A Novel Aziridine-based Bruton's Tyrosine Kinase Inhibitor Induces Apoptosis Through Down-regulation of p65/RelA Phosphorylation on Serine 536 and ERK1/2 in Mantle Cell Lymphoma

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Abstract. *Background:* Mantle cell lymphoma (MCL) is an aggressive non-Hodgkin's lymphoma characterized by hyperactive neoplastic B-cells and extended tumor cell survival. Bruton's tyrosine kinase (BTK), a crucial kinase in the B-cell antigen receptor signaling pathway, has emerged as a novel target of MCL therapy. A novel BTK-targeting inhibitor, JuSt-23F was prepared. *Materials and Methods:* The WST-8 assay was used to determine cytotoxicity and half-maximal inhibitory concentration (IC₅₀) values for JuSt-23F against the MCL cell lines Mino and Maver-1. JuSt-23F-mediated apoptosis was assessed using the annexin V assay. We detected phosphorylation of p65/RelA on serine 536 in whole Jurkat, Mino and Maver-1 cells treated with JuSt-23F and stimulated with tumor necrosis factor (TNF α). We assessed JuSt-23F-mediated phosphorylation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) in T-cell lymphoma and MCL cells stimulated by phorbol-12-myristate-13-acetate (PMA). *Results and Conclusion:* Our study suggests that JuSt-23F inhibits apoptosis selectively in B-cell lymphoma cells. JuSt-23F exerts its antiproliferative effects on MCL cells through targeting the downstream BTK signaling cascade via down-regulation of nuclear factor kappa-light-chain-enhancer of activated B-cells and ERK1/2 pathways. Thus, our findings propose the novel BTK inhibitor JuSt-23F as an attractive potential agent for investigation and treatment of MCL.

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Mantle cell lymphoma (MCL), a rare and aggressive form of B-cell non-Hodgkin's lymphoma, is usually diagnosed when it has already spread to the lymph nodes, bone marrow and other organs. Development of targeted cancer therapies, such as Bruton's tyrosine kinase (BTK) in the B-cell antigen receptor (BCR) pathway, allowed significant advances in the treatment of MCL. It was shown that inhibition of BTK induces apoptosis and reduces levels of the anti-apoptotic proteins, such as BCL2 and BCL-xL, which sensitize B-cell lymphoma to chemotherapy (1-4). Biochemical studies have shown that phospholipase C- γ 2 plays an important role in B-cell signaling (5). Cell line experiments revealed that BCR cross-linking activates three distinct families of non receptor protein tyrosine kinases, SRC family kinases, SYK and BTK, and initiated downstream events. BCR cross-linking in B-cells activated distinct mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), p38 MAPK, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and as a result, affected regulation of apoptosis (6-9). Different kinases of BCR signaling, including SYK, LYN, and BTK, have been found to be phosphorylated in primary MCL, suggesting activation of this pathway (10). Further studies with the knockdown of BTK in MCL cells revealed significant inhibition of the NF- κ B pathway, which is most important for MCL proliferation and cell migration (11-13). Rudelius and colleagues demonstrated constitutive activation of the PI3K/AKT signaling pathway in a subset of aggressive blastoid MCL variants. This signaling resulted in the activation of a number of well-known target genes and pathways, including the NF- κ B pathway, enhancing survival and cell proliferation, and inhibiting apoptosis (14).

There is evidence suggesting that NF- κ B is required for efficient transcription of the *BTK* gene (15). NF- κ B is activated by a wide variety of stimuli, including inflammatory cytokines, such as tumor necrosis factor alpha (TNF α), and

is critical for apoptotic responses in cancer progression (16, 17). NF- κ B, which is a family of five transcriptional factors, including p50, p52, p65 (RelA), RelB and c-Rel, mediates their dimerization, nuclear localization and DNA binding (18). The association of the NF- κ B p65/p50 dimer plays a pivotal role in regulating its nuclear translocation and gene transcription. Sakurai *et al.* demonstrated that TNF α induces the phosphorylation by IKK of p65 on serine 536 *in vitro* kinase assay and experiments *in vivo* (19). Furthermore, some groups investigating the regulatory mechanisms for p65 phosphorylation found that serine 536 phosphorylation was widely observed in accordance with the phosphorylation of I κ B α (20-23).

Study of the pathogenesis of MCL revealed that the development of MCL was associated with the dysregulation of different signaling pathways involved in cell proliferation and survival, including those related to transcription factor NF- κ B, AKT and ERK1/2 (24). Specific blockade of ERK1/2 pathway potentiates chemotherapy-induced apoptosis in lymphoma cells (25). It was shown that the compound 8-NH2-Ado, which was efficacious in preclinical models of MCL, dramatically reduced phosphorylation of kinases in the AKT and ERK1/2 pathways (26). Enhanced apoptosis in MCL cell lines and primary MCL cells was associated with JNK1/2 activation, and ERK1/2 and AKT1/2 inactivation (26, 27). Antitumor activity in animal models and, recently, significant clinical activity, was observed with small molecule inhibitors of protein kinases in the BCR pathway. High response rates were reported in patients with MCL (28, 29).

