The phosphatidylinositol-3-kinase inhibitor NVP-BKM120 overcomes resistance signals derived from microenvironment by regulating the Akt/FoxO3a/Bim axis in chronic lymphocytic leukemia cells

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ABSTRACT

Phosphatidylinositol-3-kinase pathway is constitutively activated in chronic lymphocytic leukemia mainly due to microenvironment signals, including stromal cell interaction and CXCR4 and B-cell receptor activation. Because of the importance of phosphatidylinositol-3-kinase signaling in chronic lymphocytic leukemia, we investigated the activity of the NVP-BKM120, an orally available pan class I phosphatidylinositol-3-kinase inhibitor. Sensitivity to NVP-BKM120 was analyzed in chronic lymphocytic leukemia primary samples in the context of B-cell receptor and microenvironment stimulation. NVP-BKM120 promoted mitochondrial apoptosis in most primary cells independently of common prognostic markers. NVP-BKM120 activity induced the blockage of phosphatidylinositol-3-kinase signaling, decreased Akt and FoxO3a phosphorylation leading to concomitant Mcl-1 downregulation and Bim induction. Accordingly, selective knockdown of *BIM* rescued cells from NVP-BKM120-induced apoptosis, while the kinase inhibitor synergistically enhanced the apoptosis induced by the BH3-mimetic ABT-263. We also found NVP-BKM120 to inhibit B-cell receptor- and stroma-dependent Akt pathway activation, thus sensitizing chronic lymphocytic leukemia cells to bendamustine and fludarabine. Furthermore, NVP-BKM120 down-regulated secretion of chemokines after B-cell receptor stimulation and inhibited cell chemotaxis and actin polymerization upon CXCR4 triggering by CXCL12. Our findings establish that NVP-BKM120 effectively inhibits the phosphatidylinositol-3-kinase signaling pathway and disturbs the protective effect of the tumor microenvironment with the subsequent apoptosis induction through the Akt/FoxO3a/Bim axis. We provide here a strong rationale for undertaking clinical trials of NVP-BKM120 in chronic lymphocytic leukemia patients alone or in combination therapies.

Introduction

Chronic lymphocytic leukemia (CLL), the most common form of adult leukemia in Western countries, is a heterogeneous disease with variable clinical presentation and evolution. The status of somatic hypermutations in the variable region of immunoglobulin genes (*IGHV*), high expression of ZAP-70 or CD38, and the presence of certain cytogenetic abnormalities have all been associated with poor prognosis. CLL is characterized by the progressive accumulation of mature, monoclonal $CD5^+$ B lymphocytes in the peripheral blood and tissue compartments (bone marrow and lymph nodes). These specialized compartments constitute the tumor microenvironment, where malignant cells encounter supporting cells and receive signals to proliferate, progress and acquire drug resistance.[1] *In vivo*, signaling pathways activated by tumor microenvironment interactions include the B-cell receptor (BCR) and NF-κB pathways. In the last years, new approaches for molecular targeting of the microenvironment have been developed, including CXCR4 antagonists[2] and specific inhibitors of kinases essential for BCR signal transduction, such as LYN, SYK, BTK, and phosphatidylinositol-3-kinase (PI3K). All of them disrupt regulatory loops between CLL cells and the microenvironment and have shown encouraging results both at pre-clinical and clinical trials.[3] PI3K pathway is at the central core of the signaling network engaged by microenvironment crosstalk and constitutes a key component of cell survival, growth and homing. Of note, PI3K axis is among the most commonly activated signaling pathways in human cancers. Particularly in CLL, PI3K pathway has been found to be constitutive activated in freshly isolated CLL cells.[4]

The PI3K family of lipid kinases consists of 3 classes of which, to date, only class I has been implicated in regulation of hematopoietic cells. Class I includes 4 catalytic isoforms divided into class IA (p110α, p110β, p110δ) and class IB (p110γ). The PI3K isoforms α and β are ubiquitously expressed, whereas PI3Kδ is primary expressed in leukocytes. In transformed cells, however, the dominant role of a specific isoform may be lost and different isoforms can assume redundant functions.[5] PI3K phosphorylates phosphatidylinositol lipids, catalyzing the production of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) in the cell membrane. This lipid product is the docking site for cytoplasmic kinases that include PDK1 and Akt, which triggers a co-ordinated set of events leading to cell survival.[6] Thus, as a result of PI3K activity, Akt is activated, this protein being a major downstream effector of PI3K.[7] Multiple Akt substrates have been identified, including members of the FoxO subfamily of Forkhead

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transcription factors and the serine/threonine kinase mammalian target of rapamycin (mTOR).[8] In CLL, the selective p110δ PI3K inhibitor GS-1101 (CAL-101) has shown efficacy both in pre-clinical[9] and clinical studies.[10] However, there is some evidence to suggest possible redundancies between the different PI3K isoforms, appointing for additional therapeutic implications in B-cell malignancies.[11] In this way, NVP-BKM120, a 2,6-dimorpholino pyrimidine derivative, is a potent, orally available, pan-class I PI3K inhibitor.[12,13] It has shown efficacy both in *in vitro* and *in vivo* models.[14-18] In addition, in a recently completed phase I trial in advanced solid tumors, NVP-BKM120 has been shown to be safe at its maximum-tolerated dose showing a favorable pharmacokinetic profile and preliminary antitumor activity.[19] Moreover, NVP-BKM120 is currently being tested in a phase I trial in patients with advanced leukemias (NCT01396499).

In this context, because of the importance of the PI3K pathway in transducing a variety of external, microenvironment-derived migratory, growth, and survival signals, here we investigated the activity of the pan-class I PI3K inhibitor NVP-BKM120 under microenvironment cross-talk conditions.

