Therapeutic Synergy in Esophageal Cancer and Mesothelioma is Predicted by Dynamic BH3 Profiling

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ABSTRACT

Approximately 20,000 patients per year are diagnosed with esophageal adenocarcinoma (EAC) and malignant pleural mesothelioma (MPM); fewer than 20% survive five years. Effective therapeutic strategies are limited even though patients receive a combination of chemotherapeutics. These tumors harbor thousands of mutations that contribute to tumor development. Downstream of oncogenic driving mutations, altered tumor mitochondria promote resistance to apoptosis. Dynamic Bcl-2 homology-3 profiling (DBP) is a functional assay of live cells that identifies the mitochondrial proteins responsible for resistance to apoptosis. We hypothesized that DBP will predict which protein to target to overcome resistance thereby enhancing combinatorial therapy.

DBP predicted that targeting either Mcl-1 or Bcl-xL increases the efficacy of the chemotherapeutic agent, cisplatin, whereas targeting Bcl-2 does not. We performed these assays by treating EAC and MPM cells with a combination of BH3 mimetics and cisplatin. Following treatments, we performed efficacy assessments including apoptosis assays, IC\textsubscript{50} calculations, and generation of a combinatorial index. DBP confirmed that targeting mitochondria with BH3 mimetics alters the threshold of apoptosis. These apoptotic effects were abolished when the mitochondrial pathway was disrupted. We validated our findings by developing knockdown models of anti-apoptotic proteins Mcl-1, Bcl-xL, and the mitochondrial effector proteins Bax/Bak. Knockdown of Mcl-1 or Bcl-xL recapitulated the results of BH3 mimetics. Additionally, we report an approach for BH3 profiling directly from patient tumor samples. We demonstrate that the DBP assay on living tumor cells measures the dynamic changes of resistance mechanisms, assesses response to combinatorial therapy, and provides results in a clinically feasible timeframe.
INTRODUCTION

Esophageal adenocarcinoma (EAC) and malignant pleural mesothelioma (MPM) have poor 5-year survival rates of 10 – 20%.[1, 2] Effective therapy, especially targeted therapy, for EAC and MPM is limited.[3, 4] Both are treated with doublet chemotherapy. Even when treatment leads to tumor responses, these cancers usually develop resistance. The common link between these cancers is chronic exposure to the environmental carcinogens, bile acid and asbestos.[5-7] These carcinogens induce thousands of mutations which account for their aggressive phenotypes.[8-10] Whether treating with chemotherapy or targeting a specific mutation, these tumors circumvent death through other somatic mutations or bypass pathways to enable therapeutic resistance.[11] Therefore, treatment strategies that target these resistance mechanisms are urgently needed.[12]

Recently, we showed that chronic exposure of pre-neoplastic Barrett’s esophageal cells to bile salt induced malignant transformation through a mitochondrial mechanism termed, ‘Minority MOMP’ (mitochondrial outer membrane permeabilization).[5, 13, 14] MOMP is the critical event mediated by the colocalization of the membrane channel proteins, Bax and Bak, to the outer mitochondrial membrane which releases cytochrome c (cyt c) to activate the caspase system resulting in cell death.[15, 16] Minority MOMP activates the intrinsic pathway of the apoptotic machinery in roughly 8% of the cancer cells’ mitochondria, which is not sufficient to result in cell death. Instead, minority MOMP promotes genomic instability, cellular transformation, and tumorigenesis.[17-19] Since mitochondrial pathways are downstream of oncogenic driver proteins, targeting these pathways is a strategy that can overcome the mechanisms that enable tumor cell resistance.[15, 20]

MOMP is a switch-like event regulated by the balance of anti-apoptotic and pro-apoptotic B-cell lymphoma 2 (Bcl-2) family members [21]. Toxic stimuli may make cells more susceptible to MOMP; however, cells may withstand stressful stimuli and avoid apoptosis by upregulating anti-apoptotic proteins [22]. During Minority MOMP, we noted that the expression of the anti-apoptotic protein, Myeloid Cell Leukemia (Mcl)-1, doubled.[6, 23] Mcl-1 is a critical anti-apoptotic protein that prevents the activation of the intrinsic mitochondrial apoptotic pathway.[24-27] Bcl-xL is also a clinically promising target because it is highly expressed in both EAC and MPM.[5, 28] Mcl-1 and Bcl-xL may have redundant functions. Studies on Bcl-xL and McI-1 in EAC and MPM as the potential therapeutic targets are relatively limited.[24, 25]

Bcl-2 homology-3 (BH3) profiling is a functional assay of live cells that identifies which of these family of proteins are responsible for resistance to apoptosis.[29] The pro- and anti-apoptotic Bcl-2 proteins that regulate MOMP interact at hydrophobic BH3 domains.[22] BH3 profiling measures the relative interactions of these proteins to determine whether a tumor cell is near the threshold to activate apoptosis.[29-31] Dynamic BH3 profiling (DBP) is a live cell assay that measures changes in the BH3 profile before and after drug treatment to predict clinical response.[32] Drugs that inhibit Bcl-2 proteins are a class of compounds termed ‘BH3 mimetics.’[33] To be a BH3 mimic, a drug must selectively inhibit an anti-apoptotic protein, bind with high affinity, and induce MOMP in a Bak/Bax dependent manner. Recently, Villalobos-Ortiz and colleagues developed a biochemical ‘toolkit’ and utilized DBP to identify the appropriate BH3 mimetic in murine cells that overexpressed Bcl-2 proteins.[34]

