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Identification of chemosensitivity nodes for vinblastine through small interfering RNA high-throughput screens.

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Running title: Vinblastine sensitization by BCL-xL siRNA and ABT-263

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Abbreviations: ABT-263, (4-[4-[[2-(4-chlorophenyl)-5,5-dimethylcyclohexen-1-yl]methyl]piperazin-1-yl]-N-[4-[[(2R)-4-morpholin-4-yl-1-phenylsulfanylbutan-2-yl]amino]-3-(trifluoromethylsulfonyl)phenyl]sulfonylbenzamide); BME, Basal Media Eagle; BCL-2, B-cell lymphoma 2; BCL-xL, B-cell lymphoma-extra-large; DMSO, dimethyl sulfoxide; EMEM, Eagle's Minimum Essential Medium; FDR, False discovery rate; GBM, glioblastoma multiforme; HA, Human astrocytes; MAD, Median absolute deviations; HTS, high throughput screening; PBS, phosphate buffered saline; RFU, relative fluorescent units; siRNA, small interfering RNA; SCR, scrambled; STE, 250 mM sucrose, 10 mM Tris and 1 mM EGTA at pH 7.4; VR, viability ratio; VBL, vinblastine

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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 drugs targets. Therefore, we implemented a small interfering RNA (siRNA) synthetic lethal screen targeting 5,520 unique druggable genes to identify novel chemosensitivity nodes for vinblastine, a clinically used microtubule destabilizing agent. We transiently transfected human glioblastoma cells with siRNAs for 48 h and then treated cells with a sub-lethal concentration of vinblastine. Forty-eight h 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, BCL-xL, because of its role in the intrinsic apoptosis signaling pathway, as well as the availability of pharmacological inhibitors. We found nontoxic concentrations of 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) (Dumontet and Jordan, 2010, Jordan and Wilson, 2004). 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, Kavallaris, et al., 2001, Ross, et al., 1999). 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 gliomas; 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). While the addition of chemotherapy to resection and radiotherapy has increased the two-year survival rate from 1 to 20%, a successful treatment, much less a cure, for GBM remains elusive. Patients receiving chemotherapeutic agents, such as the 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 chemotherapy

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treatments that can specifically target these chemoresistant 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 (Iorns, et al., 2007, Kaelin, 2005). Additionally, 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. As 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 sub-lethal concentration of the BCL-2 family member inhibitor 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.

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MATERIALS AND METHODS

Reagents

The *Silencer* Druggable Genome siRNA Library (Version 1.1), *Silencer Select* secondary library, *Silencer Select* Negative Control No. 1 and BCL-xL *Silencer Select* siRNAs (siRNA ID s1920, s1921, and s1922) were purchased from Ambion (Austin, TX). AllStars Hs Cell Death Control siRNA was purchased from Qiagen (Valencia, CA). DharmaFECT2 transfection reagent, 5x siRNA resuspension buffers, and the siGENOME Non-Targeting siRNA #1 were purchased from Dharmacon (Lafayette, CO). Tissue culture-treated 384-well microtiter plates were from Greiner Bio-One (GMBH, 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 (Bedford, MA). Eagle's Minimum Essential Medium (EMEM), Opti-MEM, Basal Medium Eagle (BME), phosphate buffered saline (PBS), L-glutamine, penicillin/streptomycin, Hoechst 33342, and 1.0 mm Novex 4-20% Tris-Glycine Gels were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Cellgro (Manassas, VA). ECL Western blotting substrate was from Pierce Biotechnology (Rockford, IL). The T98G, A549,U87, U3T3, LNZ 428, MDA-MB-231, HeLa cell lines and human astrocytes (HA) 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 (4-[4-[[2-(4-chlorophenyl)-5,5-dimethylcyclohexen-1-yl]methyl]piperazin-1-yl]-N-[4-[[(2R)-4-morpholin-4-yl-1-phenylsulfanylbutan-2-yl]amino]-3-(trifluoromethylsulfonyl)phenyl] sulfonylbenzamide) was obtained from ChemieTek (Indianapolis, IN). GAPDH rabbit monoclonal (#2118), BCL-xL rabbit polyclonal (#2762) and BAX rabbit monoclonal (#5023)

antibodies were purchased from Cell Signaling (Danvers, MA). BCL-xL rabbit monoclonal [E18] (ab32370), cytochrome *c* mouse monoclonal (ab13575) and ERAB [5F3] - Mitochondrial Marker mouse monoclonal were purchased from Abcam (Cambridge, MA). Rabbit and mouse peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (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 EMEM 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 BME 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% CO_2. T98G and A549 cell lines were validated during the study by RADIL Research Animal Diagnostic Laboratory (University of Missouri-Columbia).

VBL and ABT-263 were dissolved in 100% DMSO. Compound treatments were added 48 h after initial cell seeding for both the synthetic lethal screens and drug combination studies. Compound treatment and DMSO vehicle controls were diluted in medium with 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 Ambion *Silencer* Druggable Genome siRNA library at a final concentration of 20 nM per target in a one gene per well format as previously reported (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

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incubator at 37°C with 5% CO_2. 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 was then measured by incubating the cells with the CellTiter-Blue viability assay (1:5 ratio of CellTiter-Blue to medium) for three h, according to manufacturer's protocol. Plates were read on the Spectramax M5 (Molecular Devices, Sunnyvale, CA).

Data analysis for the siRNA synthetic lethal screen

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

$$\%\ Cell\ Viability = 100\left(\frac{x_{RFU}}{\bar{y}_{RFU}}\right) \qquad (1)$$

where x_{RFU} is the RFUs for each sample and $\bar{y}_{RFU}$ is the average RFUs for the DMSO/SCR siRNA control within each plate.

We employed the median absolute deviations (MAD) analysis, an outlier detection method, which is resistant to the presence of outliers within the samples as previously reported (Thaker, et al., 2010). Samples with an 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.

