Systematic mapping of BCL-2 gene dependencies in cancer reveals molecular determinants of BH3 mimetic sensitivity

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While inhibitors of BCL-2 family proteins (BH3 mimetics) have shown promise as anti-cancer agents, the various dependencies or co-dependencies of diverse cancers on BCL-2 genes remain poorly understood. Here we develop a drug screening approach to define the sensitivity of cancer cells from ten tissue types to all possible combinations of selective BCL-2, BCL-XL, and MCL-1 inhibitors and discover that most cell lines depend on at least one combination for survival. We demonstrate that expression levels of BCL-2 genes predict single mimetic sensitivity, whereas EMT status predicts synergistic dependence on BCL-XL+MCL-1. Lastly, we use a CRISPR/Cas9 screen to discover that BFL-1 and BCL-w promote resistance to all tested combinations of BCL-2, BCL-XL, and MCL-1 inhibitors. Together, these results provide a roadmap for rationally targeting BCL-2 family dependencies in diverse human cancers and motivate the development of selective BFL-1 and BCL-w inhibitors to overcome intrinsic resistance to BH3 mimetics.
The process of intrinsic apoptosis is tightly regulated by the BCL-2 family of proteins. In human cancers, the anti-apoptotic BCL-2 proteins play a critical role in protecting cells, which are often "primed" for apoptosis, from committing to irreversible cell death. To date, the most well described of the anti-apoptotic BCL-2 genes are BCL-2, BCL-XI, and MCL-1, and recently, following over a decade of extensive research effort, potent and selective inhibitors of each of these proteins were developed. Much is known about the cancer types that respond well to selective BCL-2 inhibitors, and indeed the BCL-2 inhibitor venetoclax (ABT-199) is now FDA approved to treat certain leukemias such as chronic lymphocytic leukemia (CLL). In contrast, outside of a small number of studies in select cancer types, little is known regarding which cancers might respond well to single agent BCL-XI or MCL-1 inhibition. Finally, to the best of our knowledge, no studies have systematically examined the dependencies of cancers on combinations of BCL-2 family proteins.

With these limitations in mind, we set out to address the following questions: What are the dependencies of diverse human cancers with respect to BCL-2, BCL-XI, MCL-1, and their combinations? What are the molecular features of tumors that drive these dependencies? Finally, which cancers fail to respond to BH3 mimetics, and how can this intrinsic resistance be overcome? To answer these questions, we developed a screening strategy to assess the sensitivity of cancer cell lines to all possible combinations of a selective BCL-2 inhibitor (ABT-199), a selective BCL-XI inhibitor (WEHI-539), and a selective MCL-1 inhibitor (A-1210477). Using this approach, we mapped cellular dependencies and co-dependencies on BCL-2, BCL-XI, and MCL-1 across a large number of primary and established cancer cell lines representing 10 distinct cancer types. These data provide new insights into the landscape of sensitivity to BH3 mimetics in human cancers, revealing molecular determinants of sensitivity and a role for a novel endoplasmic reticulum (ER) stress-epithelial-mesenchymal transition (EMT) axis in dictating the frequently observed synergy between BCL-XI and MCL-1 inhibitors in solid tumors. Collectively, these findings may help guide the use of BH3 mimetics as precision therapies in defined cancers.

Results

Mapping of BCL-2 gene dependencies. To begin, we first made several assumptions regarding the BH3 mimetic drugs ABT-199, WEHI-539, and A-1210477 based on prior literature and our own experience. First, we elected to perform screens using a concentration of 1 µM for both ABT-199 and WEHI-539, as complete target inhibition is observed at these concentrations, and concentrations above this level may have off-target effects or may not be achievable in patients. A-1210477 is a first-in-class probe compound, and as such is less potent than ABT-199 or WEHI-539. Therefore, a concentration of 10 µM was selected for this compound, as at this dose MCL-1 is fully inhibited without inhibitory effects on BCL-2 and BCL-XI. A drug panel consisting of all possible single, double, and triple agent combinations of these drugs, at these concentrations, was then constructed and assayed in cell lines after a 72 h treatment using a conventional viability assay (see Methods) (Fig. 1a). To ensure that this assay accurately reveals BCL-2 family dependencies, we assembled several cell lines previously reported to be dependent on BCL-2, BCL-XI, MCL-1, or combinations of these proteins, then verified the recovery of expected dependencies (Fig. 1b) [6,9–11]. In prior studies, we identified Panc 03.27 cells as BCL-XI dependent, and as such this line was included as a control. To further validate this BCL-2 family dependency assay, we compared its results to conventional BH3 profiling assays (Supplementary Fig. 1A–C).

Consistent with the reported selective, on-target activities of the BH3 mimetics above, these assays revealed that BCL-XI dependency levels from viability assays correlate strongly on a cell line by cell line basis with the activity of the HRK peptide, which selectively inhibits BCL-XI. Similarly, MCL-1 dependency correlated with the activity of the NOXA peptide, a selective and direct inhibitor of MCL-1. Lastly, we tested structurally independent BCL-XI (A-1331852) and MCL-1 (S63845) inhibitors, as well as a dual BCL-2/BCL-XI inhibitor (ABT-737), in cell lines exhibiting single agent BCL-2, BCL-XI, or MCL-1 inhibitor sensitivity, or a cell line resistant to the inhibition of all three genes (Supplementary Fig. 2). Importantly, the sensitivity of each of these cell lines to these BH3 mimetics recapitulated the sensitivities observed following treatment with ABT-199, WEHI-539, or A-1210477, providing confidence that the latter drugs can be used as reliable probes of BCL-2 family dependencies.

