Thiabendazole Inhibits Glioblastoma Cell Proliferation and Invasion Targeting Mini-chromosome Maintenance Protein 2

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ABSTRACT

Thiabendazole (TBZ), approved by the US Food and Drug Administration (FDA) for human oral use, elicits a potential anticancer activity on cancer cells in vitro and in animal models. Here, we evaluated the efficacy of TBZ in the treatment of human glioblastoma multiforme (GBM). TBZ reduced the viability of GBM cells (P3, U251, LN229, A172, and U118MG) relative to controls in a dose- and time-dependent manner. However, normal human astrocytes (NHA) exhibited a greater IC50 than tumor cell lines and were thus more resistant to its cytotoxic effects. 5-Ethynyl-2'-deoxyuridine (EdU)-positive cells and the number of colonies formed were decreased in TBZ-treated cells (at 150 μM, P < 0.05 and at 150 μM, P < 0.001, respectively). This decrease in proliferation was associated with a G2/M arrest as assessed with flow cytometry, and the downregulation of G2/M checkpoint proteins. In addition, TBZ suppressed GBM cell invasion. Analysis of RNA sequencing data comparing TBZ-treated cells with controls yielded a group of differentially expressed genes, the functions of which were associated with the cell cycle and DNA replication. The most significantly downregulated gene in TBZ-treated cells was mini-chromosome maintenance protein 2 (MCM2). siRNA knockdown of MCM2 inhibited proliferation, causing a G2/M arrest in GBM cell lines and suppressed invasion. Taken together, our results demonstrated that TBZ inhibited proliferation and invasion in GBM cells through targeting of MCM2.

SIGNIFICANCE STATEMENT

TBZ inhibits the proliferation and invasion of glioblastoma cells by downregulating the expression of MCM2. These results support the repurposing of TBZ as a possible therapeutic drug in the treatment of GBM.
Flubendazole has been shown to inhibit glioma proliferation and tumorigenesis. Mebendazole was shown to be cytotoxic to glioma and significantly prolonged mean survival in syngeneic and xenograft orthotopic animal glioma models (Bai et al., 2011). In a phase 1 clinical trial, mebendazole demonstrated long-term safety and acceptable toxicity at doses of up to 200 mg/kg (Gallia et al., 2021). Another benzimidazole, thiabendazole (TBZ; tiabendazole; 2-(thiazol-4-yl) benzimidazole), has been used to treat gut parasites in humans for over 50 years (Campbell and Cuckler, 1969; Whalen et al., 1971). TBZ inhibits blastocysts, candida albicans, penicillium, and psoriasias and prevents the formation of aflatoxin in plant feed, but it does not affect carcinogenesis and fertility in animals (Gosselin et al., 1984). A previous study demonstrated that TBZ reduced the growth of human fibrosarcoma (Cha et al., 2012). Therefore, as a non-toxic member of the family of benzimidazole compounds, TBZ has gained interest for its potential as an anticancer therapy in humans.

In this study, we examined the anticancer effects of TBZ and investigated its potential molecular mechanisms in GBM cells in vitro and in vivo. We demonstrated that TBZ induces G2/M arrest in GBM cells and inhibits invasion. We performed RNA sequencing on the tumor cells treated with TBZ to identify differentially expressed genes and found mini-chromosome maintenance protein 2 (MCM2) to be a key transcriptional factor downregulated by TBZ, showing the MCM2 as a molecular target of TBZ. Finally, we determined that TBZ inhibits GBM cell growth in vivo.

Materials and Methods

Ethics Statement. Nude mice (age, 4 weeks; weight, 14 to 17 g) were purchased from the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and maintained in the animal facility of Qilu Hospital, Shandong University under pathogen-free conditions. Ethical approval was granted by the Ethics Committee/Laboratory Animal Research Center protocols of Qilu Hospital, Shandong University (Jinan, China).

Cell Lines and Cultures. GBM cell lines, U251, LN229, A172, U118MG, and normal human astrocytes (NHA) were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and authenticated by short tandem repeat (STR) profiling. The cells are anchor-dependent cells in the absence of any stress. Complete medium for NHA, U251, LN229, A172, and U118MG is made up of the following reagents: Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS; Thermo Fisher Scientific), streptomycin (100 U/ml) and penicillin (100 U/ml). The cells were cultured in a humidified incubator (HERAcell 204i, Thermo Fisher Scientific) at 37°C, 5% CO2.

