ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
February 2025; 24(1):89-99.
DOI: 10.18502/ijaai.v24i1.18024

Effects of c-Kit Receptor, AKT, and NF-κB Inhibitors on Immune Evasion in Multiple Myeloma Cells

Abbas Ranjbar[1,2], Saeid Taghiloo[1], Parvin Nozari[1], Akbar Hedayatizadeh-Omran[3], and Hossein Asgarian-Omran[1,3]

[1] Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[2] Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran
[3] Gastrointestinal Cancer Research Center, Non-Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran

Received: 14 March 2024; Received in revised form: 14 September 2024; Accepted: 30 September 2024

ABSTRACT

Up-regulation of immune checkpoint ligands and secretion of soluble factors in the tumor microenvironment led to the survival of cancerous plasma cells in the bone marrow milieu. Therefore, we investigate the relationship between the inhibition of c-Kit receptor, AKT, and NF-κB signaling pathways and the regulation of immune escape mechanisms in multiple myeloma.

The U266B1 cell line was treated with Masitinib as a c-Kit receptor inhibitor, Perifosine as AKT inhibitor, and Bortezomib as NF-κB inhibitor either in single or combined form. Apoptosis and cell viability were evaluated using flow cytometry and MTT assays, respectively. The relative expression of programmed death-ligand 1 (*PD-L1*), poliovirus receptor (*PVR*), and interleukin 6 (*IL-6*) were determined by real-time PCR. Also, the secretion of IL-6 was measured by ELISA.

Our findings demonstrated decreased proliferation of U266B1 cells after co-treatment with Masitinib, Perifosine, and Bortezomib. An increase in apoptosis was observed in the co-treatment of Masitinib and Perifosine. Furthermore, results elucidated that the expression of *PD-L1* and *IL-6* decreased after treatment with Masitinib, Perifosine, and Bortezomib in both single and co-treatments. Regarding *PVR*, combined treatment of U266B1 cells with Masitinib, Perifosine, and Bortezomib decreased the expression level of *PVR*.

We showed that c-Kit receptor, AKT, and NF-κB pathway inhibitors not only serve as cytotoxic drugs but also inhibit the immune escape mechanisms of malignant plasma cells by disrupting signaling pathways.

Keywords: Immune evasion; Immune checkpoint; Multiple myeloma; Signaling pathways; Small molecule inhibitor

INTRODUCTION

Multiple myeloma (MM) is a group of plasma cell malignancies in the final stages of differentiation, derived from post–germinal-center B cells.[1] Clonal proliferation and the accumulation of malignant plasma

Corresponding Author: Hossein Asgarian-Omran, PhD; Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran. Tel: (+98 11) 3354 3091, Fax: (+98 11) 3354 3249, Email: asgarianhossein@yahoo.com, hasgarian@mazums.ac.ir

cells in the bone marrow result in anemia, bone lesions, hypercalcemia, and renal failure, collectively known as CRAB features.[2] The current general treatment for MM is hematopoietic stem cell transplantation accompanied by chemotherapy drugs and proteasome inhibitors.[3] Moreover, the 5-year survival rate for transplant-eligible patients is over 70%, while it is about 50% for elderly transplant-ineligible patients.[4] Nevertheless, MM remains an incurable disease due to evolving genetic and epigenetic alterations, the immunosuppressive microenvironment in the bone marrow, and deregulated signaling pathways.[5,6]

