Profound, durable and MGMT-independent sensitivity of glioblastoma cells to cyclin-dependent kinase inhibition

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TG02 is a novel cyclin-dependent kinase (CDK) inhibitor and thought to act mainly via CDK-9 inhibition-dependent depletion of short-lived oncoproteins such as MCL-1 or c-MYC. We studied the activity of TG02 in 9 human long-term glioma cell lines (LTC) and 5 glioma-initiating cell lines (GIC) using various cell death assays in vitro and in the LN-229 LTC and ZH-161 GIC models in vivo. TG02 exhibits strong anti-tumor cell activity with EC50 concentrations in the nanomolar range. Median survival in the LN-229 and ZH-161 models was moderately prolonged by TG02. Neither constitutive CDK levels nor those of MCL-1 or c-MYC correlated with sensitivity to TG02. Cdk-9 or cdk-5 gene silencing alone did not fully reproduce the effects of TG02. C-myc gene silencing inhibited cell growth, but did not modulate TG02 activity. Electron microscopy revealed cell death to be essentially apoptotic. High concentrations of TG02 induced annexin V binding and minor caspase 3 cleavage, but the pan-caspase inhibitor, ZVAD-fmk, or BCL-2 or MCL-1 gene transfer only moderately attenuated TG02-induced cell death, and caspase inhibition did not prevent loss of MCL-1 or c-MYC. TG02 activity was independent of O6-methylguanine DNA methyltransferase expression. Repetitive exposure to TG02 did not generate an acquired TG02 resistance phenotype, but accumulation of MCL-1, loss of c-MYC, or senescence. TG02 is a highly potent apoptosis-inducing agent in glioma cells in vitro. Caspase inhibition does not rescue TG02-treated cells and repetitive exposure fails to confer acquired resistance, supporting the clinical evaluation of TG02 in glioblastoma.

Introduction

Glioblastoma is an inevitably lethal intrinsic brain tumor thought to derive from neuroglial progenitor cells that affects more than 3/100,000 individuals per year worldwide.1,2 The standard of care, neurosurgical resection as safely feasible followed by radiotherapy with concomitant and subsequent maintenance temozolomide chemotherapy (TMZ/RT → TMZ) results in a median survival of 16 months in clinical trial populations.3 All pharmacological and immunotherapeutic approaches to improve outcome beyond this standard of care have failed.4-5

Cyclin-dependent kinases (CDK) have recently attracted interest as targets for cancer therapy, e.g., CDK-5 as a mediator of transforming growth factor (TGF)-β signaling and programmed death ligand 1 expression,6,7 CDK-7 as a transcriptional regulator,8,9 and CDK-9 for its control of c-myc expression.10

Key words: glioma, resistance, MGMT, senescence, chemotherapy

Abbreviations: AmxV: annexin V; CDK: cyclin-dependent kinase; cmr-A: cytokine response modifier; GIC: glioma-initiating cell lines; hprt1: hypoxanthine phosphoribosyltransferase 1; HRP: horseradish peroxidase; LTC: long-term glioma cell lines; MGMT: O6-methylguanine DNA methyltransferase; PI: propidium iodide; pRNAPII: phosphorylated RNA polymerase II; RT: radiotherapy; TCGA: The Cancer Genome Atlas; TGF: transforming growth factor; TMZ: temozolomide

Additional Supporting Information may be found in the online version of this article.

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TG02 is a brain-penetrating oral multi-kinase inhibitor that inhibits CDK-1, −2, −5, −7 and −9 and other tyrosine kinases. Inhibition of CDK-9, the kinase most sensitive to TG02, results in a decrease in the phosphorylation of RNA polymerase II (pRNAPII). Through CDK-9 inhibition, TG02 depletes short-lived proteins such as c-MYC or MCL-1, a survival protein of the BCL-2 family, and induces apoptosis in myeloma cells. c-MYC has been validated as a therapeutic target in a genetic experimental glioma model with conditional c-MYC inhibition in vitro and in vivo by reduced proliferation, increased apoptosis and ineffective mitosis. We have previously linked increased MCL-1 protein levels to inferior outcome in glioblastoma, and its expression may be increased in recurrent tumors. Further, genetic or pharmacological suppression of MCL-1 may sensitize glioblastoma cells to tumor necrosis factor-related apoptosis-inducing ligand. The first clinical studies of TG02 in human cancer patients revealed overall good safety and tolerability, with fatigue, vomiting, abdominal pain, decreased appetite and diarrhea as the dose-limiting toxicities (Tragara, data on file). Taken together, these observations support the clinical development of TG02 as a novel approach of (multi)targeted pharmacotherapy to glioblastoma, and early phase I/II studies in recurrent and newly diagnosed glioblastoma are ongoing. Here, we provide a detailed analysis of molecular determinants of sensitivity to TG02 in an extended panel of human glioma models.

