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

NADEZHDA ROMANCHIKOVA1, ARNIS STRODS2, JULIJA STRAZDINA1, BORISS STRUMFS1 and PETERIS TRAPENCERIS1

1Latvian Institute of Organic Synthesis, Riga, Latvia;
2Latvian Biomedical Research and Study Centre, Riga, Latvia

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 (TNFα). 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 (NF-κB) 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.

Correspondence to: Dr. Nadezhda Romanchikova, Latvian Institute of Organic Synthesis, Department of Experimental Chemotherapy, Aizkraukles Str.21, Riga, LV-1006, Latvia. Tel: +371 66155119, e-mail: nadezhda@farm.osi.lv

Key Words: Mantle cell lymphoma, Bruton’s tyrosine kinase inhibitor, NF-κB, ERK1/2, apoptosis, drug discovery.
is critical for apoptotic responses in cancer progression (16, 17). NF-κB, 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-κB p65/p50 dimer plays a pivotal role in regulating its nuclear translocation and gene transcription. Sakurai et al. demonstrated that TNFα 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 IκBα (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-κB, AKT and ERK1/2 (24). Specific blockade of ERK1/2 pathway potentiates chemotheraphy-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-GloTM (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-GloTM 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 μg peptide substrate Poly(Glu4Tyr1), 50 μM ATP, and the tested compound, or without it as control. After incubation for 30 min, an equal volume of ADP-GloTM 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) ×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 5×104 cells/ml in 100 μl per well of 96-well plates. Apoptosis was induced by treatingJurkat, Mino and Maver-1 cells with JuSt-23F at 12.5-100 μM. 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, 5×104 cells/well were seeded in a 96-well plate in 100 μl of growth medium with JuSt-23F at 3.1-100 μM 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 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.
IC 50 values for Mino and Maver-1 cells were 32 μM and lymphoma cell lines Mino and Maver-1 and T-cell lymphoma Jurkat. The concentrations ranging from 3.1 to 100 μM on MCL cell 37 μM, respectively, while that for Jurkat cells was 81 μM.

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 μM 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-κB is a transcription factor that plays a central role in many physiological processes, e.g. inflammation, tumorigenesis and apoptosis. NF-κB is activated by a wide variety of stimuli, including inflammatory cytokines such as TNFα. A number of reports have demonstrated that NF-κB activation can maintain lymphoma cell viability (33-35). Inhibition of NF-κB 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-κB pathway. We found that JuSt-23F did not affect NF-κB phosphorylation in Jurkat cells (Figure 2A). However, we detected 1.4-fold inhibition of NF-κB phosphorylation in Mino cells and 2.8-fold inhibition in Maver-1 cells treated with 20 μM JuSt-23F (Figure 2B and C). TNFα-induced down-regulation of pNF-κB 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-κB in Mino cells and 6.7-fold in Maver-1 cells induced with TNFα 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 B- and T-lymphocytes. Inhibition of MAPK pathways prevented proliferation and transcriptional regulation of genes in B-lymphocytes (37). T-Cell lymphocytes treated with 0.2-20 μM 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-
dependent manner \( (p<0.001; \text{Figure 3B and C}) \). 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 \( \mu \text{M in vitro} \). 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...
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 IC₅₀ 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 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.

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.

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.
pathway. Since the NF-κB pathway is essential for B-cell development, proliferation and survival, targeting the NF-κB pathway is an attractive approach for MCL therapy (45). NF-κB 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-XI, IkBa, cIAP1, cIAP2 and survivin (46). Serine phosphorylation at various sites of the p65 subunit of NF-κB has been shown to be important in initiating transcription (47). It was shown that transduction of canonical NF-κB signaling pathway via phosphorylation and degradation of IkBα, IKKs (especially IκkB), also phosphorylates p65/RelA at the serine 536 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 IkBα (47, 48).

Recently, phosphorylated serine 536 on p65 was detected in the cytoplasm of four MCL tested cell lines, suggesting that a noncanonical NF-κB pathway may be activated in mantle cell lymphoma. Furthermore, the data suggest that at least some genes transcribed selectively by an NF-κB 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-κB 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-κB. The NF-κB 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-κB 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-κB in MCL. Apoptotic processes could be engaged using a pharmacological inhibitor of NF-κB 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-κB, 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.

**Acknowledgements**

This project was supported by European Regional Development Fund, ERAF grant 2DP/2.1.1.1.0/14/APIA/VIAA/064.

**References**