Recent advances have demonstrated aberrant intracellular signaling in multiple myeloma patients due to genetic instabilities, physical interactions with cellular components, and the secretion of numerous soluble factors in the bone marrow.[7] C-Kit, a critical member of the receptor tyrosine kinase family, is involved in intracellular signaling and plays a crucial role in the development of some cancers.[8] Prior studies have indicated that growth factors such as stem cell factor, binding to the c-Kit receptor (CD117) or the mutated form of c-Kit, activate several signaling pathways such as RAS/MAPK, PI3K/AKT, and JAK-STAT in multiple myeloma cells resulting in expression of genes which regulate cell differentiation, proliferation, and resistance to apoptosis.[9] Furthermore, AKT, the central node of the PI3K/AKT/mTOR pathway, contributes to multiple oncogenic functions in multiple myeloma cells, ultimately leading to protein synthesis, resistance to apoptosis, increased proliferation, and survival in MM.[10] NF-κB is another known transcription factor activated by many cytokines with identified mutations in MM patients, which activates without ligands in both canonical and non-canonical pathways.[11] Previous investigations showed that NF-κB plays a vital role in controlling the expression of cytokines, cell adhesion molecules, and molecules involved in resistance to cell death in cancers, including MM.[12] In recent years, several small molecule inhibitors (SMI) such as c-Kit receptor and AKT inhibitors have been used in various studies and clinical trials in combination with other drugs such as NF-κB inhibitors, to inhibit the signaling pathways in MM.[13] Furthermore, many investigations have revealed a link between cancer cell immune evasion and the aberrant activation of these signaling pathways.[14]

Tumor cells recruit various strategies to circumvent the immune system, including immunoediting of tumor cells, and the suppression of the immune responses, which contributes to immune escape.[15] Malignant plasma cells in MM can exploit these mechanisms to induce exhaustion in immune cells, particularly $CD8^+$ T cells, through the up-regulation of ligands such as programmed death-ligand 1 (PD-L1), PD-L2, poliovirus receptor (PVR), which are known as the immune checkpoint molecules.[16,17] Furthermore, cancerous plasma cells could create favorable conditions for survival and proliferation and even downregulating immune function through the secretion of soluble factors such as interleukin 6 (IL-6).[18,19] Hence, there has been widespread interest in studying signaling pathways associated with tumorigenesis and immune escape mechanisms, we have investigated the relationship between c-Kit receptor, AKT, and NF-κB signaling pathway inhibitors with immunological escape mechanisms in multiple myeloma cells.

MATERIALS AND METHODS

Chemical Compounds and Reagents

Two SMIs, including Masitinib (c-Kit Receptor inhibitor) and Perifosine (AKT inhibitor), were supplied from the Cayman chemical company (Michigan, USA). Bortezomib (proteasome inhibitor) and Doxil (pegylated liposomal doxorubicin) as the conventional chemotherapies' agents, were obtained from the Sobhan Oncology company (Rasht, Iran). Masitinib and Bortezomib were dissolved in cell culture grade dimethyl sulfoxide (DMSO), while Perifosine was dissolved in ethanol and stored frozen in aliquots.

Cell Line and Culture

U266B1, a human myeloma cell line, was purchased from the Iranian Biological Resource Center (IRBC code: C10148). Cells were cultured in RPMI-1640 medium (Biowest, Nuaille, France) supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Nuaille, France), 100 U/mL penicillin G, 100 µg/mL streptomycin, and maintained at an atmosphere of 5% carbon dioxide at 37°C.

Colorimetric Cytotoxic Assay

MTT assay was conducted to determine the half-maximal inhibitory concentration (IC_{50}) of the drugs mentioned above. Briefly, U266B1 cells were seeded in 96-well at a density of 30×10^3 cells/well and treated with increasing concentrations of Masitinib (0 to 100 µM), Perifosine (0 to 100 µM), Bortezomib (0 to 200 nM), and

Doxil (0 to 25 µM) for 24, 48, and 72 hours. Based on the results, the 72-hour time point was selected for further analysis. After treatments, 20 µL of MTT reagent (5mg/mL) was added to each well, and the cells were incubated for an additional 4 hours at 37°C. The microplate was then centrifuged (300 g for 10 min), and the supernatant was discarded. Subsequently, 150 µL DMSO was added to each well and shaked for 20 min. Each well's optical density (OD) was measured at 570 and 720 nm using an ELISA plate reader (Synergy H1 BioTek, Winooski, USA). The IC_{50} values were calculated to demonstrate the potency of each agent in inhibiting cell growth. The proliferation of U266B1 cells was evaluated via MTT assay following treatment with Masitinib, Perifosine, and Bortezomib, both individually and in combination. Also, cells without treatment and treated those with Doxil were used as negative and positive control, respectively. All experiments were conducted in triplicates. Finally, the mean OD values for each group were divided by the OD values obtained from the control group to determine the relative cell proliferation.

