

Ibrutinib and idelalisib target B cell receptor- but not CXCL12/CXCR4-controlled integrin-mediated adhesion in Waldenström macroglobulinemia

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SUPPLEMENTAL DATA

Supplemental Materials & Methods

Materials

The following reagents were used in this study: the phosphorylation state-specific antibodies phospho-p44/42-MAPK [T202/Y204] against p-ERK-1 and -2, phospho-AKT [Ser473] against p-AKT (Cell Signaling Technology), phospho-BTK [Y551] against p-BTK (BD Biosciences), phospho-BTK [Y223] against p-BTK (Epitomics); anti-ERK2 (Santa Cruz Biotechnology), anti-AKT (Santa Cruz Biotechnology), anti-BTK (BD Bioscience), goat F(ab')₂ anti-human IgM (LE/AF; Southern Biotech), horseradish peroxidase (HRP)-conjugated rabbit anti-mouse and HRP-conjugated goat anti-rabbit (Dako); phycoerythrin-conjugated rabbit F(ab')₂ anti-IgM (Dako), mouse anti-CD79 β (SN8; Dako), mouse anti-integrin α_4 (HP2/1; Immunotech), mouse anti-integrin β_1 (4B4; Coulter), phycoerythrin-conjugated goat F(ab')₂ anti-kappa (Cytognos), and rabbit F(ab')₂ anti-lambda (Dako). Control mouse IgG₁ or IgG_{2a} (Dako), phycoerythrin-conjugated goat anti-mouse IgG₁ or IgG_{2a} (Southern Biotech), mouse anti-CXCR4 (BD Biosciences), allophycocyanin-conjugated mouse anti-CD19 (HD37; Dako); the pharmacological inhibitors ibrutinib and idelalisib (Selleck Chemicals), wortmannin and chelerythrine (Sigma-Aldrich); human plasma fibronectin (Sigma-Aldrich), BSA (fraction V; Roche), and recombinant human sVCAM-1 (R&D Systems), carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen), poly-L-lysine (PLL; Sigma-Aldrich).

Cell lines and primary material

The Waldenström macroglobulinemia (WM) cell lines MWCL-1¹ and BCWM.1², which both carry the WM-characteristic MYD88^{L265P} mutation (Figure S1A), are IgL- κ^+ and IgL- λ^+ , respectively (Figure S1C), and are both monoclonal, as determined by *IGH* GeneScan analysis (Figure S1B), were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine and pen/strep. Bone marrow aspirations from 2 WM patients were obtained after routine diagnostic or follow-up procedures at the departments of Hematology of the Academic Medical Center (AMC) Amsterdam, and mononuclear cells were purified using Ficoll. This study was conducted and approved by the AMC Medical Committee on Human Experimentation. Informed consent was obtained in accordance with the Declaration of Helsinki.

Signal transduction assays

Immunoblotting was performed essentially as described.^{3,4} In detail, 10⁷ cells/ml serum free RPMI were pretreated with DMSO, ibrutinib or idelalisib at 37°C for 1h. After stimulation with 500ng/ml goat F(ab')₂ anti-human IgM or 100ng/ml CXCL12, after 1,2 and 5 minutes the cells were directly lysed in SDS-PAGE sample buffer. Lysates (2.10⁵ cells/lane) were applied on a 10% SDS-PAGE gel, blotted and incubated with rabbit anti-phospho-ERK1/2, rabbit anti-phospho-AKT, mouse anti-phospho-Y551-BTK, and rabbit anti-phospho-Y223-BTK in 5% milk/TBST followed by HRP-conjugated goat anti-rabbit or rabbit anti-mouse and developed by enhanced chemoluminescence (Amersham Pharmacia). To confirm equal expression and loading, the blots were stripped, and incubated with the antibodies rabbit anti-ERK-2

rabbit anti-AKT, and mouse anti-BTK. Blots are representative of at least three independent experiments.