The P3 cells used in the study were derived from primary human GBM cells and were cultured in Neurobasal Medium (NBM; 21103-049, Thermo Fisher Scientific) supplemented with 2% B27 (cat#: A-1563401, ThermoFisher Scientific), 1% L-glutamine (BE17-605E, BioNordika; Oslo, Norway), 1% penicillin/streptomycin (17-003E, BioNordika), 20 ng/ml of EGF (AF-100-15, Peprotech; Rocky Hill, NJ, USA), and 20 ng/ml of bFGF (100-18B, Peprotech) in a humidified incubator at 37°C, 5% CO2. The cells were sub-cultured every 3 days at a sub-culture ratio of 1:3. A PCR-based assay was used to detect mycoplasma contamination.

Antibodies and Reagents. Mini-chromosome maintenance complex component 2 (MCM2), western-blotting dilution 1:1000), cyclin dependent kinase 1 (CDK1, western-blotting dilution 1:1000), cyclin B1 (western-blotting dilution 1:1000), N-cadherin (western-blotting dilution 1:1000), zinc finger E-box binding homeobox 1 (ZEB1, western-blotting dilution 1:1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, western-blotting dilution 1:1000) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Actin beta (ACTB, β-actin, western-blotting dilution 1:1000), proliferating cell nuclear antigen (PCNA, western-blotting dilution 1:1000), cyclin B2 (western-blotting dilution 1:1000) and Ki67 (Immuno- histochemistry dilution 1:200) were purchased from Abcam (Cambridge, UK).

Mini-chromosome maintenance complex component 5 (western-blotting dilution 1:1000), matrix metalloproteinase 2 (MMP2, western-blotting dilution 1:1000) were purchased from Proteintech (Rosemont, IL, USA).

Ubiquitin with PHD and ring finger domains (western-blotting dilution 1:1000) were purchased from Santa Cruz (Dallas, TX, USA).

Horse-radish peroxidase-labeled goat anti-rabbit secondary antibodies were provided by Zhongshan Golden Bridge Bio-technology (Beijing, China). Proteins on western blots were measured with enhanced chemiluminesence (Millipore; Burlington, MA, USA). The P4/RNase staining buffer kit was provided from BD Pharmingen (San Diego, CA, USA).

TBZ (45684, Sigma-Aldrich; St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; D2650, Sigma-Aldrich) at a concentration of 100 mM, stored at −20°C, and diluted to working concentrations in culture medium when needed. The diluted TBZ was used within 1 month.

The 96-well 3D spheroid cell reagent kit (3500-096-K, Cultiux) was purchased from Trevigen (Gaithersburg, MD, USA).

Cell Viability Assay. Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) was used to assess cell viability. GBM cells (4 × 104) were seeded into each well of 96-well plates (Corning; Corning, NY, USA) and cultured in a humidified incubator at 37°C, 5% CO2. After 24 hours, the medium was replaced with 100 μl of fresh culture medium containing different concentrations of TBZ or vehicle control (DMSO). At 24, 48, 72, and 96 hours after dosing, GBM cells were incubated with 10 μl of CCK-8 reagent in 100 μl of serum-free DMEM at 37°C for 1 hour and an EnSight Multimode Plate Reader (PerkinElmer; Singapore) was used to measure the absorbance at 450 nm. Cell viability of GBM cells transfected with MCM2 siRNA and overexpression constructs was also assessed with the CCK-8 assay.

Cell Proliferation Assay. GBM cells (1 × 104) were seeded into each well of 24-well plates (Corning) and cultured in a humidified incubator at 37°C, 5% CO2. 5-ethyl-2’-deoxyuridine (EdU) was diluted 1:1000 in DMEM complete medium, and GBM cells were treated with the EdU mixed medium for 1 hour. EdU was detected following 7 days of culturing. The medium was then replaced with fresh medium, and cells were continued culture for an additional 7 days. The culture medium was discarded, each well of the 6-well plate was fixed with 4% paraformaldehyde for 15 minutes. Each well of the 6-well plate was rinsed with 500 μl of PBS (3×), and the cells were rinsed with 500 μl of PBS (3×), and the cells were fixed with 4% paraformaldehyde for 15 minutes. Each well of the 6-well plate was rinsed with 500 μl of PBS (3×), and cells were stained for 30 minutes with crystal violet. The wells were slowly rinsed with double distilled water. After air drying the wells, clones were counted if the number of cells was >50.
Protein Lysates and Western Blotting. GBM cells were lysed with RIPA Lysis Buffer (Beyotime; Shanghai, China) supplemented with phenylmethanesulfonyl fluoride (PMSF, Beyotime) for 30 minutes after 48 hours of treatment. Lysates were centrifuged at 13,000 rpm/min for 20 minutes (Centrifuge 5804R, Thermo Fisher Scientific), and protein concentrations were assessed with the BCA assay according to the manufacturer’s instructions (Beyotime, China).