Materials and Methods

Cell culture

The human long-term cell lines (LTC) (LN-18, LN-428, D247MG, LN-319, A172, U87MG, T98G, LN-308, LN-229) and human glioma-initiating cell lines (GIC) (T-325, T-269, ZH-161, S-24, ZH-305), LN-428, ZH-161 and ZH-305 cells with lentiviral c-met gene silencing, LN-229 cells with O6-methylguanaine DNA methyltransferase (MGMT) overexpression, and LN-18 and LN-229 cells with acquired resistance to TMZ have been described. LTC were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% glutamine (10 μM/mL) (Invitrogen, Basel, Switzerland). GIC were isolated after obtaining informed consent and approval of the local ethics committee from freshly resected tumors using the Papain Dissociation System (Worthington Biochemical Corporation, Lakewood, NJ). Briefly, tumor tissue was minced and tissue was digested in DNase/Papain mixture (vial #2 and #3) for 30 min at 37 °C. After incubation, the tissue was filtered twice through a 70 μm mesh and washed with phosphate-buffered saline. Cells were centrifuged, the supernatant was removed and the pellet was separated into cells and membrane fragments by using a gradient consisting of sterile Earle’s Balanced Salt Solution/inhibitor solution mix (vial #1 and vial #3) and albumin inhibitor (vial #4). Cells were resuspended in phenol red-free complete Neurobasal Medium (NB) with B-27 supplement (20 μM/mL) (Thermo Fisher Scientific, Waltham, MA), t-glutamine (10 μM/mL), fibroblast growth factor-2 and epidermal growth factor (20 ng/mL each; Peprotech, Rocky Hill, PA) and penicillin/streptomycin (pen-strep, Sigma-Aldrich/Merck, Darmstadt, Germany) and cultured overnight. The next day medium was exchanged and cells were cultured in phenol red-free complete NB. All cells were sent for short tandem repeat analysis (DSMZ, Braunschweig, Germany) and are regularly tested for mycoplasma contamination.

Reagents

TG02 was provided by Tragara (Carlsbad, CA). Its activity against various kinase targets was assessed at S’BIO Pte Ltd (Singapore) according to standard techniques. Human CD95 ligand (mega-CD95L) was provided by Topotarget (Copenhagen, Denmark). Cycloheximide was purchased from Sigma (St. Louis, MO), staurosorpine was from AppliChem (Darmstadt, Germany). Tepotinib was provided by Merck (Darmstadt, Germany). zVAD-FMK and ac-DEVD-amc were purchased from Bachem (Bubendorf, Switzerland). siRNA (ON-TARGET plus human siRNA-SMART pool) to cdk-5, cdk-7, cdk-9 and c-myc was purchased from Dharmacon (Lafayette, CO).

Transfections

LN-229 cells expressing the viral gene product, crm-A, which blocks caspases 8 and 1, were generated as described. Stable transfection of glioma cells with the sequence-verified human bcl-2/pDNA3 plasmid was performed using the X-tremeGENE™ HP DNA Transfection Reagent (Penzberg, Germany). Clones with transgene expression were selected by G418 sulfate. Lentiviral expression vector containing human MCL-1 (NM_021960.4) open reading frame cDNA was purchased from GeneCopoeia (Rockville, MD). Infectious particles were produced in HEK 293 T cells using the expression vector, pCMV-dR8.91 packaging and pMD2.G envelope plasmids. Glioma cells were transduced with lentiviral particles and stably transfected cells were selected by puromycin.
transfection of LN-229 and ZH-161 cells was done by electroporation (Neon transfection system, Invitrogen) and non-targeting siRNA pool (Dharmacon) was used as a negative control.

