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ARAŞTIRMA/RESEARCH

PRIMA-1 induces apoptosis of leukemic cells via inhibiting bruton's tyrosine kinase

PRIMA1 ile lösemi hücrelerinde bruton tirozin kinaz inhibisyonu aracılığı apoptoz uyarımı

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Abstract

Purpose: Bruton's tyrosine kinase (Btk) is known to be critical for B-lymphocyte development, proliferation and differentiation of B-cell lineages, convey signal transduction through B cell receptor (BCR). The present study examined the anti-tumor effects of PRIMA-1 on Btk activity and explored the underlying mechanism, such as apoptosis.

Materials and Methods: Western blot analysis and Quantitative real-time polymerase chain reaction were performed to check the effects of PRIMA-1 on Btk expression level in KBM3 and Namalwa cells. Wild-type Btk and Btk-NLS (nuclear targeted Btk) expression plasmids were transfected in COS-7 cells to establish and characterize the role of Btk in apoptosis using fluorescent microscopy and FACS assay.

Results: İn this study, we observed that exposure of acute myelomonocytic leukemia cells to anti-leukemic drug (PRIMA-1) suppressed Btk expression at mRNA and protein level. Consequently, we also noticed a reduction in the expression of Nrf2 and HO-1 proteins. Remarkably, Btk nuclear localization was increased in response to low PRIMA-1 exposure, while higher concentrations of PRIMA-1 suppressed Btk expression. Furthermore, overexpression of nuclear targeted decreased apoptosis and increased cell viability compared to the wild-type Btk.

Conclusion: Our findings suggest that nuclear Btk reduces the cell apoptosis in response to PRIMA-1 exposure through oxidative response via Nrf2 and HO-1.

Key words: Leukemia, apoptosis, nuclear Btk, Nrf2, HO-1.

Öz

Amaç: Bruton's tirozin kinaz (Btk) , B lenfositlerinin gelişimi, proliferasyonu ve B hücre serilerinin farklılaşmasında, B hücre reseptörü (BCR) aracılı sinyal iletiminde kritik rol oynadığı bilinmektedir. Bu çalışmada PRIMA-1'in Btk aktivitesi üzerindeki antitumor etkisi ve altta yatan apopitoz mekanizması araştırılmıştır.

Gereç ve Yöntem: KBM3 ve Namalwa hücrelerinde Btk ekpresyon seviyesi üzerine PRIMA 1'in etkisini kontrol etmek için kantitatif real time PCR ve Western Blot analizi yapılmıştır. FACS ve flouresan mikroskobi kullanılarak apoptozda Btk 'nın rolünü belirlemek için, wild tip Btk ve Btk-NLS (Nükleus hedefli Btk) ekpresyon plazmidleri COS-7 hücrelerine transfekte edildi.

Bulgular: Bu çalışmada, akut myelomonositik lösemi hücrelerine anti lösemik ilaç uygulanması sonucunda, Btk ekspresyonun mRNA ve protein seviyesinde baskılanmasını gözlemledik. Böylece Nrf2 ve HO-1 proteinlerinin ekpresyonlarında bir azalma olduğunu da farkettik. Dikkat çekici bir şekilde Btk nükleer lokalizasyonu, düşük dozda PRIMA-1 uygulaması ile artıyorken, yüksek dozda PRIMA -1 uygulaması ile Btk ekspresyonu baskılanıyor. Buna ek olarak, wild tip Btk ile kıyaslandığında, nükleer hedefli aşırı ekspresyonda, apoptozisde azalma ve hücre canlılık oranında artış ile sonuçlanıyor.

Sonuç: Bulgularımıza göre, PRIMA-1 uygulaması ile Nrf2 ve HO-1 oksidatif cevap aracılığı ile nükleer Btk, hücre apoptozunu azalmaktadır..

Anahtar kelimeler: Lösemi, apoptoz, nükleer Btk, Nrf2, OH-1.

