BRAIN TUMORS

BCL3 expression promotes resistance to alkylating chemotherapy in gliomas

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The response of patients with gliomas to alkylating chemotherapy is heterogeneous. However, there are currently no universally accepted predictors of patient response to these agents. We identify the nuclear factor κB (NF-κB) co-regulator B cell CLL/lymphoma 3 (BCL-3) as an independent predictor of response to temozolomide (TMZ) treatment. In glioma patients with tumors that have a methylated O6-methylguanine DNA methyltransferase (MGMT) promoter, high BCL-3 expression was associated with a poor response to TMZ. Mechanistically, BCL-3 promoted a more malignant phenotype by inducing an epithelial-to-mesenchymal transition in glioblastomas through promoter-specific NF-κB dimer exchange. Carbonic anhydrase II (CAII) was identified as a downstream factor promoting BCL-3–mediated resistance to chemotherapy. Experiments in glioma xenograft mouse models demonstrated that the CAII inhibitor acetazolamide enhanced survival of TMZ-treated animals. Our data suggest that BCL-3 might be a useful indicator of glioma response to alkylating chemotherapy and that acetazolamide might be repurposed as a chemosensitizer for treating TMZ-resistant gliomas.

INTRODUCTION

Diffuse gliomas encompass both lower-grade gliomas (LGGs), including grade II and III tumors, and glioblastoma (GBM). Within each grade, subgroups of tumors that have significantly different responses to therapy exist (1, 2). Despite this heterogeneity, virtually all glioma patients receive alkylating chemotherapy as part of their treatment regimen. Although alkylators like temozolomide (TMZ) improve overall patient survival (3), many patients experience minimal benefit from their use. These observations underline the critical need for predictors of response to alkylating therapy.

Glioma cells are distinguished by the presence of recurrent copy number alterations (CNAs) affecting broad chromosomal regions (4). Although these alterations primarily involve driver genes, they can also affect nearby passenger genes not directly involved in malignant progression (5). It has been suggested that targeting such flanking genes might have therapeutic effects (6). However, passenger genes, unlike drivers, have not yet been shown to mediate therapeutic susceptibility in clinical glioma (7).

The best described mechanism of resistance to TMZ involves direct cytotoxic lesion repair by the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) (8). However, even in tumors with low MGMT expression, other pathways have been shown to independently modulate response to therapy and patient outcome (9).

Nuclear factor κB (NF-κB) plays an important role in promoting resistance to DNA-damaging agents in GBM (10, 11). In addition to the five primary NF-κB subunits, p50 (NF-κB1, p105), p52 (NF-κB2, p100), p65 (RelA), RelB, and c-Rel (12), multiple co-regulators also contribute to the overall downstream response. B cell CLL/lymphoma 3 (BCL-3) is an atypical inhibitor κB (IκB) protein that both activates and inhibits NF-κB signaling (13). BCL-3 is a candidate oncoprotein shown to be up-regulated in several malignancies (14, 15) that regulates NF-κB signaling in conjunction with p50- and p52-containing dimers (16–18).

Here, we show that BCL-3 reduced TMZ-induced cytotoxicity in gliomas through the activation of carbonic anhydrase II (CAII). In addition, we found that the CA inhibitor acetazolamide (ACZ) potentiated the effect of TMZ in mouse models of glioma, identifying a potential repurposing strategy for TMZ chemosensitization.

RESULTS

BCL3 is an independent predictor of response to TMZ in GBM

To examine the role of BCL-3 in response to TMZ, we altered its expression in patient-derived glioma stem-like cells (GSCs) (table S1) using vectors expressing human BCL-3 or short hairpins (sh) targeting BCL3 (fig. S1, A and B). Knockdown of BCL3 in GSCs with high basal BCL-3 increased cell death after treatment with TMZ (Fig. 1A and fig. S1C). Conversely, overexpression of BCL-3 in GSCs with low basal BCL-3 decreased TMZ-induced death (Fig. 1B and fig. S1D). Moreover, in U87, U251, and T98 GBM cell lines, knockdown of BCL3 with short interfering RNA (si-RNA) increased the TMZ-induced effect on clonal survival (fig. S1E), whereas overexpression of BCL-3 reduced the efficacy of TMZ (Fig. 1C and fig. S1F). These findings indicate that BCL-3 modulates cytotoxicity induced by TMZ.

Next, to investigate BCL3 in clinical GBM, we examined four independent expression databases. In these data sets, patients with high BCL3 expression had reduced survival compared to patients with low BCL3 expression (Fig. 1D, fig. S1G, and table S2). BCL3 expression was significantly associated with survival on multivariate analysis, taking the primary GBM prognostic factors into consideration [hazard ratio (HR), 1.455; P = 0.017; Table 1]. The expression of none of the primary NF-κB subunits correlated with survival (fig. S1H). Similarly, NFKBIA, the gene encoding IκBα, a potential tumor suppressor in GBM (19), also had no prognostic value on its own (fig. S1I). These results indicate that BCL3 expression plays an independent role in GBM patient outcome.

To understand whether BCL3 is specifically relevant in the setting of alkylaation damage, we looked at TCGA to analyze whether BCL3 expression affected survival of GBM patients who were untreated, treated with ionizing radiation (IR) alone, or treated with IR in combination
with TMZ. BCL3 expression did not segregate GBM patients into survival groups if they were untreated or treated with IR alone (Fig. 1E and fig. S1I). Consistent with this latter finding, overexpression of BCL3 in GBM cells did not alter clonal survival in response to IR (Fig. 1E, inset graphs). To validate this role of BCL3, we examined a second GBM expression data set, the Repository of Molecular Brain Neoplasia Data (REMBRANDT) (20). In this data set, patients also received procarbazine, lomustine, and vincristine (PCV) as alkylating chemotherapy. In REMBRANDT, BCL3 expression was only informative of survival in patients whose treatment regimen included alkylating chemotherapy (fig. S1I). These data indicate that BCL3 expression was not an intrinsic prognostic factor in GBM or informative of response to IR but specifically modulated the response to alkylating chemotherapy.

