INTRODUCTION

Apoptosis or programmed cell death is an evolutionarily conserved cellular process that is required for normal embryonic development and maintenance of tissue homeostasis. The B-cell lymphoma protein-2 (BCL-2) family of proteins are essential regulators of apoptosis, functioning as either activators or inhibitors of cell death primarily at the mitochondrial level. This family of proteins consists of three groups that each contain at least one BCL-2 homology (BH) motif (BH1-4). The pro-apoptotic BH3-only proteins, BID, BIM, PUMA, NOXA, BAD, BIK, BMF and HRK are activated or induced by cell death stimuli that, in turn, may activate the pro-apoptotic ‘multidomain effector’ proteins BAX and BAK. Once activated, these proteins homo-oligomerize to induce mitochondrial outer membrane permeabilization. Mitochondrial outer membrane permeabilization results in the release of pro-apoptotic factors such as cytochrome c from the mitochondria into the cytosol leading to apoptosis formation, caspase activation and DNA fragmentation. The anti-apoptotic members (BCL-2, BCL-XL, MCL-1, BCL-W and BFL-1) contain multiple BH motifs and function to inhibit apoptosis by direct interaction with the ‘BH3-only’ and multi-domain effectors via their BH3-binding grooves. Aberrant expression and/or function of BCL-2 family members results in deregulation of apoptosis that contributes to the development of a variety of human pathologies including cancer, neurodegeneration and autoimmunity.1, 2

Non-Hodgkin’s lymphoma (NHL) represents a heterogeneous group of lymphoid-derived malignancies that include follicular lymphoma, diffuse large B-cell lymphoma and mantle cell lymphoma (MCL). The t(14;18) chromosomal translocation results in BCL2 hyperexpression by juxtaposing it to the immunoglobulin heavy chain gene enhancer, representing the primary tumorigenic event in most follicular lymphomas that is also found in ~20% of diffuse large B-cell lymphomas.3–4 Elevated expression of BCL-2 in diffuse large B-cell lymphoma is also associated with BCL2 gene amplification or transcriptional upregulation through constitutive activation of the NFκB pathway.5–6 BCL-2 overexpression is associated with poor prognosis5, 7 in NHL by promoting cell survival and resistance to anti-tumorigenic agents.1, 2, 8 Transgenic mouse models also reveal that MCL-1 and BCL-XL hyperexpression contribute to the onset and maintenance of hematological malignancies.

Navitoclax (ABT-263) is an orally bioavailable anti-tumorigenic agent that targets BCL-2, BCL-XL and BCL-W but not MCL-1 or BFL-1(ref. 13) and is being evaluated in clinical trials as a single agent or in the adjuvant setting. However, BCL-XL-driven thrombocytopenia has been dose limiting in patients with hematological malignancies or small cell lung cancer.14–19 Consequently, we developed the BCL-2-selective inhibitor venetoclax (ABT-199) that shows superior affinity to BCL-2 relative to navitoclax and circumvents BCL-XL-driven thrombocytopenia.20 This attribute may permit attainment of higher plasma concentrations that translate into improved response rates in patients with BCL-2-dependent malignancies. Despite this, some cell lines of hematologic origin remain resistant to both venetoclax and navitoclax.20

Although BCL2 is frequently mutated in NHL,21–22 these mutations do not affect sensitivity to ABT-737(ref. 22) and are unlikely to affect navitoclax or venetoclax efficacy. Mutations have been described in murine BCL2 following ABT-737/venetoclax acquired resistance,23 however the analogous mutations in human...
BCL2 have not been reported in NHL patients. Therefore, potential inherent resistance factors may reside elsewhere in the apoptotic pathway. For example, MCL-1 has been identified by us and numerous other investigators as a factor that contributes to both intrinsic and acquired resistance to ABT-737, navitoclax and venetoclax.24–28 Merino et al.29 have suggested that navitoclax is not an efficient antagonist of BCL-XL in lymphoid cells, indicating that BCL-XL is in fact a resistance factor for ABT-737 (refs 29,30) and potentially navitoclax as well as, more obviously, venetoclax. Using highly potent and selective inhibitors of BCL-2,30,31 BCL-XL32,33 and MCL-1,34–36 and combinations thereof, we sought to further classify the survival dependency of NHL for anti-apoptotic BCL-2 family members. Consequently, these pre-clinical data inform on strategies to potentially improve on the clinical efficacy of venetoclax through co-inhibition of MCL function.