Experience with DBP in solid tumors is limited and, to our knowledge, reports of DBP in EAC and MPM do not exist. Ni Chonghaile and colleagues reported that BH3 profiling successfully predicted response to chemotherapy in cell culture models.[31] Montero and colleagues noted that DBP predicted the emergence of resistance to multiple tyrosine kinase inhibitors (TKI) in lung cancer cells.[32] Bhola and colleagues reported that DBP predicts response in colon cancer.[35] Interestingly, they found that apoptotic sensitizers differed between tumors of the same histology. Priming of the mitochondria in solid tumors, especially EAC and MPM, is difficult secondary to variability of anti-apoptotic proteins per tumor, lack of response to single agent therapy, and difficulty with ex vivo culture of solid tumors. To overcome these obstacles,
we developed a proof-of-concept model wherein DBP can predict which BH3 mimic will sensitize tumor cells to cisplatin. We hypothesized that DBP profiling is predictive of EAC and MPM response to combined treatment with BH3 mimetics and cisplatin.

MATERIALS AND METHODS

Cell lines and treatments

EAC lines FLO-1, Eso26, and OE33 were purchased from European Collection of Authenticated Cell Cultures via Sigma Aldrich. Mesothelioma cell lines H28, H226, H2052, and H2452 were purchased from American Type Culture Collection. All cell lines were authenticated yearly with human leukocyte antigen (HLA) analysis and tested for mycoplasma contamination every six months. Cells were cultured in RPMI (ThermoFisher, 11875-093) with 10% fetal calf serum and 1% penicillin/streptomycin (Gibco #15140-122).

Cells were treated as described with Bcl-2 inhibitor, ABT199 (Apexbio)[36], Bcl-xL inhibitor, A1155463 (MedChemExpress)[37], or Mcl-1 inhibitor, S63845 (Active Biochem) and AZD-5991 (MedChemExpress) [33, 38], and CDDP (APP Pharmaceuticals, NDC 63323-103-65).

Primary cells

Tumor samples were placed on ice in serum-free RPMI (Corning, 15-040-CV). Tissues were minced and dissociated using the human tumor dissociation kit (Miltenyi Biotec). Samples were either incubated at 37 degrees with gentle rocking for one hour or dissociated using the gentleMACS system following the manufacturer’s protocol. The suspension was filtered through a 70 µm cell strainer (BD Falcon) and washed with PBS.

BH3 Profiling and Dynamic BH3 Profiling

Intracellular BH3 profiling has been described in detail previously [29]. Briefly, primary cells were stained with viability dye, LIVE/DEAD™ Fixable Aqua (Thermo Fisher), washed, then incubated with FcR blocking reagent (Miltenyi Biotec) and surface stained with PE-conjugated anti-human epithelial cell adhesion molecule (EpCAM) antibody (clone HEA-125, Miltenyi Biotec) or PE-conjugated D2-40 antibody (clone NC-08, Biolegend) and common leukocyte antigen CD45 antibody (clone HI30, BioLegend). Peptide treatments and controls were mixed at 2X desired concentration in MEB2 buffer + 20 µg/ml digitonin (Sigma, D5628) and 50 µl of this combination was added per well in a 96 well, non-binding plate (Celltreat, 229590).

Cells, in 50 µl MEB2, were incubated with peptides for one hour at room temperature then formaldehyde fixed. Anti-cyt-c antibody (Biolegend, 612308) was added at 1:2000 final dilution at 4°C overnight. Viable EpCAM or D2-40 positive and CD45 negative cells were analyzed by multiparameter flow cytometry (MACSQuant, Miltenyi Biotec). Retained cyt c was measured and percent release was calculated from the MFI of cyt c stain normalized to that of wells containing 25 µM alamethicin (ALM25) (Enzo, BML-A150-0005) as 100% release control. For cultured lines, cells were harvested using TrypLE Express (Gibco, 12605-010), washed twice in PBS, and added at 5 x 10⁴ cells per well in 50 µl of MEB2.

Lentiviral knockdowns

For double knockdowns, cells were transduced with commercially validated lentiviral shRNA targeting Bak (Sigma NM_001188, TRCN00000033466), Bax (Sigma NM_004324, TRCN0000312625), or scrambled sequences (Sigma #SHC002V) at multiplicity of infection (MOI)=5. The bulk population was...
cloned by limiting dilution. Stable pools were expanded under puromycin (Sigma #P9620-10ML) selection at 2 µg/mL. Individual clones were tested by qPCR and western blot to identify double knockdowns. Mcl-1 and Bcl-xL knockdowns were generated (Sigma #NM_021960, TRCN0000194663, #NM_001191. TRCM0000033499) using MOI=5 and grown in 2 µg/mL puromycin.