Given the link between alkylation damage and MGMT promoter methylation (8), we divided patients based on MGMT promoter methylation, as assessed by the MGMT-3P27 model (21). BCL3 expression only identified distinct survival groups in tumors with high MGMT promoter methylation (Fig. 1F). Specifically, in GBM with high MGMT promoter methylation, the HR based on BCL3 expression was greater than in the entire GBM population (1.88 versus 1.41, respectively; univariate Cox regression; Fig. 1F). The data

**Fig. 1.** BCL3 predicts response to alkylating chemotherapy in GBM. (A and B) Trypan blue assays in GSCs expressing the indicated construct treated with TMZ (72 hours). Data show fold change in percent dead cells relative to cells expressing a scrambled RNA sequence (sh-SCR), or empty vector (EV), treated with vehicle, ± SD (n = 3). Inset: Immunoblotting (IB) with anti-BCL-3. (C) Clonogenic assay in U87 cells stably expressing BCL-3 or EV treated with vehicle or TMZ. Data show mean value relative to vehicle ± SD (n = 3). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (D to F) Kaplan-Meier overall survival curves in GBM patients. (D) Survival based on median MGMT promoter methylation and upper and lower quartile (TCGA). (E) TCGA GBM patients separated by median BCL3 expression and treatment modality. n = 490 patients total: no treatment, IR alone, and IR + TMZ; inset graphs: IR versus IR + TMZ in patients with high or low BCL3 mRNA (median cutoff). (F) TCGA GBM patients separated by median MGMT promoter methylation and median BCL3 expression. (G) Representative IHC staining for BCL-3 in GBM. (H) Survival curves in GBM patients separated by BCL-3 IHC staining. Kaplan-Meier curves analyzed by the log-rank test. *P < 0.05 and **P < 0.01.
shown that the prognosis of patients with MGMT promoter methylated tumors that also had high BCL3 expression was similar to that in patients with an unmethylated MGMT promoter (Fig. 1F).

As public data sets primarily examine mRNA, to study the role of BCL-3 protein, we obtained specimens from a consecutive series of glioma patients. Individual slides and tissue microarrays (TMAs) were established, and BCL-3 protein was examined by immunohistochemistry (IHC) in 86 confirmed GBMs. IHC grading was performed in a blinded fashion based on a four-tier system that was converted into a binary scale (Fig. 1G). Clinical characteristics are noted (table S4); greater than 90% of patients received TMZ. Data analysis showed that GBM patients with low BCL-3 staining survived significantly longer than those with high staining [P < 0.0001; HR, 5.418; 95% confidence interval (CI), 2.747 to 9.647; Fig. 1H], a finding independent of age. These results support the mRNA data and emphasize the importance of BCL-3 protein in modulating the response to alkylation damage.

**BCL3 is predictive in all diffuse gliomas**

Alkylating chemotherapy is also important in the management of LGGs. Examination of multiple LGG data sets demonstrated that BCL3 expression separated these patients into distinct survival groups (Fig. 2A, fig. S2A, and table S5). In addition, our analysis showed that there was no difference in BCL3 expression between grade II and III tumors (fig. S2, B and C). Multivariate analysis that included age, isocitrate dehydrogenase 1 (IDH1) mutation, 1p/19q co-deletion, and MGMT promoter methylation showed that BCL3 expression remained associated with survival (Table 2); moreover, BCL3 expression separated LGG patients into different survival groups in tumors with high MGMT promoter methylation (Fig. 2B). We next partitioned patients by treatment modality. Consistent with a predictive effect, BCL3 expression was not informative in LGG patients who did not receive an alkylating agent or those treated with IR alone, whereas in patients treated with TMZ (either alone or with PCV) in combination with IR, BCL3 expression identified distinct survival groups (Fig. 2C). When data from patients treated with alkylating chemotherapy were analyzed on multivariate analysis, low numbers of patients achieving the endpoint of death precluded meaningful statistical analysis (table S6). To validate the predictive nature of BCL3, we examined LGGs from REMBRANDT. In this data set, BCL3 expression was informative of patient survival only in LGG patients who received alkylating chemotherapy, of which >95% included TMZ (fig. S2D).

In addition, given the importance of randomized studies for identifying predictive factors, we looked at European Organization for Research and Treatment of Cancer (EORTC) study 26951, a randomized phase III clinical trial that examined addition of alkylating chemotherapy to IR in LGG (22). In this study, performed before TMZ was routinely used, BCL3 expression was not informative of survival time in patients treated with IR alone. However, among patients who received alkylating chemotherapy in addition to IR, those with low BCL3 expression survived significantly longer (HR, 2.051; P < 0.044; Fig. 2D). Moreover, multivariate analysis among patients that received chemotherapy showed that BCL3 expression remained significant (P = 0.006) when factoring in age and 1p/19q status (table S7; IDH1 mutation was not included due to incomplete patient data). These findings indicate that BCL3 is an independent predictor of response to alkylating chemotherapy in LGG.

