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Survivin Inhibitor YM155 Induces Mitochondrial Dysfunction, Autophagy, DNA Damage, and Apoptosis in Bcl-xL Silenced Glioma Cell Lines

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Abstract

Because the anti-apoptotic protein Bcl-xL is overexpressed in glioma, one might expect that inhibiting or silencing this gene would promote tumor cell killing. However, our studies have shown that this approach has limited independent activity, but may tip the balance in favor of apoptosis induction in response to other therapeutic interventions. To address this issue, we performed a pharmacological screen using a panel of signaling inhibitors and chemotherapeutic agents in Bcl-xL silenced cells. Although limited apoptosis induction was observed with a series of inhibitors for receptor tyrosine kinases, PKC inhibitors, Src family members, JAK/STAT, histone deacetylase, the PI3K/Akt/mTOR pathway, MAP kinase, CDK, heat shock proteins, proteasomal processing, and various conventional chemotherapeutic agents, we observed a dramatic potentiation of apoptosis in Bcl-xL silenced cells with the survivin inhibitor, YM155. Treatment with YM155 increased the release of cytochrome c, smac/DIABLO and apoptosis inducing-factor, and promoted loss of mitochondrial membrane potential, activation of Bax, recruitment of LC3-II to the autophagosomes and apoptosis in Bcl-xL silenced cells. We also found an additional mechanism for the augmentation of apoptosis due to abrogation of DNA double-strand break repair mediated by Rad51 repression and enhanced accumulation of γ H2AX. In summary, our observations may provide a new insight into the link between Bcl-xL and survivin inhibition for the development of novel therapies for glioma.

Keywords

glioma; Bcl-xL silencing; signaling inhibitors; synergy; caspase-dependent cell death

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

INTRODUCTION

GBM is the most aggressive and common malignant primary brain tumor, and generally responds poorly to current therapy with surgery, radiation, and conventional chemotherapy. The characteristics of glioma include aggressive cell proliferation, widespread genomic instability, and resistance to apoptosis. During malignant transformation, deregulation of retinoblastoma (RB)/cell cycle control pathways, p53 pathways, receptor tyrosine kinase (RTK) function, and phosphoinositide 3-kinase (PI3K)/AKT/phosphatase and tensin homolog (PTEN) pathways are very common [1] which leads to resistance to signaling inhibitors. The multifaceted genomic alterations that characterize these tumors remains the predominant factor limiting the clinical success of single agent therapies, regardless of drug target.

The Bcl-2 family of proteins are key regulators of apoptosis, and have been associated with chemotherapy resistance in various human cancers. This family includes both antiapoptotic (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and A-1) and proapoptotic (Bax, Bak, Bik, Bad, and Bid) proteins with opposing biological functions in either promoting or inhibiting cell survival. Targeted therapies affecting the Bcl-2 family of proteins as a strategy for overcoming chemotherapy drug resistance have been well characterized [2–7]. Using a large-scale siRNA screening approach to identify critical “nodes” for cell death signaling, we identified several targets, including nuclear factor κ B (NF- κ B) and the proteasome, as well as Akt and Bcl-xL that, when inhibited, promoted apoptotic signaling in glioma cells [8–12]. Therefore, we hypothesized that the functional blockade of Bcl-xL (genetic inhibition) should sensitize these cancer cells to chemotherapies or signaling inhibitors by restoring the apoptotic process.

To address the hypothesis, we used lentiviral-based RNA interference expression vectors to silence Bcl-xL function and treated glioma cells with signaling inhibitors or chemotherapeutic agents. We found that the survivin inhibitor YM155, but not the other signaling inhibitors, enhanced apoptosis in Bcl-xL silenced cells. This is due to the increased release of cytochrome c, smac/DIABLO and apoptosis-inducing factor to the cytosolic fraction, increased loss of mitochondrial membrane potential, activation of Bax and DNA damage.

MATERIALS AND METHODS

Cell Lines

The malignant human glioma cell lines U87, U373, T98G, A172, and LN229 were obtained from the American Type Culture Collection (Manassas, VA). LN18, LN2428, and LN2308 were provided by Dr. Nicolas de Tribolet (Lausanne, Switzerland). LN444 cell line was from Dr. Shi-Yuan Cheng (Northwestern University Feinberg School of Medicine, Chicago, IL). Cell culture conditions of these cell lines were as previously described [13]. Primary cultures were obtained from freshly resected tissues after surgical removal under an Institutional Review Board-approved protocol for acquisition and use of tumor tissue collected at the time of tumor resection. We also obtained primary GBM cells from Conversant Biologics (Huntsville, AL), which were cultured in DMEM/F12 (Invitrogen,

Carlsbad, CA). GIBCO® Human Astrocytes, normal human cells derived from human brain tissue, were purchased from Gibco Life Technologies and cultured using GIBCO® Astrocyte Medium (ThermoFisher Scientific, Pittsburgh, PA).

Reagents and Antibodies

Ribociclib, palbociclib, gefitinib, sorafenib, dasatinib, vorinostat, panobinostat, rapamycin, bortezomib, cucurbitacin-I, AZD-6244, YM-155, NVP-AUY922, AMG-925, ABT-737, ABT-263, MK-2206, XL-147, PI-103, obatociclib, and NVP-BEZ235 were purchased from Chemie Tek (Indianapolis, IN). Ponatinib, axitinib, foretinib, sotrastaurin, enzastaurin, cilengitide, barasertib, tozasertib, everolimus, WP-1066, GSK690693, JQ-1, 10058-F4, and U0126 were from Selleck (Houston, TX). Chemotherapeutics and all other chemicals used in this study were from Sigma (St. Louis, MO). The following antibodies were used: β -actin (#4970), Bcl-2 (#2872), Bcl-xL (#2764), phospho-Akt (#4051), total Akt (#9272), cyclin B1 (#4135), cyclin D1 (#2922), cyclin D3 (#2926), CDK4 (#2906), CDK6 (#3136), KU70 (#4104), PTEN (#9559), Phospho-RB (#9307), survivin (#2808), XIAP (#2042), Bim (#2819), Bid (#2002), Bak (#3814), Bax (#2774), Bcl-w (#2724), Bik (#4592), Mcl-1 (#4572), PUMA (#4976), cIAP-1 (#7065), cIAP-2 (#3130), Cytochrome c (#4280), total mTOR (#2972), phospho-mTOR (#2971), total ULK-1 (#4773), phospho-ULK1 (#5869), LC3B (#3868), Beclin-1 (#4122), smac/DIABLO (#2954), total H2AX (#2595), phospho-H2AX (#2577), SQSTM1/p62 (#8025), caspase-3 (#9664), caspase-7 (#9494), caspase-8 (#9746), caspase-9 (#9501), and PARP (#9546) were from Cell Signaling Technology (Beverly, MA). DDK antibody was purchased from Origene (Rockville, MD). Noxa (sc-26917) and AIF (sc-5586) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Bax (#556467) and RAD51 (#ab63801) were from BD Pharmingen (San Diego, CA) and Abcam (Cambridge, MA), respectively.

