MCL-1 is a key determinant of breast cancer cell survival: Validation of MCL-1 dependency utilizing a highly selective small molecule inhibitor.

Yu Xiao¹,*, Paul Nimmer¹, George S. Sheppard¹, Milan Bruncko¹, Paul Hessler¹, Xin Lu¹, Lisa Roberts-Rapp¹, William N. Pappano¹, Steven W. Elmore¹, Andrew J. Souers¹, Joel D. Leverson² & Darren C. Phillips¹, ³,*

¹ R&D, Oncology Discovery, AbbVie Inc., 1 North Waukegan Road, North Chicago, IL 60064, USA
² Oncology Development, AbbVie Inc., 1 North Waukegan Road, North Chicago, IL 60064, USA
³ To whom correspondence should be addressed. E-mail: Darren.Phillips@AbbVie.com
* Contributed equally

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Abbreviations

B-cell lymphoma protein-2, BCL-2; BCL-2 homology , BH; immunoprecipitation, IP;
mitochondrial outer membrane permeabilisation, MOMP; non-Hodgkin’s lymphoma, NHL ;
small interfering RNAs, siRNA;
Abstract

Hyperexpression of anti-apoptotic BCL-2 family proteins allows cells to survive despite the receipt of signals that would ordinarily induce their deletion, a facet frequently exploited by tumors. Tumors addicted to the BCL-2 family proteins for survival are now being targeted therapeutically. For example, navitoclax, a BCL-2/BCL-X<sub>L</sub>/BCL-W inhibitor, is currently in phase-I/II clinical trials in numerous malignancies. However, the related family member, MCL-1, limits the efficacy of navitoclax and other chemotherapeutic agents. In the present study, we identify breast cancer cell lines that depend upon MCL-1 for survival and subsequently determine the mechanism of apoptosis mediated by the MCL-1 selective inhibitor A-1210477. We demonstrate that apoptosis resulting from a loss in MCL-1 function requires expression of the pro-apoptotic protein BAK. However, expression of BCL-X<sub>L</sub> can limit apoptosis resulting from loss in MCL-1 function through sequestration of free BIM. Finally, we demonstrate substantial synergy between navitoclax and MCL-1 siRNA, the direct MCL-1 inhibitor A-1210477, or the indirect MCL-1 inhibitor flavopiridol, highlighting the therapeutic potential for inhibiting BCL-X<sub>L</sub> and MCL-1 in breast cancer.
Introduction

Programmed cell death or apoptosis is an evolutionarily conserved process that is critical for normal embryonic development and tissue homeostasis. Its dysregulation contributes to developmental malformations, neurodegeneration, autoimmunity and cancer (1). Apoptotic signals originate from a variety of cell stressors and converge at the mitochondria. Here, the BCL-2 (B-cell lymphoma protein-2) family of proteins function as guardians of cell fate, dictating the decision to live or die through the regulation of mitochondrial outer membrane permeabilisation (MOMP). The anti-apoptotic members BCL-2, BCL-X\textsubscript{L}, MCL-1, BCL-W, A1 and BCL-B contain four BCL-2 homology (BH) domains (BH1-4) and maintain cell integrity by directly binding and inhibiting the pro-apoptotic BCL-2 family members. The pro-apoptotic members can be further subdivided into the multi-domain BH1-3 proteins (BAX, BAK, and BOK) and BH3-only proteins (BIM, BIK, BID, BAD, BMF, BNIP3, HRK, PUMA and NOXA). Activation of BAX and/or BAK results in MOMP, cytochrome-c release and the commitment to apoptosis. Precisely how BAX and BAK are activated still requires elucidation, although it is likely to involve both direct activation by BH3-only proteins in addition to indirect activation through inhibition of pro-survival BCL-2 family members by BH3-only proteins (2) (3) (4) (5) (6) (7). Importantly, expression of functional BAX and BAK are required for further propagation of apoptotic signals from the mitochondria (8) and since neither individual loss of BAX nor BAK completely inhibits apoptosis, these two pro-apoptotic proteins are frequently considered to be functionally redundant (8), (9).

Given the central role of BCL-2 family members in governing cell fate, it is not surprising that their aberrant expression and function are associated with both the initiation and maintenance of the malignant state (10) (11) (12) (13) (14) (15). Oncogene addiction proposes that cancer cells rely on a single oncogene to permit cell proliferation in the absence of
apoptosis (16). Such a scenario has been described for BCL-2 and subsequently exploited therapeutically with the anti-tumorigenic agent navitoclax in non-Hodgkin’s lymphoma (NHL; (14) (15)). Navitoclax binds with high affinity to BCL-2, BCL-Xₐ and BCL-W, but not MCL-1 or A1, and induces apoptosis in many cancer cell types (17). Although single agent activity is reported in various cancer models and in clinical trials (18) (17) (19) (20) (21), expression of MCL-1 can limit the efficacy of this class of agents (19) (22) (23) and other anti-tumorigenic agents such as vincristine (24) and TRAIL (25). Additionally, MCL-1 is a key driver of the malignant state in certain tumors. For example, overexpression of Mcl-1 in mice results in the development of B-cell lymphomas (12) and hematopoietic stem/progenitor cell tumors, and co-operates with Myc in lymphomagenesis (13). Strikingly, MCL-1 copy number is amplified in numerous human malignancies (26) and its elevated expression is associated with high tumor grade and poor survival of breast cancer patients (27). It is therefore of considerable therapeutic interest to inhibit the expression and/or function of MCL-1 in cancer and in particular, breast cancer. We recently described the synthesis of small molecules that selectively inhibit MCL-1 function (28). Here, we describe the identification of breast cancer cell lines that are addicted to the oncogene MCL-1, evaluate the mechanism of cell death resulting from selective loss of MCL-1 function, and discuss the consequences for breast cancer therapy.