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The samples were ranked according to their viability ratio, $VR = \frac{\bar{x}_{CV}}{\bar{y}_{CV}}$, where $\bar{x}_{CV}$ 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 VR and the top 2.5% of genes (138 genes) were selected. A 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 siRNA plus VBL as previously described (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 employed to decrease the possibility of false positives due to multiple comparisons (Benjamini and Hochberg, 2000). The FDR ranks the samples according to their p-value, where the lower the rank, the less strict the 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:

$$FDR_k = \frac{k\alpha}{m} \qquad (2)$$

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

Western blots and lysate preparation

Western blots were performed as previously described (Thaker, et al., 2010). Membranes were probed with antibodies at various dilutions targeting: BCL-xL (1:500), β tubulin (1:10,000), GAPDH (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 according to

manufacturer's protocol. Membranes were imaged using the FujiFILM LAS-3000 imager (R&D Systems, Minneapolis, MN).

Concentration response curves

For the 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 to 10 nM) as described in the siRNA screen. Forty-eight h post-transfection, the cells were treated with VBL (25 pM to 50 nM) with a final DMSO concentration of 0.5%. Percent cell viability of each sample was determined by CellTiter Blue as described above. All samples were normalized to the DMSO/scrambled negative control, which was termed 100% cell viability. Toxicity of siRNA alone was determined by comparing the siRNA/DMSO percent cell viability to the SCR/DMSO negative control. The "Cell Death" siRNA (AllStars Hs Cell Death Control siRNA) was used as a positive control for transfection efficiency and for determining cytotoxicity. Only experiments with >90% cell death with the "Cell Death" positive control samples were considered for analysis.

For the drug combination studies with both T98G and A549 cells, we first determined their sensitivity to VBL (25 pM to 50 nM) and ABT-263 exposure (0.8 to 50 μM). Based on these data, the two drugs were titrated in a "checkerboard" format on a 384-well plate where the concentration range of VBL was arranged in the columns and the concentration range of ABT-263 was arranged in the rows in the plate with DMSO in the last rows and columns, providing a standard concentration response curve for each drug. Cells were plated at a density of 500 cells per well. We treated plates after 48 h with VBL and ABT-263 and after an additional 48 h cell viability was determined using the CellTiter-Blue assay. All wells were normalized to the DMSO control and the percent cell viability of each sample was determined as described above.

Mitochondrial fractionation

Mitochondria isolation by differential centrifugation was adapted from previously described methods (Shiva, et al., 2001). Briefly, T98G cells were plated and treated in 100-mm dishes. Samples were placed on ice and washed with ice-cold PBS to inhibit cellular activity. Samples were scraped on ice with 500 µL of ice-cold PBS into 1.5 mL Eppendorf tubes. The samples were centrifuged at 1,500 x g for 5 min at 4°C to generate a cellular pellet. The supernatant was discarded and the pellet was homogenized in 500 µL of STE (250 mM Sucrose, 10 mM Tris, 1 mM EGTA, pH 7.4 at 4°C) on ice. Samples were centrifuged at 1,000 x g for 5 min to remove cellular debris and collect the supernatant. To separate the mitochondrial fraction from the cytosolic fraction, we centrifuged samples at 10,000 x g for 10 min. The supernatant fraction was collected for the cytosolic fraction and the pellet was collected for the mitochondrial fraction. The mitochondrial pellet was homogenized in 100 µL of STE, centrifuged at 10,000 x g for 10 min, the supernatant discarded and the pellet collected for the mitochondrial fraction. The mitochondrial pellet was resuspended in 50 µL of STE and the cytosolic and mitochondrial fractions were frozen at -80°C overnight. Samples were prepared for Western Blot analysis as described above.

Caspase-Glo 3/7 Assay

The Caspase-Glo 3/7 assay was performed according to the manufacture's protocol. Briefly, T98G cells were plated as described previously in the drug combination studies. Cells (500 cells per well) were plated in BD Falcon 384-well white/clear bottom plates. Forty-eight h after cell seeding, plates were treated with the VBL/ABT-263 combination. After an additional 48 h, the medium containing drugs was removed and the Caspase-Glo 3/7 reagent was added to wells in a 1:1 ratio of reagent to medium (25 µL of reagent and 25 µL of medium per well). Plates were incubated in the dark at room temperature for one h. The plates were read using an EnVision Multipliable Plate Reader (PerkinElmer Waltham, MA). Levels of caspase 3/7 were normalized to DMSO controls.

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siRNA sequences

The Ambion Druggable Genome consists of 16,560 *Silencer* siRNA duplexes with three siRNAs targeting each of 5,520 gene products. For the initial screen we pooled the three individual siRNA at a 1:1:1 ratio. The sequences for BCL-xL siRNAs are available in Supplementary Table 2.

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RESULTS

Identification of gene products that sensitize T98G cells to VBL using siRNA HTS methodology.

We performed a siRNA HTS, as previously described (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 scrambled 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 Figure 1 to determine the gene products that sensitized T98G cells to VBL. Briefly, MAD analysis was performed to determine outliers with 95% confidence within the screen. After all 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 ratio and the top 2.5% of gene products were selected (138 genes). We performed a 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 ($p \leq 0.01$). This process yielded 65 high confidence gene products that sensitized cells to VBL (Figure 1). To control for false positives due to multiple comparisons, we employed the Benjamini-Hochberg's false discovery rate (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 (Supplementary 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

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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 (Kang and Reynolds, 2009, Tse, et al., 2008), 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 (Figure 2). We treated T98G cells with increasing concentrations of VBL in the presence and absence of decreasing concentrations of pooled BCL-xL siRNA (sequences of siRNAs in Supplemental Table 2). We found that cells were sensitized to the higher VBL concentrations (3.13 to 25 nM) when treated in combination with non-toxic concentrations of BCL-xL siRNAs (0.63 to 10 nM, Figure 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 the 48 h (time of drug addition) and 98 h (end point of the assay) time points, BCL-xL protein levels were undetectable at all concentrations tested relative to scrambled siRNA controls (Figure 3).