**Immunoblotting**

Cell lysates (10 µg) were resolved on 4%-20% SDS-polyacrylamide gels, electrotransferred onto nitrocellulose membranes, and blocked for 1 hour at 25°C in 5% nonfat dry milk / 0.1% Tween. Blots were incubated with antibodies at 4°C overnight, washed in 0.05% Tween, and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. The blots were washed and immunocomplexes were detected using SuperSignal West Femto (Thermo #34095). Antibodies: Bak (ab32371, Abcam), Bax (ab32503, Abcam), Bcl-2 (sc-492, Santa Cruz), Bcl-xl (sc8392, Santa Cruz), and Mcl-1 (sc819, Santa Cruz).

**IC50 and apoptosis assays**

Cells treated as indicated were stained with annexin V and PI according to manufacturer’s instructions (Takara, Cat# 630110). Apoptotic cells were analyzed with FACS Caliber (Becton Dickinson, Heidelberg, Germany) and AttuneNxT (Thermo Fisher). Apoptotic cell percentage corresponded to annexin V(+)/PI(−) cells. Data were analyzed using FlowJo™ 10.4.1 software.

IC50 curves were generated with 3000 cells/well in a 96 well plate. Cells were treated overnight with ABT199, A1155463, or S63845. Cisplatin was added the next day at indicated concentrations. 48 or 72 hours later, cell viability was assayed (CellTiter 96 AQueous One Solution, Promega).

**Tissue Arrays**

Using array slides of normal tissue and tumor (Biomax), IHC staining was performed. Using a standardized IHC intensity-scoring schema, a third-party pathologist (Vitro Vivo, Rockville MD) graded IHC staining as Negative (0), Low (25%), Medium (50%) or High (≥75%).

**Histological analyses and immunohistochemical staining**

Representative paraffin-embedded sections stained with hematoxylin-eosin (H&E) were analyzed by light microscopy. Immunohistochemical analyses of paraffin-embedded tissue sections were performed using a VECTASTAIN ABC kit (Vector Laboratories Inc.). Immunohistochemical staining for D2-40 (CellMarque Tissue Diagnostics, Mouse monoclonal antibody, pre-diluted, 0.13 µg/ml, Roche) was performed using standard immunohistochemical techniques for tumor specimens fixed with paraformaldehyde and paraffin-embedded.

**Immunofluorescence staining**

Cells grown on coverslips were fixed in 4% paraformaldehyde and permeabilized in PBS with 0.2% Triton X-100. After washing, cells were blocked in 3% BSA. Primary Mcl-1 (sc819, Santa Cruz), WT1 (NBP2-47858PE, NOVUS) conjugated with PE, pan-Cytokeratin (sc-8018, Santa Cruz) conjugated with PE, Vimentin (sc-66002, Santa Cruz) conjugated with FITC antibodies and TOMM20 (ab209606, Abcam) antibodies conjugated with Alexa Fluor 647 were diluted in blocking solution and incubated overnight at 4°C. DAPI was used as internal control for immunofluorescence. Images were collected with a Zeiss 780 confocal microscope.
Statistical Analysis

Quantitative data are presented as the mean ± SD. The Student *t* test compared means between two groups. One-way ANOVA followed by the *post hoc* Dunnett test compared means of more than two groups, and a multiple range least significant difference (LSD) was used for intergroup comparisons. Survival curves were plotted by the Kaplan-Meier method, and compared by the log-rank test (*, # = *P* < 0.05; **, ## = *P* < 0.01). Statistical analyses were performed with Graph Pad Prism.

RESULTS

Mcl-1 and Bcl-xL are highly expressed in EAC and MPM tumor samples and cell lines.

To investigate whether Bcl-2 family members are clinically relevant in human EAC and MPM, we examined the expression of anti-apoptotic proteins, Mcl-1, Bcl-2, and Bcl-xL, by IHC staining in microarray (TMA) slides. The TMA slides of 10 normal esophageal mucosa samples and 40 EAC tumors (Figure 1A and 1B) and 8 normal pleural and 100 MPM tumors (Figure 1C and 1D) revealed >50% expression of Mcl-1 in over 90% of tumor specimens in both histologies. Bcl-xL expression was higher in MPM compared to EAC tumor samples. Despite our hypothesis that function, not protein expression levels, are relevant for this mechanism, we assessed differences in survival based on expression from The Cancer Genome Atlas (TCGA). As expected, expression levels did not correlate with survival (Supplemental Figure S1).

While most cells undergo MOMP, not all exhibit this mechanism. Therefore, we confirmed Bax and Bak expression by immunoblots and found both proteins in all EAC and MPM cell lines tested (Figure 1E and 1F). Additionally, the anti-apoptotic function of Bcl-2, Bcl-xL, and Mcl-1 is often redundant. When we tested the protein expression levels, the expression differed significantly, yet at least one of the proteins was present in each line. In summary, these results show that the necessary proteins are present in EAC and MPM tissue arrays and cell lines for the intrinsic mitochondrial apoptotic pathway to function. Therefore, pursuing this mechanism to overcome treatment resistance is a clinically relevant strategy.

Targeting Mcl-1 or Bcl-xL selectively primes mitochondria and sensitizes EAC and MPM to cisplatin.