MATERIALS AND METHODS

Reagents, cell culture and treatment

NHL cell lines were obtained from the American Type Culture Collection or Deutsche Sammlung von Mikroorganismen und Zellkulturen and were cultured in Iscove’s Modified Dulbecco’s Media containing 10% human serum and 10 mM l-glutamine (all from Invitrogen Corporation, Carlsbad, CA, USA). All cell lines were tested for authenticity by short tandem repeat profiling and mycoplasma by the AbbVie Core Cell Line Facility. Cells were plated at a density of 0.25 × 10^6 cells/well in 6-well plates for apoptosis assays, at 0.1 × 10^6/ml for cell viability assays, and at 3 × 10^6 per 10 cm² petri dish for western blots. Navitoclax, venetoclax, A-1210477 and A-1155463 were dissolved in anhydrous dimethyl sulfoxide to a stock solution of 10 mM. After overnight attachment, cells were treated for up to 48 h with vehicle alone, navetociax, venetoclax, A-1155463, flavopiridol or A-1210477, or in the described combinations. Where indicated, cells were pre-treated for 60 min with z-VAD-fmk (50 μM; MP Biomedicals, Santa Ana, CA, USA). Navitoclax, venetoclax, A-1155463 and A-1210477 were synthesized as described.30–33

Cell viability

Cells (0.1 × 10^6/ml) were treated in 96-well plates for 72 h and cell viability determined by CellTiter-Glo as described by the manufacturer’s instructions (Promega Corporation, Madison, WI, USA). Responses were determined as a percentage of the control treated cells and EC50 determined from sigmoidal dose-response curves using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Annexin-V/7-AAD staining

Apoptosis was determined by flow cytometric evaluation of Annexin-V/7-AAD staining as described in detail elsewhere.34

Western blot analysis

After treatment, cells were washed twice with ice-cold PBS containing 10% fetal bovine serum, centrifuged at 1000 r.p.m. for 5 min, and lysed in 50 μl of ice-cold Cell Lytic (Sigma) supplemented with protease (Roche Diagnostics Corporation, Indianapolis, IN, USA) and phosphatase (Sigma) inhibitors. Protein concentrations were determined by the BSA assay (Invitrogen) and 50 μg of protein electrophoresed by SDS–PAGE (Invitrogen). Separated proteins were transferred to nitrocellulose membranes utilizing an IBlot (Invitrogen) device. Blots were probed with MCL-1 (clone 5-19; Santa Cruz Biotechnology, La Jolla, CA, USA), PARP (clone C2-10) and BCL-2 (Clone 7; both BD Biosciences, CA, USA), caspase-3 (clone 31A1067, Abcam, Cambridge, UK) or β-actin (Sigma) antibodies followed by IRDye 680/800CW-conjugated secondary antibodies (LI-COR Biosciences, NE, USA). Proteins were visualized using the Odyssey infrared imaging system (LI-COR Biosciences) and were not further manipulated with imaging software.

Fluorescent in situ hybridization (FISH)

PBS-washed cells (2–3 × 10^5 cells/ml) were isolated on BioGenex dual spot barrier slides (100 μl per spot) for 5 min at 500 r.p.m. before fixation with 1% formaldehyde. Slides were washed twice in PBS, air dried and stored at 4°C before FISH. FISH was performed using a custom protocol on a Biogenex Xmaxtrix automated staining instrument. Briefly, slides underwent cell dehydration with ethanol, heat denaturation (96°C, 5 min) and incubation with Vysis LSI IgH/BCL2 translocation fusion probe set (Abbott Molecular Diagnostics, 05J71-001) at 42°C for 14 h, followed by a stringency wash with 2X SSC, and application of 4’,6-diamidino-2-phenylindole to stain nuclei. The IgH/BCL2 translocation status was then determined by fluorescence microscopy at ×100 magnification (Zeiss AxiosPhot 2 fluorescence microscope; Oberkochen, Germany).