Given that IDH mutation status is as important a prognostic marker as tumor grade (23), we examined a pan-glioma data set (grade II, III, and IV tumors) in which patients were separated by IDH1 mutation status (24). Notably, IDH1-mutant (IDH-mt) gliomas had lower BCL3 expression than IDH-wild type (wt) (Fig. 2E). Similar results were seen with CpG island methylator phenotype (CIMP)-positive tumors compared to CIMP-negative tumors (fig. S2E). Our analysis also showed that BCL3 expression was reduced in LGG compared to GBM (P < 0.001, fig. S2F). In this pan-glioma data set, BCL3 was correlated with survival on multivariate analysis, taking IDH1 mutation, MGMT methylation, 1p/19q co-deletion, and age into consideration (Table 3). Consistent with a predictive effect, BCL3 expression separated pan-glioma patients into distinct survival groups only if they received alkylating chemotherapy in their treatment regimen (Fig. 2F). In these patients, BCL3 expression remained significant (P = 0.023) on multivariate analysis incorporating IDH1 mutation and MGMT promoter methylation (table S8). In summary, these data indicate that BCL3 is an independent predictor of response to alkylating chemotherapy in diffuse gliomas.

**BCL3 loss is a passenger event associated with survival**

Multiple transcription factors can contribute to BCL3 mRNA expression (13); however, it is notable that BCL3 is found on 19q13, a chromosomal band deleted in 20 to 40% of all types of glioma (25–29). We therefore examined the relationship between BCL3 copy number (CN) and expression. In both LGGs and GBMs, tumors with BCL3 CN loss had significantly lower BCL3 mRNA expression (P < 0.003; Fig. 3A). Moreover, in an independent data set, LGGs and GBM with 19q deletion had significantly lower BCL3 expression than nondeleted tumors (P < 0.01; Fig. 3B). To examine the link between CN

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Table 1. Cox regression analysis in TCGA GBM patients.
Fig. 2. BCL3 is relevant in LGGs and pan-glioma patients. (A and B) Survival curves from (A) LGG data sets separated by median BCL3 expression and (B) TCGA LGG patients separated by median BCL3 expression and median MGMT promoter methylation. (C) Survival curves in TCGA LGG patients based on median BCL3 expression and treatment (n = 338 patients total). For patients treated with TMZ, 202 received only TMZ, and 57 received TMZ and PCV. No chemotherapy group includes patients with and without IR. IR alone patients received no alkylating agents. (D) Survival curves in EORTC 26951 based on median BCL3 expression. Patients were separated by treatment modality into those treated with IR alone versus IR and alkylating chemotherapy. (E) BCL3 mRNA expression in pan-glioma patients based on IDH1 mutation status in the indicated data set. For TCGA: IDH1-wt, n = 184; IDH1-mt, n = 406. For GSE16011: IDH1-wt, n = 143; IDH1-mt, n = 83. (F) Survival curves in TCGA pan-glioma patients separated by median BCL3 expression and by alkylating chemotherapy treatment (yes/no). Kaplan-Meier curves analyzed by the log-rank test.

and protein expression, we performed fluorescence in situ hybridization (FISH) on TMA samples. Tumors with hemizygous BCL3 deletion had significantly lower BCL3 than nondeleted tumors, as assessed by IHC (P = 0.0007; Fig. 3C). The association between CN and BCL3 expression raised the question of whether CN is also linked to survival. In both LGG and GBM groups, patients who had tumors with BCL3 deletion survived significantly longer than those with non-deleted tumors (P < 0.05; Fig. 3D). Moreover, BCL3 CN remained significantly associated with survival on multivariate analysis (P = 0.021; Table S9). These results indicate that genetic deletion of BCL3 is associated with reduced BCL3 expression and improved survival.

The prevalence of BCL3 loss suggests either that BCL3 is a driver of glioma formation or that it is inadvertently affected by alterations targeted to a large chromosomal region. The lack of reported BCL3 mutations or translocations in glioma suggested that BCL3 loss is not a driver of these tumors (fig. S3A). Conversely, CNAs of BCL3 correlated with those of nearby genes (fig. S3B), suggesting that their CNAs are closely linked. Therefore, we examined the CN status of the genes surrounding BCL3 in 19q13.31-32. Analysis of TCGA data showed that in both GBM and LGG, loss of all the genes in this band correlated not only with BCL3 but also with the other genes in the region (fig. 3E and fig. S3C), suggesting that BCL3 deletion occurs as a result of modifications targeted to a broad region of 19q13. This finding is consistent not only with the fact that the entire 19q arm is lost in oligodendrogliomas but also with the observation that, in astrocytic tumors and GBM, CNAs within 19q13 involve a broad region (26, 30). The above data support the hypothesis that BCL3 loss is a passenger event seen in all types of glioma.

Despite the importance of deletion, some BCL3 nondeleted gliomas also had low mRNA expression. Given the role of epigenetic modifications in regulating expression, we examined BCL3 promoter methylation. In TCGA pan-glioma data, a significant inverse correlation between BCL3 promoter methylation and expression was seen (P < 0.0001; fig. S3D). Although the correlation was evident in IDH-wt and IDH-mt tumors, this was stronger in IDH-wt tumors (fig. S3D). Analysis of the pan-glioma data set demonstrated that BCL3 promoter methylation
that have extremely low basal BCL-3, overexpression of BCL-3 for

To understand how BCL-3 promotes resistance to alkylation damage, we

BCL3 findings indicate that the correlation between

Loss and survival

1p/19q co-deletion (Fig. 3F), a finding

BCL3 promoter methylation was

status, rated by the patients'

P co-deletion (\(= 0.018\); table S10). Moreover, when the data were sepa-

radiotherapy 0.404 0.202–0.807 0.010

Procurement method 1.402 0.911–2.159 0.125

Age 1.060 1.042–1.079 0.000

Table 2. Cox regression analysis in TCGA LGG patients. BCL3 expression is also significant with inclusion of grade into analysis (\(P = 0.042\)).