Stable Cell Line Generation

Bcl-xL and non-target control shRNA MISSION shRNA Lentiviral transduction particles used in this study were obtained from Sigma. LN2308 and U87 human glioma cells were seeded in six-well plates and allowed to reach 70–80% confluence and infected according to the manufacturer's recommendations (Sigma). The day after infection, medium was changed and cells were incubated with complete media containing puromycin (1 μ g/mL). After 14 days, cell extracts were separated by SDS–PAGE and subjected to Western blotting analysis with Bcl-xL antibody.

Cell Proliferation (MTS) Assay

Cells were seeded in 96-well plates (5000 cells/well) in 100 μ L of growth medium and incubated at 37°C for 24 h before the addition of inhibitors or vehicle for 3 days. Cell growth assays were done using CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI), per the manufacturer's instructions, to evaluate the effect the inhibitors and doses as described previously [14]. Absorbance was measured at a wavelength of 490 nm, and the absorbance values of treated cells are presented as a percentage of the absorbance of untreated cells. Drug concentrations required to inhibit cell growth by 50% (IC50) were determined by interpolation of dose–response curves using IBM® SPSS® Statistics 21.

Annexin V Apoptosis Assay

Apoptosis induction in vehicle- or inhibitor-treated cells was assayed by the detection of membrane externalization of phosphatidylserine using an Annexin V assay kit (Molecular Probes, Invitrogen) as described previously [15]. About 2×10^5 cells were harvested at various intervals after treatment, washed with ice-cold phosphate-buffered saline (PBS) and resuspended in 200 μ L of binding buffer. Annexin V-FITC and 1 μ g/mL propidium iodide were added and cells were incubated for 15 min in a dark environment. Labeling was analyzed by flow cytometry with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Annexin V binds to phosphatidylserine, which translocates from the inner leaflet to the outer leaflet of the plasma membrane in apoptotic cells, so cells that are positive for annexin V staining (i.e., high Annexin V signal) are undergoing apoptosis. PI staining provides a measure of cell viability and is used to distinguish between cells in early and late apoptosis.

DiOC6 Labeling and Detection of Mitochondrial Membrane Depolarization

Mitochondrial membrane depolarization was measured as described previously [16,17]. In brief, floating cells were collected, and attached cells were trypsinized and resuspended in PBS. Cells were loaded with 50 nmol/L 3',3'-dihexyloxacarbo-cyanine iodide (DiOC6, Molecular Probes, Invitrogen) at 37°C for 15 min. The positively charged DiOC6 accumulates in intact mitochondria, whereas mitochondria with depolarized membranes accumulate less DiOC6. Cells were spun at 3000g, and rinsed with PBS twice and resuspended in 1 mL of PBS. Following acquisition of data (CellQuest software (Becton Dickinson, NJ)), the cell fluorescence information was saved in the Flow Cytometry Standard (.fcs) format. These files were then accessed with the FlowJo analysis software (Tree Star, Inc., Ashland, OR). Through this software, the fluorescence data were plotted as histograms, which were converted into and saved as Scalable Vector Graphics (.svg) files. Using Inkscape (The Inkscape Team), an Open Source vector graphics editor, the data was compiled into two-dimensional histogram overlays for comparative analysis. The loss of mitochondrial membrane potential was quantified in FlowJo by gating any left-shifted populations and subtracting from control and the percentage of cells with decreased fluorescence was determined.

Subcellular Fractionation

Cells were treated with or without inhibitors and cytosolic proteins were fractionated as described previously [18]. Briefly, cells were resuspended in a lysis buffer containing 0.025% digitonin, sucrose (250 mM), HEPES (20 mM; pH 7.4), MgCl₂ (5 mM), KCl (10 mM), EDTA (1 mM), phenylmethylsulfonyl fluoride (1 mM), 10 μ g/mL aprotinin, 10 μ g/mL leupeptin. After 10 min incubation at 4°C, cells were centrifuged (2 min at 13 000g) and the supernatant (cytosolic fraction) was removed and frozen at -80°C for subsequent use.

Immunoprecipitation and Western Blotting Analysis

Cells were washed in cold PBS and lysed in buffer containing 30 mM HEPES, 10% glycerol, 1% Triton X-100, 100 mmol/L NaCl, 10 mmol/L MgCl₂, 5 mM EDTA, 2 mM Na₃VO₄, 2 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, 0.8 μ mol/L aprotinin, 50 μ mol/L bestatin, 15 μ mol/L

E-64, 20 $\mu\text{mol/L}$ leupeptin, and 10 $\mu\text{mol/L}$ pepstatin A for 15 min on ice. Samples were centrifuged at 12 000g for 15 min, supernatants were isolated, and protein was quantified using Protein Assay Reagent (Pierce Chemical, Rockford, IL). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto a nylon membrane (Invitrogen). Nonspecific antibody binding was blocked by incubation of the membranes with 4% bovine serum albumin in Tris-buffered saline (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 three times in TBS/Tween 20 and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody in TBS/Tween 20 at room temperature for 1 h. 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 Bax immunoprecipitation, cell extracts were prepared by lysing 5×10^6 cells on ice for 30 min 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 000g for 10 min at 4°C, and the protein concentrations in the supernatants were determined. Equal amounts of protein extracts were incubated overnight with primary antibody (active Bax, 6A7, Sigma). Afterward, Dynabeads Protein G (Invitrogen) was added for 2 h, followed by magnetic separation of the immunoprecipitated fraction; Western blot analysis was carried out as described above.

