Identification of Chemosensitivity Nodes for Vinblastine through Small Interfering RNA High-Throughput Screens

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ABSTRACT

Discovering chemosensitivity pathways or nodes is an attractive strategy for formulating new drug combinations for cancer. Microtubules are among the most successful anticancer drug targets. Therefore, we implemented a small interfering RNA (siRNA) synthetic lethal screen targeting 5520 unique druggable genes to identify novel chemosensitivity nodes for vinblastine, a microtubule-destabilizing agent used clinically. We transiently transfected human glioblastoma cells with siRNAs for 48 h and then treated cells with a sublethal concentration of vinblastine. Forty-eight hours later, we analyzed cell viability and, using a series of statistical methods, identified 65 gene products that, when suppressed, sensitized glioblastoma cells to vinblastine. After completion of the secondary assays, we focused on one siRNA, B-cell lymphoma extra large (BCL-xL), because of its role in the intrinsic apoptosis signaling pathway as well as the availability of pharmacological inhibitors. We found that nontoxic concentrations of 4-[4-[[(2-[4-chlorophenyl]-5,5-dimethylcyclohexen-1-yl)[methyl]piperazin-1-yl]-N-[4-[[2R]-4-morpholin-4-yl-1-phenylsulfonylbutan-2-yl]amino]-3-[trifluoromethylsulfonyl]phenyl)sulfonylbenzamide (ABT-263), an inhibitor of the BCL-2 family members (BCL-2, BCL-xL, and BCL-w), sensitized glioblastoma and non–small-cell lung cancer cells to vinblastine and induced apoptosis through the intrinsic cell death pathway. These results illustrate the usefulness of unbiased siRNA screens as a method for identifying potential novel anticancer therapeutic combinations.

Introduction

Cancer cells are highly dependent on microtubule dynamics, making microtubules an excellent target for anticancer treatment (Jordan and Wilson, 2004). To date, microtubule-destabilizing agents are among the most successful anticancer therapies, with multiple microtubule-destabilizing agents in clinical use, including the vinca alkaloids, which are used for the treatment of Hodgkin’s lymphoma, non-Hodgkin’s lymphoma, non–small-cell lung cancer, breast cancer, and glioblastoma multiforme (GBM) (Jordan and Wilson, 2004; Dumontet and Jordan, 2010). Despite the general success of these vinca alkaloids, resistance remains a serious clinical problem in a variety of cancers, including GBM (Feun et al., 1994; Ross et al., 1999; Kavallaris et al., 2001). Therefore, there is a need for the development of novel combination chemotherapeutics to help decrease the clinical resistance associated with microtubule-destabilizing agents (Jordan and Wilson, 2004). Identifying chemosensitivity pathways, or nodes, provides an attractive strategy for enhancing the therapeutic potential of established anticancer agents.

GBM is the most common and aggressive form of glioma; a majority of patients die within a year of being diagnosed (Adamson et al., 2009). These primary brain tumors arise from normal human astrocytes and are especially difficult to treat due to their location, aggressive biological behavior, infiltrating growth, and resistance to current anticancer therapies (Eramo et al., 2006). Although the addition of chemotherapy to resection and radiotherapy has increased the 2-year survival rate from 1 to 20%, a successful treatment...

ABBREVIATIONS: GBM, glioblastoma multiforme; ABT-263, 4-[4-[[(2-[4-chlorophenyl]-5,5-dimethylcyclohexen-1-yl)[methyl]piperazin-1-yl]-N-[4-[[2R]-4-morpholin-4-yl-1-phenylsulfonylbutan-2-yl]amino]-3-[trifluoromethylsulfonyl]phenyl)sulfonylbenzamide; BCL-2, B-cell lymphoma 2; BCL-xL, B-cell lymphoma extra large; DMSO, dimethyl sulfoxide; FDR, false discovery rate; MAD, median absolute deviation; HTS, high-throughput screening; PBS, phosphate-buffered saline; RFU, relative fluorescent unit; siRNA, small interfering RNA; SCR, scrambled; VBL, vinblastine.