Methods

Isolation and culture of primary cells

Peripheral blood mononuclear cells (PBMCs) were obtained from 37 CLL patients who had not received treatment for the previous three months and 4 healthy donors. Written informed consent was obtained from all patients in accordance with the Ethics Committee of the Hospital Clínic, University of Barcelona and the Declaration of Helsinki. This study has been approved by the local Institutional Review Board (2009/4206). The characteristics of the patients are listed in the *Online Supplementary Table S1*. Primary CLL cells were isolated and cultured as described in the Online Supplementary Methods.

The percentage of tumoral cells ($CD19^+$, $CD5^+$) as well as the expression levels of ZAP-70 and CD38 was analyzed by flow cytometry. The *IGHV* gene mutational status was verified according to the European Research Initiative on CLL guidelines.[20] Cytogenetic alterations were assessed by fluorescence *in situ* hybridization (FISH). In cases with 17p deletions, the mutational analysis of the second allele was carried out by direct sequencing, according to the International Agency for Research on Cancer TP53 consortium (*http://p53.iar.fr*). *SF3B1*, *NOTCH1* and *MYD88* mutations have been previously reported.[21,22]

Drugs and assessment of apoptotic features by flow cytometry

CLL cells were incubated as indicated with different concentrations of NVP-BKM120 (kindly provided by Novartis). For drug combination studies, cells were simultaneously treated with ABT-263 (Selleck Chemicals), bendamustine (Mundipharma) or fludarabine (Teva) for 48 h. Cell viability was quantified by flow cytometry analysis by double labeling of phosphatidylserine (PS) exposure with Annexin V-fluorescein isothiocyanate (FITC), and cell permeabilization with propidium iodide (PI; Bender Medsystems). Cytotoxicity against PBMCs was evaluated by staining with anti-CD3-FITC (Becton Dickinson), anti-CD19-phycoerythrin (Becton Dickinson) antibodies and Annexin V-Pacific Blue (Life technologies). Labeled cells were analyzed on a FACScan (Becton Dickinson) or Attune (Life Technologies) cytometers. Mitochondrial hallmarks of apoptosis were evaluated as previously described.[23] Combination index (CI) values were calculated with the CalcuSyn software version 2.0 (Biosoft) by using the Chou and Talalay algorithm. The interaction between 2 drugs was considered synergistic when CI was less than 0.8.

Protein isolation and Western blot analysis

Whole protein extraction and Western blot analysis were carried out as described previously.[24] Membranes were probed with the antibodies specified in the *Online Supplementary Methods*.

BCR stimulation by anti-immunoglobulin M antibodies crosslinking

To determine the efficacy of the inhibitor to antagonize BCR-derived prosurvival signals, BCR triggering was performed by adding anti-human immunoglobulin M (anti-IgM; 25 μg/mL) for 30 min, as described in the *Online Supplementary Methods*. When indicated, cells were treated with NVP-BKM120 simultaneously to the addition of anti-IgM. .

Migration and actin polymerization assays

Chronic lymphocytic leukemia cells were treated with NVP-BKM120 for 1 h before CXCL12-induced migration and actin polymerization assay with phalloidin-tetramethyl rhodamine isothiocyanate staining were determined. Detailed methods for these assays are provided in *Online Supplementary Methods.*

Statistical analysis

Data are represented as mean ± standard error of the mean (SEM) of the indicated experiments. Non-parametric Mann-Whitney test or paired t-tests were used to assess differences between 2 groups. Two-way ANOVA was used to determine how response was affected by two factors. $P<0.05$ was considered statistically significant.

Detailed methods for PIP_3 ELISA assay, RT-PCR, RNA interference assay and stromal cell co-culture are provided in the *Online Supplementary Methods*.

Results

NVP-BKM120 induces apoptosis in CLL cells independent of prognostic markers

To explore the antitumoral effect of the PI3K inhibitor NVP-BKM120, cells from 6 CLL cases were exposed to increasing concentrations of the drug for 48 h. NVP-BKM120 induced a dose-dependent cytotoxic effect, ranging from 27.12%±18.09 (at 1 uM) to 60.70%±13.30 at the higher dose tested (10 μM). At 2 μM the cytotoxic effect was 31.63%±15.34 (Figure 1A) and was confirmed in 37 CLL cases where the compound induced a mean cytotoxicity of 31.41%±15.69 (Figure 1B). It is important to note that no cytotoxic effect was observed in PBMCs isolated from healthy donors (**, $P<0.01$) (Figure 1B). A phase I clinical study reported that the maximum tolerated dose of the drug was around 5 μM[19] and in view of this we performed most of the studies at doses of 1 and 2 μM in order to avoid off-target effects and toxicities. In addition, NVP-BKM120 also induced cytotoxicity in bone marrow-derived (n=3, 45.9%±14.30) and lymph node-derived (n=3, 39.35%±5.74) CLL cells at the dose of 2 μM (*data not shown*).

As shown in the *Online Supplementary Table S1*, there were no significant differences in NVP-BKM120 cytotoxicity in cells from patients with *IGHV*-mutated *versus* *IGHV*-unmutated status or expression of ZAP-70 and CD38. Moreover, we did not find any association