Growth, proliferation and viability assays

Cell number, CFSE, and viability were measured in individual wells. Cells were washed with PBS, and labeled with 1 μ M CFSE for 15 minutes at 37°C. CFSE was quenched with FCS, and cells were plated (10⁴) in a 96-well plate with 10%FCS/RPMI and DMSO, ibrutinib, and/or idelalisib. At day 0 and 5, numbers of viable cells were counted using a FACS Canto II flow cytometer system (BD Biosciences, San Jose, CA, USA) interfaced to FACS Diva software (v 6.0), and analyzed with Flow Jo (v7.2.1), the percentage of growth of untreated cells at day 5 was normalized to 100%; CFSE was measured in FITC channel, the geometric mean of the untreated cells at day 5 was normalized to 1.0; and viability was determined using FSC-SSC gating (which correlated completely with Annexin V- or DiOC₆-stainings in all cell lines), the percentage viable cells of untreated cells at day 5 was normalized to 100%. The bars represent the means + SEM of at least three independent experiments, each assayed in triplicate.

Adhesion assays

The cell adhesion assays were performed essentially as described.^{3,4} In detail, adhesion assays were performed in triplicate on EIA/RIA 96-well plates (Costar) coated with PBS containing 10 μ g/ml fibronectin or 500ng/ml VCAM-1 at 4°C overnight, or with 1mg/ml poly-L-lysine (PLL) at 37°C for 15 minutes, and blocked with 4% BSA/RPMI at 37°C for 1h. Cells were pretreated with DMSO, 100nM ibrutinib, 1 μ M idelalisib, 100nM wortmannin, or 1 μ M chelerythrine in 1%BSA/RPMI at 37°C for 1h. If indicated, unbound ibrutinib was washed out 3 times with 1% BSA/RPMI. Subsequently, cells were stimulated with either 500ng/ml goat F(ab')₂ anti-human IgM, or 50ng/ml PMA, and 1,5.10⁵ BCWM.1 or MWCL.1 cells, or 4.10⁵ bone marrow mononuclear cells from WM patients in 100 μ l were immediately plated and incubated at 37°C for 30 minutes. After extensive washing of the plate with 1% BSA/RPMI to remove non-adhered cells, the adherent cells were fixed for 10 minutes with 10% glutaraldehyde in PBS and subsequently stained for 45 minutes with 0.4% crystal violet/20% methanol/water. After extensive washing with water, the dye was eluted in methanol and absorbance was measured after 40 minutes at 570nm on a spectrophotometer (Multiskan RC spectrophotometer, Thermo labsystems). Background absorbance (no cells added) was subtracted. Absorbance due to nonspecific adhesion, as determined in wells coated with 4% BSA, was always less than 10% of the absorbance of anti-IgM-stimulated cells. Maximal adhesion (100%) was determined by applying the cells to wells coated with PLL, without washing the wells before fixation. For analysis of primary WM cells, cells were detached with 2mM EDTA/0.5% BSA/0.02% NaN₃/PBS, followed by an allophycocyanin-conjugated anti-CD19 staining. The numbers of adhered CD19⁺ WM cells were analyzed on a FACScanto II flow cytometer system (BD Biosciences, San Jose, CA, USA) interfaced to FACS Diva software (v 6.0), and analyzed with Flow Jo (v7.2.1). Adhesion of the nonpretreated anti-IgM-stimulated cells was normalized to 100% and the bars represent the means + SEM of at least three independent experiments, each assayed in triplicate, or means +SEM of at least three independent experiments, each assayed in triplicate.

Chemokine-mediated adhesion was assayed as described above, except that the chemokine CXCL12 (50ng/ml) was co-immobilized with 500ng/ml VCAM-1. The

plates were spun directly after applying the cells, and the cells were allowed to adhere for 5 minutes.

Migration assays

The cell migration assays were performed essentially as described.⁵ In detail, migration assays were performed in triplicate with transwells (pore size 8µm) coated with 1µg/ml VCAM-1 or uncoated. The lower compartment contained 100ng/ml CXCL12 in 0.5%BSA/RPMI. $5 \cdot 10^5$ cells/100ul 0.5%BSA/RPMI, pretreated with DMSO, ibrutinib and/or idelalisib at 37°C for 1h, were applied to the upper compartment and allowed to migrate for 5h at 37°C. The amount of viable migrated cells was determined by FACS and expressed as a percentage of the input. The migration of nonpretreated cells on VCAM-1-coated transwells in the presence of CXCL12 was normalized to 100%, and the bars represent the means + SEM of at least three independent experiments, each assayed in triplicate.