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INTRODUCTION

Bruton's tyrosine kinase (Btk) is a cytoplasmic enzyme, which belongs to the TEC family of kinases (TFKs)[1,2]. Btk is expressed in the B cell lineage as well as in other hematopoietic cells and plays important roles in B cell development, differentiation and signaling[3-5]. Btk is indispensable for intracellular signaling pathways, its depletion or malfunction leads often to severe disease. Accordingly, loss-of-function mutations in the Btk gene are responsible for the primary immunodeficiency X-linked agammaglobulinemia (XLA) in humans, and the related but remarkably milder condition, X-linked immunodeficiency (xid) in mice[6-10]. The XLA disease is caused by a B-cell developmental defect with a reduction in serum Ig of all classes, thus leading to recurrent bacterial infections[11, 12]. To-date, more than 700 different mutations in the Btk gene have been identified.

Btk structure consists of different domains, a pleckstrin homology (PH) domain followed by a TH (Tec homology) domain. Next, lies the Src homology (SH) domains, SH3, SH2 and SH1[13]. The catalytic activity resides in the C-terminal, SH1 (kinase) domain[4]. The PH domain has been shown to be important for membrane targeting in many signaling proteins including phosphoinositide phosphatidylinositol-3, 4, 5-triphosphate triphosphate (PIP3), protein kinase C and G-proteins[14-17].

Since cloning the gene for the Btk kinase, the connection between Btk and cancer was elusive. Btk up-regulation has been reported in many B cell leukemias and lymphomas, which is shown to be important regulator for the tissue homing, adhesion and migration of tumor cells[18-22]. Therefore, targeting Btk for treating various B cell malignancies using inhibitors has been intensively studied. Pharmacologic inhibitors of Btk have demonstrated highly promising treatment effects for several non-Hodgkin lymphomas and B-cell lymphomas[19, 23]. However, the recent Btk inhibitor (Ibrutinib) acting on multiple B-cell-derived tumors was not exempted from resistance and side effects [24, 25]. Therefore, developing a new treatment that targets Btk for treating B cell malignancies in patients with acquired resistance would be an important strategy.

PRIMA-1^{MET} (also known as APR-246) is a low-molecular-weight anti-cancer drug and a methylated derivate of PRIMA-1. PRIMA-1 has been shown to restore the activity of mutated and unfolded wild-type p53 protein to prevent tumor cell growth in mice[26,27]. PRIMA-1 exposure leads to modulation of many wild-type p53 inducible-target genes, such as p21, Noxa, Puma, GAD45, Caspases (Caspase 2, 3 and 9) and MDM2[28,29]. Increased transcription activity of miRNA34a is also reported in cells exposed to PRIMA-1 [30], which is subsequently induced apoptosis.

Patients with chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), Waldenström Macroglobulinemia (WM) and colorectal cancer (CRC) treated with PRIMA-1 increase cytotoxic response to this drug with no differences between homozygous p53 deleted and non-deleted in CLL, WM and CRC patients[31-34]. Moreover, induced apoptosis of breast cancer cells with p53 mutation has been linked to the involvement of pro-apoptotic genes Bax and Puma[35].

Recently, we have reported that global gene expression using Affymetrix arrays on KMB3 AML cells after PRIMA-1 exposure has led to up regulation of genes preferentially related to defense mechanisms occurring in response to oxidative stress and heat shock[36].

To date, the effects of PRIMA-1 in various hematological malignancies due to elevated activity of Btk have not been explored. The present study examined the anti-tumor effects of PRIMA-1 on Btk activity and explored the underlying mechanism, such as apoptosis. In addition, we showed that PRIMA-1 is a promising drug for treating tumors mainly dependent on overactive Btk since this drug significantly affected the expression of Btk and Nrf2/HO-1. Additionally, increased nuclear localization of Btk induced cell survival and reduced cell death.

MATERIALS AND METHODS

Cell lines

KBM3 (human acute myelomonocytic leukemia) cells were a kind gift from Dr. Anderson BS, Houston, Texas, USA[37]. Namalwa cells (human Burkitt lymphoma B-cells), and COS-7 (African green monkey fibroblast-like kidney) cells were obtained from the American Type Culture Collection (ATCC). KBM3 cells were cultured in IMDM medium (GIBCO®, Life Technologies) supplemented with 15% Fetal Bovine Serum (FBS)

(Life Technologies) and 2% L-glutamine. Namalwa cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. COS-7 cells were cultivated in Dulbecco´s Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS. All cell lines were incubated in a humidified 5% CO_2 incubator at 37 °C.

