ORIGINAL ARTICLE

Loss in MCL-1 function sensitizes non-Hodgkin's lymphoma cell lines to the BCL-2-selective inhibitor venetoclax (ABT-199)

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As a population, non-Hodgkin's lymphoma (NHL) cell lines positive for the t(14;18) translocation and/or possessing elevated *BCL2* copy number (CN; *BCL2*[High]) are exquisitely sensitive to navitoclax or the B-cell lymphoma protein-2 (BCL-2)-selective inhibitor venetoclax. Despite this, some *BCL2*[High] cell lines remain resistant to either agent. Here we show that the MCL-1-specific inhibitor A-1210477 sensitizes these cell lines to navitoclax. Chemical segregation of this synergy with the BCL-2-selective inhibitor venetoclax or BCL-X$_L$-selective inhibitor A-1155463 indicated that MCL-1 and BCL-2 are the two key anti-apoptotic targets for sensitization. Similarly, the CDK inhibitor flavopiridol downregulated MCL-1 expression and synergized with venetoclax in *BCL2*[High] NHL cell lines to a similar extent as A-1210477. A-1210477 also synergized with navitoclax in the majority of *BCL2*[Low] NHL cell lines. However, chemical segregation with venetoclax or A-1155463 revealed that synergy was driven by BCL-X$_L$ inhibition in this population. Collectively these data emphasize that *BCL2* status is predictive of venetoclax potency in NHL not only as a single agent, but also in the adjuvant setting with anti-tumorigenic agents that inhibit MCL-1 function. These studies also potentially identify a patient population (*BCL2*[Low]) that could benefit from BCL-X$_L$ (navitoclax)-driven combination therapy.

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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 apoptosome formation, caspase activation and DNA fragmentation. The anti-apoptotic members (BCL-2, BCL-X$_L$, 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 prognosis[5,7] 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-X$_L$ hyperexpression contribute to the onset and maintenance of hematological malignancies.[9–12]

Navitoclax (ABT-263) is an orally bioavailable anti-tumorigenic agent that targets BCL-2, BCL-X$_L$ 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-X$_L$-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-X$_L$-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

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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-X$_L$ in lymphoid cells, indicating that BCL-X$_L$ 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,[(ref. 20)] BCL-X$_L$[(ref. 31)] or MCL-1,[(refs 27,28,32)] 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-1 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 mycoplasm by the AbbVie Core Cell Line Facility. Cells were plated at a density of 0.25×10^6 cells/well in six-well plates for apoptosis assays, at 0.1×10^6/ml for cell viability assays, and at 3×10^6 per 10 cm^2 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. Flavopiridol was dissolved in dimethyl sulfoxide at 1 mM. After overnight attachment, cells were treated for up to 48 h with vehicle alone, navitoclax, 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.[20,31–33] Unless otherwise indicated, all chemical reagents were obtained from Sigma Aldrich (St. Louis, MO, USA).

Cell viability

Cells $(0.1 \times 10^6$/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 EC$_{50}$s 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 S-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 (LICOR Biosciences, NE, USA). Proteins were visualized using the Odyssey infrared imaging system (LICOR Biosciences) and were not further manipulated with imaging software.

Fluorescent *in situ* hybridization (FISH)

PBS-washed cells $(2–3 \times 10^6$ 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 Xmatrx 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 AxioPhot 2 fluorescence microscope; Oberkochen, Germany).

Determination of *BCL2*, *BCL2L1* 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 manufacture'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-X$_L$ 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-X$_L$ 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-X$_L$ 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. B0807-067), biotin-labeled anti-BCL-X$_L$ (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 protease 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 *BCL2*^{High} (t(14;18)$^+$ and/or high *BCL2* CN) and *BCL2*^{Low} groups identifies the former group as being particularly sensitive to venetoclax.[20] Here we have characterized additional NHL cell lines for sensitivity to venetoclax and navitoclax as well as