Since BTK has been shown to play an important role in a variety of cellular processes, including cell-cycle regulation and apoptosis, the BTK signaling pathway may represent an attractive approach for therapy of MCL. In this study, we investigated a novel small molecule BTK inhibitor JuSt-23F.

Materials and Methods

Synthesis of JuSt-23F and preparation of solutions. JuSt-23F was synthesized under the leadership of Dr. P. Trapencieris at the Latvian Institute of Organic Synthesis.

Stock solution was prepared by dissolving JuSt-23F in at 10 mM concentration. Depending on the experimental conditions, the stock solution of JuSt-23F was diluted with appropriate solvent.

Cell lines and cultivation. The following cell lines were obtained from the American Type Cell Culture Collection (ATCC): MCL cells Mino- (ATCC, CRL-3000) and Maver-1 (ATCC, CRL-3000); Jurkat, acute T-cell leukemia, T lymphocyte (ATCC, TIB-152). All cell lines were routinely maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum, and were grown in culture in incubator at 37°C with 5% CO₂ in a humidified atmosphere.

Assay of BTK activity *in vitro*. The luminescent kinase assay ADP-Glo™ (Promega, Madison, WI, USA) was used for the screening of

BTK inhibitors. The assay detected the effects of novel chemical compounds on the catalytic activity of purified recombinant BTK. In the first step, ADP formed from a BTK reaction was converted into ATP which was further converted into light by Ultra-Glo™ Luciferase. The luminescent signal was measured and positively correlated with BTK activity. The assay was performed in white 96-well plate. Briefly, in the first step of the BTK kinase reaction: buffer (40 mM Tris pH 7.5, 20 mM MgCl₂, and 0.1 mg/ml BSA) was mixed with 10 ng recombinant kinase, 1 μ g peptide substrate Poly(Glu₄Tyr₁), 50 μ M ATP, and the tested compound, or without it as control. After incubation for 30 min, an equal volume of ADP-Glo™ reagent was added to terminate the kinase reaction and deplete the remaining ATP. In the second step, the addition of double volume of the Kinase Detection reagent for 30 min resulted in simultaneous conversion of ADP to ATP. We then measured luminescence (RLU) on an Infinite M100 microplate reader (Tecan Group Ltd, Mannedorf, Switzerland) with integration time 750 ms.

Calculation percentage of BTK activity and IC₅₀. The percentage of inhibition of BTK activity was calculated as: (RLU_{sample}/RLU_{control}) $\times$ 100. The IC₅₀ values were quantified using the percentage data and GraphPad Prism 5.03 software (GraphPad Software Inc., San Diego, CA, USA). All data are given as the mean of three independent experiments.

Apoptosis assay. Apoptosis detection was performed using the annexin V-FITC/propidium iodide fluorescent staining assay. Cells were plated at a density of 5 $\times$ 10⁵ cells/ml in 100 μ l per well of 96-well plates. Apoptosis was induced by treating Jurkat, Mino and Maver-1 cells with JuSt-23F at 12.5-100 μ M. All samples were set up in triplicate. After 24 h of incubation, the cells were harvested, measured on a BD FACSAria II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with Flowing Software (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland). Furthermore, the percentage of early plus late apoptotic untreated and JuSt-23F-treated cells was calculated. Percentage of apoptosis induction was calculated as the difference of apoptosis of JuSt-23F-treated cells and untreated cells for each JuSt-23F concentration.

WST-8 cell proliferation assay. To evaluate the effect of JuSt-23F on Jurkat, Mino and Maver-1 cell viability, we performed WST-8 assays (Cayman Chemical, Michigan, USA) according to the manufacturer's protocol. This proliferation assay was based on extracellular reduction of tetrazolium dye and formation of soluble formazan which positively correlates with cell viability (30). In brief, 5 $\times$ 10⁴ cells/well were seeded in a 96-well plate in 100 μ l of growth medium with JuSt-23F at 3.1-100 μ M or without it as control. Each concentration of compound was used in triplicate wells with cells and one well as medium control. Plates were incubated in at 37°C with 5% CO₂. After 24 h, 10 μ l of WST-8 reagent was added to each well and incubation was continued for another 4 h. Soluble formazan was measured in cultivation medium at 450 nm using an Anthos HTII plate reader (Anthos Labtec instruments, Salzburg, Austria). The cytotoxicity and IC₅₀ value were quantified using the absorbance data and GraphPad Prism 5.03 software (GraphPad Software Inc.). All data are given as the mean of three independent experiments