The data described herein identify a number of breast cancer cell lines that depend on MCL-1 for survival. MCL-1 dependency accurately predicted the efficacy of flavopiridol, an anti-tumorigenic CDK inhibitor described to downregulate MCL-1 expression. To further investigate the mechanisms of MCL-1 dependency, we utilized the recently described MCL-1 inhibitor A-1210477. Inhibition of MCL-1 function in cell lines identified to be MCL-1 dependent resulted in caspase-dependent apoptosis that required BAK but not BAX. However, MCL-1 gene expression alone did not predict susceptibility to apoptosis in response to loss of MCL-1 function. Rather,
we found the ratio of *MCL-1: BCL-X* to be critical. In this regard, BCL-X, but not BCL-2
overexpression afforded greater protection against apoptosis resulting from loss in MCL-1
function. Further, MCL-1 siRNA, A-1210477 or flavopiridol was synergistic with navitoclax.
Materials and Methods

Reagents, cell culture and treatment

Breast cancer cell lines obtained from the ATCC or DSMZ were cultured in the recommended media containing 10% FBS (Invitrogen Corporation, Carlsbad, CA, USA) and 10mM L-glutamine. All cell lines were tested for authenticity by short tandem repeat (STR) profiling by the AbbVie Core Cell Line Facility. For apoptosis assays and determination of JC-1 fluorescence, cells were plated at a density of 250 000 cells/well in 6-well plates, in cell viability assays at 0.05x10^6/ml, and for protein extraction assays and microarrays, 3x10^6 per 10cm² petri dish. A-1210477 was dissolved in anhydrous DMSO to a stock solution of 10mM and flavopiridol in DMSO at 100μM. After overnight attachment, cells were treated for up to 72 hrs with either vehicle alone, navitoclax, flavopiridol, or A-1210477 or the described combinations. Where indicated, cells were pretreated for 60 min with z-VAD-fmk (ICN). Navitoclax and A-1210477 were synthesized as described (28) (29). Unless otherwise indicated, all chemical reagents were obtained from Sigma Aldrich (St. Louis, MO, USA).

Small interfering RNA system and transfection

Small interfering RNAs (siRNA) were synthesized by Dharmacon Research Inc. (Lafayette, CO, USA). Breast cancer cells were transfected with 20nMol of siRNA utilizing Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The sense sequences of the siRNAs used are MCL-1 CDS: GCATCGAACCATTAGCAGATT (#2; CAT # D004501-11) and MCL-1 3’UTR: CGAAGGAAGUAUCGAAUUU (#5; Cat # J004501-17), with non-targeting siRNA duplexes used as a negative control (On-target plus non-targeting siRNA, Cat # D-001810-01-20). Additional MCL-1 siRNA sense sequences utilized were GGCAGTCGCTGGAGATTATTT (#1; Cat #
D004501-02), GCTAAACACTTGAAGACCAATT (#3; Cat # D004501-12) and GATTGTGACTCTTTCTTT (#4: Cat # D004501-13). The sense sequence of BCL-2 On-Target Plus siRNA is GGAGGAUUGUGGCCUUCUU (Cat # J-003307-18) and BCL-XL On-Target Plus siRNA is UUAGUGAUGGAAGAGAA (Cat # J-003458-14). Bim silencing was induced using SMARTpool ON-TARGETplus BIM siRNA (Cat # M004383-02).

Generation of MCL-1, BCL-2 or BCL-XL overexpressing or BAK-silenced cells

BAK silenced cells were generated as described elsewhere (30). Briefly, stable BAK silencing was attained via a short hairpin RNA for BAK (complementary sense and antisense oligonucleotides 5′-GGACGACATCAACCGACGC-3′) cloned into the retroviral vector pRETRO-SUPER (Oligoengine) with a puromycin selection cassette. The BCL-2 construct (pEF) was kindly provided by Dr. David Huang (WEHI, Australia; (31)). Flag-tagged Mcl-1 constructs were generated in the pLHCX retroviral backbone (Clontech, Mountain View, CA) from the human cDNA. The lentiviral construct pLOC-BCL-XL was obtained from Thermo-Open Biosystems (Pittsburgh, PA, U.S.A). All constructs were verified by dideoxy Sanger sequencing. Retroviral supernatants were produced through transfection of the GP2-293 packaging cell line (Clontech) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany). Lentiviral supernatants were produced with MISSION Lentiviral Packaging Mix (Sigma) in 293T cells. After 48hrs of culture, virus-containing supernatant was harvested and applied to SKBR3 cells in 75cm² culture flasks (0.5x10⁶ per flask) for a further 48hrs in the presence of polybrene (8μg/ml; Sigma). Cells were washed and selected after 3 days with 300μg/ml hygromycin B (Invitrogen; pLHCX constructs), 5μg/ml blasticidin (Sigma; pLOC constructs) or 1μg/ml puromycin (Sigma) in the case of transduction with pRETRO-SUPER or pEF constructs in the full
complement of media. The expression of BCL-2, BCL-XL, MCL-1 or BAK was confirmed by western blot analysis and compared to vector control transduced cells and the parental line.