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much less a cure, for GBM remains elusive. Patients receiving chemotherapeutic agents, such as vinca alkaloids, still have poor survival rates indicative of a tumor cell population that is resistant to current therapies (Parney and Chang, 2003; Stupp et al., 2005). Thus, there is a desperate need for treatments that can specifically target these chemotherapy-resistant GBM cells and potentially increase the survival rate of what is currently a devastating disease.

Small interfering (siRNA) technology has been used to identify genes that are essential for cancer cell survival (Kaelin, 2005; Iorns et al., 2007). In addition, siRNA synthetic lethal approaches can be used to uncover proteins that suppress the growth inhibitory and cytotoxic effects of anticancer agents (Kaelin, 2005; Whitehurst et al., 2007). Druggable genome siRNA libraries comprising siRNAs against gene products that are theoretical targets for drug development have the potential to streamline the identification of small molecule inhibitors. Because approximately two thirds of the proteins encoded in the human genome are thought to be highly problematic as drug targets, specifically focusing on the druggable genome can eliminate a significant subset of nontherapeutic genes (Hopkins and Groom, 2002).

To help identify novel treatment combinations for GBM, we implemented a siRNA high-throughput screen targeting the druggable genome to identify gene products that sensitized human T98G GBM cells to vinblastine (VBL). In this study, we used a series of statistical methods to identify 65 unique gene products that were toxic to cancer cells in combination with VBL. We found that reduction of the prosurvival protein B-cell lymphoma extra large (BCL-xL) by siRNA sensitized T98G cells to VBL. A sublethal concentration of the B-cell lymphoma 2 family member inhibitor 4-[4-[2-(4-chlorophenyl)-5,5-dimethylcyclohexen-1-yl]methyl]piperazin-1-yl]-N-4-[[2(R)-4-morpholin-4-yl-1-phenylsulfonylbutan-2-yl][aminol]-3-(trifluoromethylsulfonyl)phenyl]sulfonylbenzamide (ABT-263) (Tse et al., 2008) mimicked the results seen with BCL-xL siRNA in both human T98G GBM and A549 non–small-cell lung cancer cells. Our study illustrates how siRNA high-throughput screening (HTS) technology can be an efficient, unbiased method to identify novel combination anticancer treatments and thereby potentially increase the efficacy of established anticancer drugs such as VBL.

Materials and Methods

Reagents. The Silencer Druggable Genome siRNA Library (version 1.1), Silencer Select secondary library, Silencer Select Negative Control 1, and BCL-xL Silencer Select siRNAs (siRNA identification s1920, s1921, and s1922) were purchased from Ambion (Austin, TX). AllStars He Cell Death Control siRNA was purchased from QIAGEN (Valencia, CA). DharmaFECT 2 transfection reagent, 5x siRNA re-suspension buffers, and the SiGENOME Non-Targeting siRNA #1 were purchased from Dharmacon RNA Technologies (Lafayette, CO). Tissue culture-treated 384-well microtiter plates were from Greiner Bio-One (Freckenhausen, Germany). CellTiter-Blue Cell Viability Assay and Caspase-Glo 3/7 Assay were purchased from Promega (Madison, WI). BD BioCoat Collagen I 384-well microplates and BD Falcon 384-well white/clear bottom plates were purchased from BD Biosciences (San Jose, CA). Eagle’s minimum essential medium, Opti-MEM, Eagle’s basal medium, phosphate-buffered saline (PBS), l-glutamine, penicillin/streptomycin, Hoescht 33342, and 1.0-mm Novek 4x to 20% Tri-glycine gels were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Cellgro (Manassas, VA). ECL Western blotting substrate was from Thermo Fisher Scientific (Waltham, MA). The T98G, A549, U87, U3T3, LN2, MDA-MB-231, and HeLa cell lines and human astrocytes were purchased from the American Type Culture Collection (ATCC; Manassas, VA). VBL and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). ABT-263 was obtained from ChemieTek (Indianapolis, IN). Glyceraldehyde-3-phosphate dehydrogenase rabbit monoclonal, BCL-xL rabbit polyclonal, and BAX rabbit monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA). BCL-xL rabbit monoclonal (E18), cytochrome c mouse monoclonal, and ERAB [5F3] mitochondrial marker mouse monoclonal were purchased from Abcam Inc. (Cambridge, MA). Rabbit and mouse peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Mini EDTA-free Protease Inhibitor Cocktail Tablets were purchased from Roche (Nutley, NJ).

