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

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The Bruton tyrosine kinase (BTK) inhibitor ibrutinib and the phosphatidylinositol-3-kinase δ (PI3Kδ) inhibitor idelalisib show promising clinical efficacy in the treatment of Waldenström macroglobulinemia (WM), a lymphoplasmacytic lymphoma. Very recently, ibrutinib became the first-ever FDA- and EMA-approved treatment for WM patients. Here, we investigated the molecular and cellular mechanisms underlying the clinical efficacy of ibrutinib and idelalisib in WM patients. We show that, at clinically relevant concentrations, idelalisib, but not ibrutinib, reduced proliferation of WM cells, whereas cytotoxicity was not observed. Furthermore, we demonstrate that WM cells express a signaling-competent B-cell antigen-receptor (BCR) which controls integrin-mediated adhesion, and that ibrutinib and idelalisib both inhibit BCR-controlled signaling and integrin-mediated adhesion, whereas chemokine (CXCL12/CXCR4)-controlled signaling, adhesion and migration are not affected. Our data indicate that ibrutinib and idelalisib target BCR-controlled retention of WM cells in the lymphoid organs, resulting in the clinically observed mobilization of malignant cells from these protective niches into the circulation; this may deprive the WM cells from essential microenvironmental growth and survival factors, resulting in WM regression. In addition, our results provide a molecular explanation for the relative ibrutinib-resistance of WM patients with gain-of-function CXCR4 mutations.

WM, a lymphoplasmacytic lymphoma, is characterized by the accumulation of post-germinal center B cells in bone marrow, spleen, liver and lymph nodes, which produce a monoclonal IgM M-protein. Apart from genetic lesions in the malignant cells, such as MYD88 and CXCR4 mutations, the bone marrow and lymphoid microenvironments play a critical role in the survival and proliferation of these cells. The CXCL12/CXCR4-axis plays a major role in homing of WM cells to these protective niches. Furthermore, WM cells express a biased IgH V repertoire, suggesting that (antigen-dependent) BCR signaling plays a role in the pathogenesis of WM.
In CLL and MCL, BCR signaling plays a prominent role in the regulation of integrin-mediated retention of malignant cells in lymphoid organs. In these patients, the BCR-signalosome inhibitors ibrutinib and idelalisib induce a rapid decrease in lymphadenopathy, accompanied by transient lymphocytosis. In CLL and MCL, we have previously demonstrated that ibrutinib and idelalisib target BCR-controlled - and ibrutinib also chemokine-controlled - integrin-mediated adhesion, resulting in mobilization of the malignant cells from their protective niches in the lymphoid organs into the circulation, followed by lymphoma regression. Recently, ibrutinib received FDA- and EMA-approval for the treatment of CLL and MCL, and idelalisib for small lymphocytic lymphoma and follicular lymphoma. Also for WM, the clinical trials were very promising, with an overall response rate of 90.5% (n=63) for ibrutinib, and 55-80% (n=9 and n=10) for idelalisib, and recently, ibrutinib became the first-ever FDA-approved treatment for WM patients. Interestingly, in WM-patients ibrutinib also induces lymphocytosis, but patients with CXCR4 mutations are relatively resistant against ibrutinib. Here, we investigated the molecular and cellular mechanisms underlying the clinical efficacy of ibrutinib and idelalisib in WM patients.

First we assessed the possible effect of ibrutinib and idelalisib on cell growth in the WM cell-lines MWCL-1 and BCWM.1, which both carry the WM-characteristic MYD88L265P mutation (Figure S1A). Cell growth was already reduced at 10-100nM idelalisib, but only at 1μM ibrutinib (Figure 1 A and S2 A-B). The observed dose-dependency of ibrutinib was in agreement with Yang et al. Distinguishing between proliferation and viability revealed that at clinically relevant/achievable concentrations (i.e., Cmax ibrutinib 170nM (dosis 420mg/day) and idelalisib 6μM (350mg/day)) only idelalisib inhibited proliferation, whereas neither drug affected cell viability (Figure 1 B-C and S2 A-B). The differential effect of idelalisib and ibrutinib may reflect the capacity of PI3K to regulate not only BTK- but also AKT-mediated signaling (Figure 2A), including mTOR-, GSK3- and FOXO-pathways.
Furthermore, it is tempting to suggest that aberrant NFκB activation by mutant MYD88 may compensate for ibrutinib treatment, since combining IRAK-inhibitors with ibrutinib enhances NFκB inhibition and WM cytotoxicity.\(^{13}\)