ABT-263 sensitizes T98G and A549 cells to VBL.

To determine whether ABT-263, a pro-survival BCL-2 family inhibitor, sensitized cells to VBL, we treated T98G cells with increasing concentrations of VBL in the presence or absence of a nontoxic concentration of ABT-263 (Figure 4A). As seen in Figure 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 normal human astrocytes (HA) by Western blotting (Supplementary Figure 2). We found that relative to the normal human astrocytes, BCL-xL

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protein was overexpressed in the majority of the cancer cell lines (MDA-MB-231, A549, T98G, U87 and U3T3). To determine whether the 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 or absence of nontoxic concentrations of ABT-263. We found that in the presence of 12.5 μM ABT-263, A549 cells were also sensitized to VBL at concentrations greater than 3.13 nM (Figure 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% while the addition of ABT-263 greatly reduced cell viability (Figure 4). We, therefore, 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 determine 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 conditions tested (BCL-xL siRNA in T98G cells and ABT-263 in T98G and A549 cells), VBL induced a cytostatic effect at concentrations of ≥1.56 nM (Supplemental Figure 3). In the presence of BCL-xL siRNA in the T98G cells, however, the loss of BCL-xL protein sensitized the cells to VBL and we propose that the cytostatic concentrations became cytotoxic based on (Supplemental Figure 3A). In the T98G and A549 cell lines the addition of a non-toxic concentration of ABT-263 also sensitized the cells to VBL, inducing a cytotoxic effect at cytostatic concentrations of VBL alone (Supplemental Figure 3B and C, respectively).

ABT-263 and VBL induce cell death through mitochondria-dependent apoptosis.

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We examined caspase-3/7 activation to assess the possible role of mitochondrial-dependent apoptosis (Lakhani, et al., 2006) in the presence of nontoxic concentrations of ABT-263 and VBL in T98G cells (Figure 5A). From the time of drug addition up to eight h, we did not observe any significant increase in caspase-3/7 activity by nontoxic concentrations of VBL or ABT-263; however, at 24 h we noted caspase-3/7 activation at 25 nM VBL and 1.56 μM ABT-263 (Figure 5A). We then treated cells with increasing concentrations of VBL in the presence or absence of 1.56 μM ABT-263 and measured caspase-3/7 activity (Figure 5B). At 24 h, there was a VBL concentration-dependent increase in caspase-3/7 activation, which was greatly enhanced after the addition of 1.56 μM ABT-263 (Figure 5B).

We also examined cytochrome *c* localization to the mitochondria to determine whether the observed cell death was occurring through mitochondrial-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 (Figure 6A and B), which was further decreased in the presence of VBL (Figure 6C and D). This is indicative of cytochrome *c* release into the cytoplasm and ultimately the induction of mitochondria-dependent apoptosis.

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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 a 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 HTSs are 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.

The MAD analysis has previously been utilized 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 with the overall results. In removing these replicates, we uncovered a subset of possible false-negatives that were being heavily biased by the presence of outliers. To determine gene products that sensitized GBM cells to VBL, we used a similar statistical approach to that of Whitehurst *et al.*(Kaelin, 2005, Whitehurst, et al., 2007) employing both the Student's t-test and the viability ratios as a measure of "hit" determination. In contrast to the strategy used by Whitehurst *et al.*, (Kaelin, 2005, Whitehurst, et al., 2007), we performed a linear analysis, where we first limited the 5,520 gene products of the druggable genome by their viability ratios (top 2.5%), then performed Student's t-tests on the remaining 138 genes. By first limiting the number of Student's t-tests from 5,520 to 138, we decreased the possibility of a false

positive due to multiple comparisons from 100% to 75%. As indicated by the FDR with which we used an α of 0.02, we confirmed that all 65 high confidence gene products identified by performing 138 Student's t-tests, versus 5,520 tests, were likely true positives. Further evaluation with different siRNAs produced nine prioritized gene products. Assuming that an inhibitor targeting these gene products could have the same molecular effect as decreasing the protein expression by siRNA, we were particularly interested in the top gene products with commercially available inhibitors to mimic the effect of the siRNA. Of particular interest was BCL-xL, a member of the BCL-2 pro-survival proteins that plays an essential role in the intrinsic cell death pathway. When the pro-survival proteins, BCL-2 and BCL-xL are overexpressed in cancer, the ratio of pro- and anti-apoptotic proteins is disturbed and the intrinsic cell death pathway can be evaded (Hockenbery, 2010, Kang and Reynolds, 2009, Li, et al., 2009, Trask, et al., 2002). This is significant as the majority of cancer chemotherapies, including cytotoxic drugs such as VBL, induce cell death through the intrinsic signaling pathway. In some cases, overexpression of the BCL-2 pro-survival proteins can actually enhance the resistance of the cells to these anticancer therapies (Tse, et al., 2008).

Oblimersen is a BCL-2 antisense oligonucleotide that has been used clinically to specifically decrease the expression of BCL-2 protein in cancer cells. BCL-2 pro-survival protein siRNAs, like oblimersen, can resensitize chemotherapy resistant cancer cells overexpressing the BCL-2 pro-survival proteins: BCL-2, BCL-xL and BCL-w (Castilla, et al., 2006, Gazitt, et al., 1998, Liu, et al., 1999). Decreasing the expression of the BCL-2 pro-survival proteins by siRNA predisposes the cells to an apoptotic phenotype that reestablishes the balance between the pro-survival and pro-apoptotic proteins (Supplementary Figure 4). While the combination of oblimersen with the microtubule stabilizing agents, paclitaxel and docetaxel, has had clinical success in small cell lung cancer, non-small cell lung cancer and hormone refractory prostate cancer (Kang and Reynolds, 2009, Liu, et al., 2008, Tolcher, et al., 2005), oblimersen has not,

to our knowledge, been tested clinically in combination with VBL and other microtubule destabilizing agents.