Next, we used this assay to profile a large panel of 78 established cell lines from diverse cancer types, including acute myeloid leukemia (LAML), high-grade serous ovarian cancer (OV), colo-rectal adenocarcinoma (COAD), pancreatic ductal adenocarcinoma (PAAD), non-small cell lung cancer (LUAD), cutaneous melanoma (SKCM), liver hepatocellular carcinoma (LIHC), bladder cancer (BLCA), breast cancer (BRCA), and glioblastoma multiforme (GBM) (The Cancer Genome Atlas (TCGA) abbreviations used throughout; Fig. 1c and Supplementary Table 1). Further, we complemented these findings with assays performed in a panel of 13 primary patient-derived cultures (Fig. 1d), including primary COAD cells established directly from patient tumors, suggesting correspondence between in vitro and in vivo sensitivities in these models (Fig. 1e).

Collectively, pan-cancer cell line response behaviors revealed a number of notable patterns. First, whereas many of the LAML cell lines tested exhibited some dependency on BCL-2, a finding that coheres with published literature [14], the majority of solid cancers did not. Exceptions to this observation were SKCM cell lines and one LIHC cell line (SNU 423), which exhibited a modest BCL-2 dependency. This finding likely explains why single agent BCL-2 inhibitors such as ABT-199 have been less efficacious in solid cancers compared to LAML. LAML cells did not depend on BCL-XI for survival but did exhibit a robust MCL-1 dependency, again consistent with recent evidence [15]. In solid cancer cell lines, a fraction were dependent on BCL-XI, MCL-1, or the combination of the two. In particular, we observed a specific dependence on BCL-XI in select tumor types, in particular in subsets of BLCA, SKCM, and PAAD lines. This finding was unexpected given that solid tumors have only rarely been associated with single agent sensitivity to BH3 mimetics and suggests the possibility of defining subsets of these tumors vulnerable to selective BCL-XI inhibition. We also observed MCL-1 dependence in subsets of BRCA, SKCM, and PAAD cell lines, with the sensitivity of BRCA lines to single agent MCL-1 inhibition being consistent with recent reports [16]. Finally, and surprisingly, we observed that 49 of 78 cell lines were sensitive (defined as a viability loss of >50%) to the combined inhibition of BCL-XI + MCL-1. Consistent with the lack of activity of single agent BCL-2 inhibition, the addition of ABT-199 to the three conditions above (i.e. BCL-2+BCL-XI, BCL-2+MCL-1, and the triple combination) did not cause a significant increase in viability loss, suggesting that BCL-2 does not significantly contribute to intrinsic
BH3 mimetic resistance in solid cancers, and that BCL-2 selective inhibitors may be unlikely to improve the apoptotic responses of solid tumors to various drug therapies. Finally, we observed that cell lines from OV, COAD, and LUAD tumors were frequently insensitive to all combinations of BH3 mimetics, suggesting either lower overall degrees of apoptotic priming or different anti-apoptotic BCL-2 protein dependencies in these tumors. To distinguish between these two possibilities, we selected cell lines that were singly dependent on BCL-2, BCL-XL, or MCL-1 (single-gene), synergistically dependent on BCL-XL + MCL-1, or resistant to all tested BH3 mimetics. BH3 profiling was then performed on these cell lines using the BIM and PUMA peptides, which measure a cell’s ability to undergo apoptosis and overall BCL-2 priming, respectively (Supplementary Figure 3). Importantly, resistant cell
lines were still depolarized by the BIM peptide, indicating that they have intact BAX/BAK machinery and are capable of undergoing apoptosis. Similarly, the PUMA peptide also induced depolarization in the resistant cell lines, indicating that these cell lines indeed have some degree of dependence on BCL-2 family anti-apoptotic proteins. However, the single-gene dependent group of cell lines exhibited a significantly higher PUMA depolarization signal than either the synergistic or the resistant groups, indicating that this group of cell lines had an abnormally high amount of BCL-2 family priming. Taken together, these data indicate that resistant cell lines (i.e., cell lines that do not respond to any of the tested combinations of BH3 mimetics) are apopotopically competent and exhibit comparable overall BCL-2 family priming relative to the cell lines that synergistically respond to BCL-XL + MCL-1 co-inhibition. As such, these resistant cell lines likely rely on some combination of additional BCL-2 genes (e.g., BFL-1, BCL-w, etc.) which are not inhibited by the available BH3 mimetics. In summary, the results of this large-scale profiling effort reveal that many solid tumor types are dependent upon some combination of BCL-2 family anti-apoptotic proteins, including a large subset of tumors with targetable dependencies on BCL-XL, MCL-1, or the combination, motivating the search for specific biomarkers of these vulnerabilities.