Cell Culture and Compounds. T98G glioblastoma cells were maintained in Eagle’s minimum essential medium supplemented with Earle’s basic salt solution, nonessential amino acids, sodium pyruvate, 1% L-glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum. The non–small-cell lung cancer cell line A549 was maintained in Eagle’s basal medium supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum. Cells were grown in a humidified incubator at 37°C with 5% CO2. T98G and A549 cell lines were validated during the study by the Research Animal Diagnostic Laboratory (University of Missouri at Columbia, Columbia, MO).

VBL and ABT-263 were dissolved in 100% DMSO. Compound treatments were added 48 h after initial cell seeding for both synthetic lethal screens and drug combination studies. Compound treatment and DMSO vehicle controls were diluted in medium to a final DMSO concentration of 0.5%. Cells were incubated for an additional 48 h in the presence of compounds or vehicle controls.

siRNA Synthetic Lethal Screen. T98G cells were wet reverse-transfected with the Silencer Druggable Genome siRNA Library at a final concentration of 20 nM per target in a one gene per well format as reported previously (Thaker et al., 2010). Each gene was targeted with three pooled siRNAs to ensure efficient protein suppression. The transfected cells were incubated for 48 h in a humidified incubator at 37°C with 5% CO2. After 48 h, the medium was removed and replaced with medium containing either 1.2 nM VBL or 0.5% DMSO vehicle control. Cells were incubated for an additional 48 h in the presence of compounds, and cell viability then was measured by incubating the cells with CellTiter-Blue Cell Viability Assay (1:5 ratio of CellTiter-Blue to medium) for 3 h, according to the manufacturer’s protocol. Plates were read on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA).

Data Analysis for the siRNA Synthetic Lethal Screen. The siRNA synthetic lethal screens were performed in three independent replicates to uncover sensitizers of T98G cells to VBL. Relative fluorescence units (RFU) from each targeted siRNA well were normalized to in-plated scrambled (SCR) negative control values treated with DMSO (DMSO/SCR siRNA), which allowed for plate-to-plate comparisons. The percentage cell viability was determined using the following equation:

\[
\% \text{Cell Viability} = 100 \left( \frac{x_{\text{RFU}}}{y_{\text{RFU}}} \right)
\]

where \(x_{\text{RFU}}\) is the RFUs for each sample and \(y_{\text{RFU}}\) is the average RFUs for the DMSO/SCR siRNA control within each plate.

We used median absolute deviation (MAD) analysis, an outlier detection method, which is resistant to the presence of outliers within the samples as reported previously (Thaker et al., 2010). Samples with a MAD score >3.5 were defined as outliers and were discarded. The viability of cells targeted by the siRNA was calculated by averaging the remaining values from the screen.

The samples were ranked according to their viability ratio, \(VR = \left( \frac{X_{\text{CV}}}{Y_{\text{CV}}} \right)\), where \(X_{\text{CV}}\) is the average cell viability of the siRNA plus
compound treatment and \( \bar{y}_{CV} \) is the average cell viability of the siRNA plus vehicle control. The targeted genes were ranked from lowest to highest according to their viability ratio, and the top 2.5% of genes (138 genes) were selected. Student’s \( t \) test was performed for these 138 targeted genes to determine the significant difference between cells treated with siRNA plus vehicle control and cells treated with siRNA plus VBL as described previously (Whitehurst et al., 2007). Genes with a \( p \) value \(< 0.01 \) were selected, yielding 65 siRNAs that sensitized T98G cells to VBL. The Benjamini-Hochberg false discovery rate (FDR) was used to decrease the possibility of false positives due to multiple comparisons (Benjamini and Hochberg, 2000). The FDR ranks the samples according to their \( p \) values, where the lower rank corresponds to less strict criteria (Devlin et al., 2003). By ranking the \( p \) values of the Student’s \( t \) test for each sample from smallest to largest, the FDR can be calculated using the following equation:

\[
FDR_k = \frac{k \alpha}{m}
\]

where \( k \) is the rank of the sample, \( \alpha \) is the confidence interval, and \( m \) is the total number of \( t \) tests performed. Each ordered \( p \) value \((p_k)\) is compared with the corresponding FDR, where any test with a \( p \) less than the FDR is declared significant.

