YM-155 Potentiates the Effect of ABT-737 in Malignant Human Glioma Cells via Survivin and Mcl-1 Downregulation in an EGFR-Dependent Context

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Abstract
Antiapoptotic proteins are commonly overexpressed in gliomas, contributing to therapeutic resistance. We recently reported that clinically achievable concentrations of the Bcl-2/Bcl-xL inhibitor ABT-737 failed to induce apoptosis in glioma cells, with persistent expression of survivin and Mcl-1. To address the role of these mediators in glioma apoptosis resistance, we analyzed the effects of YM-155, a survivin suppressant, on survival on a panel of glioma cell lines. YM-155 inhibited cell growth and downregulated survivin and Mcl-1 in a dose- and cell line-dependent manner. While U373, LN18, LNZ428, T98G, LN229, and LNZ308 cells exhibited an IC_{50} of 10 to 75 nmol/L, A172 cells were resistant (IC_{50} ~ 250 nmol/L). No correlation was found between sensitivity to YM-155 and baseline expression of survivin or cIAP-1/cIAP-2/XIAP. However, strong correlation was observed between EGF receptor (EGFR) activation levels and YM-155 response, which was confirmed using EGFR-transduced versus wild-type cells. Because we postulated that decreasing Mcl-1 expression may enhance glioma sensitivity to ABT-737, we examined whether cotreatment with YM-155 promoted ABT-737 efficacy. YM-155 synergistically enhanced ABT-737–induced cytotoxicity and caspase-dependent apoptosis. Downregulation of Mcl-1 using short hairpin RNA also enhanced ABT-737–inducing killing, confirming an important role for Mcl-1 in mediating synergism between ABT-737 and YM-155. As with YM-155 alone, sensitivity to YM-155 and ABT-737 inversely correlated with EGFR activation status. However, sensitivity could be restored in highly resistant U87-EGFRvIII cells by inhibition of EGFR or its downstream pathways, highlighting the impact of EGFR signaling on Mcl-1 expression and the relevance of combined targeted therapies to overcome the multiple resistance mechanisms of these aggressive tumors. Mol Cancer Ther; 12(3); 1–13. ©2013 AACR.

Introduction
Glioblastoma is the most common primary malignant brain tumor and one of the most aggressive forms of cancer. Glioblastoma exhibits a high resistance to standard chemotherapy and radiotherapy. Resistance of tumor cells to the induction of apoptosis is an important reason for the failure of anticancer treatments in patients with gliomas, and several factors working in concert have been implicated as sources of this treatment resistance (1). We, among others, have indicated that dysregulation of the NF-kB, Bcl-2, and Akt pathways may be integrally involved in mediating glioma resistance to apoptotic signaling (1–9). Bcl-2 is a prime target for novel therapeutics because it is overexpressed in many forms of cancer and contributes to cancer progression and therapy resistance based on its ability to inhibit apoptosis. New anticancer therapeutics, such as ABT-737, are being developed to specifically target the prosurvival members of the Bcl-2 family by using small-molecule mimetics of BH3-only proteins (10). As a single agent, ABT-737 triggers apoptosis in various types of human cancers including multiple myeloma, leukemia, and lymphoma (11–15). We, among others, have shown only weak activity against various solid tumor cell lines, such as gliomas (6, 16, 17). In our studies, ABT-737 in combination with bortezomib induced apoptosis (6); however, inactivation of PTEN limited the therapeutic efficacy of ABT-737 in glioma. Others have shown that sensitivity to ABT-737 depends on the expression levels of Mcl-1, Bcl-2, Bcl-xL,
Bim, and Noxa (12–14, 18–20), and that Mcl-1 downregulation dramatically increases ABT-737 lethality in diverse malignant cell types (14, 16, 21, 22). Moreover, Bak and Bax activation is essential in potentiation of ABT-737 lethality by agents that downregulate Mcl-1 (12, 14, 22).

