases). Importantly, GSH biosynthesis exhibited a critical protective role in cells with mutant IDH1 enzyme. A loss-of-function experiment showed that 72 hours after genetic silencing of GCLC/GCLM resulted in remarkable apoptotic changes. The annexin V/PI apoptosis assay showed that apoptotic cell population increased remarkably upon siRNA treatment (R132H, siCont = 0.45% vs. siGCLM.2 = 31.2%; Fig. 2D and E).

The transcription factor Nrf2 is a basic leucine zipper (bZIP) protein that regulates the cellular responses to oxidative stress by activating the expression of antioxidant genes (24). Under physiologic conditions, Nrf2 is tightly controlled by interaction with Kelch-like ECH-associated protein 1 (Keap1), which is a protein adaptor for E3 ubiquitin ligases, and proteasomal degradation. When cells are challenged by oxidative stress, Keap1–Nrf2 interaction is disrupted and the dissociated Nrf2 translocates into the nucleus for transcriptional activation (25). In IDH1-mutated cells, the increased ROS levels may trigger Nrf2 stabilization and gene transcription, which could be relevant to prompt GSH synthesis. To test this hypothesis, we first evaluated Nrf2-associated gene transcription via ARE-luciferase reporter assay. We found that mutant IDH1 expression was associated with enhanced Nrf2-dependent transcriptional activation (Fig. 3A). Furthermore, through ChIP assay, we demonstrated that the affinity of Nrf2 to antioxidant gene promoters was strongly enhanced after mutant IDH1 introduction, indicating that Nrf2 transactivation plays a central role in ROS homeostasis in IDH1-mutated cells (Fig. 3B). Consistent with these findings, genetic silencing of Nrf2 resulted in downregulation of antioxidant genes, such as GCLC, GCLM, HMOX1, NQO1, and SLC7A11 in IDH1-mutated cells (Fig. 3C; Supplementary Fig. S1E). The induction of Nrf2 transcription activity was accompanied by its prolonged protein stability. Immunoprecipitation assay showed that Nrf2 ubiquitination is compromised in the presence of mutant IDH1 (Fig. 3D). Furthermore, the protein stability of Nrf2 was elevated when mutant IDH1 enzymes were expressed (Fig. 3E and F). The protein half-lives of Nrf2 were prolonged from 22.4 to 49.2 minutes (R132C) or 62.3 minutes (R132H).

Suppressing Nrf2/GSH axis results in oxidative damage in IDH1-mutated cells

Considering the central role of Nrf2 in the physiology of IDH1-mutated cells, blockade of Nrf2/GSH axis may be an effective therapeutic approach for tumors with IDH1 R132 variants. To investigate this, we tested a Nrf2 inhibitor, brusatol, in IDH1-mutated cells. Brusatol has been shown to strongly reduce Nrf2 transcriptional activity and enhance chemosensitivity in transformed cells (21, 26). Here, we confirmed that brusatol promoted Nrf2 degradation in IDH1-mutated cells, as evidenced by increased Nrf2 protein ubiquitination (Fig. 4A). Moreover, cycloheximide pulse chase assay confirmed that Nrf2 protein stability is compromised upon brusatol treatment (Fig. 4B). The protein half-lives decreased for both IDH1 R132C (67.1 vs. 11.1 minutes) and R132H (41.6 vs. 10.34 minutes) variants (Fig. 4C). Furthermore, Western blot analysis showed that Nrf2 protein levels drastically decreased after brusatol treatment (Fig. 4D). Accordingly, ChIP-PCR assay showed that the Nrf2 affinity for DNA sharply decreased in the presence of brusatol (Fig. 4E).