Adenovirus Infection

PTEN wild-type adenovirus (Ad-PTEN) and Ad-CMV were kindly provided by Dr. Craig Henke (University of Minnesota, Minneapolis, MN) and Dr. Christopher Kontos (Duke University Medical Center, Durham, NC), respectively. Glioma cells were infected with adenovirus vectors at 50 MOI (multiplicity of infection) for 48 h at 37°C. The medium was changed and treated with inhibitors. Cells were processed for Western blot or annexin V apoptosis analysis as described above.

Transient Transfection

Logarithmically growing glioma cells were transfected using FuGENE HD transfection reagent as recommended by the manufacturer (Promega). Optimal 29mer-pRS-shRNA constructs were obtained from Origene (Rockville, MD). Sequences specific for human Bcl-2 (catalog number TR316461) and non-target control shRNA (catalog number TR30012) sequences were used for this study. For overexpression studies, pCMV-6 vector (Myc-DDK-tagged, catalog number PS100001) or Myc-DDK tagged Bcl-2 expression plasmid (catalog number RC204498) were obtained from Origene. Cells were seeded in six-well plates (for Western blotting and annexin V/PI analysis) and allowed to reach 70–80% confluence. About 1 μg of shRNA or DNA in 100 μL Opti-MEM medium was mixed with 2 μL of FuGENE HD transfection reagent. After the mixture was incubated at room temperature for 10 min, complete medium was added to make the total volume up to 2 mL. For cell proliferation analysis, cells were seeded in 96-well plates in 100 μL of growth medium and transfected with 50 ng of shRNA or DNA per well. After 24 h post-

transfection, medium was changed and cells were incubated with inhibitors for the indicated period of time. Cell proliferation (colorimetric tetrazolium MTS assay), cell viability (annexin V/propidium iodide binding) or Western blot analysis were carried out as described above.

Fluorescence Microscopy

Cells were grown on chamber slides (Nalge Nunc, Naperville, IL) in growth medium, and, after an overnight attachment period, were exposed to selected concentrations of inhibitor or vehicle (DMSO) for various intervals. To label mitochondria, cells were incubated with Mitotracker red (MitoTracker® probe, Invitrogen, catalog number M 22425) for 30 min. Then cells were washed once with PBS, fixed with 3.7% formaldehyde for 30 min. After washing two times in PBS, cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were washed with PBS, blocked with 0.5% bovine serum albumin for 1 h and then incubated with primary antibodies overnight at 4°C. After PBS wash, the slides were incubated with secondary antibody and Hoechst 33342 (Invitrogen) for 2 h at room temperature. The slides were then washed in PBS, mounted, and examined under a fluorescent microscope. Changes in response to inhibitor treatment were evaluated by microscopic (EVOS, Thermo Fisher Scientific) inspection.

Statistical Analysis

All data represent at least three independent experiments and are expressed as mean±S.D. For annexin V/PI assays and loss of mitochondrial membrane potential analysis (ψ_m), experiments were repeated three times at least in duplicates. Western and immunoblot analysis were repeated three times. Scanning densitometry was performed on Western blots using acquisition into Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) followed by image analysis (UN-SCAN-IT gel TM, version 6.1, Silk Scientific, Orem, UT). Values in arbitrary number shown in the Western blots represent densitometer quantification of band normalized to loading control. The significance of differences between experimental conditions was determined using a two-tailed Student's *t* test. Differences were considered significant at *P* values <0.05.

RESULTS

Targeting Bcl-xL Sensitizes Glioma Cells to the Survivin Inhibitor, YM-155

Overexpression of antiapoptotic members of the Bcl-2 family strongly correlates with chemoresistance in a variety of neoplasms including glioma [19–21]. Our small interfering RNA (siRNA) synthetic lethal screen targeting 5520 unique druggable genes identified multiple gene products, including Bcl-xL and Bcl-2 that contribute to the glioma cell survival and proliferation [8,9,11]. We assessed the expression level in a panel of nine established glioma cell lines (LN444, LN2428, T98G, U373, A172, LN18, LN229, U87, and LN2308), four patient-derived cultures (GBM-1, GBM-2, GBM-3, and GBM-4) and human astrocytes. Western blot analysis revealed an up-regulation of both Bcl-xL and Bcl-2 in glioma cell lines compared with non-neoplastic human astrocytes (***P* < 0.005; human astrocyte vs. representative glioma cell line, Supplementary Figure S1A–C).

Then, to experimentally identify agents that could be therapeutically combined with Bcl-xL inhibitors, we generated stable cell lines targeting Bcl-xL protein (Figure 1A) and screened signaling inhibitors and/or cancer therapeutics for their effect on cell viability. The panel of inhibitors selected for the study includes receptor kinase inhibitors (gefitinib and sorafenib), multi-target kinase inhibitors (ponatinib, axitinib, and foretinib), PKC inhibitors (Sotrastaurin, enzastaurin, and rottlerin), Src family kinase inhibitor (dasatinib), histone deacetylase inhibitors (vorinostat and panobinostat), proteasomal inhibitor (bortezomib), DNA-PK inhibitor (NU70206), CDK inhibitors (palbociclib and ribociclib), integrin inhibitor (cilengitide), aurora kinase inhibitors (barasertib, and tozasertib), heat shock protein inhibitors (17-AAG and NVP-AUY922), MEK inhibitors (U0126 and AZD6244), Bcl-2/Bcl-xL inhibitors (ABT-737, ABT-263, and obatoclax), PI3K/Akt/mTOR pathway inhibitors (GSK690693, MK2206, XL-147, PI-103, and everolimus), Myc inhibitor (10058-F4), BET bromodomain inhibitor (JQ-1), JAK/STAT inhibitor (WP-1066), chemotherapeutic agents (topoisomerase inhibition, topotecan and etoposide; DNA alkylation, temozolomide, carmustine and doxorubicin; DNA strand termination, gemcitabine and 5-Fluorouracil; disruption of microtubule dynamics, taxol, vincristine, and vinblastine), and survivin inhibitor (YM155) for their effect on cell viability. As shown in Figure 1B and C, cell death in Bcl-xL silenced LNZ308 and U87 cells showed a strong increase following YM155 (survivin inhibitor) treatment compared to the control group (stable cell line generated using non-target shRNA) as determined by annexin V/PI flow cytometry. Surprisingly, we did not observe a clear annexin V/PI positive population of cells treated with other classes of inhibitors.