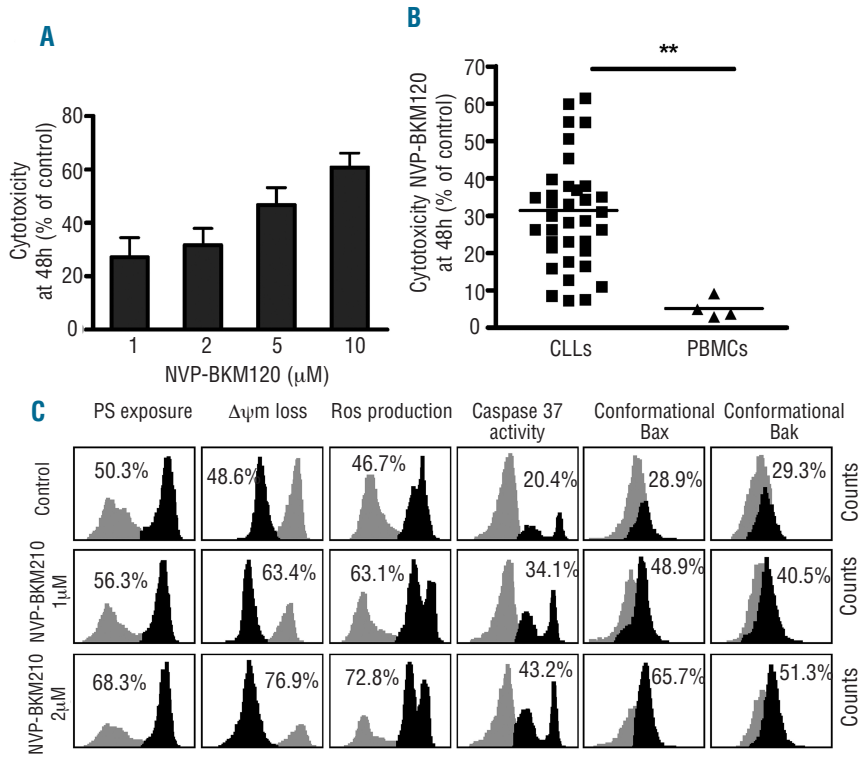

Figure 1. NVP-BKM120 cytotoxicity in primary CLL cells. (A) CLL cells (n=6) were treated with increasing doses of NVP-BKM120 (range 1-10 μM) for 48 h and cytotoxicity was measured by Annexin V. Mean ± SEM of all the samples analyzed. (B) Primary CLL cells (n=37) and PBMCs from healthy donors (n=4) were incubated with 2 μM NVP-BKM120 for 48 h before cytotoxicity was assessed by Annexin V labeling. **, $P<0.01$. (C) CLL cells were treated with NVP-BKM120 (1 and 2 μM) for 48 h and apoptosis hallmarks were determined by flow cytometry. A representative case was shown (CLL n. 5). Percentages inside each chart refer to the population in black.

between NVP-BKM120 sensitivity and the most frequent cytogenetic (17p, 11q and 13q deletions and trisomy 12) and genomic (*SF3B1, NOTCH1* and *MYD88* mutations) alterations encountered in CLL cells, despite the low number of cases in each group (*Online Supplementary Table S1*).

Importantly, NVP-BKM120 activated the typical mitochondrial hallmarks of apoptosis, including PS exposure, mitochondrial depolarization (Δψm), reactive oxygen species (ROS) production, caspase-3/7 activity and Bax/Bak conformational changes (Figure 1C).

These results indicated that NVP-BKM120 selectively induced mitochondrial apoptosis in the majority of CLL cases, including those bearing adverse cytogenetic and/or genomic alterations.

NVP-BKM120 blocks PI3K/Akt/FoxO3a signaling pathway while inducing Bim and down-regulating Mcl-1

As NVP-BKM120 is considered a pan-PI3K inhibitor, we then sought to determine its potential on inhibiting PI3K activity. As shown in Figure 2A, by measuring the amount of PIP_3 extracted from control and treated CLL cells, we confirmed that NVP-BKM120 significantly decreased PI3K activity in CLL cells after 30 min (81.69%±19.41 of inhibition, *, $P<0.05$).

To further evaluate the effect of this compound in the PI3K-mediated signaling, we analyzed the phosphorylation status of its main downstream effector Akt. We found that short time exposure (6 h) to NVP-BKM120 (1 and 2 μM) induced a decrease in the phosphorylation levels of Akt at Ser473 in CLL cells (Figure 2B). As FoxOs proteins are important targets of the PI3K/Akt pathway and the phosphorylation by Akt is one of the major regulatory mechanisms by which FoxO-mediated transcription is repressed,[25] we next addressed if FoxO3a was a target for NVP-BKM120-mediated apoptosis in CLL cells. Indeed, we found a downregulation of FoxO3a phosphorylation that led to the induction of the proapoptotic BH3-only protein Bim in NVP-BKM120-treated CLL cells, according to the role of FoxO3 as a transcription factor of Bim (Figure 2B). We then analyzed the antiapoptotic members of the Bcl-2 family (Bcl-2, Bcl-X_L and Mcl-1) and we found a downregulation of Mcl-1 after exposure to NVP-BKM120 (Figure 2B).

In order to determine whether NVP-BKM120 modulation of Bim and Mcl-1 was transcriptional, we monitored *BIM* and *MCL-1* mRNA levels by qRT-PCR. Exposure to 2 μM NVP-BKM120 for 6 h resulted in no significant changes in *MCL-1* transcripts whereas a significant increase in *BIM* mRNA levels was observed (**, $P<0.01$) (Figure 3A). As NVP-BKM120 was not interfering with MCL-1 transcription, we then determined whether its downregulation was due to inhibition of the translation, as it has been described that mTOR regulates translation of mRNAs containing long 5'-UTRs, including Mcl-1.[26] Accordingly, it has recently been reported that the multikinase inhibitor sorafenib is able to block Mcl-1 at translational level.[27,28] As shown in Figure 3B, treatment of primary CLL cells with 2 μM NVP-BKM120 decreased the phosphorylation levels of several kinases implicated in the translational machinery such as mTOR, S6 ribosomal protein, the eIF4E-binding protein 1 (4E-BP1) and the translation initiator factor eIF4E, thus arguing in favor of a translational-dependent regulation of Mcl-1 levels.