Viability assays and flow cytometry
The cells were seeded in 96-well plates, allowed to recover for 24 h in complete medium, and were then exposed to TG02 for acute 72 h growth inhibition assays (high seeding density) or for assessment of clonogenic survival (low seeding density). Metabolic activity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). It was previously confirmed to reflect numbers of colonies or spheres. Induction of cell death at 72 h treatment was evaluated by annexin V (AnxV) and propidium iodide (PI) (BD PharMingen and Sigma) flow cytometry. Staurosporine-induced apoptosis was used as a positive control. Fluorescence for each condition was recorded in a BD FACSuite™ software (BD Biosciences) flow cytometer and analyzed by BD FACSuite™ software (BD, Allschwil, Switzerland). For cell cycle analysis, the cells were fixed, permeabilized with ice-cold 70% ethanol, washed, RNA was digested with RNase A (Gibco), and DNA was stained with PI (Sigma-Aldrich).

Transmission electron microscopy
The cells including supernatant were fixed in 2x fixation solution (2.5% glutaraldehyde and 1.6% formaldehyde in a 100 mM sodium cacodylate buffer, pH 7.4, final concentration), dehydrated in a graded ethanol series and embedded into epon. Sections of 60 nm were imaged with a Tecnai Spirit transmission electron microscope (FEI, Hillsborough, OR).

Real-time PCR
mRNA was isolated using the NucleoSpin® RNA II system (Macherey-Nagel, Düren, Germany) and DNase treatment. cDNA was prepared using the cDNA reverse transcription kit (Applied Biosystems, Forster City, CA). Target gene expression was determined by real-time PCR using hypoxanthine phosphoribosyltransferase 1 (hprt1) as a housekeeping gene with a variation of the 2^ΔΔCT method for relative quantification (Livak and Schmittgen, 2001). The primer sequences are summarized in Supporting Information Table S1.

Immunoblot
Cell lysates were prepared and quantification of protein was performed as described. Proteins were separated by SDS-PAGE under reducing conditions and then transferred to nitrocellulose membranes (Biorad, Munich, Germany) which were blocked in Tris-buffered saline containing 5% skim milk and 0.1% Tween 20 prior to antibody incubation, using the following primary antibodies: CDK-9 and CDK-5 (Santa Cruz Biotechnology, Inc., Dallas, TX), CDC-2 (POH1, CDK-1), CDC-2, CDC-7, c-MYC, MCL-1, X-linked inhibitor of apoptosis (XIAP), pRNAPII, BCL-2, caspase 3, LC3A/B (all Cell Signaling Technology, Leiden, The Netherlands) and MGMT (Thermo Fisher Scientific). Protein bands were visualized using horseradish peroxidase (HRP)-coupled secondary antibodies (Santa Cruz Biotechnology) and enhanced chemoluminescence (Pierce/Thermo Fisher, Madison, WI). Quantification of bands for correlation analyses was done using ImageJ software (Open Source).

Generation of TG02-resistant GIC sublines
LN-229, LN-308, ZH-161 or ZH-305 were split into two fractions labeled as parental (naïve) or resistant (R) cells and continuously processed in parallel. They received fresh medium without or with TG02 at the same time. R cells underwent serial TG02 stimulation at increasing concentrations every 3–4 weeks (10, 20, 40, 80 nM). Before starting the next stimulation cycle, a sub-fraction of naïve and R cells were seeded to assess growth inhibition using 72 h drug exposure assays.

Senescence staining
Non-irradiated or irradiated (20 Gy) naïve or TG02-R LN-229 or LN-308 cells were cultured on Slide & Chamber microscopy slides (Watson Bio Labs, Tokyo, Japan) and β-galactosidase activity was determined at pH 6 using the Senescence β-galactosidase Staining Kit (Cell Signaling Technology). The cells were incubated for 3 days at 37 °C without CO2, then covered with 70% glycerol, and blue coloration was observed under the microscope (20x magnification).

Animal studies
Tissue distribution was assessed in female Balb/c nude mice after a single oral administration of 75 mg/kg TG02. At 0, 10 and 30 min, and 1, 2, 4, 8 and 24 h after dosing, 3 mice per sampling point were sacrificed by administering an overdose of carbon dioxide. Blood samples were collected by cardiac puncture and tissue samples were collected and extracted in methyl tert-butyl ether. Samples were analyzed for TG02 using a validated LC-MS/MS method.