YM155-Induced Caspase-Dependent Cell Death in Bcl-xL Silenced Cells

To investigate the mechanisms underlying enhanced apoptosis in Bcl-xL silenced cells, we examined the IAP family protein expression in Bcl-xL silenced U87 and LNZ308 and corresponding non-target vector cell lines. We found that the primary target survivin was significantly down-regulated by YM155 in both Bcl-xL silenced and non-target control cells. However, there was a minimal or modest change in the expression levels of cIAP1, cIAP2, or XIAP (Figure 2A and B). Recently, Eno et al. [22] have shown that Mcl-1 and Bcl-xL are capable of compensating for the loss of one another in the cellular response to oxidative stress-induced apoptosis. Because anti-apoptotic Bcl-2 sub-family proteins share significant homology, we speculated that the loss of Bcl-xL could be compensated for by another anti-apoptotic Bcl-2 family member. To investigate that, we ran a series of Western blots and examined the expression levels in both U87 and LNZ308 cell lines. As evident from the immunoblot, there was no significant change or “compensation” observed for the loss of Bcl-xL in both LNZ308 and U87 cell lines (Figure 2C; compare lanes 1 vs. 5). Regardless of Bcl-xL status or YM155 treatment, a minimal expression of Bik, Noxa, and Bcl-w was seen. Immunoblotting also confirmed a modest level of increase in Bim in the YM155-treated Bcl-xL silenced cells but not in the non-target vector control cells (Figure 2C). In accordance with our previous report [14,16], Mcl-1 protein was down-regulated by YM155 in both Bcl-xL silenced cells as well as the vector control.

At the clinically achievable concentrations (5–25 nmol/L) [23,24], YM155 led to 10–15% cell death in U87 and LNZ308. However, Bcl-xL silenced cells were much more sensitive to

YM155-induced apoptosis than control counterparts. YM155 induced 25%, 60%, and 80% apoptosis for Bcl-xL silenced LNZ308 cells at 10, 25, and 50 nmol/L compared to 10%, 13%, and 17% apoptosis for control cells under the same conditions (Figures 2D,2E). A similar result was seen in U87 cells (Figure 2E). In agreement with the annexin V/PI apoptosis analysis, Western immunoblotting revealed the appearance of cleaved fragments of caspases and PARP in Bcl-xL silenced cells in a concentration dependent manner but not in stable cell lines expressing vector backbone only (Figure 2F). Similarly, kinetics studies revealed that activation of caspases 7, 3, and PARP occurred within 8 h in Bcl-xL silenced LNZ308 and U87 cells, suggesting that Bcl-xL plays a prominent role in inhibiting apoptosis induction with YM155 (Figure 2G).

To assess the contribution of Bcl-2 in YM155-induced toxicity, LNZ308 and U87 cells were transiently transfected with Bcl-2 expression plasmid or control vector. Western blotting confirmed over-expression of Bcl-2 in these transfected cells (Supplementary Figure S2A). To investigate the effect of Bcl-2 on cell proliferation and viability, U87 and LNZ308 cells were incubated with increasing concentrations of YM155 for 72 h. It is clear that YM155 has a dose-dependent effect, with IC₅₀ values of 26 ± 5.5 , 25 ± 4.9 , and 29 ± 5.2 nM for mock, empty vector and Bcl-2 transfected LNZ308 cell line (Supplementary Figure S2B, upper panel). A similar result was observed for U87 cell line (Supplementary Figure S1B, lower panel). Annexin V and propidium iodide analysis revealed that treatment with YM155 (100 nM for 24 h) resulted in 34%, 39%, and 32% cell death in mock transfected, vector transfected or Bcl-2 transfected LNZ308 cell line (Supplementary Figure S2C, upper panel). A similar result was observed in U87 cell line (Supplementary Figure S2C, lower panel) cell lines. These results suggest that high levels of Bcl-2 expression did not provide any protection to YM155 in glioma cells.

To examine the role of Bcl-2 silencing in YM155-induced apoptosis, an RNA interference approach was employed to knockdown Bcl-2 in LNZ308 and U87 cells. Twenty-four hours after transfection, the knockdown efficacy of Bcl-2 shRNAs was determined by Western blot analysis. As shown in Supplementary Figure S3A, Bcl-2 shRNAs suppressed the expression of Bcl-2 in both LNZ308 and U87 cells transfected with Bcl-2-specific shRNA compared with non-target vector control. Cell proliferation assay and cell viability assay was performed. There was a concentration dependent inhibition of YM155-induced cell proliferation (Supplementary Figure S3B) and induction of apoptosis was observed in both U87 and LNZ308 cell lines (Supplementary Figure S3C). However, our results also demonstrate that there was no significant change between vector transfected and Bcl-2 shRNA transfected U87 or LNZ308 cells, suggesting Bcl-xL but not Bcl-2 can directly confer resistance to YM155-induced apoptosis in glioma cells.

Bcl-xL Depletion Induces Loss of Mitochondrial Membrane Potential, Release of Cytochrome c, and Enhances Bax Conformational Changes During YM155-Induced Apoptosis

In our previous studies we have shown that Bcl-2 family proteins are key regulators of the mitochondrial apoptotic pathway, and changes in mitochondrial membrane potential (ψ_m) are thought to represent an early event in the induction of apoptosis [13,16,17]. Because Bcl-

xL is predominantly located in the mitochondria, we examined the effect of Bcl-xL knockdown on the mitochondrial apoptotic events triggered by YM155. YM155 caused a minimal or no change of mitochondrial membrane potential in LNZ308 and U87 cells stably expressing non-target shRNA vector (Figure 3A and B, left panels). On the other hand, YM155 caused a significant change in the reduction of mitochondrial membrane potential in a concentration dependent manner (i.e., appearance of a population to the left suggesting the loss of mitochondrial membrane potential, ψ_m) in Bcl-xL silenced cells (Figure 3A and B, right panels). Quantitative analysis of multiple experiments revealed that as low as 25–50 nmol/L resulted in 35–70% loss of mitochondrial membrane potential respectively in Bcl-xL silenced LNZ308 cells (bottom panel of Figure 3A). A similar result was observed in U87 cells (bottom panel of Figure 3B). Furthermore, Bcl-xL silenced cells enhanced YM155-induced cytochrome c, smac/DIABLO, and apoptosis-inducing factor (AIF) release into the cytosol from mitochondria (Figure 3C).