The PAGE Gel Fast Preparation Kit (PG112, Epizyme, Shanghai, China, 4°C storage) was used to prepare 1.5-mm-thick, 10% gels with 15 wells according to the manufacturer’s instructions.

Cell lysates (20 μg protein) were subjected to western blot analysis, according to previously described protocols (Kong et al., 2019). Membranes were incubated with primary antibody at 4°C overnight followed by incubation with appropriate secondary antibodies (1:2000) for 1 hour at room temperature. Chemiluminescent signals were imaged using the Chemiluminescence Imager (Bio-Rad; Hercules, CA, USA) according to the manufacturer’s protocol. Band density was quantified with ImageJ software and normalized to GAPDH or β-actin. All experiments were repeated three times.

Cell Cycle. U251 and P3 cells were diluted to 4 × 10^5 cells/ml, and 2 ml of cell suspension was seeded into each well of a 6-well plate and cultured overnight. TBZ was added to cells at the following concentrations: 0 μM (DMSO), 150 μM, and 300 μM. After 2 days, cells were rinsed and harvested at 4000 rpm/min for 5 minutes, and gently fixed in 300 μl of fresh PBS and 700 μl of 75% ethanol. Cells were incubated at 4°C overnight, harvested at 4000 rpm/min for 5 minutes, and rinsed with PBS at 4000 rpm/min for 5 minutes. The cell pellets were stained with propidium iodide (PI, BD Biosciences; San Jose, CA, USA) dye solution for 20 minutes and then followed by flow cytometry for cell cycle analysis. In gate P1, the linear relationship was set for P2L-A and P2L-H. Gate P2 is looped to select diploid and tetraploid cells, and 10,000 events were collected under P2 conditions. Data were exported, and ModFit 2.0 software was used to determine the cell cycle distribution. GBM cells transected with MCM2 siRNA and MCM2 overexpression constructs were similarly processed to obtain cell cycle parameters.

Trans-well Invasion Assay. U251 and P3 cells were diluted to 4 × 10^5 cells/ml, and 2 ml of cell suspension was seeded into each well of a 6-well plate. The cells were incubated under different conditions for 48 hours. Transwell migration plates with an 8-μm pore size (Corning; Sigma-Aldrich) were coated with Matrigel (Becton-Dickinson; Bedford, MA, USA) for 4 hours. The upper chamber of a transwell apparatus was seeded with 20,000 cells in 100 μl of DMEM without FBS and 600 μl of medium containing 10% FBS was added to the lower chamber. After incubation at 37°C, the remaining cells were removed from the top side of the insert with a cotton swab, and the migrated cells were fixed with 4% paraformaldehyde for 15 minutes, rinsed twice with PBS, and stained with crystal violet for 30 minutes. The dye was removed, and cells were rinsed with double distilled water. Images from three random views under a light microscope were acquired and used to count migrated cells.

Cell Invasion in 3D Culture. Three thousand cells were seeded into each well of the 3D Culture Qualified 96-well spheroid formation plates (Trevigen) with 100 μl of medium and cultured at 37°C, 5% CO2 for 72 hours in a humidified incubator. After 72 hours, U251 and P3 cells formed tumor spheroids. Plates were placed on ice for 15 minutes, and 50 μl of invasion matrix (Trevigen, 3500-096-03) was added to each well in the plates. Plates were centrifuged at 300xg at 4°C for 4 minutes and incubated at 37°C for 1 hour. Conditioned medium (100 μl) with different concentrations of TBZ was added to each well of the plates. P3 tumor spheroids were incubated for 3 days, and U251 tumor spheroids were incubated for 10 days. Images of the spheroids were captured every 24 hours under bright field microscopy with a 4× objective. The 192-hour images of U251 and the 48-hour images of P3 were analyzed with the software ImageJ. GBM cells after transfection with MCM2 siRNA or MCM2 overexpression constructs were also assessed in 3D invasion culture as described above.

RNA-Seq and Bioinformatics Analysis. The RNA-Seq libraries were prepared using the Illumina TruSeq RNA sample preparation kit (Illumina, San Diego, CA) and sequenced through paired-end (150 base paired-end reads) sequencing performed on the Illumina NovaSeq 6000 platform. Raw data were then quality filtered to generate “clean reads” for further analysis. The clean reads were aligned to the human genome reference (hg19) using STAR software and the reference-based assembly of transcripts was conducted using HISAT2. We used picard to compare the results and to remove redundancy and used Sematic software to detect single-nucleotide variations and InDels. All previously identified single-nucleotide variations and InDels were determined using the dbSNP database. Gene expression values were expressed as reads per kilobase of exon per million fragments mapped using kallisto software. To identify truly differentially expressed genes, the false discovery rate was used for the rectification of the P values. The differentially expressed genes (P value ≤ 0.05, |log2FC| ≥ 1) were subjected to enrichment analyses of gene ontology and Kyoto Encyclopedia of Genes and Genomes pathways. Protein–protein interaction networks were visualized with Cytoscape software.

