Constitutive and B-cell receptor-induced activation of STAT3 are important signaling pathways targeted by bortezomib in leukemic mantle cell lymphoma

Fanny Baran-Marszak,[1,2,3] Mohand Boukhiar,[1,2] Stéphanie Harel,[3] Christelle Laguillier,[3] Claudine Roger,[1-3] Remy Gressin,[5-6-7-8] Antoine Martin,[1-2-4-8] Remi Fagard,[1-2] Nadine Varin-Blank,[1-2] Florence Ajchenbaum-Cymbalista,[1-2-3] and Dominique Ledoux[1-2]

[1]INSERM U978, Bobigny, France; [2]Université Paris 13, UFR SMBH, Bobigny, France; [3]AP-HP, Service d'Hématologie Biologique, Hôpital Avicenne, Bobigny, France; [4]AP-HP, Service d'Anatomopathologie, Hôpital Avicenne, Bobigny, France; [5]INSERM U823, Grenoble, France; [6]Université Joseph Fourier, Grenoble I, Grenoble, France; [7]CHU de Grenoble, Service d'Hématologie Clinique, Grenoble, France, and the [8]French GOELAMS Group

Funding: this work was supported by a grant from Université Paris13, by the Association pour la Recherche sur le Cancer, by the Ligue contre le Cancer-Comité Seine St. Denis and by the GOELAMS group.

Manuscript received on November 15, 2009. Revised version arrived on July 14, 2010. Manuscript accepted on July 14, 2010.

Correspondence: Dominique Ledoux /Florence Ajchenbaum-Cymbalista, UMR U978 INSERM-Université Paris 13, UFR SMBH, 74 rue Marcel Cachin, 93000 Bobigny, France. Email: ledoux@univ-paris13.fr/ florence.cymbalista@avc.aphp.fr

The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
The deregulation of several transcription factors contribute to the aggressive course of mantle cell lymphoma. This study focuses on survival signals emanating from the tumor environment and involving the signal transducer and activator of transcription (STAT) 3 through cytokines or antigen recognition.

Design and Methods
Primary mantle cell lymphoma cells were isolated from 20 leukemic patients. The phosphorylation status of STAT3 was evaluated by immunoblottting and immunofluorescence, the levels of cytokine secretion by enzyme-linked immunosorbent assay and the cell survival signals by apoptosis and cell viability assays.

Results
STAT3 was constitutively phosphorylated in the Jeko-1 mantle cell lymphoma cell line and in 14 out of 20 (70%) cases of leukemic mantle cell lymphoma as the result of an autocrine secretion of interleukin-6 and/or interleukin-10. In addition, B-cell receptor engagement resulted in an increase of both *in vitro* cell survival and STAT3 phosphorylation in primary mantle cell lymphoma cells. Inhibition of the Janus-activated kinase/STAT3 pathway increased spontaneous apoptosis and suppressed B-cell receptor-induced cell survival in all cases analyzed. The impact of *in vitro* exposure to the proteasome inhibitor bortezomib was next evaluated in primary mantle cell lymphoma cells. Bortezomib induced apoptosis and a decrease of both interleukin-6/interleukin-10 secretion and STAT3 phosphorylation. In addition, bortezomib inhibited B-cell receptor-triggered STAT3 phosphorylation and cell survival.

Conclusions
We demonstrated that STAT3 was activated in primary mantle cell lymphoma cells either constitutively through a cytokine autocrine loop or in response to B-cell receptor engagement, both processes leading to a survival signal inhibited by bortezomib. STAT3 appears, therefore, to play a pivotal role in mantle cell lymphoma and represents a promising therapeutic target.

Key words: mantle cell lymphoma, B-cell receptor, STAT3, bortezomib.

Citation: Baran-Marszak F, Boukhiar M, Harel S, Laguillier C, Roger C, Gressin R, Martin A, Fagard R, Varin-Blank N, Ajchenbaum-Cymbalista F, and Ledoux D. Constitutive and B-cell receptor-induced activation of STAT3 are important signaling pathways targeted by bortezomib in leukemic mantle cell lymphoma. Haematologica 2010;95(11):1865-1872. doi:10.3324/haematol.2009.019745

Introduction

Mantle cell lymphoma (MCL) is an aggressive and incurable malignant lymphoma, representing approximately 5% of non-Hodgkin's lymphomas, with a median survival of 3 to 5 years. Despite new chemotherapeutic combinations, MCL is characterized by a poor overall response due to rapid relapse after initial treatment or primary resistance to conventional drugs.[1] However, phase II studies are currently evaluating the efficacy of the proteasome inhibitor bortezomib in MCL with encouraging results in relapsed and refractory cases.[2] MCL was initially depicted as a proliferative pool of pre-germinal center (GC) naïve B cells with germline immunoglobulin heavy chain variable-region (*IGHV*) genes. However, numerous studies showed that about 20-30% MCL carry somatic mutations in their *IGHV* genes.[3] By analogy with pre-GC and post-GC cells, a subset of MCL might derive from B cells exposed to the GC environment, thus reflecting a molecular heterogeneity of MCL.

Gene profiling studies in MCL cells have revealed overexpression of oncogenic factors such as c-Myc as well as a simultaneous deregulation of multiple genes implicated in the regulation of nuclear factor kappa B (NF-κB).[4] Furthermore, a previous immunochemistry study showed that the oncogenic transcription factor signal transducer and activator of transcription 3 (STAT3) was constitutively phosphorylated on tyrosine residues in 20/43 (47%) lymph node biopsies.[5] Constitutively active STAT3 contributes to the malignant phenotype in numerous human cancer cell lines and primary tumors by promoting uncontrolled cell growth and survival through dysregulated protein expression, including that of interleukin (IL)-10 and STAT3 itself.[6] Moreover, STAT3 induces tumor angiogenesis by up-regulating the expression of vascular endothelial growth factor, and modulates immune functions towards tumor immune evasion.[6,7] Overall, several studies point to STAT3 as a promising target for anticancer therapy.[8] STAT proteins are usually phosphorylated on tyrosine 705 by Janus-associated kinases (JAK) upon cytokine receptor engagement. Both IL6 and IL10 are known to phosphorylate STAT3. It was also shown that the MCL molecular signature included overexpression of IL10 receptor[4] and that IL10 was able to sustain cell proliferation in MCL primary cells,[9] suggesting an autocrine/paracrine role for IL10 in MCL cell survival or proliferation. Activation of STAT3 in B cells may also result from B-cell receptor (BCR) engagement through two possible pathways: a delayed and indirect phosphorylation of STAT3[10,11] or alternatively a JAK-independent rapid and transient phosphorylation of STAT3 by Lyn.[12] After BCR engagement, human circulating normal $CD5^+$ B cells produce more IL10 than $CD5^-$ B-cells,[13] and in animal models a strong BCR signal is responsible for the specific expansion of $CD5^+$ B cells.[14] In our study, we deciphered the signals generated by cytokines and BCR engagement resulting in STAT3 phosphorylation and subsequent MCL cell survival.