PCR

The MYD88 mutation was determined as described.⁶ DNA was isolated with the QIAamp DNA Micro kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Screening DNA for the *MYD88* L265P mutations was performed with allele-specific PCR assays, employing primers that were designed to specifically anneal with their 3'-terminal nucleotide to either the mutated or wild-type base. The primers were: *MYD88* (L265P; T794C) Fw: TGCCAGGGGTACTTAGATGG + Rv: CCTTGTA CTTGATGGGGA-TCG and *MYD88* (WT): Fw: GTGCCCATCAGAA-GCGACT + Rv: GGGCCTCAGAACAGTCTTCA. The *IGH* GeneScan analysis was performed as described.⁷

Flow cytometry

10^5 cells were stained with phycoerythrin-conjugated anti-IgM, anti-kappa, anti-lambda, or with mouse anti-CXCR4, anti-CD79β, anti-α₄-integrin, anti-β₁-integrin, or control mouse IgG₁ or IgG_{2a} (isotype control), and secondary stained with phycoerythrin-conjugated goat anti-mouse IgG₁ or IgG_{2a} for 30 min on ice and washed. Cell staining was measured on a FACScanto II flow cytometer system (BD Biosciences, San Jose, CA, USA) interfaced to FACS Diva software (v 6.0), and analyzed with Flow Jo (v7.2.1). For CXCR4 internalization, the cells were pretreated with DMSO, 100nM ibrutinib, and/or 1µM idelalisib for 1h, and subsequently with 100nM CXCL12 for 5 minutes, and immediately kept on ice.

Synergy calculations

CompuSyn (ComboSyn, Inc.) was used for calculating combination indices, based on the Chou-Talalay method.⁸

Statistical analysis

Graphpad Prism (GraphPad Software Inc.) was used for all graphs and statistics. The one sample *t*-test was used to determine the significance of differences between means and normalized values. All multicomparisons were analyzed by a one-way analysis of variance (ANOVA). A *post hoc* Dunnett's *t*-test was carried out following a significant ANOVA, comparing the drugs treatments to the DMSO-controls. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

References

1. Hodge LS, Novak AJ, Grote DM, et al. Establishment and characterization of a novel Waldenstrom macroglobulinemia cell line, MWCL-1. *Blood* 2011;117(19):e190-7
2. Ditzel Santos D, Ho AW, Tournilhac O, et al. Establishment of BCWM.1 cell line for Waldenström's macroglobulinemia with productive in vivo engraftment in SCID-hu mice. *Exp Hematol.* 2007;35(9):1366-75.
3. de Rooij MF, Kuil A, Geest CR, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* 2012;119(11):2590-2594.
4. Spaargaren M, Beuling EA, Rurup ML, et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J.Exp.Med.* 2003;198(10):1539-1550.
5. de Gorter DJ, Beuling EA, Kersseboom R, et al. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity* 2007;26(1):93-104.
6. Kraan W, Horlings HM, van KM, et al. High prevalence of oncogenic MYD88 and CD79B mutations in diffuse large B-cell lymphomas presenting at immune-privileged sites. *Blood Cancer J.* 2013;3:e139.
7. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia.* 2003;17(12):2257-317.
8. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* 2010;70(2):440-446.

