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The BCL2 antagonist ABT-199 triggers apoptosis, and augments ibrutinib and idelalisib mediated cytotoxicity in *CXCR4*$^{Wild\text{-}type}$ and *CXCR4*^{WHIM} mutated Waldenstrom macroglobulinaemia cells

Whole genome sequencing has revealed *MYD88*^{L265P} and *CXCR4*^{WHIM} somatic mutations in Waldenstrom's Macroglobulinemia (WM) (Treon *et al*, 2012; Hunter *et al*, 2014). *MYD88*^{L265P} supports WM cell survival through divergent pathways including Bruton Tyrosine Kinase (BTK) and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta (PI3KCD, also termed PI3Kδ) (Yang *et al*, 2013a,b). Ibrutinib targets BTK, while idelalisib targets PI3KCD. Ibrutinib and idelalisib are active in previously treated WM patients, with overall response rates of 87% and 70%, respectively (Treon *et al*, 2013; Gopal *et al*, 2014). Most responses are partial and no complete responses have been observed.

CXCR4^{WHIM} mutations confer *in vitro* ibrutinib and idelalisib resistance (Cao *et al*, 2014; Roccaro *et al*, 2013) and CXCR4^{WHIM}-mutated WM patients have lower overall and major responses to ibrutinib (Treon *et al*, 2013). AKT1 and MAPK1 are hyper-activated in *CXCR4*^{WHIM}-engineered WM cells following CXCL12 stimulation, and AKT1 is constitutively activated in WM cells from *CXCR4*^{WHIM} patients on ibrutinib (Cao *et al*, 2014). The hyper-activation of AKT1 and MAPK1 may contribute to ibrutinib resistance in *CXCR4*^{WHIM}-mutated WM patients (Cao *et al*, 2014).

BCL2 is an anti-apoptotic protein that confers resistance to many anti-neoplastic agents used in B-cell malignancies. *BCL2* is overexpressed in primary WM cells (Chng *et al*, 2006). ABT-199 is a highly selective BCL2 antagonist that is clinically active in B-cell malignancies, with major responses observed in 3 of 4 previously treated WM patients (Davids *et al*, 2014). These findings suggest that *BCL2* contributes to WM cell survival.

We therefore investigated whether BCL2 also protects against ibrutinib- and idelalisib-related apoptosis in CXCR4^{WT} and CXCR4^{WHIM} WM cells. Written consent was obtained for use of patient samples per our Institutional Review Board. Development of CXCR4^{WT} and CXCR4^{S338X} BCWM.1 and MWCL-1 cells was previously reported (Cao

et al, 2014). Ibrutinib and idelalisib were obtained from MedChem Express (Monmouth Junction, NJ, USA), and ABT-199 from Selleck Chemicals (Houston, TX, USA). BCL2 levels were determined by immunoblotting (Abcam, Cambridge, MA, USA). For survival assessment, WM cells underwent immunoblotting for cleaved PARP and caspase-3