Molecular determinants of single-gene dependencies. Next, we sought to establish the molecular determinants of the key responses observed in our cell line profiles: single agent BCL-XL and MCL-1 dependency and synergy between BCL-XL and MCL-1. Two of the most dominant phenotypic drivers in a given cancer are oncogenic mutations (oncogene or tumor suppressor status) and tissue of origin. As such, we first sought to determine which of these two properties best predicted BCL-2 gene dependencies. To address this question, we filtered our list of 78 cell lines to only those that are in the Cancer Cell Line Encyclopedia (CCLE) and therefore can be linked to publicly available genomic annotation data. These cell lines were then grouped based on their predominant BCL-2 gene dependencies (with a 25% viability loss threshold): BCL-XL dependent, MCL-1 dependent, BCL-XL + MCL-1 co-dependent, or mostly resistant (Fig. 2a). We selected 10 of the most commonly mutated genes in human cancers (TP53, PIK3CA, PTEN, KRAS, EGFR, NFI, BRAF, RBI, ATM, BRCA2, and BRCA1) and determined the status of each of these genes in all cell lines. To determine if tissue of origin or mutation status was a better predictor of dependency phenotype, we first performed a linear regression analysis (Supplementary Table 2) for each dependency phenotype (BCL-XL dependence, MCL-1 dependence, etc.), and the goodness-of-fit for each model (Fig. 2b) was used to determine the better predictor of response. In all cases, tissue of origin was a superior predictor relative to oncogene/tumor suppressor mutation status, as indicated by higher \( R^2 \) values.

A key, distinguishing feature of cells from distinct tissues are their unique gene expression patterns. We and others have previously demonstrated that expression levels of BCL-2 genes often correlate, and at times even drive, dependencies on various BCL-2 genes. For example, a recent report demonstrated that expression of NOXA elicited a dependence on BCL-2 in a subset of neuroblastomas\(^{17}\). We therefore hypothesized that tissue of origin may dictate BCL-2 family dependencies via its effects on BCL-2 family gene expression patterns. To determine if expression levels of BCL-2 genes predicted single-gene dependencies across diverse cell lines, we mined mRNA expression levels of the predominate BCL-2 family genes for each cell line, then performed a linear regression analysis to identify those strongly correlated with functional dependencies (Fig. 2c and Supplementary Table 3). In agreement with prior reports, we found that BCL-2 gene expression positively and strongly correlated with BCL-2 dependency across cell lines. MCL-1 dependency is strongly associated (anti-correlated) with BCL-XL mRNA levels, a finding consistent with recent data from our group and others\(^{18-20}\). Lastly, expression levels of NOXA strongly correlated with BCL-XL dependence, with BCL-XL dependent cell lines having higher expression levels of NOXA than non-dependent lines. To confirm the functional relevance of the identified associations relevant to BCL-XL and MCL-1 dependencies, we first stably transduced three cell lines with a strong MCL-1 dependence (Mia PaCa-2, H23, and SK-BR-3) using a BCL-XL overexpression construct, then treated cells with A-1210477 or the combination of this drug with WEHI-539 (Fig. 2d). Consistent with the correlation data, overexpression of BCL-XL reduced sensitivity to the MCL-1 inhibitor, resistance that could be reversed with the addition of the BCL-XL inhibitor. Similarly, three BCL-XL dependent cell lines (HT-1376, SCaBER, and T24) were stably transduced with shRNA targeting NOXA. Knockdown of NOXA expression attenuated sensitivity to BCL-XL inhibition, protection that could be reversed with the addition of A-1210477.

These findings demonstrate that across heterogeneous solid tumors from diverse tissue and oncogenic mutational backgrounds, sensitivity to single agent BCL-XL and MCL-1 inhibitors is strongly associated with the expression levels of defined BCL-2 family members. To determine whether the expression levels of these genes have predictive value, we used a sliding scale analysis to identify threshold expression levels of BCL-XL and NOXA that best segregated tumor cell lines on the basis of MCL-1 or BCL-XL dependency, respectively. This analysis identified an expression threshold for NOXA that robustly segregated WEHI-539 responsive and non-responsive lines (Fig. 2e, \( p = 0.005 \)). Further, this analysis also identified a BCL-XL expression threshold that robustly segregated A-1210477 responsive and non-responsive lines (Fig. 2e, \( p < 0.0001 \)). To independently validate this finding, we used data from a recent, large cell line chemogenomic profiling effort which determined the sensitivity of hundreds of cell lines to the dual BCL-2 and BCL-XL inhibitor ABT-263, among other agents\(^{18}\). The NOXA expression threshold identified...
above also effectively segregated sensitive and resistant lines ($p < 0.0001$), with lines expressing high levels of NOXA being more sensitive to ABT-263 than cell lines below the threshold, and sensitive lines exhibiting submicromolar median inhibitory concentration-50% (IC50) values (Fig. 2f). Together, these data demonstrate that expression levels of specific BCL-2 family members can be used to identify solid tumors sensitive and resistant to single agent BH3 mimetics, independent of tissue type or oncogenic mutational background.

**EMT underlies synergistic co-dependence on BCL-X$_L$+MCL-1.** Perhaps the most striking and unexpected finding from our cell line profiling effort (Fig. 1c) is the extent to which inhibiting BCL-X$_L$, MCL-1, or the combination is efficacious in solid
tumor-derived lines. Specifically, of the established cell lines for which genomic data are available, only 7 of 69 lines failed to respond (<20% viability loss) to some combination of these inhibitors. Approximately 50% of these responding lines exhibited single agent sensitivity to BCL-XL or MCL-1 inhibitors, while the remaining lines showed pronounced synergy between BCL-XL and MCL-1 inhibitors, with the combination being substantially more potent than expected on the basis of additivity alone (Fig. 3a). To identify signaling events that may underlie this BCL-XL/MCL-1 synergy, gene set enrichment analysis (GSEA) was performed (Fig. 3b). Surprisingly, this analysis revealed that the top four pathways associated with BCL-XL/MCL-1 inhibitor synergy were all related to the epithelial–mesenchymal transition (EMT). To further examine this relationship, we formulated “mesenchymal” and “epithelial” expression signatures (see Methods). Using these signatures, we observed that BCL-XL/MCL-1 inhibitor synergy is anti-correlated with the mesenchymal state and positively correlated with the epithelial state, both across individual cell lines (Fig. 3c) and in tissue-based groupings of cell lines (Fig. 3d).