Generally, the activation of Bax is inferred by its translocation from cytosol to mitochondria. To confirm YM155-induced mitochondrial translocation of Bax, control (DMSO), and YM155-treated cells were fixed and immunostained for active Bax by using a conformation-specific antibody (6A7, anti-Bax monoclonal antibody) that selectively recognizes only the proapoptotic form of Bax, and mitochondria were stained with MitoTracker Red. As shown in Figure 3D, YM155 induced no “active” Bax immunofluorescence, shown by the green signal in vector expressing cells. However, upon YM155 treatment, “active” Bax (green signal, cells stained with 6A7 anti-active Bax antibody) was clearly identified in Bcl-xL silenced cells. Image overlay indicates the presence of conformationally changed Bax co-localized with mitochondria (Figure 3E, lower panel).

Next, to validate our previous observation that combining YM155 with pharmacological inhibition of Bcl-2/Bcl-xL (ABT737) induces Bax conformational changes [16], we examined the Bcl-xL genetically silenced cell line in conjunction with YM155 treatment. Cell lysates were immunoprecipitated with anti-Bax 6A7 antibody, which recognizes the conformationally active form of Bax, and were analyzed by immunoblotting using a non-conformation-dependent Bax antibody. As shown in Figure 3F, the active form of Bax was readily detected in Bcl-xL silenced cells treated with YM155 for 6 h and also to a much lower extent in vector control cells (stable cell line generated from non-target shRNA) treated with YM155 for 24 h.

YM155 Initiates Autophagy by Suppressing the PI3K/Akt/mTOR Axis in Bcl-xL Silenced Cells

Although autophagy is primarily a protective process for the cell, many investigators have shown that YM155 treatment induced autophagy and promoted apoptosis instead of protecting against cell death [25–27]. Because Bcl-xL plays an important role in the crosstalk between autophagy and apoptosis [28], we wanted to understand the role of Bcl-xL/YM155 interplay in glioma. Western blot analysis with an antibody to Ser 473 p-Akt, but not total AKT, showed a marked decrease in response to YM155 treatment in the Bcl-xL silenced cell line (Figure 4A). To test whether Akt inhibition by YM155 had functional consequences, we examined the phosphorylation status of mTORC1 at Ser2448 and ULK1

at Ser757. As shown in Figure 4A, upon treatment with YM155, the phosphorylation of mTORC1 at Ser2448 was efficiently dephosphorylated. Phosphorylation of ULK1 (at Ser757), which inhibits ULK1 function, was decreased. Furthermore, YM155 treatment downregulated the expression of SQSTM1, a 62 KD protein degraded only by autophagy, in Bcl-xL silenced U87 and LN2308 cells but not in the vector carrying control group. However, the expression of Beclin-1 (autophagy marker) was found to be significantly reduced in the Bcl-xL silenced cell line (Figure 4A). Densitometric analysis showed that there was a 47%, 80%, and 75% decline in the ratio of pmTOR, phospho-ULK1 and Beclin-1 respectively in Bcl-xL silenced LN2308 cells when treated with YM155 at 25 nmol/L for 24 h. Similar results were obtained for Bcl-xL silenced U87 cells (Supplementary Figure S4).

Because Bcl-xL and Beclin-1 proteins are key regulators of cell proliferation, autophagy and apoptosis [29], we thought that Bcl-xL silencing may have a direct role in initiating autophagy and cell death. Western blot analysis revealed that YM155 increased the conversion of LC3I to LC3II in a concentration dependent manner (Figure 4A). Similarly, cells immunostained with LC3B antibody to identify the accumulation of autophagosomes, double-membrane structures with cytoplasmic materials subsequently transported to be degraded, clearly revealed YM155-induced LC3 puncta in a concentration dependent manner in Bcl-xL silenced cells, suggesting the presence of more mature autophagosomes. In contrast, LC3B puncta could hardly be found in the YM155-treated non-target shRNA transduced stable cell line, suggesting that the induction of autophagy was concomitant with Bcl-xL silencing (Figure 4B). Then to examine whether autophagy was directly involved in the induction of apoptosis or protects against cell death, cells were pretreated with two different autophagy inhibitors (3-methyladenine, 3-MA or bafilomycin A1, BafA1) alone or with YM155, and observed for cell death. The annexin V/propidium iodide analysis revealed that YM155 reduced cell viability of Bcl-xL silenced LN2308 and U87 cells as expected. 3-MA or BafA1 alone caused minimal or no apoptosis. However, 3-MA and BafA1 partially but significantly inhibited YM155-induced cell death (Figure 4C), indicating that autophagic cell death may contribute in part to YM155-induced cell death in Bcl-xL silenced cells.

PTEN gene is mutated or deleted in 30–40% of gliomas [30]. As shown in Supplementary Figure S1, phosphorylated AKT was expressed at much higher levels in LN444, U373, A172, U87, LN2308, GBM-2, GBM-3, and GBM-4 cells. PTEN expression was identified in human astrocytes, LN428, T98G, LN18, and LN229 and all primary glioma cell lines but not detected in other cell lines. Because inhibition of some targets in PI3K/AKT pathway are critical to autophagy and cell viability, we hypothesized that inhibiting the PI3K pathway by enforced overexpression of PTEN (in a PTEN deleted cell line) would improve the effectiveness of YM155. We used PTEN deleted LN2308 cell line [31]. Overexpression of PTEN efficiently led to the dephosphorylation of Akt/PKB kinase, a downstream target that is dephosphorylated and inactivated by PTEN [32]. There was no change in total Akt and β -actin protein level (Figure 4D, left panel). Treatment of cells with YM155 resulted in reduction of survivin in Ad-CMV (compare lanes 1 vs. 2; Figure 4D, left panel) or Ad-PTEN (compare lanes 3 vs. 4; Figure 4D, left panel) infected cells. LC3-II expression/conversion (autophagosomal marker) increased in the YM155-treated groups, whereas ectopic expression of Ad-PTEN further potentiated the effect (compare lanes 2 vs. 4; Figure

4D, left panel). Forty-eight hours after infection, apoptosis was determined by FACS analysis. As expected, treatment with YM155 in Bcl-xL silenced cells infected with Ad-CMV resulted in an increase in apoptosis, whereas overexpression of PTEN further potentiated the susceptibility of cells to YM155 (Figure 4D, right panel), suggesting that Akt signaling plays an important role in YM155-induced cytotoxicity. To investigate whether the increased cell death with PTEN overexpression was due to increased autophagy, cells were pretreated with 3-MA and cell viability was assessed. As shown in Figure 4D, pretreatment of cells with 3-MA effectively protected cells (Figure 4D, right panel). Together, these data show that modulation of AKT signaling pathway could play a pivotal role in apoptotic susceptibility to YM155 in Bcl-xL silenced cells.