Importantly, the suppression of Nrf2 activity resulted into exacerbated oxidative damage and cell death in IDH1-mutated cells. Annexin V/PI flow cytometry assay showed that brusatol increased apoptotic rates by 1.9-fold and 2.7-fold in IDH1 R132C and R132H U251 cells, respectively (Fig. 4F and G). Consistently, brusatol also resulted in cell apoptosis in IDH1-mutated BTIC TS603, but the trend was much less in IDH1 wild-type BTICs (Supplementary Fig. S2D and S2E). We noticed that brusatol treatment resulted in reduced ARE-luciferase activity, suggesting that Nrf2 activity is suppressed in these cells (Fig. 4H). Quantification of GSH revealed that brusatol further reduced GSH availability in IDH1-mutated cells. Brusatol decreased the GSH/GSSG ratio by 75.8% and 75.9% in IDH1 R132C and R132H cells, respectively (Fig. 4I). Accordingly, the cytoplasmic levels of ROS were significantly elevated by brusatol treatment (Fig. 4J).

Targeting Nrf2/GSH axis suppresses IDH1-mutated xenografts

The aforementioned in vitro experiments strongly indicate that the blockade of GSH metabolism could be a valuable approach to suppress malignancies with mutant IDH1 enzymes. To better test this hypothesis, we established a xenograft mice model based on a patient-derived IDH1-mutated cell line TS603 (Fig. 5A). Patient-derived TS603 glioma cells with intrinsic mutant IDH1 enzyme were injected into NSG immunocompromised mice to establish xenograft tumor. When the tumor mass approaches 50 mm², mice were treated with either brusatol and/or the exogenous antioxidant NAC. Tumor growth curve showed that brusatol significantly reduced the expansion of tumor mass (Fig. 5B and C). Notably, NAC abolished the suppressive effect of brusatol, suggesting ROS played a critical role in brusatol effects on tumor growth. No significant loss of body weight was observed during the treatment (Supplementary Fig. S1F). Histologic analysis revealed that brusatol treatment reduced the expression of antioxidant genes such as Nrf2, SLC7A11, GCLC, and GCLM (Fig. 5D). NAC slightly restored antioxidant gene expression in the xenografts. On the other hand, brusatol led to reduced expression of Ki67, but elevated levels of DNA damage markers, γH2A.X and TUNEL (Fig. 5E).

Similarly, NAC treatment minimized cytotoxicity in IDH1-mutated xenografts.

Discussion

IDH1-mutated malignancies and therapeutic approaches

Mutations of IDH1/2 genes are widespread genetic abnormalities detected in several types of human malignancies, including lower grade glioma, leukemia, chondroma, chondrosarcoma, and cholangiocarcinoma. Cancer-associated IDH mutations cause amino acid substitution of an arginine residue in the IDH enzyme catalytic center.
Biochemical studies showed that IDH1 R132 mutants have elevated affinity for both NADPH ($K_m = 0.44 \mu M$) and $\alpha$-ketoglutarate ($K_m = 965 \mu M$), indicating that the mutant enzyme prefers NADPH and $\alpha$-ketoglutarate, whereas wild-type IDH enzyme prefers NADP$^+$ and isocitrate for its catalytic function (7, 27).

Several pioneering studies showed that direct targeting mutant IDH1 enzyme is an effective treatment for IDH1-mutated hematopoietic malignancies, such as relapsed or refractory AML (28). Regarding IDH1-mutated solid tumors, Rohle and colleagues (13) showed that the inhibition of mutant IDH1 delayed IDH1-mutated xenograft
Suppressing Nrf2/GSH axis results in oxidative damage in IDH1-mutated cells. A, Immunoprecipitation (IP) assay measures Nrf2 ubiquitination in U251 cells with IDH1-mutant expression after brusatol (Bru) treatment (40 nmol/L, 12 hours). B, Cycloheximide (CHX) pulse chase assay measures Nrf2 protein stability in IDH1-mutated U251 cells after brusatol treatment. C, Quantification of Nrf2 half-lives from results in B. D, Western blot measures the expression of GSH synthesis enzymes with brusatol treatment (40 nmol/L, 24 hours) in IDH1-mutated U251 cells. E, ChIP PCR assay showed antioxidant genes promoter affinity of Nrf2 with brusatol (40 nmol/L, 24 hours) in IDH1-mutated U251 cells. **, P < 0.05; ***, P < 0.01. F, Annexin V/PI apoptosis assay showed apoptotic changes in IDH1-mutated U251 cells with brusatol (40 nmol/L, 72 hours). Exogenous antioxidant catalase (Cata) was used as exogenous ROS scavenger. G, Quantification of apoptotic cells in F. ***, P < 0.001. H, ARE-Luciferase reporter assay showed Nrf2-associated gene transcription with brusatol (40 nmol/L, 24 hours) in IDH1-mutated U251 cells. **, P < 0.05; ***, P < 0.01. I, GSH/GSSG measurement in IDH1-mutated U251 cells with brusatol treatment (40 nmol/L, 24 hours). **, P < 0.01. IB, immunoblot.