Determination of BCL2, BCLL1 and MCL1 CN

DNA was isolated from NHL cell lines using DNeasy blood and tissue kit (Qiagen, Venlo, Netherlands; #69506) per manufacturer’s protocol, except eluted in reduced EDTA TE buffer (Teknova, Hollister, CA, USA; T0223) and quantitated with PicoGreen assay (Molecular Probes, Thermo-Fisher, Waltham, MA, USA). Copy number was determined by SNP 6.0 assay (500 ng DNA input) per manufacturer’s protocol (Affymetrix cytogenetics copy number assay rev. 2) followed by data smoothing and quantitation of CEL files in Partek software (Partek Inc., St Louis, MO, USA).

Protein expression

BCL-2, BCL-XL, and MCL-1 protein expression were measured using an assay developed based on the Luminex technology (Austin, TX, USA). In brief, MCL-1, BCL-2 and BCL-XL, capture antibodies were custom conjugated to Luminex carboxyl beads (bead region 9, 33 and 64, respectively) by Millipore (St. Charles, MO, USA). MCL-1 detection antibody was also conjugated to biotin through a custom service provided by Millipore. BCL-2 and BCL-XL detection antibodies conjugated to biotin were included in the DuoSetic kits from R&D Systems (Minneapolis, MN, USA). Cells were lysed in MILLIPLEX MAP lysis buffer 1 (Millipore Cat. no. 43-040, Danvers, MA, USA) containing protease inhibitor cocktail (Sigma). Protein expression was determined using a Luminex FlexMap 3D system (Luminex) as described in depth elsewhere.35 Data are presented as median fluorescent intensity.

Electrochemiluminescent ELISA

Streptavidin multi-array 96-well plates (Meso Scale Discovery (MSD), Gaithersburg, MD, USA) were used to immobilize biotin-labeled anti-BCL-2 (US Biological, catalog no. B807-067), biotin-labeled anti-BCL-XL (Abcam, catalog no. ab25062), biotin-labeled anti-MCL-1 (NeoMarker, catalog MS-681-B) and biotin-labeled IgG1 (US Biological, catalog no. 11904-6A2). Protein samples (75 μg; extracted with CHAPS buffer containing 10 mM iodoacetamide and phosphatase inhibitors; Roche and Sigma, respectively), were subsequently added to each plate in duplicate. The plate was incubated overnight at 4°C to pull down BCL-2. After three washes with PBS–Tween, anti-BIM (Epitomics; catalog no. 1036-1) was added and incubated for 1 h in the dark at room temperature with rotation at 650 r.p.m. Subsequently, sulfo-tagged goat anti-rabbit antibody (MSD; Rockville, MD, USA) was added to each well and incubated for a further 30 min as mentioned above and then washed three times with PBS–TWEEN. Finally, 150 μl of 2× MSD read buffer T was added per well and fluorescence measured with a MSD Sector Imager 6000 (MSD, Gaithersburg, MD, USA).

Statistical analysis

Data are represented as the mean ± s.e.m. In all cases, the number of independent experiments is described within the figure legend. The Mann–Whitney U-test was used to determine statistical significance. Spearman’s rank correlation co-efficient was used to determine statistical dependence between two variables. The Bliss independence model was used to evaluate synergy.36

RESULTS

We recently described the BCL-2-selective inhibitor venetoclax to show superior potency to navitoclax in pre-clinical models of hematological cancers. Venetoclax in vitro potency correlates with the expression of BCL-2 in NHL cell lines. Furthermore, segregation of NHL cell lines into BCL2high (t(14;18) and/or high HCL2 CN) and BCL2low groups identifies the former group as being particularly sensitive to venetoclax.20–22 Here we have characterized additional NHL cell lines for sensitivity to venetoclax and navitoclax as well as
their CN and/or t(14;18) translocation status (Supplementary Table 1). To assess the contribution of BCL-XL for survival, we treated all NHL cell lines with BCL-XL-selective inhibitor A-1155463. As expected, BCL2High cell lines were resistant to the BCL-XL-selective inhibitor A-1155463 (EC50 > 2 μM). Although the majority of NHL cell lines were also resistant to navitoclax, SU-DHL-8 and RCK8 were sensitive. Importantly, navitoclax sensitivity in SU-DHL-8 and RCK8 was driven by BCL-XL inhibition since both cell lines were sensitive to A-1155463 and resistant to venetoclax (Figure 1a and Supplementary Figure 1). We next assessed protein expression of the anti-apoptotic BCL-2 family members in these NHL cell lines using the Luminex FlexMap 3D