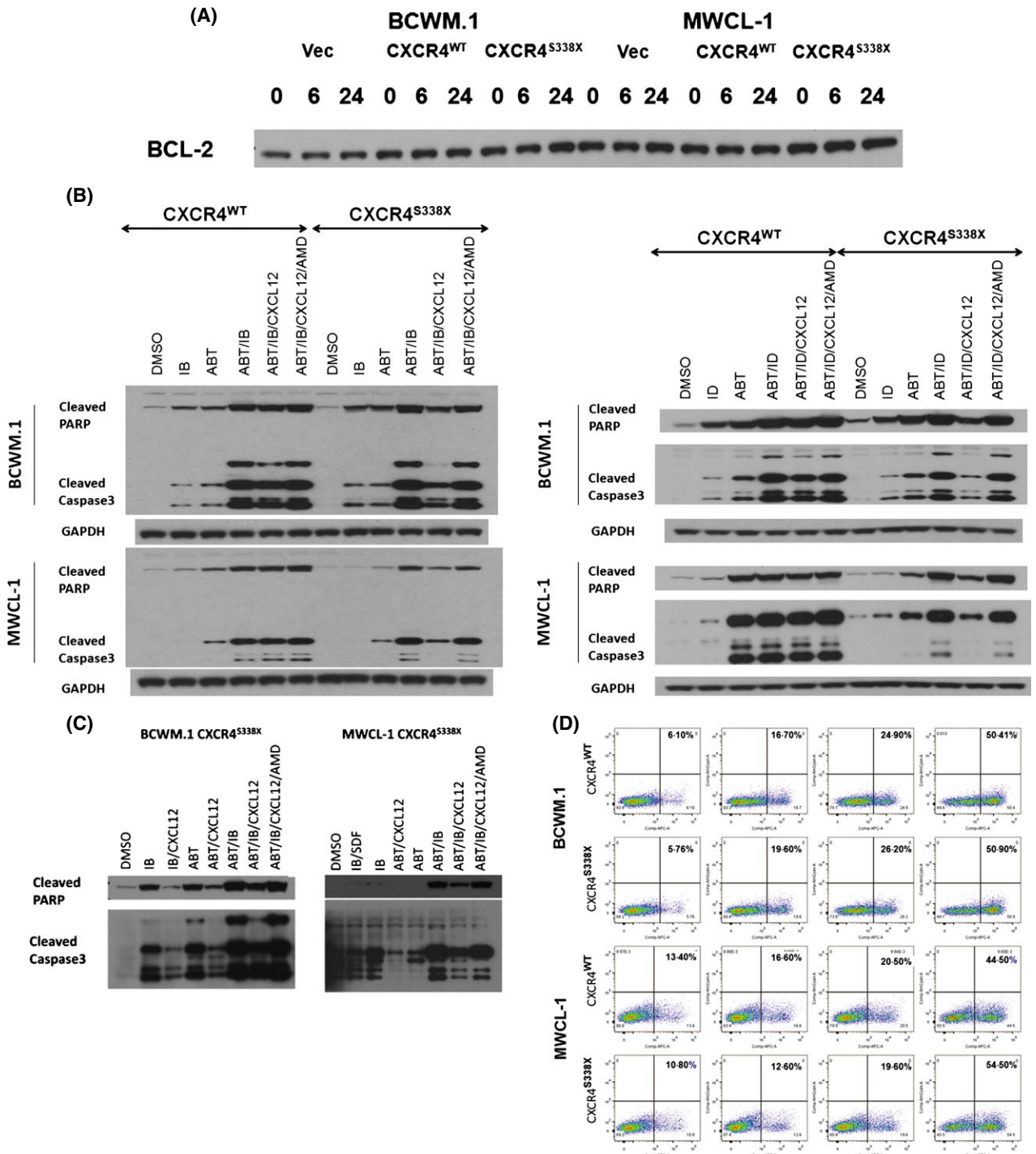

Fig 1. Apoptotic changes related to ABT-199 alone, and in combination with ibrutinib or idelalisib in CXCR4^{WT}- and CXCR4^{S338X}-engineered WM cells. (A) Protein levels of BCL2 following CXCL12 in vector only, CXCR4^{WT} and CXCR4^{S338X} BCWM.1 and MWCL-1 cells; (B) Caspase-3 and PAPR cleavage following ibrutinib (IB), idelalisib (ID), ABT-199 (ABT), in the presence of absence of CXCL12 (SDF) and AMD3100 (AMD); (C) Caspase-3 and PARP cleavage in CXCR4^{S338X} BCWM.1 and MWCL-1 cells treated with ABT-199 in the absence or presence of CXCL12 and AMD3100 (AMD); (D) CXCR4^{WT} and CXCR4^{S338X} expressing WM cells were treated for 18 h with vehicle control (dimethylsulphoxide; DMSO), ibrutinib (IB) or ABT-199 (ABT) alone and in combination in the presence of CXCL12. Annexin V staining was performed to assess apoptosis. Results from a representative study set of are shown. Similar results were obtained with idelalisib.