Design and Methods

Mantle cell lymphoma samples and cell lines

Peripheral blood mononuclear cells (PBMC) were obtained from 20 MCL leukemic patients by Ficoll-Hypaque density gradient. The diagnosis of MCL was ascertained by immunophenotyping, cytogenetics, fluorescence *in situ* hybridization (FISH) analysis of t(11;14) and overexpression of cyclin D1. All patients provided written informed consent, validated by the Ethics Committee from the GOELAMS group, in accordance with the Declaration of Helsinki. Patients usually received treatment very quickly after sampling, making it difficult to repeat all experiments several times. Nonetheless, reproducibility of the results was ensured in eight out of 20 cases by repeating experiments two to six times. For BCR stimulation, plates were coated with rabbit anti-human IgM antibody (10 μg/mL) as previously described.[15] The cell lines, cell cultures and reagents are described in the *Online Supplementary Design and Methods.*

Determination of IGHV mutational status

Amplification and sequence analysis of *IGHV* rearrangements were performed on either DNA or cDNA as previously described.[16] A homology cut-off value of 98% to the germline sequence was used to discriminate between unmutated (≥98%) and mutated (<98%) *IGHV* gene status.

Apoptosis and cell viability assays

Cell apoptosis was analyzed by annexin V-FITC and propidium iodide staining and cell viability was evaluated by the methyltetrazolium salt (MTS) assay. Further details are provided in the *Online Supplementary Design and Methods*.

Quantification of IL6 mRNA and cytokine proteins

The expression of *IL6* mRNA was analyzed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using the RT^2 profiler PCR arrays. Cytokines in cell culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA). Further details are provided in the *Online Supplementary Design and Methods*.

Analysis of STAT3 by western blotting and immunofluorescence

Total protein extracts from 3×10^6 cells were separated on 10% polyacrylamide denaturing gel, transferred to a nitrocellulose membrane and incubated overnight either with rabbit polyclonal anti-STAT3 antibody (1/1000) or with rabbit polyclonal anti-phospho-Tyr^{705}STAT3 antibody (1/1000) (Cell Signaling, Beverley, MA, USA), followed by a secondary horseradish peroxidase-conjugated antibody (Bio-Rad). Detection was performed using ECL and autoradiography. Densitometric analysis of immunoblots was performed using the Quantity One software (Bio-Rad). The immunofluorescence analysis is described in the *Online Supplementary Design and Methods*.

Statistical analyses

Differences between groups were determined using the unpaired Student's t-test or the Wilcoxon-signed rank test as appropriate. Statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA).

Results

The constitutive phosphorylation of STAT3 in mantle cell lymphoma cells is dependent on a cytokine autocrine secretion

Constitutive Tyr^{705}-phosphorylation of STAT3 was detected by western blotting in the MCL cell line Jeko-1 (Figure 1A). We, therefore, investigated whether this constitutive phosphorylation of STAT3, as observed in MCL tumors,[5] might be dependent on a cytokine autocrine secretion. This signal was considerably reduced when Jeko-1

cells were washed. A gradual recovery of the phosphorylation was observed as early as 30 min when the cells were re-incubated with their own supernatant (Figure 1A). Similar results were observed in primary peripheral lymphocytes from a MCL patient (UPN1). Indeed, constitutive STAT3 phosphorylation was already observed upon initial cell isolation and this phosphorylation markedly increased within 3 h of cell culture and remained high for up to 24 h (Figure 1B). Additionally, incubation of the MCL cell line Rec-1 and of primary MCL lymphocytes (UPN7), both lacking basal STAT3 phosphorylation, in a Jeko-1 culture supernatant led to rapid phosphorylation of STAT3 (*Online Supplementary Figure S1*) confirming that STAT3 activating factors are released by Jeko-1 cells. Treatment of Jeko-1 cells with cycloheximide or brefeldin A, well-known inhibitors of protein synthesis and secretion, respectively, led to complete inhibition of STAT3 phosphorylation and incubation of these treated cells with supernatant from untreated Jeko-1 cells restored STAT3 phosphorylation (*Online Supplementary Figure S2*). Secretion of both IL6 and IL10 was measured by ELISA in MCL cell culture supernatants after 24 h (*Online Supplementary Figure S3*). Jeko-1 cells secreted high amounts of IL10 alone (1250±210 pg/mL), cells from UPN1 and UPN10 secreted both IL6 and IL10 (IL6 ≥ 750 pg/mL; IL10 ≥ 250 pg/mL for both cases) while cells from UPN12 secreted only IL6 (450 pg/mL). Conversely, Rec-1 cells and primary cells from UPN7 did not secrete significant amounts of these cytokines. To ascertain that IL6 and/or IL10 secretion was responsible for STAT3 phosphorylation, cells were treated with cytokine-neutralizing or receptor-blocking antibodies. Exogenous treatment of Jeko-1 cells with anti-IL10 neutralizing antibodies decreased STAT3 phosphorylation whereas exogenous anti-IL6 antibodies did not (Figure 1C). Disruption of the IL10 signaling pathway by anti-IL10 receptor-α (anti-IL10-Rα) antibody totally blocked STAT3 phosphorylation, confirming that, in Jeko-1 cells, STAT3 activation was dependent on the IL10 activation pathway (Figure 1C). Similar results were obtained with MCL patients' samples (Figure 1D). In UPN10, addition of anti-IL6-Rα and anti-IL10-Rα antibodies led to an almost complete inhibition of STAT3 phosphorylation while in UPN12, whose cells secreted only IL6, anti-IL6-Rα antibodies only reduced STAT3 phosphorylation. In UPN1, blocking the IL10 pathway markedly decreased STAT3 phosphorylation indicating a higher sensitivity to IL10 of these cells or a lower expression of IL6-Rα. Finally, we observed induction of STAT3 phosphorylation upon IL10 treatment in MCL cell lines (Rec-1, HBL-2) lacking basal STAT3 phosphorylation (*Online Supplementary Figure S4*). Collectively, these results indicate that the constitutive phosphorylation of STAT3 in MCL cells is mainly dependent on IL6 and/or IL10 autocrine secretion.