### Western Blot Analyses and Lysate Preparation

Western blot analyses were performed as described previously (Thaker et al., 2010). Membranes were probed with antibodies at various dilutions targeting BCL-xL (1:500), \( \beta \)-tubulin (1:10,000), glyceraldehyde-3-phosphate dehydrogenase (1:1000), BAX (1:200), cytochrome c (1:200), and ERAB (1:200). Positive antibody reactions were visualized using either rabbit or mouse peroxidase-conjugated secondary antibodies (1:1000) and chemiluminescence by ECL Western Blotting Substrate (GE Healthcare, Piscataway, NJ) according to the manufacturer’s protocol. Membranes were imaged using a FujiFILM LAS-3000 imager (R&D Systems, Minneapolis, MN).

### Concentration-Response Curves

For VBL sensitization assays using BCL-xL siRNAs, cells were transfected in collagen-coated 384-well plates with increasing concentrations of BCL-xL siRNA (0.63–10 nM), as described in the siRNA screen. Forty-eight hours post-transfection, the cells were treated with VBL (25 pM to 50 nM) with a final DMSO concentration of 0.5%. The percentage cell viability of each sample was determined by CellTiter-Blue as described above. All of the samples were normalized to the DMSO/SCR siRNA negative control, which was termed 100% cell viability. Toxicity of siRNA alone was determined by comparing the DMSO/SCR per-

### Identification of Gene Products That Sensitize T98G Cells to VBL Using siRNA HTS Methodology

We performed siRNA HTS, as described previously (Thaker et al., 2010), to identify gene products that sensitize T98G GBM cells to VBL. Three pooled siRNAs were plated in 384-well plates in a one gene per well format with SCR and cell death controls on each plate, which allowed for plate-to-plate comparisons. We wet reverse-transfected T98G cells with three pooled siRNAs against each target in two identical plates and determined cell viability. We applied a series of statistical methods outlined in Supplemental Fig. 1 to determine the gene products that sensitized T98G cells to VBL. In brief, MAD analysis was performed to determine outliers with 95% confidence within the screen. After all of the outliers were removed, we determined the viability ratios of each gene product and averaged all three screens. The targeted genes were ranked according to their viability ratios, and the top 2.5% of gene products was selected (138 genes). We performed Student’s \( t \) test on the 138 genes, comparing the siRNA to siRNA plus VBL treatments, and determined the gene products that, when suppressed, sensitized T98G cells to VBL with 99% confidence \((p < 0.01)\). This process yielded 65 high-confidence gene products that sensitized cells to VBL (Fig. 1). To control for false positives due to multiple comparisons, we used the Benjamini-Hochberg FDR procedure, which decreased the probability of at least one false positive due to multiple comparisons when performing \( t \) tests on...
numerous samples (Benjamini and Hochberg, 1995). Applying an α of 0.02, we found that all 65 gene products identified by the viability ratios and Student’s t test were declared significant according to the FDR (Supplemental Table 1).

To confirm positive siRNAs from the primary HTS, we performed a secondary assay using two unique siRNAs rather than the pooled siRNAs from the primary high-throughput screen. We found 40 siRNAs targeting 29 different gene products that sensitized T98G cells to VBL (Table 1). For nine of the 29 gene products, both unique siRNAs sensitized cells (Table 1).