High expression of survivin has been also associated with the increasing malignancy grade among gliomas (23–25). Survivin is a member of the inhibitor of apoptosis protein (IAP) family and has gained interest as a potential molecular target in the treatment of cancer (26). Recent reports suggest that survivin protein levels are upregulated by coexpression of HER2 and EGF receptor (EGFR) through the PI3K/Akt signaling pathway in breast cancer cells (27). In previous studies, we have shown that overexpression of survivin is found in more than 70% of pediatric and adult malignant gliomas (28).

YM-155 is a novel survivin suppressant that is currently in clinical trials (29, 30). YM-155 has been shown to suppress survivin expression, with little effect on expression levels of other IAP family members, and to inhibit growth and viability of diverse human cancer cell lines (31, 32). Because YM-155-induced apoptosis is associated with downregulation of Mcl-1 (33), which is a significant mediator of resistance to ABT-737, we hypothesized that combining YM-155 and ABT-737 would potentiate apoptosis induction in gliomas, either by direct inhibitory effects on survivin levels or by associated effects on Mcl-1. Our results validated this hypothesis; moreover, we observed a strong association between treatment response and EGFR expression status, which was enhanced by targeted inhibition of this pathway.

Materials and Methods

Cell lines

U87, T98G, U373, A172, and LN229 were obtained from the American Type Culture Collection. LN18, LNZ308, and LNZ428 were provided by Dr. Nicolas de Tribolet (University of Lausanne, Switzerland). The establishment of the parental human glioblastoma cell line, U87, and its derivatives, which overexpress exogenous wild-type (WT) EGFR (U87-EGFR), or constitutively active EGFR with a genomic deletion of exons 2–7 (U87-EGFRvIII) has been described elsewhere (34). The cell lines were kindly provided by Dr. W.K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA). Human astrocytes and growth media were obtained from ScienCell Research Laboratories. Cell lines used in this study were not authenticated. Cell culture conditions of these cell lines were as previously described (3, 35).

Reagents and antibodies

ABT-737, YM-155, BEZ-235, gefitinib, and PI-103 were purchased from Chemie Tek. N-Acetyl-l-cysteine (NAC) and LY294002, U0126 were purchased from Cell Signaling Technology. Pan-caspase inhibitor, Z-VAD-fmk was purchased from R&D Systems. The following antibodies were used: Bcl-2, Bcl-xL, Mcl-1, Bim, Bak, Bax, cleaved PARP, cleaved caspase 3, cleaved caspase-7, survivin, cIAP-1, cIAP-2, XIAP, Mcl-1, Bax, Bak, phospho extracellular signal-regulated kinase (pERK), total ERK, pAkt, total Akt, p56K (Ser235/236), and β-actin were from Cell Signaling Technology. Monoclonal anti-Bax (6A7) was from Sigma. Phospho EGFR antibodies were from Invitrogen. All primary antibodies were used at 1:1,000 dilutions. Secondary antibodies conjugated to horseradish peroxidase, used at 1:2,000 dilutions, were purchased from Santa Cruz Biotechnology, and visualized by enhanced chemiluminescence (Cell Signaling Technology).

Cell proliferation assay

Cells (5 × 10^5/well) were plated in 96-well microtiter plates (Costar) in 100 μL of growth medium, and after overnight attachment exposed for 3 days to inhibitors or vehicle [dimethyl sulfoxide (DMSO)]. After the treatment interval, cells were washed in medium, and the number of viable cells was determined using a colorimetric cell proliferation assay (CellTiter96 Aqueous NonRadioactive Cell Proliferation Assay; Promega) as described previously (3).

Annexin V apoptosis assay

Apoptosis was evaluated using fluorescein isothiocyanate (FITC)–conjugated Annexin V/propidium iodide (PI) assay kit (Molecular Probes, Invitrogen) based on Annexin V binding to phosphatidylserine exposed on the outer leaflet of the plasma membrane lipid bilayer of cells entering the apoptotic pathway as described previously (3, 6). Briefly, cells were treated with or without inhibitors for the indicated duration, collected by trypsin–EDTA, pelleted by centrifugation (1,000 rpm for 5 minutes), washed in ice-cold PBS, and resuspended in the Annexin V-FITC and 1 μg/mL PI reagent in the dark for 15 minutes before flow-cytometric analysis. Labeling was analyzed by flow cytometry with a FACSCalibur flow cytometer (BD Biosciences). A minimum of 20,000 cells per sample was collected.