NF- κ B phosphorylation assay. Cell-based ELISA (BioAssay Systems, Hayward, CA, USA) was used to measure the phosphorylation of

p65/RelA on serine 536 (pNF- κ B) in whole Jurkat, Mino and Maver-1 cells according to the manufacturer's protocol. For this assay, cells were plated at a density of 5×10^5 cells/ml into 96-well black plates and treated with different concentrations of JuSt-23F for 4 h and then induced by TNF α (5 ng/ml) for 15 min. Cells were then fixed and permeabilized in the wells. Phosphorylated and total NF- κ B was measured using a double immunoenzymatic labeling procedure. pNF- κ B fluorescence (FL) was read at Ex/Em of 535/585 nm and NF- κ B at Ex/Em of 360/450 nm. The quantity of pNF- κ B was normalized as: $(\Delta FL_{pNF-\kappa B} / \Delta FL_{NF-\kappa B}) / (\Delta FL_{pNF-\kappa B} / \Delta FL_{NF-\kappa B})_0$, where $(\Delta FL_{pNF-\kappa B} / \Delta FL_{NF-\kappa B})_0$ was the control reference value (untreated wells).

ERK1/2 phosphorylation assay. Cell-based ELISA (BioAssay Systems) measured dually phosphorylated ERK1/2 in mitogen-stimulated whole Jurkat, Mino and Maver-1 cells as described elsewhere (31). In brief, cells were seeded at a density of 5×10^5 cells/ml in 96-well black plates in 100 μ l, treated with JuSt-23F for 4 h and then induced by 50 nM phorbol-12-myristate-13-acetate (PMA) for 30 min. Cells were then fixed and permeabilized in the wells. Phosphorylated and total ERK was measured using a double immunoenzymatic labeling procedure. The fluorescence was read at Ex/Em of 535/590 nm for pERK and at Ex/Em of 360/450 nm for ERK detection. The quantity of pERK was normalized as: $(\Delta FL_{pERK} / \Delta FL_{ERK}) / (\Delta FL_{pERK} / \Delta FL_{ERK})_0$, where $(\Delta FL_{pERK} / \Delta FL_{ERK})_0$ was the control reference value (untreated wells).

Statistical analysis. Student's *t*-test was used to analyze the difference between control and JuSt-23F-treated samples. Statistical significance was set at $p < 0.05$. All data are given as the mean of three independent experiments.

Results

JuSt-23F inhibits BTK catalytic activity *in vitro*. Firstly, we inhibited BTK catalytic activity *in vitro* using luminescent kinase assay ADP-Glo™ and estimated IC₅₀ values. Our experiments revealed that compound JuSt-23F, which belongs to the class of bis-2-arylaziridines, inhibited BTK enzymatic activity in a dose-dependent manner, with an IC₅₀ value of 37.3 μ M. Thus, our findings show that JuSt-23F is a potential BTK inhibitor.

JuSt-23F cytotoxicity towards MCL cells. A previous report demonstrated that the expression of BTK protein was detected in B-cells but not in T-cells (32). It was also shown that inhibition of BTK activity resulted in inhibition of cell proliferation and cell survival in various B-cell malignancies (32). We evaluated the cytotoxicity of JuSt-23F at concentrations ranging from 3.1 to 100 μ M on MCL cell lines Mino and Maver-1 and T-cell lymphoma Jurkat. The IC₅₀ values for Mino and Maver-1 cells were 32 μ M and 37 μ M, respectively, while that for Jurkat cells was 81 μ M. Thus, we have shown that a 24 h treatment with JuSt-23F revealed a modest inhibitory effect on proliferation of MCL cells. JuSt-23F had no significant influence on T-cell survival, but shows antiproliferative effects against B-cell lymphoma cell lines Mino and Maver-1.

JuSt-23F selectively induces apoptosis of B-cell lymphoma cells. We further investigated whether JuSt-23F selectively inhibited B-cell growth by inducing cell death. We assayed for apoptosis in B-cell lymphoma cell lines Mino, Maver-1 and in Jurkat T-cells treated with JuSt-23F at 12.5-100 μ M for 24 h. Analysis of apoptosis by flow cytometry demonstrated that T-cells did not significantly undergo apoptosis (Figure 1), being about 4% in both treated and untreated JuSt-23F cells. However, JuSt-23F treatment revealed extensive apoptosis of Mino and Maver-1 cells in a dose-dependent manner in comparison with untreated cells (Figure 1). JuSt-23F induced about 30% apoptosis of Mino cells and 40% of Maver-1 cells, but not of Jurkat cells (Figure 1B). JuSt-23F selectively induces apoptosis of B-cell lymphoma cells under our experimental conditions.