Cell Apoptosis Assay

U266B1 cells were seeded in 24-well plates at a density of 150×10^3 cells per well and treated with an optimal concentration of Masitinib (5.14 µM), Perifosine (9.3 µM), and Bortezomib (0.9 nM) for 72 hours. Subsequently, programmed cell death (PCD) was examined by dual-color FITC-labeled Annexin V/Propidium Iodide (PI) detection kit (IQ products, Groningen, The Netherlands). Briefly, untreated and treated cells were harvested by centrifugation (400 g for 5 min) followed by washing with calcium buffer. Next, 5 µL Annexin V/FITC was added to the cell suspension and incubated for 20 minutes at 4°C. Following incubation, cells were washed again with calcium buffer and incubated with 2 µL PI for an additional 10 minutes at 4°C. Finally, the presence of phosphatidylserine on the external layer of the cell membrane and the permeability to PI were analyzed using a Partec PAS flow cytometer system (Partec GmBH, Germany). Unstained cells were processed separately to remove background staining and establish appropriate gating. Additionally, cells treated with Doxil served as the positive control.

RNA Isolation, cDNA Synthesis, and Semi-Quantitative Real-Time PCR

U266B1 cells were seeded in 6-well plates at a density of 450×10^3 cells per well and treated with an optimal concentration of Masitinib (5.14 µM), Perifosine (9.3 µM), and Bortezomib (0.9 nM) for 72 hours. To measure the expression level of genes of interest, total RNA was isolated using the Denazist kit (Mashhad, Iran). Subsequently, complementary DNA (cDNA) was synthesized from total RNA using the Yekta-Tajhiz cDNA synthesis kit (Tehran, Iran). To assess the relative expression level of PD-L1, PVR, and IL-6, semi-quantitative Real-Time PCR was performed by the Amplicon (Copenhagen, Denmark) SYBER Green PCR Master Mix Reagents on a Step One Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All primers were designed by AllelID software, and obtained from metabion international AG (Planegg, Germany) (Supplementary Table 1). A melting curve analysis was performed after each run to validate that there were no primer dimers and that the amplification was selective. The cycles were set at 95°C for 5 minutes as initial denaturation, followed by 40 cycles at 94°C for 30 seconds, 57°C (β-actin), 61°C (PD-L1), 62°C (IL-6), and 60°C (PVR) for 30 seconds, and extension at 72°C for 30 seconds. Finally, the transcript levels of PD-L1, PVR, and IL-6 were normalized to β-actin, and the relative expression of each molecule was calculated using the Pffafl method. The primer sequences are shown in Supplementary Table 1.

Cytokine Measurement

The level of IL-6 cytokine was measured in the supernatant of U266B1 cells. For this purpose, cells were seeded in 6-well plates at a density of 450×10^3 cells per well and treated with an optimal concentration of Masitinib (5.14 µM), Perifosine (9.3 µM), and Bortezomib (0.9 nM) for 72 hours. The IL-6 level was measured by ELISA according to the manufacturer's instruction (Sanquin, Amsterdam, The Netherlands). Finally, the absorbance of the samples was recorded at 450 nm, with 630 nm used as a reference wavelength with a multi-scan plate reader (Synergy H1 BioTek, Winooski, USA).

Statistical Analysis

Results were analyzed using GraphPad Prism 8 software. All data are presented as the mean±SD. The Shapiro-Wilk test was conducted to assess the normality of the data distribution. One-way Analysis of variance (ANOVA) was performed, followed by the proper post hoc test for multiple comparisons between the treated

and untreated groups. A *p*-value of less than 0.05 ($p<0.05$) was considered statistically significant difference, with the following designations: as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