**Cell Viability.**

Cells (0.05x10^6/ml) were treated in 96-well plates for 72hrs and cell viability determined by CellTiterGlo™ as described by the manufacturer’s instructions (Promega Corporation, Madison, WI, USA).

**Flow cytometry.**

Apoptosis was determined by flow cytometric evaluation of the Sub-G0/G1 content of DNA cell cycle histograms as described in detail elsewhere (32). Inner mitochondrial membrane potential was determined by flow cytometric evaluation of JC-1 fluorescence. Briefly, 15 minutes prior to the termination of drug incubation periods, cells were loaded with 2.5µg/ml JC-1 and incubated at 37°C in a humidified 95% air/5% CO2 atmosphere for 15 minutes. Samples were harvested, washed twice with ice cold PBS, and resuspended in 500µl of PBS containing 0.25% BSA. Samples were immediately analyzed for the ratio of JC-1 aggregates to JC-1 monomers by flow cytometry (BD FACSCalibur).

**Cytochrome c release assay.** Cytochrome c release from the mitochondria to the cytosol following cell treatment was determined as described elsewhere (33).

**Immunoprecipitations and Mesoscale Discovery (MSD)-ELISA assays.**

All protein lysates were extracted with 1% CHAPS buffer containing protease and phosphatase inhibitors (Roche and Sigma, respectively). For MSD-ELISA assays, streptavidin
multi-array 96-well plates (MSD, Gaithersburg, MD, USA) were used to immobilize Biotin labeled-anti-BCL-2 (US biological). Protein samples (75μg), were subsequently added to each plate in duplicate and incubated overnight at 4°C to pull down BCL-2 (US biological, Swampscott, MA, USA), BCL-X<sub>i</sub> (Abcam, Cambridge, USA), MCL-1 (LabVision/NeoMaker, Fremont, CA), all Biotin labeled. After 3 washes with PBS-Tween, anti-BIM (Epitomics, Burlingame, CA, USA) was added and incubated for 1hr in the dark at room temperature with rotation at 650rpm. Subsequently, sulfo-tagged goat anti-rabbit antibody (MSD) was added to each well and incubated for a further 30 minutes as above and then washed three times with PBS-Tween. Finally, 150mL of 2xMSD read buffer T was added per well and fluorescence measured with a MSD Sector Imager 6000. For immunoprecipitations, protein lysates (500μg; input) were subjected to immunoprecipitation with biotin labeled anti-MCL-1 (Labvision/Neomakers, Fremont, CA) or rabbit monoclonal anti-BAK antibodies (Epitomics, Burlingame, CA) overnight at 4 °C with rotation. Streptavidin beads or protein G sepharose were added to each lysate and incubated at 4 °C for a further 2 hrs with gentle rocking. Samples were then centrifuged for 30 seconds to separate beads and supernatant. Beads were subsequently washed three times with ice-cold 1% CHAPS buffer and then eluted with SDS sample buffer prior to western blot analysis as described below. Supernatants were collected for control western blots.

**BAX and BAK activation**

BAK activation was determined with an antibody that specifically recognizes the active form of BAK (clone TC-100; EMD Chemicals, USA) and detected with goat anti-mouse Alexa Fluor® 488 (Invitrogen) secondary antibody by flow cytometry. BAX activation was determined as described in detail elsewhere (33).
Western blot Analysis

After treatment, cells were detached with trypsin, centrifuged at 1,000 r.p.m. for 5min, and lysed in 50μl of ice-cold Cell Lytic™ (Sigma) supplemented with protease (Roche Diagnostics Corporation, Indianapolis, IN) and phosphatase inhibitors (Sigma). Protein concentrations were determined by the BSA assay (Invitrogen) and 50μg of protein electrophoresed by SDS-PAGE (Invitrogen). Separated proteins were transferred to nitrocellulose membranes utilizing iBlot® (Invitrogen). Blots were probed with anti-MCL-1 (clone S-19), anti-BAX (clone N-20; both Santa Cruz Biotechnology, La Jolla, CA, USA), anti-MnSOD (clone 19), anti-cytochrome C (clone 7H8.2C12), anti-PARP (clone C2-10), anti-BCL-2 (clone 7; all BD Biosciences, CA, USA), anti-BAK (clone Y164), anti-BIM (clone Y36) and anti-BCL-XL (clone E18; all Epitomics), anti-CASPASE-3 (clone 31A1067, Abcam) or β-ACTIN (Sigma), followed by IRDye 680/800CW-conjugated antibodies (LICOR Biosciences, Ne, USA). Proteins were visualized utilizing the Odyssey® infrared imaging system (LI-COR Biosciences).

RNA isolation, microarray analysis and copy number (CN) determination

Total RNA was extracted from cell lines using Qiazol lysis reagent and the RNeasy 96 universal tissue kit (Qiagen). RNA purity and integrity were checked on Agilent Bioanalyzer 2100. RNA used for microarray analysis with Affymetrix protocols. Whole genomic expression profile of the cell lines were obtained using Affymetrix HumanGenome U133A 2.0 arrays with 3 biological replicates, normalized with GCRMA algorithm and log2 transformed using Partek software.

Total DNA was extracted from cell lines in triplicates using Qiagen DNeasy kits. Genome-wide DNA profiles were obtained from 500ng of DNA using the Affymetrix Genome-Wide Human Genome-Wide Human SNP 6.0 arrays, per manufacturer’s instructions. Raw CEL files of
other cell lines were downloaded from Sanger Institute COSMIC cell line project. The raw CN was extracted from CEL files using pooled HapMap samples as a reference and smoothed by segmentation algorithm with Partek software.