Inhibition of STAT3 constitutive phosphorylation is associated with increased apoptosis

Since IL6 and IL10 signaling is mediated by JAK proteins, we studied the effect of AG490, a chemical inhibitor of JAK proteins, on STAT3 phosphorylation. Treatment of Jeko-1 cells with AG490 resulted in a decrease of the tyrosine phosphorylation level of STAT3 (*Online Supplementary Figure S5*) and JAK3 (*Online Supplementary Figure S6*). Moreover, the decrease in STAT3 phosphorylation was associated with a dose- and time-dependent loss of Jeko-1 cell viability and a 3.3-fold increase of apoptosis (from 18%

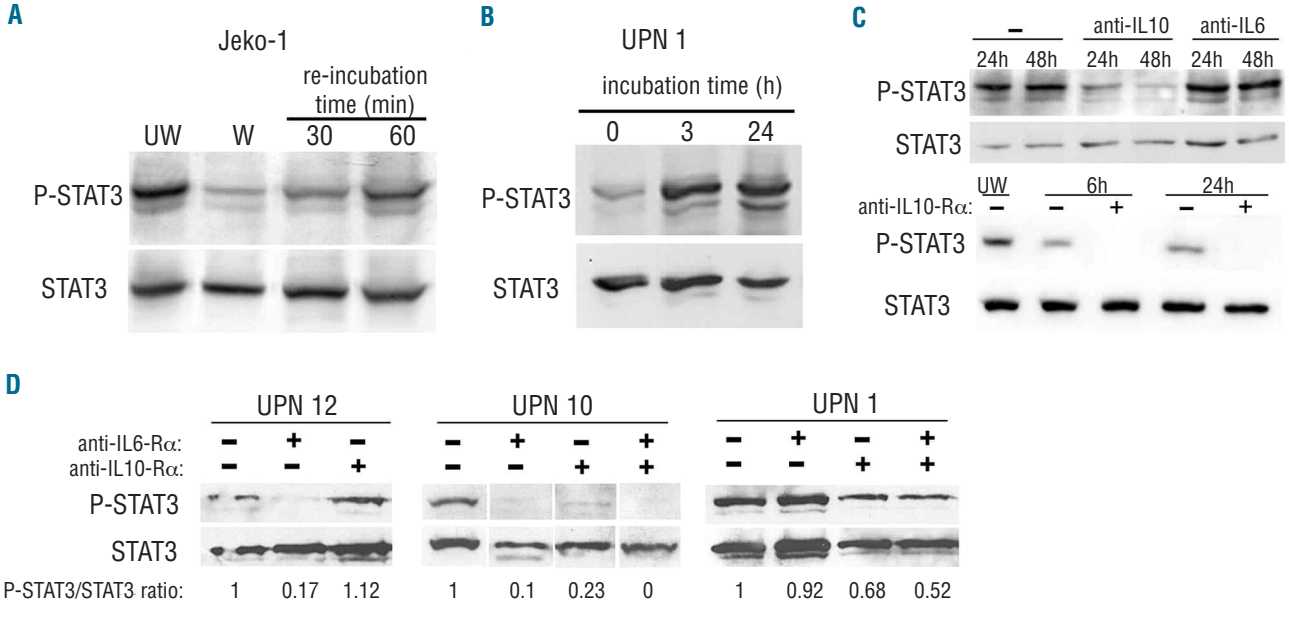

Figure 1. STAT3 is constitutively phosphorylated in MCL cells and depends on a cytokine autocrine loop. (A) Jeko-1 cells were either not washed (lane 1, UW) or washed for 45 min in RPMI medium (lane 2, W) and then reincubated in their own culture medium for 30 min (lane 3) or 60 min (lane 4). Total protein extracts were analyzed by western blotting with anti-phosphotyrosine705 STAT3 antibody and by reblotting with anti-STAT3 antibody. (B) Peripheral blood mononuclear cells (PBMC) from a MCL patient (UPN1) were separated from blood by Ficoll, washed and immediately lysed (lane 1) or cultured for 3 or 24 h before lysis. (C) *Top panel*: Jeko-1 cells were washed and incubated in the absence (-) or in the presence (+) of either anti-IL10 or anti-IL6 neutralizing antibodies (40 μg/mL); *Bottom panel*: Jeko-1 cells were either not washed (UW) or washed and incubated in the absence (-) or in the presence (+) of anti-IL10 receptor-α (anti-IL10-Rα) blocking antibody (40 μg/mL). (D) Decrease of STAT3 phosphorylation in primary MCL cells by disrupting the IL10/IL6 pathways. PBMC were purified from blood and cultured for 24 h in the absence (-) or in the presence (+) of anti-IL10-Rα and/or anti-IL6-Rα antibodies (40 μg/mL).

to 60% of apoptotic cells) (Figure 2 A-B). A very limited effect of AG490 on apoptosis was observed in Rec-1 cells and normal B cells (Figure 2B), neither of which displayed constitutive STAT3 phosphorylation (Figure 3 for B cells). For primary MCL lymphocytes showing constitutive STAT3 phosphorylation, treatment with AG490 (100 μM) led to the complete disappearance of STAT3 phosphorylation and an increased rate of apoptosis (24% and 72% of apoptotic cells for untreated and treated cells, respectively) (*Online Supplementary Figure S7*). Moreover, we treated cells showing constitutive phosphorylation of STAT3 with Stattic, a small-molecule inhibitor of STAT3 dimerization and activation.[17] Stattic abolished the STAT3 phosphorylation (*Online Supplementary Figure S8*) and induced a dose-dependent increase of apoptosis (Figure 2C) while it had a limited effect on apoptosis in normal B cells lacking basal STAT3 phosphorylation. Altogether, these results indicate that survival of MCL cells is dependent at, least in part, on the JAK/STAT3 signaling pathway.

Phosphorylation status of STAT3 in lymphocytes from mantle cell lymphoma patients with a leukemic presentation