The standard operating procedures for the therapeutic animal studies were approved by the Swiss Cantonal Veterinary office under the Animal license permission number ZH105/2015. The care and treatment of all animals was in accordance with the Swiss Federal Law on the Protection of Animals, the Swiss Federal Ordinance on the Protection of Animals and the guidelines of the Swiss confederation. Tumor cell implantation was performed as described. Briefly, 7.5 × 10^6 LN-229 or 10^7 ZH-161 cells were implanted intracranially into the right striatum of immunocompromised CD1 nude mice (Charles River, Sulzfeld, Germany). Systemic treatment with TG02 was performed by oral gavage twice weekly (30 mg/kg) from day 10 post surgery onwards. The mice were observed daily and euthanized when developing neurological symptoms.

For immunohistochemistry, primary antibodies were polyclonal rabbit anti-pRNAPII (Cell Signaling Technology, 4,735,
Furthermore, expression of cdk was quantified in the TCGA dataset. This was not the case except for longer survival associated with higher gene expression of cdk-1, cdk-2 and c-myc when the cut-off of highest significance was chosen (Supporting information Fig. S1a). Furthermore, expression of mcl-1, c-myc and xiap did not correlate with survival (Supporting information Fig. S1b). Next we assessed cdk mRNA expression and protein levels in our glioma cell line panel. All cdk were expressed in all cell lines, albeit at different expression levels, without correlation between mRNA and protein levels (data not shown), suggesting major post-transcriptional regulation (Figs. 1a and 1b). We also explored the constitutive expression of bona fide indirect targets of TG02, mcl-1, c-myc and xiap mRNA as determined by q-RT-PCR and respective protein levels by immunoblot: these were rather homogeneous for XIAP, but not for MCL-1 or c-MYC (Figs. 1c and 1d). There was uniform activity of TG02 in the cell line panel with EC_{50} concentrations between 43 nM and 188 nM in acute growth inhibition assays and between 14 nM and 73 nM in clonogenic cell death assays. GIC were particularly sensitive in clonogenic cell death assays (Figs. 1e and 1f). EC_{50} values for TG02 in either assay did not correlate with mRNA expression or protein levels of any direct or indirect target antigen included in Figures 1a–1d. Moderate single agent activity of TG02 alone in vivo was confirmed in one LTC (LN-229) (p = 0.050) and one GIC (ZH-161) (p = 0.056) model (log-rank Mantel-Cox test) (Figs. 1g and 1h). Pharmacodynamic activity in vivo was verified by reduction of pRNAPII and a less substantial reduction of c-MYC protein in TG02 treated animals as assessed by immunohistochemistry (Figs. 1i and 1j).

Modulation of target gene expression by TG02
TG02 expectedly decreased mRNA levels of its indirect target, mcl-1. In contrast, c-myc mRNA levels were altered in a less predictable manner by TG02, and showed major variation by cell line, concentration and time of exposure (Fig. 2a). At the protein level, exposure to TG02 resulted in rapid loss of pRNAPII and the bona fide target protein, MCL-1. XIAP levels were also reduced, albeit later than MCL-1. In contrast, changes in c-MYC levels were less consistent among cell lines, with uniform suppression in LN-229 and ZH-305, but increased rather than decreased levels at some concentrations in LN-308 and ZH-161, all in a reproducible fashion. CDK-9 levels were progressively reduced by TG02 in all cell lines (Fig. 2b and Supporting Information Fig. S2). Cdk-9 or cdk-5 gene silencing was then performed to assess whether the changes induced by TG02 could be largely attributed to CDK9 or CDK5 inhibition. CDK9 protein depletion was incomplete at protein level in both models. Yet, there was no consistent MCL-1 or c-MYC depletion in response to cdk-9 or cdk-5 gene silencing alone or in combination, neither on mRNA nor protein level (Supporting Information Fig. S3b–S3d). Conversely, c-myc gene silencing inhibited cell growth, but did not confer specific sensitization to TG02 (Supporting Information Fig. S3e and Figs. 2c–2f).