Figure 4.

expansion in vivo. However, preliminary data from several early phase clinical trials showed modest impact on objective response rate and delay of progression of IDH1-mutated solid tumors. More effective therapies, such as developing refined inhibitors of mutant IDH1, or targeting IDH-related pathways, have been urged to improve disease outcome of IDH1-mutated malignancies. Besides direct targeting the mutant enzyme, several lines of evidence show that metabolic reprogramming in IDH1-mutated cells could be targeted to synergize with conventional chemo/radiotherapies. We and other colleagues demonstrated that NAD⁺ depletion, as well as 2-HG-mediated deficiency...
in homologous DNA recombination establish vulnerability to PARP inhibitors in IDH1-mutated cells (29–32). The glutaminase inhibitor, CB-839, has also been proposed to be useful for the treatment of IDH1-mutated cancers (33, 34). In this study, we extended the investigation of effective therapy for IDH1-mutated cancer and discovered that Nrf2/GSH metabolism could be another therapeutic vulnerability in malignancies that harbor IDH1 mutation.

IDH-mutated cells develop dependency on GSH ROS scavenging

The production of ROS is involved in several aspects of cancer biology, such as genomic instability, loss of growth control, cellular motility, and tumor invasiveness (35, 36). On the other hand, excessive ROS is harmful to biological molecules, resulting in oxidative damage to DNA, lipid, and proteins (37). Maintaining appropriate ROS levels is key to cancer cells during oncogenesis and therapeutic resistance. GSH is an endogenous antioxidant tripeptide that participates in the elimination of reactive molecules, such as free radicals, peroxides, lipid peroxides, and metals. The thiol group in reduced GSH is responsible for its reducing activity, which alleviates oxidative stress through direct reduction of disulfide bonds in the cysteine residues of cytoplasmic proteins and eliminates ROS through the GSH-ascorbate cycle (38). For IDH1-mutated malignancies, several pioneered studies suggested the correlation with GSH depletion and ROS accumulation (39). Although failed control of intracellular ROS has been indicated with tumorigenesis process (40), there is still lack of a direct evidence showing IDH1-mutant–derived ROS promote tumor development.

In this study, we found that IDH1-mutated malignancies exhibit a tendency to suffer oxidative stress, as the introduction of mutant IDH1 is closely associated with elevated ROS levels in cytoplasm and mitochondria (Fig. 1C–H). Similarly, recent research suggests that tumoral GSH levels negatively correlate with 2-HG, suggesting that IDH1-mutated cells have elevated demands for GSH (41). Our findings showed that, in IDH1-mutated cells, the key regulatory enzymes of GSH biosynthesis were upregulated to meet the increased demands of endogenous antioxidant systems (Fig. 2A–C). Loss-of-function experiments demonstrated that blocking GSH synthesis led to remarkably elevated ROS levels, oxidative stress, and apoptotic changes (Fig. 2D and E). Overall, our findings suggest that upregulation of
GSH-based ROS scavenging pathways play a central role to maintain cellular homeostasis in IDH1-mutated cancers.