Bim contributes to NVP-BKM120-induced mitochondrial apoptosis in CLL cells

To ascertain if the increase in the BH3-only protein Bim was functionally important for NVP-BKM120-induced apoptosis in CLL cells, we used a siRNA-mediated approach to knock-down BIM. Figure 3C shows that

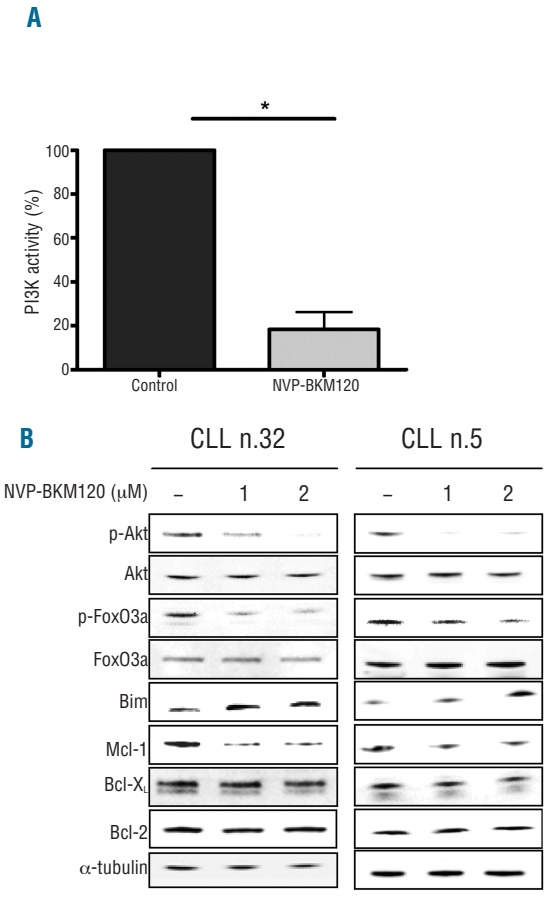

Figure 2. Modulation of PI3K/Akt/FoxO3a pathway and Bcl-2 anti-apoptotic family in CLL cells exposed to NVP-BKM120. (A) Primary CLL cells (n=3) were treated with 2 μM NVP-BKM120 for 30 min and PI3K activity was assessed. Data represent the mean ± SEM of the 3 cases analyzed. *, $P<0.05$. (B) CLL cells were incubated with NVP-BKM120 (1 and 2 μM) for 6 h before Western blot analysis was performed. Two representative cases out of 9 were showed.

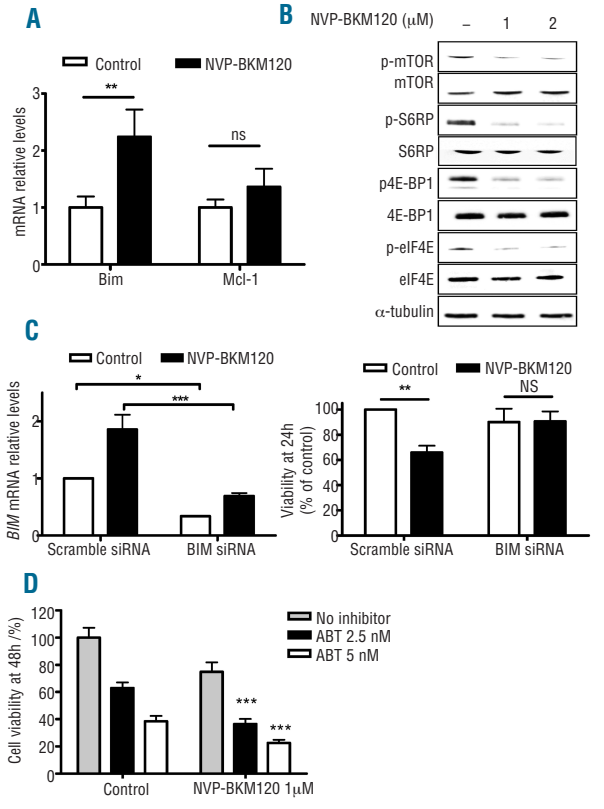

Figure 3. Role of Bim and Mcl-1 in NVP-BKM120-induced apoptosis in CLL cells. (A) Analysis of mRNA expression by quantitative RT-PCR in primary CLL cells (n=8) incubated with 2 μM NVP-BKM120 for 6 h. Mean ± SEM of the cases analyzed. **, $P<0.01$; ns=not significant. (B) Western blot analysis of several kinases implicated in the translational machinery in CLL primary cells exposed to NVP-BKM120 (1 and 2 μM) for 6 hours. A representative case was showed (CLL n. 32). (C) Primary CLL cells were transfected by electroporation with BIM siRNA and non-silencing siRNA in 3 independent experiments. Transfected cells were then incubated with 2 μM NVP-BKM120 for 24 h. Viability was assessed by flow cytometry labeling of AnnexinV and knockdown of Bim protein was quantified by RT-PCR. Mean ± SEM of the cases analyzed. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$; ns=not significant. (D) Primary CLL cells (n=5) were simultaneously exposed to NVP-BKM120 (1 μM) and ABT-263 (2.5 and 5 nM) for 48 h. Bars represent the mean ± SEM of cell viability referred to untreated control cells. ***, $P<0.001$.

transfection with siRNA oligonucleotides directed toward this gene significantly reduced mRNA levels (*, $P<0.05$) providing significant protection against NVP-BKM120-induced cell death when compared to scramble siRNA (***, $P<0.001$).

To provide further evidence of the role of Bcl-2 family of proteins in NVP-BKM120 antitumoral activity in CLL cells, we examined the effect of combining NVP-BKM120 with the BH3-mimetic ABT-263. Simultaneous exposure of CLL cells to NVP-BKM120 (1 μM) and ABT-263 (2.5 and 5 nM) for 48 h led to a notable reduction in cell viability that was more effective than single drug treatment (Figure 3D). Interestingly, combination of NVP-BKM120 1 μM and ABT-263 2.5 and 5 nM was found to induce significant cytotoxic effect (***, $P<0.001$), with CI values of 0.528 and 0.607, respectively.