To determine whether targeting Bcl-2 family proteins sensitized cells to chemotherapeutics, we chose the well-established BH3 mimetics, S63845, A1155463, and ABT-199, which selectively target Mcl-1, Bcl-xL, and Bcl-2, respectively (Figure 2A). We performed DBP on EAC cells, FLO-1 and Eso26, treated with these drugs versus DMSO control (Figure 2B). The outcome measurement of DBP is cyt c release. On the heatmap, green represents a lack of cyt c release and red represents 100% release. When the cells were treated with S63845 that targets Mcl-1, cyt c release increased noted by the BAD peptide showing red with S63845 compared to green with DMSO. This effect also was noted for Eso26, but not as robust. Similarly, treatment with A1155463 resulted in increased cyt c release. In contrast, ABT-199 targeting Bcl-2 showed no differences in the cyt c release compared to DMSO. These findings suggest that targeting Mcl-1 or Bcl-xL, but not Bcl-2, primed the mitochondria.

To determine whether the results of DBP are phenotypically relevant, we asked whether priming the mitochondria changed the effects of cisplatin (CDDP) on the cells. We used CDDP because it is the most common backbone of chemotherapy regimens for EAC and MPM. We performed IC50 calculations for FLO-1 and noted that at the IC50 level, S63845 and A1155463, but not ABT-199, increased the sensitivity to CDDP (Figure 2C). With an annexin V assay to determine whether BH3 mimetics enhanced apoptosis with CDDP, we noted that combining S63845 or A1155463 with CDDP significantly increased apoptosis.
when compared to CDDP alone (Figure 2D). In contrast, ABT-199 in combination with CDDP was not different from CDDP alone. To further characterize apoptotic cell morphological changes, we used Hoechst 33342 to stain pyknotic nuclei which revealed that the relative apoptotic cells are consistent with the Annexin V analysis (Supplemental Figure S2). To determine whether the effects of S63845 where synergistic with CDDP, we performed a combinatorial index (CI) evaluation. We use the method of Chou and Talalay to quantify synergy between S63845 and CDDP.[39] The CI at different dose levels that caused growth suppression (fraction affected) revealed synergy between S63845 and CDDP at most doses in two cell lines. In contrast, only 1 out of the 8 doses showed synergy between ABT199 and CDDP (Supplemental Figure 3). We repeated the experiments in Eso26 and noted similar findings (Supplemental Figure S4A, 4B and 4C).

EAC and MPM have different embryologic origins, different cell surface IHC markers, and different sensitivities to drug treatment. Therefore, we repeated these experiments in the two MPM cell lines, H28 and H2452. S63845 or A1155463 increased mitochondrial cyt c release in the MPM cell line, H28, whereas ABT-199 did not (Figure 2E). Similar to EAC lines, the IC_{50} level decreased and relative apoptosis increased with S63845 or A1155463 but not ABT-199 (Figure 2F, 2G, and Supplemental Figure S2). Of note, the mitochondrial priming was reduced with both S63845 and A1155463, however, A1155463 was particularly effective noted by a high percentage of black and red on the heatmap. Additionally, the CI index revealed synergy between S63845 and CDDP in H28 (Supplemental Figure 3B). Similar results were noted with H2452 (Supplemental Figure 4E and 4F). Of note, the IC_{50} levels of MPM were substantially higher compared to EAC cells which is expected given that MPM response to therapy is particularly poor (Supplemental Figure S5).

Together, these results indicate that DBP was predictive of synergistic responses of Bcl-2 family inhibition with CDDP. The results of DBP are phenotypically relevant given that treatment with S63845 or A1155463 and CDDP, the effects of CDDP were enhanced. These results are specific given that when we treated the cells with ABT-199 and CDDP, no enhancement of CDDP effects were noted. In summary, DBP can predict which anti-apoptotic protein(s) to target to overcome therapeutic resistance.

**BH3 mimetic enhances the effects of CDDP through the intrinsic mitochondrial apoptotic pathway.**

As mentioned previously, a BH3 mimetic must induce MOMP in a Bak/Bax dependent manner. Therefore, we tested whether the results were abrogated when this pathway is disrupted. We developed double knockdowns (DKD) of Bak and Bax in two EAC cell lines, FLO-1 and OE33, by lentiviral transduction of shRNA (shBax/Bak) (Figure 3A and Supplemental Figure 6). We performed a DBP with the shBax/Bak versus non-targeted control (shCTL). Cyt c release was completely abrogated in the shBak/Bak cells (Figure 3B). When the shCTL cells were treated with S63845, cyt c release occurred. As further support, the heatmap with shBax/Bak displayed green with every peptide, which suggests that no baseline level of MOMP occurred without these proteins.

To confirm that results of DBP were functionally relevant, the cells were treated with S63845 and CDDP. If S63845 enhances the efficacy of CDDP via the intrinsic pathway, loss of Bak and Bax should abrogate apoptosis. Treatment of shBax/Bak cells with S63845 and CDDP alone or in combination resulted in complete loss of apoptosis compared to controls (Figure 3C). The lack of cyt c release in the DBP and the loss of apoptosis without Bak and Bax indicate that S63845 kills the cells through the intrinsic mitochondrial apoptotic pathway.

