p110α inhibition overcomes stromal cell-mediated ibrutinib resistance in mantle cell lymphoma

Running title: Stromal cell-mediated ibrutinib resistance

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Conflicts of Interest: The authors have no conflict of interest to declare.

Abstract

Acquired resistance to cancer drugs is common, also for modern targeted drugs like the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib, a new drug approved for the treatment of the highly aggressive and relapsing mantle cell lymphoma (MCL). The tumor microenvironment often impacts negatively on drug response. Here we demonstrate that stromal cells protect MCL cells from ibrutinib-induced apoptosis and support MCL cell regrowth after drug removal by impairing ibrutinib-mediated down-regulation of phosphoinositide-3-kinase (PI3K)/AKT signaling. Importantly, the stromal cell-mediated ibrutinib resistance was overcome *in vitro* by inhibiting AKT activity using the PI3K catalytic p110α subunit specific inhibitor BYL719. This was seen both for MCL cell lines and primary MCL cells. Furthermore, inhibition of p110α activity by BYL719 potentiated the ability of ibrutinib to inhibit MCL tumor growth *in vivo* in a mouse xenograft model. The stromal cell-mediated ibrutinib resistance was found to be due to a direct interaction with MCL cells and involves the integrin VLA-4, since disrupting stromal cell-MCL cell interaction using a VLA-4 blocking antibody abrogated the ibrutinib resistance. This suggests that combined treatment with ibrutinib and a p110α inhibitor, alternatively by disrupting stromal cell-MCL cell interaction, may be a promising therapeutic strategy to overcome stromal cell-mediated ibrutinib resistance in MCL.

Introduction

The tumor microenvironment (TME) with its non-malignant cells and stromal components has a major influence on tumor cell proliferation, survival, dissemination and resistance to therapy. This includes Non-Hodgkin lymphomas (NHL) (1,2). Furthermore, recent results suggest that the interaction between the TME and lymphoma cells is bidirectional, for example by the lymphoma cells secreting cytokines which in turn attract non-malignant cells like macrophages, immune and stromal cells that influence the tumor cells (2).

Mantle cell lymphoma (MCL), a B cell NHL, which comprises 5-7% of NHLs, and most often presents itself in an aggressive manner, is considered incurable. In MCL, a constitutive activation of the B cell receptor (BCR) signaling pathway has been shown to be essential in MCL pathogenesis. This involves increased levels of phosphorylated (active) Bruton tyrosine kinase (BTK) and SYK, two key signaling components of the BCR pathway (3-5). BCR signaling activates several downstream signaling pathways including nuclear factor-κB (NF-κB), PI3K/AKT, RAS and mitogen-activated protein kinase (MAPK), which all contribute to cell survival, proliferation, adhesion and migration of B cells (6,7). Particularly, high constitutive NF-κB activity is seen in both MCL cell lines and patient samples and is considered to play a central role in the pathogenesis of MCL (8,9). One of the most recently developed targeted drugs inhibiting the BCR/NF-κB signaling is ibrutinib (binds covalently to the active site of BTK at cysteine 481), which in clinical studies has been shown to have unprecedented effects on several B cell malignancies (10). Due to its significant efficacy, specificity and limited side effects, ibrutinib has been approved for treatment of relapsed/refractory MCL and chronic lymphocytic leukemia (CLL) (11).

Ibrutinib has been evaluated in clinical studies of relapsed/refractory MCL showing an overall response rate (partial or complete) of 50-68% (12,13). Still, initial primary resistance (32-50%) or acquired resistance to ibrutinib is common (13-16). Furthermore, most of the initially responsive MCL patients eventually acquire ibrutinib resistance and relapse, with a median duration of response limited to 3.5-17.5 months (12,13). Importantly, only in very few cases of MCL could development of ibrutinib resistance in relapsing MCL patients be attributed to an acquired mutation in the BTK (the enzyme targeted by ibrutinib), which was not present before the start of the ibrutinib treatment, that could explain ibrutinib resistance. For example, it was reported that only 1 out of 8 patients with non-mutated BTK post ibrutinib treatment experienced durable response >1 year (13). Taken together, acquired BTK

mutations in MCL during ibrutinib treatment seem to explain only a few cases in which ibrutinib resistance arises. This is indicative that other mechanisms probably are more relevant in MCL for the development of ibrutinib resistance. Considering the general impact of the TME on cancer drug sensitivity, it is not unlikely that the TME may play a role in the development of ibrutinib resistance. In fact, MCL cells have been shown to become resistant to conventional anti-cancer drugs in the presence of stromal cells (17-20). This suggests that TME-MCL interaction most likely contributes to drug resistance *in vivo*, which in turn underlies the cause for minimal residual disease (MRD) and relapse. However, the mechanism for acquired resistance to ibrutinib is largely unclear and may be complex. For example, PI3K/AKT or ERK1/2 activity, rather than BTK activity, may correlate to clinical response to ibrutinib in MCL (4,21). A more detailed understanding of the mechanism(s) of ibrutinib insensitivity will be very useful to find ways of overcoming it, thereby improving therapeutic outcome.

In this study, we have investigated the mechanism for stromal cell-mediated ibrutinib resistance and demonstrate how it can be overcome by targeting PI3K signaling by inhibiting the catalytic subunit p110α or by disrupting stromal cell-MCL cell interaction.

Materials and Methods

Chemicals and antibodies

Ibrutinib (PCI-32765, BTK-inhibitor), Idelalisib (CAL101, p110δ inhibitor), TGX221 (p110β inhibitor), CZC24832 (p110γ inhibitor), and LY294002 (pan-p110α/β/δ inhibitor) were obtained from Selleckchem. The structures, characteristics and references to these compounds can be found on the company homepage (sellleckchem.com). BYL719 (p110α inhibitor) was from Active Biochem. Integrin alpha4/CD49d (VLA-4) antibody (HP2/1) and mouse IgG Isotype control were from Thermo Fisher Scientific. Anti-Human CD19-APC (#302212) and anti-human/mouse phospho-AKT (473)-APC (#17-9715-42) antibodies were obtained from eBioscience. Human BD Fc Block™ antibody (#564220) was from BD Pharmingen™.

Cell lines and primary MCL cells

Human MCL cell lines Mino and Rec-1 as well as the murine stromal cell line MS-5 were from Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh (DSMZ), Leibzig, Germany. All the cells in the repository of and provided by DSMZ have undergone authentication by DNA (STR) profiling. The human follicular dendritic cell (FDC) line HK-2m was provided by Dr. Y.S. Choi (22). All the cell lines were tested mycoplasma free by the MycoAlert™ mycoplasma detection kit from Lonza. The primary MCL cells derived by blood sampling were from Prof. Anders Österborg, Hematology Center, Karolinska University Hospital, Solna, Sweden. All the cell lines were grown in in RPMI 1640 (GIBCO) supplemented with 10% FBS (GIBCO) and 100 IU penicillin/mL and 100ug/mL streptomycin (GIBCO).

Mice, tumor grafting and treatment

NOD/SCID IL-2Rγ^{null} (NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ) mice (23) also denoted NSG, were bred and kept under pathogen free conditions in the animal facility at Karolinska University Hospital (Huddinge, Sweden) as previously described (24). Xenografts were obtained by injecting male NSG mice subcutaneously in the right flank with 1x10^7 MCL cells, followed by intraperitoneal treatment once daily with vehicle, ibrutinib (5mg/kg/day), BYL719 (20mg/kg/day) or ibrutinib plus BYL719 from the day when the volume of the tumors reached 40-60mm^3. The tumor growth was measured daily and tumor volume was

calculated as 0.5 x length (mm) x width^2 (mm) using a caliper.