The expression and phosphorylation status of STAT3 were further evaluated in a series of 20 MCL patients. The characteristics of the cases studied are summarized in *Online Supplementary Table S1*. All these MCL patients had a leukemic phase with a lymphocyte count superior to 4.6×10^9/L (mean lymphocytosis 51.6×10^9/L) and 18 out of 20 samples contained at least 59% of tumor cells. Moreover, 45% (9/20) of the cases displayed mutated *IGHV* gene status. All cases expressed STAT3 protein and variable levels of STAT3 constitutive phosphorylation were detected in 70% (14/20) of the cases (Figure 3). Constitutive STAT3 phosphorylation was significantly higher in *IGHV* mutated cases (n=9) than in *IGHV* unmutated cases (n=11) ($P<0.001$). All cases with mutated *IGHV* genes (n=9/9) and half of the cases with unmutated *IGHV* genes (n=5/11) exhibited STAT3 constitutive phosphorylation. The presence of unphosphorylated STAT3 was restricted to *IGHV* unmutated cases (n=6/6). Similarly, STAT3 phosphorylation was not detected in purified normal B-lymphocytes or in PBMC obtained from healthy subjects. In order to analyze the cellular localization of phosphorylated STAT3, phosphotyrosine STAT3 staining was performed by immunofluorescence in primary MCL cells, Jeko-1 cells and normal B-lymphocytes (*Online Supplementary Figure S9*). Staining was equivalent for all the tumor cells from a given patient. In terms of localization, primary MCL cells showed strong phosphorylated STAT3 staining in both the cytoplasm and the nucleus whereas Jeko-1 cells exhibited a predominant nuclear localization. No staining was detected in normal B-lymphocytes. All tested cases (n=12) showing constitutive STAT3 phosphorylation secreted significant levels of IL6 (from 200 to 1700 pg/mL). Secretion of IL10 was more variable (from 0 to 450 pg/mL) (*Online Supplementary Figure S10*). Secretion of IL6 and/or IL10 by MCL cells was not always correlated with a detectable phosphorylation of STAT3 (see UPN19), thus suggesting that the expression level of the cytokine receptors could also influence the level of STAT3 phosphorylation. In conclusion, leukemic MCL cells, notably those harboring mutated *IGHV* genes, often exhibit constitutively phosphorylated STAT3 and secrete significant amounts of IL6 and/or IL10, suggesting a role for these cytokine-dependent pathways in extranodal and in peripheral blood MCL cell survival.

B-cell receptor engagement induces STAT3 phosphorylation in primary mantle cell lymphoma cells and is associated with an inhibition of spontaneous apoptosis

Since lymph nodes are the most commonly affected lymphoid structures in MCL, the impact of BCR engage-

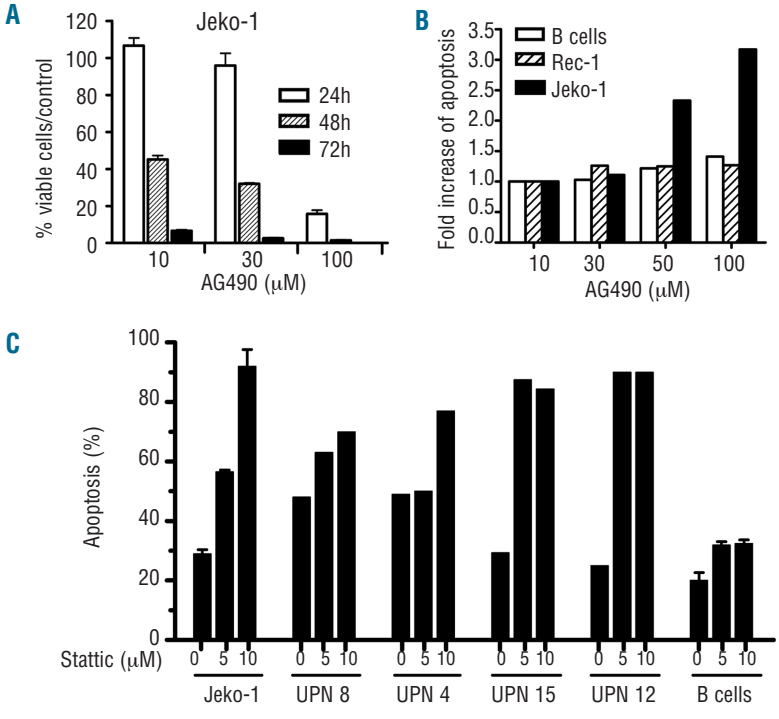

Figure 2. Blockade of STAT3 phosphorylation induces apoptosis of MCL cells. (A) Treatment of Jeko-1 cells with the JAK inhibitor AG490 induced a dose- and time-dependent decrease of cell viability measured by a MTS assay after 24, 48, and 72 h of treatment (n=3). (B) Jeko-1, Rec-1 and normal B cells were incubated for 24 h with increasing concentrations of AG490 and apoptosis was measured by flow cytometry. Fold increase of apoptosis was normalized to apoptosis of untreated cells. The percentage of apoptotic cells corresponded to the percentage of annexin V-positive, including propidium iodide (PI)-negative and PI-positive cells. (C) Jeko-1 cells (n=3), primary MCL cells (UPN) and purified B cells (n=3) were incubated for 24 h with Stattic and apoptosis was measured by flow cytometry.

ment on primary MCL cell survival was next investigated. Mean *in vitro* spontaneous apoptosis in unstimulated cells was 32% (range, 21% to 63%) (Figure 4A). Subsequent stimulation via the BCR induced significant inhibition of apoptosis (mean inhibition=25%, n=20; $P<0.001$). Increased survival after BCR ligation was not statistically different between *IGHV* mutated (mean=31%) and unmutated cases (mean=33%). We next considered the impact of BCR ligation on STAT3 phosphorylation status (Figure 4B). In contrast with the rapid phosphorylation via the IL6/IL10 receptor pathways (Figure 1A), BCR-induced STAT3 phosphorylation was delayed for up to 3 h (*data not shown*). In all cases exhibiting no constitutive STAT3 phosphorylation, BCR ligation further induced it (group A, n=6). In cases displaying medium to low levels of STAT3 constitutive phosphorylation, noticeable BCR-induced phosphorylation was observed (8 out of 10 cases, group B). Finally, in cases exhibiting high levels of constitutive STAT3 phosphorylation (group C, n=4), no additional increase was detected, possibly reflecting some saturation of the phosphorylation signal.

We next investigated whether BCR-induced STAT3 phosphorylation might result from an indirect effect via IL6 and/or IL10 secretion. Blocking the IL6 pathway with an anti-IL6-Rα antibody inhibited both constitutive and BCR-induced STAT3 phosphorylation while blockade of the IL10 pathway had no effect (*Online Supplementary Figure S11*). Accordingly, a 2- to 4.5-fold increase in *IL6* mRNA level was observed following 3 h of BCR ligation (*Online Supplementary Figure S12*). This suggests that the cytokine-dependent pathway is required for BCR-induced phosphorylation of STAT3. In addition, disruption of the JAK/STAT3 signaling pathway using AG490 or Stattic led to the suppression of both BCR-induced STAT3 phosphorylation and BCR-induced survival signals (*Online Supplementary Figure S13*). Altogether, these results indicate that STAT3 is involved in both inherent and BCR-induced survival of MCL cells.