Deciphering the TG02-induced cell death pathway
Next, we assessed whether TG02 induced cell cycle arrest or cell death as its primary mode of action. Cell cycle studies showed a relatively uniform pattern: there were few changes at 6 or 24 h, but a reduction of the G0/1 fraction and less prominently of the G2/M fraction when the sub-G0/1 fraction representing dead cells emerged at 72 h; no additional peaks, corresponding to >4n, e.g., 8n, DNA content indicative of mitotic catastrophe emerged (Figs. 3a and Supporting Information S4). High concentrations of TG02 exceeding the EC_{50}...
Figure 1. TG02 target gene expression and sensitivity to TG02. (a) Cdk-1,2,5,7,9 mRNA expression was determined by q-RT-PCR using hprt1 as a house-keeping gene. (b) CDK-1,2,5,7,9 protein levels were assessed by immunoblot. The specificity of the double band for CDK-7 was verified by gene silencing (Fig. S3A, Supporting Information). (c) The expression of c-myc, mcl-1 and xiap mRNA was determined by q-RT-PCR. (d) Protein levels of c-MYC, MCL-1 and XIAP were assessed by immunoblot. (e) The cells were exposed to TG02 in triplicate for 72 h and metabolic activity was assessed by MTT assay. (f) The cells were exposed to TG02 in clonogenic survival respectively sphere formation assays in triplicate. Metabolic activity was assessed by MTT assay. Data in e and f are expressed as EC_{50} values (n = 3–5, SEM of 3–5 independent experiments). (g,h) Survival of nude mice implanted intracranially with LN-229 or ZH-161 cells treated with TG02 (30 mg/kg) twice weekly from day 14 onwards. Time to neurological deterioration was monitored daily. (i,j) Tissues harvested at 14 h after the last of a total of 11 injections of TG02 were assessed for levels of phosphorylated RNA polymerase II or c-MYC by immunohistochemistry. Inserts show isotype controls, size bar correspond to 50 μm.
induced annexin V binding to the cell membrane and moderate caspase 3 cleavage (Figs. 3b and Supporting Information Fig. S5). Electron microscopy revealed induction of typical apoptotic changes by TG02 that were seen earlier and more extensively in LN-229 than in ZH-161 cells. Features of mitotic catastrophe were seen in single cells in ZH-161, but not in LN-229 cells. Autophagy was not seen with TG02 treatment in either cell line, but readily with salinomycin that was used as a positive control (Fig. 3c). Accordingly, even high concentrations of TG02 did not induce LC3 cleavage, a
surrogate marker of autophagy, to an extent detectable by immunoblots, yet, consistent with apoptosis, TG02 induced caspase 3 cleavage and DEVD-amc-cleaving activity (Figs. 4a and 4b). However, co-exposure to the pan-caspase inhibitor, zVAD-fmk, only moderately attenuated the cytotoxic effects of TG02 (Fig. 4c) at concentrations where caspase 3 cleavage was blocked by zVAD-fmk (data not shown). Blocking specifically the extrinsic cell death pathway by gene transfer-mediated expression of the viral protein, crm-A, which blocks caspases 8 and 1, abrogated CD95 ligand-induced apoptosis, but had no effect on cell death induced by TG02 (Supporting Information Fig. S6). Next we explored whether apoptosis regulators of the BCL-2 family modulated TG02-induced cytotoxicity. We generated LN-229 and ZH-161 cell lines overexpressing BCL-2 or MCL-1 (Supporting Information Figs. S7a and S7b). Indeed, BCL-2 gene transfer into LN-229 surrogates marker of autophagy, to an extent detectable by immunoblots, yet, consistent with apoptosis, TG02 induced caspase 3 cleavage and DEVD-amc-cleaving activity (Figs. 4a and 4b). However, co-exposure to the pan-caspase inhibitor, zVAD-fmk, only moderately attenuated the cytotoxic effects of TG02 (Fig. 4c) at concentrations where caspase 3 cleavage was blocked by zVAD-fmk (data not shown). Blocking specifically the extrinsic cell death pathway by gene transfer-mediated expression of the viral protein, crm-A, which blocks caspases 8 and 1, abrogated CD95 ligand-induced apoptosis, but had no effect on cell death induced by TG02 (Supporting Information Fig. S6). Next we explored whether apoptosis regulators of the BCL-2 family modulated TG02-induced cytotoxicity. We generated LN-229 and ZH-161 cell lines overexpressing BCL-2 or MCL-1 (Supporting Information Figs. S7a and S7b). Indeed, BCL-2 gene transfer into LN-229
or ZH-161 cells moderately attenuated TG02-induced cell death (Fig. 4d) although DEVD-amc cleavage activity was completely suppressed (Supporting Information Figs. S7c and S7f). Moreover, overexpression of MCL-1 had only minor protective effects from TG02-induced cell death in LN-229 cells (Supporting Information Figs. S7e and S7f), although,
Interestingly, lentivirally encoded MCL-1 protein appeared not to be regulated by TG02-mediated transcriptional suppression (Fig. 4e). Accordingly, when caspase 3 processing was inhibited by zVAD-fmk, RNAPII dephosphorylation was unaffected, and MCL-1 and MYC proteins were still depleted by TG02, confirming that cell death induced by TG02 may proceed in the absence of caspase activation (Fig. 4f).