Taken together, these findings support the contribution of Bim to the mitochondrial apoptosis induced by NVP-BKM120.

NVP-BKM120 abrogates BCR-derived signaling

To determine the effects of NVP-BKM120 on CLL cell signaling mediated via the BCR, we stimulated cells with anti-IgM in the presence of NVP-BKM120. As shown in Figure 4A, NVP-BKM120 2 μM induced apoptosis with similar efficiency in IgM-stimulated than in non-stimulated CLL cells (**, $P<0.01$, ***, $P<0.001$). In response to BCR engagement, CLL cells increased the expression of phospho-Akt, phospho-FoxO3a and Mcl-1. NVP-BKM120 was able to completely block both basal and IgM-induced phospho-Akt, phospho-FoxO3a and Mcl-1 expression (Figure 4B). Additionally, NVP-BKM120 was also able to induce Bim, at both transcriptional (*, $P<0.05$) and translational levels, even in the presence of anti-IgM (Figure 4C).

We next evaluated whether NVP-BKM120 could block T-cell chemokines CCL3 and CCL4 that are secreted by CLL cells in response to BCR stimulation. Figure 4D shows that BCR stimulation increased CCL3 and CCL4 mRNA levels (CCL3: 12.09±5.28; CCL4: 10.00±5.05), whereas NVP-BKM120 incubation significantly blocked this induction (CCL3: 5.43±3.32, **$P<0.01$; CCL4:

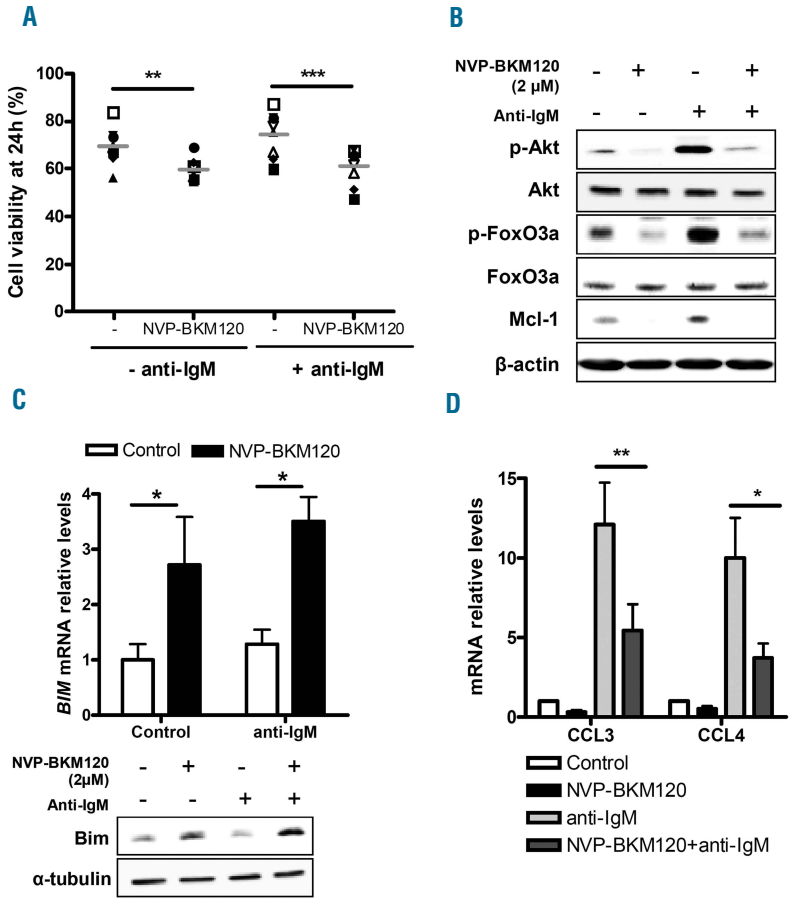

Figure 4. NVP-BKM120 abrogates BCR-derived signals. (A) Primary CLL cells (n=8) were incubated simultaneously with 2 μM NVP-BKM120 and anti-IgM and cell viability was assessed at 24 h by Annexin V/PI flow cytometry. Horizontal lines represent the mean. **, *P*<0.01, ***, *P*<0.001. (B) Western blot analysis after stimulation of CLL cells for 30 minutes with anti-IgM in the presence of 2 μM NVP-BKM120. A representative case was showed (CLL n. 4). (C) Primary CLL cells (n=6) were incubated with 2 μM NVP-BKM120 in presence or absence of anti-IgM for 6 h. Analysis of BIM was then determined by RT-PCR and Western blot analysis. Mean ± SEM of the cases analyzed. *, *P*<0.05. A representative case was shown (CLL n. 4). (D) Analysis of mRNA expression by RT-PCR of CCL3 and CCL4 chemokines in 4 CLL cases incubated simultaneously with NVP-BKM120 and anti-IgM for 6 h. Mean ± SEM of the cases analyzed. *, *P*<0.05, **, *P*<0.01.

3.70±1.83, *P<0.05). These findings highlight NVP-BKM120 ability to inhibit BCR-derived responses in CLL cells.

NVP-BKM120 induces cytotoxicity in the presence of microenvironment survival signals on CLL cells

It is well documented that stromal microenvironment contributes to CLL cell proliferation, survival and drug resistance.[2] Consistent with previous results,[29] we observed that co-culture with the stromal cell line HS-5 protected CLL cells from spontaneous apoptosis, thus cell viability in control samples was 67.80%±9.86 and increased up to 81.93%±6.0 after HS-5 co-culture (***, P<0.001, Figure 5A). NVP-BKM120 treatment (2 μM, 24 h) of CLL cells induced cytotoxicity both with and without HS-5 co-culture (Figure 5A; **, P<0.01). At the molecular level, NVP-BKM120 efficiently down-regulated stroma-induced phospho-Akt, phospho-FoxO3a and Mcl-1 expression (Figure 5B), and still induced Bim at both mRNA (**, P<0.01) and protein levels (Figure 5C).