The microarray expression and CN data of all breast cancer cell lines is available at GEO with accession number GSE66072.

**Statistical analysis**

Line and column graphs are presented as the mean ± s.e.m. The Bliss independence model was used to evaluate synergy (34). Spearman rank correlation co-efficient and associated statistical significance was determined using GraphPad Prism.
Results

The anti-apoptotic BCL-2 family protein MCL-1 has been identified as both an intrinsic and acquired resistance factor that limits the efficacy of a variety of anti-tumorigenic agents. However, over-reliance on a single anti-apoptotic gene to maintain survival, as has been described for BCL-2 hyperexpression in NHL, may actually serve as the cancer cell’s Achilles’ heel, a phenotype coined oncogene addiction. Selective inhibition of such oncogene targets is therefore a promising means of inducing cancer cell death (14) (15). In light of this hypothesis, we aimed to determine the MCL-1 dependency of a panel of breast cancer cell lines utilizing gene silencing. Interpretation of siRNA screens is often complicated by off-target activities. To address this issue, we first evaluated the efficacy of a panel of MCL-1 specific siRNAs in SKBR3 cells, correlating degree of MCL-1 silencing with degree of apoptosis (Supplemental Figure 1a). MCL-1 siRNA # 5 induced the most dramatic phenotypic response, targeting the 3’UTR of MCL-1. Overexpression of MCL-1 inhibited cell death induced by MCL-1 siRNA # 5, but did not rescue the cell death induced by MCL-1 siRNA that targeted the CDS (MCL-1 siRNA #2) as measured by caspase-3 activation and PARP cleavage (Figure 1a) or the appearance of sub-G0/G1 DNA content (Supplemental Figure 1b). Subsequently we screened a large panel of breast cancer cell lines for susceptibility to cell death following treatment with MCL-1 siRNA # 5. MCL-1 dependence was defined as a greater than 30% loss in cell viability upon MCL-1 siRNA treatment when compared to control-treated cells. 47% (15/32) of breast cancer cell lines were found to depend on MCL-1 for survival (viability < 70% following MCL-1 siRNA treatment; Figure 1b & Supplemental Table 1). Susceptibility to MCL-1 siRNA or MCL-1 copy number did not segregate according to breast cancer histological subtype (Supplemental Figure 2a & b). Gene expression analysis revealed that cell survival specifically correlated with the ratio of MCL-1:BCL-X\textsubscript{i} expression but not MCL-1:BCL-2 (Figure 1c), indicating that BCL-X\textsubscript{i} may act redundantly with
MCL-1 in maintaining the survival of breast cancer cells. Neither expression of *BCL-2* nor *MCL-1* alone correlated with breast cancer cell response to MCL-1 siRNA, although a weaker correlation was observed with *BCL-X*_i (*BCL2L1*) gene expression alone (Supplemental Figure 2c) than with the *MCL-1:BCL-X*_i ratio. Notably, *MCL-1* copy number did not predict breast cancer cell line susceptibility to cell death following treatment with MCL-1 siRNA (Supplemental Figure 2d).

SKBR3 was further validated as a MCL-1 dependent cell line through overexpression of BCL-X_i or BCL-2 (Supplemental Figure 1c & 1d). BCL-X_i overexpression limited apoptosis of SKBR3 cells treated with MCL-1 siRNA to a greater extent than that of BCL-2 overexpression, corroborating BCL-X_i expression as a factor that most dramatically alleviates the survival dependency of breast cancer cells for MCL-1 (Figure 1d). Furthermore, siRNA directed against *BCL-X*_i, but not *BCL-2*, sensitized breast cancer cell lines such as HCC-1806 to cell death as a result of *MCL-1* silencing (Figure 1e).

To further validate the dependence of breast cancer cell lines on MCL-1 for survival, we examined the effect of flavopiridol on MCL-1 dependent versus MCL-1 independent cell lines (determined herein with MCL-1 siRNA). Flavopiridol inhibits various cyclin dependent kinases (CDKs) and has been reported to reduce expression of MCL-1 (35) (36). Flavopiridol caused a rapid downregulation of MCL-1, to a similar extent and with similar kinetics, in both MCL-1-dependent (SKBR3) and MCL-1 independent (HCC-1806) cell lines (Supplemental Figure 3a). Importantly, apoptosis occurred with more rapidly in SKBR3 cells than in HCC-1806 cells. Cell death, as indicated by PARP cleavage and appearance of sub-G0/G1 DNA content, more closely paralleled loss of MCL-1 expression in SKBR3 cells than in HCC-1806 (Supplemental Figure 3a), recapitulating the rapid kinetics of apoptosis that result from MCL-1 gene silencing in SKBR3 (Supplemental Figures 3b & 3c).
Recently we described a new class of selective MCL-1 inhibitors that bind to the BH3 binding groove of MCL-1, including the small molecule A-1210477 ([28]; Figure 2a). A-1210477 treatment disrupted the association of BIM with MCL-1 in SKBR3 cells (Figure 2b), resulting in caspase-dependent apoptosis (Figure 2c). BIM siRNA reduced the expression of BIM in SKBR3 cells and inhibited A-1210477-induced caspase-3 activation (Figure 2d). This compound also induced classical features of mitochondrial-mediated apoptosis in MCL-1 dependent cell lines that were synonymous with apoptosis resulting from MCL-1 silencing, including cytochrome c release (Figure 2e) and loss of inner mitochondrial membrane potential (Figure 2f). As with MCL-1 directed siRNA, apoptosis induced by A-1210477 was substantially inhibited by BCL-XL overexpression but to a much lesser extent by BCL-2 overexpression (Figure 3).