To functionally confirm these findings, we targeted E-cadherin with lentiviral shRNAs to induce EMT via the release of β-catenin. In five cell lines from four tissues of origin displaying variable levels of BCL-XL/MCL-1 inhibitor synergy, EMT activation via E-cadherin knockdown significantly reduced synergy (Fig. 3e, f). Taken together, these data suggest that epithelial cells maintain a state wherein apoptosis is buffered by both BCL-XL and MCL-1, and inhibition of both molecules is required to sufficiently induce apoptosis. Upon transition to a mesenchymal state, this dual dependence is lost. Due to the robustness of this phenotypic switch, we next sought to understand the molecular mechanism(s) that might explain this finding.

**EMT increases dependence on BCL-XL through PERK signaling.**

Triggering an EMT could cause a decrease in the synergy associated with the BCL-XL/MCL-1 inhibitor combination by either causing a decrease in sensitivity to the combination or by increasing sensitivity to the BCL-XL or MCL-1 inhibitor alone. In the cell line panel above, forcing EMT via shE-cadherin transduction failed to either decrease sensitivity to the drug combination or increase sensitivity the MCL-1 inhibitor (Supplementary Fig. 4). However, an increase in sensitivity to the BCL-XL inhibitor was observed in all tested lines (Fig. 4a). Consistent with this finding, BCL-XL dependence was significantly and positively correlated with the mesenchymal signature across individual cell lines ($p = 0.0009$) and in tissue-based groupings of cell lines (Fig. 4b). To further test this correlation between mesenchymal status and BCL-XL dependence, we selected a canonical EMT gene,Slug (SNAI2), and probed for its expression via western blotting in 15 representative cell lines (Supplementary Fig. 5). Slug protein expression was correlated with BCL-XL dependence (Fig. 4c). Collectively, these data support a model where EMT causes a loss of BCL-XL/MCL-1 inhibitor synergy via an increase in dependency on BCL-XL alone. Thus, EMT status, as measured by a mesenchymal signature or Slug expression, predicts a cell’s position along the continuum between BCL-XL dependence and BCL-XL/MCL-1 synergistic co-dependence.

The regulation of apoptosis by BCL-2 family proteins is a tightly regulated process which can be altered dramatically by stochiometric changes in these proteins’ expression levels or cellular availability. Therefore, the simplest explanation for how EMT status could shift BCL-XL dependence would be that this process somehow impacts the expression/availability of a BCL-2 protein(s). Since BCL-XL and NOXA expression were the strongest correlates of BCL-XL/MCL-1 dependence, we first analyzed protein expression for these genes following induction of an EMT (via shE-cadherin). In PC-9 cells, E-cadherin knockdown did not affect BCL-XL protein expression, but caused a dramatic increase in NOXA protein expression (Fig. 5a). This finding is in line with our previous finding that knockdown of NOXA is sufficient to protect BCL-XL dependent cell lines from the effects of BCL-XL inhibition (Fig. 2d). While there are several signaling pathways that are known to induce NOXA, we sought to identify which of these pathways may also be regulated by EMT. Toward this end, a recent publication demonstrated that EMT signaling can activate the PERK signaling pathway (a component of the ER stress pathway). Importantly, several reports have also demonstrated that PERK/ER stress signaling can increase the expression of NOXA via a pathway involving activation of PERK, phosphorylation of eIF2α (which inhibits cap-dependent mRNA translation), induction of ATF4 and ATF3, and ATF4/ATF3-dependent induction of NOXA expression. Indeed, this pathway was induced in PC-9 cells transduced with multiple independent shE-cadherin hairpins, suggesting this might be the mechanism of NOXA induction (Fig. 5b). This hypothesis was confirmed by treating cells with the PERK inhibitor GSK2606414, which prevented the activation of the PERK signaling pathway and the induction of NOXA (Fig. 5c). Together, these data support a model in which epithelial cells depend synergistically on BCL-XL and MCL-1 for survival, whereas in mesenchymal cells the induction of the PERK signaling pathway, and consequently of NOXA, inhibits MCL-1, resulting in BCL-XL dependence (Fig. 5d).