RNA Interference. GenePharma (Shanghai, China) used BLAST (www.ncbi.nlm.nih.gov/BLAST?) to select an appropriate target sequence and synthesized siRNAs. Interfering RNA sequences (siRNA) targeting human MCM2 (GenePharma Gene; Shanghai, China) were transfected into cells with Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. Western blot was used to determine the knockdown efficiency 48 hours after transfection. siRNA sequences used were the following: Negative control (sense 5′-UCG GCC UAG CAC GUU CTA CAC-3′, antisense 5′-GCU UCC GAA CCG ACA GCG GGT-3′), MCM2-Homo-709 (sense 5′-GUG GUG AUC AAC UAU GAG GAC UTT-3′, antisense 5′-AGU GUG ACA UCC CAA UGA CCA CTT-3′), MCM2-Homo-1211 (sense 5′-CCA UAC AGU ACA ACC ATT-3′, antisense 5′-UGG UAC UGC UAG AUG GUG CTT-3′), MCM2-Homo-1355 (sense 5′-GCA UAC ACA ACC ATT-3′, antisense 5′-UAG UUG UUG UAG AUG ACC-3′).

Lentiviral Transduction. Lentiviral vectors expressing human mRNA targeting MCM2 (GenePharma, Shanghai) or scrambled-control (negative control) were used to generate stable cell clones overexpressing MCM2 or a nonspecific RNA as the control. Transfected clones were selected in 1 mg/ml of puromycin (Selleckchem; Houston, TX, USA) for 2 weeks. Western blot analysis was used to evaluate the transduction efficiency.

Orthotopic Xenograft Model. P3 cells expressing luciferase-GFP (X×10X; OBio Technology; Shanghai, China) were implanted into the brains of nude mice. After 7 days, tumor was determined using bioluminescence imaging (PerkinElmer IVIS Spectrum; Walltham, MA, USA), and the mice were divided into the following two groups: control, n = 5; TBZ, n = 5. Mice were intraperitoneally injected with diluted DMSO alone (control) and TBZ (50 mg/kg per day) every day. Tumor volume was monitored using the bioluminescence imaging every week for 3 weeks, and the weight of each mouse was recorded every week for 4 weeks. Tumor-bearing nude mice were treated until severe symptoms or death appeared imminent: the body weight had decreased > 10%, and mice were unable to return upright after being pushed down. Survival (in days) was determined as the number of days starting from implantation (day 1) to death. Mice were euthanized at the end of the experiment. Excised tumor tissue was snap frozen in liquid nitrogen or formalin-fixed for further analysis.

Liquid Chromatography–Tandem Mass Spectrometry Analysis. The nude mice were separated into TBZ-treated group (intraperitoneal injection, three mice) and control group (three mice). Two hours after the injection, the mice were anesthetized with chloral hydrate solution (Qiliu hospital, China). PBS was perfused through the heart. The mice were sacrificed by CO2 inhalation and the brain samples were collected and stored at −80°C. The TBZ and the brain samples were further analyzed by liquid chromatography–tandem
Fig. 1. Thiabendazole (TBZ) promotes G2/M arrest in glioblastoma multiforme cells in vitro. (A) Cell Counting Kit-8 assays to measure cell viability of P3, U251, LN229, A172, U118MG, and NHA treated with TBZ at different concentrations for 48 hours. Data points represent the percentage (%; OD450 treated/OD450 untreated) relative to untreated cells. (B) IC50 value of cell lines P3, U251, LN229, A172, U118MG, and NHA calculated with GraphPad Prism 8. (C) Cell Counting Kit-8 assays to measure cell viability of P3 and U251 with TBZ at different concentrations. 

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<tr>
<th>Cell Line</th>
<th>IC50 (µM)</th>
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<tr>
<td>P3</td>
<td>215.1</td>
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<tr>
<td>U251</td>
<td>346.0</td>
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<tr>
<td>LN229</td>
<td>266.0</td>
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<tr>
<td>A172</td>
<td>160.4</td>
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<td>U118MG</td>
<td>455.5</td>
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<td>NHA</td>
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mass spectrometry to examine the distribution of TBZ within the brain tissue. In brief, the tissue samples were weighed, and appropriate amounts of methanol (chromatographically pure; Thermo Fisher, USA) and zirconia grinding beads were added (Servicebio, Wuhan, China). The samples were ground for 5 minutes after vortexing for 10 minutes and then centrifuged at 15,000 RPM for 10 minutes (centrifuge: D3024R, Dragonlab, Beijing, China). The supernatant was collected and diluted for the analysis on the UltiMate 3000 RS (ThermoFisher Scientific, USA) and TSQ Quantum (ThermoFisher Scientific, USA) instruments.