Molecular determinants of sensitivity to TG02
To determine whether any of the hallmark molecular lesions of glioblastoma are linked to relative resistance to TG02, we correlated results from molecular profiling of the cell line panel obtained from gene panel sequencing27 (von Achenbach et al. submitted), with TG02 sensitivity (Figs. 1e and 1f). No clear pattern emerged for TG02 sensitivity and mutational or deletional status of the tp53, pten or cdk-2 genes (data not shown). In contrast, we noted that cell lines with high p-c-MET levels assessed by LumineX assay (Szabo et al. unpublished data) were more resistant to TG02 than cell lines with lower p-c-MET levels (r = 0.65, p = 0.017). However, neither exposure to the c-MET inhibitor tepotinib nor c-met gene silencing affected cellular sensitivity to TG02 to a relevant extent (Supporting Information Fig. S8).

TG02-induced cell death is independent of MGMT
MGMT mRNA and protein levels determined by RT-PCR and immunoblot correlated (r = 0.8, p = 0.017) (Figs. 5a and 5b), but were not linked to sensitivity to TG02. MGMT levels were not affected by exposure to TG02 (Fig. 5c), and combinations of TG02 and TMZ induced additive, but never synergistic suppression of clonogenic survival (Figs. 5d–5f). The latter was true also for the combination of TG02 with single fractions of irradiation (data not shown). Moreover, LN-18 or LN-229 cell lines generated to exhibit acquired resistance to TMZ, or LN-229 cells engineered to overexpress MGMT, demonstrated resistance to TMZ, but not to TG02 (Fig. 5g).

No acquired resistance to TG02
We were also interested in determining potential escape pathways from repeated exposure to TG02 following a paradigm used previously to study acquired resistance to TMZ.21 Yet, using the LTC LN-229 and LN-308 and the GIC ZH-161 and ZH-305 as models, we did not succeed in establishing stable cell lines more resistant to TG02 than parental cell lines. In contrast, we noted that repeated exposure to TG02 reduced cell growth in GIC and that the LTC models even showed a progressive increase in TG02 sensitivity after a washout of 3–4 weeks (Fig. 6a). Assessment of TG02 targets after repetitive exposure revealed different, but profound changes: LN-229 exhibited a strong increase in MCL-1 whereas LN-308 showed a complete loss of c-MYC protein (Fig. 6b). Furthermore, long-term TG02-treated LN-229, but not LN-308 cells (data not shown) displayed β-galactosidase staining indicative of senescence (Fig. 6c).

Discussion
The failure of multiple targeted treatment approaches in glioblastoma suggests that pharmacological agents should have a broader, but still tumor-specific or at least tumor-selective activity to improve outcome beyond the moderate efficacy of the current standards of care.4,5

Here we report that the novel orally available CDK inhibitor TG02 uniformly inhibits proliferation and decreases clonogenicity respectively spherogenicity in human LTC and GIC models (Fig. 1). GIC were particularly sensitive to TG02 which has been considered essential for novel agents to hold promise in glioblastoma.28 Expectedly, phosphorylation of RNA polymerase II was inhibited by TG02 in a concentration-dependent manner, and transcription of the indirect TG02 target, mcl-1, was uniformly suppressed. In contrast, changes in c-myc expression were unpredictable, and c-MYC protein levels even increased occasionally in a cell line-, time- and concentration-dependent manner (Figs. 2a and 2b). It has previously been proposed that suppression of CDK9 may trigger a compensatory increase in c-myc expression, mediated by bromodomain-containing protein 4.29 We confirm that c-MYC may be a suitable target in glioblastoma, yet, the apparent lack of interaction between constitutive c-MYC levels and TG02 sensitivity, and the failure of c-myc gene silencing to modulate TG02 activity do not support the notion that c-MYC alone is the major pharmacological target of TG02 in glioblastoma, but rather indicate complex regulatory, potentially compensatory mechanisms of c-myc expression (Figs. 2c–2f). Of note, loss of c-MYC protein upon chronic exposure to TG02 appears to support a role for c-MYC at least in LN-308 cells (Fig. 6b).

