Cell Death and Survival

PI3K and Bcl-2 Inhibition Primes Glioblastoma Cells to Apoptosis through Downregulation of Mcl-1 and Phospho-BAD

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Abstract
Glioblastoma multiforme (GBM) is a highly malignant human brain neoplasm with limited therapeutic options. GBMs display a deregulated apoptotic pathway with high levels of the antiapoptotic Bcl-2 family of proteins and overt activity of the phosphatidylinositol 3-kinase (PI3K) signaling pathway. Therefore, combined interference of the PI3K pathway and the Bcl-2 family of proteins is a reasonable therapeutic strategy. ABT-263 (Navitoclax), an orally available small-molecule Bcl-2 inhibitor, and GDC-0941, a PI3K inhibitor, were used to treat established glioblastoma and glioblastoma neurosphere cells, alone or in combination. Although GDC-0941 alone had a modest effect on cell viability, treatment with ABT-263 displayed a marked reduction of cell viability and induction of apoptotic cell death. Moreover, combinatorial therapy using ABT-263 and GDC-0941 showed an enhanced effect, with a further decrease in cellular viability. Furthermore, combination treatment abrogated the ability of stem cell–like glioma cells to form neurospheres. ABT-263 and GDC-0941, in combination, resulted in a consistent and significant increase of Annexin V positive cells and loss of mitochondrial membrane potential compared with either monotherapy. The combination treatment led to enhanced cleavage of both initiator and effector caspases. Mechanistically, GDC-0941 depleted pAKT (Serine 473) levels and suppressed Mcl-1 protein levels, lowering the threshold for the cytotoxic actions of ABT-263. GDC-0941 decreased Mcl-1 in a posttranslational manner and significantly decreased the half-life of Mcl-1 protein. Ectopic expression of human Mcl-1 mitigated apoptotic cell death induced by the drug combination. Furthermore, GDC-0941 modulated the phosphorylation status of BAD, thereby further enhancing ABT-263–mediated cell death.

Implications: Combination therapy with ABT-263 and GDC-0941 has novel therapeutic potential by specifically targeting aberrantly active, deregulated pathways in GBM, overcoming endogenous resistance to apoptosis. Mol Cancer Res; 12(7); 987–1001. ©2014 AACR.

Introduction
Glioblastoma, the most common primary malignant brain tumor, displays a remarkable resistance toward therapy. The current mainstay of treatment is surgery followed by radiation/chemotherapy (1). In the last decade, the most promising therapeutic regimens include the oral chemotherapeutic drug, temozolomide (Temodar). This DNA modifying compound shows efficacy particularly in patients who have silenced expression of the DNA—repair enzyme O6-methylguanine DNA methyltransferase (MGMT). Testing patient samples for MGMT promoter methylation status predicts responses to temozolomide treatment. Certain therapeutic regimens, involving temozolomide, offer slight survival benefits in the range of 3 months. Thus, there is an urgent need for novel pathway-related rational therapies.

One of the mechanisms as to why glioblastoma escapes various treatment modalities is the fact that they exhibit high resistant to apoptosis (2–4). Multiple factors mediating this resistance have been described, including the aberrant expression of the bcl-2 family of proteins. Glioblastomas display high levels of bcl-2 (5) and bcl-xL (6). Therefore, these tumors are potential candidates for treatment with BH3 mimetics, such as ABT-263 (Navitoclax; ref. 7), an orally available inhibitor of bcl-2 and bcl-xL. From a mechanistic point of view, BH3 mimetics bind to the inhibitory bcl-2 family of proteins and prevent their interaction with proapoptotic Bcl-2 family members, such as Bad, Bid, and...
Bim. This allows them to drive intrinsic apoptosis with translocation of cytochrome c to the cytosol, formation of the apoptosome, cleavage of caspase-9, and subsequent activation of effector caspase-3 to complete programmed cell death. This class of molecules is more effective in tumors with high levels of bcl-2/bcl-xL. ABT-263 is currently under investigation in various malignancies, either as a single drug, compound, or in combination with other antitumor reagents (8, 9) and has already reached clinical trials (10). Specifically, preclinical studies, involving ABT-263, have shown that it has activity against small cell lung cancer (SCLC) as well as acute lymphoblastic leukemia (7). Especially, the efficacy in SCLC, which despite treatment nowadays still have a poor prognosis, is of significant relevance because this type of lung cancer is not amenable to the modern approaches of personalized medicine so far as compared with non–small cell lung cancer (NSCLC) in which certain mutations of the EGFR gene (deletions in exon 19 and point mutations in exon 21) render these tumors sensitive to the tyrosine kinase inhibitors, gefitinib or erlotinib. The toxicity profile of ABT-263 is favorable compared with standard chemotherapies. However, one caveat of ABT-263 and related molecules is that tumors with high protein expression levels of Mcl-1, another member of the bcl-2 family of proteins with antiapoptotic properties, exhibit marked resistance against them. Glioblastomas display high levels of Mcl-1 expression, which is driven by several upstream cascades, such as NOTCH (11) or CREB3L2-ATF5 (12) signaling pathways. To overcome this resistance, several strategies have been devised, such as the targeting of a second molecular pathway that allows the depletion of high levels of endogenous Mcl-1 in cancer cells. Along these lines, we herein show that GDC-0941, a recently developed and orally available thienopyrimidine derivative that inhibits PI3K in the nanomolar range (13), lowers Mcl-1 levels in glioblastoma cells, rendering them more sensitive to the apoptotic properties of ABT-263. In addition, GDC-0941 affects the phosphorylation status of BAD, thereby modulating the sensitivity of glioblastoma cells to BH3 mimetics.