**BCL-xL siRNA Sensitizes T98G Cells to VBL at Cytostatic Concentrations.** Due to the availability and the biological relevance of inhibitors targeting the prosurvival protein BCL-xL (Tse et al., 2008; Kang and Reynolds, 2009), we focused further on BCL-xL-specific siRNAs to determine whether they could sensitize T98G cells to increasing concentrations of VBL as indicated by the primary and secondary assays (Fig. 2). We treated T98G cells with increasing concentrations of VBL in the presence and absence of decreasing concentrations of pooled BCL-xL siRNAs (sequences of siRNAs in Supplemental Table 2). We found that cells were sensitized to the higher VBL concentrations (3.13–25 nM) when treated in combination with nontoxic concentrations of BCL-xL siRNAs (0.63–10 nM; Fig. 2). We performed Western blot analyses to determine whether the sensitization of VBL to T98G cells was due to specific knockdown of BCL-xL protein. At both 48- (time of drug addition) and 96-h time points (end point of the assay), BCL-xL protein levels were undetectable at all of the concentrations tested relative to those of SCR siRNA controls (Fig. 3).

**ABT-263 Sensitizes T98G and A549 Cells to VBL.** To determine whether ABT-263, a prosurvival BCL-2 family inhibitor, sensitized cells to VBL, we treated T98G cells with increasing concentrations of VBL in the presence and absence of a nontoxic concentration of ABT-263 (Fig. 4A). As seen in Fig. 4B, the toxicity of VBL at concentrations ≥1.56 nM was increased by the addition of 1.56 μM ABT-263.

We compared expression levels of BCL-xL protein in T98G cells, other human glioblastoma cells (U87, U373 and LNZ 428), and other human cancer cells (MDA-MB-231 breast, HeLa cervical, and A549 lung cancer cells) to those of normal human astrocytes by Western blot analyses (Supplemental Fig. 2). We found that, relative to the normal human astrocytes, BCL-xL expression was lower in glioblastoma and lung cancer cells and higher in breast cancer cells. In addition, the expression levels of BCL-xL were lower in VBL-treated cells compared to untreated cells.

**TABLE 1**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>p Value a</th>
<th>p Value b</th>
<th>Gene Symbol</th>
<th>p Value a</th>
<th>p Value b</th>
<th>Gene Symbol</th>
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<td>NS</td>
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NS, not significant; siRNAs in the secondary assay with p > 0.05.

**Fig. 1.** Reduction in cell viability with the top 65 gene products from the primary siRNA HTS. Percentage cell viabilities were determined for the siRNA plus DMSO (white bars) and siRNA plus VBL (black bars), as described under Materials and Methods, for all of the genes in the druggable genome library. The top hits from the screen, according to the viability ratios and p values (65 genes), are listed on the abscissa. In most cases, the siRNAs were not toxic to the cells when used alone; however, upon the addition of a nontoxic concentration of VBL, the combination was toxic. Each value is the mean of three independent experiments. Bars, S.D.
cytes, BCL-xL protein was overexpressed in the majority of the cancer cell lines (MDA-MB-231, A549, T98G, U87, and U3T3). To determine whether BCL-xL sensitization of VBL was unique to T98G cells, we treated A549 non-small lung cancer cells with increasing concentrations of VBL in the presence and absence of nontoxic concentrations of ABT-263. We found that, in the presence of \( 12.5 \) nM ABT-263, A549 cells also were sensitized to VBL at concentrations \( \leq 3.13 \) nM (Fig. 4B).

It is notable that a 48-h exposure of either T98G or A549 cells to VBL alone did not reduce cell viability markedly below 50%, whereas the addition of ABT-263 greatly reduced cell viability (Fig. 4). Therefore, we performed growth inhibition studies using cell death siRNAs, which were known to induce >90% cell death in these cells (Thaker et al., 2010). By comparing the cell viability at the time of VBL addition and the 48-h end point of the assay, we could distinguish between no growth inhibition, which was defined as having the same cell viability as the negative control, and complete growth inhibition, which was defined as having the cell viability of the negative control at the time of plating. We defined cytotoxicity in terms of added loss of cell viability and normalized cell viability to that of the cell death control after subtracting the cytostatic effect. In all of the conditions tested (BCL-xL siRNA in T98G cells and ABT-263 in T98G and A549 cells), VBL induced a cytostatic effect at concentrations of \( \geq 1.56 \) nM (Supplemental Fig. 3). In the presence of BCL-xL siRNA in T98G cells, however, the loss of BCL-xL protein sensitized the cells to VBL, and we propose that the cytostatic concentrations became cytotoxic (Supplemental Fig. 3A). In the T98G and A549 cell lines, the addition of a nontoxic concentration of ABT-263 also sensitized the cells to VBL, inducing a cytotoxic effect at cytostatic concentrations of VBL alone (Supplemental Fig. 3, B and C, respectively).