Reagents and antibodies

Protease inhibitor Complete Mini EDTA-free tablets was purchased from Roche, phosphatase inhibitor cocktail was obtained from Sigma, The SDS-PAGE (4-12% Bis-Tris gels) and nitrocellulose membranes of the iBlott Dry-Blotting system were purchased from Life Technologies. PRIMA-1^{Met} was purchased from Merck Millipore. The antibodies used in this work were as follows: anti-pY551-Btk (1:1,000) from BD pharmingen; anti-Btk (1:2,000) and anti-actin (1,100,000) from Sigma; anti-Nrf2 (1:1,000) and anti-HO-1 (1:1,000) from Santa Cruz Biotechnology; anti-Histon1 from Abcam. The plasmids GFP-Btk-wt, GFP-Btk-NLS, wt-pSGT-Btk, and DsRed-Liar were previously described[38].

Plasmid transfection

Transient transfections of the COS-7 cells using plasmids encoding GFP-Btk-wt (wild-type) and GFP-Btk-NLS (nuclear targeted form) proteins were performed in 6-well plates using the cationic polymer polyethyleneimine (PEI) (Polysciences Inc.), according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction (q-RT-PCR)

cDNAs were obtained from total RNA isolated from KBM3 cells using SuperScript™ RT-PCR system (Invitrogen). Q-RT-PCR was performed on StepOne plus Sequence Detector (Applied Biosystems) using the SYBR green PCR master mix (MM) together with Btk primer. Samples were normalized to the housekeeping gene β-actin or GAPDH and the relative expression of the Btk gene in the PRIMA-1 treated samples was calculated and normalized to the DMSO-treated samples using the comparative CT method.

Western blotting

Cells were washed once with PBS and the cell pellets were lysed using lysis buffer and then suspended in 4x sample buffer, heated for 5 minutes at 65 °C prior electrophoresis. Proteins were separated on 4-12% SDS-Bis-Tris NuPAGE gels (Life Technologies) and transferred to nitrocellulose membranes using the Iblot system (Life Technologies). Membranes were then blocked for 1 hour at room temperature in LI-COR Blocking Buffer (LI-COR Biosciences GmbH) followed by overnight incubation at 4 °C with specific primary antibodies mentioned in materials and methods. Membranes were washed five times with PBST buffer (0.01% Tween-20). Then the following secondary antibodies were employed for 1 h at room temperature: Goat anti-mouse-800CW, or goat anti-rabbit-680 (1:20000) dilutions from (LI-COR Biosciences GmbH). Membranes were scanned using the Odyssey infrared imaging system (LI-COR Biosciences GmbH).

Immunofluorescence

After 48 hours of GFP-Btk-wt, GFP-Btk-NLS and GFP mock transfection, COS-7 cells were washed three times with PBS. Then the cells were fixed and permeabilized with 3% (w/v) formaldehyde for 15 min at room temperature. Cells were incubated with DAPI stain at a dilution of 1:300 for 5 min and then washed intensively with PBS. Images were captured using Olympus microscope (Olympus-IX81). For image processing, the cellSense Dimension software (Olympus, Tokyo) was used.

Nuclear and cytoplasmic fractionation

Nuclear and cytoplasmic extracts of KBM3 cells were prepared using the NE-PER kit (Pierce), according to the manufacturer's protocol.

ATP cytotoxicity assay

Following Btk-constructs transfection in COS-7 cells for 48 hours, 1.25% trichloracetic acid (TCA) was added to the cells in a dilution of 1:1 and an automated bioluminescence assay was used to determine the ATP levels using ATP Kit SL 144-041 (Bio Thema) as previously described [39].

Assessment of Apoptosis using flow cytometry

48 hours post-transfection of COS-7 cells with GFP-Btk-wt, GFP-Btk-NLS and GFP mock (control), the cells were harvested, centrifuged and

washed in PBS. Apoptosis was assessed using Annexin-V (Becton Dickinson) and propidium iodide (PI) (Sigma Aldrich) staining and flow cytometry. One hundred thousand cells were labeled with Annexin-V and PI in final concentrations of 125 ng/ml and 5 μg/ml, respectively. Cell death was analyzed using FACScan flow cytometer and CELL Quest software (Becton Dickinson).

