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ORIGINAL ARTICLE

The WHIM-like CXCR4^{S338X} somatic mutation activates AKT and ERK, and promotes resistance to ibrutinib and other agents used in the treatment of Waldenstrom's Macroglobulinemia

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CXCR4^{WHIM} somatic mutations are common Waldenstrom's Macroglobulinemia (WM), and are associated with clinical resistance to ibrutinib. We engineered WM cells to express the most common WHIM (Warts, Hypogammaglobulinemia, Infections and Myelokathexis), CXCR^{S338X} mutation in WM. Following SDF-1a stimulation, CXCR4^{S338X} WM cells exhibited decreased receptor internalization, enhanced and sustained AKT kinase (AKT) and extracellular regulated kinase (ERK) signaling, decreased poly (ADP-ribose) polymerase and caspase 3 cleavage, and decreased Annexin V staining versus CXCR4 wild-type (WT) cells. CXCR4^{S338X}-related signaling and survival effects were blocked by the CXCR4 inhibitor AMD3100. SDF-1a-treated CXCR4^{S338X} WM cells showed sustained AKT and ERK activation and decreased apoptotic changes versus CXCR4^{WT} cells following ibrutinib treatment, findings which were also reversed by AMD3100. AKT or ERK antagonists restored ibrutinib-triggered apoptotic changes in SDF-1a-treated CXCR4^{S338X} WM cells demonstrating their role in SDF-1a-mediated ibrutinib resistance. Enhanced bone marrow pAKT staining was also evident in CXCR4^{WHIM} versus CXCR4^{WT} WM patients, and remained active despite ibrutinib therapy in CXCR4^{WHIM} patients. Last, CXCR4^{S338X} WM cells showed varying levels of resistance to other WM relevant therapeutics, including bendamustine, fludarabine, bortezomib and idelalisib in the presence of SDF-1a. These studies demonstrate a functional role for CXCR4^{WHIM} mutations, and provide a framework for investigation of CXCR4 inhibitors in WM.

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INTRODUCTION

Whole-genome sequencing has revealed MYD88 L265P and CXCR4 mutations as the most prevalent somatic mutations in Waldenstrom's Macroglobulinemia (WM).[1,2] Approximately 90–95% and 30% of WM patients harbor somatic mutations in MYD88 and CXCR4, respectively.[3–8] Both nonsense and frameshift somatic mutations in the C-terminal domain of CXCR4 have been reported in WM patients.[2,8] MYD88 triggers downstream signaling through Bruton's tyrosine kinase (BTK) and interleukin-1 receptor-associated kinases, which promote nuclear factor κB signaling.[6,9] The impact of CXCR4 somatic mutations remains to be clarified in WM. The location of somatic mutations in the C-terminal domain in WM patients is similar to that observed in the germline of patients with WHIM (Warts, Hypogammaglobulinemia, Infections and Myelokathexis) syndrome, a congenital immunodeficiency disorder characterized by chronic noncyclic neutropenia.[10] Mutations in the C-terminal domain block CXCR4 receptor internalization following SDF-1a stimulation, thereby allowing the receptor to remain in an activated state.[11,12] AKT kinase (AKT), extracellular regulated kinase (ERK) and possibly BTK are activated in response to WT CXCR4 signaling by SDF-1a, and activation of these pathways has been shown to support the growth and survival of WM cells.[9,11–14] Ibrutinib is a novel anti-neoplastic agent that binds to and inactivates BTK. Ibrutinib also suppresses phosphatidylinositol 3-kinase-δ-triggered AKT and ERK signaling.[15] We therefore examined the impact of WHIM-like CXCR4 mutations by engineering WM cells to express the most common somatic mutation (CXCR4^{S338X}) identified by whole-genome sequencing in WM patients, and studied WM cell growth and signaling of AKT, ERK and BTK in response to ibrutinib.

MATERIALS AND METHODS

CXCR4^{WT} and CXCR4^{S338X} cDNAs were subcloned into plenti-IRES-GFP vector, and transduced using an optimized lentiviral-based strategy into BCWM.1 and MWCL-1 WM cells.[1,9] Five days after transduction, green fluorescent protein (GFP)-positive cells were sorted and used for functional studies. Surface expression of CXCR4 was determined by flow cytometric analysis using a phycoerythrin-cyanine 5-conjugated anti-CXCR4 monoclonal antibody (BD Biosciences, San Jose, CA, USA). Transduced cell lines were stimulated with or without SDF-1a (10–100 nM), and cell surface expression of CXCR4 determined on CXCR4^{WT}- and S338X-transduced cells. CXCR4 expression levels were calculated as: [(receptor geometric mean fluorescence intensity [MFI] of treated cells-MFI of isotype IgG control)/(receptor geometric MFI of unstimulated cells-MFI of isotype IgG control)] × 100. For phosphoflow experiments, cells were fixed with BD Phosflow Fix Buffer1 (BD Biosciences) at the indicated time point at 37 °C for 10 min followed by two washes with 1 × perm/wash buffer I. Fluorescence-activated cell sorting analysis was performed using conjugated antibodies to phospho-ERK1/2 (T^{202}/Y^{204}), phospho-AKT(S^{473}) (BD Phosflow) and phospho-BTK (Y^{223}) (BD Pharmigen, San Jose, CA, USA). Results were confirmed by immunoblotting. Cell signaling and survival studies related to CXCR4 signaling were performed in the presence or absence of SDF-1a (20 nM; R&D Systems, Minneapolis, MN, USA), ibrutinib (0.5 μM), bendamustine (5–10 μM), fludarabine (3 μM), bortezomib (5 nM), idelalisib (0.5 μM; MedChem Express, Monmouth, NJ, USA), the CXCR4 inhibitor AMD3100 (30 μM) and pertussis toxin (500 ng/ml), a G-protein-coupled receptor (GPCR) antagonist that blocks CXCR4 signaling (Sigma-Aldrich, St Louis, MO, USA). Cell signaling and survival studies related to AKT and ERK