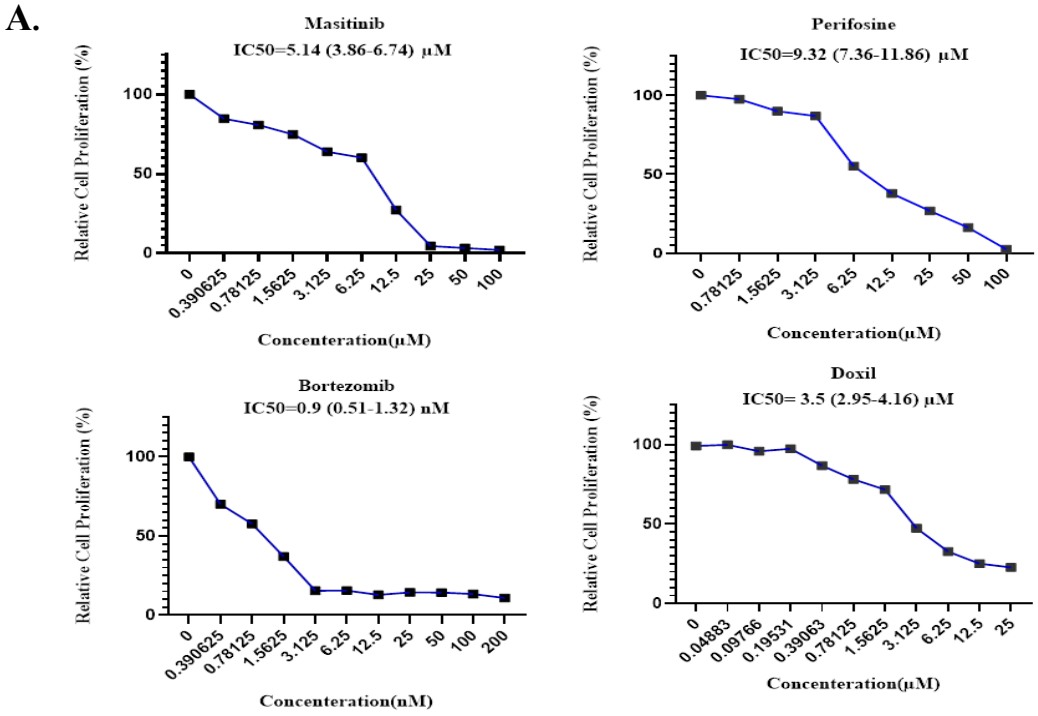
RESULTS

Cell Viability of U266B1 Cells Following Treatment with Masitinib, Perifosine, Bortezomib, and Doxil

The effects of Masitinib, Perifosine, and Bortezomib on growth inhibition were measured in U266B1 Cells. Initially, the IC_{50} values of the Masitinib, Perifosine, Bortezomib, and Doxil were determined using the MTT Assay following 72 hours of exposure to increasing drug concentrations (Figure 1A). Next, the anti-proliferative activity of single or combined compounds against U266B1 cells was measured based on the IC_{50} results. The relative cell proliferation index was calculated for all samples. Our experiment revealed that the proliferation of U266B1 cells decreased significantly following treatment with Masitinib, Perifosine, and Bortezomib (Figure 1B). Moreover, co-treatment with compounds resulted in more growth inhibition than a single treatment compared to the control group. However, there was no significant difference in growth inhibition between combined treatments of two or three compounds, except for the Masitinib/Perifosine co-treatment, which displayed enhanced effects (Figure 1B).

The Combination of Masitinib, Perifosine, and Bortezomib Induces Significant Apoptosis

To measure the levels of apoptosis resulting from the inhibitory effects of Masitinib, Perifosine, and Bortezomib, U266B1 cells were treated with these compounds in both single and combined groups. Following treatment, the U266B1 cells were stained with Annexin V-FITC/PI for flow cytometric assay (Figure 2A). The results showed that only treatment with Bortezomib in the single groups resulted in significant apoptosis. Furthermore, the combination of compounds led to a significantly higher level of apoptosis compared to the control group (Figure 2B). However, there was no significant difference between a single treatment with Bortezomib and the combination treatment of Bortezomib with either Masitinib or Perifosine (Figure 2B).