Cell Viability assay

After treating the cells with the PRIMA-1 drug, the viability of the cells was measured using trypan blue exclusion method. The absolute number of viable cells (proliferative cells) was counted using countess instrument (Life Technologies) according to the manufacturer's protocol.

Statistical Analysis

All data are expressed as means ± standard error means (M ± SEM) and data analysis was carried out using statistically available software Graph prism version 6. Student t-test was used for the comparison between two groups. Comparisons between more than two groups were made using one-way analysis of variance (ANOVA) in combination with Dunnett t-test post hoc analysis. Dunnett t- test compares all groups against control group. When P values <0.05, the results were considered significant.

RESULTS

PRIMA-1 downregulates Btk expression

Progression of many B cell leukemias and lymphomas has been related to the activity of Btk in these cells[40,41]. Thus, targeting Btk to treat various cell malignancies, especially Burkitt lymphoma B cells and acute myeloid leukemia would be an important strategy. Recently, we have found that PRIMA-1 increases the cell death rate of acute myeloid leukemia cells (KBM3) cell line[36]. To achieve this goal of treating cell malignancies based on Btk consequences, we used PRIMA-1 inhibitor. Here, we identified that KBM3 cells treated with PRIMA-1 for 24 hours significantly inhibited the transcription of the Btk mRNA expression compared to the control (Figure 1A).

To identify whether the protein level of Btk is also affected in response to PRIMA-1 exposure. Therefore, we performed another set of experiment in KBM3 cell lines. As shown in Figure 1B, treating KBM3 cells with PRIMA-1 for 24 hours displayed a remarkable reduction of Btk Y551 phosphorylation level compared to DMSO treated cells. Additionally, the total level of Btk was also decreased.

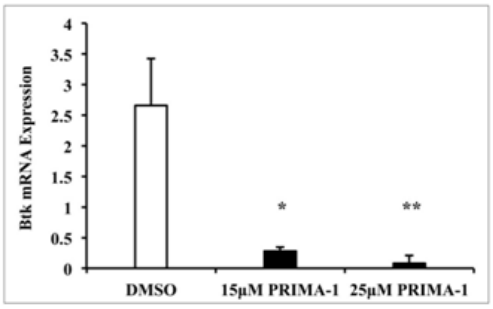
Previously, it has been shown that Btk regulates the expression level of Nrf2 gene [42]. To examine the effect of Btk on Nrf2/HO-1 expression in response to PRIMA-1 exposure, we probed the membranes with both Nrf2 and HO-1 antibodies. Interestingly the reductions of the Nrf2/HO-1 levels were correlated with Btk downregulation (Figure 1B).

PRIMA-1 induces nuclear translocation of Btk

In addition to the plasma membrane recruitment, Btk has been shown to traffic from the cytoplasm to the nucleus[43,44] presumably, to regulate potential targets inside the nucleus that might lead to up-regulation or down-regulation of certain genes. Nucleocytoplasmic transport of proteins plays an important role in the regulation of many cellular processes. To gain more insight of the PRIMA-1 effect on the subcellular distribution and dynamic localisation of Btk, KBM3 cells were used and treated with PRIMA-1 (15 μM or 25 μM) and nucleocytoplasmic fractionation assay was performed. Interestingly, KBM3 cells treated with PRIMA-1 (15 μM) caused an increase in the Btk nuclear import compared to DMSO treated cells (Figure 2, second lane). While PRIMA-1 (25 μM) completely blocked the expression of Btk, therefore Btk nuclear translocation was not observed (Figure 2, third lane). These results suggested that Btk might be necessary as a protective response against the anti-leukemic effects of PRIMA-1. Moreover, to address the response of an increased nuclear Btk translocation was due to protective response, we used Wortmannin (pan PI3K-inhibitor), which is known as Btk upstream regulator and increases cell stress and apoptosis [45]. To achieve this, KBM3 cells were treated with Wortmannin alone or in combination with PRIMA-1. As presented in Figure 2, Wortmannin increased the nuclear resident of Btk in response to stress (lane 4-6). Surprisingly, co-exposure of Wortmannin with PRIMA-1 (25 μM) abrogated the effect of the PRIMA-1 on the reduction of the Btk expression and restored Btk expression (lane 5-6), which was also translocated to the nucleus

Figure 1.