YM155 Modulates DNA Damage Response Genes in Bcl-xL Silenced Cells

Because RAD51 (Figure 5A and Supplementary Figure S5) inhibition generates DNA double-strand breaks that accumulate and trigger apoptosis [33–35], we questioned whether the increase in YM155-induced apoptosis in Bcl-xL silenced cells could be due to an increase in DNA double-strand breaks. To test this hypothesis, we examined whether phospho- γ H2AX, a surrogate marker for double-strand DNA breaks and Rad51 (marker for homologous DNA recombination and repair) protein levels correlate with double-strand DNA damage and repair, respectively. Our results demonstrated that incubation with YM155 increased the fluorescence intensity of γ -H2AX immunostaining in a concentration dependent manner. The increase in γ -H2AX immunostaining and increased foci induced by YM155 were significantly higher and remained elevated throughout the experimental period in the Bcl-xL-silenced cells when compared with the stable cells generated with non-target shRNA (Figure 5B and Supplementary Figure S6). Because Rad51 forms discrete foci (functional complexes at DNA lesions), which can be visualized in cells with DSBs [36], we used an immunocytofluorescence assay to evaluate the effect of YM155 on focus formation. Abundant Rad51 nuclear foci (detected in 100% of cells) were present in the DMSO treated (vehicle control) cells. Interestingly, upon YM155 treatment, Rad51 nuclear foci were not detectable in Bcl-xL-silenced cells when compared with the stable cells expressing non-target shRNA (Figure 5C). Quantitative analysis clearly showed that the percentage of cells that focally concentrated at the nuclei decreased after YM155 treatment (Supplementary Figure S7).

DISCUSSION

Resistance to chemotherapeutics and molecularly targeted agents is a major problem facing current cancer research. Activation of prosurvival signaling and/or the inactivation of proapoptotic signaling pathways can lead to drug resistance. A diverse range of molecular mechanisms have been implicated in drug resistance; among these, Bcl-xL has an important role in the control of cell death through its inhibition of apoptosis [37,38]. Because genetic knockdown effectively silences a target gene by the selective inactivation of its corresponding mRNA (and hence the protein) from the cell, whereas pharmacological inhibition only blocks the function of a protein and has a propensity to cross react with multiple targets, we used shRNA to knock down Bcl-xL and generated stable glioma cell lines. A non-target shRNA-mediated stable cell line served as a control. First, we screened a

panel of clinical and experimental cancer therapeutic agents, and observed that suppression of Bcl-xL significantly enhanced YM155-induced cell death through the induction of apoptosis. Importantly, silencing Bcl-xL did not affect the endogenous levels of Bcl-2 and IAP family proteins. YM155 inhibits survivin expression at a low nanomolar concentration in both Bcl-xL silenced and vector control groups of cells. However, in this study, we found an increase in the annexin V and propidium iodide positive cell populations and the activation of the caspase cascade in YM155-treated Bcl-xL silenced cells but not in the vector carrying control group.

YM155 potently inhibits cell growth and induces apoptosis in a wide variety of human cancer cell lines and xenograft models [39–42]. While YM155 does suppress survivin levels, we and others have shown that the potent anti-tumor activity seems to be mediated by the activation of the mitochondrial membrane dysfunction, ER stress response, abrogation of STAT3 signaling, inhibition of Akt, and Mcl-1 expression [16,43–47]. Recently, Wagner et al [45] have shown that the apoptotic cell death induced by YM155 was rescued by ectopic expression of Mcl-1 but not survivin, suggesting that Mcl-1 as the pivotal downstream target of YM155. In this paper, we have shown for the first time that Bcl-xL is a crucial mediator of apoptosis sensitivity in response to YM155. We have also demonstrated that enforced overexpression or silencing Bcl-2 does not influence YM155-induced cell death in glioma cell lines.

Because BCL-2 family proteins are important regulators of the intrinsic mitochondrial pathway of apoptosis, we evaluated whether YM155 affects mitochondrial outer membrane permeability in the Bcl-xL silenced cells. Treatment with YM155 resulted in the loss of mitochondrial membrane potential, release of cytochrome c, smac/DIABLO and apoptosis-inducing factor (AIF). In Bcl-xL silenced cells, upon YM155 treatment, we observed that “active Bax” localized to the mitochondria around the time of cytochrome c release. This effect was not seen in the non-target shRNA cell line. The signals responsible for this change in Bax conformation and distribution are not known. Nonetheless, once inserted into mitochondria, Bax likely triggers cell death by permeabilization of the outer mitochondrial membrane, thereby releasing cytochrome c into the cytosol resulting in the activation of caspases and PARP [48]. The appearance of the active subunit of caspases and PARP, corresponding with the decrease of its precursor form was detected only in Bcl-xL silenced cells treated with YM155, suggesting that activation of caspases and PARP are crucial components of cell death pathways.

Recently, Wang et al. [26] have demonstrated that YM155 could inhibit adenoid cystic carcinoma cell proliferation and induce apoptosis by promoting autophagy in vitro as well as in vivo. In agreement with recent observations [25–27], our study demonstrated that treatment with YM155 displayed morphologic and functional features of autophagy and apoptosis. The molecular mechanisms of apoptosis and autophagy are quite different and the regulatory molecules involved in the control of autophagy and apoptosis are varied [49]. For example, TRAIL [50], histone deacetylase inhibitors [51], tyrosine kinase inhibitors [52], and proteasome inhibitors [53] can induce autophagy and apoptosis, despite their different mechanisms of action. Much evidence suggests that autophagy protects cells from energy starvation, genotoxic and metabolic stress. On the other hand, excessive autophagy