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were performed in CXCR4^{S338X}-expressing cells as described above, in the presence or absence of either AKT- (MK-2206, 0.5 μM and AZD-5363, 0.5 μM) or MEK- (AS-703026, 0.25 μM; AZD-6244, 0.5 μM and UO126, 5.0 μM) specific inhibitors at their IC_{50} dose (Selleck Chemicals Inc., Houston, TX, USA). For survival studies, WM cells were incubated for 6 h and apoptosis assessed by immunoblotting using antibodies for cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase 3 (Abcam, Cambridge, MA, USA), and also by Annexin V staining (R&D Systems) in the presence of 5.0 μM of BCL-2 inhibitor (GDC-0199; Selleck Chemicals Inc.) to optimize ibrutinib-related apoptotic effects in SDF-1a rescue experiments. Bone marrow core biopsies from WM patients whose aspirates were used to sort for CD19$^+$ cells and Sanger sequencing for the C-terminal domain were stained for phospho-AKT and phospho-ERK (Cell Signaling Technologies, Danvers, MA, USA) before and after ibrutinib therapy.

RESULTS

Non-transfected BCWM.1 and MWCL-1 cells express very low levels of CXCR4. We therefore transfected these cell lines with plenti-IRES-GFP vector alone, CXCR4^{WT} or CXCR4^{S338X} WHIM-like protein-expressing vectors. Flow cytometric analysis confirmed expression, as well as similar levels of cell surface CXCR4 expression for CXCR4^{WT} and CXCR4^{S338X}-engineered WM cells (Figure 1a). Stimulation of transfected WM cells with the ligand for CXCR4 (SDF-1a) for 30 min resulted in significantly greater downregulation of cell surface CXCR4 expression on CXCR4^{WT} versus CXCR4^{S338X}-expressing WM cells ($P<0.001$; Figure 1b). Because AKT, ERK and possibly BTK are known downstream signal mediators of CXCR4, we next interrogated their signaling by phosphoflow and immunoblotting. WM cells were stimulated with SDF-1a for 2, 15 and 30 min and evaluated by phosphoflow analysis. Stimulation with SDF-1a resulted in enhanced and prolonged AKT and ERK activation in CXCR4^{S338X} versus GFP vector only and CXCR4^{WT}-expressing BCWM.1 and MWCL-1 WM cells (Figure 2a). In contrast, only minimal changes in BTK activation were observed between vector only, CXCR4^{WT} and CXCR4^{S338X}-expressing cells stimulated with SDF-1a using phosphoflow analysis. Immunoblotting after 2 min of SDF-1a stimulation confirmed enhanced AKT and ERK phosphorylation in

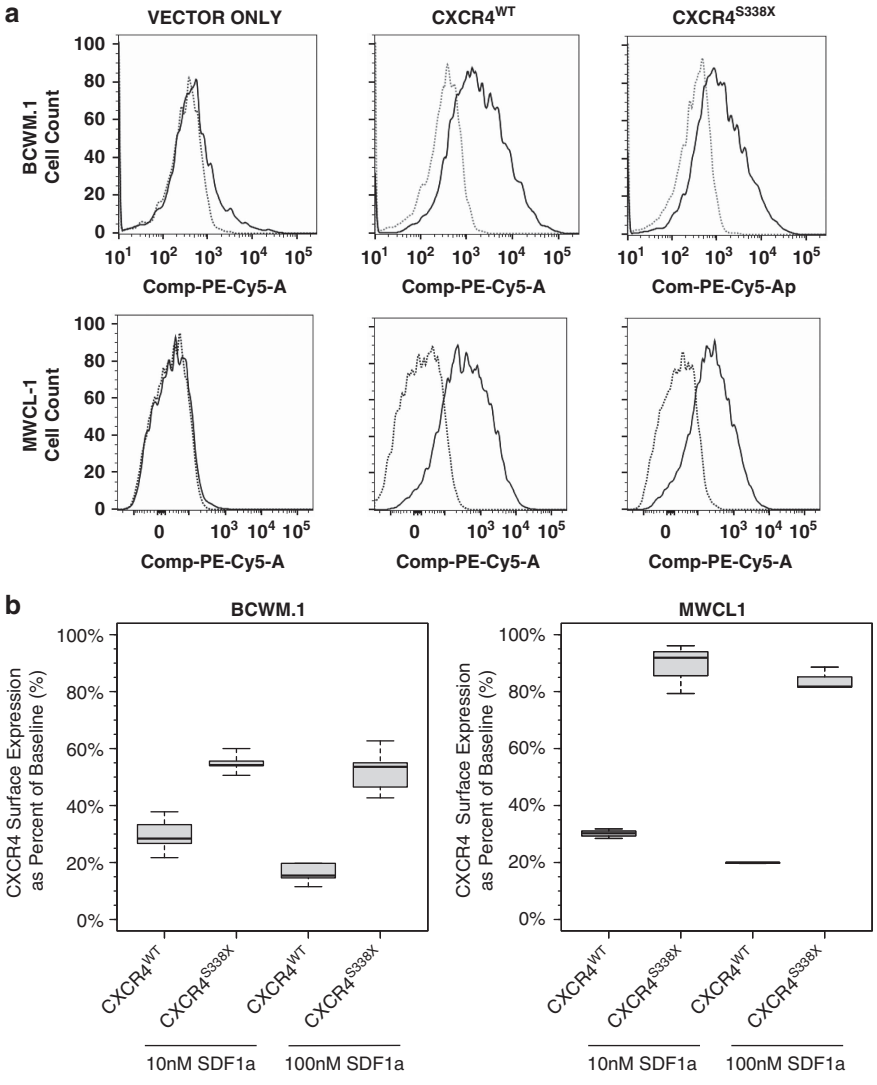

Figure 1. CXCR4 cell surface expression following SDF-1a stimulation of CXCR4^{WT} and CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells. (**a**) Cell surface expression of CXCR4 in vector only, CXCR4^{WT} and CXCR4^{S338X}-engineered BCWM.1 and MWCL-1 cells by flow cytometry using PE-Cy5-conjugated anti-CXCR4 mAb (12G5) (dark line) or isotype control (gray line). (**b**) Changes in cell surface CXCR4 expression following stimulation of CXCR4^{WT} and CXCR4^{S338X}-engineered BCWM.1 and MWCL-1 cells for 30 min at 37 °C with SDF-1a (10 nM, 100 nM). Surface CXCR4 expression was assessed by flow cytometry and expression relative to baseline levels are shown. Data represent the median of at least three independent experiments; *$P<0.001$ for CXCR4^{S338X} versus CXCR4^{WT}-expressing BCWM.1 and MWCL-1 cells at both 10 nM and 100 nM dose of SDF-1a. PE-Cy5, phycoerythrin-cyanine 5.