Supplemental Figures

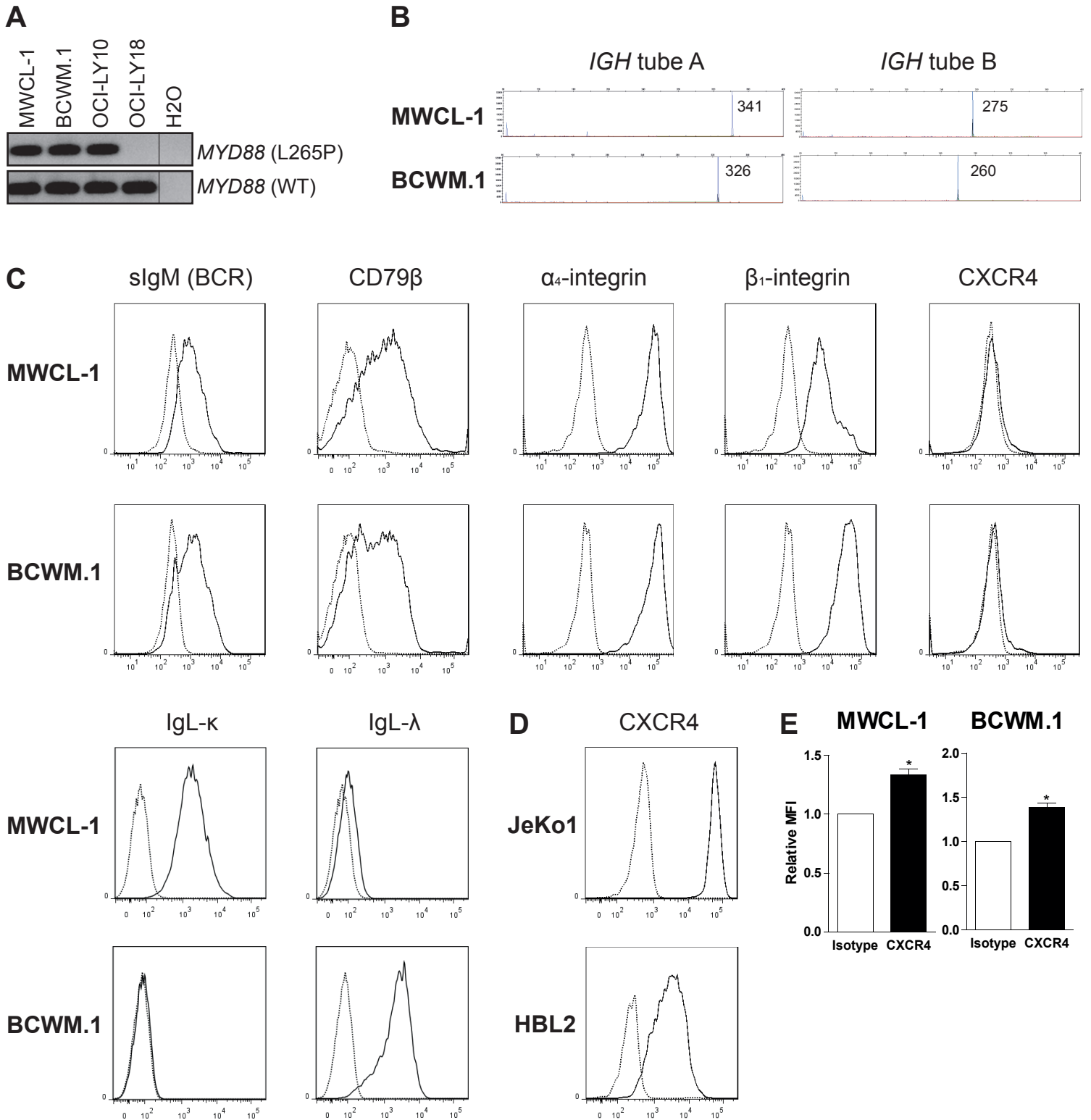


Figure S1. BCR, integrin, and CXCR4 expression in WM

(A) MWCL-1 and BCWM.1 were heterozygous for MYD88 (L265P; T794C), a characteristic of WM. The DLBCL samples OCI-LY10 and OCI-LY18 are positive and negative controls for MYD88 (L265P), respectively. (B) GeneScan analysis of Ig heavy chain (*IGH*) rearrangements from MWCL-1 and BCWM.1. (C) MWCL-1 and BCWM.1 were stained for surface IgM (BCR), the BCR-coreceptor CD79 β , α_4 -integrin, β_1 -integrin, the chemokine receptor CXCR4, and the kappa and lambda immunoglobulin light chains (solid lines), or isotype controls (dashed lines) and analyzed by flow cytometry. (D) The MCL cell lines JeKo1 and HBL2 (used as positive control) were stained for CXCR4 (solid lines), or isotype control (dashed lines) and analyzed by flow cytometry. (E) MWCL-1 (n = 3) and BCWM.1 (n = 4) were stained for CXCR4 or isotype control and analyzed by flow cytometry. Graphs are presented as normalized means + SEM (1.0 = MFI from isotype staining). *: p < 0.05 significantly different (one sample t-test).