can reduce cell survival by promoting autophagic cell death. The Bcl-2 protein family proteins play a dual role in the control of apoptosis and autophagy [54]. Because Beclin-1 colocalizes with Bcl-xL within mitochondria via its BH3 domain and has been proposed to coordinate both apoptosis and autophagy through direct interaction with anti-apoptotic family members Bcl-2 and/or Bcl-xL [55], we examined the effect of YM155 in both Bcl-xL silenced and control cells. Recently, Zhang et al [56] have shown that YM155 treatment (and/or survivin inhibition by siRNA) significantly enhanced autophagy by upregulating Beclin-1. In contrast to what was seen by Zhang et al. [56], we found Beclin-1 was significantly reduced in Bcl-xL silenced cells after YM155 treatment. Because Beclin-1 is a caspase substrate [57], the reduction may be due to the increased level of caspase activation (in Bcl-xL cells after YM155 treatment) that could lead to Beclin-1 cleavage. Because Bcl-xL is an endogenous binding partner for Beclin-1 [55], we cannot exclude additional factors influencing Beclin-1 expression under these conditions (i.e., in our model, we have silenced Bcl-xL, a binding partner of Beclin-1). p62/SQSTM1, a multifunctional adapter protein that has been implicated in autophagic processes was significantly reduced following the treatment with YM155. Our results also demonstrate that YM155 treatment resulted in the reduced phosphorylation of AKT, mTOR and Ulk-1. Our observation of enhanced cell death in PTEN overexpressed cells supports the role of Akt signaling in YM155-induced cell death. This effect was significantly potentiated in Bcl-xL silenced cells. Furthermore, pretreating cells with autophagy inhibitors (3-MA and BafA1) protected Bcl-xL silenced cells, suggesting that an increase in autophagic flux induced by YM155 might contribute to the enhanced cell death observed in Bcl-xL silenced cells.

Increased expression of Rad51 is associated with a wide range of human tumors. Welsh et al. [58] have shown that Rad51 protein was elevated in 50% and 70% of primary and recurrent GBM tumor patients respectively. Our earlier studies revealed that inhibition of HDAC enzymes by vorinostat led to a significant reduction in RAD51 expression and potentiated bortezomib-induced cytotoxicity [18]. Similarly, targeting Rad51 increased glioma cells' sensitivity to temozolomide [58]. Here, we demonstrated that, exposure to YM155 caused a significant reduction of nuclear foci in Bcl-xL silenced cells, indicating that the Bcl-xL—Rad51 axis might play a critical role in YM155-induced cell death.

In summary, we have shown that silencing Bcl-xL enhances YM155-induced cytotoxicity. These results raise the possibility of “multiple hits,” one directly on the mitochondria to trigger apoptosis and the other in the cell nucleus, resulting in DNA damage, and DNA repair. We speculate that the principle site of action of apoptosis regulation by YM155 is probably the mitochondrial membrane because Bcl-xL mainly resides in mitochondria, protecting against mitochondrial membrane leakiness [59]. Our results demonstrate that YM155 activates the intrinsic (mitochondria-dependent) apoptotic pathway in Bcl-xL silenced cells. This is based on several pieces of evidence: (i) loss of mitochondrial membrane potential; (ii) induction of cytochrome c release; (iii) activation of Bax, which influences the potentiation of apoptosis signaling [48]. Silencing Bcl-xL also (i) reduced phospho-Akt/phospho-mTOR expression levels; and (ii) increased conversion of autophagic vesicles (autophagosomes) to trigger autophagy, lead to eventual cell death. An additional mechanism for the augmentation of apoptosis may be due to abrogation of DNA double-strand break repair mediated by Rad51 repression. The fact that YM155 markedly enhanced

both the accumulation of γ H2AX and loss of RAD51 in Bcl-xL silenced cells may in part explain the dramatic sensitizing effect. As a sensitive oxidative DNA damage marker, the striking increases of H2AX phosphorylation that were observed in Bcl-xL silenced cells after YM155 treatment imply the promotion of DNA double-strand break formation, which may play an important role in the enhancement in DNA damage and apoptosis.

Finally, it is important to mention that we did not observe a clear annexin V/PI positive population in Bcl-xL silenced cells treated with other classes of inhibitors. Several studies have provided compelling evidence of the importance of overexpression of anti-apoptotic proteins in regulating apoptotic resistance following chemotherapy or radiation-therapy. A possible reason for the failure may be due to the inability of the drug to target the complete repertoire of anti-apoptotic proteins or the activation of multiple compensatory pathways by glioma cells that may have built-in redundancies, making the inhibition of any single pathway ineffective.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AIF	apoptosis-inducing factor
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
DMSO	dimethyl sulfoxide
EGFR	epidermal growth factor receptor
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
IAP	inhibitor of apoptosis protein
PI3K	phosphatidylinositol 3-Kinase
PBS	phosphate-buffered saline
PTEN	phosphatase and tensin homolog deleted from chromosome 10
PAGE	polyacrylamide gel electrophoresis
PDGFR	platelet-derived growth factor receptor

TBS	Tris-buffered saline
PARP	poly ADP-ribose polymerase
PI	propidium iodide
PTEN	phosphatase and tensin homolog on chromosome 10
TBS	Tris-buffered saline
VEGFR	vascular endothelial growth factor receptor

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Figure 1.

(A) LNZ308 and U87 Bcl-xL or non-target shRNA expressing stable cell lines were generated as described in the Methods. Cell extracts were separated by SDS-PAGE and subjected to Western blotting analysis with Bcl-xL antibody. β -actin served as loading control. Bcl-xL silenced or non-target shRNA vector containing LNZ308 cells (B) or U87 (C) were seeded at 60% confluence, allowed attachment overnight, and treated with the indicated signaling inhibitor for 24 h. Control cells received an equivalent amount of DMSO. Apoptosis was analyzed by flow cytometry as described in the Methods. The result represents the mean of three independent experiments (** $P < 0.005$ values considered statistically significant).