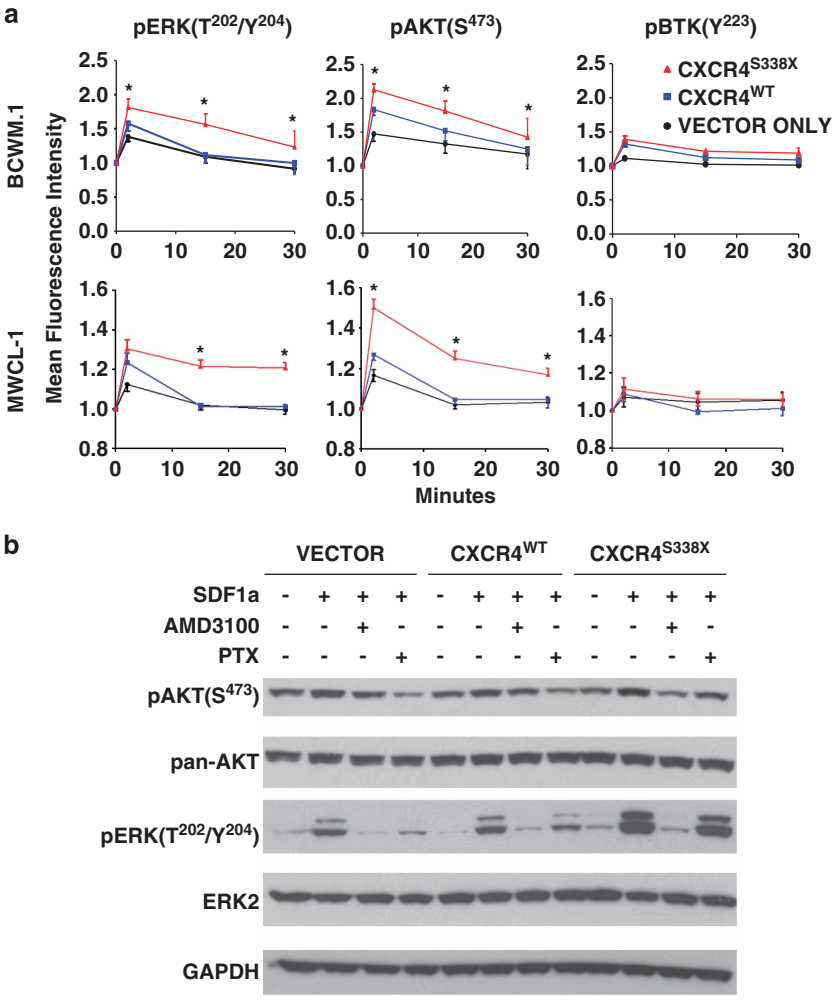

Figure 2. Impact of SDF-1a on AKT, ERK or BTK activation in plenti-GFP vector only, CXCR4^{WT} and CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells. (**a**) plenti-GFP vector, CXCR4^{WT} and CXCR4^{S338X}-expressing WM cells were treated with SDF-1a (20 nM) for 2, 15 and 30 min and phosphoflow analyses performed using conjugated anti- phospho-ERK (T^{202}/Y^{204}), phospho-AKT (S^{473}) and phospho-BTK(Y^{223}) antibodies. Data represent the mean of at least three experiments ± s.e.m.; *$P < 0.05$ for CXCR4^{S338X} versus CXCR4^{WT}. (**b**) Immunoblotting studies depicting differences in phospho-ERK (T^{202}/Y^{204}), total ERK, phospho-AKT (S^{473}), total AKT in plenty-GFP vector only, CXCR4^{WT} and CXCR4^{S338X}-expressing BCWM.1 cells stimulated for 2 min with SDF-1a (20 nM) after either no pre-treatment or pre-treatment for 2 h with AMD3100 (30 μM) or pertussis toxin (500 ng/ml; PTX). Membranes were stripped following pERK and pAKT staining, and were then probed for total ERK and AKT as shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

CXCR4^{S338X} BCWM.1 cells relative to vector only, and CXCR4^{WT} cells (Figure 2b). Total AKT and ERK protein levels remained the same in vector only, CXCR4^{WT} and CXCR4^{S338X}-expressing BCWM.1 cells following SDF-1a stimulation in these studies, denoting that activation of AKT and ERK occurred in the absence of changes in total protein expression for these transcription factors (Figure 2b). Importantly, both AMD3100 and pertussis toxin blocked both AKT and ERK activation confirming their SDF-1a-triggered transactivation via GPCR/CXCR4 signaling in both CXCR4^{WT} and CXCR4^{S338X}-expressing BCWM.1 cells (Figure 2b).