Materials and Methods

Cell lines and reagents

Human glioblastoma cell lines, LN229 (p53 mutated, PTEN wild-type), U87 (p53 wild-type, PTEN mutated), and U373 (p53 mutated, PTEN mutated) with defined mutation status were cultured as described previously (14) and were obtained from the American Type Culture Collection (ATCC). The ATCC confirmed the identities of the glioblastoma cells (except for U373, which is no longer verified by the ATCC). The stem cell-like glioma (neurosphere) NCH421K, NCH644, and NCH690 cells were verified and obtained from Cell Line Services (Heidelberg, Germany) and were cultured in neutral stem cell medium containing DMEM/F12 (Gibco-Invitrogen), recombinant human epidermal growth factor (rhEGF, 20 ng/mL; Sigma), basic fibroblast growth factor (bFGF, 20 ng/mL; Upstate), BIF: bovine serum albumin, transferrin, insulin, and penicillin G and streptomycin (Gibco-Invitrogen) in the absence of serum as previously described (15, 16). Primary neurosphere GS9-6 stem-like glioma cells (12) are p53 wild type and were cultured in the same media as NCH421K cells. Cycloheximide and MG132 were purchased from Sigma Aldrich. GDC-0941 and ABT-263 were purchased from LC Laboratories.

Neurosphere formation assay

NCH421K cells were mechanically dissociated and plated at a density of 500 cells per well in 24-well plates. Twenty-four hours later, cells were treated with GDC-0941 and ABT-263, singly or in combination. Following 10 days, neurosphere formation was assessed by counting the number of neurospheres that harbor at least 25 cells per sphere as described in refs. 17 and 18). Experiments were performed at least in duplicates and statistical analysis was performed.

Antibodies and Western blotting/immunoblotting

Antibodies to Mcl-1 (1:500; CST), phospho-Akt [Serine 473 (Ser 473); 1:500; CST], pan Akt (1:1,000; CST), cleaved caspase-3 (1:250; CST), human caspase-9 (1:1,000; CST), 14–3–3 (1:1,000; Santa Cruz Biotech), BAD (1:200; CST), phospho-BAD 112 (1:200; CST), phospho-BAD 136 (1:200; CST), and actin (1:2,000, clone AC15; Sigma Aldrich) were used. Western blotting was performed as described previously. Samples were lysed either in RIPA cell lysis buffer (CST) supplemented with protease inhibitor cocktail and phosphatase inhibitors or directly lysed in Laemmli-sample buffer. Lysates were boiled and equal amounts of protein were loaded onto a gradient (4%–12%) poly-acrylamide precast-gel (Invitrogen). Samples were resolved at 120 V (constant voltage) for 15 minutes and subsequently for 45 minutes at 180 V (constant voltage). The gel was transferred (wet-transfer) to a PDVF membrane (Bio-Rad) at 45 V (constant) for 90 minutes. Protein transfer was confirmed by Ponceau S staining. Membranes were washed 3 times in TBS-T and blocked with 5% milk in TBS-T. After blocking, membranes were washed once and incubated with the primary antibody according to the vendor’s instructions. Secondary antihorse or anti-rabbit antibodies conjugated with horseradish-peroxidase were used at dilutions ranging from 1:2,000 to 1:10,000. For immunodetection, the Pierce ECL Western blotting substrate was utilized. Membranes were developed either by exposure to a film or by using the C-DiGit Blot Scanner (LI-COR).

Real-time PCR and cDNA synthesis

Glioblastoma cells were harvested after indicated treatments and RNA was isolated with a Column-Based Isolation Kit (RNeasy Mini Kit; Qiagen), as described in the manual of instructions. Subsequently, 500 ng of cDNA was reverse transcribed, using the “First Strand cDNA Synthesis Kit” (Origene). Real-time PCR was performed, utilizing a “Omnimix HS Kit” (Cepheid). The following primers were used: 18S forward: AGT CCT TGC CCT CTT TTG TAC ACA; 18S reverse: GAT CGG AGG GCC TCA CTA AAC; Mcl-1 forward: CCA AGA AGG ATG CAT GGA ACC AT; and
Mcl-1 reverse; CAG CAC ATT CCT GAT GCC ACC T. The PCR cycle parameters are available upon request. Data analysis were performed by the "Delta-Delta CT METHOD" and data are provided as percentages relative to the control.

**Analysis of cellular viability, apoptosis, and mitochondrial membrane potential**
3(4,5-Dimethyl-thiazolyl-2-yl)2,5 diphenyltetrazolium bromide (MTT) colorimetric assay was conducted as previously described. In brief, 2,000 cells per well were seeded in 96-well plates at least in duplicate 24 hours before treatment. After treatment cells were incubated with MTT for 2 hours at 37°C and assayed for absorbance at 570 nm. For apoptosis determination, cells were stained with Annexin V (FITC)/propidium iodide as described in the manufacturer’s instructions (BD Bioscience). Following staining, cells were run on a FACS Calibur machine (Becton Dickinson). Flow cytometric plots were generated by FlowJo software. To analyze mitochondrial membrane potential (MMP) in response to treatment, cells were stained with JC-1 (Molecular Probes) according to the manufacturer’s instructions. Cells were analyzed on a FACS Calibur machine (Becton Dickinson) on FL1 (green channel) and FL2 (red channel) and the number/percentage of cells with loss of MMP was determined as described in ref. 19.

**Transfections, plasmids, and siRNAs**
pcDNA3, pcDNA3-hMcl1 (Addgene ID: 25375), pcDNA3 Bad S112A S136A (phosphorylation deficient; Addgene ID: 8778), and pcDNA3 Bad (Addgene ID: 8778) were used. For transient transfections, glioblastoma cells were seeded in 12-well dishes at a density of 50,000 cells per well. Twenty-four hours later, cells were transfected with the designated plasmids, using Lipofectamine 2000 or Fugene HD according to the manufacturer’s instructions. Cells were assayed for protein expression 48 to 72 hours after transfection, and following confirmed ectopic expression of the protein of interest experiments were performed. Nontargeting human siRNA pools and human siRNA Mcl-1 pools were obtained from Thermo Fisher Scientific.