Figure 2. Non-target shRNA or Bcl-xL silenced LNZ308 or U87 cells were (A) treated with YM155, indicated concentrations for 24 h; (B) treated with 25 nM YM155 for indicated duration, h. Control cells received equivalent concentrations of vehicle, DMSO (O). Cell extracts were separated by SDS-PAGE and subjected to Western blotting analysis with the indicated antibodies. β -actin served as the loading control. (C) LNZ308 and LNZ308-Bcl-xL silenced or U87 or U87-Bcl-xL silenced cells were treated with YM155 (25 nM) for the indicated duration. Western blot analysis was performed with the indicated antibodies. β -actin served as loading control. (D) Logarithmically growing cells were treated with YM155 (nM) for 24 h. Apoptosis was analyzed by flow cytometry as described in the Materials and Methods. The results represents the mean of three independent experiments. Non-target shRNA or Bcl-xL silenced LNZ308 or U87 cells were (F) treated with YM155 indicated concentrations for 24 h; (G) treated with 25 nM YM155 for indicated duration, (h). Equal

amounts of protein were separated by SDS-PAGE and subjected to Western blotting analysis with the indicated antibodies. β -actin served as the loading control (CF, cleaved fragment; FL, full length).

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Figure 3.

LNZ308 and U87 (non-target shRNA or Bcl-xL shRNA) cells were treated with the indicated concentrations of YM155 for 18 h. DMSO served as vehicle control (0). The integrity of the mitochondrial membranes of the cells was examined by DiOC6 staining and flow cytometry. Decrease in fluorescence intensity reflected loss of Ψ_m . Data represent mean $\pm$ SD of three independent experiments carried out in triplicate. A representative FACS plot (A, LNZ308; B, U87) and a histogram obtained from three independent experiments were shown in the bottom panel (** $P < 0.001$, compared with vehicle control). (C) Logarithmically growing LNZ308 (non-target shRNA or Bcl-xL shRNA) cells were treated with YM155 (nM) for 24 h. Cytosolic extract was prepared after 12 h of treatment, and equal amounts of protein were separated by SDS-PAGE and subjected to Western blotting analysis with the indicated antibodies. (D and E) Non-target shRNA or Bcl-xL silenced LNZ308 cells were grown on chamber slides in growth medium, and, after an overnight

attachment period, were exposed to YM155 (25 nM) or vehicle (DMSO) for 12 h. To label mitochondria, cells were incubated with Mitotracker red for 30 min. Then cells were washed once with PBS, fixed and permeabilized as described in the Materials and Methods. Cells were then incubated with anti-Bax antibody (6A7 monoclonal antibody at 1:100 dilutions, catalog number 556467, BD Pharmingen) overnight at 4°C. After PBS wash, the slides were incubated with secondary antibody (anti-mouse IgG-alexa 488, 1:200) and Hoechst 33342 (Invitrogen) for 2 h at room temperature, then washed in PBS, mounted, and examined under a fluorescent microscope. Changes in response to inhibitor treatment were evaluated by microscopic (EVOS, Thermo Fisher Scientific) inspection. (F) LNZ308 cells (stable cells generated from Non-target shRNA or Bcl-xL shRNA) were treated with YM155 at the indicated concentration for the indicated duration and lysed with 1% CHAPS buffer. An equal amount of protein was immunoprecipitated (IP) with monoclonal anti-Bax (6A7; Sigma–Aldrich) antibody and then immunoblotted with polyclonal anti-Bax antibody (Cell Signaling Technology). The results of a representative study are shown; three additional experiments produced similar results.

Figure 4.

(A) LNZ308 and U87 (non-target shRNA or Bcl-xL silenced) cells were treated with the indicated concentrations of YM155 for 24 h. DMSO served as vehicle control (0). Whole cell extract was prepared, and equal amounts of protein were separated by SDS-PAGE and subjected to Western blotting analysis with the indicated antibodies. (B) LNZ308 (non-target shRNA or Bcl-xL silenced) cells were grown on chamber slides in growth medium, and, after an overnight attachment period, were exposed to YM155 (indicated concentration) or vehicle (DMSO) for 24 h. Cells were fixed, washed and permeabilized as described in the Materials and Methods. Then cells were incubated with LC3B (polyclonal antibody at 1:100 dilutions, catalog number 3868, Cell Signaling Technology) overnight at 4°C. After PBS wash, the slides were incubated with secondary antibody (anti-rabbit IgG-alexa 488, 1:200) and Hoechst 33342 (Invitrogen) for 2 h at room temperature. (C) Bcl-xL silenced LNZ308 or U87 cells were incubated with 3-MA (1.0 mM) or bafilomycin A1 (BafA1, 50 nM) in the

presence or absence of YM155 (25nM) for 24 h. Apoptosis was analyzed by flow cytometry. Bar chart representing three independent experiments are shown (* $P < 0.05$; ** $P < 0.005$, values considered statistically significant). (D) Logarithmically growing LNZ308-Bcl-xL silenced stable cells were infected with Ad-CMV- or Ad-PTEN at 50 multiplicity of infection. Forty-eight hours after infection, cells were incubated in the presence of YM155 (25 nM) or DMSO (vehicle control) for 20 h. Cells were lysed and equal amount of protein was separated by SDS-PAGE and subjected to Western blotting analysis with the indicated antibodies (left panel). In parallel, cells were pretreated with 3-MA (1.0 mM) for 2 h, followed by YM155 (25 nM) for 20 h. At the end of the incubation period, the viable cell numbers were determined by flow cytometric analysis. Histogram (right panel) represents the mean number of apoptotic cells acquired from three independent experiments. The values represent the mean $\pm$ S.D. ** $P < 0.005$ values considered statistically significant.

Figure 5.

LNZ308 and U87 (non-target shRNA or Bcl-xL silenced) cells were treated with the indicated concentrations of YM155 for 24 h. DMSO served as vehicle control (0). Whole cell extract was prepared, and equal amounts of protein were separated by SDS-PAGE and subjected to Western blotting analysis with the indicated antibodies. Total H2AX served as loading control (A). (B and C) LNZ308 (non-target shRNA or Bcl-xL silenced) cells were grown on chamber slides in growth medium, and, after an overnight attachment period, were exposed to YM155 or for 6 h (B) or 24 h (C). Control cells received vehicle, DMSO. Cells were fixed, washed and permeabilized as described in the Materials and Methods. Then cells were incubated with γ -H2AX (polyclonal antibody at 1:100 dilutions, catalog number 7631, Cell Signaling Technology, (B) or RAD51 (polyclonal antibody at 1:100 dilutions, catalog number 63801, Abcam, (C) overnight at 4°C. After PBS wash, the slides were incubated with secondary antibody (anti-rabbit IgG-alexa 488, 1:200) and Hoechst 33342 (Invitrogen) for 2 h at room temperature.