JuSt-23F down-regulates p65/RelA phosphorylation on serine 536 in MCL cells. NF- κ B is a transcription factor that plays a central role in many physiological processes, *e.g.* inflammation, tumorigenesis and apoptosis. NF- κ B is activated by a wide variety of stimuli, including inflammatory cytokines such as TNF α . A number of reports have demonstrated that NF- κ B activation can maintain lymphoma cell viability (33-35). Inhibition of NF- κ B resulted in lymphoma cells apoptosis *in vitro* and in clinical studies (11, 36). We assessed whether JuSt-23F mediates apoptosis through regulation of the NF- κ B pathway. We found that JuSt-23F did not affect NF- κ B phosphorylation in Jurkat cells (Figure 2A). However, we detected 1.4-fold inhibition of NF- κ B phosphorylation in Mino cells and 2.8-fold inhibition in Maver-1 cells treated with 20 μ M JuSt-23F (Figure 2B and C). TNF α -induced down-regulation of pNF- κ B in Mino and Maver-1 cells was also determined in a dose-dependent manner ($p < 0.001$; Figure 2B and C). We detected 1.9-fold inhibition of pNF- κ B in Mino cells and 6.7-fold in Maver-1 cells induced with TNF α and treated with JuSt-23F (Figure 2B and C). Thus, we assume that JuSt-23F induction of apoptosis is regulated through down-regulation of p65/RelA phosphorylation of serine 536 in MCL cells.

JuSt-23F reduces ERK 1/2 phosphorylation in MCL cells. It was recently shown that inhibition of BTK effectively abrogates downstream survival pathways activated by BTK, including ERK1/2, in B-cell lymphoma (37). Stimulation by mitogens, such as PMA, eventually leads to phosphorylation of ERK1/2 in T- and B-lymphocytes. Inhibition of MAPK pathways prevented proliferation and transcriptional regulation of genes in B-lymphocytes (37). T-Cell lymphocytes treated with 0.2-20 μ M JuSt-23F did not reduce ERK1/2 phosphorylation in PMA-induced cells (Figure 3A). However, JuSt-23F treatment and PMA stimulation resulted in inhibition of ERK 1/2 phosphorylation 1.1- to 1.8-fold in Mino cells and 1.3- to 2.1-fold in Maver-1 cells in a dose-

Figure 1. JuSt-23F-mediated apoptosis after 24 h in cell lines Jurkat, Mino and Maver-1 stained with annexin V/propidium iodide. A: Control, untreated cells (top panel); JuSt-23F-treated 25 μ M (bottom panel). The percentage of early apoptotic cells are shown in the bottom square and of late apoptotic cells in the top square. B: Kinetics of apoptosis induction (%) in Jurkat, Mino and Maver-1 cells according to JuSt-23F concentration. Statistical significance was set at $p < 0.01$. Data were prepared from the results of flow cytometric analysis.

dependent manner ($p < 0.001$; Figure 3B and C). Thus, the BTK-activated ERK1/2 survival pathway is down-regulated in JuSt-23F-treated MCL cells Mino and Maver-1.

Discussion

We described the efficacy of JuSt-23F, a novel small molecule aziridine-based BTK inhibitor, in inhibition of

MCL cell proliferation and survival. Our results demonstrated that JuSt-23F has BTK inhibitory activity with IC_{50} value of 37.2 μ M *in vitro*. Moreover, our novel BTK inhibitor is selectively cytotoxic against B-cells compared to T-cells. Genetic studies of many BCR signaling pathways have highlighted complex redundancies as well as pleiotropic effects on cell types other than B-cells (38, 39). Nevertheless, it was found that BTK was primarily expressed

Figure 2. JuSt-23F-mediated phosphorylation of NF-κB in Jurkat T-cell lymphoma (A) and mantle cell lymphoma Mino (B) and Maver-1 (C) cell lines. Normalized phosphorylation of p65/RelA on serine 536 (pNF-κB) in cells is shown. Cells were untreated; treated with 20 μM JuSt-23F; stimulated with TNFα (5 ng/ml); or treated with TNFα (5 ng/ml) plus 2 μM or 20 μM of JuSt-23F. Statistical significance was set at $p < 0.05$. Data represent the mean ± standard deviation of triplicate experiments.

in B-cells, but not in T-cells or plasma cells (40). Thus, BTK represents a uniquely attractive kinase target for selective inhibition of B-cell survival.

Small-molecule inhibitors of BTK have shown anticancer activities *in vitro* and in animal models. As a result, BTK inhibitors have become an area of substantial clinical interest. Some years ago LFM-A13 was described as a BTK inhibitor with an IC_{50} of 17 μM *in vitro* (41), and has been widely used in preclinical studies as a probe for BTK involvement in various cellular functions. The proteasome inhibitor bortezomib (Velcade, 2006) and lenalidomide (Revlimid, 2013) which target ubiquitin E3 ligase cereblon were approved for the treatment of MCL. Ibrutinib was the third

Figure 3. JuSt-23F-mediated phosphorylation of ERK1/2 in Jurkat T-cell lymphoma (A) and mantle cell lymphoma Mino (B) and Maver-1 (C) cell lines. Normalized ERK1/2 phosphorylation (pERK) is shown. Cells were untreated; treated with 20 μM JuSt-23F, stimulated with 50 nM phorbol-12-myristate-13-acetate (PMA), or treated with 50 nM PMA plus 0.2, 2 or 20 μM JuSt-23F. Statistical significance was set at $p < 0.05$. Data represent the mean ± standard deviation of triplicate experiments.

drug approved by the US Food and Drug Administration in 2013 for the treatment of MCL. Ibrutinib was shown to completely and irreversibly inhibit B-cell activation and block signaling pathways downstream of BTK (42-44).