Navitoclax is a small molecule inhibitor of BCL-2 and BCL-XL that is currently being assessed in phase-I/II clinical trials for the treatment of multiple malignant states. However, MCL-1 has been identified as a key resistance factor that limits the efficacy of this class of anti-tumorigenic agent (22) (19) (23), with approximately 66% (21/32) of breast cancer cell lines studied here being resistant to navitoclax (EC50>5µM; Figure 4a, & Supplemental Table 2). Reciprocally, the data described here implicate BCL-XL as a factor that limits breast cancer cell death in response to pharmacological blockade or genetic loss of MCL-1 function. Treatment of multiple breast cancer cell lines with A-1210477 sensitized them to navitoclax (Figure 4a) with significant synergy (Figure 4b) that was recapitulated with MCL-1 specific siRNA (Figure 4c, 4d & Supplemental Table 2).

The hierarchical interaction of the BCL-2 family members is under considerable debate and several models exist that attempt to explain their regulation of apoptosis. The “direct activation” model proposes that the anti-apoptotic BCL-2 family members act as a sink to sequester certain BH3-only proteins, thereby preventing their activation of BAX and/or BAK (2).
To further understand the mechanism of synergistic cell death induced by A-1210477 and navitoclax, we utilized HCC-1806 cells. HCC-1806 is resistant to apoptosis in response to single agent treatment with navitoclax, A-1210477, MCL-1 siRNA, BCL-2 siRNA or BCL-XL siRNA (Figure 4d & Supplemental Figure 4). However, simultaneously targeting BCL-XL with navitoclax or siRNA, and MCL-1 with siRNA or A-1210477, results in rapid apoptosis in this cell line (Figure 4 & Supplemental Figure 4). Treatment of HCC-1806 cells with navitoclax results in disruption of BCL-2:BIM and BCL-XL:BIM complexes that is accompanied by elevated MCL-1:BIM interaction (Figure 5a), consistent with a lack of cell death (Figure 4d). Conversely, treatment of cells with MCL-1 siRNA enhances BIM interaction with BCL-2 and BCL-XL without apoptosis induction (Figure 5b). In accordance with the binding mode of A-1210477 (28), treatment of HCC-1806 cells with A-1210477 disrupted MCL-1:BIM interaction and, as observed with MCL-1 siRNA, promoted the association of BIM with BCL-2 and BCL-XL, (Figure 5c) as well as the protection against MCL-1-mediated apoptosis (Figure 4). The total amount of BIM bound to BCL-2, BCL-XL and MCL-1 is not altered by BCL-2/BCL-XL inhibition with navitoclax, MCL-1 inhibition with A-1210477 or MCL-1 silencing (Figures 5d & e) and suggests that these anti-apoptotic factors act as redundant sinks to bind free BIM and prevent apoptosis. Only when BIM’s interactions with MCL-1 and BCL-XL (and/or BCL-2) are simultaneously disrupted (Figure 5d & e) does apoptosis ensue in HCC-1806 cells (Figure 4).

Expression of BAX and/or BAK is critical for the efficient propagation of apoptotic signals that converge at the mitochondria and regulate the release of mitochondrial related pro-apoptotic factors such as cytochrome c. Treatment of MCL-1 dependent SKBR3 cells with MCL-1 siRNA or A-1210477 resulted in upregulation of BAK protein levels (Figure 6a) independent of any changes in BAK mRNA (Figure 6b). Although caspase inhibition abrogated A-1210477-induced apoptosis (Figure 2c), z-VAD-fmk did not prevent BAK protein upregulation following
MCL-1 inhibition (Figure 6c). Both MCL-1 siRNA and A-1210477 induced BAK activation as visualized by flow cytometry using an antibody that specifically recognizes the active confirmation of BAK (Figure 6d). Treatment of MCL-1 dependent SKBR3 cells with A-1210477 resulted in dissociation of the MCL-1:BAK interaction (Figure 6e). Together, these data suggested that BAK is of significant importance to cell death that results from inhibition of MCL-1 function. We therefore generated SKBR3 cells with diminished BAK expression using shRNA that specifically targets BAK (Figure 7a). BAK silencing resulted in protection against apoptosis mediated by MCL-1 siRNA or A-1210477 in SKBR3 cells (Figure 7b). Interestingly, BAX siRNA did not protect against apoptosis resulting from A-1210477 or MCL-1 siRNA treatment in SKBR3 cells and did not afford further protection in the presence of diminished BAK expression (Figure 7c & 7d). BAX was only activated in MCL-1 dependent SKBR3 cells in the context of MCL-1 inhibition combined with navitoclax treatment (Figure 7e). These data in MCL-1-dependent cell lines indicate that cell death following loss in MCL-1 function requires BAK and not BAX.
Discussion