**Resistance to BCL-XL+MCL-1 Inhibition from BFL-1 or BCL-w.**

Although most tested cell lines responded to some combination...
of BCL-2/BCL-X<sub>L</sub>/MCL-1 inhibitors, we noted a minority of lines that were innately resistant (<10% viability loss) to every combination. This implies that there are additional mechanisms that protect these cell lines from apoptosis, even when BCL-2, BCL-X<sub>L</sub>, and MCL-1 are inhibited. To find additional molecular targets to sensitize these resistant cell lines, we employed a negative-selection CRISPR screen using a library of sgRNAs that target a variety of cancer-related genes, including BCL-2 genes. The innately resistant cell line TYKNU was transduced with the CRISPR library at a low multiplicity of infection of 0.3, which
Fig. 3 EMT status predicts BCL-X\textsubscript{L}/MCL-1 synergy. a A heatmap of synergy scores (−0.5 to 0.99) for the combined inhibition of BCL-X\textsubscript{L}+MCL-1, compared to the BCL-X\textsubscript{L} or MCL-1 dependency scores (0-100% viability loss) from each line. Synergy was calculated according to the Bliss equation. b A pie chart showing the distribution of BCL-X\textsubscript{L}/MCL-1 dependent, BCL-X\textsubscript{L}/MCL-1 synergistically co-dependent, or insensitive cell lines. A GSEA was performed on cell lines ranked by the synergy score of the BCL-X\textsubscript{L}+MCL-1 co-inhibition and the top four enriched pathways are shown. c Mesenchymal and epithelial gene signature scores were calculated from each cell line and compared to the BCL-X\textsubscript{L}/MCL-1 synergy scores from each cell line in a Pearson correlation analysis. d Tissue-average mesenchymal/epithelial signature scores were calculated from each tissue type and compared to the tissue average BCL-X\textsubscript{L}/MCL-1 synergy score via Pearson correlation analysis. e Genetic validation of EMT status and BCL-X\textsubscript{L}/MCL-1 synergy. Three cell lines from the BCL-X\textsubscript{L}/MCL-1 synergy group (T47D, Hep G2, BT-474) and two resistant cell lines (Panc 08.13, PC-9) were forced to undergo an EMT via the genetic knockdown of E-cadherin. The transduced cell lines were treated with WEHI-539, A-1210477 or both for 3 days, and percentage viability loss was assessed via CTG. Synergy scores for all conditions were calculated (n = 3) to determine what effect forcing an EMT has on BCL-X\textsubscript{L}/MCL-1 synergy and data are presented as mean synergy scores ± SEM. A student’s t-test was performed between each shECADHERIN vs the shSCRAMBLE (* = p < 0.05). f An example of E-cadherin knockdown is shown via western blotting.

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Fig. 4 Mesenchymal status predicts BCL-X\textsubscript{L} dependence. a, Changes in BCL-X\textsubscript{L} dependence score in cells that underwent an EMT. The 5 cell lines that exhibited a decrease in BCL-X\textsubscript{L}/MCL-1 synergy (Panc 08.13, Hep G2, BT-474, T-47D, PC-9) following EMT were treated with WEHI-539 for 3 days. Percentage viability loss was assessed via CTG and the change in BCL-X\textsubscript{L} dependence was calculated by normalizing the shECADHERIN transduced cells (n = 3) to the shSCRAMBLE (n = 3) and data are presented as the mean change (LOG2) in BCL-X\textsubscript{L} dependence ± SEM. A student’s t-test was performed between each shECADHERIN vs the shSCRAMBLE (* = p < 0.05). b The tissue-average mesenchymal and BCL-X\textsubscript{L} dependence scores were compared via Pearson correlation analysis. c The cell line protein expression (see Supplementary Fig. 5) of SNAI2 (SLUG), one of the genes from the mesenchymal signature, was correlated to BCL-X\textsubscript{L} dependence or BCL-X\textsubscript{L}/MCL-1 synergy via a Pearson correlation analysis.

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reduces the likelihood of a single cell receiving multiple knockouts. Screens were performed in the presence of combined BCL-X\textsubscript{L} and MCL-1 inhibition given that no additional toxicity was conferred by BCL-2 inhibition (Fig. 1). After a 2-week treatment with the drug combination, samples were sent for sequencing and the depletion scores for each gene were calculated relative to the vehicle control sample (Supplementary Table 4). Interestingly, among the top sensitizers were two relatively understudied BCL-2 family members BFL-1 and BCL-w (Fig. 6a). The fact that these genes—which are not inhibited by any of the drugs used in the BH3 mimetic panel—scored as sensitizers suggests that they play important roles in preventing apoptosis in this cell line. To validate this finding, we used two individual sgRNAs for each gene to generate isogenic derivatives of TYKNU and two additional innately resistant cell lines (WiDR and A549), then subjected them to the BH3 mimetic panel (Fig. 6b, c). In TYKNU cells, knockout of either of these genes was sufficient to sensitize cells to varying degrees to inhibition of BCL-X\textsubscript{L}, MCL-1, and the combination, with BCL-w knockout having the most potent effects. Consistent with this finding, in both WiDR and A549 cells, BCL-w or BFL-1 knockout conferred sensitivity to BCL-X\textsubscript{L}, MCL-1, and the combination, with BCL-w knockout conferring particularly strong sensitization (Fig. 6b, c). Together, these data demonstrate that intrinsic resistance to selective inhibition of BCL-X\textsubscript{L}, MCL-1, and their combinations is mediated by the BCL-2 family members BFL-1 and BCL-w. Given that the knockout of these proteins led to complete viability loss in all intrinsically resistant cell lines following treatment with BH3 mimetics, these data provide a strong rationale for developing selective pharmacological inhibitors of these relatively understudied BCL-2 family proteins.