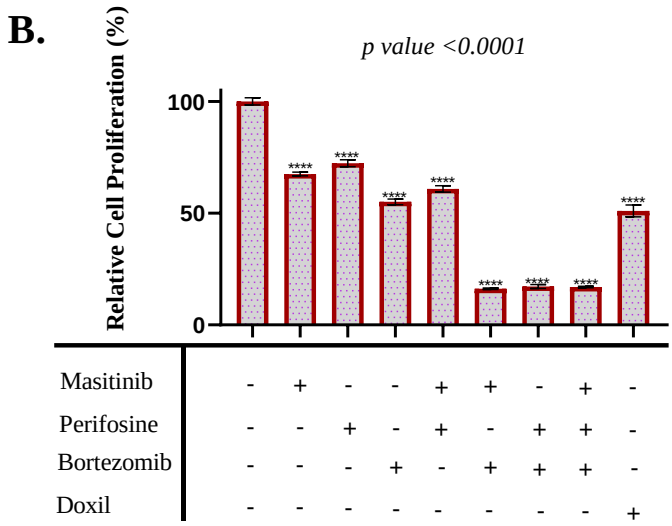
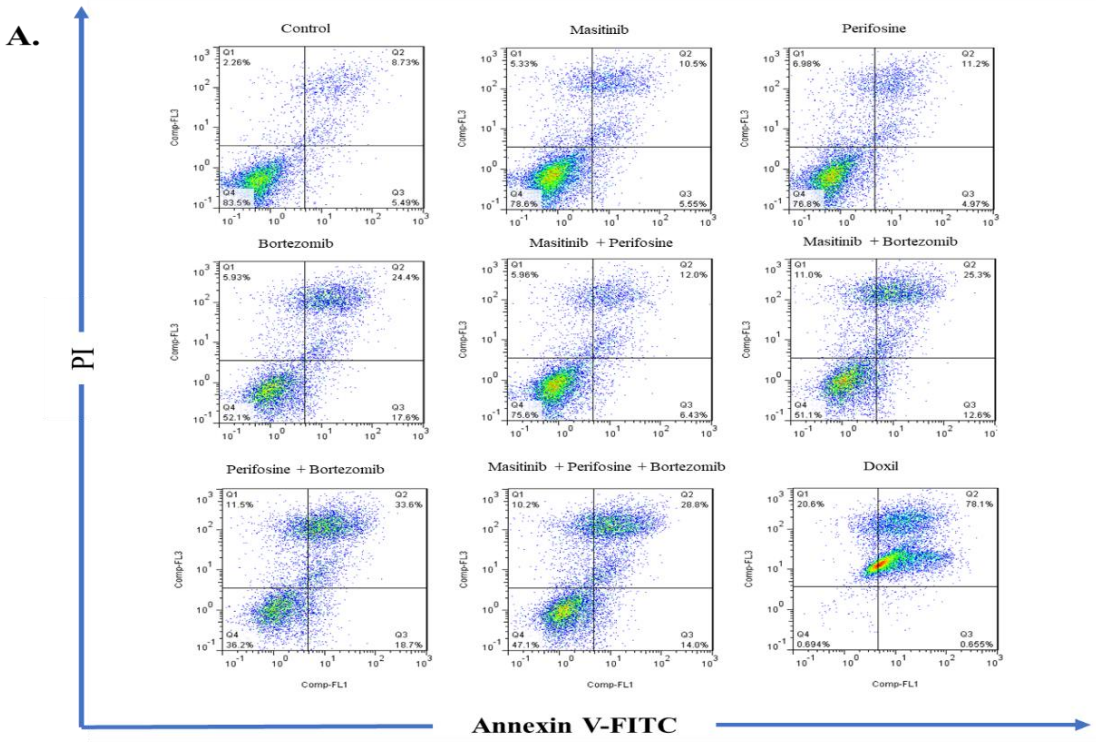


Figure 1. Determination of half-maximal inhibitory concentration (IC50) values and proliferation levels of U266B1 cells following treatment with Masitinib, Perifosine, Bortezomib, and Doxil. A) U266B1 cells were treated with various concentrations Masitinib (0 to 100 µM), Perifosine (0 to 100 µM), Bortezomib (0 to 200 nM), and Doxil (0 to 25 µM) for 72 hours. Cell viability was measured using MTT assay. The calculated IC50 values of Masitinib, Perifosine, Bortezomib and Doxil were 5.14 (3.86-6.74) µM, 9.32 (7.36-11.86) µM, 0.9 (0.51-1.32) nM and 3.5 (2.95-4.16) µM, respectively. B) U266B1 cells were cultured in the absence or presence of Masitinib, Perifosine, and Bortezomib for 72 hours either in single or combined treatments. Cell proliferation was determined using MTT assay. The results are expressed as the mean±SD of three independent experiments. One-way ANOVA with Dunnett's post hoc test was used for multiple comparisons between treated groups and untreated group, with significance indicated as**p<0.0001.**