Figure 1. Chemical segregation of navitoclax activity in NHL cell lines; requirement for BCL-2 or BCL-XL for survival. The efficacy of navitoclax, venetoclax or A-1155463 was determined in NHL cell lines as described in the Materials and Methods section. EC50s were calculated from the resulting sigmoidal dose/response curves (see Supplementary Table 1) and segregated according to their BCL2High or BCL2Low status. Data are presented as the mean of at least three independent experiments. Cell lines with navitoclax EC50s > 2 μM were deemed resistant. Data are presented as the mean of at least three independent experiments (a). Expression of anti-apoptotic BCL-2 family proteins was determined by Luminex as described and segregated according to the BCL2High or BCL2Low status. The median is shown in red and the Mann–Whitney U-test was used to determine statistical significance. NS, not significant (b). Navitoclax-resistant BCL2High cells were treated with navitoclax or venetoclax (both 1 μM) for 8 h and the interaction of BIM with BCL-2 or MCL-1 assessed using an Electrochemiluminescent ELISA (MSD) as described in the Materials and Methods section. Data are presented as the mean ± s.e.m. of three independent experiments (c).
As expected, BCL-2 protein expression was significantly higher in BCL2High cell lines relative to BCL2Low cell lines. However, MCL-1 and BCL-XL protein levels were approximately the same in either population (Figure 1b). Furthermore, the BCL-XL protein expression in the A-1155463-sensitive cell lines RCK8 and SU-DHL-8 was 4721 and 719 median fluorescent intensity, respectively (Figure 1b), and did not reflect the EC50 of A-1155463 in each cell line (446.4 nM and 167.4 nM, respectively; Figure 1a and Supplementary Figure 1).

MCL-1 can be considered an intrinsic as well as an acquired resistance factor that limits the efficacy of navitoclax, ABT-737 and venetoclax. However, MCL-1 protein expression does not directly correlate with the sensitivity of NHL cell lines to navitoclax or venetoclax (Figure 1b and Supplementary Figure 2). Since some BCL2High NHL cell lines are relatively resistant to navitoclax (EC50 > 2 μM; Figure 1a and Supplementary Table 1), we treated BCL2High NHL cell lines (SU-DHL-4, WSU-DLCL2, WSU-NHL) with navitoclax or venetoclax and evaluated interactions of BIM with BCL-2 and MCL-1. Both navitoclax and venetoclax disrupted BCL-2-BIM interactions; however, this was accompanied by an enhanced association of BIM with MCL-1 (Figure 3b).

The CDK inhibitor flavopiridol has been assessed clinically in NHL patients and is a transcriptional repressor of MCL-1 expression. We have demonstrated that A-1210477 induces a cellular phenotype similar to that observed following repression of MCL-1 expression through inhibition of transcriptional elongation by flavopiridol. Flavopiridol therefore represents a clinically relevant surrogate for inhibiting MCL-1 function.

The MCL-1 inhibitor A-1210477 synergizes with navitoclax in BCL2High NHL cell lines via BCL-2 and not BCL-XL inhibition. NHL BCL2High cell lines were co-treated with navitoclax (0–20 μM); (a), the BCL-2-selective inhibitor venetoclax (0–20 μM) or the BCL-XL-selective inhibitor A-1155463 (0–20 μM); (b), in combination with the MCL-1-specific inhibitor A-1210477 (0, 5, 10 and 15 μM) for 48 h and the effect on viability determined. Synergy was quantified using the Bliss algorithm (c). Data are presented as the mean ± s.e.m. of three independent experiments.
of MCL-1 and, in combination with navitoclax or venetoclax, resulted in enhanced PARP cleavage and caspase-activation indicating an apoptotic phenotype (Figure 5a and b). To further validate the mechanism of cell death and synergy, we evaluated annexin-v/7-AAD staining by flow cytometry in the presence or absence of the broad spectrum caspase inhibitor z-VAD-fmk. A-1210477 potentiated the degree of annexin-v/7-AAD staining induced by either navitoclax or venetoclax. This staining was inhibited by z-VAD-fmk, indicating that caspase activity is required for this apoptotic phenotype. Similarly, flavopiridol-sensitized BCL2High NHL cells sensitized to venetoclax-mediated apoptosis as evidenced by high annexin-v/7-AAD staining that was caspase-dependent (Figure 5c and d).