From this observation, we further evaluated whether NVP-BKM120 could enhance CLL cell killing when these cells were co-cultured with HS-5 in the presence of fludarabine and bendamustine, both drugs currently used in CLL treatment. Figure 5D depicts the mean relative viabilities of CLL cells co-treated with NVP-BKM120 1 μM, a dose that did not affect HS-5 cells viability (*data not shown*), and fludarabine (0.5 μg/mL) or bendamustine (10 μM) for 48 h. Interestingly, the combination of both agents was significantly more effective than single drug alone, both in the presence and absence of HS-5 (***, P<0.001). Thus, a synergistic effect was observed both in combination with fludarabine (CI: 0.598) and bendamustine (CI: 0.675) without stroma, and also in co-culture with stromal cells (CI: 0.471 for fludarabine and CI: 0.462 for bendamustine).

NVP-BKM120 inhibits CXCL12-induced CLL migration and actin polymerization

CXCL12 is a chemokine secreted by different types of stromal cells that has a direct prosurvival effect on CLL cells and may guide migration of CLL cells to the stroma microenvironment.[30] Thus, we sought to analyze the effect of NVP-BKM120 in the presence of CXCL12. Figure 6A indicates that NVP-BKM120 2 μM overcame CXCL12 (200 ng/mL) stimulation and induced apoptosis with the same efficiency in CXCL12-stimulated cells (***, P<0.001) as in non-stimulated CLL cells (***, P<0.001).

To assess the effect of NVP-BKM120 on the migratory capacity of CLL cells induced by CXCL12, CLL cells were assayed for chemotaxis toward CXCL12 (200 ng/mL) after 1 h of pre-incubation with NVP-BKM120 2 μM. Figure 6B shows that NVP-BKM120 significantly reduced the number of migrating CLL cells from peripheral blood in the presence of the chemokine (43.77%±21.93 of inhibition, *, P<0.05). Significant inhibition of migration cells (*, P<0.05) were also observed in the presence of CXCL12 in CLL cells derived from bone marrow (26.04%±14.32 of inhibition, Figure 6C) and lymph node (27.61%±19.67 of inhibition, Figure 6D).

As cell migration in response to chemokines requires

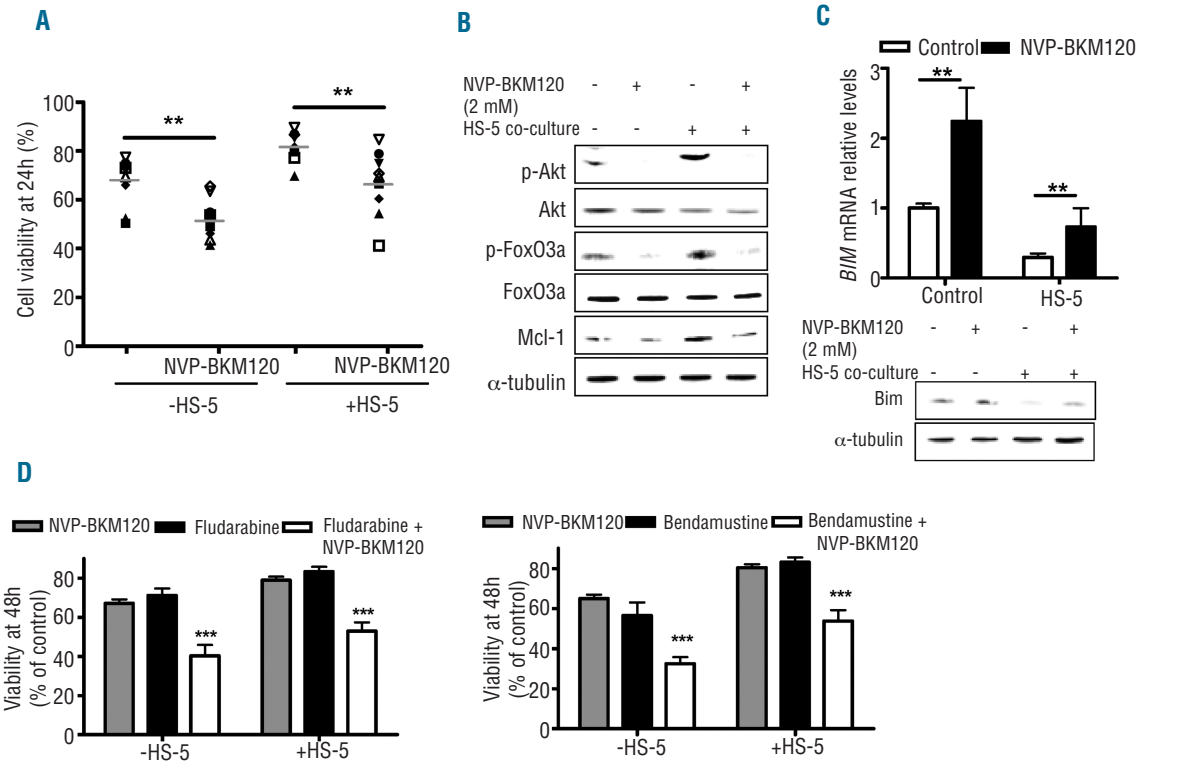

Figure 5. NVP-BKM120 induces cytotoxicity in the presence of microenvironment survival signals on CLL cells. (A) Primary CLL cells (n=8) were co-cultured with or without HS-5 and incubated with 2 μM NVP-BKM120. Cell viability was assessed at 24 h by Annexin V/PI flow cytometry. Horizontal lines represent the mean. **, *P*<0.01. (B) Western blot analysis after co-culture of CLL cells with HS-5 in the presence of 2 μM NVP-BKM120 for 6 h. A representative case was showed (CLL n. 4). (C) Primary CLL cells (n=8) co-cultured with or without HS-5 were incubated with 2 μM NVP-BKM120 for 6 h. Analysis of Bim was then determined by RT-PCR and Western blot analysis. A representative case was shown (CLL n.4). Mean ± SEM of the cases analyzed. **, *P*<0.01. (D) CLL cells (n=6) were simultaneously incubated with 1 μM NVP-BKM120 and fludarabine (0.5 μg/mL) or bendamustine (10 μM) with or without stroma. Viability at 48 h was calculated relative to the respective untreated control, with or without stroma. Mean ± SEM of the cases analyzed. *, *P*<0.001.**