Finally, throughout the course of this study, we observed several cell lines whose responses to certain BH3 mimetic combinations are exceptionally strong or exceptionally weak relative to the rest of the cell lines in that tissue type (Supplementary Table 5). Given that our results demonstrate...
that sensitivity to BH3 mimetic combinations is largely dependent upon the expression of BCL-2 family genes, we assessed whether these exceptionally responsive or non-responsive cell lines were outliers with regard to BCL-2 family expression (Fig. 6d). Specifically, for each outlier cell line, we calculated the percentage difference in both BCL-2 dependence and BCL-2 family gene expression compared to the averages for cell lines from its tissue of origin. In several cases, outlier expression of BCL-2 family genes was consistent with the observed changes in BCL-2 dependence. For example, OCI AML3 cells are resistant to BCL-2 inhibition (compared to the other tested LAM1 lines) and also express higher than tissue average levels of MCL1 and BFL1 mRNA. To functionally validate a selection of these exceptional outliers, we selected two cell lines: H441 and Panc 03.27. H441 cells are highly dependent on BCL-XL and also express higher levels of NOXA compared to tissue average (LUAD), a result which would be expected to lead to BCL-XL dependence via MCL-1 suppression. Similarly, PANC 03.27 cells are also exceptionally dependent on BCL-XL and express higher levels of NOXA compared to tissue average (PAAD). In each case, shRNA-mediated knockdown of NOXA was sufficient to reverse BCL-XL dependence, reverting these cells to a state more resembling the tissue average (Fig. 6e, f). Thus, the relationships between BCL-2 family expression and sensitivity the BH3 mimetics identified in this study can be used to identify exceptional responders, a finding that may allow for the prospective identification of patients likely to respond to specific BH3 mimetic therapies. As a resource to guide the identification of additional outlier-expression relationships, we have broken down the heatmap of BCL-2 gene dependencies into separate “snapshot” figures based on tissue of origin, including mRNA expression data for key BCL-2 genes, in Supplementary Figures 6-15.

**Discussion**

The identification of ABT-737 as the first true BH3 mimetic initiated a paradigm shift in terms of how we think about targeting apoptotic machinery in cancer cells. In the first decade following this shift, great progress was made targeting BCL-2 in a variety of leukemias, ranging from CLL to LAML. Since the development of ABT-737, much research has focused on synthesizing new BH3 mimetics which can inhibit other BCL-2 family members, with a particular emphasis on BCL-xL and MCL-1. Although many of these putative BH3 mimetics were later found to work through non-specific pathways,22-25, several on-target inhibitors of BCL-xL (WEHI-539, A-1155463, A-1331852) and MCL-1 (A-1210477, S63845) have recently been described13,26,27. Importantly A-1155463, A-1331852, and S63845 have all demonstrated safety and efficacy in early in vivo models, suggesting that these agents (or derivatives thereof) may someday make it to the clinic. These findings have culminated in the first FDA approved BCL-2 inhibitor (venetoclax), which has
demonstrated clinical efficacy in hematological malignancies. However, analogous approaches targeting BCL-2 in solid malignancies have been far less successful, with only a handful of success stories17,28. Thus, there is a critical need to understand the contexts in which BH3 mimetics targeting BCL-2, BCL-XL, MCL-1, and other family members have activity, either as single agents or in defined combinations.

Here, addressing this need, we defined the landscape of BCL-2 gene dependencies in cancers derived from ten distinct tissues of origin. This effort identified both known and novel single-gene dependencies as well as combinatorial co-dependencies. Surprisingly, we observed unexpected cases of single gene dependencies across cell lines from a variety of tissues. For example, we identified a novel BCL-XL dependence in the majority of tested BLCA...
cell lines as well as roughly 50% of PAAD cell lines. These findings demonstrate that, although rare, acute sensitivity to single agent BH3 mimetics can occur in solid cancers. Further, we discovered that the expression levels of the BCL-2 family proteins BCL-2, BCL-XL, and NOXA are associated with, and functionally regulate, sensitivity to single agent BCL-2, MCL-1, and BCL-X1 inhibitors across tumors from diverse tissues of origin. These findings confirm and extend previous findings. For example, in LAML, it is appreciated that BCL-2 expression levels predict sensitivity to BCL-2 inhibition, and recent studies in breast cancer imply that BCL-X1 function is critical for sensitivity to MCL-1 inhibition3–6. Together, the discovery that solid tumors often harbor exquisite sensitivities to single agent BH3 mimetics targeting MCL-1 or BCL-X1, and the discovery that these dependencies can be accurately predicted based on the expression of the BCL-2 family members BCL-XL and NOXA, respectively, may have direct translational implications.

Another striking feature of this dependency landscape is the pervasive and synergistic efficacy of BCL-X1 + MCL-1 co-inhibition across cell lines from multiple tissues of origin. Specifically, this combination was synergistically active in roughly 50% of solid tumor cell lines, a discovery that extends the results of recent reports identifying a BCL-XL/MCL-1 co-dependency in breast and small cell lung cancers4,7. To better understand the basis for this synergy, we performed GSEA analysis, coupled with functional validation experiments, ultimately identifying a signaling pathway linking EMT to PERK signaling which culminates in increased NOXA expression and functional dependence on BCL-X1. Thus, mesenchymal-like cells have increased BCL-X1 dependence, whereas epithelial-like cells are characterized by synergistic co-dependence on BCL-X1 and MCL-1. It is well established that EMT can promote resistance to a variety of chemotherapies and cellular stresses through the acquisition of stem cell-like properties, including resistance to apoptosis, and recent reports imply that BCL-X1 may drive EMT-induced resistance to apoptosis19,29. Our results extend these findings by demonstrating that the process of EMT renders cells dependent on BCL-X1 for their survival. As such, although cells that have undergone an EMT are more resistant to diverse chemotherapies, they exhibit increased sensitivity to BCL-X1 inhibition. Finally, prior studies have established that the PERK-NOXA signaling pathway driving BCL-X1 dependence can be induced by specific chemotherapies, suggesting that these agents may be broadly useful as potentiators of sensitivity to BCL-X1 inhibitors31,12,4,30.