**DISCUSSION**

Although navitoclax has shown encouraging activity in hematologic malignancies as a single agent and in the adjuvant setting, its clinical utility is limited by thrombocytopenia driven by inhibition of BCL-X	extsubscript{L}. We recently described the development of the BCL-2-selective inhibitor venetoclax that shows superior affinity for BCL-2 and excellent selectivity over BCL-X	extsubscript{L}. This translates into increased potency and efficacy in pre-clinical models of lymphoid malignancies that are dependent on BCL-2 for survival. Importantly, its lack of affinity for BCL-X	extsubscript{L} circumvents thrombocytopenia, a dose-limiting toxicity associated with navitoclax. Subsequently, objective responses have been obtained in clinical trials of venetoclax in chronic lymphocytic leukemia and NHL patients. MCL-1 and BCL-X	extsubscript{L} are intrinsic and acquired resistance factors that limit the efficacy of navitoclax or ABT-737 and therefore may impact the clinical utility of venetoclax. We therefore sought to understand the functional roles of MCL-1 and BCL-X	extsubscript{L} in NHL cell lines with intrinsic resistance to venetoclax.

We have segregated NHL cell lines into two populations; BCL2High represents lines with high BCL2 expression and/or the BCL2 translocation t(14;18), whereas lines without these lesions were defined as BCL2Low. As a population, BCL2High NHL cell lines are largely sensitive to navitoclax- or venetoclax-induced apoptosis. Despite this, some BCL2High NHL cell lines are relatively resistant to venetoclax, with EC_{50} > 2 μM in vitro, a facet we hypothesized here to be a consequence of MCL-1 function and not simply expression. Expression of MCL-1 at the protein level does not directly correlate with resistance to navitoclax or venetoclax in NHL cell lines herein, or at the gene level with ABT-737 in chronic lymphocytic leukemia. Treatment of resistant NHL cell lines with navitoclax, venetoclax or A-1155463 resulted in enhanced MCL-1: BIM interactions that we hypothesized to inhibit BAX/BAK activation and subsequently limit the efficacy of these compounds. This capacity of MCL-1 to function as a ‘sink’ for additional free or displaced BIM serves as a survival response to cellular stress mediated by BCL-2 and/or BCL-X	extsubscript{L} inhibition. However, this process

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**Figure 3.** The MCL-1 inhibitor A-1210477 synergizes with navitoclax in BCL2Low NHL cell lines via BCL-X	extsubscript{L} and not BCL-2 inhibition. NHL BCL2Low cells were treated as in Figure 2 and the degree of synergy determined by Bliss analysis (a). BCL2Low NHL cell lines were treated with navitoclax or A-1155463 (all 1 μM) for 8 h and the interaction of BIM with BCL-X	extsubscript{L} or MCL-1 assessed using an electrochemiluminescent ELISA (MSD) as described in the Materials and Methods section (b). Data are presented as the mean ± s.e.m. of three independent experiments.
also primes these cells for death by agents that inhibit MCL-1 function.

Loss of MCL-1 function through gene silencing or indirect pharmacological inhibition sensitizes many tumor types to navitoclax.24-28 A-1210477 is a MCL-1-specific inhibitor that induces apoptosis in a phenotypically identical fashion to MCL-1 siRNA.27,28,32 Herein, we have used A-1210477 and other selective BCL-2 family inhibitors to define the contributions of MCL-1, BCL-2 and BCL-X\textsubscript{L} in maintaining the survival of various NHL cell lines. These ‘chemical parsing’ experiments demonstrated that sensitization of BCL\textsubscript{2High}\textsubscript{L} NHL cell lines to navitoclax in response to direct MCL-1 inhibition with A-1210477 or indirectly through loss in MCL-1 expression mediated by flavopiridol, is driven by BCL-2 inhibition with no contribution from BCL-X\textsubscript{L}. The BCL-2-selective inhibitor venetoclax was equivalent, if not slightly superior, to navitoclax in inducing caspase-dependent cell death in synergy with A-1210477; perhaps because loss in MCL-1 function achieved through cellular exposure to A-1210477 is mechanically distinct from that of flavopiridol. A-1210477 binds to the BH3-binding groove of MCL-1 and results in stabilization of MCL-1 protein levels.28,32 This is analogous to that observed with BIM BH3 peptides.46 In contrast, flavopiridol treatment results in a loss in MCL-1 protein expression (Figure 5a and b) through transcriptional repression of MCL-1.41,42 However, CDK9 regulates several other genes that dictate cellular survival,47 and flavopiridol’s effect on their expression may also contribute to synergy with navitoclax, venetoclax or A-1155463.