**Plotting and Statistical Analysis.** Each assay was independently conducted at least three times. All analyses were performed using GraphPad Prism 8.02 software (San Diego, CA, USA). Data were reported as the mean ± S.D. The statistical significance of data were evaluated using a Student's t-test, and the following P values were considered significantly different: *P < 0.05; **P < 0.01; ***P < 0.001.

**Results**

**Thiabendazole Induces G2/M Arrest in GBM Cells.** To determine whether TBZ is cytotoxic to GBM, we first exposed GBM cell lines and NHA to TBZ in vitro. The viability of all cells tested, including P3, U251, LN229, A172, U118MG, and NHA, decreased in a dose-dependent manner with increasing concentrations of TBZ (Fig. 1A). The IC₅₀ of NHA was at least 100 μM greater than for all other cell lines, indicating that TBZ might be selective for tumor cells at certain concentrations (Fig. 1B). In the functional experiments, we then chose P3, representing a primary GBM cell line and U251, representing one of most common GBM laboratory cell lines. In the colony-forming assay, colony numbers were decreased by ~50% for P3 and U251 cells with 150 μM of TBZ and decreased by 90% for P3 and 75% for U251 with 300 μM of TBZ (Supplemental Fig. 1, A and B). Finally, the viability of P3 and U251 cells under treatment with different TBZ concentrations was less at 96 hours compared with 48 hours (Fig. 1C). These results indicated that TBZ potently reduced viability of GBM cells in a dose- and time-dependent manner.

Cell proliferation and cell cycle parameters were also examined in P3 and U251 cells under TBZ treatment. In P3 cells, EdU incorporation was reduced by 70% with 150 μM of TBZ and 90% with 300 μM of TBZ. In U251 cell lines, EdU incorporation was reduced by 40% with 150 μM of TBZ and by ~70% with 300 μM of TBZ. The EdU reduction in both P3 and U251 cell lines occurred in a dose-dependent manner with increasing TBZ concentrations (0, 150, and 300 μM) at 48 hours (Fig. 1D and Supplemental Fig. 1C). Cell cycle analysis further demonstrated that the percentage of GBM cells accumulated in G2/M increased under TBZ treatment in a dose-dependent manner (Fig. 1E and Supplemental Fig. 1D). Finally, in western blot analysis, proteins associated with cell proliferation were decreased in cells treated with TBZ. Protein levels were all reduced by more than 60% in 300 μM of TBZ (Fig. 1F). These results demonstrated that levels of key checkpoint proteins paralleled cell cycle arrest induced by TBZ in GBM cell lines.

**TBZ Inhibits Invasion of GBM Cells.** To determine whether TBZ might inhibit infiltration capabilities of GBM cells, we examined GBM cells under TBZ treatment in transwell and Matrigel assays. In transwell assays, the number of P3 cells penetrating the membrane was reduced from >250 (0 μM of TBZ) to ~120 (150 μM of TBZ) and to no more than 60 (300 μM of TBZ). The number of invasive U251 cells was reduced from ~250 (0 μM of TBZ) to ~100 (150 μM of TBZ) and ~35 (300 μM of TBZ). After 48 hours of TBZ treatment, the number of cells in both cell lines was reduced in a dose-dependent manner relative to controls (0 μM; Fig. 2A). We also measured the invasive ability of GBM spheroids derived from P3 and U251 cells in suspension culture. The invasive areas of P3 spheres in Matrigel were also decreased to 65% (150 μM of TBZ) and 27% (300 μM of TBZ), and the invasive areas of U251 were decreased to 35% (150 μM of TBZ) and 17% (300 μM of TBZ) after exposure to TBZ relative to controls (0 μM of TBZ; Fig. 2B). Increasing TBZ concentrations further led to reduced invasion (Fig. 2B). In western blots performed on lysates prepared from P3 and U251 cells treated with TBZ, invasion-related proteins associated with EMT, such as N-cadherin, ZEB1, and MMP2, were downregulated >30% with 150 μM of TBZ and >50% with 300 μM of TBZ. Protein levels decreased in response to TBZ in a dose-dependent manner (Fig. 2C). These results indicated that TBZ suppressed invasion of GBM cells and inhibited expression of proteins involved in EMT.