In myeloma cell lines, TG02 induced the cleavage of initiator caspases 7, 8, and 9 within 6 h, leading to the activation of the effector caspase 3 and cleavage of its substrate, poly-ADP-ribose polymerase (PARP). Further, TG02 treatment depleted protein levels of the inhibitor of caspases XIAP in MM1S and some other myeloma cell lines. Myeloma cell apoptosis induced by TG02 was partially mediated by caspases since their inhibition by the pan-caspase inhibitor zVAD-fmk, or overexpression of MCL-1, reduced the percentage of apoptotic cells.12 In glioma cells, electron microscopy confirmed apoptosis as the dominant pathway of TG02-induced cell death (Fig. 3). The extent of caspase activation is limited (Fig. 4), involves the intrinsic rather than extrinsic cell death pathway since caspase 8 appeared not be required (Supporting Information Fig. S6), and caspase activation may eventually not be required for cell death to occur. This is because there was significant growth inhibition at concentrations of TG02 that were insufficient to promote caspase cleavage and since neither zVAD-fmk nor bcl-2 nor mcl-1 gene transfer were particularly effective in inhibiting cell death. Accordingly, the loss of survival proteins in TG02-treated cells was not prevented by pharmacological caspase inhibition, placing caspase activation probably down-stream of the commitment point for cell
death induction (Fig. 4f). While apoptosis is classically linked to caspase activation,30 it may evolve in the absence of caspase activation and still be sensitive to BCL-2 family proteins, e.g., in glioma cells exposed to alkylphosphocholines.31 TG02 acts independent of the MGMT status and may thus be particularly useful in glioblastoma lacking MGMT promoter
methylation, moreover, glioma cells generated to acquire resistance to TMZ\textsuperscript{21} were not cross-resistant to TG02 (Fig. 5).

Efforts to generate sub-cell lines with acquired resistance to TG02 yielded interesting observations, first, resistance was not induced, second, two cell lines repeatedly exposed to TG02 and studied in more depth revealed profound, but different changes predicted to be potential biomarkers of altered sensitivity to TG02, loss of c-MYC, potentially thereby loss of c-MYC dependence, or gain in MCL-1 levels, third, repeated exposure to TG02 may induce senescence (Fig. 6). These observations indicate that – theoretically – tumors sensitive to TG02 may not easily develop efficient escape strategies \textit{in vivo}. Our study complements and extends another recent study exploring the activity of TG02 in different glioma models \textit{in vitro} and \textit{in vivo} that focused on synergy with TMZ and changes in energy metabolism.\textsuperscript{32} These authors confirmed CDK9 as the main target of

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**Figure 6. Explorative studies of acquired resistance to TG02.**

(a) The cells were exposed to TG02 in 72 h growth inhibition assays. (b) Naïve or R cells were untreated or exposed to TG02 at 20 or 100 nM for 24 h. Protein levels of CDK9, MCL-1 and c-MYC were assessed by immunoblot. (c) LN-229 naïve or R cells were assessed for senescence by β-galactosidase staining, using irradiation at 20 Gy as a positive control (size bar full figure 100 μm, size bar full figure 25 μm).
TG02 and hypothesized that mitochondrial dysfunction and ATP depletion were the proximate cause of cell death, consistent with our assumption that caspase inhibition delays cell death, but that cell death induced by TG02 can proceed in a caspase-independent manner. While Su et al. observed no activity of TG02 alone in the GL-261 mouse glioma model, we observed moderate single agent activity in two human glioma models, including one GIC model (Figs. 1g and 1h).

Altogether, its broad activity including in TMZ-resistant models, independence of MGMT, and the low likelihood of inducing resistance support the clinical development of TG02 as a novel targeted agent for glioblastoma. Initial data indicate that the combination of TG02 with TMZ in dose-intensified regimens in patients with recurrent glioblastoma is feasible. A clinical trial exploring TG02 alone in recurrent glioblastoma, or in combination with either RT or TMZ, by MGMT promoter methylation status, in newly diagnosed elderly glioblastoma or anaplastic astrocytoma patients (NCT03224104 EORTC 1608, STEAM) is ongoing.

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References
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