BCR-signaling controls survival, proliferation, and adhesion of B cells. After having established that the BCR is expressed (Figure S1 B-C) and functional in MWCL-1 and BCWM.1 cells (Figure 2 A, C) and in primary WM cells (Figure 2B), we investigated how ibrutinib and idelalisib affect BCR-controlled signaling. Ibrutinib completely abrogated BTK autophosphorylation (Y223), whereas phosphorylation of the activating LYN/SYK substrate-site Y551 of BTK was actually augmented (Figure 2A). A similar potentiation of BTK-Y551 phosphorylation upon ibrutinib treatment, indicative for the inhibition of BTK-mediated negative feedback of proximal BCR signaling, has been observed in other B cell lines (e.g. Namalwa Burkitt’s cells,\(^5\) and MCL cell lines (manuscript in preparation)). Interestingly, this occurred in the absence of additional BCR-cross linking of the WM cells (Figure 2A), suggesting substantial basal BCR-signaling (tonic/chronic BCR-signaling). In addition, ERK activation was inhibited by ibrutinib, but AKT activation was not affected. Previously, we and others have reported that ibrutinib abrogated AKT-signaling in CLL and MCL cells,\(^5,6\) but we have recently demonstrated that this was not related to specific BTK-inhibition (manuscript in preparation). Idelalisib completely abrogated AKT activation, activation of BTK by LYN/SYK, which requires PIP\(_3\)-mediated membrane association, and activation of ERK were also inhibited, but BTK autophosphorylation was not affected (Figure 2A).

An important function of the BCR, of major relevance for the clinical efficacy of ibrutinib and idelalisib in CLL and MCL, is the control of integrin-mediated adhesion/retention. Indeed, BCR stimulation induced adhesion of the WM cell lines as well as primary WM cells to the extracellular matrix component fibronectin and the cellular adhesion molecule VCAM-1 (Figure 2 B-C and S3 A), which is mediated by integrin \(\alpha_4\beta_1\) (being
expressed on MWCL-1 and BCWM.1 cells (Figure S1C). Moreover, BCR-controlled adhesion was inhibited by 40-50% upon ibrutinib and idelalisib treatment (Figure 2 C and S3 A). Adhesion in response to the PKC-activator PMA was not attenuated (Figure 2 C and S3 A), demonstrating that the observed effects of ibrutinib and idelalisib on BCR-controlled integrin activation were selective, and not caused by cytotoxicity. Inhibition of adhesion by ibrutinib and idelalisib was already observed at 3.2nM and 100nM, respectively (Figure 2D), well within their clinically achievable ranges. Furthermore, the ibrutinib effect persisted upon wash-out (Figure S3B), indicating it is BTK-specific, involving covalent irreversible binding of ibrutinib to BTK. Unlike in CLL and MCL,8 combining the drugs did not enhance the inhibitory effect (Figure 2 E and S4 A-B). Nevertheless, combination (or sequential) therapy could still be beneficial in WM as it may possibly prevent or overcome single drug-resistance, e.g. due to mutations in BTK or PLCγ2. The partial effects of ibrutinib and idelalisib on adhesion can be explained by involvement of parallel pathways, implicating other kinases. In support of this, the pan-PI3K-inhibitor wortmannin and the more distally acting PKC-inhibitor chelerythrine completely abolished BCR-controlled adhesion (Figure 2E). Given the previously reported interaction of BTK with L265P-MYD88,13 it is tempting to speculate that BTK engaged in either the TLR- or BCR-signalosome may control different cellular functions.