In vitro co-culturing

5×10^4-1×10^5 MCL cells were added per well in a 24-well plate with stromal cells that 3 days prior had been seeded with 1×10^5 murine MS-5 stromal or human follicular dendritic cells (FDC) per well (pre-treated with 10μg/ml mitomycin C (Sigma) for 2.5h). The co-cultures were then treated with either vehicle or drug for the number of days indicated.

To address the effect of conditioned cell medium on MCL cells, 1×10^5 murine MS-5 stromal cells (pre-treated with 10μg/ml mitomycin C (Sigma) for 2.5h) were grown for 3 days in a 24-well plate where after 5×10^4 MCL cells were added to an upper hanging cell culture insert (24-Well Millicell, Millipore). Vehicle or indicated drugs were then added to the medium for the number of days as stated in the figure legend, where after the MCL cells were harvested and analyzed.

Analysis of MCL cell proliferation (CFSE and EdU labelling)

Carboxyfluorescein succinimidyl ester (CFSE, Biolegend) labeled MCL cells were cultured (with or without stromal cells) and treated as indicated. Cell proliferation was indicated by a reduced CFSE signal (CFSE dilution). Alternatively, cell proliferation was analyzed by an EdU incorporation assay as described by the manufacturer of Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Thermo Fisher Scientific).

Analysis of MCL cell apoptosis

Harvested cells from the co-cultures were first stained with anti-human CD19-APC antibody in order to identify the human MCL cells. Apoptosis was determined by FITC-Annexin V/ 7-Aminoactinomycin D (7-AAD, eBioscience) staining, and apoptosis in the stained MCL cells was quantified by flow cytometry.

Analysis of AKT activity

Cells were fixed with Intracellular Fixation Buffer (eBioscience), permeabilized with absolute methanol, blocked with Human BD Fc Block™ antibody and stained with pAKT (473) antibody followed by analysis by flow cytometry.

Flow cytometry assay

A FACS Calibur (Becton-Dickinson) was used for all of the flow cytometry experiments. For each sample, approximately 10^4 cells were analyzed and the data was evaluated using FCS Express5 software. In all figures where the result from one flow cytometric analysis is shown, this exemplifies a representative result from 2-3 independent experiments.

RNA extraction and quantitative real-time PCR (qPCR)

RNA isolation, cDNA synthesis and qPCR were performed as described previously (25). The sequences of gene primers used are shown in Supplementary Table S1.

Statistical analysis

Unpaired, two-tailed students t-test or one-way ANOVA (Tukey's multiple comparison tests) were performed for the statistical analysis. $P<0.05$ was considered significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Mean values ± standard deviation (SD) are presented.

Ethical aspects

Animal care was in accordance with the guidelines of Karolinska Institutet and all animal experiments were performed according to the approval by the local animal ethical committee (Stockholm South Animal Ethical Committee, approval S61-14). Ethical permission for the use of primary MCL material was approved by the Regional Ethical Committee in Stockholm (approval 2010/1478-32) to Prof. Anders Österborg, who provided the cells.

Results

Stromal cells mediate ibrutinib resistance of MCL cells

Both Mino and Rec-1 MCL cells are sensitive to ibrutinib *in vitro* as determined by increased apoptosis and inhibition of proliferation following treatment (Supplementary Figs. S1A, S1B and ref. (8)). To investigate whether ibrutinib sensitivity of MCL cells is affected by stromal cells, Rec-1 or Mino cells were co-cultured with or without murine MS-5 stromal cells where after the impact of stromal cells on MCL cell proliferation and apoptosis was analyzed in the CD19 positive cell population (Fig. 1A). The co-cultured MCL cells displayed reduced growth inhibition by ibrutinib (200nM or 500nM) compared to cells grown alone

assayed as reduced CFSE staining (Figs. 1B and C and Supplementary Fig. S1C). Co-culturing MS-5 stromal cells with Rec-1 or Mino MCL cells also largely reduced sensitivity of the MCL cells to ibrutinib-induced cell apoptosis (Figs. 1D and E). The same result was seen when Rec-1 cells were co-cultured with human FDC stromal cells (Supplementary Fig. S1D). These results demonstrated that stromal cells strongly impaired MCL cell sensitivity to ibrutinib. Importantly, if Mino cells which were resistant to ibrutinib-induced cell apoptosis in the presence of stromal cells were transferred into new wells without stromal cells and retreated with the same concentrations of ibrutinib, they regained sensitivity to ibrutinib-induced apoptosis (Supplementary Fig. S2). This confirmed that stromal cells, and not a tumor cell autonomous mechanism, conferred the resistance. Furthermore, the inability of stromal cell conditioned media to prevent ibrutinib-induced cell apoptosis suggested that a direct stromal cell-MCL interaction is the main cause for the resistance (Figs. 1F and G). The requirement for direct stromal cell-MCL cell interaction for generating ibrutinib resistance was further tested by the addition of an adhesion molecule VLA-4 blocking antibody to the Mino-MS-5 cell co-cultures, which resulted in a strong reduction in adhesion of Mino cells to the stromal cells (Supplementary Fig. S3A). Disrupting the interaction using the VLA-4 blocking antibody sensitized the pro-apoptotic effect of ibrutinib on Rec-1 and Mino cells, respectively, compared to when treated with ibrutinib+control IgG (Figs. 1H and I). Similar results were seen when Rec-1 cell interaction with FDC was abrogated by the VLA-4 blocking antibody (Supplementary Fig. S3B). The VLA-4 blocking antibody in itself did not show any pro-apoptotic effect compared to treatment with the control IgG antibody, neither in the presence nor absence of stromal cells (Figs. 1H and I, Supplementary Figs. S3B and S3C). This emphasizes that a direct stromal cell-MCL cell interaction is responsible for the ibrutinib resistance. Furthermore, the results support the involvement of VLA-4 in MCL cell attachment to stromal cells.

Stromal cells support MCL cell regrowth after ibrutinib removal

Rec-1 and MS-5 cell co-cultures were treated with ibrutinib as depicted in the experimental outline Fig. 2A. Rec-1 cell proliferation was largely impaired after 10 days of treatment with 500nM ibrutinib, indicated by a reduction of the EdU signal (Fig. 2B). However, a substantial recovery of proliferating cells was observed 6 days after ibrutinib removal (Fig. 2B). To exclude a cell autonomous effect, Rec-1 cells in the absence of MS-5 stromal cells were

treated for 2 days with 500nM ibrutinib where after ibrutinib was removed. In contrast to the situation when the Rec-1 cells were co-cultured with MS-5 cells, this 2 days ibrutinib treatment in the absence of stromal cells was sufficient to prevent recurrence of Rec-1 cell proliferation as analyzed 6 days later (Fig. 2C). Importantly, the lack of Rec-1 cell regrowth was not due to ibrutinib-induced cell death as no major apoptosis was detected after the 2 day treatment with ibrutinib (Supplementary Fig. S4A). This was in contrast to when the Rec-1 cells were treated with the pan-PI3K inhibitor LY294002, where a significant increase in apoptosis was detected (Supplementary Fig. S4A). Furthermore, the reappearance of proliferating cells after ibrutinib removal originated mainly from the adherent cells and not from the cells growing in suspension (Fig. 2D). The same result was seen for Mino cells (Supplementary Fig. S4B). Likewise, inhibiting Mino cell interaction with MS-5 stromal cells using the VLA-4 blocking antibody during ibrutinib treatment prevented regrowth of the MCL cells after ibrutinib removal, while cells from treatments with the agents individually did not (Supplementary Fig. S4C).