From the primary and secondary siRNA HTS, we found that, similarly to the combination of oblimersen with microtubule stabilizing agents, upon the addition of BCL-xL siRNA to T98G GBM cells, we could sensitize these cells to the microtubule destabilizing agent, VBL. Similarly, when we combined a sub-lethal concentration of ABT-263, which is a small molecule inhibitor that binds in the BH3 domain of BCL-2, BCL-xL, and BCL-w, with VBL, we observed a greater than additive toxic effect as indicated by the siRNA HTS. The activation of caspase-3/7 might have resulted from mitochondrial-dependent apoptotic pathways that are regulated by BCL-2 family members (Lakhani, et al., 2006), although we recognize effector caspases, such as caspase 3, are also activated by mitochondrial-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 and higher) that alone reduced cell viability. This is consistent with the notion that an external stimulus, such as an anticancer agent, is necessary for the activation of intrinsic apoptosis (Ackler, et al., 2008, Ackler, et al., 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, had we selected a p value of ≤0.05 instead of ≤0.01 both BCL-2 and BCL-w would have been included in the primary dataset (Supplementary Figure 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, Ackler, et al., 2010), we believe the combination of ABT-263 with VBL is worthy of further investigation, especially with those tumor types, such as GBM, which are dependent upon the overexpression of BCL-2 pro-survival proteins.

In summary, we have employed 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 CONTRIBUTION

Participated in research design: Kitchens, Shun, Lazo

Conducted experiments: Kitchens, McDonald

Contributed new reagents or analytical tools: Pollack

Performed data analysis: Kitchens, Shun

Wrote or contributed to the writing of the manuscript: Kitchens, Pollack, Lazo

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Reduction in cell viability with the top 65 gene products from the primary siRNA HTS. Percent cell viabilities were determined for the siRNA plus DMSO (white bars) and siRNA plus vinblastine (VBL, black bars), as described in the Materials and Methods section of this manuscript, for all 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 addition of a non-toxic concentration of VBL, the combination was toxic. Each value is the mean of three independent experiments. Bars equal S.D.

Figure 2. BCL-xL siRNA sensitization of T98G cells to VBL. Three siRNAs targeting BCL-xL were pooled together and tested as sensitizers of T98G cells to VBL. 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 h 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 in the Materials and Methods section. All data points were normalized to a SCR/DMSO control to allow for well-to-well comparison. 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 equal S.E.M. *p≤0.05

Figure 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 analysis. BCL-xL siRNA decreased protein levels at all concentrations tested relative to the scrambled control, at both 48 and 96 h. Blot is representative of three independent experiments.

Figure 4. Sensitization of T98G and A549 cells to VBL by ABT-263. (A) T98G cells were treated with increasing concentrations of VBL (25 pM to 25 nM) in the presence and absence of a nontoxic concentration of ABT-263 (1.56 μM). (B) A549 cells were treated with increasing concentrations of VBL (25 pM to 25 nM) in the presence and absence of a nontoxic concentration of ABT-263 (12.5 μM). At higher concentrations of VBL (1.56 to 25 nM for T98G and 3.13 to 25 nM for A549), ABT-263 significantly decreases the cell viability relative to VBL alone in both the T98G and A549 cancer cell lines. Cell viability was determined using a CellTiter Blue assay. All values were normalized to the DMSO control (No ABT-263). (○) Cells treated with increasing concentrations of VBL. (■) Cells treated with increasing concentrations of VBL in the presence of nontoxic ABT-263 concentrations. Each value is the mean of four independent experiments. Bars equal S.E.M. *p≤0.05

Figure 5. Induction of intrinsic apoptosis induced by VBL and ABT-263. (A) Activation of caspase-3/7 in T98G cells was measured at toxic (25 μM) and nontoxic (1.56 μM) concentrations of ABT-263 and toxic (25 nM) concentrations of VBL over time. Caspase-3/7 activation was measured at 0 (black), 1 (checkered), 8 (diagonal lines) and 24 (Whitehurst, et

al.) h. (B) T98G cells were treated with increasing concentrations of VBL(25 pM to 25nM) in the presence and absence of 1.56 μM ABT-263. Caspase-3/7 activation was measured at 24 h using a Caspase-3/7 Glo assay kit. (Δ) Cells treated with increasing concentrations of vinblastine. (■) Cells treated with combinations of VBL and ABT-263. Each value is the mean of three independent experiments. Bars equal S.E.M. *p≤0.05

Figure 6. ABT-263 dependent caspase-3/7 induction of intrinsic apoptosis. (A) T98G cells were treated with decreasing concentrations of ABT-263 (3.13 to 0.31 μM) and the levels of cytochrome *c* in the mitochondrial fractions were visualized by Western blot analysis. ERAB, a mitochondrial specific marker, was used as a loading control. (B) Quantification of cytochrome *c* localization to the mitochondrial fraction relative to ERAB expression levels. All 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 equal S.E.M. *p≤0.05

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TABLES

Table 1. Confirmation of VBL sensitization with individual siRNAs and T98G cells.

	p-value			p-value			p-value	
Gene Symbol	**siRNA A**	**siRNA B**	**Gene Symbol**	**siRNA A**	**siRNA B**	**Gene Symbol**	**siRNA A**	**siRNA B**
ACOT11	0.023	<0.001	ADSL	*NS**	0.003	POLR1A	*NS**	0.041
AKT3	0.033	0.002	ANKRD30A	*NS**	0.042	PRDX6	0.014	*NS**
BCL-xL	0.035	0.041	BTN3A2	*NS**	0.041	PROCR	0.002	*NS**
FUK	0.006	0.003	CDC42	*NS**	0.002	RAB9A	*NS**	0.031
ITPR3	0.003	0.010	DHFRL1	0.046	*NS**	SDHA	*NS**	<0.001
KDELR1	0.029	0.030	GSTA1	0.027	*NS**	SERPINA10	*NS**	0.024
NOS1	0.008	0.003	HSD11B1L	0.012	*NS**	SERPINB13	*NS**	0.012
NRAS	0.003	0.001	LOC136242	0.030	*NS**	SPINK1	0.004	*NS**
SERPINA3	0.004	0.008	NEIL3	<0.001	*NS**	UGT1A9	0.046	*NS**
			NIT1	*NS**	0.045	UGT2B17	*NS**	0.004
			PMM2	0.023	*NS**	WARS2	0.012	*NS**

* *NS* (not significant) represents siRNAs in the secondary assay with a p>0.05.