Our findings revealed that BTK inhibitor JuSt-23F selectively induces apoptosis of MCL cells, but not of Jurkat T-cell lymphoma cells, and targets two pathways of activated BCR, NF-κB and ERK1/2. Many findings support BTK inhibition as a therapeutic approach for the treatment of human diseases, associated with activation of the BCR

pathway. Since the NF- κ B pathway is essential for B-cell development, proliferation and survival, targeting the NF- κ B pathway is an attractive approach for MCL therapy (45). NF- κ B is now widely recognized as a key positive regulator of cancer cell proliferation and survival *via* its ability to transcriptionally activate many pro-survival and anti-apoptotic genes such as *XIAP*, *BCL2*, *BCL-XL*, *I κ B α* , *cIAP1*, *cIAP2* and survivin (46). Serine phosphorylation at various sites of the p65 subunit of NF- κ B has been shown to be important in initiating transcription (47). It was shown that transduction of canonical NF- κ B signaling pathway *via* phosphorylation and degradation of I κ B α , IKKs (especially IKK β), also phosphorylates p65/RelA at the serine 536 site within the trans-activating domain. Some research groups have recently demonstrated that p65 phosphorylation on serine 536 stimulated the translocation of p65 into the nucleus *via* the IKK signaling cascade, independently of effects on I κ B α (47, 48).

Recently, phosphorylated serine 536 on p65 was detected in the cytoplasm of four MCL tested cell lines, suggesting that a noncanonical NF- κ B pathway may be activated in mantle cell lymphoma. Furthermore, the data suggest that at least some genes transcribed selectively by an NF- κ B complex containing a form of p65 phosphorylated on serine 536 were not modulated by proteasome inhibitors (42, 47). We demonstrated that JuSt-23F did not modulate proteasome activity in MCL cells Mino and Maver-1 (data not shown).

Targeting NF- κ B in the clinic has thus far been less successful. Activation of BTK triggers a cascade of signaling events that culminates in transcriptional regulation involving NF- κ B. The NF- κ B survival pathway also has the ability to cross-talk with other survival pathways including PI3K/AKT in various cancer types (50, 51). Therefore, targeting the NF- κ B pathway alone may not be sufficient to induce apoptosis of malignant cells and combinations of various inhibitors may be required to achieve the desired effect. Few studies have reported on the role of NF- κ B in MCL. Apoptotic processes could be engaged using a pharmacological inhibitor of NF- κ B in MCL. Interestingly, Ibrutinib demonstrated a remarkable response rate in patients with relapsed/refractory MCL (43). However, approximately one-third of patients have primary resistance to the drug while other patients appear to lose response and develop secondary resistance. The ERK pathway, which is critical to the pathogenesis, proliferation and survival of MCL cells, is actually the focus of interest for targeted therapy. Recent studies of Ma *et al.* demonstrated that inhibition of ERK1/2 and AKT phosphorylation correlates well with cellular response to BTK inhibition in cell lines and primary tumors treated with Ibrutinib (44). Bernard *et al.* also showed BCR-dependent activation in MCL of several important effectors such as SYK, BTK, ERK and STAT3. Moreover, inhibition of the proximal BCR signals

results in a complete inhibition of downstream effectors such as ERK or STAT3 and induction of MCL cell apoptosis (52). Reduced migration of MCL Jeko-1 cells, as well as enhanced sensitivity to a chemotherapeutic agent, was associated with decreased phosphorylation of ERK1/2 and AKT kinases (53). Our study suggests JuSt-23F abrogates at least two downstream BTK signaling pathways, ERK1/2 and NF- κ B, involved in B-cell lymphoma survival, resulting in induction of MCL cell apoptosis. Thus, our findings coincide with the known data. JuSt-23F might be assessed by combination with the other targeted inhibitors to prevent primary resistance or overcome secondary resistance, as well as improvement of therapy efficacy. We believe that this small-molecule aziridine-based inhibitor is a promising agent for treatment and study of such B-cell malignancy as MCL.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