**MCM2 is Significantly Downregulated in TBZ-treated Glioma Cells.** To identify potential gene targets of TBZ, we performed RNA sequencing on RNA isolated from GBM cells treated with the molecule. Gene ontology and Kyoto Encyclopedia of Genes and Genomes analysis of the resultant differentially expressed genes showed that TBZ treatment altered expression of genes associated with the cell cycle, mitosis, and DNA replication (Fig. 3, A and B). Through protein–protein interaction enrichment analysis, we selected out a protein–protein interaction network involving genes regulating the G2/M phase of the cell cycle (Fig. 3, C and D). Of the top ten differentially expressed genes in both P3 and U251 cell lines, MCM2, ubiquitin with PHD and ring finger domains 1, and mini-chromosome maintenance complex component 5 showed the greatest difference in expression levels between treated and untreated cells (Fig. 3E). However, we found only MCM2 to be significantly downregulated at the protein level in both TBZ-treated P3 and U251 cell lines (Supplemental Fig. 2). Moreover, MCM2 protein levels decreased in a dose-dependent manner (Fig. 3F).

In Kaplan-Meier analysis performed with expression data from the TCGA and CGGA datasets, we found MCM2 to be

(0, 100, 200, and 300 μM) and at different time points (24, 48, 72, and 96 hours). Data points are the OD450 values. All data are expressed as the mean ± S.D. of values from triplicate experiments. (D) Analysis of EdU-positive cells of P3 and U251 treated with different concentrations of TBZ for 48 hours. The percentage of EdU-positive cells (EdU-positive/DAPI-positive × 100%) was quantified in three random fields per sample. (E) Data points represent the percentage of cells in G0/G1 and G2/M in P3 and U251 at 48 hours after TBZ treatment. (F) Western blot to detect levels of cyclin B1, cyclin B2, CDK1, PCNA, and β-actin in P3 and U251. All data are expressed as the mean ± S.D. of values from triplicate experiments. ns = none-significant, *P < 0.05, **P < 0.01, and ***P < 0.001 compared with controls.
increased in GBM and low-grade gliomas. Furthermore, high expression of MCM2 was related to poor survival of patients with glioma (Fig. 3, G and H). These results indicate that TBZ plays a role in causing G2/M cell cycle arrest in GBM cells, which may be mediated through the downregulation of MCM2. Thus, MCM might be a novel therapeutic target for the treatment of human glioma.

**Knockdown of MCM2 Inhibits Glioma Cell Proliferation and Invasion.** To determine whether loss of the putative TBZ target MCM2 suppressed GBM proliferation, we
Fig. 3. Mini-chromosome maintenance complex component 2 (MCM2) mRNA and protein are downregulated by thiabendazole (TBZ) in glioblastoma multiforme cells in vitro. (A) Gene ontology analysis on the differentially expressed mRNAs identified by RNA sequencing of RNA isolated from TBZ-treated P3 and U251 cell lines versus controls. (B) Pathway enrichment analysis of the differentially expressed mRNAs identified through RNA sequencing of RNA isolated from TBZ-treated P3 and U251 cell lines compared with controls. (C) Protein–protein interaction enrichment analysis to predict genes interacting with other genes. (D) The network of protein–protein interaction derived from the differentially
expressed genes associated with the cell cycle. (E) Top ten genes with the greatest fold change in mRNA expression levels (fold change value >1000) in P3 and U251, respectively. Intersecting genes, MCM2, mini-chromosome maintenance complex component 5, ubiquitin with PHD and ring finger domains 1, downregulated in P3 and U251 cells are highlighted in red. (F) Western blot to determine expression levels of MCM2 and GAPDH (protein loading control) in P3 and U251 cells treated with DMSO or TBZ at the indicated concentrations for 48 hours. (G) Analysis of expression levels of MCM2 mRNA in normal brain tissue samples and glioblastoma multiforme samples in the publicly available database TCGA. *P < 0.05. (H) Kaplan-Meier analyses to determine differences in overall survival for patients with low and high MCM2 expression, MCM2 OE, were increased in U251- and P3-MCM2-OE cells (Fig. 5E). In conclusion, these results indicated that overexpression of MCM2 reversed TBZ-induced inhibition of proliferation and invasion in GBM cells.