DiOC6 labeling and detection of mitochondrial membrane depolarization

Mitochondrial membrane depolarization was measured as described previously (6). In brief, floating cells were collected and attached cells were trypsinized and resuspended in PBS. Cells were loaded with 50 nmol/L 3',3'-dihexyloxacarbocyanine iodide (DiOC6, Invitrogen) at 37°C for 15 minutes. The positively charged DiOC6 accumulates in intact mitochondria, whereas mitochondria with depolarized membranes accumulate less DiOC6. Cells were spun at 3,000 × g, rinsed with PBS, and resuspended in 1 mL of PBS. Fluorescence intensity was detected by flow cytometry and analyzed with CellQuest (Becton Dickinson) and FlowJo (Tree Star, Inc.) analysis software.

Immunoprecipitation and Western blot analysis

Cells were washed in cold PBS and lysed in buffer containing 30 mmol/L HEPES, 10% glycerol, 1% Triton.
Figure 1. YM-155 sensitizes glioma cells to ABT-737 toxicity. A, chemical structure of YM-155, LY294002, PI-103, BEZ-235, ABT-737, and U0126. B, glioma cells were treated with the indicated concentrations of ABT-737 or YM-155 or both for 24 hours. Apoptosis was assessed by Annexin V-FITC and PI staining and FACS analysis. Data are representative of 3 independent experiments. C, in parallel, cell extracts were prepared, and equal amounts of protein were separated by SDS-PAGE and subjected to Western blot analysis with the indicated antibodies. D, U373 and LNZ308 cells were treated with ABT-737 (2.5 µmol/L), YM-155 (25 nmol/L), or the combination (A + Y). In parallel, cells were pretreated with 25 µmol/L Z-VAD-FMK (pan-caspase inhibitor) for 2 hours followed by the combination of ABT-737 plus YM-155 for 24 hours. Control cells received an equivalent amount of DMSO. Apoptosis (top, representative Annexin V-binding histogram; bottom, bar chart representing 3 independent experiments) was analyzed by flow cytometry. The percentages of cells in each quadrant are indicated.
X-100, 100 mmol/L NaCl, 10 mmol/L MgCl₂, 5 mmol/L EDTA, 2 mmol/L Na₃VO₄, 2 mmol/L β-glycerophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, 0.8 μmol/L aprotinin, 50 μmol/L bestatin, 15 μmol/L E-64, 20 μmol/L leupeptin, and 10 μmol/L pepstatin A for 15 minutes on ice. Samples were centrifuged at 12,000 × g for 15 minutes, supernatants were isolated, and protein was quantified using Protein Assay Reagent (Pierce Chemical). Equal amounts of protein were separated by SDS-PAGE and electrotransferred onto a nylon membrane (Invitrogen). Nonspecific antibody binding was blocked by incubation of the membranes with 4% bovine serum albumin in TBS/Tween 20 (0.1%). The membranes were then probed with appropriate dilutions of primary antibody overnight at 4°C. The antibody-labeled blots were washed 3 times in TBS/Tween 20 and incubated with a 1:2,000 dilution of horseradish peroxidase–conjugated secondary antibody in TBS/Tween 20 at room temperature for 1 hour. Proteins were visualized by Western Blot Chemiluminescence Reagent (Cell Signaling). Where indicated, the membranes were reprobed with antibodies against β-actin to ensure equal loading and transfer of proteins.

For immunoprecipitation, cell extracts were prepared by lysing 5 × 10⁶ cells on ice for 30 minutes in CHAPS lysis buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% CHAPS, protease, phosphatase inhibitors]. Lysates were clarified by centrifugation at 15,000 × g for 10 minutes at 4°C, and the protein concentrations in the supernatants were determined. Equal amounts of protein extracts were incubated overnight with primary antibody. Afterward, Dynabeads Protein G (Invitrogen) was added for 2 hours, followed by magnetic separation of the immunoprecipitated fraction; Western blot analysis was carried out as described earlier. Scanning densitometry was conducted using acquisition into Adobe Photoshop (Adobe Systems, Inc.) followed by image analysis (UN-SCAN-IT gel, version 6.1; Silk Scientific).