In addition to induction of MOMP in a Bax/Bak dependent manner, a BH3 mimetic must selectively inhibit an anti-apoptotic protein. Therefore, we established Mcl-1 and Bcl-xL knockdowns (shMcl-1, shBcl-xL) in FLO-1 cells by lentiviral transduction to determine whether these knockdowns phenocopied the effects of Mcl-1 and Bcl-xL inhibition (Figure 3D). The DBP revealed cyt c release from the shMcl-1 and shBcl-xL
cells was similar compared to drug treatment (Figure 3E). Again, to determine whether the cellular phenotype was similar, both shMcl-1 and shBcl-xL cells were treated with CDDP. The IC₅₀ level decreased with shMcl-1 versus shCTL (Figure 3F and 3H). Apoptosis significantly increased in the shMcl-1 or shBcl-xL cells (Figure 3G and 3I). These results show that drug treatment and protein knockdown of Mcl-1 or Bcl-xL have similar effects. As further support for the crucial role for Mcl-1, we tested Mcl-1 localization to the mitochondria and its inhibitory effect on MOMP. Mcl-1 localized primarily to the mitochondria of FLO-1 and H28 cells confirmed by its co-localization with mitochondrial marker TOMM20 (Supplemental Figure S7). In summary, these results indicate that the BH3 mimetics specifically enhances the effects of CDDP through the intrinsic mitochondrial apoptotic pathway in a Mcl-1 or Bcl-xL dependent manner.

**Dynamic BH3 profiling in Mesothelioma Tumor Specimens Reveals Patient-Specific Responses**

Given that targeting anti-apoptotic proteins may enhance the efficacy of standard therapeutics, we assessed whether Dynamic BH3 profiling (DBP) could be performed directly from fresh EAC and MPM tumor specimens. To our knowledge, DBP has not been performed in MPM or EAC, therefore, we first asked whether BH3 profiling could be performed in these histologies and whether the profiles differed between patients.

For patients with MPM, we performed operations called pleurectomy and decortications (Figure 4A). Specimens were sent to pathology for pathological confirmation of the laboratory findings. Both H&E and IHC are required for diagnosis; IHC revealed high expression of D2-40, WT-1, and Pan Cytokeratin (Figure 4B and 4C). The D2-40 protein enables FACS analysis to identify tumors from the stromal background which were performed on single cell suspensions for live, CD45-, D2-40+ cells to generate BH3 profiles (Figure 4D). We observed that Patient A, C and G showed a strong response to BAD and HRK BH3 peptides in addition to the ubiquitous sensitizer, PUMA. Patient B and E appear to be poorly primed, given the weak response to BH3 peptides, which suggests resistance to Bcl-2 antagonism. In summary, these results indicate that BH3 profiles can be developed directly from MPM specimens.

After performing BH3 profiles, we asked whether DBP can be performed in MPM samples when targeting the anti-apoptotic proteins with A1155463, ABT-199, and AZD-5991. Given that AZD-5991 is a Mcl-1 inhibitor that is currently in clinical trials, we switched from the cell culture model with S638452 to AZD-5991 to translate this work into clinical trials. We performed Live/Dead staining at 48 h post-surgery and noted that the majority of cells were alive (Figure 4E). Next, we performed IHC revealing high expression of WT-1, D2-40 and Pan Cytokeratin which confirms that the experiments are performed on MPM tumor cells (Figure 4F). Lastly, we performed DBP in MPM tumors with A1155463, ABT-199, and AZD-5991 (Figure 4G). The DBP indicate that mitochondrial priming in MPM is feasible and that the patterns differ between patients. Patient H showed about 50% cyt c release with AZD5991 but otherwise had relatively poor priming with BH3 mimetic. In contrast, Patient J and K appear Bcl-xL dependent as noted by a marked response to A1155463 with NOXAA, MS1 and HRK peptides. Patient K also had a >50% cyt c release with the BAD protein when treated with AZD5991 whereas patients I and J were poorly primed with AZD5991. To further confirm the predicted drug’s efficacy in Patient K, we combined the BH3 mimetics with CDDP which revealed significant increases in apoptosis with A1155463 or AZD-5991 and CDDP compared to ABT-199 or DMSO control (Figure H).

**Dynamic BH3 profiling in Esophageal Adenocarcinoma Specimens**

EAC is an epithelial tumor whereas MPM is a mesenchymal tumor; therefore, we established whether the methods developed for MPM would apply to EAC. Unlike MPM, patients with EAC receive neoadjuvant chemoradiotherapy prior to resection (Figure 5A). Therefore, tumor specimens are radiated and contain a high level of fibrosis. Regardless of this treatment, biopsies were sent for confirmation by standard H&E stains (Figure 5B). After dissociated into single cell suspensions, gating identified single, live, CD45-
population, and EpCAM+ cells. BH3 profiles were performed in three EAC patients which revealed variable patterns of cyt c release (Figure 5C). In contrast to MPM, the radiated specimens would not survive 48 h of ex vivo culture to perform DBP. However, one patient had metastatic EAC to the pleura which was biopsied and DBP was performed on this non-irradiated tumor. In the DBP of this patient, both A1155463 and AZD5991 primed mitochondria noted by about 50% increases in cyt c release. These results suggest that this patient is sensitive to both Bcl-xL and Mcl-1 inhibition (Figure 5D). Our results reveal the DBP may be successful in EAC patients, however, pre-treatment biopsies will be required given that irradiated specimens do not survive ex vivo.