Covariate Univariate regression

BCL3 expression 1.431 1.186–1.735 0.000

MGMT methylation 0.268 0.167–0.431 0.000

IDH1 mutation 0.103 0.062–0.173 0.000

1p/19q co-deletion 0.486 0.281–0.837 0.009

Grade (II or III) 3.554 2.194–5.758 0.000

Radiotherapy 0.404 0.202–0.807 0.010

Chemotherapy 0.219 0.068–0.706 0.011

Procurement method 1.402 0.911–2.159 0.125

Age 1.060 1.042–1.079 0.000

Covariate Multivariate regression

BCL3 expression 1.374 1.096–1.722 0.006

MGMT methylation 0.685 0.363–1.293 0.243

IDH1 mutation 0.401 0.186–0.864 0.020

1p/19q co-deletion 0.646 0.339–1.229 0.183

Age 1.064 1.042–1.086 0.000

was significantly associated with survival on multivariate analysis incor-

rating IDH mutation, MGMT promoter methylation, and 1p/19q co-deletion (\(P = 0.018\); table S10). Moreover, when the data were sepa-

rated by the patients’ IDH status, BCL3 promoter methylation was only informative in patients with IDH-wt tumors (Fig. 3F), a finding consistent with the stronger correlation between BCL3 methylation and expression in IDH-wt compared to IDH-mt tumors (fig. S3D). These results indicate that, in glioma, the genetic and epigenetic alter-

ations of BCL3 that regulate expression are significantly associated with survival.

Given the correlation between BCL3 CN loss and that of other 19q13 genes, we examined whether the expression of other 19q13 genes was also associated with survival. Unlike BCL3, the mRNA expres-

sion of none of the surrounding 19q13 genes (fig. S3C) could separate TCGA GBM patients into significantly distinct survival groups, including the expression of RELB, an NF-kB subunit located on 19q13 whose CNAs are tightly linked to BCL3 and have been previously associated with GBM (fig. S1H) (2, 31) (GlioVis data portal for visualization and analysis of brain tumor expression data sets) (32). These findings indicate that the correlation between BCL3 loss and survival does not extrapolate to all genes in the 19q13 chromosomal region.

BCL3 induces epithelial-to-mesenchymal transition in GBM

To understand how BCL3 promotes resistance to alkylation damage, we modulated its expression in GSCs and GBM cell lines. In A172 cells that have extremely low basal BCL-3, overexpression of BCL-3 for 2 weeks changed the cell morphology to a spindle-like appearance, suggestive of epithelial-to-mesenchymal transition (EMT) (Fig. 4A). To further study this finding, we examined GSCs with low and high basal BCL-3 expression. Overexpression of BCL-3 in GBM44 GSCs and A172 cells increased the expression of several mesenchymal markers, including vimentin (VIM), SNAI1, and TWIST1, and reduced the expression of the epithelial marker E-cadherin (CDH1) (Fig. 4B and fig. S4A). Conversely, knockdown of BCL3 in GSCs expressing high basal BCL-3, GBM43S and GBM34, resulted in decreased mesenchymal marker expression (Fig. 4B). One prominent feature of mesenchymal differentiation is acquisition of a more stem cell–like phenotype (1, 33) that can be demonstrated in vitro by anal-

ysis of neurosphere formation. Loss of BCL3 reduced neurosphere formation ability in GBM34 cells, whereas overexpression of BCL-3 had the opposite effect in GBM44 GSCs, supporting the role of BCL-3 in mesenchymal differentiation (Fig. 4C). Consistent with the above, in TCGA GBM and LGG, a correlation between the expression of BCL3 and multiple EMT markers was seen (Fig. 4D and fig. S4B). Moreover, mesenchymal (MES) GBMs, as determined by the Phillips or Verhaak classification systems (1, 2), had significantly higher BCL3 expression than proneural (PN) tumors (\(P < 0.005\); Fig. 4E and fig. S4C). Correlation of BCL3 with the Phillips MES or PN signature genes (1) demonstrated that, although there was a strong positive correlation between BCL3 and all the MES signature genes (mean correlation is 0.59, ±0.13; table S11), the correlation between BCL3 and PN signature genes was negative (mean correlation is −0.39, ±0.17; table S11) (GlioVis data portal for visualization and analysis of brain tumor expression data sets) (32). These data suggest that, in glioma, BCL-3 promotes mesenchymal differentiation.

BCL-3 induces mesenchymal differentiation via promoter-specific NF-κB dimer exchange

BCL-3 has no known enzymatic function and mediates its effects by regulat-

ing downstream gene expression. To understand how BCL3 promotes mesenchymal differentiation, we examined genome-wide mRNA expression in GBM cells expressing si-BCL3 compared to si-control, a nontargeted sequence (table S12). As BCL3 primarily acts by modulating NF-κB signaling, we looked at NF-κB–dependent factors previously identified as being specific to the MES phenotype (10). Knockdown of BCL3 caused a decrease of more than 80% of NF-κB–dependent MES genes (Fig. 5A). Among the transcripts most significantly down-regulated (>2-fold, adjusted \(P < 0.001\)) were CD44, chemokine (C-C motif) ligand 2 (CCL2), colony-stimulating factor 2 (CSF2), NF-κB–dependent factors critical for mesenchymal differen-

tiation (10), and leukemia inhibitory factor (LIF), a primary MES signature gene (1). The BCL-3 dependence of these and several other MES genes was verified after depletion and overexpression of BCL-3 in GSCs and GBM cells (Fig. 5B and fig. S5A). In addition, in clinical GBM, a strong correlation between the expression of these factors and BCL3 was seen (Fig. 5C).