Many components of the BCR-signalosome are also involved in CXCL12/CXCR4-signaling. CXCR4 is expressed on MWCL-1 and BCWM.1 cells, although at low levels (Figure S1 C-E). Membrane expression and CXCL12-induced internalization of CXCR4 were not affected by ibrutinib and idelalisib (Figure S5). Treatment with ibrutinib completely abrogated BTK autophosphorylation (Y223), whereas phosphorylation of Y551 was augmented; however, both already in the absence of CXCL12, indicating it may rather reflect inhibition of tonic/chronic BCR-signaling (Figure 3A). Furthermore, ibrutinib did not affect
CXCL12-induced activation of ERK and AKT. Idelalisib reduced AKT phosphorylation, but again already in the absence of CXCL12, most likely reflecting inhibition of tonic/chronic BCR-signaling (Figure 3A). Activation of BTK and ERK were not affected. Moreover, CXCL12-induced adhesion and migration were not (specifically) inhibited by ibrutinib and idelalisib (Figure 3B-D); the effect of ibrutinib on CXCL12-induced adhesion was reversible upon washout, which demonstrates it does not reflect a BTK-specific action of ibrutinib (Figure 3C). Together, these data indicate that the BCR-signalosome components are not critical for CXCL12-induced responses in WM.

Although CXCL12 is important for homing, most likely it is not involved in retention: in lymphoid tissues CXCL12 is abundantly expressed, causing CXCR4 desensitization and internalization. Interestingly however, approximately 30% of WM patients carry WHIM-like mutations in CXCR4 (e.g., S338X); this mutation prevents CXCR4 desensitization, which may result in aberrant CXCL12-controlled adhesion and sustained retention of WM cells in lymphoid organs (Figure 3E). Combined with our observation that ibrutinib (or idelalisib) does not target CXCL12-controlled adhesion of WM cells, this may explain why WM patients with these gain-of-function CXCR4 mutations show a strongly reduced lymphocytosis upon, and are less responsive to, ibrutinib treatment as compared to patients with wild type CXCR4.³

Taken together, our data show that ibrutinib and idelalisib target tonic and antigen-controlled BCR-signaling in WM cells, thereby inhibiting BCR-controlled integrin-mediated adhesion (Figure 3E). In vivo this would result in impaired retention of WM cells in lymphoid tissues, explaining the lymphocytosis observed upon ibrutinib treatment.³ Thus, our data indicate that ibrutinib and idelalisib do not directly kill WM cells but rather target their BCR-controlled adhesion, thereby causing mobilization of the malignant cells from the protective
niches in the lymphoid organs into the circulation, resulting in deprivation of microenvironmental growth- and survival-factors, and clinically evident WM regression.


Figure 1. Idelalisib, but not Ibrutinib, strongly inhibits WM proliferation

MWCL-1 and BCWM.1 cells were labelled with CFSE and cultured in the presence of different concentrations of ibrutinib or idelalisib. After 5 days, the numbers of viable cells were counted (A), proliferation was measured by analyzing the CFSE-dilution (B), and the viability was determined (C), (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 2. Ibrutinib and idelalisib target BCR-controlled signaling and integrin-mediated adhesion of WM cells

(A) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib or 1μM idelalisib were stimulated with 500ng/ml αIgM, and immunoblotted for p-BTK (pY551 and pY223), p-AKT, and p-ERK. Total BTK, AKT, and ERK2 were used as loading controls. (B) Bone marrow mononuclear cells from 2 WM patients were stimulated with αIgM or PMA, and allowed to adhere to fibronectin-coated surfaces for 30 minutes. Adherence of CD19+ WM cells was quantified by flow cytometry. (C) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib or 1μM idelalisib were stimulated with αIgM or PMA, and allowed to adhere to fibronectin-coated surfaces for 30 minutes. The means are from 6 (BCWM.1) or 10 (MWCL-1) independent experiments. (D) MWCL-1 cells pretreated with different concentrations of ibrutinib or idelalisib were stimulated with αIgM and allowed to adhere to fibronectin-coated surfaces for 30 minutes. (E) MWCL-1 cells pretreated with different BCR-signalosome inhibitors were stimulated with αIgM or PMA, 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 3. Ibrutinib and idelalisib do not target CXCL12-controlled integrin-mediated adhesion and migration of WM cells**