Bortezomib inhibits constitutive activation of STAT3

Bortezomib (PS-341, Velcade) is a proteasome inhibitor currently in use for the treatment of MCL.[18,19] We, therefore, considered the impact of bortezomib on STAT3 phosphorylation and MCL cell survival. *In vitro* treatment of MCL cells with bortezomib (10 nM) induced a variable but significant increase of apoptosis (mean increase in apoptosis=45%, n=14; $P=0.001$) (Figure 5A) but also abolished constitutive phosphorylation of STAT3 (Figure 5B) and decreased IL10 and IL6 secretion to variable extents (n=8, $P<0.01$) (*Online Supplementary Figure S14*). Moreover, exogenous addition of IL10 overcame bortezomib-mediated blockade of STAT3 phosphorylation (*Online Supplementary Figure S15*). These results were compared to those following *in vitro* exposure to fludarabine, another therapeutic agent in MCL known to interfere with the p53-dependent apoptosis pathway.[20] Even exposed to a high concentration of fludarabine (20 μM), MCL cells showed stronger resistance to apoptosis compared to bortezomib (mean increase in apoptosis=19%, n=14, $P=0.002$), and phosphorylation of STAT3 was sustained (Figure 5 A-B). Since bortezomib was previously shown to inhibit constitutive activation of NF-κB in MCL[21] and *IL6* and *IL10* are target genes of NF-κB[22], our results were in line with a possible NF-κB-dependent phosphorylation of STAT3. To assess the specific impact of NF-κB on STAT3 phosphorylation, we used the BAY 11-7082 specific inhibitor of IκBα phosphorylation. BAY 11-7082 inhibited altogether the constitutive STAT3 phosphorylation, IL6 and IL10 secretion and induced cell apoptosis (*Online Supplementary Figure S16*). Altogether, these data indicate that inhibition of STAT3 constitutive phosphorylation by bortezomib may occur through a disruption of the autocrine secretion loop of IL6/IL10. They also suggest a link between NF-κB and STAT3, which are both constitutively activated in MCL cells.

Bortezomib inhibits B-cell receptor-induced survival signals and B-cell receptor-induced STAT3 phosphorylation

Bortezomib-induced *in vitro* apoptosis was further analyzed in the context of BCR stimulation. Bortezomib treatment significantly abrogated the BCR-induced survival response in all cases (n=14, $P<0.001$) (Figure 6A).

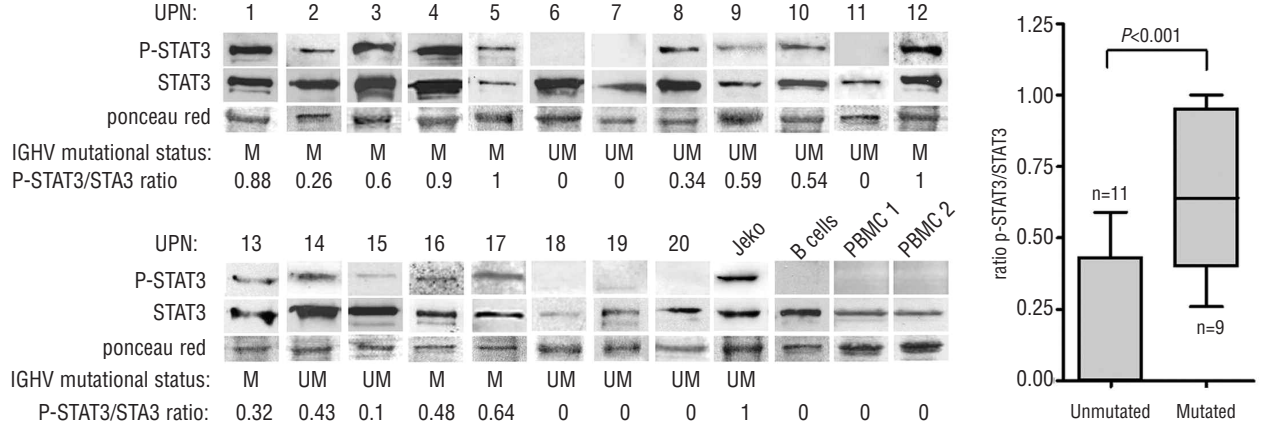

Figure 3. Status of STAT3 phosphorylation in MCL cells from a series of 20 leukemic patients. Total protein extracts from PBMC of either leukemic MCL patients (UPN1-20) or healthy individuals (PBMC1, PBMC2), from purified normal B lymphocytes (B-cells) or from the Jeko-1 cell line were obtained after 24 h of culture. p-STAT3 and total STAT3 expression were analyzed by western blotting (left panel). Mutated (M) and unmutated (UM) *IGHV* status was noted. Densitometric results expressed as pSTAT3/STAT3 ratios are shown for each MCL sample and in a scatter graph associating both *IGHV* mutated and unmutated cases (right panel) [median ± quartile (*box*) ± SE (*bars*)].

Meanwhile, BCR-induced phosphorylation of STAT3 was decreased or abolished after bortezomib treatment whereas it was unchanged upon fludarabine treatment (Figure 6B). BCR-induced STAT3 phosphorylation was also abrogated in the presence of BAY 11-7082, supporting the existence of a link between NF-κB and STAT3 pathways in the context of BCR stimulation (*Online Supplementary Figure S17*).

Discussion

This study was focused on the exploration of pathways, emanating from the microenvironment (antigen, cytokines) and contributing to MCL cell survival and chemoresistance, in particular through STAT3 activation. The study, performed on fresh primary MCL samples, showed that 70% (14/20) of leukemic MCL cases presented constitutive phosphorylation of STAT3 on tyrosine residues and that its selective inhibition induced apoptosis. STAT proteins usually become rapidly activated upon cytokine receptor engagement via associated JAK. In particular, IL6 and IL10 both activate STAT3. In this study, we demonstrated that the constitutive STAT3 phosphorylation resulted from autocrine IL6 and/or IL10 secretion probably dependent on NF-κB constitutive activation. In the other six out of 20 (30%) MCL cases without indication of STAT3 phosphorylation, the absence of activation may have reflected low levels of IL6/IL10 secretion (see UPN7) and/or weak expression of IL6/IL10 receptors thereby resulting in a non-functional cytokine autocrine loop. An autocrine IL6/IL10 loop leading to the activation of STAT3 was previously shown to promote tumor growth in other malignant disease such as multiple myeloma[23] and squamous cell carcinoma of the head and neck.[24] Furthermore, bortezomib-induced apoptosis of MCL cells is associated with a decrease of IL6/IL10 secretion and with inhibition of the constitutive phosphorylation of STAT3. Given that bortezomib is also able to inhibit NF-κB in MCL[21] but does not affect STAT3 expression and DNA binding activity,[25] our results suggest that bortezomib-induced suppression of STAT3 phosphorylation in MCL may occur through disruption of the NF-κB-dependent cytokine autocrine loop. They also suggest that NF-κB and STAT3 pathways may cooperate to play an important role in MCL proliferation, as described in diffuse large B-cell lymphoma.[22]