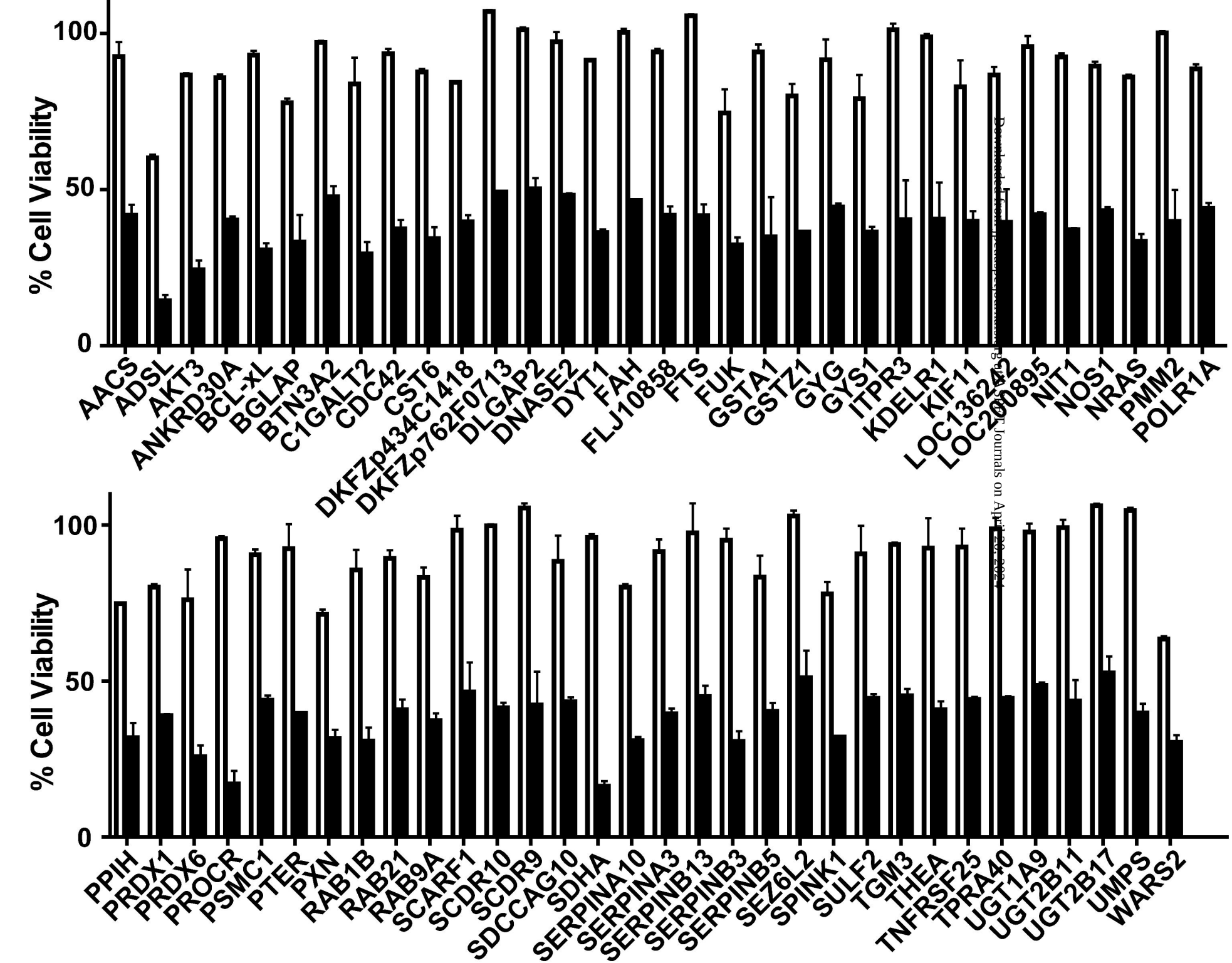

Figure 1.