A.



B.

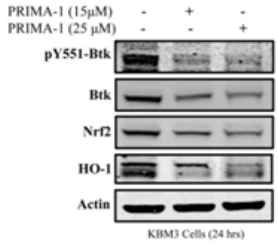


Figure 1. PRIMA-1 suppresses the expression of Btk. (A) KBM3 cells were treated with PRIMA-1 (15 μM) for 24 h.

Figure shows fold changes in Btk mRNA level (+SEM) detected by Q-RT-PCR analysis (Quantitative reverse transcription polymerase chain reaction) The data are presented as mean ± SEM. $P<0.05$ versus vehicle considered significant. *= $P<0.05$. (B) KBM3 cells were left untreated or treated with PRIMA-1 inhibitor and incubated for 24 h. Total cell lysates (TCLs) were processed for Western blotting. The effect of PRIMA-1 inhibitor on the pY551-Btk, total Btk, Nrf2, HO-1 and Actin levels was monitored using antibodies against each of them

Nuclear resident Btk increases cell survival and protects the cells from apoptosis

Since PRIMA-1 exposure induced the nuclear resident of Btk, we wondered whether the nuclear Btk would have any biological role for the cells. Therefore, we engineered a nuclear resident form of the protein and fused it with GFP (GFP-Btk-NLS) to address this effect (Figure 3A). Interestingly, the addition of a single NLS motif at the C-terminal region of Btk rendered the protein entirely nuclear in 100% of the transfected COS-7 cells compared to the Btk-wt, which was mainly cytoplasmic and translocated to the membrane (Figure 3B). Additionally, we transiently transfected COS-7 cells with GFP-Btk-wt, GFP-Btk-NLS or GFP-Mock as a control. 48 hours post transfection; the harvested cells were subjected to ATP cytotoxic assay to measure the survival rate of the cells as the possible physiological role of Btk in the nucleus. As shown in Figure 4A, GFP-Btk-NLS significantly increased the cell survival compared to GFP-Btk-wt. To further assess the effect of nuclear-localized Btk on the cells, we performed FACS assay. In this experiment, after transfection of GFP-Btk-wt, GFP-Btk-NLS or GFP-mock into COS-7 cells, we used both Annexin-V and PI to determine the apoptotic rate effect of these constructs on the cells. Interestingly, the nuclear resident form of Btk (GFP-Btk-NLS) significantly protected the cells from apoptosis compared to the cytoplasmic Btk (GFP-Btk-wt) (Figure 4B). These results demonstrated that translocation of Btk to the nucleus followed by increased cell survival and decreased apoptosis

Figure 2.

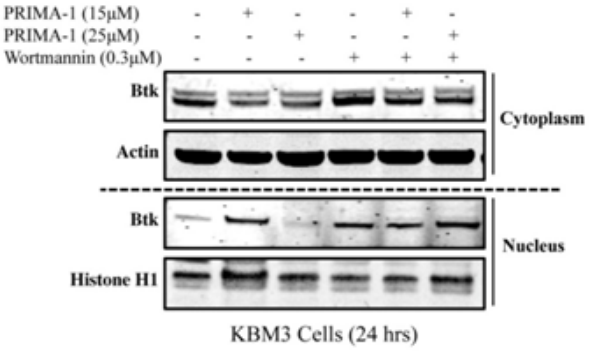


Figure 2. Increases translocation of Btk in response to PRIMA-1 exposure. KBM3 cells were treated in the presence or absence of inhibitors (PRIMA-1 or Wortmannin) for 24 h and then lysed. Nuclear and cytoplasmic fractions were prepared, and equal amounts of total protein was resolved by 4-12% Bis-Tris SDS-PAGE and transferred to a nitrocellulose membrane. The steady-state levels of Btk in each fraction were analyzed by Western blot analysis. The purity of the nuclear and cytoplasmic fractions was verified with antibodies against Histone1 and β-actin, respectively.