The mitochondria function as a point of convergence for apoptotic cell signaling initiated in response to a variety of physiological, environmental or pharmacological agents. BCL-2 family proteins regulate the decision to live or die at the mitochondrial level and their aberrant expression can contribute to the malignant state, providing a survival advantage by which cancerous cells can escape deletion and consequently flourish (2) (3) (7). Many lymphomas solely depend on the hyperexpression of BCL-2 for survival (14) (15), an “oncogene addiction” achieved through enhanced BCL-2 copy number or the t(14;18) chromosomal translocation (37) (38). Herein we utilize a highly selective small molecule inhibitor of MCL-1 to demonstrate that MCL-1 is a key determinant of breast cancer cell survival. Consequently we describe for the first time, the in vitro efficacy and mechanism of action of A-1210477 in breast cancer cell lines. We further define MCL-1 dependency as a BAK–dependent process. Additionally, we demonstrate synergy between A-1210477 and the BCL-2/BCL-X\textsubscript{L}/BCL-W inhibitor navitoclax in breast cancer cell lines, and describe the mode by which breast cancer cell lines are able to resist apoptosis by navitoclax treatment.

Utilizing MCL-1 specific siRNA, we found approximately 47% (15/32) of breast cancer cell lines to depend on MCL-1 for survival. Although the mRNA expression of any single anti-apoptotic factor was not predictive of susceptibility to MCL-1 siRNA, the ratio of MCL-1 to BCL-X\textsubscript{L} mRNA was. Overexpression of BCL-X\textsubscript{L}, but not BCL-2, conferred significant resistance to MCL-1 siRNA in the MCL-1 dependent cell line SKBR3, validating this correlative analysis and confirming similar findings reported in NSCLC (39). Moreover, gene silencing experiments confirm that synergy between loss in MCL-1 function and navitoclax is BCL-X\textsubscript{L} driven. Classification of breast cancer cell lines according to MCL-1 dependency was also predictive of susceptibility to flavopiridol, a CDK9 inhibitor and transcriptional repressor of MCL-1. MCL-1-dependent lines
were more sensitive to flavopiridol-mediated apoptosis, and cell death occurred more rapidly than in cells that do not depend upon functional MCL-1 for survival. Amplification of MCL-1 (>3 copies) was present in 44% (14/32) of breast cancer cell lines studied (Supplemental Table 1), reflecting data obtained in primary breast cancer samples (26). However, MCL-1 copy number did not predict dependency upon MCL-1 for survival in breast cancer cell lines treated with MCL-1 siRNA (Supplemental Figure 2d). Similarly, MCL-1 copy number or susceptibility to MCL-1 siRNA did not segregate according to breast cancer sub-type (Supplemental Figure 2a & 2b).

We recently described the development of highly selective inhibitors of MCL-1 (28) that can be further utilized to examine the mechanism of cell death that results from inhibition of MCL-1 function. A-1210477 induced apoptosis in a mechanistically identical manner to death resulting from MCL-1 gene silencing. A-1210477 and MCL-1 siRNA induced caspase-dependent apoptosis in SKBR3, a MCL-1 dependent cell line, which was associated with the release of mitochondrial cytochrome c, loss in ΔψM and BAK activation. BAK has been described to associate with MCL-1 (40) (41) (42) and in vivo deletion of BAK rescues the survival of Mcl-1 deficient murine thymocytes (43). Accordingly, we found that apoptosis induced via a loss in MCL-1 function required BAK expression. Furthermore, BCL-XL overexpression in SKBR3 prevented MCL-1-mediated apoptosis to a greater extent than that observed with BCL-2 overexpression, further substantiating the notion that the ratio of MCL-1:BCL-XL gene expression and not the ratio of MCL-1:BCL-2 is a determinant of MCL-1 dependency in breast cancer cell lines. These conclusions are consistent with previous data indicating that BAK does not significantly bind BCL-2 but is sequestered by MCL-1 and BCL-XL (44). Similarly, BCL-2 overexpression does not rescue MCL-1-deficient mouse thymocytes (43). BAX was not activated by A-1210477 or MCL-1 siRNA, and BAX silencing did not prevent the ensuing cell death, consistent with the low binding affinity of MCL-1 for BAX and the fact that overexpression of
MCL-1 does not block BAX-induced apoptosis (45). Unlike in vivo deletion of BAK, BAX deletion does not rescue MCL-1-deficient thymocytes (43). BAX was only activated upon loss of MCL-1 function in the context of coincident BCL-2/BCL-X<sub>L</sub> inhibition with navitoclax. Collectively, these data indicate that apoptosis resulting from a loss in MCL-1 function requires BAK and not BAX and that these two gatekeepers of the intrinsic apoptotic pathway do not foster complete functional redundancy in breast cancer cell lines.

We used three approaches to inhibit MCL-1 function: MCL-1 gene silencing with specific siRNA, direct inhibition with A-1210477, and indirect knockdown through transcriptional repression of MCL-1 expression with the CDK9 inhibitor flavopiridol (Supplemental Figures 3 & 5). Inhibition of MCL-1 function was synergistic with navitoclax in multiple breast cancer cell lines irrespective of the approach. Utilizing the cell line HCC-1806, a cell line that does not depend solely on MCL-1 for survival and is resistant to navitoclax (EC<sub>50</sub> > 5 μM), we demonstrate a mechanism by which the anti-apoptotic BCL-2 family members are capable of compensating for loss in expression or function of a related anti-apoptotic protein. Inhibition of MCL-1 function pharmacologically with A-1210477 or genetically with MCL-1 siRNA resulted in redistribution of the BH3-only protein BIM from MCL-1 to BCL-2 and BCL-X<sub>L</sub>. Thus, in response to a loss of MCL-1 function, BCL-2 and BCL-X<sub>L</sub> collectively acted as a sink to sequester displaced BIM and prevent the initiation of apoptosis. This process serves to prime the cells for BCL-2/BCL-X<sub>L</sub>-dependent death as evidenced by their subsequent vulnerability to navitoclax. Conversely, MCL-1 serves as a sink to bind free BIM upon BCL-2/BCL-X<sub>L</sub> inhibition with navitoclax, making these cells impervious to navitoclax-mediated apoptosis. Notably, A-1210477 did not activate BAK in MCL-1-independent cells despite being able to displace BIM (data not shown).