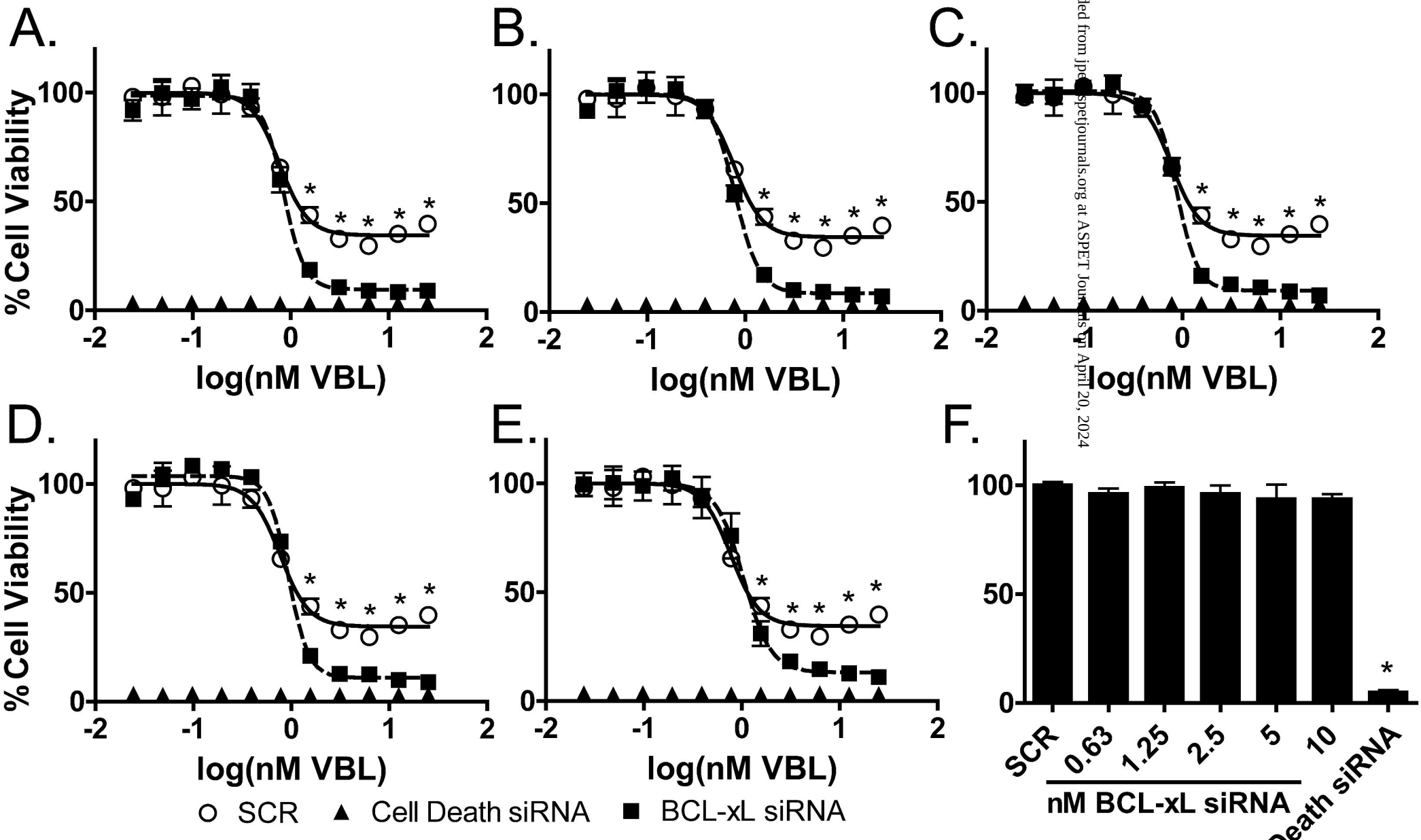

Figure 2.

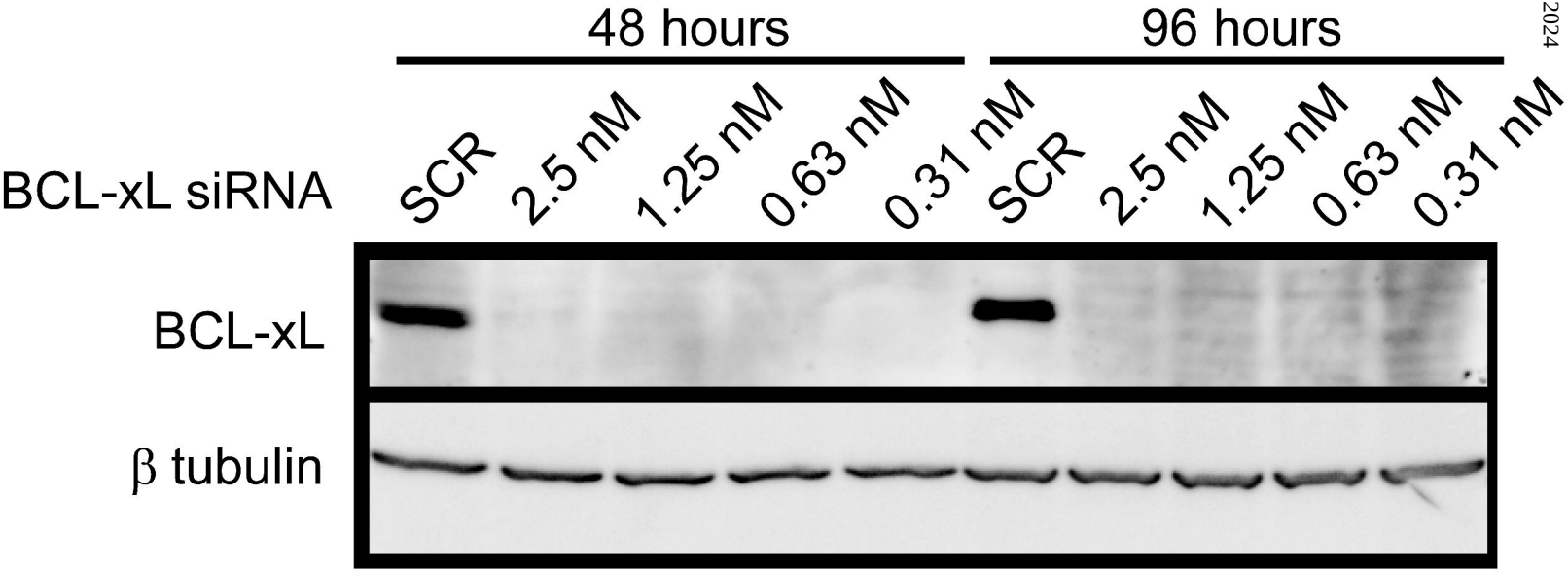
48 hours
96 hours
BCL-xL siRNA
SCR
2.5 nM
1.25 nM
0.63 nM
0.31 nM
SCR
2.5 nM
1.25 nM
0.63 nM
0.31 nM
BCL-xL
β tubulin

Figure 3.