Finally, although the growing armamentarium of drugs targeting BCL-2, BCL-X1, and MCL-1 has broadened the landscape of tumors amenable to targeting with BH3 mimetics, our findings demonstrate that some tumors remain insensitive to the combination of BCL-2, BCL-X1, and MCL-1 inhibitors. The PERK inhibitor GSK2606414 was purchased from Tocris Bioscience and used at the indicated concentrations.

BH3 mimetic panel. Concentrations of ABT-199, WEHI-539, and A-1210477 were carefully selected to yield maximal inhibition (close to 100%) of their annotated targets without significant off-target effects. For ABT-199 and WEHI-539, 1 µM was used, and these concentrations are in line with those used by other groups20,22,33. A-1210477 is less potent than ABT-199 and WEHI-539 and thus a concentration of 10 µM was selected, which is consistent with previous reports5. A 500x stock of each of the following combinations of drugs was made at the selected concentrations: ABT-199, WEHI-539, A-1210477, ABT-199 + WEHI-539, ABT-199 + A-1210477, WEHI-539 + A-1210477, ABT-199 + WEHI-539 + A-1210477. All of these combinations yielded a roughly 50% DMSO concentration of 0.2%, and therefore 0.2% DMSO was used as a vehicle control in the panel. Cell lines were then seeded in triplicate at 5000 cells per well in Greiner white-bottom 96 well plates, and allowed to adhere overnight. The next day, cells were incubated with the BH3 mimetic panel and viability was assessed via Cell Titer Glo (Promega) after a 72 h incubation in drug. This drug incubation period was selected based on previous optimization experiments in our lab. Percentage viability loss was calculated as follows: 100%{1 – [(average signal for a given treatment) / (average signal from untreated wells)]}. This turned viability loss into a positive signal ranging from 0 to 99 (with 0 indicating no apparent viability loss) and this signal was used in the subsequent analysis and also represents a “dependence/dependency” score. For calculating synergy of the BCL-X1 + MCL-1 combination, we first divided all dependence scores by 100 to convert each value to a 0 to 1 scale, and used the Bliss formula: Synergy = (1 – BCL-X1 dependence) × (1 – MCL-1 dependence) – (observed BCL-X1 + MCL-1 co-dependence).

Methods

Cell culture and reagents. Refer to Supplementary Table 1 for a full list of all cell lines used in this study and their growth media. All commercially available cell lines were obtained from ATCC, except OVS400 which was obtained from the JRCB, tested for mycoplasma contamination, and authenticated with the Promega PowerPlex 18D kit for STR profiling. A subset of patient-derived cell lines were either generated at Duke University12 or at Johns Hopkins University13. All cell lines were grown at optimal confluence (no lower than 30% or higher than 80%) and were given fresh media every 2–3 days. For BH3 mimetics, ABT-199 (obtained from Selleckchem), WEHI-539 (obtained from ApexBio), and A-1210477 (obtained from Active Biochem) were used as BCL-2, BCL-X1, and MCL-1 inhibitors, respectively. The PERK inhibitor GSK2606414 was purchased from Tocris Bioscience and used at the indicated concentrations.

Statistical analysis: tissue vs. mutation status. To test if oncogene or tumor suppressor status correlated with BCL-2 gene dependency data, cell lines were first binned as either wild-type or mutant (including amplifications/deletions) for the following genes: TP53, PIK3CA, PTEN, KRAS, EGFR, NF1, RAF1, BAT2, and BRCA1. To investigate whether genetic mutation status or tissue of origin is a better predictor of drug effectiveness, we used the following linear model setting up:

\[ y = X\beta + \epsilon, \quad \epsilon \sim N(0, \sigma^2I) \]  

(1)

where \( y \) is an \( n \)-vector of drug sensitivity scores, \( X \) is an \( n \times p \) design matrix jointly holding both cell line tissue of origin and mutation status of select oncogenes, \( \beta \) is the corresponding \( p \)-dimensional vector of additive effect sizes, and \( \epsilon \) is normally distributed residual noise with variance \( \sigma^2 \) and identity matrix \( I \). For each single agent and drug combination, we compute a \( p \)-value denoting the significance of association for each tissue type and oncogene mutation of interest (See Supplementary Table 2). To further prove the robustness of these results, we also conducted an alternative analysis where we compared how well each biomarker explained the variation across the drug sensitivity scores (see “Goodness of fit”, Supplementary Table 2). Here, we used the following to compute an R-squared (\( R^2 \)) statistic, which assessed how well each data type fit model:1:

\[ R^2 = 1 - \frac{SS_{bet} + SS_{res}}{SS_{tot}} = \frac{\sum (y_i - \hat{y}_i)^2}{\sum (y_i - \bar{y})^2} \]  

(2)

where \( y_i \) is the mean of the observed drug sensitivity scores. Note the greater the \( R^2 \) statistic, the better the model fit.