**Immunohistochemistry of glioblastoma specimens**
Tissue microarrays (TMA), containing 34 de-identified glioblastoma specimens (in triplicate), were provided by the Division of Neuropathology at the Columbia University Medical Center. TMAs were created by removing three one-millimeter cores of glioblastoma tumors. Four-micrometer-thick sections were cut and deparaffinized in xylene. For antigen, retrieval sections were pretreated by boiling samples in 0.01 M citrate buffer (pH 6.0) for 5 minutes. TMAs were stained with a dilution of 1:50 of phosphorylated Akt (ph-Akt; Ser 473; clone D9E; CST Inc.), and with a dilution of 1:100 of phosphorylated BAD (ph-BAD; Ser 112; clone 40A9; CST, Inc.), respectively. The secondary antibodies included anti-Rabbit IgG (1:200; purchased from Vector Laboratories), and antimouse polymeric antibodies (purchased from Dako) and diaminobenzidine was used as chromogene. TMAs were scored by 3 board-certified pathologists (J.F. Cray, P.D. Canoll, and M.D. Siegelin), using a 3-tier scoring system (0: no expression, 1: weak expression, 2: strong expression). When there was a disagreement, the majority of scores (“2 of 3 wins”) was taken. In the rare case, where the pathologists span the full range (0, 1, 2), then a score of 1 was assigned. A final score for each tumor was reached based on the highest scoring core (out of 3 cores per glioblastoma specimen). Statistical analysis was performed by Spearman correlation analysis. Representative photographs of the TMAs were taken.

**Statistical analysis**
Data were analyzed by 2-sided unpaired t tests using a GraphPad Prism or one-way analysis of variance followed by Tukey Multiple Comparison Test. Values are provided as mean ± SD or mean ± SEM of replicates of a representative experiment out of at least 2 independent determinations. A P value of less than 0.05 (P < 0.05) was accepted as statistical significant.

**Results**
**Glioblastoma cells display high expression of Mcl-1, ph-Akt, and ph-BAD**
To demonstrate that glioblastoma cells display high levels of the antiapoptotic Bcl-2 family protein, Mcl-1, as well as a deregulated PI3K pathway, we determined the expression levels of ph-Akt (Ser 473) and Mcl-1 in 3 established and 3 glioblastoma neurosphere cultures [NCH644, NCH690, and G39-6 (a low passage ex vivo neurosphere cell line), respectively (Supplementary Fig. S1A)]. Consistent with their known PTEN mutation status, U87 and U373 glioblastoma cells displayed higher levels of ph-Akt as compared with the wild-type LN229 glioblastoma cells. About the expression status of Mcl-1, U373 revealed the lowest expression, whereas U87 showed the highest levels (Supplementary Fig. S1A). The glioma neurosphere cells showed similar protein levels of Mcl-1 (Supplementary Fig. S1A). BAD is an important sensitizer for the intrinsic pathway of apoptosis and multiple phosphorylation sites were identified, for example at amino acid 112 and 136, that when phosphorylated render cells more resistant to apoptosis. Our glioblastoma cell line panel showed detectable levels of ph-BAD at S136 and S112 (Supplementary Fig. S1A), suggesting that BAD is in a rather inactive state in malignant glioma and that targeting this molecule might counteract the intrinsic apoptotic resistance associated with high-grade gliomas.

**The combination of GDC-0941 and ABT-263 elicits enhanced loss in cellular viability in glioblastoma cells**
First, we determined the effect of the single reagents, GDC-0941 and ABT-263, on the viability of U373 and LN229 cells, respectively. Treatment of LN229 glioma cells with increasing concentrations of GDC-0941 did not result in a significant reduction in cellular viability after 24 hours (Fig. 1B). In contrast, increasing concentrations of ABT-263 (1 μmol/L: 63.51% ± 1.89%, P = 0.0064; 2 μmol/L: 69.61% ± 0.5927, P = 0.0078; 4 μmol/L: 61.32% ±
1.528, $P = 0.0046$) statistically significantly reduced cellular viability in LN229 cells (Fig. 1A). Next, we aimed to determine the effect of GDC-0941 and ABT-263 combination therapy on cellular viability. Treatment of LN229 cells with the combination of these drugs exerted a statistically significant decrease of cellular viability (38.76% ± 0.3905) compared with single treatments with either ABT-263 ($P < 0.0001$) or GDC-0941 ($P < 0.0001$; Fig. 1C). Contrasting the results in LN229 cells, treatment of U373 glioma cells with increasing dosages of GDC-0941 (1 μmol/L: 86.62 ± 0.9061, $P = 0.0279$; 2 μmol/L: 83.66 ± 2.039, $P = 0.0210$; 4 μmol/L: 75.74 ± 3.256, $P = 0.0085$) elicited a mild, but statistically significant reduction in cellular viability with respect to the vehicle-treated cells (Fig. 1E).

Increasing concentrations of ABT-263 (1 μmol/L: 87.40 ± 1.512, $P = 0.0028$; 2 μmol/L: 101.3 ± 2.867, $P = 0.4285$; 4 μmol/L: 75.57 ± 1.187, $P = 0.0001$) resulted in a minimal reduction of cellular viability in U373 that was not as pronounced as in LN229 glioblastoma cells (Fig. 1D). The combination regimen revealed a statistical significant
decrease of cellular viability (56.49 ± 2.861) compared with single treatments with either ABT-263 (P = 0.0108) or GDC-0941 (P = 0.0113; Fig. 1F).