**Transient transfection**

Optimal 29mer-pRS-shRNA constructs were obtained from Origene. Sequences specific for human Mcl-1 (ACC TAG AAG GTG GCA TCA GGA ATG TGC TG) and control sequences [GCA CTA CCA GAG CTA ACT CAG ATA GTA CT; nontarget short hairpin RNA (shRNA)] were used for this study. Glioma cells were seeded in 6-well plates and allowed to reach 70% confluence. Transfection of targeting or control shRNA was conducted by using FuGene 6 according to the manufacturer’s recommendations (Roche Applied Science). One microgram of Mcl-1 or nontargeting shRNA in 100 μL Opti-MEM medium was mixed with 2 μL of FuGene 6. After the mixture was incubated at room temperature for 20 minutes, complete medium was added to make the total volume up to 2 mL. After 48 hours, media was changed and cells were incubated with inhibitors for 24 hours. Cell viability (Annexin V binding) or Western blot analysis was carried out as described earlier.

**Statistical analysis**

Unless otherwise stated, data are expressed as mean ± SD. The significance of differences between experimental conditions was determined using a two-tailed Student t test. Differences were considered significant at P values less than 0.05.
Results

YM-155 sensitizes glioma cells to ABT-737 but not non-neoplastic astrocytes

Glioma cells were treated with ABT-737 or YM-155 or both (Fig. 1A) and apoptotic cell death was examined by Annexin V/PI staining. As shown in Fig. 1B, YM-155 significantly increased the sensitivity of LN18, U373, LNZ428, LN229, T98G, and LNZ308 cells to ABT-737 treatment compared with cells treated with ABT-737 alone. Simultaneous treatment with ABT-737 and YM-155 resulted in a significant increase in the appearance of cleaved fragments of caspase-7, -3, PARP (Fig. 1C). This apoptotic response was circumvented by the broad-specificity caspase inhibitor z-VAD-fmk (Fig. 1D). In contrast to the above cell lines, a more modest effect was seen in A172 cells (Fig. 1E). As shown in Supplementary Fig. S1A, ABT-737 had no effect on normal human astrocytes even at high concentrations (72 hours; IC_{50} > 25 μmol/L). Simultaneous treatment of ABT-737 plus YM-155 also had little or no effect on cell proliferation (Supplementary Fig. S1A), caspase or PARP activation (Supplementary Fig. S1B), suggesting selectivity against glioma cells versus non-neoplastic astrocytes.

Sensitivity of glioma cell lines to YM-155

To investigate the independent activity of YM-155 in glioma cells, we examined the cytotoxic efficacy of YM-155 in a panel of glioma cell lines under normal culture conditions. After a 24-, 48-, and 72-hour treatment, inhibition of cell proliferation and viability was assessed by MTS assay. All glioma cell lines responded in a dose-dependent manner (Fig. 2A) and time-dependent (data not shown) manner to YM-155 with reduction of cell numbers. U373, LN18, LNZ428, T98G, LN229, and LNZ308 cells exhibited an IC_{50} of approximately 10 to 75 nmol/L, whereas A172 cells were resistant to YM-155 with IC_{50} approximately 250 nmol/L. To investigate the mechanisms underlying the differential sensitivity to YM-155, a series of Western blot analyses were done to compare the expression of various IAP family proteins. No correlation was found between the sensitivity of cells to YM-155 and the expression of survivin, cIAP-1, cIAP-2, and XIAP (Fig. 2B). We then examined the ability of YM-155 to inhibit survivin expression. In accordance with previous reports (32, 33), survivin was downregulated in a concentration-dependent manner (Fig. 2C). However, YM155 had no effect on XIAP, cIAP-1, and cIAP-2 (data not shown). Because survivin expression is regulated by EGFR via the PI3K/Akt signaling pathway (27), we investigated the levels of EGFR phosphorylation (activation of phosphorylation of tyrosine residues at 845, 1,068, 1,086, 1,148, and 1,173) by Western immunoblot analysis. EGFR phosphorylation levels varied substantially among the cell lines tested, and strong correlation was observed between EGFR activation levels and response to YM-155 (Fig. 2D).