(Abcam), and Annexin V staining (R&D Systems, Minneapolis, MN, USA) by flow cytometry. Synergistic drug interactions were determined by the Caspase-Glo® 3/7 assay (Promega, Madison WI, USA) in the presence of CXCL12 (50 nmol/l).

CXCR4^{WT} and CXCR4^{S338X} BCWM.1 and MWCL-1 cells expressed BCL2 protein, with higher levels observed in the latter. BCL2 levels were not impacted by CXCR4 mutation status, or CXCL12 stimulation (Fig 1A). Treatment of CXCR4^{WT} and CXCR4^{S338X} BCWM.1 and MWCL-1 cells with ibrutinib or idelalisib induced caspase-3 and PARP cleavage at 6 h (Fig 1B), though modest Annexin V staining, denoting full apoptotic progression, was observed at 18 and 40 h (data not shown). CXCL12 abrogated ibrutinib- or idelalisib-related PARP and/or caspase-3 cleavage, and was more pronounced in CXCR4^{S338X} WM cells. Similarly, ABT-199 induced PARP and caspase-3 cleavage in CXCR4^{S338X} and CXCR4^{WT} BCWM.1 and MWCL-1 cells. PARP and caspase-3 cleavage was less pronounced in MWCL-1 cells regardless of CXCR4 mutation status, and probably reflected higher BCL2 protein levels (Fig 1A). CXCL12 also abrogated PARP and caspase-3 cleavage in ABT-199 treated CXCR4^{S338X} BCWM.1 and MWCL-1 cells despite unaltered BCL2 protein levels (Fig 1A, C).

ABT-199 augmented PARP and caspase-3 cleavage in CXCR4^{S338X} and CXCR4^{WT} BCWM.1 and MWCL-1 WM cells treated with ibrutinib or idelalisib (Fig 1B), and led to increased Annexin V staining (Fig 1D). CXCL12 abrogated PARP and/or caspase-3 cleavage (Fig 1B), and Annexin V staining (data not shown) more so in CXCR4^{S338X} following