Stromal cells impair ibrutinib-mediated down-regulation of PI3K/AKT signaling in MCL cells

Our initial results showed that ibrutinib-mediated repression of NF-κB activity remained intact in MCL cells when co-cultured with MS-5 stromal cells, as indicated by retained down-regulation of some typical NF-κB target genes (Supplementary Fig. S5A). However, when co-culturing Mino or Rec-1 cells with MS-5 cells, the latter strongly impaired the ibrutinib-mediated reduction of pAKT (Ser473) levels as compared to when MCL were grown in the absence of stromal cells (Figs. 3A and B). Similar result was seen when Rec-1 cells were incubated with human FDC (Supplementary Fig. S5B). These results suggest that stromal cells impair ibrutinib-mediated down-regulation of AKT activity.

p110α inhibition enhances ibrutinib-mediated down-regulation of pAKT levels and helps to overcome stromal cell-mediated ibrutinib resistance *in vitro*.

In order to test if the stromal cell-mediated impaired ability of ibrutinib to reduce AKT activity involved PI3K/AKT signaling, we screened different PI3K isoform specific inhibitors for their ability to restore Mino cell sensitivity to ibrutinib-induced apoptosis in MS-5 stromal

cell-Mino cell co-cultures. When treating the co-cultures with increasing concentration of PI3K catalytic subunit inhibitors BYL719 (p110α), TGX221 (p110β), CZC24832 (p110γ) or CAL101 (p110δ) in the absence or presence of 500nM ibrutinib, we observed that combined treatment with BYL719 most effectively restored the ability of ibrutinib to induce Mino cell apoptosis (Fig. 4A). A weaker effect was observed by CAL101 (Fig. 4A, compare the degree of apoptosis ± ibrutinib at 5μM BYL719 and 20μM CAL101 which show the same degree of apoptosis in the absence of ibrutinib). Treatment with TGX221 or CZC24832 did not effectively restore ibrutinib sensitivity of the Mino cells. 5μM BYL719 also effectively restored Rec-1 cell sensitivity to ibrutinib in the presence of MS-5 stromal cells (Supplementary Fig. S6A). Importantly, BYL719 also enhanced the ability of ibrutinib to induce apoptosis in 3 out of 4 cases when primary MCL cells were co-cultured with MS-5 stromal cells (Fig. 4B). Similarly to the sensitivity of the Mino MCL cells to the various p110 catalytic PI3K subunit inhibitors in the presence of stromal cells, the sensitivity of the Mino cells in the absence of stromal cells was most profound to BYL719. A weaker sensitivity to CAL101 was seen, while the Mino MCL cells were largely resistant to CZC24832 and TGX221 (Supplementary Fig. S6B). Furthermore, combined ibrutinib+BYL719 treatment of Mino cells in the presence of MS-5 stromal cells resulted in at least an additive reducing effect on pAKT levels compared to single drug treatment (Fig. 4C). Similar results were seen for Rec-1 cells co-cultured with FDC (Supplementary Fig. S6C).

To investigate the effect of combined ibrutinib+BYL719 treatment on MCL cell proliferation and regrowth, cells were treated as outlined in Fig. 2A. Upon treatment with 500nM ibrutinib alone, restart of Mino cell proliferation was observed 6 days after the ibrutinib was removed. In contrast, restart of proliferation of Mino cells that initially had been treated with ibrutinib+BYL719 was largely impaired after drug removal (Fig. 4D). The same result was seen for Rec-1 cells (Supplementary Fig. S6D). The impaired regrowth of the Mino cells correlated to reduced pAKT levels in the cells (Fig. 4E).

p110α inhibition helps to overcome stromal cell-mediated ibrutinib resistance *in vivo*.

To test whether BYL719 also can enhance the ibrutinib effect *in vivo*, Mino cells were grafted to immunocompromised mice and tumor growth was measured following treatment with 5mg/kg/day of ibrutinib or 20mg/kg/day of BYL719 alone or in combination. BYL719

treatment alone did not show significant inhibition as compared to vehicle treatment at any time point while ibrutinib only significantly inhibited tumor growth at early time points (Fig. 5A). However, when ibrutinib and BYL719 were administered together, a significant inhibition of tumor growth was observed, also at the later treatment period when none of the drugs alone significantly inhibited tumor growth (Fig. 5A). Tumor weight at the endpoint confirmed the effects (Fig. 5B). Mouse weight was not affected by the treatments (Supplementary Fig. S7A). Furthermore, when we analyzed mRNA expression of some typical NF-κB target genes from the above tumor tissues, we found that ibrutinib alone still effectively repressed gene expression, while repression by BYL719 alone was absent or minor (Supplementary Fig. S7B). For the NF-κB target genes analyzed, no enhanced repression was seen when the cells were treated with ibrutinib+BYL719 compared to when cells were treated with ibrutinib alone.

Discussion

Tumor recurrence due to development of resistance to cancer drugs is an obstacle for long-term treatment effects. This is also the case for the BTK inhibitor ibrutinib, a recently FDA approved targeted drug for relapsing MCL and CLL. In some recent clinical studies it was shown that development of ibrutinib resistance in MCL is not uncommon (13,15,16). Furthermore, in the study by Martin et al. (13) it was shown that development of ibrutinib resistance was associated with failed improvement of MCL outcome also when treated with other front-line drugs. This demonstrates the need to find ways to overcome ibrutinib resistance. Considering that intrinsic mechanisms, e.g. BTK mutations, explaining ibrutinib resistance are rare in MCLs (13), alternative mechanisms are likely responsible. Tumor resistance to cancer drugs have to a large extend been attributed to the TME, thereby contributing to MRD and relapse (26). In this report we describe that stromal cells protect MCL cells from ibrutinib effects and that ibrutinib sensitivity was established if TME-mediated “ibrutinib resistant” MCL cells were re-treated with ibrutinib following removal of stromal cells. This demonstrates a non-autonomous tumor cell mechanism as responsible for the resistance. This observation also resembles the finding in other cancers, e.g. in

glioblastoma, that recruited stromal cells after radiation facilitate tumor regrowth (27). Furthermore, although stromal cell-derived soluble factors have been attributed to targeted drug resistance in cancers (28,29), we show that a direct contact between MCL and stromal cells is central in conferring the ibrutinib resistance, since growth of MCL in stromal cell conditioned media was nonsufficient to effectively confer ibrutinib resistance.

Our results which show a stromal cell-mediated impairment of the ability of ibrutinib to repress pAKT levels in the MCL cells are in line with described association between PI3K/AKT signaling and resistance to other drugs used in treatment of cancers (30,31). In contrast, we demonstrate that ibrutinib-mediated repression of NF-κB target genes remained fully functional in MCL cells also when co-cultured with MS-5 stromal cells. This shows that TME-mediated ibrutinib resistance is not due to abrogated NF-κB activity. This is supported by an observation that ibrutinib still can down-regulate BTK activity in ibrutinib resistant MCL cells (4). That the PI3K catalytic subunit p110α specific inhibitor BYL719 restored the ability of ibrutinib to induce apoptosis in both MCL cell lines and in 3 out of 4 primary MCL leukemic cell samples supports that PI3Kα/AKT signaling is involved in the stromal cell-mediated ibrutinib resistance. Furthermore, we also observed that ibrutinib+BYL719 pre-treated MCL cells (in the presence of stromal cells) showed impaired capacity for regrowth after drug removal. This correlated to reduced pAKT levels. However, whether this represents a more permanent inhibition or just a shorter delay in proliferation restart was not examined in detail. Importantly, we also showed that BYL719 treatment enhanced the ability of ibrutinib to inhibit Mino cell derived tumor growth *in vivo* in a xenograft mouse model. Likewise, PI3Kα signaling was recently also demonstrated to play a role in activated B cell like Diffuse Large B-cell Lymphoma (ABC-DLBCL) as inhibition of both p110α and p110δ was required to efficiently repress tumor growth of ABC-DLBCL in a mouse model (32). Furthermore, dual inhibition of p110α/δ synergized with ibrutinib in causing remission in the DLBCL models.