Collectively our data also potentially inform on the hierarchical interaction of the BCL-2 family members. Several models have been proposed to describe their dynamic interactions...
that consequently modulate apoptotic function. We hypothesize that a balance between MCL-1 and BCL-X\textsubscript{L} expression, and therefore the status of MCL-1 dependency, can switch the mode by which the BCL-2 family of proteins interact to initiate apoptosis: from mode 2 in MCL-1 dependent cells to mode 1 in those cells dependent upon two or more anti-apoptotic BCL-2 family members for survival (2) (5) (6) (7). The combined use of highly selective inhibitors of BCL-2 (ABT-199; (46)), BCL-XL (A-1155463; (47)) or MCL-1 as described herein, may facilitate resolution of the complex question as to precisely how BCL-2 family members interact to dictate cell fate and hence therapy in specific clinical oncology settings.

Taken together, these data provide a pre-clinical rationale for targeting MCL-1 in breast cancer. We have demonstrated MCL-1 dependency in a significant proportion of breast cancer cell lines utilizing gene silencing techniques and have validated our observations with the selective MCL-1 inhibitor A-1210477. Subsequently, we demonstrated that apoptosis resulting from a loss in MCL-1 function requires BAK and not BAX. Expression of BCL-X\textsubscript{L} provides a means by which breast cancer cell lines can escape MCL-1 dependent death, although this leaves them susceptible to navitoclax treatment. In this regard, we demonstrate substantial apoptotic synergy between agents that impair MCL-1 function and navitoclax in breast cancer.
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Figure legends

Figure 1 The efficacy of MCL-1 siRNA on the viability of breast cancer cell lines correlates with the ratio of MCL-1:BCL-X<sub>i</sub> expression. SKBR3 cells were treated with siRNA that specifically targets either the 3'UTR (#5) or coding sequence (CDS; #2) of MCL-1 or scrambled siRNA (Sc; all 20nMol) for 24hrs. The effects on MCL-1, PARP, caspase-3, β-actin and PARP protein were determined by western blot (a). The described breast cancer cell lines were treated with MCL-1 siRNA (#5; 20nMol) for 72hrs and the effect on viability determined using CellTiterGlo™. The effect of Sc siRNA compared to non-treated cells is described in Supplemental Table 1. Data are presented as the mean ± s.e.m. of at least three independent experiments (b). The expression of MCL-1:BCL-X<sub>i</sub> or MCL-1:BCL-2 mRNA obtained from a panel of breast cancer cell lines are shown correlated with the viability of breast cancer cell lines treated with MCL-1 siRNA (#5; c). SKBR3 cells overexpressing BCL-2 or BCL-X<sub>i</sub>, the vector ctrl (Vct Ctrl) or parental cell line (wild type, WT) were treated with MCL-1 siRNA (#5) or scrambled siRNA (Sc) for 24hrs and the percentage apoptosis determined from the sub-G0/G1 content of DNA cell cycle histograms by flow cytometry. Data are presented as the mean ± s.e.m. of three independent experiments. Western blots confirm the degree of BCL-2 or BCL-X<sub>i</sub> overexpression and degree of MCL-1 silencing (d). HCC-1806 cells (MCL-1 independent) were pre-treated with BCL-2 or BCL-X<sub>i</sub> specific siRNA prior to exposure to MCL-1 specific siRNA (#5; all 20nMol) for a further 24hrs. The expression of MCL-1, BCL-2, BCL-X<sub>i</sub>, caspase-3, β-actin and PARP protein were determined by western blot and apoptosis determined by flow cytometry (e).

Figure 2 The MCL-1 inhibitor A-1210477 inhibits MCL-1:BIM interaction and induces classical features of apoptosis that are synonymous with MCL-1 silencing. The chemical structure of the MCL-1 inhibitor A-1210477 is shown in (a). SKBR3 cells were treated with the MCL-1 inhibitor A-1210477 for 4hrs and the interaction of BIM with MCL-1 determined by immunoprecipitation (IP) followed by western blot. Western blots of the IP input and supernatants are shown (b). SKBR3 cells were pre-treated with the caspase inhibitor z-VAD-fmk (75µM) for 1 hour prior to treatment with A-1210477, MCL-1 siRNA (#5, 20nMol) or scrambled siRNA (Sc, 20nMol) for a further 24hrs and the induction of apoptosis compared to the non-treated controls. The degree of MCL-1 silencing in the presence or absence of z-VAD-fmk is shown by western blot, along with the associated changes in caspase-3 & PARP cleavage (c). SKBR3 cells were pre-treated with
BIM siRNA or Sc siRNA (both 20nMol) for 48hrs prior to treatment with A-1210477 for an additional 6hrs, and expression of BIM, MCL-1, caspase-3 and β-actin determined by western blot (d). Cytochrome C (Cyt C) release (e) or dissipation of the inner mitochondrial membrane potential utilizing JC-1 (2.5µg/ml; f) were determined in SKBR3 cells treated with Sc siRNA (20nMol), MCL-1 siRNA (#5; 20nMol) or treatment with A-1210477 for 6hrs. MnSOD and BAX expression were also determined and serve as loading controls.