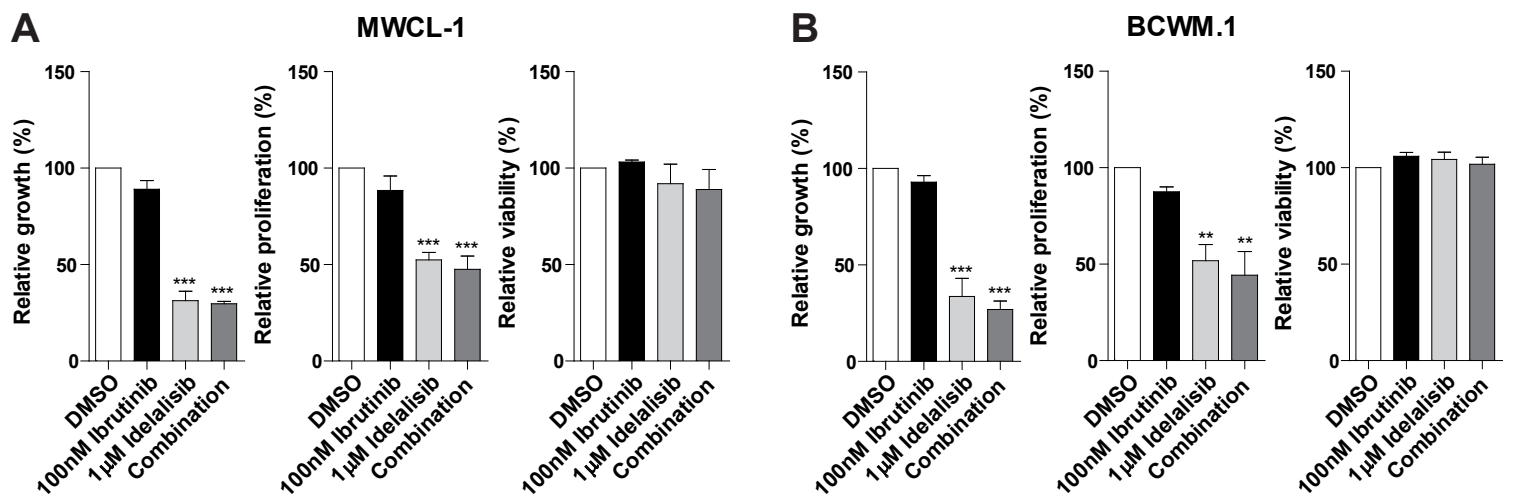


Figure S2. Idelalisib, but not Ibrutinib, strongly inhibits WM proliferation

MWCL-1 (A) and BCWM.1 (B) cells were labelled with CFSE and cultured in the presence of ibrutinib and/or idelalisib in combination. After 5 days, the numbers of viable cells were counted (left), proliferation was measured by analyzing the CFSE-dilution (middle) and the viability was determined (right) (n = 3 independent experiments). Graphs are presented as normalized means + SEM (100% = cells treated with only DMSO). * p < 0.05; ** p < 0.01; *** p < 0.001, significantly different from DMSO controls (one-way ANOVA followed by Dunnett's *t*-test).