Because AKT, ERK and BTK signaling are impacted by ibrutinib, we next cultured CXCR4^{WT} and CXCR4^{S338X}-expressing WM cells in the presence or absence of SDF-1a and/or ibrutinib. We examined cell surface expression of CXCR4 in transfected WM cells following ibrutinib treatment at 0.5, 1, 2 and 6 h and observed little or no significant changes in cell surface CXCR4 expression in either CXCR4^{WT} or CXCR4^{S338X}-expressing WM cells (data not shown). Furthermore, addition of ibrutinib did not affect SDF-1a-related changes in cell surface CXCR4 expression in vector only, CXCR4^{WT} and CXCR4^{S338X}-transfected WM cells (data not shown). We then examined the impact of SDF-1a-triggered AKT, ERK and BTK

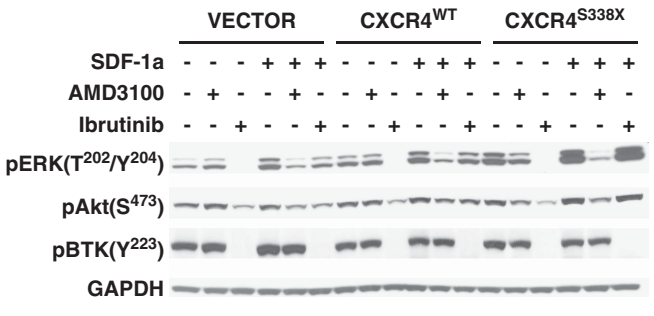

Figure 3. Impact of ibrutinib on phospho-AKT, ERK and BTK expression following SDF-1a stimulation of plenti-GFP vector, CXCR4^{WT} and CXCR4^{S338X}-expressing BCWM.1 cells. Plenti-GFP vector, CXCR4^{WT} and CXCR4^{S338X}-expressing BCWM.1 cells were pretreated for 2 h with either ibrutinib (0.5 μM) or AMD3100 (30 μM) before stimulation with SDF-1a (20 nM) for 2 min. Results depict differences in phospho-AKT, phospho-ERK and phospho-BTK obtained by immunoblotting following SDF-1a stimulation in the absence or presence of ibrutinib (0.5 μM). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

signaling following ibrutinib treatment in CXCR4^{WT} and CXCR4^{S338X} BCWM.1 cells. Ibrutinib attenuated SDF-1a-triggered AKT and ERK activation in plenti-GFP vector only and CXCR4^{WT}-expressing WM cells, whereas in CXCR4^{S338X}-expressing WM cells both AKT and ERK signaling remained robust and did not show attenuation in the presence of ibrutinib (Figure 3). Conversely, in both SDF-1a-treated CXCR4^{WT} and CXCR4^{S338X}-expressing WM cells, ibrutinib blocked BTK signaling (Figure 3). These studies therefore show that SDF-1a triggered AKT and ERK, but not BTK activation despite treatment with ibrutinib in CXCR4^{S338X}-expressing BCWM.1 cells.