(A) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib or 1μM idelalisib were stimulated with 100ng/ml CXCL12, and immunoblotted for p-BTK (pY551 and pY223), p-AKT, and p-ERK. Total BTK, AKT, and ERK2 were used as loading controls. (B) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib and/or 1μM idelalisib were allowed to adhere to VCAM-1- and CXCL12-cocoated surfaces for 5 minutes (n = 3 independent experiments). (C) MWCL-1 cells pretreated with 100nM ibrutinib were subsequently washed (to remove any non-covalently bound ibrutinib) and allowed to adhere to VCAM-1- and CXCL12-cocoated surfaces for 5 minutes (n = 3 independent experiments). (D) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib and/or 1μM idelalisib were allowed to migrate towards CXCL12 in VCAM-1-coated transwells for 5 hours (n = 3 independent experiments). (E) Model of the mechanism of action of ibrutinib and idelalisib in WM. Inhibition of BTK by ibrutinib or PI3Kδ by idelalisib impairs BCR-controlled integrin-mediated adhesion of WM cells in bone marrow (BM) and lymph nodes (LN), which results in their egress from these protective niches into the circulation, resulting in WM regression. The homing receptor CXCR4 is normally desensitized upon binding of CXCL12, which is highly expressed within the lymphoid organ microenvironment; however, this is prevented by the WHIM-like mutation (S338X), lacking the regulatory domain. Consequently, CXCR4 S338X might aberrantly support retention of WM cells in the lymphoid organs. Since CXCR4-controlled integrin-mediated adhesion is insensitive to ibrutinib and idelalisib, this would
counteract their ability to inhibit BCR-controlled integrin activation, thus explaining the clinically observed ibrutinib-resistance of WM patients with the CXCR4\textsuperscript{S338X} mutation.

Graphs are presented as normalized means $\pm$ SEM (100% = stimulated cells without inhibitors). NS: not significantly different from DMSO controls (one-way ANOVA).
Figure 1 (de Rooij et al)
Figure 2 (de Rooij et al)
Figure 3 (de Rooij et al)
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-CD79β (SN8; Dako), mouse anti-integrin α4 (HP2/1; Immunotech), mouse anti-integrin β1 (4B4; Coulter), phycoerythrin-conjugated goat F(ab′)2 anti-kappa (Cytognos), and rabbit F(ab′)2 anti-lambda (Dako). Control mouse IgG1 or IgG2a (Dako), phycoerythrin-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotech), mouse anti-CXCR4 (BD Biosciences), allophycoerythrin-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 succinimidy ester (CFSE; Invitrogen), poly-L-lysine (PLL; Sigma-Aldrich).

Cell lines and primary material
The Waldenström macroglobulinemia (WM) cell lines MWCL-1 and BCWM.1, 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. In detail, 10^7 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 (2x10^5 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 1µM CFSE for 15 minutes at 37°C. CFSE was quenched with FCS, and cells were plated (10^5) 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 10µg/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, 1µM idelalisib, 100nM wortmannin, or 1µM 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 1.5.10^5 BCWM.1 or MWCL.1 cells, or 4.10^5 bone marrow mononuclear cells from WM patients in 100µl 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 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). 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. 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. 5.10⁵ 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. 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: CTTTGACTTTGATGGGA-TCG and MYD88 (WT): Fw: GTGCCCATCAGAA-GCGACT + Rv: GGGCCTCAGAACAGTCTTCA. The IGH GeneScan analysis was performed as described.

**Flow cytometry**
10⁵ 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 IgG₂a (isotype control), and secondary stained with phycoerythrin-conjugated goat anti-mouse IgG₁ or IgG₂a 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.

**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


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 CD79β, α4-integrin, β1-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 αIgM, 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 αIgM, 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).