Additional support for using a p110α inhibitor in the treatment of MCL can be derived from data by Psyrri et al. (33), who showed that in 68% of the cases of primary MCL and in several MCL cell lines, increased *PIK3CA* expression due gene amplification is present, resulting in enhanced AKT activity. Notably, we found that inhibition of p110α was more effective than inhibition of p110δ in sensitizing cells to ibrutinib in the presence of stromal cells, while inhibition of p110β or p110γ did not influence ibrutinib sensitivity. This together

with the observation that MCL tumor cells with increased p110α expression can sustain constitutive PI3K signaling despite p110δ inhibition (34), further supports a rationale for targeting p110α in MCL. However, although the PI3Kα/AKT signaling pathway seems to have a key role in stromal cell-mediated ibrutinib resistance, other signaling pathways may contribute (35).

Adhesion molecules, including VLA-4, are highly expressed on MCL cells and have a central role in MCL-stromal cell interaction (36). As demonstrated by us (Supplementary Fig. 3A) and others (17), blocking VLA-4 disrupts the stromal-MCL cell interaction. Likewise, Herman et al. (37) described that ibrutinib partially blocked CLL cell adhesion to stroma or stromal components and may reduce VLA-4-dependent pro-survival signals in the TME. Importantly, we here demonstrate that disruption of MCL-stromal cell interaction by blocking VLA-4 led to regained ibrutinib sensitivity, supporting that the stromal cell-mediated ibrutinib resistance involves a direct stromal cell-MCL cell interaction. Similarly, it has been shown that VLA-4 blocking can overcome stromal cell-mediated rituximab resistance of NHL cells *in vitro* (38). Furthermore, a high VLA-4 expression has been shown to be associated in leukemia with adverse outcome and shorter survival (39,40). Although not demonstrated in this report, a direct MCL-stromal cell interaction which involves VLA-4 has been reported to activate PI3K/AKT signaling (35,41). Combined with our results, this may explain why a direct stromal-MCL cell interaction results in ibrutinib resistance. This is consistent with another finding showing that VLA-4 blocking antibody treatment combined with the cytotoxic drug Ara-C enhanced reduction of pAKT levels (42). Furthermore, it was demonstrated very recently that in MCL cells that had been selected for ibrutinib resistance (and were devoid of BTK mutations), ibrutinib was unable to reduce PI3K/AKT signaling in comparison to sensitive MCL cells (35). In addition, it was also demonstrated that co-culturing MCL cells with stromal cells resulted in an upregulated expression of integrin β1 (subunit of VLA-4). Moreover, a reduction of AKT in MCL cells lead to reduced integrin β1 expression. Notably, it has been shown that PI3Kα signaling activates VLA-4 in the lymph node niche (43). These results show that PI3K/AKT signaling is a central hub for both inside-out as well as outside-in signaling in MCL-stromal cell interaction (35). It has also been demonstrated that the transcriptional program of mesenchymal stromal cells in the bone marrow microenvironment is altered by leukemia cells (44), further supporting a bidirectional cross-talk between the cancer and the TME cells, with the lymphoma cells reshaping the TME and the TME supporting lymphoma cell proliferation and survival, contributing to the

ibrutinib resistance. Although we did not observe an effect following p110α inhibition on stromal cell survival (Supplementary Fig. S8), a possible impact of altered PI3Kα/AKT signaling in the stromal cells on stromal cell-lymphoma cell cross-talk cannot be excluded. Indeed, it has been suggested that inhibition of PI3K/AKT signaling in cells of the TME could contribute to the effects seen by PI3K inhibitors (45). Taken together, these results support that PI3K/AKT signaling has a central role in stromal cell-mediated ibrutinib resistance and support our results that targeted inhibition of PI3Kα/AKT signaling or MCL-stromal cell interaction can overcome stromal cell-mediated ibrutinib resistance.

Acknowledgements

We thank Prof. Yong Sung Choi and Prof. Anders Österborg for providing us with the human FDC line and primary MCL cells, respectively. We thank Dr. Gergely Talaber, Xiaogang Feng, Dr. Peng Zhang and Dr. Hongya Han for providing experimental advice and Dr. Mattias Berglund for valuable comments on the manuscript.

This research was supported by funding's from Cancerfonden (Swedish Cancer Society, CAN 2014/593 and CAN2017/404) to S. Okret and Barncancerfonden (Swedish Childhood Cancer Foundation, PR2014-00135 and PR2016-0038) to S. Okret. Scholarships from China Scholarship Council (CSC) supported J. Guan (2011617056) and D. Huang (201600160069), respectively.

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Figure legends

Figure 1. Bone marrow stromal cells mediate ibrutinib resistance of MCL cells.

(A) shows the outline of analysis of MCL cell proliferation and apoptosis by flow cytometry. For analysis of apoptosis, collected cells were stained with anti-human CD19-APC antibody in order to distinguish the human MCL cells from the stromal cells. Cell proliferation was assayed by staining the MCL cells with CFSE dye prior to incubation with stromal cells or by labelling cells with EdU 2.5 h before harvesting where after cells were stained with Alexa Fluor 488-azide according to the manufactures manual. In the cases of assaying cell proliferation by CFSE, lymphoma cell proliferation is indicated by CFSE dilution. For analysis of cell apoptosis, the cells were stained with Annexin V-FITC. (B-C) 5×10^4 CFSE labeled Rec-1 or Mino MCL cells were added per well in a 24-well plate that 3 days prior had been seeded or not seeded with murine MS-5 stromal cells (pre-treated with 10μg/ml mitomycin C for 2.5h). Cells were then treated with either vehicle or ibrutinib (200nM or 500nM) for 10 days where after cells were collected and labeled with anti-human CD19-APC antibody. Cell proliferation of the MCL cells was determined by flow cytometry analysis based on the dilution of the CFSE signal. (D-E) Rec-1 or Mino MCL cells grown with or without MS-5 stromal cells were treated with either vehicle or ibrutinib (200nM or 500nM) for 10 days where after cells were collected. Apoptosis was determined in CD19 positive lymphoma cells by flow cytometry followed by Annexin V staining. (F-G) 5×10^4 Rec-1 or Mino MCL cells grown with or without conditioned medium from MS-5 stromal cell cultures were treated with either vehicle or ibrutinib (200nM and 500nM) for 10 days where after cells were collected and CD19 positive cells were analyzed for apoptosis by Annexin V staining. Data analysis for Figs. 1B-G was performed by unpaired, two-tailed students t-test, and error bars show the SD (n=3, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns, not significant). (H-I) Co-cultures of Rec-1 or Mino cells with MS-5 stromal cells were treated for 10 days with IgG (control, 4μg/ml), ibrutinib (500nM)+IgG, anti-VLA-4 blocking antibody (4μg/ml), or ibrutinib+anti-VLA-4 blocking antibody where after cell apoptosis of the MCL cells was determined by analyzing CD19 positive cells by Annexin V staining. Data analysis was performed by one-way ANOVA Tukey's test (n=3, *** $P<0.001$).