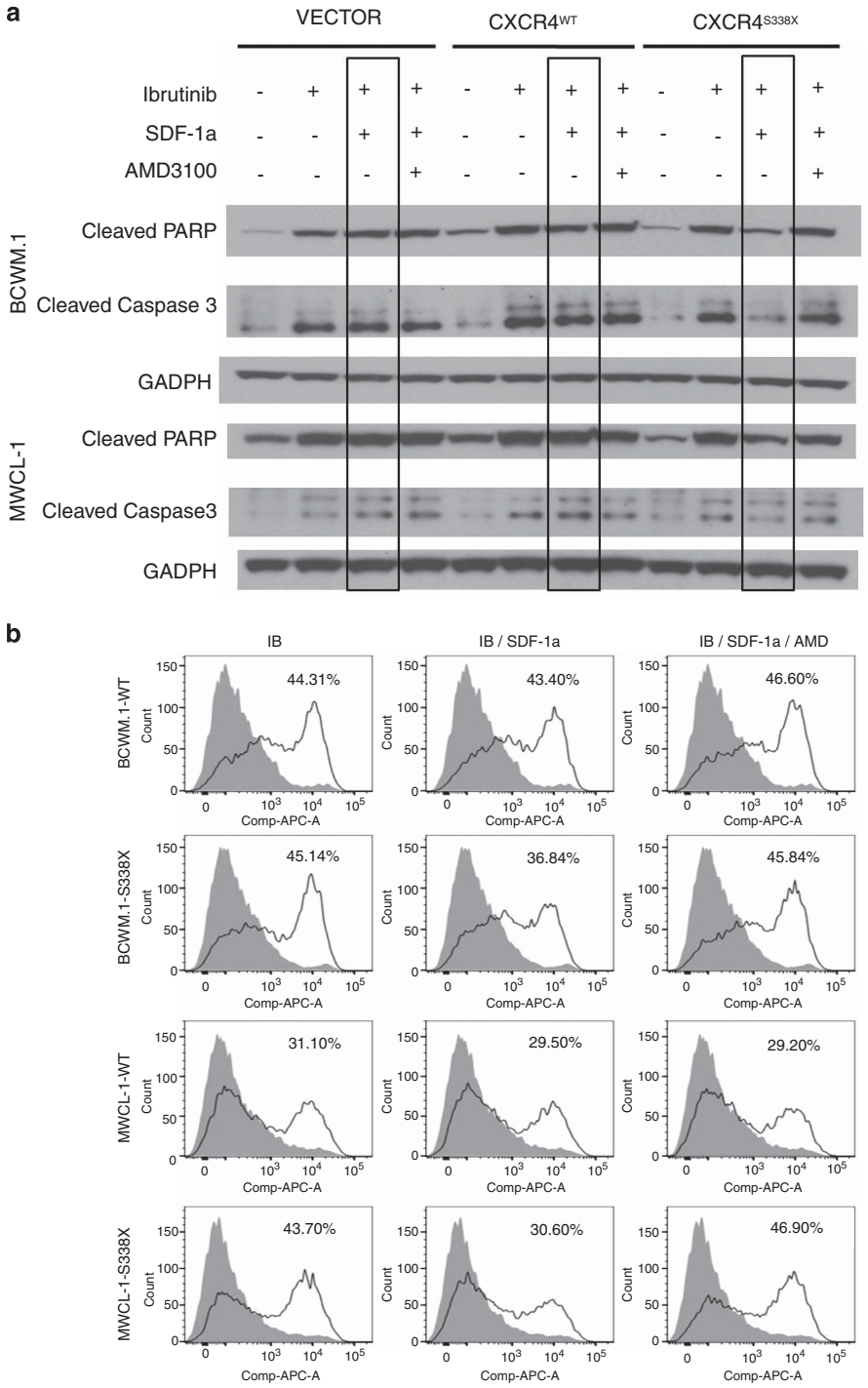

Figure 4. Impact of CXCR4^{S338X} expression on ibrutinib triggered apoptosis. (**a**) CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells show resistance to ibrutinib (IB)-induced PARP and caspase 3 cleavage in the presence of SDF-1a and reversed by AMD3100. Plenti-GFP vector, CXCR4^{WT} and CXCR4^{S338X}-expressing WM cells were treated for 6 h with IB (0.5 μM) in the presence or absence of SDF-1a (20 nM) and/or the CXCR4 receptor antagonist AMD3100 (30 μM). PARP and caspase 3 cleavage were assessed by immunoblotting at 6 h. (**b**) Annexin V staining of wild-type and CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells following treatment with dimethyl sulfoxide (DMSO) vehicle control (shaded curve), IB, IB plus SDF-1a (IB/SDF-1a), or IB plus SDF-1a and the CXCR4 inhibitor AMD3100 (IB/SDF-1a/AMD) at 18 h (non-shaded curves). Percentages shown denote treatment-related Annexin V staining (outside of DMSO vehicle control). Study was performed in triplicate, and results from a representative study set are shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

To clarify if the expression of the CXCR4^{S338X} mutant protein conferred enhanced survival against ibrutinib, we next cultured SDF-1a-treated vector only, CXCR4^{WT} and CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells in the presence or absence of ibrutinib (0.5 μM) and/or AMD3100 for 6 h. We then assessed apoptotic changes by evaluating for cleaved PARP (a caspase 3 substrate) and cleaved caspase 3 given prior studies establishing caspase-mediated killing for ibrutinib.[15] As shown in Figure 4, treatment with ibrutinib for 6 h led to increased PARP and caspase 3 cleavage in vector only, CXCR4^{WT} and CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells. Co-culture with SDF-1a failed to protect plenti-GFP vector only and CXCR4^{WT}-expressing cells from ibrutinib-induced PARP and caspase 3 cleavage. Conversely, SDF-1a rescued CXCR4^{S338X}-expressing WM cells from ibrutinib-induced apoptosis, a finding that could be reversed by co-treatment of CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells with both ibrutinib and the CXCR4 receptor antagonist AMD3100 (Figure 4). Annexin V staining confirmed rescue of ibrutinib-mediated apoptotic changes by SDF-1a, as well as restoration of apoptotic changes by addition of AMD3100 to BCWM.1 and MWCL-1 cells treated with ibrutinib and SDF-1a (Figure 4).

As AKT and ERK, but not BTK, show continued SDF-1a-triggered activation in CXCR4^{S338X}-expressing WM cells treated with ibrutinib, we next sought to clarify if AKT and ERK contributed to the enhanced survival of these cells. We therefore treated SDF-1a-cultured CXCR4^{S338X} BCWM.1 cells with either AKT- (MK-2206 and AZD-5363) or MEK- (AS-703026, AZD-6244 and UO126) specific inhibitors with and without ibrutinib (0.5 μM) for 6 h so as to clarify the contribution of AKT and ERK to ibrutinib resistance. The inhibitory activity of MK-2206, as well as AS-703026, AZD-6244 and UO126 was confirmed by western blot analysis for pAKT (S^{473}) and pERK (T^{202}/Y^{204}), respectively (Figure 5). The inhibitory effect of AZD-5363 on AKT, which is known to paradoxically hyper-phosphorylate pAKT(S^{473}) was confirmed by inhibition of the phospho-activity for the downstream AKT targets glycogen synthase kinase 3β and pS6 (Figure 5).[16,17] As before, SDF-1a blocked ibrutinib-triggered PARP and caspase 3 cleavage in CXCR4^{S338X}-expressing BCWM.1 cells. Conversely, addition of either AKT or ERK inhibitors to ibrutinib resulted in augmented PARP and caspase 3 cleavage versus ibrutinib alone in SDF-1a cultured CXCR4^{S338X} BCWM.1 cells (Figure 5).

As pAKT and pERK are enhanced in SDF-1a-stimulated CXCR4^{S338X} versus CXCR4^{WT} WM cells, we next sought to determine whether these transcription factors were differentially activated in samples from WM patients with and without CXCR4^{WHIM} mutations. Bone marrow samples from six patients (three CXCR4^{WT} and three CXCR4^{WHIM}) with relapsed/refractory disease who underwent daily ibrutinib therapy, as previously described by us, were selected for these studies.[18] As shown in Figure 6, robust immunohistochemical staining for pAKT was present at baseline in bone marrow tumor samples from CXCR4^{WHIM} patients, whereas by comparison marginal staining for pAKT was present in CXCR4^{WT} patients. pERK staining was present at low levels in both CXCR4^{WT} and CXCR4^{WHIM} patients, without any discernible differences (data not shown). Importantly, in CXCR4^{WHIM} patients, pAKT staining remained robust without any changes from baseline despite these patients being on continuous ibrutinib therapy for 6 months. In contrast, CXCR4^{WT} patients on continuous ibrutinib therapy showed marginal pAKT staining relative to CXCR4^{WHIM} patients. As before, low levels of pERK staining were present despite ibrutinib therapy in all patients, regardless of CXCR4 mutation status (data not shown).