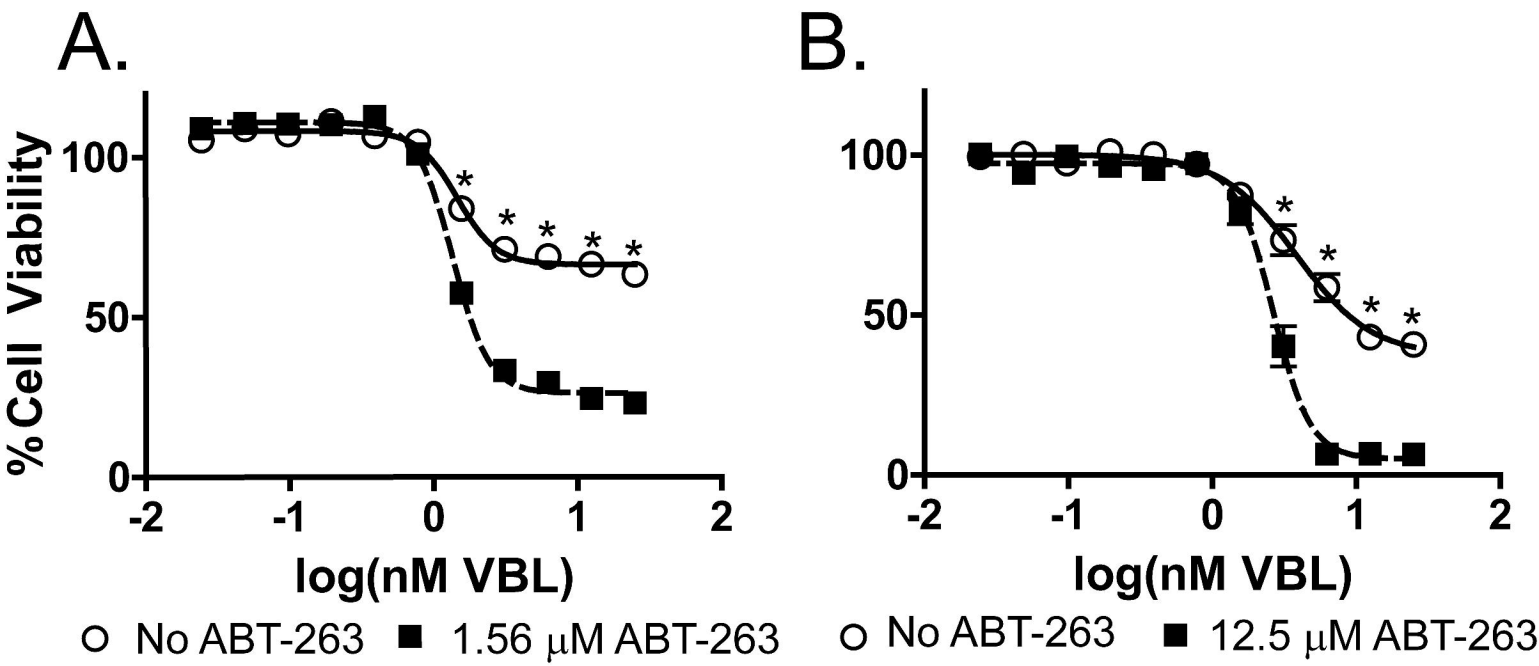
A.
B.
% Cell Viability
100
50
0
-2
-1
0
1
2
log(nM VBL)
No ABT-263
1.56 μM ABT-263
12.5 μM ABT-263

Figure 4.

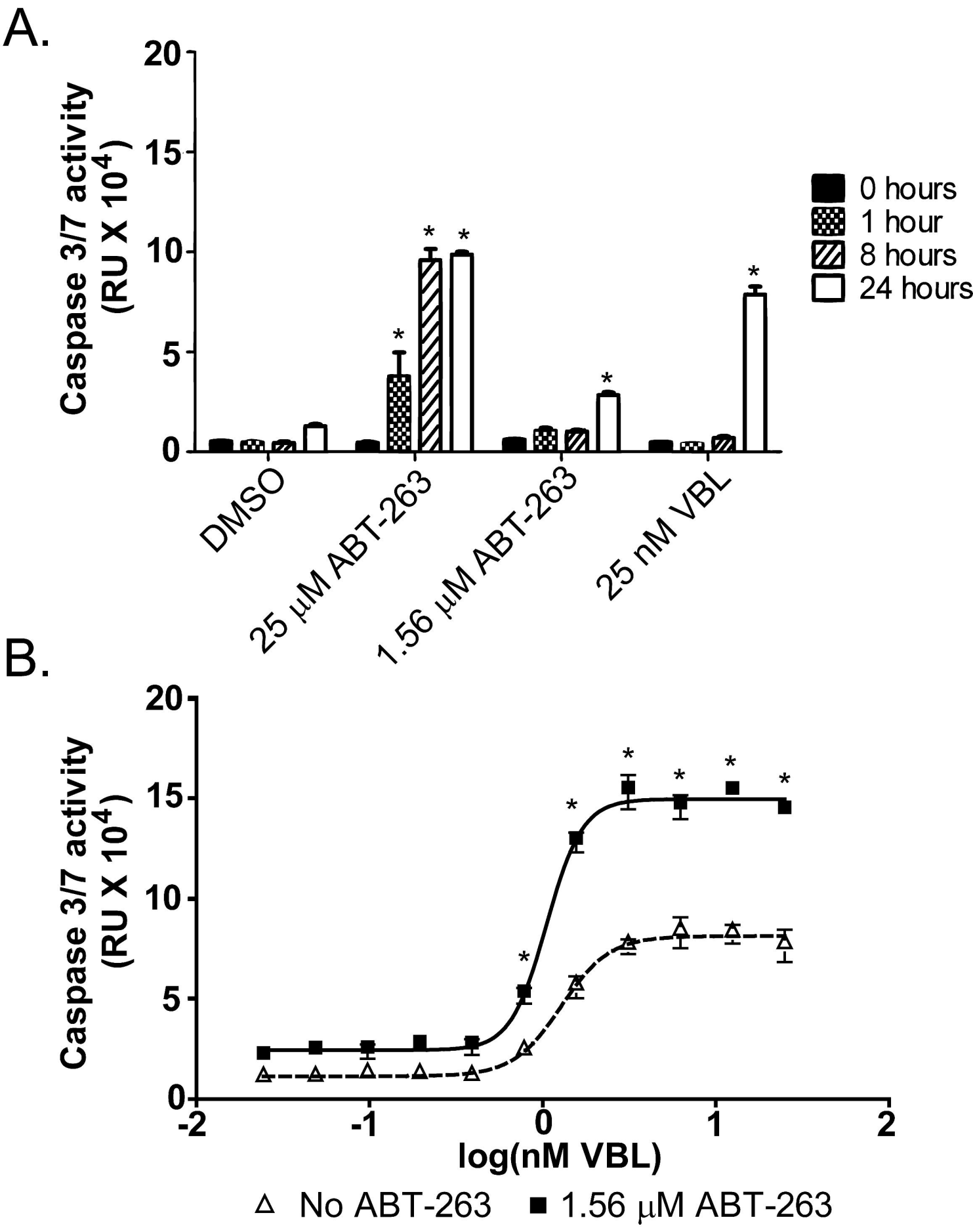
A.
Caspase 3/7 activity (RU X 10^4)
20
15
10
5
0
0 hours
1 hour
8 hours
24 hours
DMSO
25 μM ABT-263
1.56 μM ABT-263
25 nM VBL
B.
Caspase 3/7 activity (RU X 10^4)
20
15
10
5
0
-2
-1
0
1
2
log(nM VBL)
No ABT-263
1.56 μM ABT-263