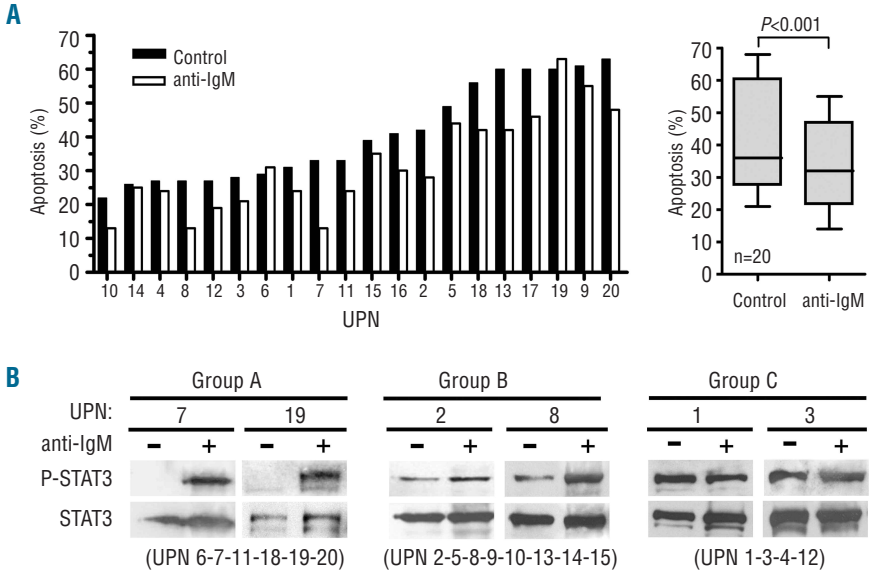

Figure 4. BCR engagement induces inhibition of spontaneous apoptosis and STAT3 phosphorylation in primary MCL cells. (A) Spontaneous apoptosis was measured by flow cytometry of PBMC from 20 leukemic MCL patients. PBMC were purified from blood and cultured for 24 h in the absence (control) or in the presence of 10 μg/mL of immobilized anti-IgM antibody (left panel). BCR-induced apoptosis of the whole sample population (n=20) is also shown as median ± quartile (*box*) ± SE (*bars*) (right panel). (B) STAT3 phosphorylation was analyzed following 24 h of BCR stimulation (anti-IgM). Groups A, B and C were defined depending on constitutive and BCR-induced phosphorylation of STAT3. Corresponding UPN cases are noted.

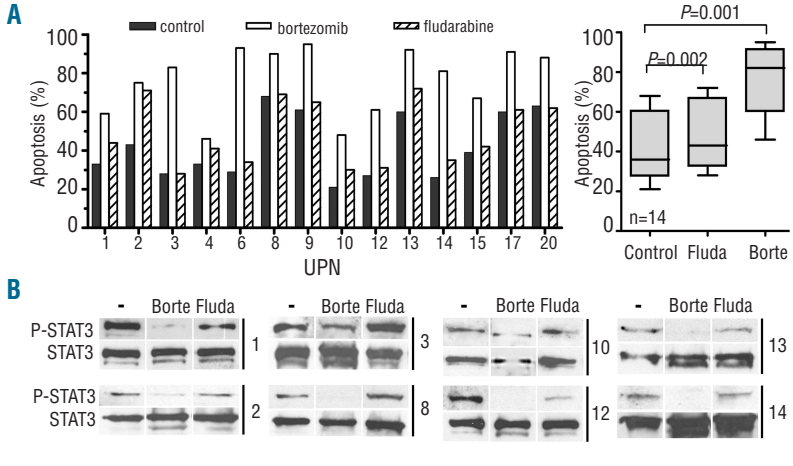

Figure 5. Bortezomib induced-apoptosis is associated with a decrease of constitutive STAT3 phosphorylation. (A) Apoptosis rates were measured by flow cytometry after treatment with bortezomib (10 nM) or fludarabine (20 μM) for 20 h (left panel). Fludarabine- and bortezomib-induced apoptosis are also shown as median ± quartile (*box*) ± SE (*bars*) (n=14) (right panel) (B) STAT3 phosphorylation status after treatment with bortezomib (borte, 10 nM, 20 h) as compared to treatment with fludarabine (Fluda, 20 μM, 20 h).

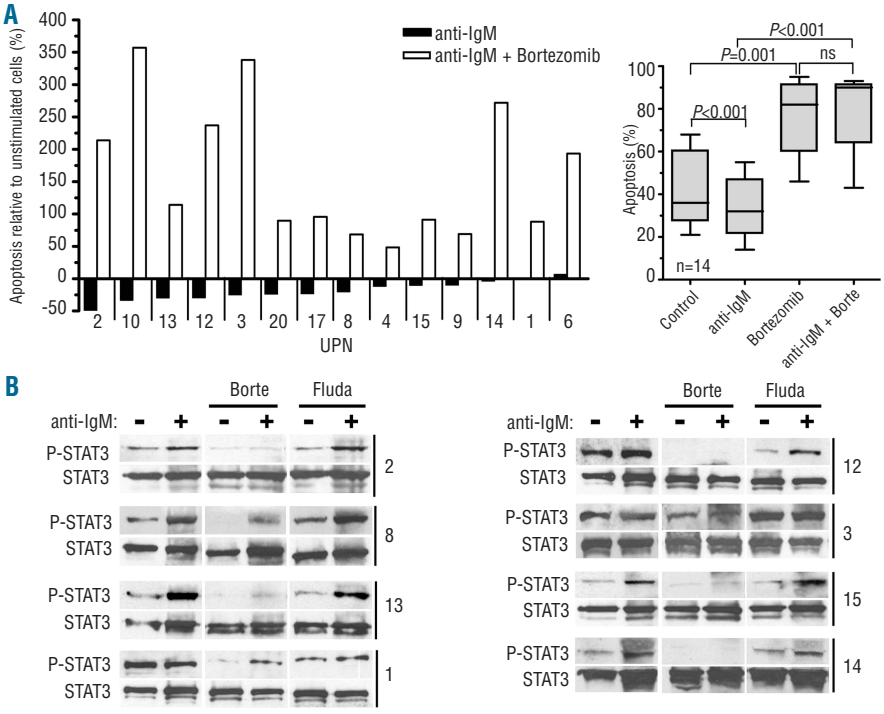

Figure 6. Bortezomib inhibits both BCR-induced survival signal and STAT3 phosphorylation. (A) Primary MCL cells were either stimulated for 20 h with an anti-IgM antibody or left unstimulated, both in the absence or in the presence of bortezomib (10 nM). Apoptosis rates were then measured by flow cytometry. For each condition (± bortezomib), the percentage of apoptotic cells was normalized to that of unstimulated cells and calculated as relative numbers as follows: [(% apoptosis BCR-stimulated cells - % apoptosis BCR-unstimulated cells) / (% apoptosis BCR-unstimulated cells)] x100 (left panel). Apoptosis resulting from BCR-stimulated cells (anti-IgM), bortezomib-treated cells and BCR-stimulated cells treated with bortezomib is also shown as median ± quartile (*box*) ± SE (*bars*) (n=14) (right panel) (B) STAT3 phosphorylation status upon BCR engagement was analyzed in parallel under bortezomib treatment (Borte, 10 nM, 20 h) or fludarabine treatment (Fluda, 20 μM, 20 h).