The combination of GDC-0941 and ABT-263 is effective in ex vivo glioblastoma neurosphere cultures

Glioblastomas are bona-fide examples of heterogeneous tumors, harboring different cell populations. Among these, the so-called "cancer stem cells" are of particular interest as they are implicated in the rapid recurrence of glioblastomas in patients, and are highly resistant to treatment. To this end, we asked the question if stem cell–like glioblastoma cells are susceptible to the ABT-263 and GDC-0941 combination regimen. With this aim, we treated the neurosphere forming NCH421K glioblastoma cells with ABT-263 and GDC-0941, singly or in combination, and conducted neurosphere formation assays. Although single reagents had a modest effect on the formation of NCH421K cell neurospheres, the combination regimen had a marked inhibitory effect on neurosphere formation (Fig. 1G and H). This finding suggests that combination treatment may influence tumorigenicity of these glioblastoma cells and, more importantly, that it not only affects the bulk of the tumor, but also targets the potential stem cell–like cellular fraction within malignant gliomas.

The combination of GDC-0941 and ABT-263 as well as ABT-199 causes enhanced apoptotic cell death with rapid loss of MMP and increased activation of initiator and effector caspases

To test the hypothesis that combination of GDC-0941 and ABT-263 works through enhanced apoptosis, we performed Annexin V staining with subsequent flow cytometry in one low-passage ex vivo glioblastoma neurosphere cell line, GS9-6, 3 glioblastoma neurosphere cell lines, NCH421K, NCH690, and NCH644, and in 1 established glioblastoma cell line, LN229. Glioblastoma cells were treated with 2 μmol/L of GDC-0941, ABT-263, or the combination of both reagents for 24 hours. Although single treatments with GDC-0941 and ABT-263 revealed modest induction of apoptosis, the combination treatment induced a significantly higher proportion of Annexin V positive cells as compared with the single treatments in all cell lines tested (Fig. 2A and B). Furthermore we also tested as to whether another orally available Bcl-2 inhibitor, ABT-199, which causes fewer side effects (thrombocytopenia) will induce enhanced cell death in the presence of GDC-0941. To test this hypothesis, 2 glioblastoma neurosphere cell lines, NCH644 and NCH690 as well as the established glioblastoma LN229 cells were treated with suboptimal dosages of ABT-199 in combination with GDC-0941. Akin to ABT-263, ABT-199 cooperates with GDC-0941 to enhance cell death (Fig. 2C and D). Our findings again suggest that our proposed treatment combination exerts activity against the stem-like cells fraction of glioblastoma. Apoptosis is accompanied by loss of mitochondrial integrity, and because ABT-263 affects intrinsic apoptotic signaling, we hypothesized that the enhanced apoptotic cell death induced by GDC-0941 and ABT-263 could be accompanied by a concomitant loss of MMP. LN229 cells were treated under the same conditions as above, stained with JC-1 and subjected to flow cytometric analysis. Although LN229 cells treated with ABT-263 (mean: 46.33 ± 1.667) and GDC-0941 (mean: 24.67 ± 0.8819) showed a modest loss in MMP, the combined treatment led to a significant higher proportion of cells with loss in MMP (mean: 80.33 ± 0.8819; <0.0001; n = 3; Fig. 2E and F). Because the initiator and effectors of apoptosis are caspases, we wished to determine if caspase-9 and caspase-3 show activation in the various treatment settings. We theorized that given that the combined treatment of GDC-0941 and ABT-263 caused more apoptosis, and more cells with loss of MMP, cells treated with the drug combination would exhibit a stronger activation of initiator as well as effector caspasas. To this end, LN229 and U373 glioblastoma cells were treated with ABT-263, GDC-0941, or the combination of both reagents for 7 hours and subjected to immunoblot analysis for cleavage/activation of caspases. We found a slight activation of both initiator caspase-9 and effector caspase-3 in ABT-263–treated cells. However, the combination of ABT-263 and GDC-0941 exerted further activation of caspase-9 and caspase-3 compared with the single treatments in both LN229 and U373 glioblastoma cells (Fig. 3C and D), in keeping with the enhanced apoptosis and loss of MMP observed.

GDC-0941 inhibits PI3K signaling in glioblastoma cells

To elucidate the mechanism as to why ABT-263 and GDC-0941 demonstrate enhanced apoptotic cell death, loss of MMP and activation of caspases when compared with the single treatments, we investigated if GDC-0941 could lower the apoptotic threshold by suppression of Akt signaling. All, U373, LN229 (Fig. 3A and B), and U87 (Fig. 4C) cells showed complete inhibition of Akt phosphorylation at Ser 473 upon GDC-0941 treatment, in the presence or absence of ABT-263, showing that GDC-0941 is a potent and inhibitor of the PI3K signaling cascade in malignant glioma cells.

GDC-0941 depletes Mcl-1 protein levels in a posttranscriptional manner in glioblastoma cells