actin polymerization, we therefore analyzed the effects of NVP-BKM120 on this cellular response. As shown in Figure 6E, CXCL12 induced a notable increase in actin polymerization at 15 seconds (s) of stimulation that was significantly decreased with NVP-BKM120 2 μM after only 60 s (*, *P*<0.05; **, *P*<0.01). These data suggest that NVP-BKM120 could block CXCL12-induced CLL chemotaxis and actin polymerization.

Discussion

BCR signaling plays an important role in the CLL pathogenesis and disease progression, as promotes maintenance and expansion of tumoral cells.[31] Besides from BCR signalling, many other factors have been identified to enhance CLL cell survival, such as Toll-like receptors, cytokines, chemokines, CD40, BAFF, integrins and components of extracellular matrix.[3] Many of these factors activate similar intracellular signalling pathways, being one of the most prominent the PI3K/Akt/mTOR pathway. In our study, we have examined the response of primary CLL cells to the PI3K inhibitor NVP-BKM120. Our data indicate that NVP-BKM120 selectively activates the intrinsic apoptotic pathway in B cells and that its cytotoxic activity in CLL occurs independently of the most common prognostic markers such as mutational status of *IGHV*, ZAP-70 and CD38 expression, the adverse cytogenetic alterations and the new recurrent mutations described in CLL cells.[21,22] Furthermore, this study suggests that NVP-BKM120 mediates cytotoxicity both by directly inhibiting PI3K signalling in CLL cells and by disabling the supportive effect of many microenvironmental factors including co-culture with stromal cells, activation of the BCR and of CXCL12. More specifically, NVP-BKM120 is able to induce cytotoxicity despite IgM-mediated stimulation of BCR and to inhibit BCR-dependent induction of the T-cell attracting chemokines CCL3 and CCL4. In addition, we show that NVP-BKM120 sensitizes CLL cells to cytotoxic drugs such as fludarabine and bendamustine even in the presence of protective bone marrow-derived stromal cells. Several PI3K inhibitors are in pre-clinical studies and only few results have been reported in clinical trials. Among them, a phase I study of the PI3Kδ inhibitor GS-1101 showed an overall response (OR) of 26%. However, 80% of patients had a reduction in lymphadenopathy by ≥50%.[10] Interestingly, it has recently been reported that combination of GS-1101 with rituximab and/or bendamustine induces an OR higher than 78%.[32] In line with this, clinical responses to SYK[33] and BTK[34] inhibitors in CLL are also characterized by a rapid mobilization of tumoral cells from nodal masses to peripheral circulation, with a significant decrease in lymphadenopathies and splenomegaly and an increase in the number of lymphocytes in peripheral blood. Consistently, we observed that NVP-BKM120 might also be effective in cells from lymph