YM-155 downregulates Mcl-1 and sensitizes glioma cells to ABT-737 by enhancing Bax conformational changes and disrupting Bak/Bax interactions with Mcl-1

Because downregulation of Mcl-1 has been observed to confer sensitivity to ABT-737 in other tumor types (14, 22), we questioned whether the potentiating effect of YM-155 on ABT-737 response was due to an effect on Mcl-1 expression. YM-155 caused downregulation of Mcl-1 in a dose-dependent manner (Fig. 3A) but did not affect other Bcl-2 family members (data not shown). As shown in Fig. 3B, Mcl-1 shRNA also caused significant enhancement of apoptosis induction (Fig. 3B). Because Mcl-1 blocks the progression of apoptosis by binding and sequestering the proapoptotic protein Bak, we examined the association between Bak and Mcl-1. Glioma cells were treated with ABT-737 or YM-155 or both and immunoprecipitated using Bak antibody followed by Western blotting using Mcl-1 antibody. As shown in Fig. 3C, the association between Mcl-1 and Bak was not changed by ABT-737 treatment, whereas this effect was largely abrogated by the combination of ABT-737 and YM-155, suggesting that cotreatment with ABT-737 plus YM-155 was able to disrupt the Bak-Mcl-1 interaction.

To examine the effect of YM-155 and ABT-737 on Bax activation in glioma cells, we analyzed activity-related conformational changes of Bax (12) by immunoprecipitation followed by Western blot analysis with antibodies recognizing N-terminal epitopes of Bax (monoclonal anti-bax, 6A7, Sigma). When cells were separately exposed to ABT-737 (2.5 μmol/L) or YM-155 (25 nmol/L), a minimal Bax conformational change was observed. In contrast, cells coexposed to YM-155 and ABT-737 displayed a significant increase in Bax conformational change compared with cells treated with ABT-737 alone (Fig. 3D), suggesting that down-regulation of Mcl-1 and activation of Bax may be responsible for the marked induction of apoptosis in cells coexposed to YM-155 and ABT-737.

Combination of YM-155 and ABT-737 induces loss of mitochondrial membrane potential

Because Bcl-2 family proteins are key regulators of the mitochondrial apoptotic pathway, and changes in mitochondrial membrane potential (Δψ_m) are thought to represent an early event in the induction of apoptosis, and likely capture the effects of agents on various aspects of Bcl-2 family member homeostasis, we evaluated the effect ABT-737 with or without YM-155 on Δψ_m. The integrity of the mitochondrial membranes of the cells was examined by DiOC6 staining and flow cytometry; the decrease in fluorescence intensity reflected the loss of Δψ_m. As shown in Fig. 4A, exposure of U373 and LN18 (YM-155–sensitive) glioma cells to 25 nmol/L of YM-155 for 24 hours caused a marked loss of Δψ_m compared with the control group. However, no significant change in Δψ_m was seen in A172 (YM-155–resistant cell line). Cotreatment with ABT-737 and YM-155 produced an even more...
dramatic loss of $\Delta \Psi_m$ (Fig. 4B) in U373 and LN18 but only a modest change in A172 cell lines. The use of NAC, a scavenger of reactive oxygen species (ROS), almost completely rescued apoptosis (Fig. 4C) and reversed the disruption in $\Delta \Psi_m$ (Fig. 4D) induced by YM-155 + ABT-737 in the sensitive cells.