Figure 2. Stromal cells support Rec-1 cell regrowth after ibrutinib treatment is terminated.

(A) Experimental outline. MCL cells were seeded onto MS-5 stromal cells and the cells were then treated with either vehicle or ibrutinib (500nM) for 10 days. On day 10, cells were incubated with EdU for 2.5h before cell harvesting, where after cells were collected and labeled with anti-human CD19-APC antibody, followed by Alexa Fluor 488-azide staining. In a parallel set of wells, ibrutinib was removed and fresh medium without ibrutinib was added and cells were cultured for an additional 6 days, followed by labeling and staining as described above. (B) Rec-1 cells co-cultured with stromal cell were treated as described in (A) above. Cell proliferation of CD19 positive Rec-1 cells were analyzed by EdU/Alexa Fluor 488-azide staining by flow cytometry. (The weakly EdU/Alexa Fluor 488-azide stained peak (intensity $\approx 10^1$) represents non-proliferating cells while the EdU/Alexa Fluor 488-azide peak (intensity 10^2-10^3) represents proliferating cells (cells in S-phase). The figure shows one out of three independent experiments with similar results. (C) Rec-1 cells grown in the absence of MS-5 stromal cells were treated with ibrutinib for 2 days where after ibrutinib was removed and the cells were cultured for another 6 days. Cell proliferation was analyzed following EdU/Alexa Fluor 488-azide staining by flow cytometry. The figure shows one out of three independent experiments with similar results. (D) Rec-1 cells co-cultured with MS-5 stromal cells as described in A were treated with either vehicle or ibrutinib (200nM or 500nM) for 10 days where after CD19 positive cells in suspension or adherent to stromal cells were analyzed for EdU/Alexa Fluor 488-azide staining by flow cytometry (left column). In a parallel set of experiments, adherent and suspension cells were separated on day 10 and the cells were allowed to continue to grow in the absence of ibrutinib for 6 days and analyzed as above (right column) at day 16. The bar figures to the right show quantitative data and data analysis was performed by unpaired, two-tailed students t-test, and error bars show the SD (n=3, *** $P<0.001$; ns, not significant).

Figure 3. Stromal cells impair ibrutinib-mediated down-regulation of pAKT levels in MCL cells.

(A) MCL cells were labeled with CFSE before co-culturing with MS-5 stromal cells in order to distinguish MCL cells from MS-5 cells. After treatment, cells were collected and stained with an anti-pAKT-APC antibody. CFSE positive MCL cells were gated and analyzed for

pAKT staining by flow cytometry. (B) 5×10^4 Mino or Rec-1 cells were first labeled with CFSE and then added to 24-well plates pre-seeded with or without stromal cells. The cells were then treated with either vehicle or ibrutinib (500nM) for 5 days. On day 5, all the cells were collected, washed, fixed and stained for pAKT using an anti-pAKT-APC antibody. pAKT levels in CFSE labelled cells were determined by flow cytometry (left). The bar figures to the right show the relative change in pAKT level (median florescence intensity (MFI)) when treated with ibrutinib in the presence or absence of MS-5 stromal cells. Data analysis was performed by unpaired, two-tailed students t-test, and error bars show the SD (n=3, *** P<0.001).

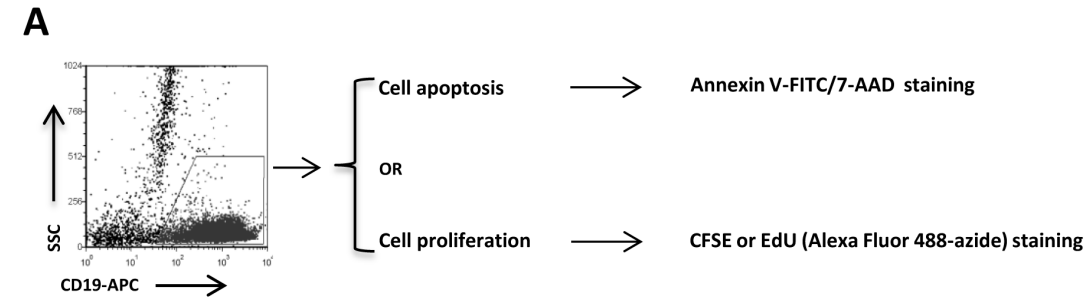
Figure 4. p110α inhibition enhances ibrutinib-mediated down-regulation of pAKT levels and helps to overcome stromal cell-mediated ibrutinib resistance *in vitro.*

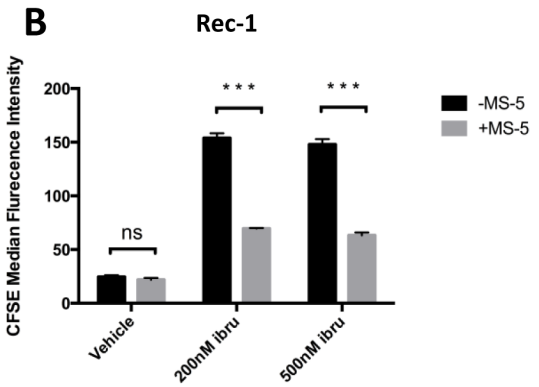
(A) Mino cell apoptosis in the presence of MS-5 stromal cells after treatment with increasing concentrations of specific p110 catalytic subunit PI3K inhibitors alone or together with 500nM ibrutinib for 10 days. BYL719 (p110α specific inhibitor), CAL101 (p110δ specific inhibitor); TGX-221 (p110β specific inhibitor) and CZC24832 (p110γ specific inhibitor). Cell labeling and analysis of apoptosis were performed as described in Fig. 1A. For each treatment, experiments were performed in triplicates. The Annexin V staining signal of gated CD19 positive cells (Mino cells) was analyzed by flow cytometry. (B) $1x10^6$ cells from 4 different MCL patient samples were co-cultured with MS-5 stromal cells and treated with vehicle, ibrutinib (500nM) and BYL719 (5μM) alone or in combination for 10 days. Apoptosis was analyzed by Annexin V staining of gated CD19 positive MCL patient cells by flow cytometry. The percentage of apoptotic cells for each condition is shown in the bar graphs. (C) The bar graph shows pAKT levels in Mino cells as analyzed by flow cytometry after 10 days of drug treatment in the presence of MS-5 stromal cells as indicated in the figure. (D and E) Analysis of cell proliferation (D) and pAKT levels (E), respectively, of the MCL cells after drug removal (experimental outline as in Fig. 2A). The figures show results from cells collected day 16, i.e. 6 days after the drug(s) were removed. Data analysis in Figs. 4C-E was performed by one-way ANOVA Tukey's test, and error bars show the SD (n=3, * P<0.05, ** P<0.01, *** P<0.001).

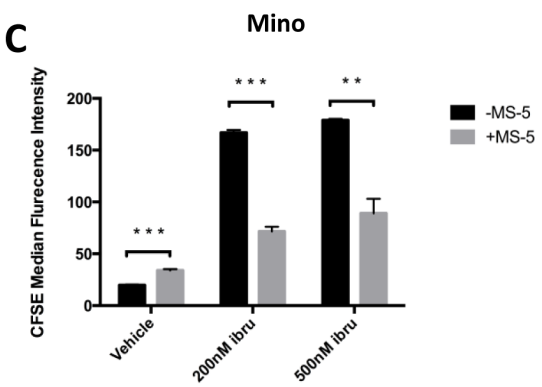
Figure 5. Treatment with the p110α inhibitor BYL719 augments the efficiency of ibrutinib to inhibit MCL tumor growth *in vivo*.