**DISCUSSION**

Even when EAC and MPM tumors respond to systemic chemotherapy, resistance eventually develops in nearly all patients. Resistance develops regardless of whether patients receive standard chemotherapy or targeted therapy. Standard pathological assessment and even molecular assessments of solid tumors have produced significant advancements in cancer treatment. Yet, in vivo, cancer cells are dynamic and overcome most therapeutic strategies, even those that were initially successful. Current molecular diagnostic assays do not account for cellular plasticity, a common feature underlying tumor heterogeneity in situ.[40] The dynamic nature of these mechanisms permits tumor cells to adapt to therapy and acquire resistance. In order to account for the dynamic cellular changes, bioassays should measure changes in the cell when exposed to therapeutics. Measurement of molecular changes after therapy is particularly important in tumors that are no longer responding to therapy. Additionally, EAC and MPM rarely respond to single agent therapy; therefore, an ideal bioassay also should assess the response to multiple agents. Lastly, an effective bioassay must produce results rapidly enough for clinical decisions in patients whose tumors often progress quickly. Dynamic BH3 profiling (DBP) has the potential to achieve these goals. Therefore, we sought to develop a proof-of-concept model with DBP as a bioassay of living tumor cells in MPM and EAC that accounts for the dynamic changes in the resistance mechanisms, assesses response to combinatorial therapy, and provides results in a clinically feasible timeframe.

Given that DBP has promise in predicting response to therapy, we asked whether DBP predicts enhanced efficacy of BH3 mimetics in combination with standard therapy. Ni Chonghaile and colleagues reported that BH3 profiling successfully predicted response to chemotherapy in cell culture.[31] The authors observed that BH3 profiling predicted initial response to chemotherapy, which suggested that BH3 profiling is likely an actionable bioassay in solid tumors. Montero and colleagues advanced these findings when they noted that DBP predicted the emergence of resistance to multiple tyrosine kinase inhibitors (TKI) in lung cancer cells.[32] They showed that DBP performed at progressive time points correlated with emergence of resistance in lung cancer cells treated with TKIs over several months. Bhola and colleagues advanced these findings with solid tumors by screening patient-derived xenografts from colon cancer with multiple therapeutics. They noted that responses were highly patient specific. Despite these advances, whether DBP could predict targets that enhanced the response to standard therapy is unknown. Since EAC and MPM are rarely treated with single agents, we asked whether DBP could foretell which resistance protein to target in combination with CDDP treatment. We chose BH3 mimetics that directly target the anti-apoptotic proteins in the intrinsic mitochondrial apoptotic pathway as a proof-of-concept approach. This pathway is downstream of mutant driver proteins; therefore, should be effective regardless of mutational burden. We chose CDDP because it is the most commonly used chemotherapeutic in both EAC and MPM. We observed that the BH3 mimetics that altered the DBP predicted which drug enhances efficacy of CDDP. We noted that targeting Mcl-1 or Bcl-xL, but not Bcl-2, showed alterations in the DBP. Subsequently, we noted that targeting Mcl-1 or Bcl-xL increased the efficacy of CDDP whereas targeting Bcl-2 did not. These results show that DBP is predictive of combinatorial therapy and that directly targeting the anti-apoptotic proteins...
is a feasible approach. This approach will help identify a multi-drug treatment strategy for treatment refractory EAC and MPM.

Considering that BH3 mimetics appear effective with CDDP, we queried whether these effects were specific to the intrinsic mitochondrial apoptotic pathway. Villalobos-Ortiz and colleagues developed a biochemical ‘toolkit’ which is a methodology to ensure that the most selective and potent BH3 mimetics are identified.[34] They used DBP in conjunction with annexin V/Hoechst viability testing to ensure that the BH3 mimetics functioned as predicted based on their anti-apoptotic target. They defined a BH3 mimetic based on selectively inhibiting an anti-apoptotic protein, binding with high affinity, and inducing MOMP in a Bak/Bax dependent manner. They overexpressed Bcl-2 anti-apoptotic proteins in murine cells and treated with a panel of putative BH3 mimetics. However, whether DBP will predict which anti-apoptotic protein to target in solid tumor cells that do not overexpress these proteins is unknown. Therefore, we asked whether the effects of combinatorial therapy with BH3 mimetics and CDDP occurred by selectively targeting these anti-apoptotic proteins. By knocking down the effector proteins, Bax and Bak, we observed complete elimination of apoptosis in these cells. Next, to determine whether the BH3 mimetics were specific for Mcl-1 or Bcl-xL, as predicted by the DBP, we knocked down Mcl-1 or Bcl-xL and observed the same effects as blocking with a drug. Taken together, by blocking effector proteins and Mcl-1 and Bcl-xL, we observed that the combined effects were specific for the intrinsic mitochondrial apoptotic pathway.