Given the protective effect of SDF-1a against ibrutinib in CXCR4^{S338X} BCWM.1 and MWCL-1 cells, we next sought to clarify if SDF-1a protected against apoptosis triggered by other WM relevant therapeutics (Figure 7). Treatment of CXCR4^{S338X} BCWM.1 and MWCL-1 cells with bendamustine, fludarabine, bortezomib or idelalisib at their EC$_{50}$ dose ranges resulted in changes in PARP and caspase 3 cleavage at 6 h, which varied based on treatment and WM cell type. CXCR4^{S338X} BCWM.1 cells exhibited moderate levels of SDF-1a-mediated rescue for bendamustine, fludarabine and bortezomib, and strong SDF-1a-mediated rescue for idelalisib, with reversal of rescue mediated by co-treatment of cells with AMD3100 for all agents. CXCR4^{S338X} MWCL-1 cells displayed a moderate level of rescue effect by SDF-1a for idelalisib, followed by lesser levels of rescue for fludarabine and bortezomib, with reversal of SDF1-a rescue mediated by co-treatment with AMD3100. Conversely, little to no SDF-1a rescue was observed in bendamustine-treated CXCR4^{S338X} MWCL-1 cells (Figure 7). Idelalisib, which targets phosphatidylinositol 3-kinase-δ, a key modulator of AKT activation that supports WM cell survival,[19] showed pronounced rescue by SDF-1a and reversal of rescue by AMD3100 for Annexin V studies as well in both CXCR4^{S338X} BCWM.1 and MWCL-1 WM cells (Figure 7).

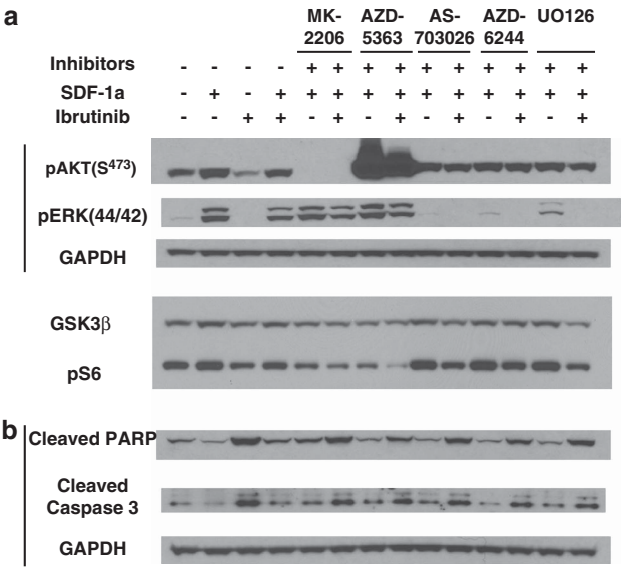

Figure 5. Inhibitors of AKT or ERK overcome SDF-1a-mediated resistance to ibrutinib-triggered PARP and caspase 3 cleavage in CXCR4^{S338X}-expressing BCWM.1 cells. CXCR4^{S338X}-expressing WM cells were treated with ibrutinib (0.5 μM) alone or in the presence of SDF-1a (20 nM) and/or the AKT inhibitors MK-2206 (0.5 μM) and AZD-5363 (0.5 μM); or the MEK inhibitors AS-703026 (0.25 μM), AZD-6244 (0.5 μM) and UO126 (5.0 μM). (**a**) Immunoblotting results for phospho-AKT (S^{473}) and phospho-ERK (T^{202}/Y^{204}) in CXCR4^{S338X}-expressing BCWM.1 cells pretreated with ibrutinib with and without AKT or ERK inhibitors, then subjected to SDF-1a stimulation for 2 min. The inhibitory effect of AZD-5363 on AKT, which is known to paradoxically hyper-phosphorylate pAKT(S^{473}) was confirmed by inhibition of the phospho-activity for the downstream AKT targets glycogen synthase kinase 3β and pS6. (**b**) Immunoblotting results for cleaved PARP and cleaved caspase 3 in CXCR4^{S338X}-expressing BCWM.1 cells treated with ibrutinib and/or AKT or ERK inhibitors for 6 h at IC$_{50}$ doses. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

DISCUSSION

We sought to address the functional significance of WHIM-like mutations in CXCR4 that are present in up to 30% of WM patients, and represent the first reporting of CXCR4 somatic mutations in cancer.[2] Hence, studies addressing the functional role of these mutations may yield important insights into the oncogenic role of WHIM-like CXCR4 mutations, as well as revelations that can be exploited therapeutically in WM. We show in these studies that the most common WHIM-like mutation (CXCR4^{S338X}) identified in WM patients conferred decreased receptor downregulation, as well as enhanced and sustained AKT and ERK, but not BTK activation