Overactivation of the EGFR pathway confers resistance to YM-155 and ABT-737–induced cytotoxicity

In view of our observation that the glioma cells with high levels of EGFR activation, such as A172 (Fig. 2D), seemed to be less sensitive to both YM-155 and the combination of YM-155 and ABT-737 (Fig. 1E), we questioned whether EGFR activation status was a key contributor to treatment response. We therefore compared dose–response relationships with YM-155 in native U87 cells with those transduced to highly overexpress EGFR-WT and EGFRvIII, a truncated constitutively active EGFR variant. As shown in Fig. 5A, cell proliferation in vitro was inhibited (assessed after 3 days of exposure to YM-155 by MTS assay) by YM-155 to a substantially greater degree in native versus EGFR-transduced cell lines, with $IC_{50}$ of approximately 60, 270, and 280 nmol/L for U87, U87-EGFR (WT), and U87-EGFRvIII, respectively (Fig. 5B). YM-155 had a significantly lower inhibitory effect on survivin and Mcl-1 expression in U87-EGFR and U87-EGFRvIII than U87 cells (Fig. 5C) and a correspondingly reduced induction of apoptosis in the EGFR-transduced cells (Fig. 5D). To examine the relation between EGFR status and treatment response to YM-155 and ABT-737, cells (U87 and U87-EGFRvIII) were treated with ABT-737 or YM-155 or both. Annexin V assay showed that U87 cells were strikingly more sensitive to the combination of
ABT-737 and YM-155 than U87-EGFRvIII cells (Fig. 5E). Western blot analysis further validated this differential efficacy. Cotreatment with YM-155 and ABT-737 induced substantially less caspase-7, -3, and PARP cleavage in U87-EGFR and U87-EGFRvIII cell lines than in parental U87 cells (Fig. 5F). Differential effects were also observed in terms of Bax activation (Fig. 5G).

EGFR provides major survival signals through both ERK and Akt in glioma cells

Because survivin is involved in multiple signaling mechanisms initiated by EGFR activation (26), we characterized whether certain components of downstream EGFR signaling might correlate with the observed resistance in EGFR-overactivated glioma cells. Cells were incubated with U0126 (MEK1 inhibitor), LY294002 (PI3K/Akt inhibitor), BEZ-235 or PI-103 (PI3K/mTOR dual kinase inhibitors), or gefitinib (EGFR inhibitor) for 24 hours, and levels of respective phosphoproteins and survivin were analyzed by Western blotting. The survivin levels were significantly decreased by U0126, LY294002, BEZ-235, PI-103, and gefitinib (Fig. 6A). To test whether inhibition of ERK or PI3K/Akt/mTOR pathways could sensitize U87-EGFRvIII cells to YM-155 + ABT-737–induced toxicity, cells were pretreated with either U0126, LY294002, BEZ235, or PI-103 and the effect of

Figure 3. YM-155 downregulates Mcl-1 and sensitizes glioma cells to ABT-737 by enhancing Bax conformational changes and disrupting Bak/Bax interactions with Mcl-1. A, logarithmically growing glioma cells were treated with the indicated concentrations of YM-155 for 24 hours and subjected to Western blot analysis with anti-Mcl-1 antibody. B, LN18, U373, and LNZ308 cells were transfected with nontarget or Mcl-1 shRNA. Forty-eight hours posttransfection, cells were treated with the indicated concentrations of ABT-737 for 24 hours and viability was assessed by Annexin V/PI apoptosis assay. In parallel, cell lysates were collected and protein was subjected to Western blot analysis using anti-Mcl-1 antibody. C, LN18, U373, LNZ29, and LNZ308 cells were treated with inhibitors for 24 hours. An equal amount of protein (400 μg) was immunoprecipitated (IP) with Bak antibody. Immunoprecipitates were subjected to Western blot analysis using anti-Mcl-1 antibody. D, lysates were subjected to immunoprecipitation using anti-Bax 6A7 (Sigma-Aldrich) that recognizes only Bax protein that has undergone conformational change. Immunoprecipitates were subjected to immunoblotting using anti-Bax polyclonal antibodies (Cell Signaling Technology). IgG, immunoglobulin G.
Figure 4. Combination of YM-155 and ABT-737 induces loss of mitochondrial membrane potential. A, U373, LN18, and A172 cells were treated with the indicated concentrations of YM-155 for 24 hours. The integrity of the mitochondrial membranes of the cells was examined by DiOC6 staining and flow cytometry. Decrease in fluorescence intensity reflected loss of Δψm. Histogram (top) and bar chart (bottom) represent the mean number of Δψm cells acquired from 3 independent experiments. B, U373, LN18, and A172 cells were treated with the indicated concentrations of YM-155 with ABT-737 (2.5 µmol/L) for 24 hours. Loss of mitochondrial membrane potential was determined. C and D, U373 and LN18 cells were treated with ABT-737 (2.5 µmol/L) or YM-155 (25 nmol/L) or the combination of both (A + Y). In parallel, cells were pretreated with NAC (5 mmol/L, ROS scavenger) for 2 hours followed by the combination of ABT-737 plus YM-155 for 24 hours. Apoptosis (C) or loss of mitochondrial membrane potential (D) was analyzed by flow cytometry.