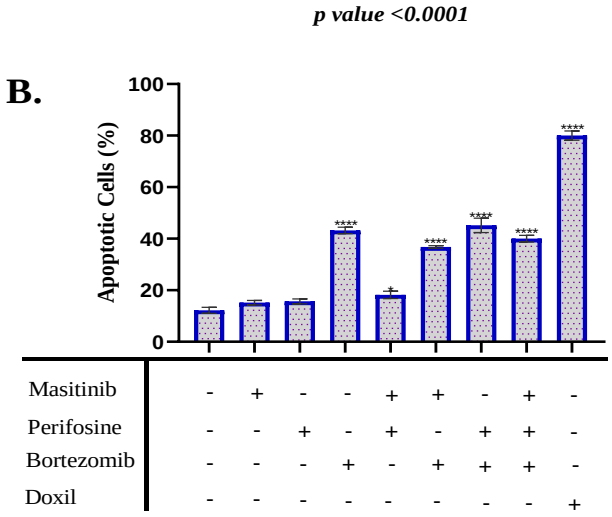


Figure 2. Effects of c-Kit receptor, AKT, and NF-kB inhibitors and Doxil on apoptosis of U266B1cells. U266B1 cells were cultured in the absence or presence of Masitinib, Perifosine, and Bortezomib for 72 hours either in single or combined treatment. Apoptotic cells were then detected by Annexin-V/PI staining assay via flow cytometry. A) A representative flow cytometric dot plot is shown. B) The percentage of apoptotic U266B1 cells is presented. Annexin V positive cells were considered as percentage of apoptotic cells. The results are expressed as the mean±SD of three independent experiments. One-way ANOVA with Dunnett's post hoc test was used for multiple comparisons between treated groups and the untreated group. *p<0.05; ****p<0.0001.

Expression of Immune Evasion Molecules Following Exposure to Masitinib, Perifosine, and Bortezomib

To further elucidate the role of signaling pathways in the expression of immune evasion molecules, the effects of Masitinib, Perifosine, and Bortezomib on the expression of PD-L1, PVR, and IL-6 were evaluated in U266B1 cells. The relative expression of these molecules on U266B1 cells were determined 72 hours after exposure with Masitinib, Perifosine, and Bortezomib by Semi-Quantitative Real-Time PCR using β-actin as a housekeeping internal control. The results showed that expression of PD-L1 was significantly reduced after treatment with Masitinib, Perifosine, and Bortezomib, both individually and in combination compared to the control group (p<0.0001; Figure 3A). The most substantial decrease in PD-L1 expression occurred with the combination of all three drugs. Additionally, U266B1 cells treated with Masitinib and Perifosine exhibited a greater reduction in PD-L1 expression than those treated with Bortezomib alone (p<0.01; Figure 3A). Moreover, obtained data demonstrated that combining two drugs, Masitinib and Perifosine, Masitinib and Bortezomib, and all three drugs, resulted in a significant reduction in PD-L1 expression compared to Bortezomib treatment alone (p<0.01; Figure 3A). As another gene involved in immune evasion, PVR demonstrated considerable downregulation in a single treatment of U266B1 cells with Masitinib and Perifosine (p<0.01; Figure 3B). Furthermore, only treatment with Perifosine and Bortezomib and a combination of three drugs indicated a significant decrease (p<0.01; Figure 3B). However, no significant differences were observed among other treatment groups.

IL-6, as a pleiotropic cytokine, is essential for the growth and survival of malignant plasma cells and also showed immune suppression characteristics in the tumor microenvironment. The data demonstrated a significant reduction in all groups following exposure to Masitinib, Perifosine, and Bortezomib except for the group treated individually with Masitinib compared to control group (p<0.0001; Figure 3C). Regarding the results, most reductions in the IL-6 expression were observed in the combination of Perifosine and Bortezomib and using the three drugs altogether. However, no significant differences were noted among the groups that exhibited reduced IL-6 expression (Figure 3C).