To determine whether this approach is clinically feasible in MPM and EAC, we performed DBP directly from patient tumors. The majority of DBP have been performed on non-solid tumors such as leukemia and lymphoma. Leukemia and lymphoma are conducive to single cell suspensions when isolated from the blood. In contrast, solid tumors have a highly developed microenvironment that contains stromal cells, neovascular cells, immune cells as well as actual tumor cells. Bhola and colleagues reported DBP from two colon cancer resections.[35] They dissociated the tumors, treated for 24 hours, and performed a DBP on EpCAM+ cells.[31] Analysis directly from the tumor cells is critical because the other cells and especially the immune cells will generate DBP that will result in misleading information. Therefore, we isolated the CD45- cells to avoid performing DBP on immune cells and stained with D2-40 and EpCAM, respectively. Prior to the DBP, we performed BH3 profiles on 10 patients’ samples that revealed substantial heterogeneity between patients regardless of histology. This heterogeneity is consistent with the report by Bhola and colleagues who tested 11 drugs on each of the two colon cancer specimens and noted substantial differences in priming between the two specimens despite the same histology. After the initial BH3 profiles, we performed DBP on the MPM tumors which revealed different responses to BH3 mimetics per patient. These findings highlight the requirement for precision-based medicine with an effective bioassay such as DBP to overcome resistance in these tumors.

Our results show that DBP can screen drug combinations for synergistic effects. Additionally, we generated BH3 profiles in ten patients and DBP in four patients with two different solid tumors, but several limitations exist. Bhola and colleagues report high-throughput DBP to screen a larger number of drugs in PDX models.[35] While the PDX model generates reliable results, production of a PDX per patient sample requires several months, which is not a clinically feasible. Developing the high-throughput technique directly from patient samples is a future direction that is critical for clinical translation. Another critical limitation is post-therapy DBP. DBP was not feasible after tumor cells were irradiated, therefore, results from pre-treatment biopsies will be required for tumors that are treated with radiation.

EAC and MPM contain thousands of mutations per cell; therefore, targeting one mutation or mutant pathway is overcome by redundant mutations that enable cancer cell growth. Targeting mitochondrial pathways holds promise because mitochondria regulate cell death through the intrinsic pathway and serve as a critical center for multiple metabolic pathways essential for tumor growth. However, this approach
requires live cell assays that measure the alterations in mitochondria when treated with therapeutics. Therefore, a dynamic bioassay is necessary for this strategy to be successful. DBP achieves all of these goals. In summary, DBP is a bioassay of living tumor cells that measures changes in resistance mechanisms, predicts response to combinatorial therapy, and produces results rapidly enough for clinical feasibility. Our future goal is to develop this technology to generate clinically meaningful results within seven days of tumor biopsy (Figure 6).

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Figure 1: Expression levels of Bcl-2 protein family members in patient tissue and EAC and MPM cell lines. A. EAC samples were categorized into four levels of expression patterns, 10 normal esophageal tissues were compared with 40 tumor samples. B. Representative samples from IHC analysis of EAC samples microarray showing examples of expression of Bcl-2, Bcl-xL, and Mcl-1(scale bar =50 µm). C. MPM samples were categorized into four levels of expression patterns; 8 normal tissues were compared with 100 tumor samples. D. Representative samples from IHC analysis of MPM samples microarray showing examples of expression of Bcl-2, Bcl-xL, and Mcl-1. E. Immunoblot of Bcl-2 family expression in EAC lines. F. Immunoblot of Bcl-2 family expression in MPM cell lines.

Figure 2: Mcl-1 and Bcl-xL are involved in mitochondrial priming to enhance response to conventional treatment. A. Interaction of Bcl-2 proteins and BH3 mimetics: the function of Bcl-xL, Bcl-2 and Mcl-1 is to prevent the activation of Bax and Bak. A1155463 inhibits Bcl-xL, ABT-199 inhibits Bcl-2 and S63845 inhibits Mcl-1. B. Heatmaps showing DBP in FLO-1 cells with 1.0 µM A1155463, S63845 or ABT-199 pretreatment for 24 h. C. cells were treated with different concentrations of CDDP (0.02-25 µg/ml) for 48 h after pre-treatment for 24 h with 1.0 µM A1155463, S63845 or ABT-199; the IC50 values of CDDP on FLO-1 cells are determined. D. After pre-treatment with 1.0 µM A1155463, S63845 or ABT199 for 24 h, the apoptotic ratio in cells at 48 h after treatment with CDDP were measured by annexin V flow cytometry; cells positive for annexin V staining were counted as apoptotic cells (*p < 0.05; ** p < 0.01 for combination treatment versus DMSO; ## p < 0.01 for combination treatment versus A1155463, ABT-199 or S63845 single treatment). E. Heatmap showing DBP in H28 cells with 1.0 µM A1155463, 5.0 µM S63845 or ABT-199 pretreatment for 24 h. F. Cells were treated with different concentrations of CDDP (0.4-400 µg/ml) for 48 h after pre-treatment for 24 h with 1.0 µM A1155463, 5.0 µM S63845 or ABT-199; the IC50 values of CDDP on H28 cells are determined. G. After pre-treatment with 1.0 µM A1155463, 5.0 µM S63845 or ABT199 for 24 h, the apoptotic ratio in cells at 48 h after treatment with CDDP were measured by annexin V flow cytometry; cells positive for annexin V staining were counted as apoptotic cells (*p < 0.05; ** p < 0.01 for treatment versus DMSO; ## p < 0.01 for combination treatment versus A1155463, ABT-199 or S63845 single treatment).