Figure 3.

A.

B.

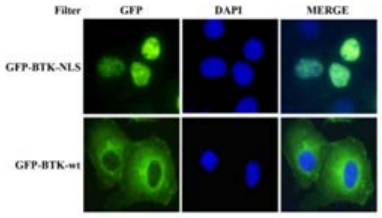
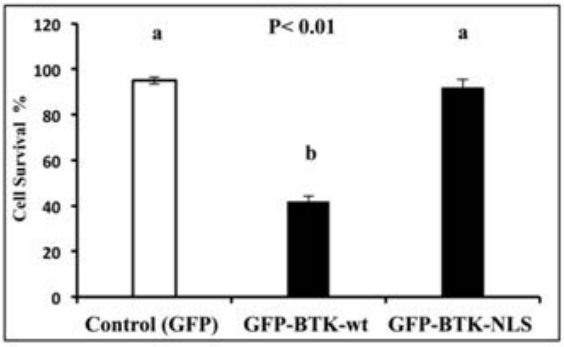


Figure 3. Localization of Btk. (A) Schematic representation of the domain structure of Btk and the location of GFP and NLS binding terminals on Btk is shown. (B) Equal amounts of GFP-Btk-NLS or GFP-Btk-wt were transiently transfected in COS 7 and localization of Btk was monitored 48 h post-transfection. Btk is shown in green, while DAPI (nuclear marker) is shown in blue.

Figure 4.

A.



B.

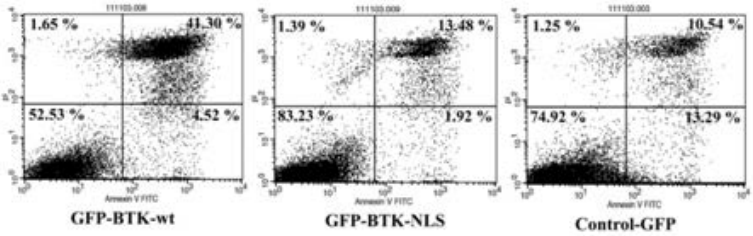


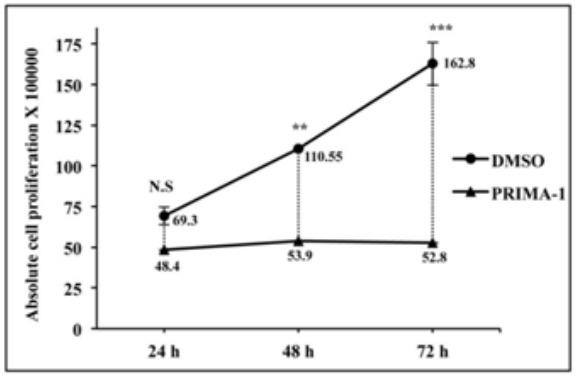
Figure 4. Nuclear Btk increases cell survival and decreases apoptosis. (A) GFP-mock, GFP-Btk-wt or GFP-Btk-NLS were transfected in COS 7 cells and tested for cell survival using the ATP cytotoxicity assay. The bars show the percentage of cell survival in the GFP-Btk-wt and GFP-Btk-NLS cells compared to the GFP control cells (Mean of 3 independent experiments ± SEM). (B) COS 7 cells were transfected with Mock, GFP-Btk-wt and GFP-Btk-NLS and the cell death level was detected using FACS analysis.

PRIMA-1 prevents cell proliferation through downregulation of Btk in Namalwa cells