Some studies identified *IGHV* mutational status as a relevant prognostic factor in MCL,[3,26-28] suggesting an involvement of antigen stimulation in the course of the disease. In these studies, patients with mutated *IGHV* appeared to have a better clinical outcome.[26] In the present study, all the *IGHV* mutated cases were associated with constitutive phosphorylation of STAT3 whereas all cases with basal unphosphorylated STAT3 bore unmutated *IGHV*. Examination of the pattern of *IGHV* rearrangements and mutational status in our series revealed an absence *IGHV3-21* rearrangement and a high proportion of *IGHV* mutated cases (55%) when compared to studies carried out on biopsies.[29] This pattern appears to be characteristic of MCL leukemic presentations and corroborates previous reports indicating that *IGHV* mutated cases are more frequent in non-nodal series than in nodal ones (56% and 10%, respectively) and that *IGHV3-21* rearrangements, associated with unmutated *IGHV*, are mostly described in nodal MCL.[27,30,31] Interestingly, *IGHV3-21* cases as well as some unmutated *IGHV* cases were not included in this functional study due to either a low lymphoma circulating cell count or a major rate of spontaneous apoptosis. Altogether, these results suggest that the constitutive STAT3 phosphorylation observed in MCL cases harboring mutated *IGHV* and with a leukemic phase may confer an intrinsic survival potential to malignant cells.

Given that BCR engagement can promote B-cell proliferation and survival,[32] we investigated its effect in both unmutated and mutated *IGHV* MCL cases. First, we observed that sustained BCR signaling could promote survival in almost all cases independently of *IGHV* mutational status. These results are quite the opposite from those observed in chronic lymphocytic leukemia in which we and others have shown that a strong BCR-induced survival signal was significantly associated with absence of somatic *IGHV* mutations and disease progression.[15] Next, we demonstrated that BCR stimulation induced phosphorylation of STAT3 in 70% of the cases (n=14/20). A link between STAT3 and BCR signaling was initially highlighted in murine B lymphocytes.[10,12,33] $CD5^+$ self-renewing B1 lymphocytes were shown to express phosphorylated STAT3 constitutively, conferring intrinsic resistance to radiation-induced apoptosis. Moreover, BCR engagement in $CD5^-$ B2 cells could induce STAT3 activation leading to proliferation.[34] The results of the present study indicate that the IL6 pathway plays a role in the BCR-induced phosphorylation of STAT3 in MCL. An anti-IL6 receptor blocking antibody abrogated this phosphorylation and BCR stimulation induced a significant increase in the mRNA level of *IL6*. These results are consistent with the effect of cycloheximide on delayed STAT3 tyrosine phosphorylation upon BCR engagement in B2 cells, probably by blocking cytokine-induced pathways.[35] Finally, BCR-induced survival was associated in the present study with enhanced STAT3 phosphorylation. Interestingly, the BCR-induced survival was abolished upon treatment with AG490 or Stattic, indicating that the JAK/STAT3 pathway is involved at least in part in this signal. We found that bortezomib treatment could block both the BCR-induced survival signal and BCR-induced STAT3 phosphorylation in contrast to what was observed in the presence of fludarabine which is active through the activation of the p53 pathway. Moreover, we also demonstrated that constitutive phosphorylation of STAT3 is abrogated by bortezomib through disruption of the cytokine autocrine loop. These new mechanisms of action of bortezomib targeting STAT3 could thus contribute to its anti-neoplastic effects on MCL tumor cells.

In conclusion, we report here that in primary MCL cells, two STAT3 activating processes, mediated through components of the tumor environment, contribute to increased cell