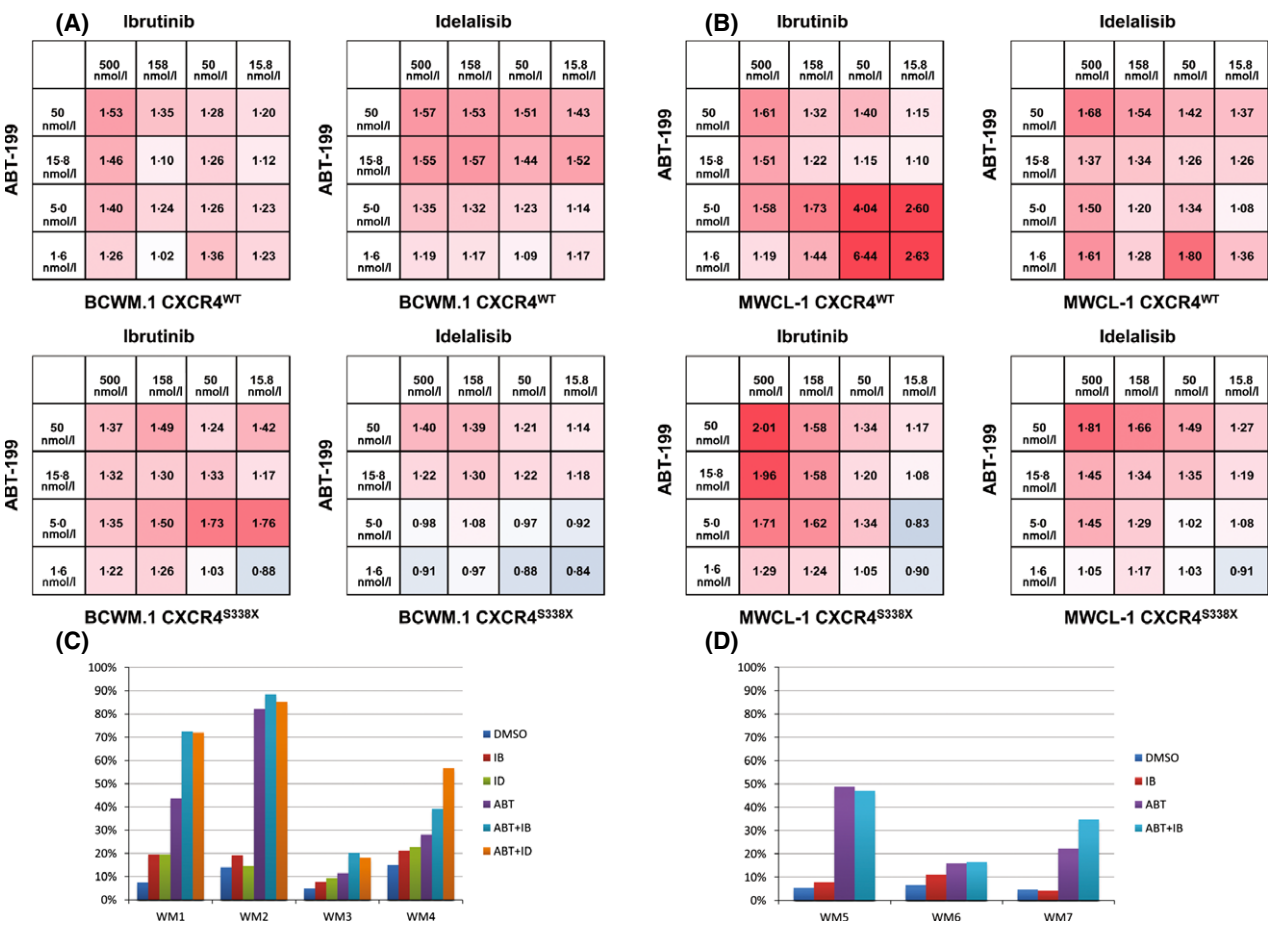

Fig 2. Treatment effects of ABT-199 in combination with ibrutinib or idelalisib in CXCR4^{WT} and CXCR4^{S338X} expressing cell lines and primary WM patient cells. Synergistic drug interactions for ABT-199 with either ibrutinib or idelalisib in CXCR4^{WT} and CXCR4^{S338X} expressing BCWM.1 (A) and MWCL-1 (B) cells were determined in triplicate sample sets in the presence of CXCL12 (50 nM) 6 h following treatment at indicated doses using a caspase-3/7 cleavage assay. Combination Index >1·0 denotes synergistic interactions. Apoptotic changes following ABT-199 alone, and in combination with ibrutinib or idelalisib in primary WM cells genotyped for MYD88 and CXCR4 mutations. Results from flow cytometric studies of *ex vivo* treated WM cells (CD19^{+} gated) from (C) untreated WM patients (n = 4) or (D) WM patients on active ibrutinib treatment (n = 3). Cells were treated with vehicle control (DMSO), ibrutinib (IB), idelalisib (ID) and ABT-199 (ABT), alone and in combination. P = 0·02 in untreated WM patient cells comparing any *ex vivo* treatment *versus* vehicle control; $P \leq 0{\cdot}05$ for ABT-199 combinations *versus* any monotherapy in untreated WM patient cells; P = 0·04 for ABT-199 *versus* vehicle control for cells taken from patients on active ibrutinib therapy. All patients expressed MYD88^{L265P}. Patients WM1, WM2, WM5, and WM6 expressed *CXCR4*^{S338X} mutations.