**ABT-263 and VBL Induce Cell Death through Mitochondria-Dependent Apoptosis.** We examined caspase-3/7 activation to assess the possible role of mitochondria-dependent apoptosis (Lakhani et al., 2006) in the presence of nontoxic concentrations of ABT-263 and VBL. VBL induced a cytostatic effect at concentrations of \( \geq 1.56 \) nM (Supplemental Fig. 3). In the presence of BCL-xL siRNA in T98G cells, however, the loss of BCL-xL protein sensitized the cells to VBL, and we propose that the cytostatic concentrations became cytotoxic (Supplemental Fig. 3A). In the T98G and A549 cell lines, the addition of a nontoxic concentration of ABT-263 also sensitized the cells to VBL, inducing a cytotoxic effect at cytostatic concentrations of VBL alone (Supplemental Fig. 3, B and C, respectively).

**Fig. 2.** BCL-xL siRNA sensitization of T98G cells to VBL. Three siRNAs targeting BCL-xL were pooled and tested as sensitizers of T98G cells to VBL. A–E, cells were transfected with negative control siRNAs (SCR), positive control siRNAs (cell death), and BCL-xL siRNAs at decreasing concentrations: (A) 10 nM, (B) 5 nM, (C) 2.5 nM, (D) 1.25 nM, and (E) 0.63 nM. Forty-eight hours post-transfection, the cells were treated with increasing concentrations of VBL ranging from 25 pM to 25 nM. After 48 h, cell viability was determined by CellTiter-Blue fluorometric assay as described under Materials and Methods. All of the data points were normalized to a SCR/DMSO control to allow for well-to-well comparisons. The effect of different concentrations of VBL on cell viability in cells treated with SCR or BCL-xL siRNA was determined. [ ], T98G cells treated with VBL in combination with siRNA negative control (SCR). [ ], T98G cells treated with VBL in combination with pooled BCL-xL siRNAs. [ ], T98G cells transfected with cell death negative control siRNA. F, SCR and BCL-xL siRNAs were nontoxic to cells. Each value is the mean of four independent experiments. Bars represent S.E.M. *, p ≤ 0.05.

**Fig. 3.** Reduction in BCL-xL protein levels in T98G cells after siRNA treatment. Cells were transfected with either negative control (SCR) siRNA or BCL-xL pooled siRNA at various concentrations (2.5, 1.25, 0.63, and 0.31 nM), and protein levels of BCL-xL were measured at 48 and 96 h by Western blot analyses. BCL-xL siRNA decreased protein levels at all of the concentrations tested relative to the SCR control, at both 48 and 96 h. The Western blot is representative of three independent experiments.
caspase-3/7 activation, which was greatly enhanced after the addition of 1.56 μM ABT-263 (Fig. 5B).

We also examined cytochrome c localization to the mitochondria to determine whether the observed cell death was occurring through mitochondria-dependent apoptosis. We initially treated T98G cells with increasing concentrations of ABT-263 and observed a concentration-dependent decrease in cytochrome c localization to the mitochondria (Fig. 6, A and B), which was decreased further in the presence of VBL (Fig. 6, C and D). This is indicative of cytochrome c release into the cytoplasm and ultimately the induction of mitochondria-dependent apoptosis.

Discussion

siRNA HTS technology has facilitated the identification of gene products that when suppressed result in decreased proliferation or even death of cancerous cells (Willingham et al., 2004; Thaker et al., 2010). To help identify novel combination treatments for cancer, we performed VBL-dependent siRNA HTS targeting the druggable genome. Using this siRNA methodology combined with a series of statistical methods, we identified gene products that sensitized GBM cells to VBL. siRNA HTS is an inherently variable type of cellular assay, where data sets can fluctuate daily due to differences in ambient conditions, cell density, cellular functionality, human or mechanical error, and siRNA transfection efficiency (Birmingham et al., 2009). Even in the most consistent siRNA screens, the transfection efficiency is challenging to regulate and monitor on a well-to-well basis, which increases the possibility of variability among replicates. Thus, complementary but independent statistical methods are necessary to identify high-confidence gene products from the primary screen.