Following Treatment whit Masitinib, Perifosine, and Bortezomib

Certain growth-promoting factors, including IL-6, are vital for the survival of multiple myeloma cells such as U266B1, which can produce this cytokine. IL-6 concentrations in the supernatant of U266B1 cells were measured 72 hours after exposure to Masitinib, Perifosine, and Bortezomib using ELISA. The results mirrored those from the mRNA expression analysis, showing a significant reduction in all groups, except for the Masitinib-only treatment in comparison to the control group (p<0.0001; Figure 4). Nonetheless, there were no significant differences observed between the groups in which the reduction of IL-6 concentration was demonstrated. (Figure 4).

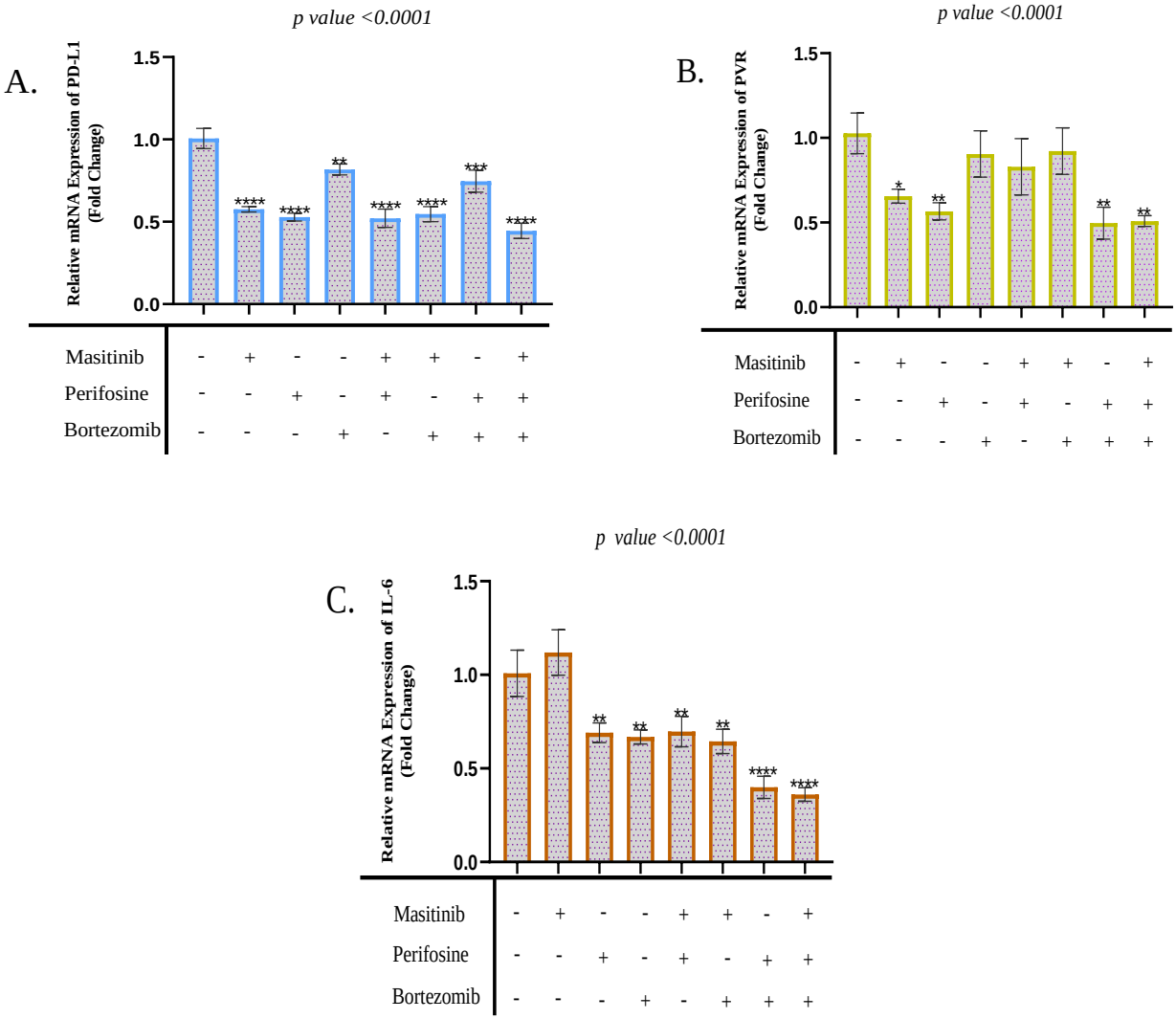


Figure 3. Effects of c-Kit receptor, AKT, and NF-kB inhibitors on the expression of programmed death-ligand 1 (PD-L1), poliovirus receptor (PVR) and interleukin 6 (IL-6). U266B1 cells were cultured in the absence or presence of Masitinib, Perifosine, and Bortezomib for 72 hours either in single or combined treatment. After that, total RNA was extracted, and single-stranded cDNA was synthesized. Real-time PCR was performed with specific primers for PD-L1, PVR, and IL-6. A) Relative mRNA transcript levels of PDL1. B) Relative mRNA transcript levels of PVR. C) Relative mRNA transcript levels of IL-6. Gene expression results are represented as mean±SD of the Pfaffl method after normalization with β-actin as an internal control. One-way ANOVA with Dunnett's post hoc test was used for multiple comparisons between treated groups and the untreated group. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