Figure 3: BH3 mimetic enhances the effects of CDDP through the intrinsic mitochondrial apoptotic pathway A. Stable double knock-down (DKD) of Bax and Bak (shBax/Bak) or scrambled control (shCTL) were generated in FLO-1 and OE33 cells by transfecting short-hairpin RNA lentiviruses. The protein levels of Bax and Bak expression were analyzed by western blot. B. Heatmap shows DBP in FLO-1 and OE33 cells treated with or without S63845 for 24 h. C. FLO-1 cells were pretreated with S63845 for 24 h; the apoptotic ratio in DKD and shCTL cells at 48 h after treatment with CDDP measured by annexin V flow cytometry; cells positive for annexin V staining were counted as apoptotic cells (** p < 0.01 for treatment versus DMSO; ## p < 0.01 for combination treatment versus S63845 single treatment). D. Stable knock-down of Mcl-1 (shMcl-1), Bcl-xL (shBcl-xL) or scrambled control (shCTL) were generated by transfecting short-hairpin RNA lentiviruses. The protein levels of Mcl-1 and Bcl-xL expression were analyzed by western blot. E. Heatmap shows DBP in shMcl-1, shBcl-xL or shCTL FLO-1 cells. F. The IC50 values of CDDP with shMcl-1 or shCTL FLO-1 cells are determined. G. The apoptotic ratio in shMcl-1, shBcl-xL or shCTL FLO-1 cells after treatment with CDDP for 48 h, measured by annexin V flow cytometry; cells positive for annexin V staining were counted as apoptotic cells. H. The IC50 values of CDDP with shMcl-1 or shCTL H28 cells are determined. I. The apoptotic ratio in shMcl-1, shBcl-xL or shCTL H28 cells after treatment with CDDP for 48 h, measured by annexin V flow cytometry; cells positive for annexin V staining were counted as apoptotic cells. (## p < 0.01 for CDDP versus DMSO; ** p < 0.01 for combination treatment versus shMcl-1, shBcl-xL versus scrambled control).

Figure 4: Dynamic BH3 profiling in Mesothelioma Tumor Specimens Reveals Patient-Specific Responses. A. Upper panel showing gross findings of the MPM during surgery, lower panel showing the appearance of mesothelioma once removed. B. H&E staining of the resected specimen shows epithelioid
component with tubule formation and solid architecture (scale bar = 200 μm). C. Representative results of D2-40, Pan-Cytokeratin and WT1 immuno-histochemical staining on MPM specimens establish the diagnosis and correlate to surface markers used for FACS analysis (scale bar = 200 μm). D. Heatmap showing BH3 profiling in seven patients reveal patient-specific profiles. E. A live/dead assay performed in ex vivo cultured tumor cells after surgery 48h with 4% PFA fixed cells for positive control. Green fluorescence denotes viable cells stained with calcein-AM, while reddish-orange fluorescence represents dead cells stained with ethidium homodimer. F. Immunofluorescence double staining of pan-cytokeratin, D2-40 and WT1 (red) and vimentin (green) in ex vivo cultured Patient K tumor cells (scale bar = 200 μm). G. DBP performed in 4 patient tumors with 0.2 μM A1155463, AZD5991 and ABT-199 for 20h ex vivo. H. An annexin V assay reveals that pre-treatment with 0.2 μM A1155463, AZD5991 and ABT199 for 24 h prior to 48 hours of CDDP treatment primed the mitochondria as noted by the apoptotic ratios. Cells positive for annexin V staining were counted as apoptotic cells (*p < 0.05 for treatment versus DMSO; #p < 0.05 for combination treatment versus A1155463, ABT-199 or S63845 single treatment).

**Figure 5: BH3 profiling in EAC patients.** A. An esophageal adenocarcinoma of the distal esophagus after surgery. Left panel shows the gross appearance of the intact esophagus and the right panel shows the mucosal surface with the tumor. B. H&E staining of the resected specimen shows atypical cytologic features including increased nuclear/cytoplasmic ratio, pleomorphism, prominent nucleoli, and intraluminal necrotic debris (scale bar = 200 μm). C. Heatmap showing BH3 profilings in three patients with EAC. D. Dynamic BH3 profiling with 0.2 μM A1155463 and AZD5991 for 20 h ex vivo from a metastatic pleural implant that was not radiated with the primary esophageal tumor.

**Figure 6: Schema of cancer cell isolation from patient.** A. An endoscopy procedure involves inserting a long, flexible tube (endoscope) down the throat and into the esophagus. A camera on the end of the endoscope shows the esophagus. B. Endoscopic view of EAC. C. Positron emission tomography of EAC shows high glucose uptake. D. Appearance of esophagus after surgery. E. H&E staining of the resected specimen. A magnified view of black-boxed area reveals large tumor nest. F-G. Flow cytometry analysis of cancer cell subsets isolates single cancer cell suspensions labeled with cell surface markers produce distinguishable BH3 profiles upon gating.
Figure 1

A

B

C

D

E

F

Esophageal Cancer Lines

Mesothelioma Lines

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Figure 5
Molecular Cancer Therapeutics

Therapeutic Synergy in Esophageal Cancer and Mesothelioma is Predicted by Dynamic BH3 Profiling

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