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References

- Cinar M, Hamedani F, Mo Z, Cinar B, Amin H and Alkan S: Bruton tyrosine kinase is commonly overexpressed in mantle cell lymphoma and its attenuation by ibrutinib induces apoptosis. *Leuk Res* 37: 1271-1277, 2013.
- Smith MR: Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance. *Oncogene* 22: 7359-7368, 2003.
- Bonavida B: Rituximab-induced inhibition of antiapoptotic cell survival pathways: implications in chemo/immunoresistance, rituximab unresponsiveness, prognostic and novel therapeutic interventions. *Oncogene* 26(25): 3629-3636, 2007.
- Pickering BM, de Mel S, Lee M, Howell M, Habens F, Dallman CL, Neville LA, Potter KN, Mann J, Mann DA, Johnson PW, Stevenson FK and Packham G: Pharmacological inhibitors of NF-kappaB accelerate apoptosis in chronic lymphocytic leukaemia cells. *Oncogene* 26: 1166-1177, 2007.
- Kim YJ, Sekiya F, Poulin B, Bae YS and Rhee SG: Mechanism of B-cell receptor-induced phosphorylation and activation of phospholipase C- γ 2. *Mol Cell Biol* 24(22): 9986-9999, 2004.
- Fluckiger AC, Li Z, Kato RM, Wahl MI, Ochs HD, Longnecker R, Kinet JP, Witte ON, Scharenberg AM and Rawlings DJ: Btk/Tec kinases regulate sustained increases in intracellular Ca²⁺ following B-cell receptor activation. *EMBO J* 17(7): 1973-1985, 1998.
- Jiang A, Craxton A, Kurosaki T and Clark EA: Different protein tyrosine kinases are required for B cell antigen receptor-mediated activation of extracellular signal-regulated kinase, c-Jun NH₂-terminal kinase 1, and p38 mitogen-activated protein kinase. *J Exp Med* 188(7): 1297-1306, 1998.