The potent inhibitory effect of PRIMA-1 on the cell proliferation of various tumor cells has been reported[46-48]. Recently, we have shown that induction of apoptosis by PRIMA-1 in AML cells is correlated to the reactive oxygen species (ROS) formation and depletion of glutathione (GSH)[36]. In order to investigate the cellular effects of PRIMA-1 on Burkitt B cell lymphoma and examine the potential effect of Btk in response to this drug, a combination of cell proliferation assay and immunoblotting was employed in Namalwa cells. Namalwa cells were either left untreated or treated with PRIMA-1 for 24, 48 or 72 hours. Remarkably, the cell proliferation was significantly decreased after 48 hours treatment with PRIMA-1 and displayed more sensitivity with a persisting influence (Figure 5A). Similar results were also obtained in KBM3 cells treated with PRIMA-1 (data not shown). Moreover, we performed immunoblotting of the Namalwa cells treated with PRIMA-1 for 72 hours. As shown in Figure 5B, we identified that Btk expression was completely blocked compared to DMSO treated cells. Additionally, we detected a significant reduction of the expression of Nrf2 and HO-1 (Figure 5B). Collectively, these data indicated that the anti-proliferative effect of PRIMA-1 on Namalwa cells was due to downregulation of Btk and Nrf2/HO-1.

Figure 5.

A.



B.

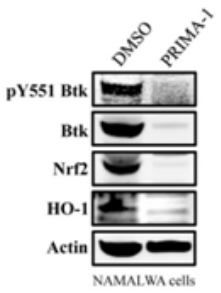


Figure 5. PRIMA-1 inhibits Namalwa cells proliferation and suppresses Btk. (A) Namalwa cells were treated with DMSO or PRIMA-1 (15 μM) and followed for 72h. A cell proliferation test was performed and the absolute number of viable cells was counted.
The data are presented as mean ± SEM. P<0.05 versus vehicle considered significant. **=P<0.01, ***= P<0.001. (B) Total cell lysates of Namalwa cells either treated or untreated with PRIMA-1 (15 μM) were resolved on 4-12% Bis-Tris SDS-PAGE and the influence of this inhibitor was detected by immunoblotting the membrane with anti-pY551-Btk, total Btk, Nrf2, HO-1 or actin antibodies.

DISCUSSION

Pharmacologic inhibitors of Btk have been demonstrated to be highly promising treatment against several non-Hodgkin lymphomas and B-cell lymphomas. Also, there is genetic evidence of Btk activity in mice with increased tumor incidence due to constitutive activation of BCR pathway[49]. Therefore, this is important and promises well for the clinical use of Btk inhibitors. Recently, it has been demonstrated that the oocyte tumors in Drosophila melanogaster develop when mutations in the corresponding fly protein, Btk29A occurs. In order to achieve silencing of transposons in the germline and rescue the oocyte tumors, a complex interplay between Wnt-signaling and the tyrosine kinase Btk29A takes place[50].

Currently, a new Btk inhibitor (PCI-320765) is available[51], which was recently renamed as Ibrutinib, binds covalently to a cysteine residue (for irreversible binding) close to the catalytic site in Btk[52]. Ibrutinib has already demonstrated efficacy in patients with various B-lineage diseases as diverse as

CLL, mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL) and multiple myeloma (MM) as well as in WM, demonstrating that rather diverse tumors respond[53-55]. The US Food and Drug Administration (FDA) has officially approved Ibrutinib as the therapeutic drug for treating patients with CLL, MCL and WM. The exact mechanism underlying the sensitivity of B-cell tumors to Ibrutinib is not known. Administration of Ibrutinib (420 mg/day) in patients with CLL, induces a rapid shrinkage of enlarged lymph nodes, lymphocytosis, and symptomatic improvement within first the few weeks of treatment[20]. When treatment is stopped this process is reversed[23].

Nevertheless, recent studies have reported acquired resistance in patients treated with Ibrutinib due to occurring mutation in the tumor cells with CLL and MCL. The common mutation that leads to resistance to the Btk inhibitors (Ibrutinib or Acalabrutinib) involves mutation of a Cysteine 481 residue substitution to Serine where Ibrutinib binding occurs, which hints to the disruption of the irreversible covalent binding and reduces the affinity of Ibrutinib to Btk[56,57].