ABT-263 binds strongly to Bcl-2 and Bcl-xL, but weakly to Mcl-1 (7). By interacting with these antiapoptotic molecules, proapoptotic bcl-2 family members, such as Bim and Bax, are released and can initiate and facilitate intrinsic apoptosis. Because of the inefficient interaction of ABT-263 with Mcl-1, the latter is likely to mediate resistance against ABT-263 therapy. Thereafter, we hypothesized that GDC-0941 could cause a reduction in Mcl-1 levels and thereby primes glioblastoma cells to the cytotoxic actions of ABT-263. To clarify this, we treated stem cell–like glioma cells (grown as neuropheres), GS9-6 and NCH421K cells as well as established glioblastoma cell lines LN229, U373, and U87 glioblastoma cells with increasing concentrations of GDC-0941 and subjected them to Western blot analysis. Although GDC-0941 did not have an effect on Mcl-1 protein levels in U373 glioma cells (Supplementary...
Figure 2. Effects of the combination treatment of ABT-263/ABT-199 and GDC-0941 on apoptosis induction and MMP in LN229 glioblastoma cells. A and B, NCH644, NCH690, NCH421K, GS9-6, and LN229 cells were treated with 2 μmol/L ABT-263, 2 μmol/L GDC-0941, and the combination of both for 24 hours. Subsequently, cells were stained with Annexin V/propidium iodide (PI) and analyzed by flow cytometry. FL1 (Annexin V) and FL3 (propidium iodide) are represented on the x-axis and y-axis, respectively. One representative plot is shown in A. (Continued on the following page.)
Fig. S1B), treatment of LN229 and U87 glioblastoma cells as well as the neurosphere forming NCH421K and GS9-6 cells (18), caused a marked reduction of Mcl-1 protein levels, which was observed to be dose dependent (Fig. 4A–D). We then asked the question as to whether the combination treatment of ABT-263 and GDC-0941 could also reduce Mcl-1 levels. Confirming this notion, the combination therapy led to a further decrease of Mcl-1 levels in LN229 cells in a time course experiment as early as 4 hours after treatment (Fig. 4E). The timeframe of the ABT-263/GDC-0941–mediated reduction of Mcl-1 protein levels coincided with the peak cleavage of both initiator caspase-9 and effector caspase-3 (Fig. 4E). Of note, phosphorylation of Akt at Ser 473 was undetectable in the presence of GDC-0941 regardless of its being administered as a single reagent or in combination with ABT-263 (Fig. 4E). We then evaluated the effect of ABT-263 on Mcl-1 levels. We did not detect any changes of expression in Mcl-1 levels in LN229 upon ABT-263 treatment (Supplementary Fig. S1D), whereas in U373 cells ABT-263 led to a marked increase in Mcl-1 protein levels (Supplementary Fig. S1C), the significance of which remains to be elucidated. Phosphorylation of Akt was minimal or not affected by ABT-263 in both LN229 and U373 glioblastoma cells 7 hours after treatment (Fig. 4A and Supplementary Fig. S1C). To determine if Mcl-1 is regulated at the transcriptional level by GDC-0941, LN229 cells were exposed to increasing concentrations of GDC-0941 for 7 hours. Despite a tendency to decrease, no statistically significant change of which the expression of Mcl-1 was determined by GDC-0941 in malignant glioma cells and enhanced activation of initiator and effector caspases by the combined treatment of ABT-263 and GDC-0941 (A–D) LN229 (A and C) and U373 (B and D) were treated with 2 μmol/L GDC-0941, 2 μmol/L ABT-263 or the combination of both reagents for 7 hours and subjected to immunoblotting. Shown are the results (A and B) for ph-Akt (Ser 473), ph-Akt and total Akt. Whole cell lysates were also resolved by Western blot analysis and probed for initiator caspase-9 (cCP9) and effector caspase-3 (cCP3) in both LN229 (C) and U373 (D) cells. ABT—ABT-263; GDC—GDC-0941; ABT + GDC—combined treatment of ABT-263 and GDC-0941.
significant alterations of Mcl-1 mRNA levels were detected at 2, 4, and 8 μmol/L of GDC-0941 ($P = 0.1441$; Fig. 5A), which suggests that a posttranscriptional mechanism may primarily contribute to GDC-0941–mediated suppression of Mcl-1 levels. Recent studies show that Mcl-1 is a protein with a short half-life, prone to degradation by the proteasome, and caspases (20–22). Hence, we postulated that a potential mechanism for GDC-0941–driven depletion of Mcl-1 protein levels could be enhanced proteasomal degradation. To test this, we treated LN229 cells for 7 hours with different concentrations of GDC-0941 in the presence or absence of MG132, a proteasomal inhibitor. Treatment with MG132 partially rescued GDC-0941–mediated Mcl-1 depletion (Fig. 5B), in keeping with the hypothesis that GDC-0941 enhances Mcl-1 degradation through the proteasome. We demonstrated that the combined treatment of ABT-263 and GDC-0941 induces apoptosis to a greater extent than single treatments, it would be thus conceivable that the combination treatment as well as GDC-0941 administered alone would impact the protein half-life of Mcl-1. To examine this postulate, we treated LN229 glioblastoma cells with the protein synthesis inhibitor, cycloheximide in the presence or absence of ABT-263 and GDC-0941. Single treatment with GDC-0941, in the presence of cycloheximide, significantly reduced the protein half-life of Mcl-1, with a marked decrease of Mcl-1 protein levels already after 1.5 hours of treatment (Fig. 5C). Similarly, combination treatment of ABT-263 and GDC-0941 significantly altered protein half-life of Mcl-1, causing a decline of Mcl-1 levels starting 45 minutes following treatment, with a substantial depletion 3 hours of therapy (Fig. 5D). These results indicate the existence of a posttranslational mechanism by which GDC-0941 alone and in combination with ABT-263 depletes Mcl-1 protein levels. Mcl-1 reduction by GDC-0941 was rescued by the proteasome inhibitor MG132, indicating that Mcl-1 protein levels are suppressed by enhanced proteasomal degradation.
Ectopic overexpression of human Mcl-1 attenuated apoptosis induced by the combination therapy of GDC-0941 and ABT-263

To further confirm the notion that Mcl-1 mediates apoptotic resistance, we tested 2 cell lines with high (U87) and low levels of Mcl-1 (LN229) protein expression for the sensitivity of ABT-263. Consistent with the expression pattern, U87 cells were more resistant to the cytotoxic effects of ABT-263 as compared with LN229 cells (Supplementary Fig. S2A). Next, we investigated if overexpression of Mcl-1 would attenuate ABT-263/GDC-0941–mediated induction of apoptosis. In these experiments, LN229 cells were transfected with a plasmid encoding the open reading frame of human Mcl-1. Forty-eight hours after transfection with either a control empty pcDNA3 plasmid, or a plasmid encoding human Mcl-1, LN229 cells were incubated with the protein synthesis inhibitor, cycloheximide (10 μg/mL), in the presence or absence of GDC-0941 (2 μmol/L) for the indicated time points and then subjected to immunoblotting. Membranes were incubated with a Mcl-1–specific antibody as well as with β-actin, serving as a loading control. Values are given as mean ± SEM of representative experiments. GDC2, GDC4, and GDC8—2, 4, and 8 μmol/L of GDC-0941.