To study the mechanism by which BCL-3 modulates NF-κB, we first examined an NF-κB–dependent luciferase reporter. Overexpression of BCL-3 in GBM cells caused an increase in basal and tumor necrosis factor–α (TNFa)–induced NF-κB activity (Fig. 5D) and an increase in TNFa–induced CD44 expression (fig. S5B), a finding previously reported to promote mesenchymal differentiation (10). We then looked at the primary NF-κB subunits p50, p52, and p65. Although overexpression of BCL-3 did not induce a change in subunit expression, it increased p65 nuclear translocation (Fig. 5E). To ex-

amine this finding further, we depleted BCL3 in GBM34 GSCs.
Knockdown of \textit{BCL3} using two distinct sh-RNA constructs decreased nuclear and increased cytoplasmic p65 (Fig. 5F). Given this finding, to study whether inherent differences in BCL-3 correlated with differences in nuclear p65, we first examined GSCs and GBM cell lines. In the cell lines examined, we found a positive correlation between BCL-3 and nuclear p65 protein (Fig. 5G). In addition, in clinical GBM specimens, we found a significant positive correlation between nuclear IHC staining of BCL-3 and p65 ($P = 0.009$; Fig. 5H). These results suggest that, in GBM, BCL-3 induces NF-κB activity and promotes p65 nuclear translocation.

Although BCL-3 did not alter the amount of nuclear p50 or p52, we examined whether it promoted MES differentiation by altering the chromatin recruitment of these subunits. We performed quantitative chromatin immunoprecipitation (qChIP) using primers spanning the \textit{κB} sites of several BCL-3–regulated MES genes. Overexpression of BCL-3 induced recruitment of p65 to the \textit{κB} sites of MES-specific genes, including \textit{CD44} and \textit{LIF} (Fig. 5I and fig. S5C). Also, whereas p50 recruitment decreased at \textit{CD44}, \textit{LIF}, and \textit{CCL2} promoters, the recruitment of p52 increased (Fig. 5I and fig. S5C). Gel shift studies using probes corresponding to the \textit{CD44} and \textit{CCL2} \textit{κB} sites showed that, in the presence of high BCL-3, p50 DNA binding was lost, whereas p52 binding was induced at both \textit{κB} sites (Fig. 5J and fig. S5D). Finally, we examined endogenous chromatin enrichment of NF-κB subunits at MES promoters in patient-derived GSCs. The predominant subunits bound to MES promoters in GBM34 GSCs (BCL-3 high) were p52 and p65 (Fig. 5K). These findings indicate that high BCL-3 promotes MES gene expression by inducing promoter-specific NF-κB dimer exchange.

The increased recruitment of p52 to MES promoters suggested that p52 is involved in mediating the MES differentiation induced by BCL-3. We therefore depleted p52/\textit{NFKB2} in GBM34 GSCs (BCL-3 high). Knockdown of p52/\textit{NFKB2} resulted in decreased expression of \textit{CD44}, \textit{CCL2}, and interleukin-8 (IL8) (fig. S5E). Moreover, in TCGA GBMs, there was a positive correlation between the expression of \textit{NFKB2} and high BCL-3 expression (fig. S6E). These findings suggest that TMZ induces\textit{CAII} via formation of cytotoxic \textit{O}^{2-}-methylguanine adducts.

No CA isoforms have yet been reported to be NF-κB target genes. Although knockdown of either p50/\textit{NFκB1} or p65 did not alter basal \textit{CAII} expression, loss of p52/\textit{NFKB2} attenuated basal and TMZ-induced \textit{CAII} expression in GBM34 GSCs (Fig. 6F and fig. S6F). Analysis of the \textit{CAII} promoter and the upstream coding region revealed three potential \textit{κB} sites (\textit{κB1}, \textit{κB2}, and \textit{κB3}; Fig. 6G). ChIP studies demonstrated that p50, p52, and p65 are recruited to \textit{κB1} and \textit{κB2}, but not \textit{κB3}, and gel shift analysis showed that NF-κB binds the \textit{κB2} probe (fig. 6G). In U87 cells, TMZ decreased enrichment of p50 at \textit{κB1} and \textit{κB2}, increased enrichment of p52 (Fig. 6H), and had no effect on either BCL-3 or p65 (fig. S6H). Consistent with this, gel shift studies showed that, in response to TMZ, p52 replaced p50 in the NF-κB dimer bound to the \textit{κB2} probe (Fig. 6I). The above findings suggested that TMZ induces \textit{CAII} via formation of cytotoxic \textit{O}^{2-}-methylguanine adducts.
to an increase in TMZ-induced cytotoxicity (Fig. 6M). Moreover, depletion of CAII and BCL3 together did not increase the anti-glioma effect of TMZ any more than knockdown of BCL3 alone (Fig. 6N), supporting the contention that CAII and BCL3 act in the same pathway in response to TMZ. These findings indicate that BCL3 promotes resistance to TMZ by induction of CAII as a result of promoter-specific NF-κB dimer exchange.

**ACZ enhances the anti-glioma effect of TMZ**

CAII is potently blocked by the CA inhibitor ACZ (35). ACZ has been reported to be effective against GBM cells (36, 37) and to enhance apoptosis by TMZ (38, 39). Consistent with this observation, we found that although ACZ alone did not alter overall clonogenic survival of GBM cells, it enhanced the ability of TMZ to reduce survival (fig. S7A). ACZ enhances the anti-glioma effect of TMZ by BCL3 dimer exchange.