Explaining drug sensitivity with gene expression. For cell lines included in the CCLE, mRNA expression data (“Gene-centric RMA-normalized mRNA expression data”) was obtained from the CCLE download portal (https://portals.broadinstitute.org).
CRISPR constructs. Brieﬂy, Qiagen plasmid mini-prep protocol. Cells were inoculated in LB amp for 16 h, and the plasmid DNA was prepped using the electroporated into e cloni bacteria, and spread over LB plates containing the packaging plasmids PSPAX2 (2.75 µg) and VSVG (0.275 µg) in 17 µL of protocol as per manufacturer. After 18 h, the media was replaced with 20 mL of harvest media (DMEM (Gibco) media was replaced with fresh media containing 2 µg/mL puromycin
to amplify each CRISPR and to attach sample-unique barcodes. Ninety ng of DNA from each barcoded sample were pooled and sent for deep-sequencing to deconvolute the chances of cells taking up more than one CRISPR construct. After selection in puromycin for ~10 days, cells were plated at >3 × 10^5 cells per condition (this ensures 100% coverage of each CRISPR construct). The conditions were performed in duplicate and were either 0.2% DMSO or 1 µM WEHI-539 + 5 µM A-1210477. After 2 weeks of treatment, DNA was harvested from each condition and cleaned via the Axygen Aspyrep Mag PCR Clean-up kit as per manufacturer’s protocol. The digoxigenin-labeled probes included an epithelial and mesenchymal gene signatures. For cell lines with mRNA expression data (CCL4 cell lines), we quantiﬁed an epithelial and mesenchymal gene signature for each cell line by comparing the levels of either selected epithelial or mesenchymal markers. For epithelial markers, we used: MUC1, CDH1 (E-cadherin), E-cadherin shRNA, BFL-1 CRISPR, BCL-w CRISPR; cells were plated in 96 well plates and treated with the BH3 mimetic panel as described in the above method section. Protein lysates were generated from each condition to provide examples of each genetic manipulation.

**Genetic manipulation using ORFs, shRNA, and CRISPR.** For overexpression of BCL-XL, we used a previously published V5-tagged BCL-XL ORF plasmid. An ORF containing HsRed was used as a control. The shRNA and CRISPR sequences used are listed in Supplementary Table 6. Functional CRISPR constructs were generated via the Gibson assembly as described. Brieﬂy, 5 µg of LC2 vector was digested with Esp3I restriction enzyme at 37 °C for 2 h, resolved on a 1% agarose gel, and extracted using a QiaGen gel extraction kit as per manufacturer’s protocol. sgRNAs were ampliﬁed using the NEB Phusion Hotstart Flex kit and the following PCR conditions: 98 °C° for 30 s, 75 °C° for 30 s, 22 °C° for 30 s × 12 cycles, 72 °C° for 3 min. The following primer arrays were used:

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**Gene set enrichment analysis.** To investigate gene set enrichment, we used the global gene expression needed to reliably predict that a given sample will exhibit dependence to a single agent. Here, we use a sliding window to bin cell lines into groups according to those that express associated genes above and below the speciﬁed threshold. We then use a t-test to compute a p-value which describes the degree of signiﬁcance between the two groups (p < 0.0001).

**Epithelial and mesenchymal gene signatures.** For cell lines with mRNA expression data (CCL4 cell lines), we quantiﬁed an epithelial and mesenchymal gene signature for each cell line by comparing the levels of either selected epithelial or mesenchymal markers. For epithelial markers, we used: MUC1, CDH1 (E-cadherin), E-cadherin shRNA, BFL-1 CRISPR, BCL-w CRISPR; cells were plated in 96 well plates and treated with the BH3 mimetic panel as described in the above method section. Protein lysates were generated from each condition to provide examples of each genetic manipulation.

**Protein lysates, western blotting, and densitometry.** Whole-cell protein lysate generation, protein quantitation via Bradford assay, and western blotting was performed as described. Brieﬂy, cell pellets were resuspended in lysis buffer, rotated at 4 °C for 10 min, and cleared of debris via 16,200 RCF centrifugation for 10 min at 4 °C. Lysates were then quantiﬁed using a standard Bradford assay and denatured using 3x NuPage buffer. Westerns blots were run on a NuPage gradient gel. The following antibodies were used at 1:1000 dilutions, except NOXA and ATF3 which were used at 1:1000: Vinculin (Cell Signaling Technologies #4600), BCL-XL (Cell Signaling Technologies #2764), NOXA (Santa Cruz Biotechnology #114207), E-Cadherin (Cell Signaling Technologies #3195), BCL-XL (Cell Signaling Technologies #2764), PERK (Cell Signaling Technologies #5683), p-eIF2α (Cell Signaling Technologies #3398), ATF4 (Cell Signaling Technologies #11815), ATF3 (Santa Cruz Biotechnology sc-188), p-actin (Cell Signaling Technologies #4970), BCL-w (Cell Signaling Technologies #2724), BFL-1 (Cell Signaling Technologies #14093), SLUG (Cell Signaling Technologies #9585). Rabbit or mouse HRP-conjugated secondary antibodies (#7074, #7076) were used at a dilution of 1:2000 and proteins were detected using standard ECL detection. To quantify relative expression of SLUG protein, we ﬁrst used densitometry to quantify SLUG and actin levels from each tested cell line. Normalized SLUG expression was then compared amongst all cell lines and these data were used for the Pearson Correlation analysis.

**BH3 proﬁling.** The BH3 proﬁling assay was performed as previously described. Brieﬂy, cells were digitonin permeabilized and incubated with ﬂuorescent mitochondrial dye (JC-1) and ﬂuorescent membranes were analyzed over time in a 384 well plate format. Mitochondrial priming was measured based on changes in depolarization over time. Peptides from the BH3 domains of BIM, PUMA, BAD, NOXA, and HRK were used to determine the priming states of the analyzed cell lines.

**Data availability**

All data are available upon request from the corresponding or lead authors.

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Author contributions

Conceptualization: R.S.S. and K.C.W.; data generation: R.S.S., E.L., M.L., A.A., G.R.A.; materials and methods generation: P.S.W.; writing of original draft: R.S.S., and K.C.W.; writing, review, and editing: all authors; funding acquisition: K.C.W. and R.S.S.; supervision: K.C.W.

Additional information

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