survival. STAT3 was found to be constitutively activated through a cytokine-dependent autocrine loop and/or induced upon BCR engagement. Bortezomib appeared to target both constitutive and BCR-induced STAT3 activation in MCL, thus uncovering a new mechanism of action. Consequently, development of STAT3 inhibitors could provide new useful therapeutic agents for the treatment of this chemotherapy-resistant lymphoma.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Zelenetz AD. Mantle cell lymphoma: an update on management. Ann Oncol. 2006;17 (Suppl 4):iv12-4.
2. Fisher RI, Bernstein SH, Kahl BS, Djulbegovic B, Robertson MJ, de Vos S, et al. Multicenter phase II study of bortezomib in patients with relapsed or refractory mantle cell lymphoma. J Clin Oncol. 2006;24(30):4867-74.
3. Camacho FI, Algara P, Rodriguez A, Ruiz-Ballesteros E, Mollejo M, Martinez N, et al. Molecular heterogeneity in MCL defined by the use of specific VH genes and the frequency of somatic mutations. Blood. 2003;101(10):4042-6.
4. Martinez N, Camacho FI, Algara P, Rodriguez A, Dopazo A, Ruiz-Ballesteros E, et al. The molecular signature of mantle cell lymphoma reveals multiple signals favoring cell survival. Cancer Res. 2003; 63(23):8226-32.
5. Lai R, Rassidakis GZ, Medeiros LJ, Leventaki V, Keating M, McDonnell TJ. Expression of STAT3 and its phosphorylated forms in mantle cell lymphoma cell lines and tumours. J Pathol. 2003;199(1):84-9.
6. Yu H, Jove R. The STATs of cancer – new molecular targets come of age. Nature reviews 2004;4(2):97-105.
7. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer. 2009;9(11): 798-809.
8. Siddiquee K, Zhang S, Guida WC, Blaskovich MA, Greedy B, Lawrence HR, et al. Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. Proc Natl Acad Sci USA. 2007;104(18): 7391-6.
9. Visser HP, Tewis M, Willemze R, Kluin-Nelemans JC. Mantle cell lymphoma proliferates upon IL-10 in the CD40 system. Leukemia. 2000;14(8):1483-9.
10. Karras JG, Wang Z, Huo L, Howard RG, Frank DA, Rothstein TL. Signal transducer and activator of transcription-3 (STAT3) is constitutively activated in normal, self-renewing B-1 cells but only inducibly expressed in conventional B lymphocytes. J Exp Med. 1997;185(6):1035-42.
11. Fan H, Rothstein TL. Lymphokine dependence of STAT3 activation produced by surface immunoglobulin cross-linking and by phorbol ester plus calcium ionophore treatment in B cells. Eur J Immunol. 2001;31(2): 665-71.
12. Wang L, Kurosaki T, Corey SJ. Engagement of the B-cell antigen receptor activates STAT through Lyn in a Jak-independent pathway. Oncogene. 2007;26(20):2851-9.
13. Gary-Gouy H, Harriague J, Bismuth G, Platzer C, Schmitt C, Dalloul AH. Human CD5 promotes B-cell survival through stimulation of autocrine IL-10 production. Blood. 2002;100(13):4537-43.
14. Casola S, Otipoby KL, Alimzhanov M, Humme S, Uyttersprot N, Kutok JL, et al. B cell receptor signal strength determines B cell fate. Nat Immunol. 2004;5(3):317-27.
15. Deglesne PA, Chevallier N, Letestu R, Baran-Marszak F, Beitar T, Salanoubat C, et al. Survival response to B-cell receptor ligation is restricted to progressive chronic lymphocytic leukemia cells irrespective of Zap70 expression. Cancer Res. 2006;66(14): 7158-66.
16. Belessi CJ, Davi FB, Stamatopoulos KE, Degano M, Andreou TM, Moreno C, et al. IGHV gene insertions and deletions in chronic lymphocytic leukemia: "CLL-biased" deletions in a subset of cases with stereotyped receptors. Eur J Immunol. 2006;36(7):1963-74.
17. Schust J, Sperl B, Hollis A, Mayer TU, Berg T. Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. Chem Biol. 2006;13(11):1235-42.
18. Belch A, Kouroukis CT, Crump M, Sehn L, Gascoyne RD, Klasa R, et al. A phase II study of bortezomib in mantle cell lymphoma: the National Cancer Institute of Canada Clinical Trials Group trial IND.150. Ann Oncol 2007;18(1):116-21.
19. Gressin R, Caulet Maugendre S, Le Gouill S, Ojeda Uribe M, Alexis Vigier M, Bouabdallah K, et al. Interim results of the RiPAD+C regimen including velcade in front line therapy for elderly patients with mantle cell lymphoma. A phase II prospective study of the GOELAMS group. Blood. (ASH Annual Meeting Abstracts) 2008;112:1575.
20. Goy A. New directions in the treatment of mantle cell lymphoma: an overview. Clin Lymphoma Myeloma. 2006;7 (Suppl 1): S24-32.
21. Pham LV, Tamayo AT, Yoshimura LC, Lo P, Ford RJ. Inhibition of constitutive NF-kappa B activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis. J Immunol. 2003;171(1):88-95.
22. Lam LT, Wright G, Davis RE, Lenz G, Farinha P, Dang L, et al. Cooperative signaling through the signal transducer and activator of transcription 3 and nuclear factor-κB pathways in subtypes of diffuse large B-cell lymphoma. Blood. 2008;111(7):3701-13.
23. Vega MI, Huerta-Yepaz S, Garban H, Jazirehi A, Emmanouilides C, Bonavida B. Rituximab inhibits p38 MAPK activity in 2F7 B NHL and decreases IL-10 transcription: pivotal role of p38 MAPK in drug resistance. Oncogene. 2004;23(20):3530-40.
24. Squarize CH, Castilho RM, Sriuranpong V, Pinto DS Jr, Gutkind JS. Molecular cross-talk between the NFkappaB and STAT3 signaling pathways in head and neck squamous cell carcinoma. Neoplasia. 2006;8(9): 733-46.
25. Dai Y, Rahmani M, Pei XY, Dent P, Grant S. Bortezomib and flavopiridol interact synergistically to induce apoptosis in chronic myeloid leukemia cells resistant to imatinib mesylate through both Bcr/Abl-dependent and -independent mechanisms. Blood. 2004;104(2):509-18.
26. Lai R, Lefresne SV, Franko B, Hui D, Mirza I, Mansoor A, et al. Immunoglobulin VH somatic hypermutation in mantle cell lymphoma: mutated genotype correlates with better clinical outcome. Mod Pathol. 2006;19(11):1498-505.
27. Kienle D, Krober A, Katzenberger T, Ott G, Leupolt E, Barth TF, et al. VH mutation status and VDJ rearrangement structure in mantle cell lymphoma: correlation with genomic aberrations, clinical characteristics, and outcome. Blood. 2003;102(8): 3003-9.
28. Letestu R, Ugo V, Valensi F, Radford-Weiss I, Nataf J, Levy V, et al. Prognostic impact of p27KIP1 expression in cyclin D1 positive lymphoproliferative disorders. Leukemia. 2004;18(5):953-61.
29. Thorselius M, Walsh S, Eriksson I, Thunberg U, Johnson A, Backlin C, et al. Somatic hypermutation and V(H) gene usage in mantle cell lymphoma. Eur J Haematol. 2002;68(4):217-24.
30. Walsh SH, Thorselius M, Johnson A, Soderberg O, Jerkeman M, Bjorck E, et al. Mutated VH genes and preferential VH3-21 use define new subsets of mantle cell lymphoma. Blood. 2003;101(10):4047-54.
31. Orchard J, Garand R, Davis Z, Babbage G, Sahota S, Matutes E, et al. A subset of t(11;14) lymphoma with mantle cell features displays mutated IgVH genes and includes patients with good prognosis, nonnodal disease. Blood. 2003;101(12): 4975-81.
32. Mizuno T, Rothstein TL. B cell receptor (BCR) cross-talk: CD40 engagement creates an alternate pathway for BCR signaling that activates I kappa B kinase/I kappa B alpha/NF-kappa B without the need for PI3K and phospholipase C gamma. J Immunol. 2005;174(10):6062-70.
33. Su L, Rickert RC, David M. Rapid STAT phosphorylation via the B cell receptor. Modulatory role of CD19. J Biol Chem. 1999;274(45):31770-4.
34. Otero DC, Poli V, David M, Rickert RC. Cutting edge: inherent and acquired resistance to radiation-induced apoptosis in B cells: a pivotal role for STAT3. J Immunol. 2006;177(10):6593-7.
35. Karras JG, Huo L, Wang Z, Frank DA, Zimmet JM, Rothstein TL. Delayed tyrosine phosphorylation and nuclear expression of STAT1 following antigen receptor stimulation of B lymphocytes. J Immunol. 1996;157(6):2299-309.