These results indicate that BCL3 is required for sensitization to TMZ by ACZ. Although ACZ is possibly the most commonly used CA inhibitor (40), many other CA inhibitors are available that can potentially enhance the efficacy of TMZ (41). We examined two other clinically used general CA inhibitors, methazolamide (MZM) and topiramate (TPM).

**Fig. 3. Loss of BCL3 is a passenger event associated with improved survival.** (A) Relationship between BCL3 expression [on RNA sequencing (RNA-seq)] and BCL3 CN in TCGA LGG and GBM. In LGG, del: n = 169; diploid: n = 155; gain: n = 16. In GBM, del: n = 17; diploid: n = 89; gain: n = 39. (B) BCL3 expression in LGG and GBM tumors in relation to tumor 19q deletion status (determined by microsatellite polymerase chain reaction (PCR)). LGG: del, n = 40; non-del, n = 33. GBM: del, n = 12; non-del, n = 58. (C) Representative FISH images using BCL3 and 19q specific probes (left) and BCL3 IHC staining (high and low, right) in human gliomas. Table shows number of tumors in each category, two-sided Fisher’s exact test. (D) Kaplan-Meier curves in LGG and GBM patients based on BCL3 CN (with and without deletion). (E) Correlogram showing correlation (r, y axis) between CN of genes from the indicated regions of chromosome 19 (Chr19) in TCGA LGG patients based on median mutation status. Pearson coefficient (r) analyzed by two-sided Student’s t test. *P < 0.003 and **P < 0.0002.
MZM enhanced cytotoxicity by TMZ in a BCL-3–dependent manner (fig. S7C), whereas the anti-epileptic agent TPM did not (fig. S7D).

Given that ACZ also acts on other CAs, albeit much less effectively (35), we also examined the requirement of CAII for the combination effect of ACZ and TMZ. Knockdown of CAII with si-RNA blocked the chemosensitizing effect of ACZ on TMZ (Fig. 7C), and overexpression of CAII in cells with low basal BCL-3 promoted chemosensitization by ACZ (fig. S7E). Moreover, in U251 GBM cells that do not express CAII, ACZ only chemosensitized these cells to TMZ when CAII was exogenously expressed (Fig. 7, D and E). The efficacy of ACZ in combination with TMZ raised the question of whether ACZ modified NF-κB activity and mesenchymal differentiation. ACZ did not affect TMZ-induced NF-κB inhibitory activity (fig. S7F), and the addition of ACZ also did not induce p65 phosphorylation or alter MES gene expression (fig. S7, G and H).

To examine combination TMZ and ACZ in vivo, we performed intracranial xenograft studies in mice. In a pilot study, U87 tumors were established and ACZ was administered for 10 days after initiation of TMZ treatment. This combination regimen did not result in a significant change in survival. However, 10 days after TMZ initiation, CAII expression was still elevated (fig. S7I), suggesting that ACZ needed to be administered for a longer period of time. We therefore altered our protocol to treat animals with daily ACZ for a total of 21 days. Using this regimen, ACZ significantly increased survival time in combination with TMZ compared to TMZ alone (P < 0.05; Fig. 7F and fig. S7I). ACZ alone did not affect survival in this model (Fig. 7F).

Next, we replicated the experiments in more clinically relevant patient-derived xenograft (PDX) models. In GBM34 and GBM43S xenografts that have high BCL-3 expression, addition of ACZ significantly increased survival compared to TMZ alone (P < 0.05; Fig. 7G and fig. S7J). ACZ alone did not affect survival in this model (Fig. 7G).

**DISCUSSION**

This work identifies BCL-3 as an indicator of sensitivity to alkylating chemotherapy in GBM. Although BCL-3 expression separated all GBM patients, it is primarily informative in tumors with high MGMT promoter methylation. GBM patients with high MGMT promoter methylation, who would be expected to respond well to alkylating agents, had similar survival to those with low MGMT promoter methylation if they also had high BCL-3 expression. This finding is likely because cytotoxic O6-methylguanine adducts are required for BCL-3 to promote resistance to TMZ. In the presence of high MGMT expression, cytotoxic adducts are repaired before they signal to BCL-3 (fig. S8).
GBM34 sh-