Figure 5.

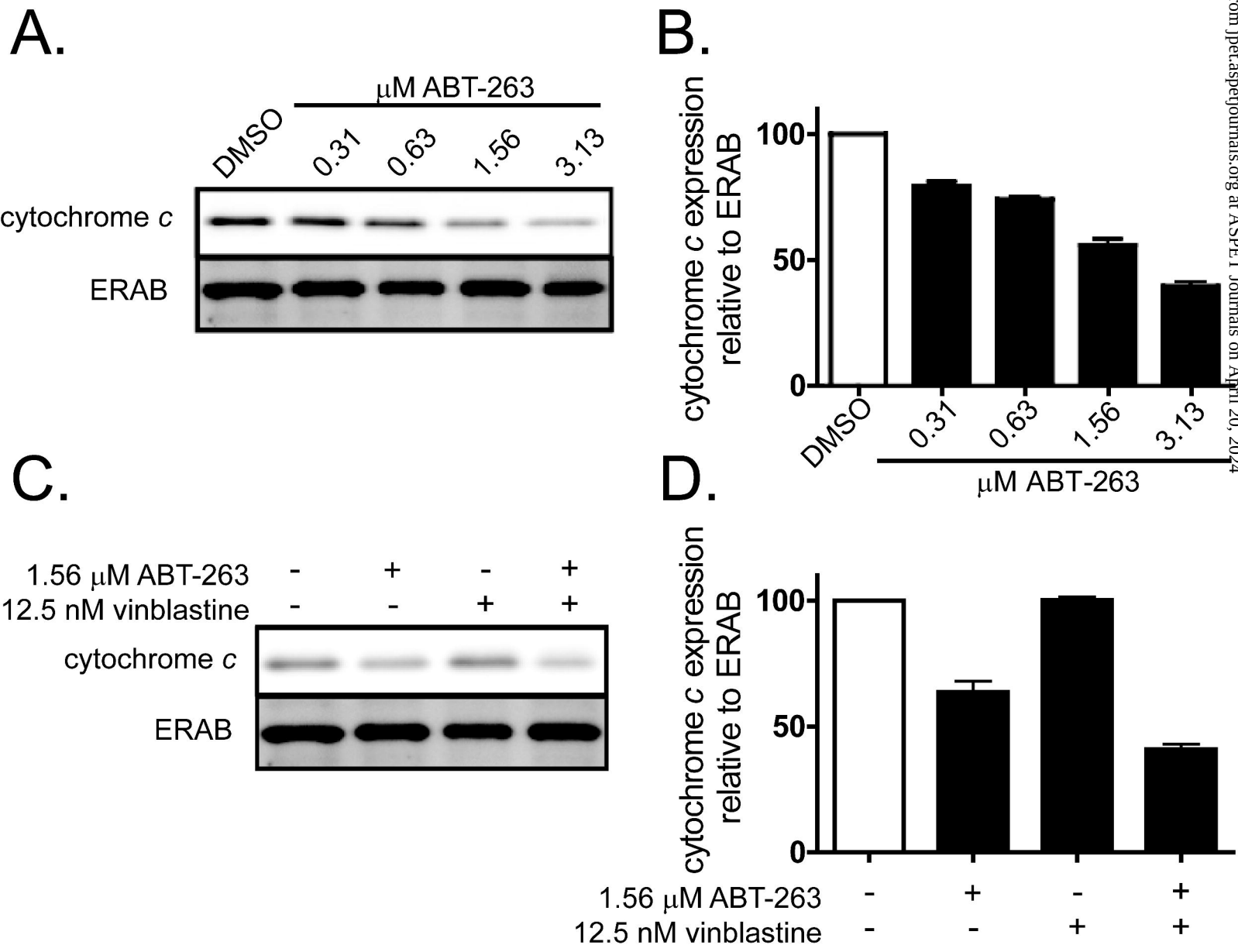

Figure 6.