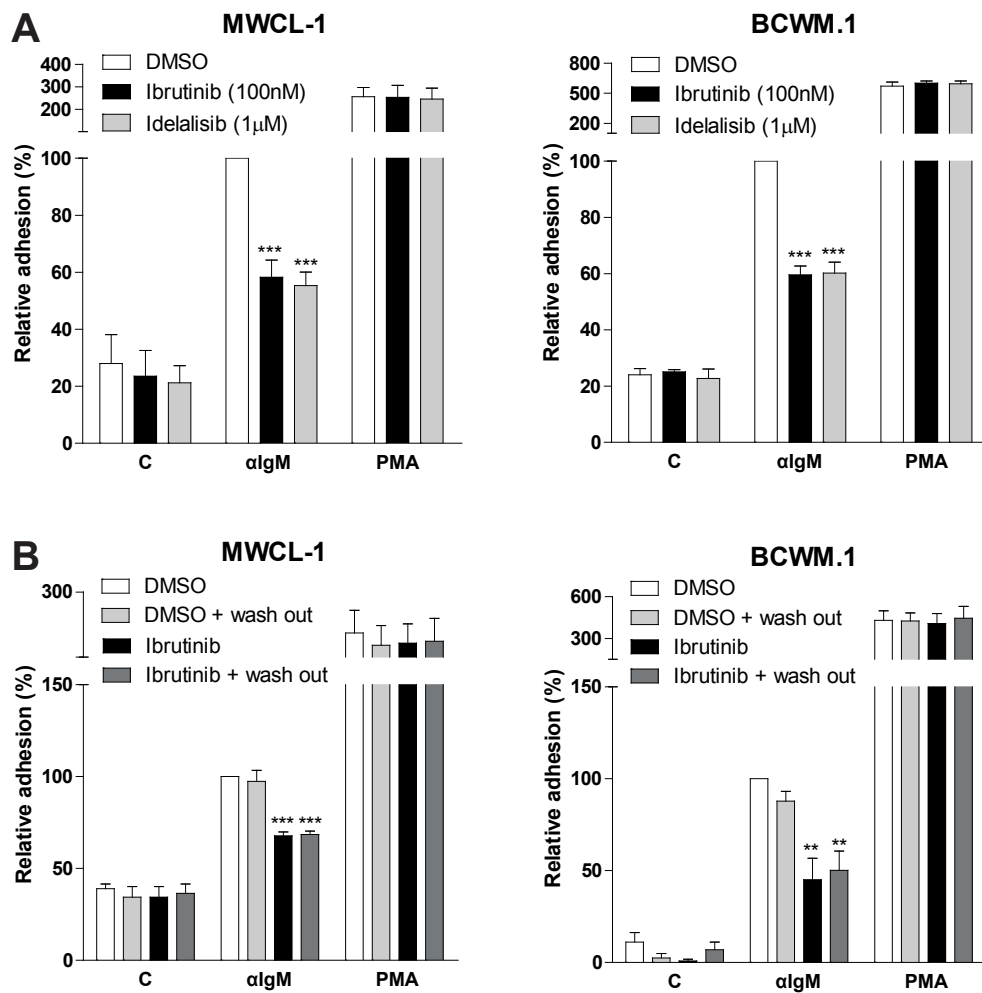


Figure S3. Ibrutinib and idelalisib target BCR-controlled integrin-mediated adhesion of WM cells
 (A) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib or 1μM idelalisib were stimulated with αIgM or PMA, and allowed to adhere to VCAM-1-coated surfaces for 30 minutes (n = 3 independent experiments). (B) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib were subsequently washed (to remove any non-covalently bound ibrutinib) and stimulated with αIgM, and allowed to adhere to fibronectin-coated surfaces for 30 minutes (n = 3 independent experiments). Graphs are presented as normalized means + SEM (100% = stimulated cells without inhibitors). * p < 0.05; ** p < 0.01; *** p < 0.001, significantly different from DMSO controls (one-way ANOVA followed by Dunnett's *t*-test).