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ABT-737 and YM-155 on viability was assessed. The viability of EGFRvIII cells was not affected by the combination of YM-155 and ABT-737. In contrast, pretreated cells showed significantly increased apoptotic sensitivity to YM-155 + ABT-737 (Fig. 6B) and increased Bax activation (Fig. 6C). As shown in Fig. 6D, exposure of U87 and U87-EGFRvIII cells to YM-155 for 24 hours caused minimal or no loss of Δψm compared with the control group. Cotreatment with ABT-737 and YM-155 produced a significant loss of Δψm in U87 but not in U87-EGFRvIII cell lines (Fig. 6E). However, pretreatment of U87-EGFRvIII cells with LY294002, BEZ235, or PI-103 (Fig. 6F) led to a significant enhancement of Δψm loss.

Discussion

Previous studies have shown that the cell killing effect of ABT-737 varies widely among cancer types or cell lines from the same type of cancers (36, 37). Our recent study (6) showed that unlike hematologic malignancies (12, 15, 38) glioma cells were resistant to apoptosis induction in response to ABT-737 alone, which may reflect activation of apoptosis-inhibitory molecules, such as Mcl-1 that are not affected by ABT-737 and survival signaling pathways that counteract apoptosis induction. In this regard, there has been much recent interest in the role of survivin as a potential apoptosis-modulating target in the treatment of cancer (26). In this study, we found that a survivin inhibitor, YM-155, potently inhibited growth of a subset of malignant human glioma cell lines. This agent significantly reduced Mcl-1 expression in sensitive cell lines, consistent with recent observations of Tang and colleagues (33), while having no effect on the expression levels of c-IAP-1, c-IAP-2, XIAP, Bcl-2, or Bcl-xL.

Because ABT-737 has been observed to be most effective against cancers in which Mcl-1, an antiapoptotic Bcl-2 family member, is downregulated or absent (12, 22), we speculated that approaches directed at inhibiting Mcl-1 could sensitize glioma cells to ABT-737. Our results showed that YM-155 in combination with ABT-737 induced cell death, mediated by mitochondrial membrane depolarization and caspase activation. Cotreatment of glioma cells with low concentrations of ABT-737 and YM-155 resulted in apoptotic cell death as verified by Annexin V staining, associated with the activation of caspases at the initiative and executive stages. The role of Mcl-1 in this process was further validated by a genetic (shRNA) approach to reduce MCL-1 levels, which independently increased the ABT-737 response.