With few exceptions, the most successful tumor treatments are based on combination therapies. This is because a tumor normally is very heterogeneous, with cells carrying multiple mutational patterns. This means that when an anti-tumor drug is used, there already exist rare resistant clones. Over time such rare resistant cells will be strongly selected for, resulting in an only transient treatment effect. When combinations of anti-tumor drugs are used, much fewer resistant tumor cells exist, in the optimal scenario resulting in that the tumor is eradicated. PRIMA-1 may also become the next line in combination with or replacement of Ibrutinib for the treatment of different malignancies because of tumor cells mutation and become resistant to Ibrutinib. Given the potent effect of PRIMA-1 might become important pharmaceutics in the future therapeutic arsenal for non-Hodgkin lymphoma and multiple myeloma (as well as in autoimmunity). Such compound could either be used in combination with Ibrutinib to treat patients developing resistance to Ibrutinib, or, they may constitute an entirely new set of drugs for B-cell tumors. We here propose to combine the analysis of treated patients with such inhibitors. We believe that intensive studies required from basic Btk research to pave the way for a deeper molecular mechanism understanding of how the newly encouraging inhibitor (PRIMA-1) works.

It has been identified that PRIMA-1 produces both oxidative stress and ER stress as p53-independent cytotoxic functions of PRIMA-1 to induce apoptosis [58]. In response to apoptotic stimuli, Bax translocates from the cytoplasm to the mitochondria, which results in permeabilization of the mitochondrial outer membrane and subsequent cytochrome c release to the cytosol and further Caspase activation[59]. Recently, we have shown that PRIMA-1 induces oxidative stress in AML cells, which are manifested by an increase in ROS, depletion of GSH as well as induction of genes that protect cells from oxidative stress. Moreover, the combination of PRIMA-1 and the PI3K/mTOR pathway inhibitor leads to synergistic cell killing of AML cells[36].

An important consequence regarding the nuclear translocation of Btk in response to PRIMA-1 exposure may involve binding of Btk to relevant substrates in the nucleus. Indeed, Btk has been shown to be an important regulator for the activity of several transcription factors including NF-κB, STAT5, FOXO1, Nrf2 and BRIGHT[42,60-63]. Notably, Btk contains an NLS-like sequence in the PH domain, but it has been shown to be functionally inactive[43]. Instead, Btk uses an exportin-1-dependent nuclear export signal to shuttle between the nucleus and cytoplasm. However, the molecular mechanism(s) of Btk shuttling are currently not known and it is under intense investigation in our laboratory. It is tempting to understand the exact physiological growth conditions that lead to the nuclear translocation of Btk due to PRIMA-1 treatment. Additionally, we found a significant correlation between sensitivity to PRIMA-1, as defined by less cell apoptosis, and an increased Btk nuclear residence. Moreover, increased cell death in Namalwa cells treated with PRIMA-1 was due to low levels of Btk and Nrf2/HO-1 level.

One of the mechanisms of increased apoptosis by PRIMA-1 might be due to the fact that Btk is necessary for the survival of the cells; therefore absence of Btk increases the cell sensitivity to the drug and leads to cell death. Moreover, it has been shown that a master regulator of the antioxidant cellular defense (HO-1) induction was abrogated in $Btk^{-/-}$ mice, and this blockade is mediated by less activity of Nrf2 due to the absence of Btk[42].

Additionally, the expression of FOXO1, a transcription factor that inhibits cell proliferation and promotes cell death, is down regulated in response to increased Btk activity during BCR cross-linking. Therefore, in Btk knockout mice the FOXO1 downregulation is impaired[64]. Now, for the first time, we observed that the suppression of Btk expression in response to PRIMA-1 exposure was correlated with the decreased expression of both Nrf2 and HO-1 protein levels, which subsequently increased the susceptibility of the cells to apoptosis. These results may provide a mechanism of action for how PRIMA-1 affects changes of cell survival kinases in tumor cells and how it inhibits Btk.

In view of these findings, we suggest that the existing data are best compatible with a dual role of Btk. Thus, depending on the phenotype of the cell and localization, Btk can be involved in signals promoting either survival or cell death. Btk may, therefore, belong to the category of important molecules that modulate apoptosis and survival during ontogeny. We also conclude that activation of the Nrf-2/HO-1 pathway is crucial cellular responses to Btk that are inhibited upon PRIMA-1 exposure. It already seems very likely that PRIMA-1, a highly promising Btk-inhibitor may become a major player in the therapeutic for the treatment of B-cell lineage-derived malignancies. Therefore, it is extremely exciting that PRIMA-1 could be proven to delineate potential targets for future treatment of such B-cell malignancies.

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