MAD analysis has been used previously as a method for “hit” detection (Chung et al., 2008) but for our purposes served as an unbiased outlier detection methodology. Using the MAD analysis, we were able to distinguish replicates within the individual screens that did not corroborate the overall results. In removing these replicates, we uncovered a subset of possible false negatives that were being biased heavily by the presence of outliers. To determine gene products that sensitized GBM cells to VBL, we used a similar
been used clinically to specifically decrease the expression of anticancer therapies (Tse et al., 2008). Actually can enhance the resistance of the cells to these chemotherapies, including cytotoxic drugs such as VBL, in some cases, overexpression of BCL-2 prosurvival proteins can reduce cell death through the intrinsic signaling pathway. Intrinsic cell death pathway can be evaded (Trask et al., 2002; Kang and Reynolds, 2009; Li et al., 2009; Hockenbery, 2002; Kang and Reynolds, 2009; Li et al., 2009; Hockenbery, 2002), although we recognize that effector caspases, such as caspase 3, also are activated by mitochondria-independent mechanisms (Guicciardi and Gores, 2009). Interestingly, the sensitization of T98G and A549 cancer cell lines to VBL by BCL-xL siRNA and ABT-263 only occurred with VBL concentrations (≥1.56 nM) that alone reduced cell viability. This is consistent with the notion that an external stimulus, such as

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**Fig. 6.** ABT-263-dependent caspase-3/7 induction of intrinsic apoptosis. A, T98G cells were treated with decreasing concentrations of ABT-263 (3.13–0.31 μM), and the levels of cytochrome c in the mitochondrial fractions were visualized by Western blot analyses. ERAB, a mitochondria-specific marker, was used as a loading control. B, quantification of cytochrome c localization to the mitochondrial fraction relative to ERAB expression levels. All of the values were normalized to the DMSO control. C, levels of cytochrome c in the mitochondrial fractions in the presence of DMSO, 1.56 μM ABT-263, 12.5 nM VBL, and ABT-263 plus VBL. D, quantification of localization of cytochrome c to the mitochondrial fraction in the presence of ABT-263, VBL, or ABT-263 plus VBL. Expression levels were normalized to ERAB expression levels as well as the DMSO control. Blots are representative of three independent experiments. Bars, S.E.M.
an anticancer agent, is necessary for the activation of intrinsic apoptosis (Ackler et al., 2008, 2010; Tse et al., 2008).

In our studies, relatively stringent criteria were used in the data analysis. The application of a more lenient viability ratio or p value would have expanded the subset of candidate gene products that sensitize VBL to cells. Indeed, if we had selected a p value of ≥0.05 instead of ≤0.01, then both BCL-2 and BCL-w would have been included in the primary data set (Supplemental Fig. 5). Due to the previous success of VBL as an individual anticancer agent (Giannakakou et al., 2000) and ABT-263 as a sensitizer to other anticancer therapies (Ackler et al., 2008, 2010), we believe that the combination of ABT-263 with VBL is worthy of further investigation, especially with those tumor types, such as GBM, which are dependent on the overexpression of BCL-2 prosurvival proteins.

In summary, we have used an unbiased siRNA HTS to expose potential drug combinations that may be of clinical utility in the treatment of cancer. In addition to BCL-xL, we identified other gene products (Table 1) that function to protect T98G cells from the cytotoxic actions of VBL and have available pharmacological inhibitors or inducers. Clearly, these are worthy of further study. Overall, these studies illustrate the value of unbiased siRNA HTS for detecting novel chemosensitivity nodes and potential new anticancer drug combinations.

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Authorship Contributions

Participated in research design: Kitchens, Shun, and Lazo.

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Contributed new reagents or analytic tools: Pollack.

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