In addition to delineating the important role of Mcl-1 in ABT-737–mediated apoptosis, our results showed that sensitivity to YM-155 and the combination of YM-155 and ABT-737 varied substantially between different glioma
cell lines, correlating strongly with levels of EGFR activation. EGFR overexpression, often resulting from gene amplification and/or mutation, has been associated with cancer progression in patients with glioma. The most common mutant form is EGFRvIII, a ligand-independent, constitutively active receptor that has been observed to correlate with poor prognosis (39, 40). In our experiments, YM-155 enhanced ABT-737–induced cytotoxicity to a...
Figure 6. EGFR provides major survival signals through both ERK and Akt in glioma cells. A, U87 and U87-EGFRvIII cells were incubated with the indicated concentrations of inhibitors for 24 hours. Control cells received an equivalent amount of DMSO. Cell extracts were subjected to Western blot analysis with the indicated antibodies. B, U87-EGFRvIII cells were incubated with U0126, LY294002 (LY), BEZ-235 (BEZ), or PI-103 (PI) for 2 hours followed by the combination of ABT-737 (2.5 μmol/L) plus YM-155 (25 nmol/L) for 24 hours (YA). Apoptosis was analyzed by Annexin V/PI assay. C, U87-EGFRvIII cells were treated with ABT-737 (2.5 μmol/L; ABT + YM) with the indicated inhibitors for 24 hours. An equal amount of protein (400 μg) was immunoprecipitated (IP) with monoclonal anti-Bax (6A7, Sigma) antibody and then subjected to Western blot analysis with polyclonal anti-Bax antibody (Cell Signaling Technology). D and E, U87 and U87-EGFRvIII cells were treated with the indicated concentrations of YM-155 (D) and with or without ABT or YM-155 (2.5 μmol/L; E) for 24 hours. Loss of mitochondrial membrane potential was determined by DiOC6 staining and flow cytometry. F, U87-EGFRvIII cells were pretreated with LY29402 (LY), BEZ-235 (BEZ), or PI-103 (PI) for 2 hours followed by the combination of ABT-737 (2.5 μmol/L) plus YM-155 (25 nmol/L) [A + Y] for 24 hours. Loss of mitochondrial membrane potential was determined as in D. **, P < 0.005.

substantially different extent depending on the cell lines examined, with significant synergistic enhancement of tumor cell killing in glioma cells without high levels of EGFR activation. Conversely, we observed that overactivation of the EGFR signaling pathway, which is noted in approximately half of malignant gliomas, was associated...
with resistance to the apoptosis induced by YM-155 and ABT-737.

Studies using various cell types imply that multiple pathways involved in EGFR signaling, including phosphoinositide 3-kinase (PI3K), JAK/STAT3, and MEK/ ERK are involved in the regulation of Mcl-1 transcription (41). We show in this study that the downstream effectors of aberrantly activated EGFR signaling pathways are involved in promoting Mcl-1 expression and result in ABT-737 and YM-155 resistance, and that EGFR, MAPK, and Akt inhibitors significantly reduced Mcl-1 protein expression levels and promoted the apoptotic effect of YM-155/ABT-737 cotreatment. Previously, our group has shown that the activation of Bax, including conformational changes and oligomerization, seems to play a crucial role in the initiation of apoptosis after other signaling-targeted therapies in gliomas (6, 42). Moreover, proapoptotic Bak is sequestered by the antiapoptotic Mcl-1 and Bcl-XL, its displacement by BH3-only proteins being required for cell death (43). The results of the present study indicate that cotreatment with YM-155 and ABT-737 indeed causes conformational change of Bax, and this event allows Bak displacement from its antiapoptotic counterparts to occur.

In summary, here we have shown that the combination of YM-155 and ABT-737 potently triggered apoptosis in a subset of glioma cells, associated with reduced Mcl-1 protein expression, loss of mitochondrial transmembrane potential, activation of Bak and Bax, and stimulation of caspase-3 activity. EGFR signaling, which has been known to activate multiple survival signaling pathways, including Ras/MAPK and PI3K/Akt signaling, was a critical modulator of this apoptotic response. Our study has shown that the sensitivity to YM-155 and ABT-737 was inversely correlated with EGFR activation status, and was partially restored in highly resistant U87-EGFRvIII cells by combining ABT-737 and YM-155 with either gefitinib, U0126, LY294002, BEZ-235, or PI-103 to inhibit various aspects of EGFR signaling. Because more than one acquired resistance mechanism may simultaneously exist in malignant gliomas, targeting a series of rationally defined survival signaling pathways in concert might provide a useful strategy to overcome the multiple nonoverlapping resistance mechanisms that characterize these highly aggressive tumors.

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

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