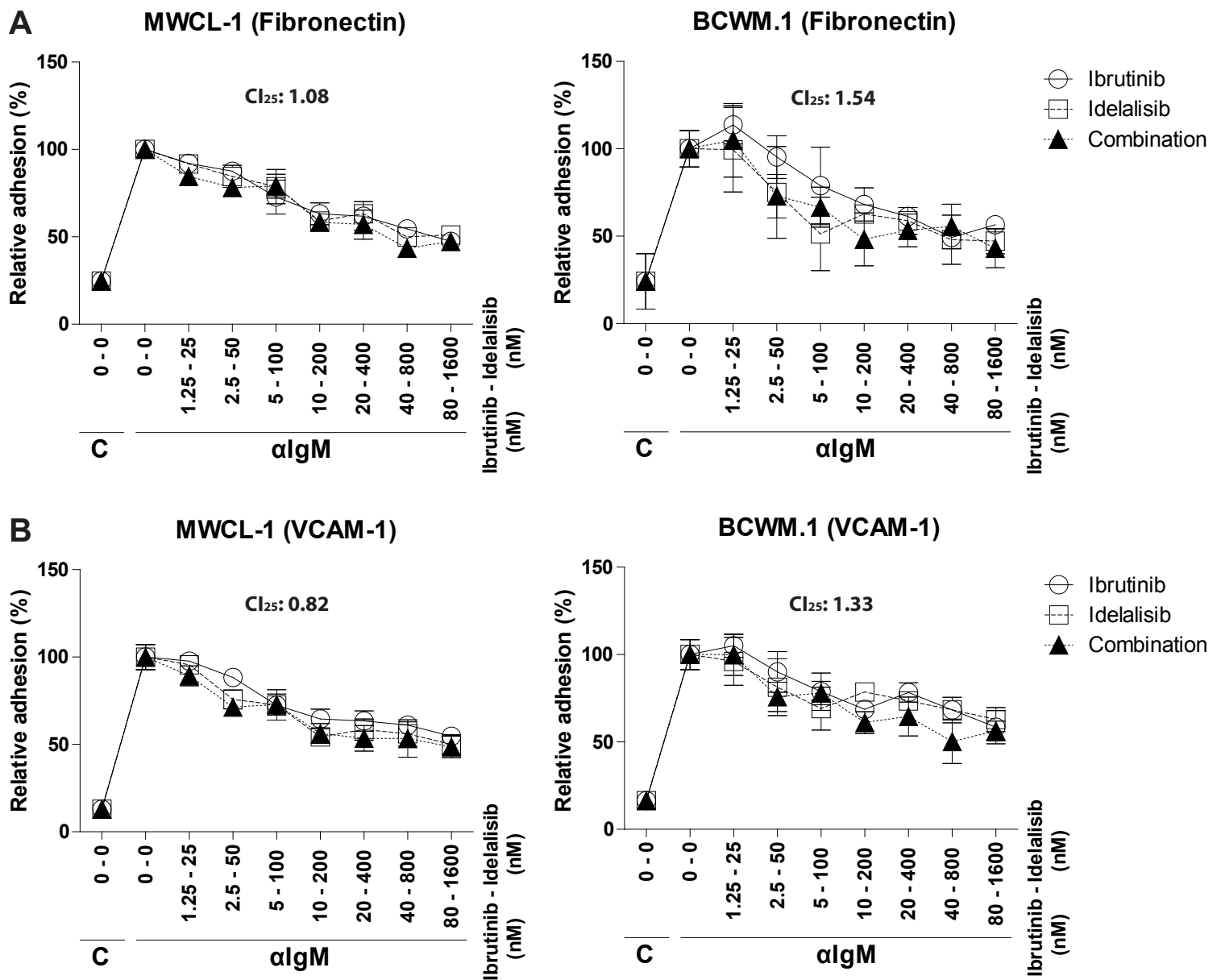


Figure S4. Ibrutinib and idelalisib do not act in a synergistic manner in WM

(A) MWCL-1 and BCWM.1 cells pretreated with different concentrations of ibrutinib, and/or idelalisib were stimulated with α lgM, and allowed to adhere to fibronectin-coated surfaces for 30 minutes. Combination indices (CI) were determined by the Chou-Talalay theorem of synergy calculations. (B) MWCL-1 and BCWM.1 cells pretreated with different concentrations of ibrutinib, and/or idelalisib were stimulated with α lgM, and allowed to adhere to VCAM-1-coated surfaces for 30 minutes. Combination indices (CI) were determined by the Chou-Talalay method of synergy calculations. CI_{25} : CI at IC_{25} (\rightarrow ~50% of maximal inhibition).

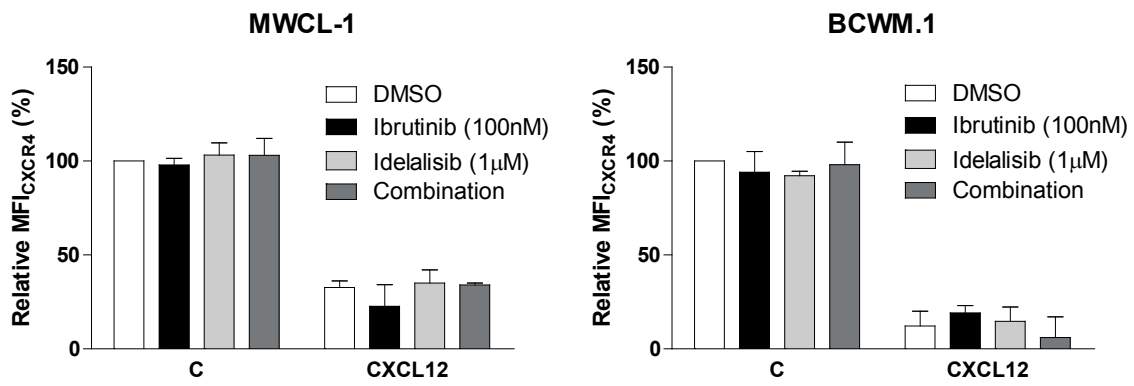


Figure S5. Ibrutinib and idelalisib do not affect CXCR4 internalization in WM

MWCL-1 (n = 3) and BCWM.1 (n = 3) cells pretreated with 100nM ibrutinib and/or 1µM idelalisib were stimulated with CXCL12 for 5 minutes, and were stained for surface CXCR4 and analyzed by flow cytometry. Graphs are presented as normalized means + SEM (100% = MFI from DMSO-treated cells). No significant differences were observed due to ibrutinib and/or idelalisib treatments (one-way ANOVA).