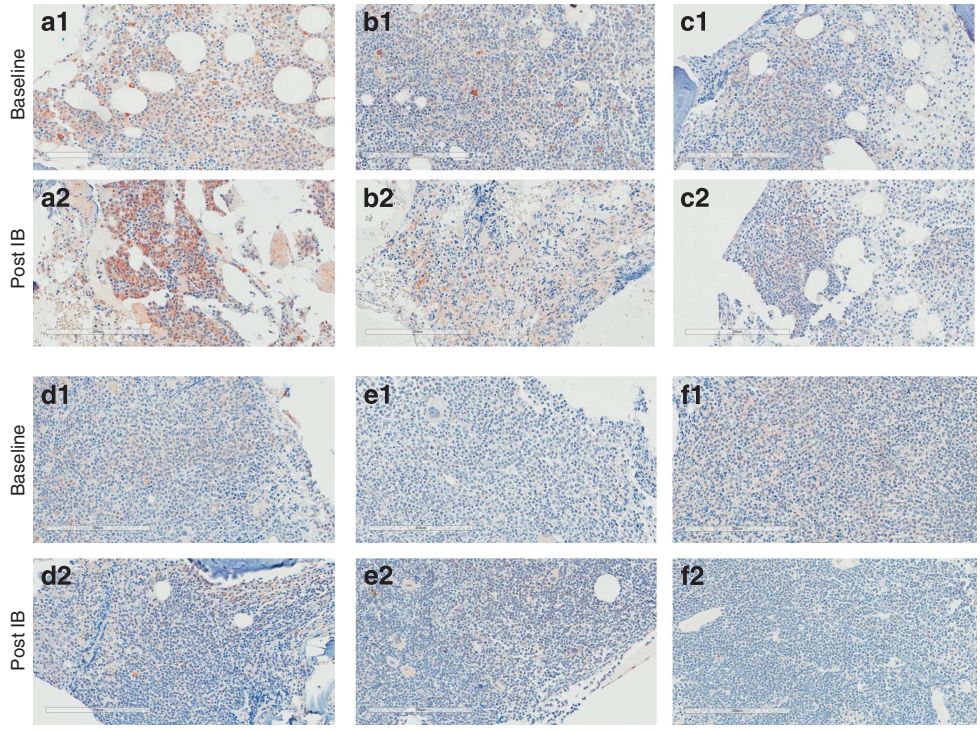

Figure 6. Immunohistochemical staining for pAKT in bone marrow samples from genotyped WM patients with $CXCR4^{WT}$ and $CXCR4^{WHIM}$ expression. Bone marrow specimens from three patients with $CXCR4^{WHIM}$ (**a**–**c**) and three patients with $CXCR4^{WT}$ (**d**–**f**) were stained for pAKT at baseline, and following 6 months of continuous ibrutinib therapy. For the depicted $CXCR4^{WHIM}$ patients, Sanger sequencing showed nonsense mutations for patient A ($CXCR4^{R334X}$) and B ($CXCR4^{S338X}$), and a frameshift mutation resulting in insertion of T at position 1013 for patient C ($CXCR4^{S338FS}$).

following SDF-1a. BTK activation following SDF-1a has been reported in myeloma cells, which show variable levels of BTK activation.[20] Conversely, BTK is activated by MYD88 L265P, and high levels of activated BTK are present in WM cell lines, which may have accounted for the minimal changes in phospho-BTK observed in response to SDF-1a stimulation.[9]

Both AKT and ERK are activated in WM cells, and inhibition of their activity leads to apoptotic changes thereby invoking a growth-promoting role for their activation in WM.[19,21] Our findings provide a putative mechanism for activation of both AKT and ERK by SDF-1a in WM through acquisition of a somatic WHIM-like ($CXCR4^{S338X}$) mutation. Enhanced AKT and ERK signaling in response to SDF-1a has also been observed in response to another WHIM-like ($CXCR4^{R334X}$) mutation, which similar to $CXCR4^{S338X}$ leads to truncation of the regulatory c-terminal domain of CXCR4.[22]

In our studies with $CXCR4^{S338X}$, as well as those by McDermott *et al.*[22] who investigated $CXCR4^{R334X}$-related signaling, use of the CXCR4 antagonist AMD3100 blocked SDF-1a-triggered AKT and ERK activation. The use of CXCR4 antagonists may therefore offer a targeted approach to therapy of WM patients with WHIM-like somatic mutations, particularly given their success in patients with WHIM syndrome patients who harbor germline CXCR4 mutations.[23] Several antagonists to CXCR4 have been developed. AMD3100 is approved for use in stem cell mobilization, whereas other CXCR4 antagonists such as BMS-936564, AMD-070, TG-0054 and others are in clinical trials. Although most cases of WM do not have CXCR4 somatic mutations, aberrant CXCR4 signaling may still exist because of either other CXCR4 path mutations as has been proposed for some WHIM-syndrome cases.[24] Investigation of other mechanisms for CXCR4 activation, as well a role for CXCR4 antagonists in patients with and without $CXCR4^{WHIM}$ mutations may be illuminating.

The central finding of these studies was that the $CXCR4^{S338X}$ WHIM-like mutation conferred resistance to ibrutinib-triggered apoptosis in WM cells, a finding that was associated with persistent AKT and ERK activation. Our findings prompted us to examine the association of CXCR4 WHIM-like mutations in patients undergoing ibrutinib therapy. These studies showed that the clinical activity of ibrutinib was muted in WM patients harboring CXCR4 WHIM-mutations.[18] Approximately 80% of relapsed/refractory WM patients who expressed $CXCR4^{WT}$ attained a major response, compared with 30% with $CXCR4^{WHIM}$ mutations following ibrutinib therapy. The finding that enhanced AKT and ERK activity following SDF-1a is present in $CXCR4^{S338X}$-expressing cells, and that inhibition of these targets potentiated ibrutinib killing provides support for a potential explanation for these clinical results, as well as a novel mechanism for ibrutinib-related resistance. Consistent with these *in vitro* findings, we observed robust pAKT staining in tumor samples from $CXCR4^{WHIM}$ patients, which contrasted against marginal pAKT staining in tumor samples from $CXCR4^{WT}$ patients. Importantly, pAKT staining remained robust despite continuous ibrutinib therapy for 6 months in $CXCR4^{WHIM}$ patients, and continued to be marginal in $CXCR4^{WT}$ patients. Conversely, low-level pERK staining was observed at baseline, and following ibrutinib therapy in bone marrow samples, without any discernible differences between $CXCR4^{WT}$ and $CXCR4^{WHIM}$ patients. These findings depict constitutive AKT activity, which functions as a powerful survival factor in WM,[19] as being relevant to *in vivo* $CXCR4^{WHIM}$ signaling, and likely in view of the aggregate findings of this study as a likely contributor to clinical resistance to ibrutinib. The absence of pERK differences in patients with and without $CXCR4^{WHIM}$ mutations, whereas a surprise could reflect either *in vivo* steady-state attainment of pERK in response to SDF-1a, dependence of pERK signaling on other (non-CXCR4) triggered pathways, as well as micro-environmental effects, which could modulate pERK activity.