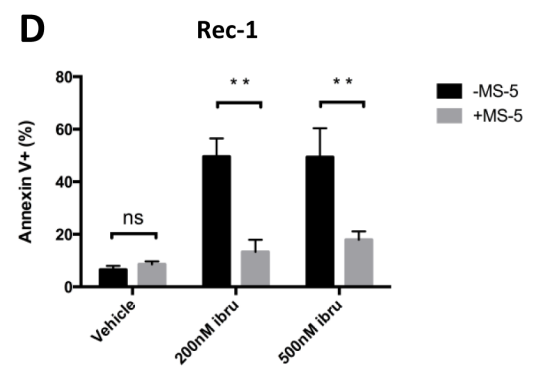
$1x10^7$ Mino cells were engrafted subcutaneously into NSG mice. Treatment started when the tumors were first palpable with daily injections with vehicle, ibrutinib (5mg/kg), BYL719 (20mg/kg) or a combination thereof. Intraperitoneal injections were given once a day and tumor volume was measured daily (A) and tumor weight at the day of sacrifice (B) was analyzed. * vehicle *vs.* ibrutinib, $^{\#}$ vehicle *vs.* ibrutinib/BYL719. Absent of a symbol or ns mean that there is no statistical significance. Data analysis was performed by one-way ANOVA Tukey's test, and error bars show the SD of 6 to 9 mice per group ($^{\#\#}$ $P<0.01$, * $P<0.05$, *** $P<0.001$).