Figure 5. Regulation of Mcl-1 by GDC-0941. A, LN229 cells were treated with increasing concentrations of GDC-0941 for 7 hours. Subsequently, mRNA was isolated, transcribed into cDNA, and real-time PCR analysis with primers spanning the Mcl-1 transcript was performed. Data are given as relative percentage to the Control and were calculated as described in the Materials and Methods section. Data were analyzed by using one-way ANOVA, followed by a Tukey Multiple Comparison Test. B, LN229 cells were treated with GDC-0941, with or without the additional of the proteasome inhibitor MG132 or the combination of both reagents for 7 hours and subjected to immunoblot analysis with antibodies against Mcl-1 or 14-3-3(β) (loading control). The vertical line on the immunoblot indicates that the first sample was noncontiguous, but run on the same gel simultaneously with the other samples. C, LN229 cells were incubated with the protein synthesis inhibitor, cycloheximide (10 μg/mL), in the presence or absence of GDC-0941 (2 μmol/L) for the indicated time points and then subjected to immunoblotting. Membranes were incubated with a Mcl-1–specific antibody as well as with 14-3-3(β), serving as a loading control. Membranes were incubated with a Mcl-1–specific antibody as well as with 14-3-3(β), serving as a loading control. Values are given as mean ± SEM of representative experiments. GDC2, GDC4, and GDC8—2, 4, and 8 μmol/L of GDC-0941.
Figure 6. Overexpression of Mcl-1 attenuates cell death induced by the combination treatment of ABT-263 and GDC-0941 and specific suppression of Mcl-1 sensitizes glioma cells to ABT-263/ABT-199-mediated cell death. A, LN229 glioma cells were transfected with a plasmid encoding human Mcl-1 or with the respective empty control plasmid. Forty-eight hours after transfection cells were harvested and subjected to immunoblotting. Membranes were incubated with a Mcl-1–specific antibody or an antibody against 14-3-3β as loading control. B, LN229 cells were transfected, selected with Geneticin and were treated with the combination of ABT-263 (ABT; 2 μmol/L) and GDC-0941 (GDC; 2 μmol/L) for 24 hours. Thereafter, cells were harvested and analyzed for cellular viability by flow cytometric analysis. LN229 cells, overexpressing human Mcl-1, displayed less dead cells in response to treatment with ABT-263/GDC-0941. A P value of less than 0.001 is indicated by 3 stars “***”. (Continued on the following page.)

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with the respective control. These results indicate that Mcl-1 is implicated in death induced by ABT-263 as a monotherapeutic as well as by the combination treatment of ABT-263 and GDC-0941.

**Specific suppression of Mcl-1 by siRNA sensitizes glioblastoma cells to ABT-263/ABT-199**

Next we assessed if specific suppression of Mcl-1 can sensitize glioblastoma cells to the cytotoxic effects of ABT-263 and ABT-199, respectively. LN229 cells transfected with a Mcl-1–specific siRNA revealed a strong reduction of endogenous Mcl-1 levels as compared with the nontargeting siRNA (Fig. 6C). Furthermore, the Mcl-1 siRNA was specific for Mcl-1 as Bcl-xL was not affected (Fig. 6C). About cell death downregulation of Mcl-1 in LN229 increased the number of dead cells as compared with LN229 cells transfected with a nontargeting siRNA (Fig. 6C). In addition, ABT-199 treatment further enhanced Mcl-1 siRNA-mediated cell death (Fig. 6D). Next we tested as to whether ABT-263 and ABT-199 enhance cleavage of initiator/effectors caspases-9/-3 in LN229 cells transfected with a Mcl-1–specific siRNA. Indeed, both ABT-199 and ABT-263 revealed a marked cleavage of caspase-9/-3 under Mcl-1–depleted conditions (Fig. 6E and F).

**GDC-0941 dephosphorylates BAD and a phosphorylation-deficient BAD mutant enhances ABT-263–mediated cell death**

Because glioblastoma cells contain high levels of phosphorylated endogenous BAD (Supplementary Fig. S1A), which is considered to be inefficient to mediate apoptosis, we seek to determine as to whether GDC-0941 modulates the phosphorylation status of BAD at Ser 112 and Ser 136. In addition, GDC-0941 and ABT-263 also cooperated to induce death of U373 glioma cells, even though there was no suppression of Mcl-1 protein levels after GDC-0941 treatment. We found that GDC-0941 led to a dephosphorylation of BAD at Ser 112 and Ser 136 (Fig. 7A–C). Consistently, GDC-0941 mediated a dramatic decrease in the phosphorylation status of Akt, paralleling the levels of BAD Ser 112 and Ser 136. Given the dephosphorylation of BAD we hypothesized that a phosphorylation-deficient BAD mutant BAD (S112A, S136A) should have stronger cell death sensitizing/inducing properties. For this purpose we transfected wild-type BAD and a phosphorylation-deficient mutant BAD (S112A, S136A) into LN229 glioblastoma cells. Transfection of both wild-type and mutant BAD increased the percentage of dead cells. However, phosphorylation-deficient BAD had a significant stronger effect on cell death as compared with wild-type BAD (Fig. 7D). Furthermore, mutated BAD cooperated with ABT-263 in apoptosis induction (Fig. 7D). These findings support the notion that GDC-0941–mediated dephosphorylation of BAD is implicated in ABT-263/GDC-0941 enhanced cell death when compared with each drug alone.