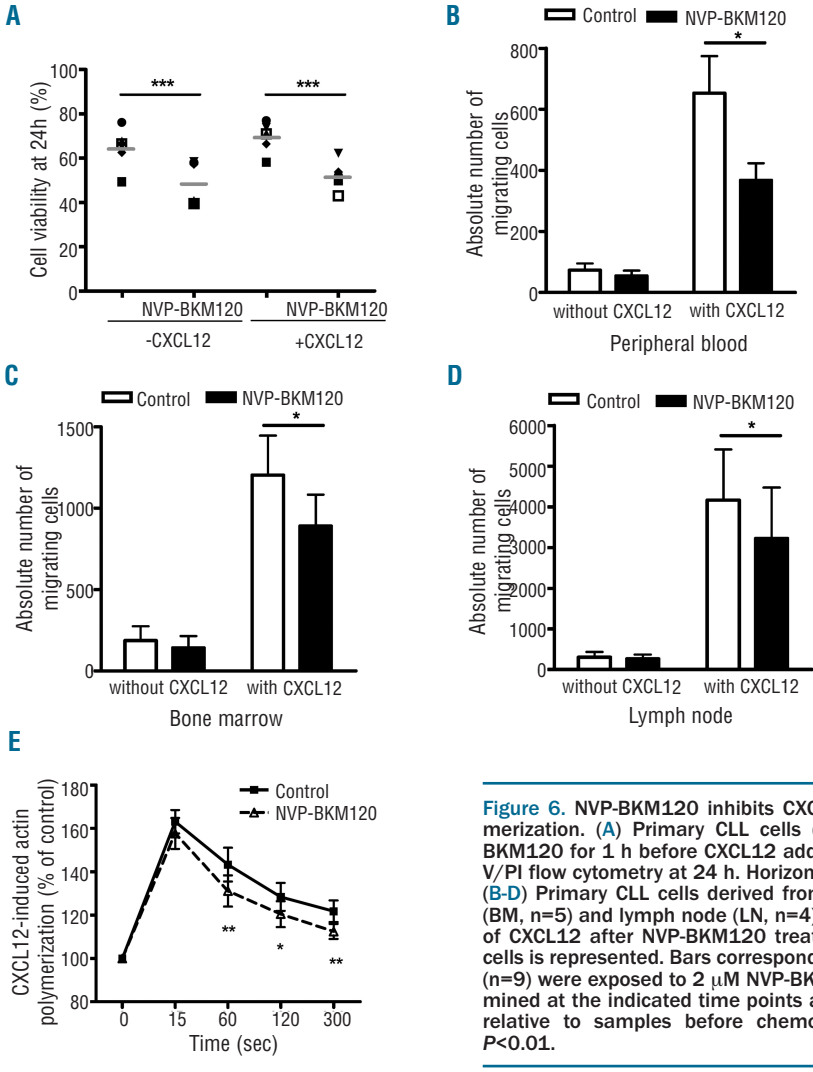

Figure 6. NVP-BKM120 inhibits CXCL12-induced CLL migration and actin polymerization. (A) Primary CLL cells (n=6) were pre-incubated with 2 μM NVP-BKM120 for 1 h before CXCL12 addition. Cell viability was assessed by Annexin V/PI flow cytometry at 24 h. Horizontal lines represent the mean. *, *P*<0.001. (B-D) Primary CLL cells derived from peripheral blood (PB, n=9), bone marrow (BM, n=5) and lymph node (LN, n=4) were assayed for migration in the presence of CXCL12 after NVP-BKM120 treatment as above. Total number of migrating cells is represented. Bars correspond to the mean ± SEM. *, *P*<0.05. (E) CLL cells (n=9) were exposed to 2 μM NVP-BKM120 for 1 h and F-actin content was determined at the indicated time points after CXCL12 addition. Results are displayed relative to samples before chemokine stimulation (100%). *, *P*<0.05, **, *P*<0.01.**

nodes and bone marrow, indicating that this compound could induce cytotoxicity to CLL cells from these compartments by deprivation from their supportive tissue microenvironment. We also found that NVP-BKM120 could inhibit CLL cell migration and signaling responses to CXCL12. These effects of NVP-BKM120 on cell migration and actin polymerization could cause interference with the cell trafficking and homing of CLL cells.

Importantly, it has recently been reported that NVP-BKM120 was 3.6 fold more toxic than GS-1101 in primary CLL cells *in vitro*,[16] thus confirming a crucial role for the non-delta PI3K isoforms in CLL to antagonize stromal cell-derived migration, survival, and drug-resistance signals[35] and pointing out the importance to target different isoforms to overcome possible redundant functions. In this sense, it has been reported that expression of p110 isoform can maintain constitutive PI3K signaling despite p110 inhibition.[36]

The inhibition of PI3K by NVP-BKM120 results in dephosphorylation of Akt and subsequent regulation of a number of proteins including FoxO3a. The tumor suppressor genes of the FoxO subfamily of Forkhead transcription factors that includes FoxO3a (or FKHRL1), FoxO1a (or FKHR), and FoxO4a (or AFX) are critical effectors downstream of Akt.[37] Activation of FoxO3a by decreasing its phosphorylation and increasing its nuclear content can upregulate the expression of genes that are involved in either apoptosis or cell cycle arrest in different types of cells.[25,38] Thus, increasing FoxO activity appears as a promising therapeutic strategy.[39] FoxO3a is an important regulator of Bim expression[40-42] and alterations in the Akt-FoxO3a axis have been described to affect Bim expression in several models.[43,44] Recently, it has been reported that plasmid-based overexpression of constitutively active FoxO3a in CLL cells reduced their survival and induced expression of Bim and p27.[45] According to this finding, we found that NVP-BKM120 is able to induce Bim in CLL cells, even in the presence of anti-IgM or co-culture with stromal cells. Moreover, Bim induction is functionally important for NVP-BKM120-mediated apoptosis in CLL cells, as an siRNA-mediated approach to knockdown *BIM* demonstrates that Bim effectively contributes to cell death after NVP-BKM120 treatment.

Bim functions as a tumor suppressor in B-cell malignancies and is a key determinant of BCR-induced apoptosis in normal B cells, where it is required for the deletion of autoreactive cells *in vivo*.[46] Moreover, Bim is the preferred dimerization partner of Mcl-1, a key target for survival sig-

nals in CLL cells. High Mcl-1 expression and a low Bim/Mcl-1 ratio is a predictive of inferior response to chemotherapeutic agents.[47,48]

Our results show that Mcl-1 is induced both in cells stimulated with anti-IgM and co-cultured with stromal cells, thereby playing an important role in microenvironment-derived signaling. In this context, it has been reported that disease in the bone marrow is less responsive to the BH3-mimetic ABT-263, which may be due to upregulation of Mcl-1 in CLL cells in contact with stroma and a decrease in Bim expression.[46,49] In our study, NVP-BKM120 induction of Bim may be able to neutralize high Mcl-1 expression and, as NVP-BKM120 also inhibits Mcl-1 translation, it accentuates even more the Bim/Mcl-1 ratio, leading to the activation of the mitochondrial apoptotic pathway. Recently, it has been reported that the combination of PI3K/Akt/mTOR inhibitors and BH3 mimetics enhances PI3K inhibition-induced apoptosis through a Bim-dependent mechanism in acute myeloid leukemia.[50] Consistently, we also found that NVP-BKM120 exerts a synergistic effect with the BH3-mimetic ABT-263, supporting the role of the Bcl-2 family of proteins in the NVP-BKM120 induced apoptosis.

Overall, our present findings establish that NVP-BKM120 effectively inhibits the PI3K signaling pathway and disturbs the protective effect of the tumor microenvironment with the subsequent apoptosis induction of CLL cells. Our data provide mechanistic insight into the cytotoxic activity of this PI3K inhibitor in CLL cells, indicating that induction of Bim is one of the key points in NVP-BKM120-mediated cytotoxicity. In addition, NVP-BKM120 inhibits CLL cell migration and actin polymerization, which may be particularly important in mobilizing CLL cells from sanctuary sites. We provide here a strong rationale for undertaking clinical trials of NVP-BKM120 in CLL patients alone or in combination therapies.

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