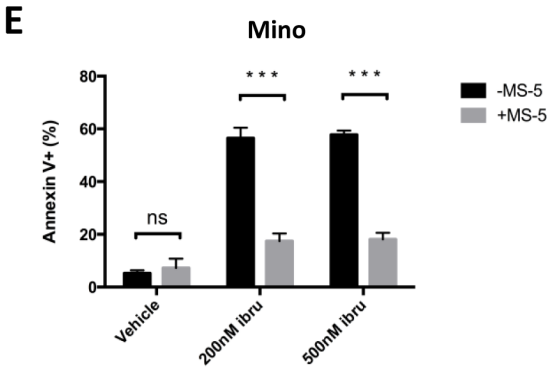
Figure 1

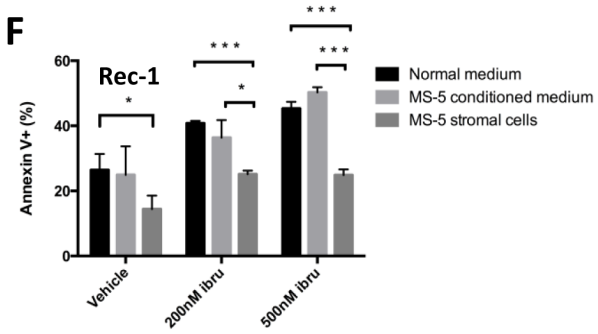


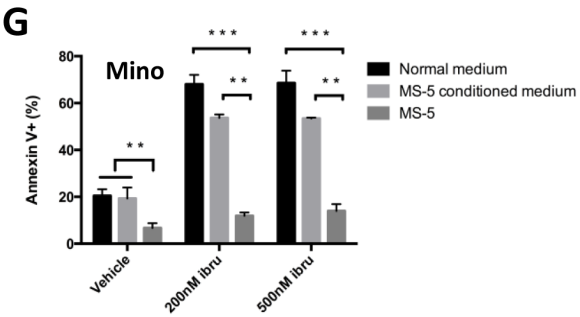


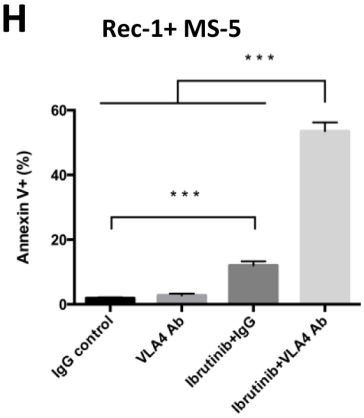












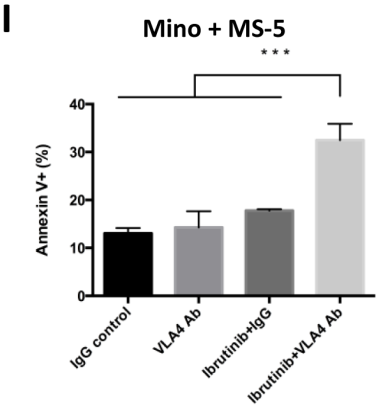


Figure 2

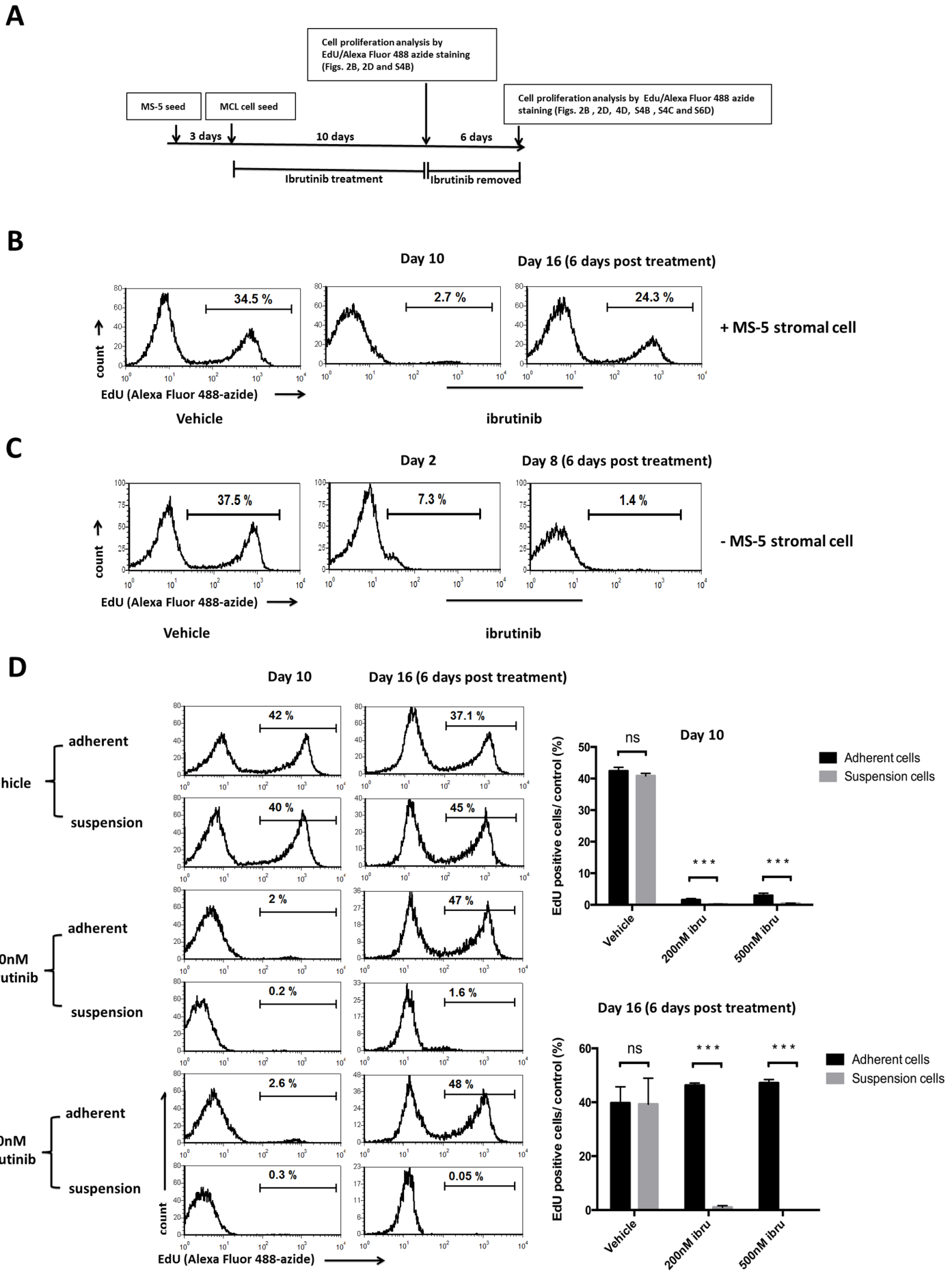


Figure 3

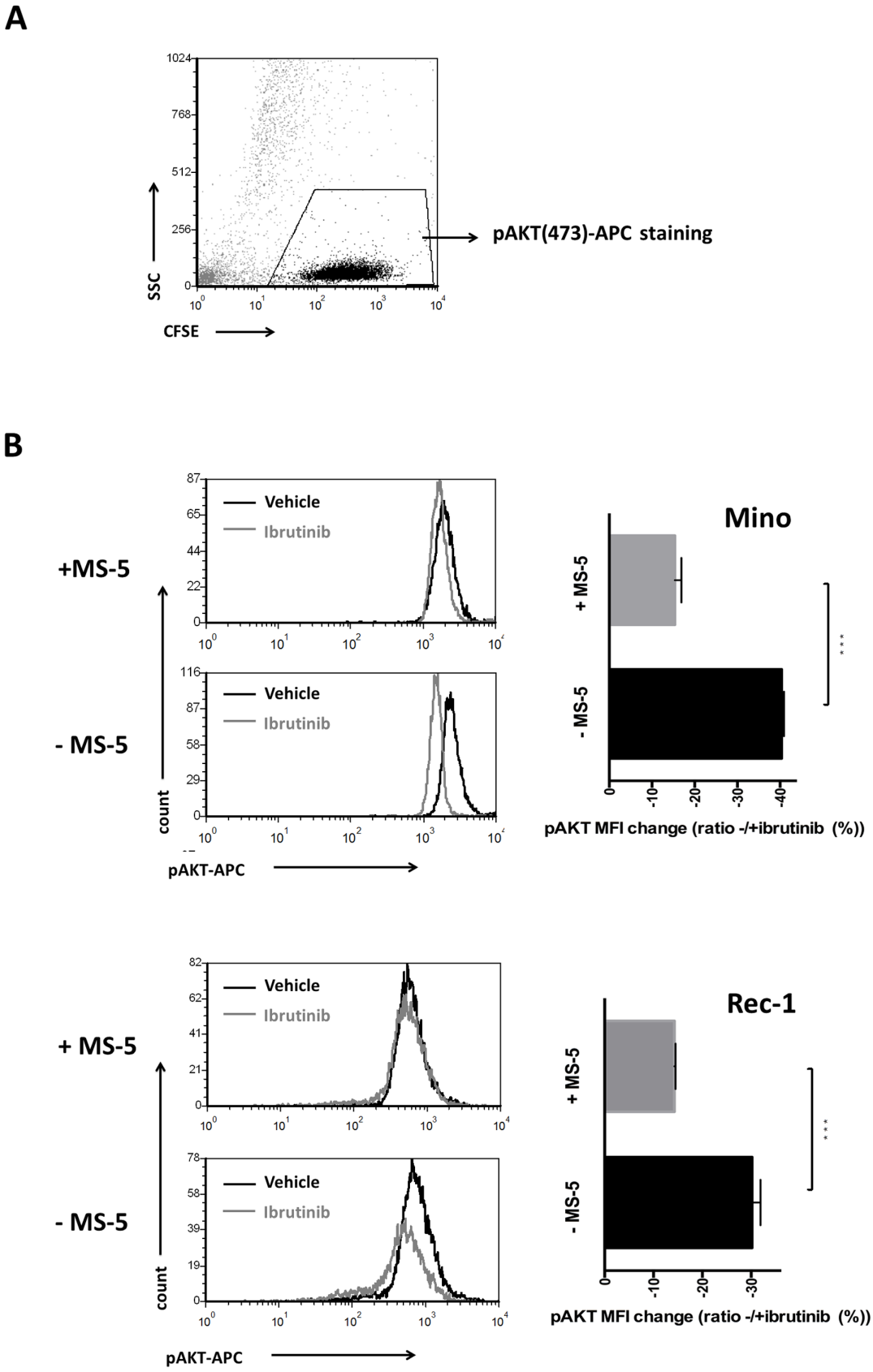
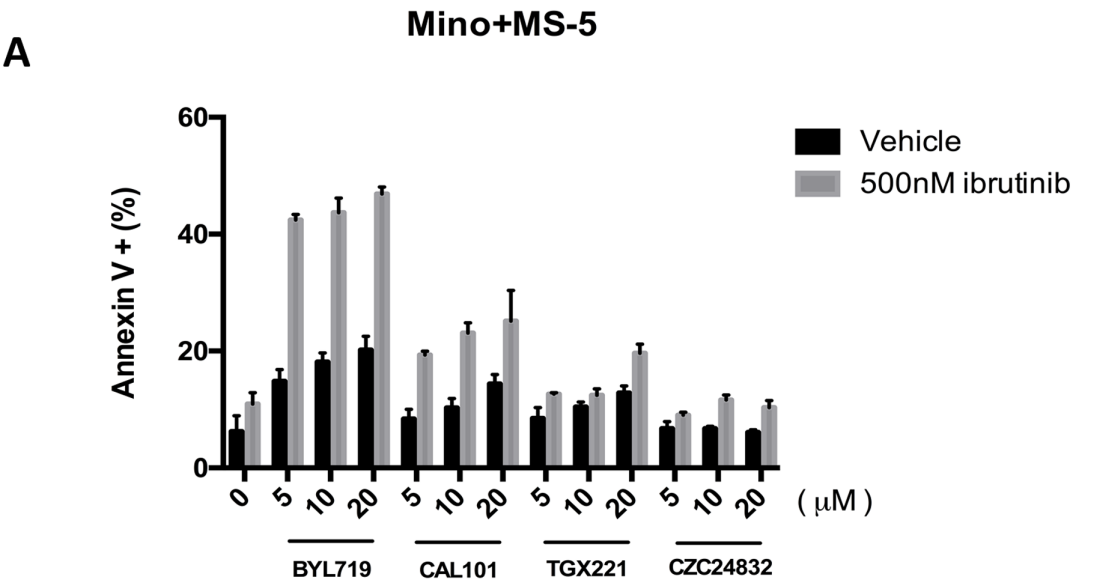
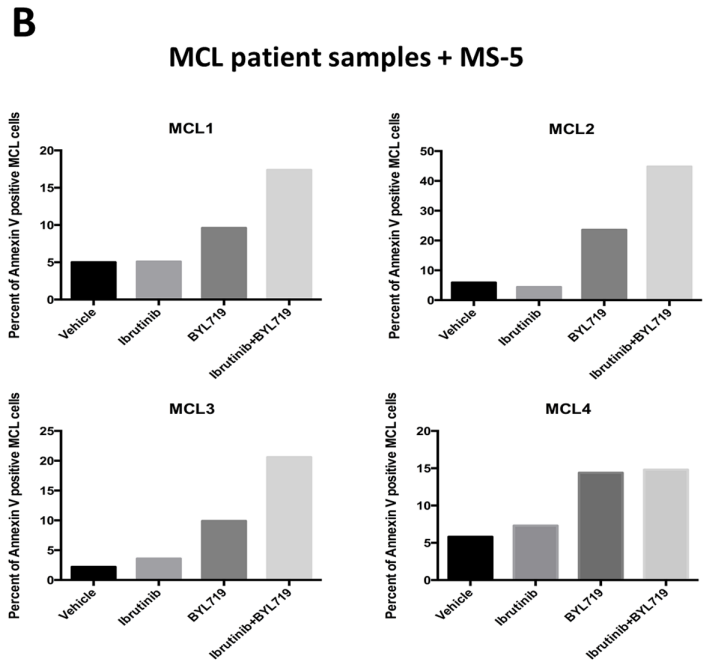
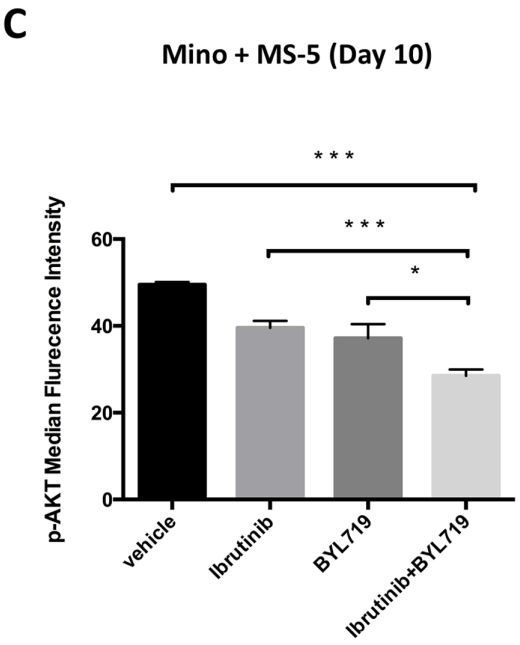
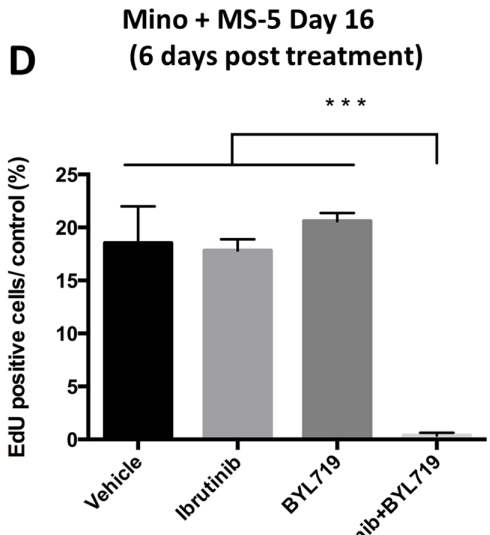