**The protein expression levels of ph-Akt (Ser 473) and ph-BAD (Ser 112) correlate in glioblastoma tissue specimens**

TMAs, containing 34 glioblastoma tissue specimens, were stained for the expression of ph-Akt and ph-BAD (Ser 112), respectively. Most glioblastomas tumor specimens showed expression of ph-Akt (Ser 473) and ph-BAD (Ser 112), whereas a minority (ph-Akt) and small fraction (ph-BAD) of cases displayed no detectable staining (Fig. 8A–C and Table 1). Although ph-Akt was both detected in the cytoplasm and nucleus of glioblastoma cells, the expression of ph-BAD (Ser 112) was localized to the cytoplasm. Tumor vasculature seemed to be mostly negative for both ph-Akt and ph-BAD, respectively. Notably, the expression levels of ph-Akt (Ser 473) and ph-BAD (Ser 112) revealed a statistically significant positive correlation (n = 34; Spearman correlation coefficient r = 0.5289; 95% confidence interval, 0.2223–0.7403; P < 0.0013). These data support the notion that the Akt pathway is active in glioblastoma and affects BAD phosphorylation, thereby inhibiting its proapoptotic properties.

**Discussion**

Glioblastoma WHO IV is a treatment-resistant cancer with a short survival time after diagnosis. Despite a significant increase in our understanding of the biology of these tumors, effective treatment strategies to overcome this disease are still unavailable. Given the heterogeneous nature of these neoplasms, it is unlikely for a "smoking-gun" therapy to be effective in all glioblastomas. A personalized approach tailored to each individual is more likely to be successful. In this context, our current understanding of glioblastoma may allow us to stratify patients into groups according to the nature of molecular aberrations in their tumors. For example, many glioblastomas harbor aberrantly active PI3K signaling and high levels of bcl-2/bcl-xL, which suggests that these tumors depend on these pathways to achieve their growth and resistance to therapy. In this work, we have targeted the 2 above-mentioned signaling networks simultaneously by administering 2 orally available drugs that...
specifically inhibit either PI3K, GDC-0941, or Bcl-2/Bcl-xL, ABT-263. ABT-263 binds to a hydrophobic groove in Bcl-2/Bcl-xL and thereby displaces proapoptotic molecules of the Bcl-2 family of proteins, such as Bim. In turn, it elicits a bax/bak-dependent cell death, which corresponds to rapid apoptosis. Similarly to ABT-737, a nonoral predecessor of ABT-263, ABT-263 reveals antiglioma activity and may thus be suitable for the treatment of glioblastoma. Plasmatic levels of 5.4 to 7.7 μmol/L were achieved in mice and higher concentrations of ABT-263 to closely resemble the clinical scenario. In this study, we used relevant plasmatic concentrations of ABT-263 to closely resemble the clinical scenario. Relevant plasma concentrations of ABT-263 to closely resemble the clinical scenario.

Figure 7. GDC-0941 modulates the phosphorylation status of BAD at Ser 112 and Ser 136 in glioblastoma cell lines. A–C, LN229, U87 and U373 cells were treated with increasing concentrations of GDC-0941 (concentrations in μmol/L) for 7 hours and subjected to immunoblotting with antibodies against phosphorylated Akt (Ser 473), Akt, ph-BAD (Ser 112 and Ser 136), and total BAD. Actin protein served as loading control. D, LN29 glioblastoma cells were transfected with plasmids, encoding wild-type BAD, a phosphorylation deficient BAD (S112A, S136A, Serine — Alanine) or the respective control plasmid. Thereafter, cells were either left untreated or treated with a suboptimal concentration of ABT-263 (1 μmol/L) and cell death was quantified by flow cytometry. Shown are the total percentages of dead cells. A P value of less than 0.01 is highlighted by 2 stars "**".
GDC-0941 Overcomes Intrinsic Apoptotic Resistance in Glioblastoma

Figure 8. Glioblastoma TMAs, containing 34 tumor samples, were stained with antibodies against ph-Akt (Ser 473) or ph-BAD (Ser 112). Representative photographs were taken. A, 0: absent expression; B, 1: low expression; and C, 2: high expression. Three representative glioblastomas (GBM1, GBM2, GBM3) are shown with different expression levels of ph-Akt (Ser 473) and ph-BAD (Ser 112).

Table 1. Expression levels of ph-Akt (Ser 473) and ph-BAD (Ser 112) in glioblastoma tissue specimens

<table>
<thead>
<tr>
<th>Protein</th>
<th>Score</th>
<th>Tumors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ph-Akt (Ser 473)</td>
<td>0</td>
<td>2/34 (6%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>15/34 (44%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17/34 (50%)</td>
</tr>
<tr>
<td>ph-BAD (Ser 112)</td>
<td>0</td>
<td>7/34 (21%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>21/34 (62%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6/34 (18%)</td>
</tr>
</tbody>
</table>

NOTE: 0, no staining; 1, low expression; 2, high expression.