ABT-199 treatment with either ibrutinib or idelalisib. Addition of the CXCR4 antagonist AMD3100 abrogated CXCL12 protective effects, and augmented PARP and caspase-3 cleavage (Fig 1B), and Annexin V staining (data not shown) in $CXCR4^{S338X}$ cells treated with ABT-199 alone and with ibrutinib or idelalisib.

To further delineate the impact of ABT-199 on ibrutinib- or idelalisib-related treatment effects, we used a caspase-3/7 cleavage assay to assess for drug synergy. Synergistic interactions (combination index >1·0) were evident at nearly all dose combinations of ABT-199 with either ibrutinib or idelalisib in $CXCR4^{WT}$ BCWM.1 and MWCL-1 cells in the presence of CXCL12. Synergistic interactions were also evident, though at higher dose combinations of ABT-199, with either ibrutinib or idelalisib in $CXCR4^{S338X}$ WM cells, reflecting the protective effects of CXCL12 in $CXCR4^{WHIM}$-mutated cells (Fig 2A, B). The impact of ABT-199 on ibrutinib- or idelalisib-related treatment effect was also assessed in bone marrow (CD19-sorted) WM cells from 4 (2 $MYD88^{L265P}CXCR4^{WT}$; 2 $MYD88^{L265P}CXCR4^{S338X}$) untreated WM patients within their microenvironment. *Ex vivo* treatment of these cells with ABT-199 resulted in both direct ($P = 0{\cdot}02$) and enhanced apoptosis when combined with either ibrutinib or idelalisib ($P \leq 0{\cdot}05$ vs. any monotherapy) regardless of *CXCR4* mutation status (Fig 2A). We also examined ABT-199 in primary WM cells within their microenvironment from three patients (1 $MYD88^{L265P}CXCR4^{WT}$; 2 $MYD88^{L265P}CXCR4^{S338X}$) on active ibrutinib treatment for ≥6 months (Fig 2C). While *ex vivo* ibrutinib treatment had little effect, treatment with ABT-199 induced apoptosis in WM cells from two of three patients (Fig 2D).

In these studies, we sought to clarify if BCL2 expression was impacted by $CXCR4^{WHIM}$ mutations, and whether BCL2 contributed to ibrutinib and idelalisib resistance in $CXCR4^{WHIM}$-mutated WM cells. Despite the lack of BCL2 modulation by CXCL12, BCL2 protected against ibrutinib- and idelalisib-triggered apoptosis in $CXCR4^{WT}$ and $CXCR4^{S338X}$ BCWM.1 and MWCL-1 cells. ABT-199 triggered apoptosis in WM cells regardless of $CXCR4^{WHIM}$ mutation status and prior ibrutinib exposure. However, more robust CXCL12 protection against ABT-199 was observed in $CXCR4^{S338X}$-mutated cells, a finding that could be related to AKT1 and MAPK1 hyper-activation (Cao *et al*, 2014). AMD3100 blocked the protective effects of CXCL12 in WM cells treated with ABT-199 alone, and with ibrutinib or idelalisib. The long-term safety and efficacy of AMD3100 administration was recently demonstrated in WHIM-syndrome patients, and provides a paradigm for CXCR4 inhibition in WM patients.

Our findings therefore show that ABT-199 induces direct apoptosis, and enhances ibrutinib and idelalisib triggered apoptosis in both $CXCR4^{WT}$ and $CXCR4^{WHIM}$ WM cells. These findings may be relevant to other therapeutics, and support further investigation of ABT-199 alone, and in combination strategies in WM patients.

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Authorship contributions

YC, GY and SPT conceived and designed the experiments. SPT, GY, ZRH performed the data analysis. JC, XL, LX, YC, GY, EH, and NT performed the laboratory experiments. SK, JJC, MSD, and SPT participated in patient care, sample and data collection. SPT wrote the manuscript.

Disclosure of conflicts of interest

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