Figure 4









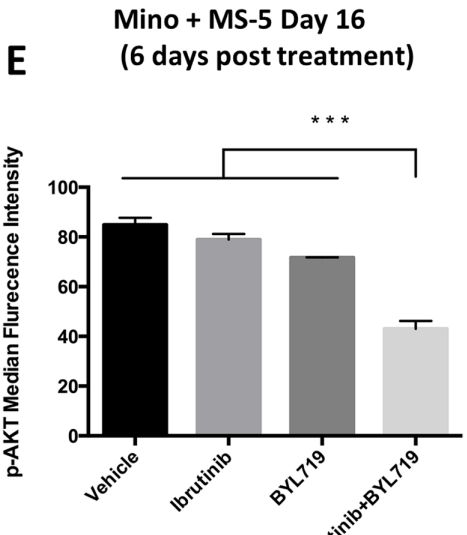
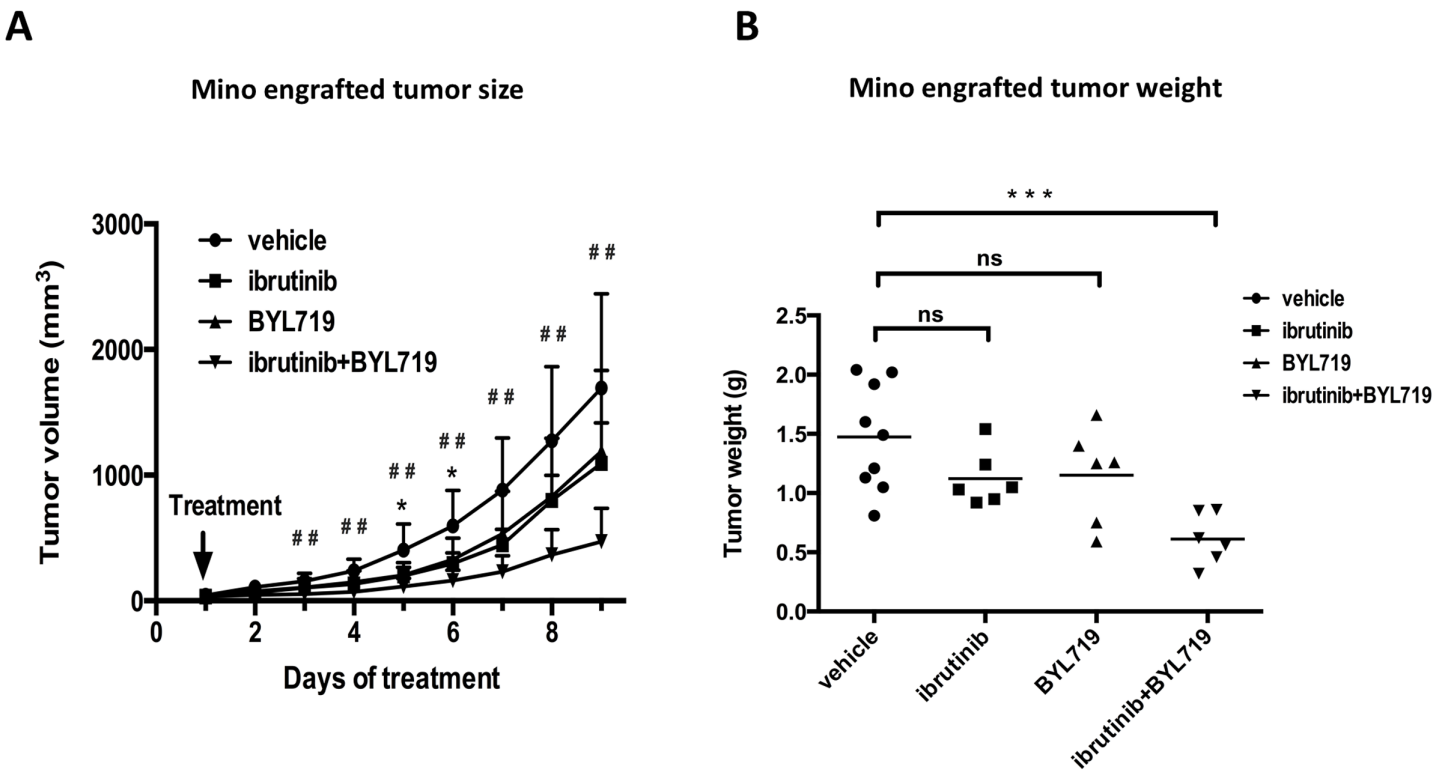


Figure 5



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Mol Cancer Ther Published OnlineFirst February 26, 2018.