- 8 Anderson JS, Teutsch M, Dong Z and Wortis HH: An essential role for Bruton's tyrosine kinase in the regulation of B-cell apoptosis. *Proc Natl Acad Sci USA* 93(20): 10966-10971, 1996.
- 9 Bajpai UD, Zhang K, Teutsch M, Sen R and Wortis HH: Bruton's tyrosine kinase links the B cell receptor to nuclear factor kappaB activation. *J Exp Med* 191: 1735-1744, 2000.
- 10 Pighi C, Gu TL, Dalai I, Larbi S, Parolini C, Bertolaso A, Pedron S, Parisi A, Ren J, Cecconi D, Chilosi M, Menestrina F, Zamo A. Phospho-proteomic analysis of mantle cell lymphoma cells suggests a pro-survival role of B-cell receptor signaling. *Cell Oncol* 34(2): 141-153, 2011.
- 11 Pham LV, Tamayo AT, Yoshimura LC, Lo P and 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* 171: 88-95, 2003.
- 12 Karin M, Cao Y, Greten FR and Li ZW: NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2(4): 301-310, 2002.
- 13 Karin M: Nuclear factor-kappaB in cancer development and progression. *Nature* 441(7092): 431-436, 2006.
- 14 Rudelius M, Pittaluga S, Nishizuka S, Pham TH, Fend F, Jaffe ES, Quintanilla-Martinez L, Raffeld M. Constitutive activation of AKT contributes to the pathogenesis and survival of mantle cell lymphoma. *Blood* 108(5): 1668-1676, 2006.
- 15 Yu L, Mohamed AJ, Simonson OE, Vargas L, Blomberg KE, Björkstrand B, Arteaga HJ, Nore BF and Smith CI: Proteasome-dependent autoregulation of Bruton tyrosine kinase (BTK) promoter *via* NF-kappaB. *Blood* 111(9): 4617-4626, 2008.
- 16 Wang SS, Purdue MP, Cerhan JR, Zheng T, Menashe I, Armstrong BK, Lan Q, Hartge P, Krickler A, Zhang Y, Morton LM, Vajdic CM, Holford TR, Severson RK, Grulich A, Leaderer BP, Davis S, Cozen W, Yeager M, Chanock SJ, Chatterjee N and Rothman N: Common gene variants in the tumor necrosis factor (TNF) and TNF receptor superfamilies and NF-kB transcription factors and non-Hodgkin lymphoma risk. *PLoS One* 4(4): e5360, 2009.
- 17 Rickert RC, Jellusova J and Miletic AV: Signaling by the tumor necrosis factor receptor superfamily in B-cell biology and disease. *Immunol Rev* 244(1): 115-133, 2011.
- 18 Dolcet X, Llobet D, Pallares J and Matias-Guiu X: NF-kB in development and progression of human cancer *Virchows Arch* 446(5): 475-482, 2005.
- 19 Sakurai H, Chiba H, Miyoshi H, Sugita T, and Toriumi W: IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J Biol Chem* 274(43): 30353-30356, 1999.
- 20 Sizemore N, Lerner N, Dombrowski N, Sakurai H and Stark GR: Distinct roles of the Ikb kinase α and β subunits in liberating nuclear factor kB (NF-kB) from Ikb and in phosphorylating the p65 subunit of NF-kB. *J Biol Chem* 277: 3863-3869, 2002.
- 21 Buss H, Dörrie A, Schmitz ML, Hoffmann E, Resch K and Kracht M: Constitutive and interleukin-1-inducible phosphorylation of p65 NF-kB at serine 536 is mediated by multiple protein kinases including Ikb kinase (IKK)- α , IKK β , IKK ϵ , TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. *J. Biol. Chem* 279(53): 55633-55643, 2004.
- 22 Lawrence T, Bebiën M, Liu GY, Nizet V and Karin M: IKK α limits macrophage NF-kB activation and contributes to the resolution of inflammation. *Nature* 434(7037): 1138-1143, 2005.
- 23 Sakurai H, Suzuki S, Kawasaki N, Nakano H, Okazaki T, Chino A, Doi T and Saiki I: Tumor necrosis factor-alpha-induced IKK phosphorylation of NF-kappaB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *J Biol Chem* 278(38): 36916-36923, 2003.
- 24 Hofmann WK, de Vos S, Tsukasaki K, Wachsman W, Pinkus GS, Said JW and Koeffler HP: Altered apoptosis pathways in mantle cell lymphoma detected by oligonucleotide microarray. *Blood* 98(3): 787-794, 2001.
- 25 Jazirehi AR, Vega MI, Chatterjee D, Goodglick L and Bonavida B: Inhibition of the RAF-MEK1/2-ERK1/2 signaling pathway, BCL-xL down-regulation, and chemosensitization of non-Hodgkin's lymphoma B-cells by Rituximab. *Cancer Res* 64(19): 7117-7126, 2004.
- 26 Dennison JB, Shanmugam M, Ayres ML, Qian J, Krett NL, Medeiros LJ, Neelapu SS, Rosen ST and Gandhi V: 8-Aminoadenosine inhibits AKT/mTOR and ERK signaling in mantle cell lymphoma. *Blood* 116(25): 5622-5630, 2010.
- 27 Dasmahapatra G, Lembersky D, Son MP, Attkisson E, Dent P, Fisher RI, Friedberg JW and Grant S: Carfilzomib interacts synergistically with histone deacetylase inhibitors in mantle cell lymphoma cells *in vitro* and *in vivo*. *Mol Cancer Ther* 10(9): 1686-1697, 2011.
- 28 Robak T and Robak P: BCR signaling in chronic lymphocytic leukemia and related inhibitors currently in clinical studies. *Int Rev Immunol* 32(4): 358-376, 2013.
- 29 Hendriks RW, Yuvaraj S and Kil LP: Targeting Bruton's tyrosine kinase in B-cell malignancies. *Nat Rev Cancer* 14(4): 219-232, 2014.
- 30 Berridge MV, Herst PM and Tan AS: Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev* 11: 127-152, 2005.
- 31 Romanchikova N, Trapencieris P, Zemitis J and Turks M: A novel matrix metalloproteinase-2 inhibitor triazolymethyl aziridine reduces melanoma cell invasion, angiogenesis and targets ERK1/2 phosphorylation. *J Enzyme Inhib Med Chem* 29(6): 765-772, 2014.
- 32 Geneviev HC, Hinshelwood S, Gaspar HB, Rigley KP, Brown D, Saeland S, Rousset F, Levinsky RJ, Callard RE, Kinnon C and Lovering RC: Expression of Bruton's tyrosine kinase protein within the B-cell lineage. *Eur J Immunol* 24(12): 3100-3105, 1994.
- 33 Cahir-McFarland ED, Davidson DM, Schauer SL, Duong J and Kieff E: NF-kappa B inhibition causes spontaneous apoptosis in Epstein-Barr virus-transformed lymphoblastoid cells. *Proc Natl Acad Sci USA* 97(11): 6055-6060, 2000.
- 34 Keller SA, Schattner EJ and Cesarman E: Inhibition of NF-kappaB induces apoptosis of KSHV-infected primary effusion lymphoma cells. *Blood* 96(7): 2537-2542, 2000.
- 35 Darnell JE Jr.: Transcription factors as targets for cancer therapy. *Nat Rev Cancer* 2(10): 740-749, 2002.
- 36 Hewamana S, Alghazal S, Lin TT, Rowntree C, Karunanithi K, Pratt G, Hills R, Fegan C, Brennan P and Pepper C: The NF-kappaB subunit Rel A is associated with *in vitro* survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target. *Blood* 111(9): 4681-4689, 2008.
- 37 Herman SE, Gordon AL, Hertlein E, Ramanunni A, Zhang X, Jaglowski S, Flynn J, Jones J, Blum KA, Buggy JJ, Hamdy A, Johnson AJ and Byrd JC: Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* 117(23): 6287-6296, 2011.