Despite their BCL\textsubscript{2Low}\textsubscript{L} NHL classification, SU-DHL-8 and RCK8 cell lines are sensitive to navitoclax. In this case, chemical parsing experiments revealed that this efficacy was driven by BCL-X\textsubscript{L}, inhibition since the BCL-X\textsubscript{L}-selective inhibitor A-1155463 was efficacious, whereas the BCL-2-selective inhibitor venetoclax was not. These data are in contrast to a recent finding by Merino et al.,29 who proposed that navitoclax does not bind to BCL-X\textsubscript{L} with sufficient avidity to kill lymphoid cells efficiently. Indeed, we found that navitoclax was able to significantly perturb the BCL-X\textsubscript{L}:BIM interactions (Figure 3b). The dependency of SU-DHL-8 and RCK8 on BCL-X\textsubscript{L} for survival is further exemplified at the protein and gene level. These cell lines are characterized as possessing low BCL-2 protein levels (Figure 1b) and high BCL-X\textsubscript{L} (BCL\textsubscript{L1}) CN (Supplementary Table 1). Furthermore, apoptosis and synergy between navitoclax and the MCL-1 inhibitor A-1210477 in BCL\textsubscript{2Low}\textsubscript{L} cell lines generally required BCL-X\textsubscript{L} inhibition and not BCL-2. We speculate that the BCL\textsubscript{2Low}\textsubscript{L} characterization may therefore represent a NHL patient population that may benefit from navitoclax rather than venetoclax treatment in the combination setting, such as with bendamustine/rituximab.20,48
NHL. Combined treatment of BCL2\(^{\text{High}}\) NHL cell lines with venetoclax and A-1210477 or flavopiridol results in the synergistic induction of apoptosis \textit{in vitro}. Importantly, the BCL-X\(_L\)-selective inhibitor A-1155463 is not efficacious as a single agent or in combination with MCL-1 inhibitors in BCL2\(^{\text{High}}\) NHL cell lines \textit{in vitro}. Collectively these data emphasize that BCL2 status is predictive of venetoclax efficacy in NHL not only as a single agent, but also in the adjuvant setting with anti-tumorigenic agents that modulate MCL-1 levels. Finally, we demonstrate that the BCL2\(^{\text{Low}}\) NHL classification predicts navitoclax combinational efficacy due to a requirement for BCL-X\(_L\) inhibition and not BCL-2. Elevated levels of MCL-1 have been described in chronic lymphocytic leukemia, MCL and multiple myeloma\(^{49-53}\) and this study in pre-clinical models of NHL paves the way to evaluate the consequence of functional inhibition of MCL-1 in combination with venetoclax in these additional hematologic malignancies.

**CONFLICT OF INTEREST**

DCP, YX, LTL, LR-R and JDL are AbbVie employees and are stock holders. DCP, YX and LTL, LR-R, AJS and JDL are AbbVie employees and contributed to experimental design, data discussions and writing the manuscript; AJS and JDL are AbbVie employees and contributed to experimental design, study conduct and financial support were provided by AbbVie. AbbVie participated in the data generation, interpretation of data, review and approval of this publication. EL is now an employee of Abbott Molecular Inc.

**AUTHOR CONTRIBUTIONS**

DCP, YX and LTL performed the experiments, they were responsible for experimental design, data discussions and writing the manuscript; EL and LR-R performed the experiments and were responsible for writing manuscript; AJS was responsible for data discussions and writing the manuscript; JDL contributed to experimental design, data discussions and writing the manuscript.

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