263 and GDC-0941 in combination induced higher levels of apoptosis as compared with each reagent on its own. Parallelizing the enhanced apoptosis observed was our finding that combination treatment provoked and increased loss of MMP. This led us to propose that GDC-0941 primes glioblastoma cells by acting on mitochondrial antiapoptotic molecules, such as the Bcl-2 family of proteins. In this context, it is well known that Mcl-1 is a bona fide inhibitor of intrinsic apoptosis playing a major role in resistance to treatment with BH3 mimetics, such as ABT-737 (27, 28) or its derivative ABT-263 (7). Mcl-1, localized to the mitochondrial outer membrane, is capable of inhibiting bax-mediated intrinsic apoptosis, and its levels in tumors correlate with prognosis. Mcl-1 may, therefore, serve both as a therapeutic marker and as a prognostic indicator. Prior studies showed that interfering with cyclin-dependent kinase (CDK) with compounds such as roscovitine, lowers Mcl-1 protein levels, and thereby sensitizes cells to the cytotoxic effects of ABT-737 (7). The effect of roscovitine was recapitulated by an shRNA-targeting Mcl-1 in human leukemia cells (7). Suppression of Mcl-1 results in activation of the proapoptotic Bcl-2 family protein bak through a conformational change (28), in line with the fact that Mcl-1 avidly binds to bak. Sorafenib (BAY 43-9006) is a compound initially identified as a broad kinase inhibitor. In contrast to other kinase inhibitors, which primarily elicit cytosoris, sorafenib also induces apoptosis in the low μmol/L range. Several molecular mechanisms have been proposed and recent studies in different tumor entities confirm that a key mechanism for cell death induction by sorafenib is the downregulation of Mcl-1. Sorafenib-mediated suppression of Mcl-1 occurs at different levels. Some reports suggest a posttranscriptional mechanism (20), whereas others favor a transcriptional one (12, 29). For instance, Yu and colleagues showed that sorafenib did not modulate Mcl-1 mRNA levels in breast cancer cells, colon cancer cells, and leukemia cells. Instead, sorafenib increased proteasomal degradation of Mcl-1 in these cells (20), in keeping with the fact that Mcl-1 was described as a protein with a short-half life and prone to proteasomal degradation. In glioblastoma, sorafenib seems to suppress Mcl-1 at the transcriptional level, involving ATF5, a transcriptional factor (12). Irrespective of the mechanism, lowering Mcl-1 levels seems to be an effective strategy to sensitize tumor cells to therapy. Indeed, therapeutic strategies, targeting Mcl-1 by sorafenib, have already reached clinical trials (clinicaltrials.gov). Our findings demonstrate that GDC-0941 lowers Mcl-1 levels in several established glioblastoma cell lines, as well as in stem cell–like glioma cells (neurosphere cultures). In addition, we also showed that the combination of ABT-263/GDC-0941 affected Mcl-1 levels. Our findings suggest that GDC-0941 regulates Mcl-1 protein levels mainly in a posttranslational manner. This is supported by our experiments, using protein synthesis inhibitor cycloheximide, which showed that in the presence of GDC-0941 the protein half-life of Mcl-1 is significantly reduced. In addition, GDC-0941–mediated suppression of Mcl-1 protein levels was rescued by the proteasomal inhibitor, MG-132, corroborating the fact that GDC-0941 regulates the proteasomal turnover of Mcl-1. GDC-0941–mediated Mcl-1 depletion occurs within a couple of hours of treatment, suggesting that the ABT-263 and GDC-0941 can be applied together without a preincubation period with the sensitizing reagent (GDC-0941). The concept of combining PI3K inhibitors or “pleiotropic kinase inhibitors” (30) as well as mTOR inhibitors with BH3 mimetics has also been exploited in other tumor entities, such as rhabdomyosarcomas (RMS) and non–small cell lung carcinomas (30–33). For instance, akin to glioblastomas, RMS harbor a deregulated PI3K pathway and targeting these tumors with AZD8055 proved to be an effective strategy to sensitize RMS cells to the cytotoxic action of ABT-737 (31). Mechanistically, the sensitizing effect of AZD8055 in this malignancy was because of
suppression of Mcl-1 (31), reminiscent of our findings with GDC-0941 in malignant glioma cells. Upstream of PI3K are the membrane bound tyrosine kinase receptors. EGFR is a classic example, which is often mutated in both lung adenocarcinomas and glioblastomas. However, the mutation sites differ between these 2 mentioned tumor entities. Although glioblastomas often harbor the EGFRvIII mutation (34), lung adenocarcinomas have mutations in exons 19 and 21, which in contrast to glioblastomas renders them remarkably sensitive to Gefitinib/Erlotinib. Upon Gefitinib treatment lung adenocarcinomas, harboring EGFR-inhibitor sensitizing mutations, undergo Bim-dependent apoptosis, which can be significantly augmented by BH3 mimetics (35). This underscores the fact that compounds interfering with molecules upstream of the PI3K signaling pathway, such as membrane bound receptors, may be favorably combined with BH3 mimetic drugs. The rational for this combination is similar to the addition of PI3K inhibitors to combinatorial regimens, because EGF, the natural ligand for EGFR, elevates Mcl-1 protein levels (36).

Currently, there are also small molecule inhibitors under development that bind to Mcl-1 and inhibit its antiapoptotic functions, for example Obatoclax (37). Therefore, it is conceivable that these compounds may be used in conjunction with BH3 mimetics, such as ABT-199 or ABT-263 (this study). The disadvantage of this strategy compared with the one presented here (the drug combination of ABT-263 + GDC-0941) is that Obatoclax acts downstream and will not affect other downstream pathways. PI3K inhibitors lower Mcl-1 levels and affect other apoptosis modulating factors, such as BAD (this study) and caspase-9. Our study nicely showed that glioblastoma tissue specimens and cell line harbor high levels of ph-BAD, that GDC-0941 has an effect on BAD phosphorylation status and that dephosphorylated BAD enhances apoptosis by BH3 mimetics. Thus, the advantage of the presented combination therapy in this study is clearly that it not only affects Mcl-1, but also additional deregulated pathways linked to the PI3K signaling axis. This becomes particularly relevant in the context of malignant glioma, in which the PI3K pathway is commonly hyperactive (primary glioblastoma).

Conclusion and Remarks
In summary, we have described a novel potential combinatorial therapy for glioblastoma, which includes the PI3K inhibitor, GDC-0941, and the BH3-mimetic, ABT-263. At clinically relevant concentrations when combined, these compounds have a significant anti-glioma effect that is stronger than each compound on its own in both established and in stem cell-like glioma cells. Furthermore, these reagents are orally available, facilitating their administration. Mechanistically, this drug combination is rational because it targets 2 aberrantly active pathways in glioblastomas. In addition, inhibition of one of these pathways (PI3K) affects the second one by lowering the expression levels of a key molecule, Mcl-1, which drives resistance to certain BH3 mimetics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Pareja, J.F. Cray, P.D. Canoll, A.H. Ross, M.D. Siegelin
Writing, review, and/or revision of the manuscript: F. Pareja, J.F. Cray, P.D. Canoll, A.H. Ross, M.D. Siegelin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Macleod, A.H. Ross, M.D. Siegelin
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Fresia Pareja, David Macleod, Chang Shu, et al.


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