**Discussion**

Benzimidazole carbamate derivatives are approved compounds for the treatment of parasitic diseases in humans.
Fig. 4. Knockdown of mini-chromosome maintenance complex component (MCM2) inhibits glioblastoma multiforme cell proliferation and invasion. (A) Western blot to assess knockdown efficiency of MCM2 by siRNA in P3 and U251 cells. (B) Cell Counting Kit-8 assays to measure cell viability of P3 and U251 transfected with MCM2 and control si-RNAs. Data points are OD450 values. **P < 0.01, ***P < 0.001. (C) Cell cycle distribution of P3 and U251 determined with propidium iodide staining by flow cytometry analysis. Data points are the percentage of cells in G0/G1 and G2/M in P3 and U251 at 48 hours after si-RNA transfection. *P < 0.05, **P < 0.01, ***P < 0.001. (D) Western blot to detect expression levels of PCNA, cyclin B1, cyclin B2, CDK1, and GAPDH (protein loading control) in P3 and U251 cells 48 hours after si-RNA transfection. (E) 3D invasion assays for P3 and U251 transfected with MCM2 or control si-RNA (scale bars: 200 μm). Graphic representation of the ratio values of the invasion area to the core area. *P < 0.05, **P < 0.01. (F) Quantification of transwell cell number for P3 and U251 cells transfected with MCM2 or control si-RNA. **P < 0.01, ***P < 0.001. (G) Western blot to determine expression levels of N-cadherin, ZEB1, MMP2, and GAPDH in P3 and U251 transfected with MCM2 or control siRNA.
Such compounds include mebendazole, albendazole, fenbendazole, thiabendazole, and flubendazole. They kill worms by binding and inhibiting beta-tubulin (Cumino et al., 2009). TBZ was approved by the United States Food and Drug Administration in 1967 for use in humans and has been used as antifungal treatment for half a century. Previous reports indicated

Fig. 5. Overexpression of mini-chromosome maintenance complex component (MCM2) rescues glioblastoma multiforme cells from thiabendazole inhibition. (A) Cell Counting Kit-8 assays to measure cell viability of P3- and U251-MCM2-OE and parental cell lines under the conditions indicated. (B) The percentage of cells in G2/M in the four treatment groups. (C) Western blot to determine expression levels of MCM2, PCNA, cyclin B1, cyclin B2, CDK1, and GAPDH in P3- and U251-MCM2-OE and parental cell lines under the conditions indicated. (D) 3D invasion assays for P3- and U251-MCM2-OE and parental cell lines under the conditions indicated (scale bars: 200 μm). Graphic representation of ratio values of the invasion area to the core area. (E) Western blot to detect expression levels of N-cadherin, ZEB1, MMP2, and GAPDH in P3- and U251-MCM2-OE and parental cell lines under the conditions indicated. All data are expressed as the mean ± S.D. of values from triplicate experiments. ns = none-significant, *P < 0.05, **P < 0.01, and ***P < 0.001.
that TBZ has antitumor effects in melanoma and fibrosarcoma, including inhibition of proliferation and migration in melanoma B16F10 cells and angiogenesis in fibrosarcoma (Zhang et al., 2013). These potential antineoplastic properties render TBZ to be of possible value as a repurposed drug for the treatment of GBM. In this study, we demonstrated that TBZ inhibited cell viability of several GBM cell lines, including P3, U251, LN229, and U118MG. The molecule suppressed proliferation of GBM cells (P3 and U251) by inducing G2/M arrest. Moreover, TBZ reduced invasion and migration of GBM cells.

Loss of cell cycle checkpoint control underlies the aggressive proliferation and dysregulation of the cell cycle associated with GBM. Thus, therapies have been designed to inhibit the cell cycle (Dominguez-Brauer et al., 2015). The mechanism of action of many microtubule inhibitors involves inhibition of the G2/M phase (Castro-Gamero et al., 2018). These drugs may be synergistic with the current standard of GBM therapy (temozolomide or radiotherapy), either by facilitating DNA damage or sensitizing malignant cells to standard therapy (Vitovcova et al., 2020). In future studies, we plan to explore...
the effect of the combination treatment of TBZ, TMZ, and radiotherapy on GBM.

RNA sequencing revealed potential targets of TBZ in GBM cells. Using gene ontology, Kyoto Encyclopedia of Genes and Genomes, and protein–protein interaction network analysis, we found that TBZ regulates the expression of proteins that function in cell proliferation and the cell cycle. MCM2 was among the most highly differentially expressed genes under TBZ treatment, and overexpression of the gene rescued TBZ-treated cells from inhibition of cell growth. As the cytotoxic effect of TBZ (>IC50) was lower in NHA than in GBM cells, the molecule might be selective for tumor cells at certain concentrations in the clinical management of patients.

The function of MCM is regulated at elongation and termination of DNA replication (Brewster and Chen, 2010; Li et al., 2015; Geo and Kang, 2018). In the process of carcinogenesis, the dysfunction of MCM generates instability in the structure of the DNA fork, thus creating conditions for the acquisition of the gene mutations driving tumor development. As a member of the MCM family, MCM2 has been shown to be overexpressed in various tumors, including hepatocellular carcinoma (Yang et al., 2018; Yang et al., 2019), pancreatic adenocarcinoma (Peng et al., 2016; Xi and Zhang, 2018), lung cancer (Cheung et al., 2017), breast carcinoma (Yousef et al., 2017; Issac et al., 2019), ovarian cancer (Deng et al., 2019), and cervical cancer (Mukherjee et al., 2007; Amaro Filho et al., 2014). MCM2 was predicted to be a valuable prognostic biomarker in cervical cancer (MCM2-regulated functional networks in lung cancer by multi-dimensional proteomics) (Castro-Gamero AM, Penak all Alexlebrasce MS, and Tome-LG, 2018) and MCM2 inhibitors are pharmacotherapeutic opportunities for glioblastoma: the old, the new, and the future. Cancer Biol Med 15:354–374.