**Figure 3 BCL-X\textsubscript{L} limits the efficacy of A-1210477 to a greater extent than BCL-2.** SKBR3 cells overexpressing BCL-X\textsubscript{L} or BCL-2 were treated with A-1210477 for 24hrs and the percentage apoptosis determined from the sub-G0/G1 DNA content of DNA cell cycle histograms and compared to vector control (Vct Ctrl) or parental cell lines (wild type, WT) . Data are presented as the mean ± s.e.m. of three independent experiments.

**Figure 4 Inhibition of MCL-1 function sensitizes breast cancer cell lines to navitoclax.** A panel of breast cancer cell lines were co-treated with the MCL-1 inhibitor A-1210477 (0, 2.5, 5, & 10µM) and navitoclax for 72hrs in 10% FBS. The effect on cell viability was determined with CellTiterGlo™ and synergy determined by Bliss analysis. Typical dose-response curves are shown in (a) and the Bliss sums (synergy) shown in (b). Alternatively, HCC-1806, MDA-MB-231, DU-4475 & SKBR3 cells were treated with navitoclax in the presence or absence of MCL-1 or scrambled (Sc) siRNA for 72hrs and viability determined with CellTiterGlo (c). The effect of MCL-1 siRNA on the efficacy of navitoclax in an extended panel of breast cancer cell lines is depicted in Supplemental Table 2. HCC-1806 (MCL-1 independent) cells were pre-treated with MCL-1 (#5) or scrambled (Sc) siRNA (20nMol each) prior to navitoclax exposure and the effect on apoptosis determined by western blot or flow cytometry (d). Alternatively, HCC-1806 cells were co-treated with navitoclax and the MCL-1 inhibitor A-1210477 at the described concentrations for 24hrs and apoptosis quantified by flow cytometry (e). Data are presented as the mean ± s.e.m. of three independent experiments.

**Figure 5 BIM redistribution mediated by loss in function of anti-apoptotic BCL-2 proteins.** HCC-1806 cells were treated for 6hrs with navitoclax (1µM; a), MCL-1 siRNA (#5; 20nMol; b & d) or A-1210477 (10µM; c & e). Experiments in (d) & (e) were performed in the presence of navitoclax (1µM). The interaction of BIM with BCL-2, BCL-X\textsubscript{L} or MCL-1 were assessed using a MesoScale
ELISA as described in the materials and methods. Scrambled siRNA (Sc; 20nMol) is used as a control for all siRNA experiments, and all data are normalized to non-treated controls. Data are presented as the mean ± s.e.m of three independent experiments.

**Figure 6 Loss of MCL-1 function modulates BAK expression.** SKBR3 cells were treated with MCL-1 siRNA (#5), scrambled siRNA (Sc; both 20nMol) or the MCL-1 inhibitor A-1210477 for 8hrs and the effect on MCL-1, PARP and BAK determined by western blot analysis (a) or by RTPCR (b). Additionally, SKBR3 cells were treated for 24hrs in the presence or absence of z-VAD-fmk (75µM) for 24hrs, and the effect on BAK and PARP expression determined by western blot (c). SKBR3 cells treated with MCL-1 siRNA (#5), Sc siRNA (both 20nMol) or the MCL-1 inhibitor A-1210477 for 6hrs and BAK activation determined by flow cytometry using an antibody that detects BAK in its activated confirmation (d). SKBR3 cells were treated with the A-1210477 for 6hrs and the interaction between BAK and MCL-1 determined by immunoprecipitation (IP) followed by western blot analysis. Western blots of MCL-1 and BAK expression from the total SKBR3 cell lysate before (Input) and after (Supernatant) IP are also shown (e).

**Figure 7 BAK is required for apoptosis resulting from loss of MCL-1 function.** SKBR3 cells stably expressing BAK shRNA were generated and expression compared to the vector ctrl (Vct Ctrl) and parental cell line by western blot (a). Wild type, Vct Ctrl and BAK shRNA expressing SKBR3 cells were treated with A-1210477, scrambled siRNA (Sc) or MCL-1 siRNA (#5; both 20nMol) for 24hrs and the effect on apoptosis determined by flow cytometric quantification of the sub-G0/G1 DNA content of DNA cell histograms. MCL-1 silencing was confirmed by western blot (b). Vct and BAK shRNA SKBR3 cells were pre-treated with BAX siRNA (20nMol) or Scrambled siRNA (Sc; 20nMol) for 24hrs prior to treatment with A-1210477 (c) or MCL-1 siRNA (20nMol; d) for a further 8hrs. Proteins were subsequently extracted and the expression of BAK, BAX, MCL-1, β-actin & PARP determined by western blot (c & d). Alternatively, SKBR3 cells were treated with A-1210477 and navitoclax for 8hrs and BAX activation determined by immunoprecipitation with activated BAX antibody (clone 6A7) and BAX expression determined by western blot (e).
Figure 1; Xiao et al.
Figure 2; Xiao et al.,
Figure 3; Xiao et al.
Figure 4: Xiao et al.,
Figure 5; Xiao et al.,
Figure 7; Xiao et al.
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Yu Xiao, Paul Nimmer, George S Sheppard, et al.

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