- 38 Texido G, Su IH, Mecklenbräuker I, Saijo K, Malek SN, Desiderio S, Rajewsky K and Tarakhovsky A: The B-cell-specific SRC-family kinase BLK is dispensable for B-cell development and activation. *Mol Cell Biol* 20(4): 1227-1233, 2000.
- 39 Lowell CA: Src-family kinases: Rheostats of immune cell signaling. *Mol Immunol* 41(6-7): 631-643, 2004.
- 40 Mohamed AJ, Yu L, Bäckesjö CM, Vargas L, Faryal R, Aints A, Christensson B, Berglöf A, Vihinen M, Nore BF and Smith CI: Bruton's tyrosine kinase (BTK): function, regulation, and transformation with special emphasis on the PH domain. *Immunol Rev* 228(1): 58-73, 2009.
- 41 Mahajan S, Ghosh S, Sudbeck EA, Zheng Y, Downs S, Hupke M and Uckun FM: Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-N-(2, 5-dibromophenyl) propenamide]. *J Biol Chem* 274(14): 9587-9599, 1999.
- 42 Honigberg LA, Smith AM, Sirisawad M, Verner E, Louny D, Chang B, Li S, Pan Z, Thamm DH, Miller RA and Buggy JJ: The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc Natl Acad Sci USA* 107(29): 13075-13080, 2010.
- 43 Wang ML, Rule S, Martin P, Goy A, Auer R, Kahl BS, Jurczak W, Advani RH, Romaguera JE, Williams ME, Barrientos JC, Chmielewska E, Radford J, Stilgenbauer S, Dreyling M, Jedrzejczak WW, Johnson P, Spurgeon SE, Li L, Zhang L, Newberry K, Ou Z, Cheng N, Fang B, McGreivoy J, Clow F, Buggy JJ, Chang BY, Beaupre DM, Kunkel LA and Blum KA: Targeting BTK with Ibrutinib in relapsed or refractory mantle-cell lymphoma. *N Engl J Med* 369(6): 507-516, 2013.
- 44 Ma J, Lu P, Guo A, Cheng S, Zong H, Martin P, Coleman M and Wang YL: Characterization of ibrutinib- sensitive and -resistant mantle lymphoma cells. *Br J Haematol* 166(6): 849-861, 2014.
- 45 Petro JB, Rahman SM, Ballard DW and Khan WN: Bruton's tyrosine kinase is required for activation of IkappaB kinase and nuclear factor kappaB in response to B cell receptor engagement. *J Exp Med* 191(10): 1745-1754, 2000.
- 46 Sethi G, Ahn KS and Aggarwal BB: Targeting nuclear factor-kappa B activation pathway by thymoquinone: role in suppression of antiapoptotic gene products and enhancement of apoptosis. *Mol Cancer Res* 6(6): 1059-1070, 2008.
- 47 Sasaki CY, Barberi TJ, Ghosh P and Longo DL: Phosphorylation of RelA/p65 on Serine 536 defines an I{kappa}B{alpha}-independent NF-{kappa}B pathway. *J Biol Chem* 280(41): 34538-34547, 2005.
- 48 Oh SM, Lee SH, Lee BJ, Pyo CW, Yoo NK, Lee SY, Kim J and Choi SY: A distinct role of neutrophil lactoferrin in RelA/p65 phosphorylation on Ser536 by recruiting TNF receptor-associated factors to IkappaB kinase signaling complex. *J Immunol* 179(9): 5686-5692, 2005.
- 49 Camara-Clayette V, Lecluse Y, Schrader C, Klapper W, Vainchenker W, Hermine O and Ribrag V: The NF-kB pathway is rarely spontaneously activated in mantle cell lymphoma (MCL) cell lines and patient's samples. *Eur J Cancer* 50(1): 159-169, 2014.
- 50 Ghosh-Choudhury N, Mandal CC and Ghosh Choudhury G: Simvastatin induces derepression of PTEN expression via NF-kappaB to inhibit breast cancer cell growth. *Cell Signal* 22(5): 749-758, 2010.
- 51 Han SS, Yun H, Son DJ, Tompkins VS, Peng L, Chung ST, Kim JS, Park ES and Janz S: NF-kappaB/STAT3/PI3K signaling crosstalk in iMyc E mu B lymphoma. *Mol Cancer* 9: 97-113, 2010.
- 52 Bernard S, Danglade D, Gardano L, Laguillier C, Lazarian G, Roger C, Thieblemont C, Marzec J, Gribben J, Cymbalista F, Varin-Blank N, Ledoux D and Baran-Marszak F: Inhibitors of BCR signalling interrupt the survival signal mediated by the micro-environment in mantle cell lymphoma. *Int J Cancer* 136(12): 2761-2774, 2015.
- 53 Kim YR and Eom KS: Simultaneous Inhibition of CXCR4 and VLA-4 Exhibits Combinatorial effect in overcoming stroma-mediated chemotherapy resistance in mantle cell lymphoma cells. *Immune Netw* 14(6): 296-306, 2014.

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