In addition, MCM2 was suggested to be a potential treatment target to breast cancer and prostate cancer (Hsu et al., 2021; Liu et al., 2021). In neuroblastoma, MCM2 expression is positively correlated with tumor growth, and thus the gene is a novel potential target for neuroblastoma pharmacological treatment (Garbati et al., 2020). Our bioinformatic analysis showed that MCM2 is upregulated in GBM tissue (TCGA) and related to decreased survival in patients with glioma (CGGA). Silencing MCM2 through siRNA knockdown inhibited proliferation and invasion of GBM cells. In contrast, the overexpression of MCM2 partially rescued GBM cells from cell cycle arrest and reduced invasion under TBZ treatment. These results suggest that MCM2 is a critical molecular target of TBZ and warrants further study as a biomarker for TBZ as a potential treatment of GBM.

In summary, TBZ inhibits the proliferation of GBM by inducing a G2/M phase cell cycle arrest and suppresses the invasion of GBM tumor cells. TBZ downregulated the expression of MCM2, which is overexpressed in GBM based on analysis of publicly available datasets. Knockdown of MCM2 decreased GBM tumor growth and invasion in vitro, and TBZ inhibits growth of GBM cells in vivo. These findings thus warrant further investigation into repurposing TBZ for the treatment of patients with GBM. However, although TBZ does slow tumor growth in our animal model, eventually the tumor kills tumor-bearing mice, possibly due to TBZ-resistant tumor cells. This recovery phase under TBZ treatment would therefore also be of interest to study in the future.

Authorship Contributions

| Participated in research design: | Yaotian Hu, Wenjing Zhou, Jian Wang. |
| References |


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Thiabendazole inhibits glioblastoma cells proliferation and invasion targeting MCM2

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**Figures and Legends**

**Supplementary Figure 1.** Thiabendazole promotes G2/M arrest in GBM cells in vitro. (S1A)
Representative images of colony formation assays for P3 and U251 under treatment with different concentrations of TBZ for 2 weeks. (S1B) Graphic representation of results from colony formation assays for P3 and U251 under treatment with different concentrations of TBZ. (S1C) Fluorescence images of EdU incorporation in P3 and U251 cells treated with TBZ (150 μM and 300 μM) or DMSO (0 μM) for 48 h. Cells were stained with Apollo 567 (red) to detect EdU and DAPI (blue) to highlight nuclei, and images were merged (magnification, 100×). (S1D) Representative images of cell cycle distribution for P3 and U251 under TBZ treatment acquired with PI staining and flow cytometry.

Supplementary Figure 2

Supplementary Figure 2. Downregulation of MCM5, UHRF1, and MCM2 in TBZ treated GBM cells. Western blot analysis performed to detect levels of MCM5, UHRF1, MCM2 and GAPDH in P3 and U251. Graphic representation of the relative expression of MCM5, UHRF1 and MCM2 in P3 and U251 under TBZ treatment relative to the control (DMSO).
**Supplementary Figure 3.** Knockdown of MCM2 inhibits glioma cell invasion.

Representative images of transwell invasion assays for P3 and U251 transfected with MCM2 or control si-RNA (scale bars: 200 µm). Cells were fixed and stained with crystal violet.

**Supplementary Figure 4.** Overexpression of MCM2 rescues TBZ-treated P3 and U251 cells from G2/M arrest. Cell cycle distribution of P3- and U251-MCM2-OE and parental cell lines acquired with PI staining and flow cytometry.
Supplementary Figure 5. Overexpression of MCM2 reverses TBZ-induced inhibition of glioma cell invasion. Representative images of transwell invasion assays for P3- and U251-MCM2-OE and parental cell lines under the conditions indicated (scale bars: 200 µm). Graphic representation of the transwell cell number for P3- and U251-MCM2-OE and parental cell lines under the conditions indicated. ns = none-significant, * P < 0.05, *** P < 0.001.
Supplementary Figure 6. TBZ distribution in brain tissue of nude mice. LC–MS spectrum analysis of the brain tissue samples obtained from nude mice (upper panel: TBZ-treated group and
middle panel: control group (3 mice per group) vs. standard TBZ sample (lower panel).