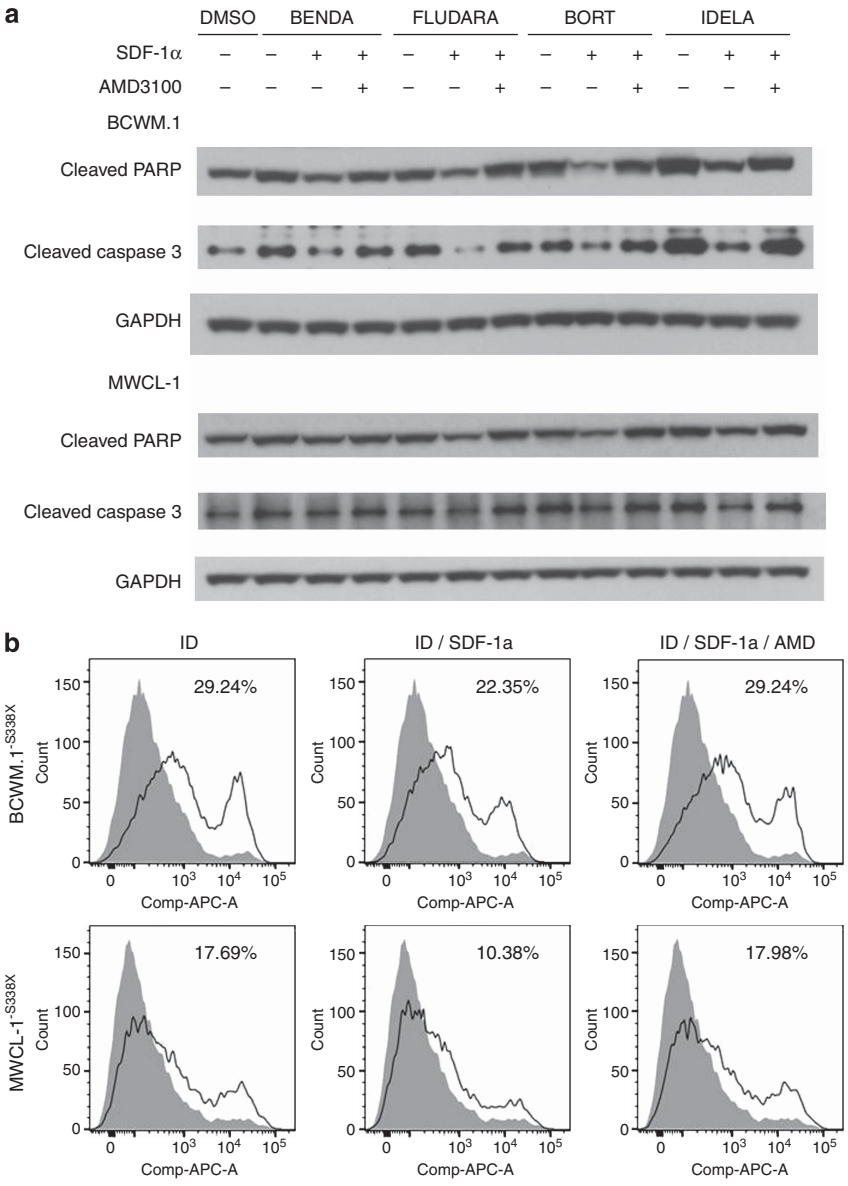

Figure 7. Impact of CXCR4^{S338X} expression on apoptosis triggered by other WM therapeutics. (**a**) CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells show variable resistance to PARP and caspase 3 cleavage mediated by WM relevant therapeutics in the presence of SDF-1a, and reversed by AMD3100. CXCR4^{S338X}-expressing WM cells were treated for 6 h with bendamustine (BENDA), fludarabine (FLUDARA), bortezomib (BORT) and idelalisib (IDELA) at their EC_{50} doses in the presence or absence of SDF-1a (20 nM) and/or the CXCR4 receptor antagonist AMD3100 (30 μM). PARP and caspase 3 cleavage was assessed by immunoblotting at 6 h. (**b**) Annexin V staining of CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells following treatment with dimethyl sulfoxide (DMSO) vehicle control (shaded curve), idelalisib (ID), idelalisib plus SDF-1a (ID/SDF-1a) or ID plus SDF-1a and the CXCR4 inhibitor AMD3100 (ID/SDF-1a/AMD) at 18 h (non-shaded curves). Percentages shown denote treatment-related Annexin V staining (outside of DMSO vehicle control). Study was performed in triplicate, and results from a representative study set are shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The additional finding in our studies that SDF-1a protected against apoptosis triggered by other WM relevant therapeutics, including bendamustine, fludarabine, bortezomib and idelalisib, in WM cells engineered to express the CXCR4^{WHIM} mutation is of great interest, and suggests the relevance of these findings against a broader array of agents used to treat WM. Rescue effects by SDF-1a in CXCR4^{S338X}-expressing BCWM.1 and MWCL-1 cells were particularly pronounced against idelalisib, a novel phosphatidylinositol 3-kinase-δ inhibitor that modulates AKT activity, and has shown promising activity in relapsed/refractory WM patients.[25] A prospective study is planned that will be evaluating the impact of CXCR4^{WHIM} mutations on idelalisib response in relapsed/refractory WM patients.

Although our study addressed intracellular signaling mediated by CXCR4^{WHIM} mutations, an impact on cell migration, adhesion and a protective microenvironment may also be relevant, as a CXCR4-blocking antibody protected mice inoculated with CXCR4^{S338X} expressing WM cells.[7] Therefore, combination strategies utilizing CXCR4 inhibitors with other chemotherapeutics could potentially be beneficial in WM patients carrying CXCR4^{WHIM} mutations and warrants further investigation. Last, these findings could also be relevant to patients with WM, as well as other B-cell malignancies despite the absence of CXCR4 WHIM mutations, as other components of CXCR4 signaling could be faulty and contribute to resistance by ibrutinib and possibly by other antineoplastic drugs.

In conclusion, our findings show that the most common CXCR4 WHIM-like somatic mutation in WM ($CXCR4^{S338X}$) confers decreased SDF-1a-triggered CXCR4 receptor internalization, enhanced AKT and ERK activation, and resistance to ibrutinib-triggered apoptosis in WM cells. Use of inhibitors targeting CXCR4 or AKT/ERK can restore the sensitivity of $CXCR4^{S338X}$-expressing WM cells to ibrutinib as well as other WM relevant agents, thereby providing a framework for the investigation of these combinations in WM.

CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

YC, ZRH and SPT conceived and designed the experiments, and wrote the manuscript. YC, ZRH and SPT performed the data analysis. LX, YC, XL, GY, NT and JC performed experiments. CJP, SK, JJC provided patient samples. SR performed immunohistochemistry studies.

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