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Fig. 5. BCL-3 promotes p65 nuclear translocation and NF-κB dimer exchange. (A) Heatmap representing expression of all MES-specific NF-κB target genes [identified in (10)] in microarray analysis of U87 cells expressing si-control (Ctrl) or si-BCL3, performed using three separate biological samples. Z score normalized expression is graded by color. (B) qPCR (upper) of MES-specific genes in GBM34 GSCs. Data show mean value relative to GAPDH normalized to sh-SCR ± SD (n = 3). Bottom: Representative immunoblots in GBM44 and GBM34 GSCs expressing the indicated construct. (C)Correlation between MES-specific genes and BCL3 mRNA in TCGA GBM samples (n = 166 for all genes). (D) Relative luciferase activity in U87 cells expressing BCL3 or EV treated with TNFα (5 ng/ml) or vehicle (12 hours). Data show mean value, normalized to vehicle-treated EV, ± SD (n = 4). (E) Representative immunoblot using the indicated cellular fractions from U87 cells expressing HA-BCL3 or EV. (F) Immunoblot analysis with the indicated antibody using cytoplasmic and nuclear extract from U87 cells expressing two separate sh-BCL3 constructs or sh-SCR. Histone 3 (H3) antibody was used as nuclear loading control. (G) Representative immunoblot using nuclear fractions from the indicated GSCs and GBM cell lines. (H) Representative IHC images (left) of GBM samples from glioma TMAs showing nuclear p65 and BCL-3 staining in two patients. Table (right) shows numbers of tumors in each category. (I) ChiP qPCR in the indicated cells after IP. Data represent enrichment at the indicated promoter as a percentage of input ± SD. (J) Representative immunoblot using the indicated cellular fractions from U87 cells expressing HA-BCL3 or EV. (K) Immunoblot analysis with the indicated antibody using cytoplasmic and nuclear extract from U87 cells expressing two separate sh-BCL3 constructs or sh-SCR. Histone 3 (H3) antibody was used as nuclear loading control. (L) ChiP qPCR in GSCs after IP with the indicated antibodies. Data represent enrichment at the indicated promoter as a percentage of input ± SD.
Fig. 6. CAII mediates BCL-3–dependent resistance to TMZ. (A) Significantly (P < 0.05) altered transcripts in U87 cells expressing si-BCL3 or si-control treated with TMZ (100 µM) or vehicle (24 hours). (B) CAII mRNA expression relative to GAPDH in GBM43S GSCs treated with TMZ (n = 3). (C) CAII mRNA in GBM34 GSCs expressing sh-BCL3 constructs or sh-control treated with vehicle or TMZ (48 hours) (n = 3). (D) CAII mRNA expression in patients from the indicated data set with lower and upper 33% BCL3 expression. REMBRANDT, n = 61 per group; GSE4271, n = 20 per group. (E) Expression of CAII, BCL3, and MGMT mRNA in GBM26 GSCs that express high MGMT (see fig. S7K) transfected with si-MGMT or si-control after treatment with 100 µM TMZ for 48 hours. Data show mean expression relative to GAPDH normalized to si-control, dimethyl sulfoxide (DMSO)–treated, ± SD (n = 2). (F) CAII expression relative to GAPDH in GBM34 GSCs expressing sh-control or two separate sh-p52/NFκB2 constructs after treatment with TMZ (48 hours). Data show mean value relative to GAPDH ± SD (n = 3). Inset: Immunoblot with anti-p52. (G) Location and sequence of putative human CAII xB sites. (H) ChIP qPCR in U87 cells treated with 100 µM TMZ or vehicle (24 hours). Data are normalized to immunoglobulin G (IgG) and represent enrichment as a percentage of input ± SD (n = 2). (I) Representative EMMA with xB2 probe in U87 nuclear lysate treated as in (H). (J) Supershift (SS) (right) and competition with specific (SC) or nonspecific (NS) probe (left). OCT1 binding confirms equal loading. (K) Co-IP in GBM34 and GBM43S GSCs (both BCL3 high) after treatment with TMZ (100 µM, 24 hours). IP and IB were performed with the indicated antibodies. Arrowheads indicate IgG heavy chain band (n = 2). (K) Reciprocal co-IP in 293T cells expressing FLAG-p50-wt, FLAG-p50-S329A, or EV after treatment with vehicle or TMZ (100 µM, 24 hours) (n = 2). (L and M) Trypan blue assays in GBM44 GSCs expressing HA-CAII or EV (L) and GBM26 GSCs expressing si-CAII or si-control (M) treated with TMZ (72 hours). Data show fold change in percent dead cells relative to vehicle ± SD (n = 3). Insets: Immunoblots with anti-CAII antibody. (N) Clonogenic assay in U87 cells expressing si-control or si-BCL3 with and without si-CAII treated with TMZ (n = 3). *P < 0.05 and **P < 0.01, two-sided Student’s t test.
BCL-3 was previously found to be elevated in gliomas and related to survival (42); however, in that study, patients with all grades of glioma were analyzed together, precluding determination of prognostic value. Our results indicate that only tumors with low BCL-3 expression will likely benefit from adding TMZ to IR, an important clinical observation. The effect of BCL-3 deletion in glioma is particularly relevant given that alterations of 19q play an important role in modulating patient outcome in these tumors. Specifically, 19q, with 1p, co-deletion is predictive of response to alkylating chemotherapy in oligodendroglioma (46), whereas loss of 19q13 alone is associated with long-term survival in GBM (29, 47). The most widely accepted hypothesis as to why 19q13 loss is so prevalent in gliomas is that a glioma-specific tumor suppressor is present in the region (48). Although no 19q tumor suppressor has yet been identified in astrocytic tumors or GBM, capicua transcriptional repressor (CIC) was recently discovered in oligodendroglioma (49).
Our studies indicate that BCL-3 promotes GBM mesenchymal differentiation, an observation consistent with previous reports linking BCL-3 to EMT (50–52). We find that, in GBM cells, BCL-3 up-regulated EMT markers and that, in clinical GBM, BCL3 expression correlated strongly with MES signature gene expression. From a mechanistic standpoint, high BCL-3 led to a change in the composition of the NF-κB dimer at MES gene promoters involving replacement of p50 by p52. In addition, BCL-3 induced p65 nuclear translocation, a finding consistent with the known importance of this subunit in MES change (10). Whereas previous work demonstrates a cell-extrinsic pathway for activation of p65 in GBM cells by cytokines released from infiltrating macrophages and microglia (10), genetic and epigenetic regulation of BCL3 represents mechanisms by which cell-intrinsic pathways also contribute to promoting NF-κB-dependent mesenchymal differentiation.