**Nrf2-regulated GSH metabolism**

The multifunctional transcriptional factor, Nrf2, governs the cellular response to oxidative stress by triggering antioxidant gene transcription. It regulates a variety of genes for electrophile and oxidant metabolism, as well as genes that support cellular survival under stress conditions (42). In this study, we recorded enhanced Nrf2 transcriptional activity that was associated with the presence of mutant IDH1 enzyme (Fig. 3A and B), suggesting that Nrf2-driven antioxidant response is a compensatory response to IDH1-associated oxidative stress. As a further validation, it was shown that several known Nrf2 transcription targets, such as GCLC, GCLM, HMOX1, NQO1, and SLCA11, were upregulated in IDH1-mutated cells (Fig. 3C). Importantly, these genes play central role in cysteine uptake and de novo GSH biosynthesis, indicating that the protective effect of Nrf2 is a result of increased intracellular GSH pool. Protein stability tests showed that Nrf2 was less ubiquitinated and degraded in IDH1-mutated cells, which would lead to the activation of antioxidant expression (Fig. 3D–F). Moreover, the activation of Nrf2/antioxidant pathway not only relieves the metabolic stress for IDH1-mutated cells but may also support cellular viability and promote growth advantage during oncogenesis. Our findings highlighted the role of Nrf2-dependent GSH metabolism in IDH1-mutated cells, indicating a selective vulnerability of IDH1-mutated malignancies.

**Targeting Nrf2/GSH metabolism as a new strategy for IDH1-mutated malignancies**

Considering the critical role of Nrf2/GSH metabolism in cancer biology and therapeutic resistance, several attempts have been made to achieve specific targeting of this pathway using small-molecular compounds. For example, by high-throughput screening assay, Singh and colleagues (43) reported that the small-molecule compound ML-385 exhibits inhibitory effect on Nrf2 transcriptional activity. However, the effective dosage of ML-385 is too high for further preclinical studies in animals. In another example, Ren and colleagues (21) reported that brusatol, a quassinoid compound, exhibits potent inhibitory effect on Nrf2 transcriptional activity. In our study, we confirmed that brusatol is able to block Nrf2 activity in IDH1-mutated cells, which strongly suppressed antioxidant pathways, such as de novo GSH biosynthesis (Fig. 4). Interestingly, brusatol has been used as a sensitiser for conventional chemotherapy (21, 26, 44). In contrast, our study showed that at a similar dosage, brusatol monotherapy is sufficient to cause apoptotic changes in IDH1-mutated cells (45). We speculate that the brusatol potent efficacy is due to the induction of ROS levels, which leads to cell death in concert with the inhibition of endogenous antioxidants expression. The xenograft experiments confirmed this hypothesis. The introduction of an exogenous ROS scavenger, NAC, compromised the tumor-suppressing effect of brusatol, engendering NAC, compromised the tumor-suppressing effect of brusatol, which would lead to the activation of antioxidant expression (Fig. 5).

Overall, our study showed that neomorphic activity of mutant IDH1 enzyme results in mitochondrial and cytoplasmic ROS accumulation by disrupting NADP+/NADPH balance. The major regulator of antioxidant responses, Nrf2, controls the GSH de novo synthesis and plays a central role in the cellular physiology of IDH1-mutated cells. Blockade of Nrf2/antioxidant pathway exhibited selective cytotoxicity in cells with IDH1 mutation (Fig. 6). Our findings highlight the importance of GSH metabolism in IDH1-mutated cells and indicate a novel therapeutic approach for malignancies with IDH1 mutation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: X. Tang, X. Fu, Y. Liu, C. Yang

Development of methodology: X. Fu, Y. Liu, C. Yang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Fu, Y. Liu, D. Yu, S.J. Cai, C. Yang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Fu, Y. Liu, D. Yu, S.J. Cai, C. Yang

Writing, review, and/or revision of the manuscript: X. Tang, X. Fu, Y. Liu, C. Yang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Tang, C. Yang

Study supervision: X. Tang, C. Yang

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