An important feature of predictors like BCL-3 is that they are also informative in that they can identify pathways to improve treatment response. MGMT promoter methylation is one such predictor; however, inhibiting MGMT has not proven to be an effective chemosensitizing strategy clinically (8). Inhibition of BCL-3 is not currently feasible in patients; therefore, we searched for downstream BCL-3–regulated targets and identified CAII as a mediator of BCL–3–dependent resistance to TMZ. Although several factors have previously been reported to contribute to the antiapoptotic effects of BCL–3 (53–55), from a clinical perspective, CAII stands out because it can be effectively inhibited by ACZ. CAII mRNA expression is not prognostic in untreated GBM (GlioVis data portal) (32), yet elevated endothelial CAII has been associated with worse outcome in astrocytoma (56). Regardless of these observations, our data demonstrate that it is not basal expression but the induction of CAII by TMZ that is important in modulating response to therapy. Although we demonstrated the predictive value of BCL3 in several independent data sets, an important limitation of our study is its retrospective nature. Ultimately, the clinical validity of using BCL–3 as a predictor in GBM will require verification in a prospective randomized trial. In addition, the question of which CA inhibitor is best for clinical use with TMZ will require further analysis. Finally, a mechanistic limitation of our study is that we have yet to determine how BCL–3 promotes p65 nuclear translocation and promoter-specific dimer exchange.

ACZ has not previously been examined in GBM xenografts; however, it was initially shown to promote chemosensitization in murine fibrosarcoma (57). More recently, ACZ was found to promote cytotoxicity of GBM cells in vitro and other cancers in vivo (37–39, 58). Although there has been significant interest in targeting other hypoxia-specific CA isoforms for GBM therapy (41, 59), ACZ is attractive because of its general clinical use and well-tolerated dosing profile (40). Using PDXs, we see that, in GBM, ACZ promoted chemosensitization specifically in the presence of high BCL–3 and low MGMT expression. Our studies suggest that, in the clinical setting, repurposing ACZ might be particularly effective in a subgroup of MGMT promoter methylated tumors that have high BCL–3 expression. Given the increase in molecular analysis of gliomas, factors such as BCL–3 might assume an important role in individualizing patient treatment, a strategy occurring with increasing frequency in cancer therapy.

**MATERIALS AND METHODS**

**Study design**

The objectives of this study were to examine the role of BCL–3 in the response of glioma to alkylating chemotherapy and to evaluate the use of ACZ as a chemosensitizer in experimental GBM. There are several design aspects relevant in this work. First, to examine the predictive role of BCL3, initially, TCGA was examined and the results were subsequently validated in other databases. Numbers of patients included or excluded in each data set are specifically noted in Materials and Methods and in each individual figure panel. To investigate survival based on BCL–3 IHC, we performed an initial power analysis based on the mRNA data from TCGA. This gave n = 74 and 32 patients per group, respectively, to have 80% power to detect a significant difference between low and high BCL–3 expression at a two-sided P < 0.05. Subsequently, after institutional review board approval, we obtained 86 consecutive GBM samples that had adequate tissue for IHC. Additional patients were not recruited. Patients were excluded from survival analysis if they died before treatment due to infection or massive hemorrhage, if no follow-up was available, or if they initially had an LGG that progressed to GBM. IHC and FISH grading was performed by two independent investigators blinded to diagnosis and to survival.

For animal studies, no statistical method was used to determine sample size. Efforts were made to achieve the scientific goals with the minimum number of animals. A sample size of five to seven animals per group was chosen on the basis of our previous experience using intracranial PDX GBMs, where we have observed 100% tumor engraftment success. After tumor implantation and before treatment, animals were randomized into the different treatment groups. Animals were excluded from the study if they were sacrificed before treatment/ randomization. Determination of the survival time was performed blinded to the specific treatment group. Experiments were repeated by more than one individual to ensure reproducibility. The survival endpoint was reached when mice lost at least 20% body weight or showed symptoms of neurological deficit. Raw data are located in table S17.

**Statistical analysis**

Data analysis was performed using Stata IC 13.0 statistical software (Stata Corporation, licensed to the University of Chicago, Research Computing Center). The Cox proportional hazard model was used for both univariate and multivariate analyses using the specific covariates noted in Results. For survival studies, Kaplan-Meier curves were plotted, and the log-rank test was performed for comparison of cohorts. HR and 95% CIs were calculated using the Mantel-Haenszel estimator model. For analysis of 19q CN correlation, correlation matrix analysis was used, and the R/corrplot package was used to display the correlation matrix. For box-and-whisker graphs, boxes show median and 25th and 75th percentiles, whereas whiskers show the 5th and 95th percentiles analyzed by unpaired t test. In vitro and other studies as indicated were analyzed by two-tailed Student’s t test with significance taken as P < 0.05. Pearson correlation was also analyzed by two-tailed Student’s t test.
expressing si-
P < 0.05) up- and down-regulated in U87 cells
Table S13. Genes that are significantly (adjusted relative to those expressing si-control (see separate Excel file).
signature gene in TCGA GBM patients.
Table S11. Correlation between
Table S10. Multivariate Cox regression of TCGA pan-glioma patients incorporating
BCL3
Table S9. Multivariate Cox regression of TCGA pan-glioma patients incorporating chemotherapy.
Table S5. Cox regression analysis using different
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BCL3 expression promotes resistance to alkylating chemotherapy in gliomas

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BCL3 dampens drug efficacy in gliomas

Chemotherapy with alkylating agents, including temozolomide (TMZ), is the most effective treatment for gliomas, but many patients do not respond to the treatment. Wu et al. now report that high B cell CLL/lymphoma 3 (BCL3) expression promoted resistance to TMZ by activating carbonic anhydrase II. Patients with high BCL3-expressing gliomas showed a poorer response to TMZ and shorter survival than did patients with low BCL3-expressing gliomas. In mice injected with high BCL3-expressing human gliomas, the carbonic anhydrase II inhibitor acetazolamide increased sensitivity to TMZ and survival in the mouse xenograft model. The results suggest that analysis of BCL3 expression might be useful in determining the best therapy for treating gliomas.