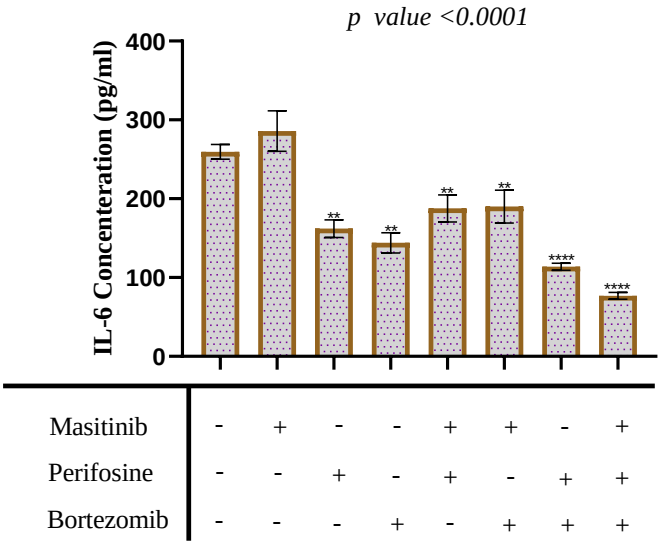


Figure 4. Effects of c-Kit receptor, AKT, and NF-kB inhibitors on interleukin (IL-6) concentration of U266B1 culture media. U266B1 cells were cultured in the absence or presence of Masitinib, Perifosine, and Bortezomib for 72 hours either in single or combined treatment. The concentration of IL-6 was measured by ELISA. The results are expressed as the Mean ± SD. One-way ANOVA with Dunnett's post hoc test was used for multiple comparisons between treated groups and the untreated group. ** p<0.01; *** p<0.001; **** p<0.0001.

DISCUSSION

A novel and potentially effective strategy for cancer treatment involves blocking signaling pathways using small molecule inhibitors. Cancer cells evade immune elimination through disrupting effector lymphocytes, recruitment of cells with immunosuppressive phenotypes, and secreting soluble mediators, all of which create barriers against the immune system.[15] Cancerous plasma cells could disrupt the immune system by expressing immune checkpoint molecules on the surface of immune cells and their ligands on multiple myeloma cells, such as PD-1/PD-L1 and TIGIT/PVR, and establishing a network of soluble mediators including IL-6, TGF-β, and IL-10.[20] Given the limited information on the blockade of signaling pathways and immune evasion, we investigated the connections between the inhibitors of c-Kit receptor, AKT, and NF-κB in regulating immune checkpoint ligand expression in U266B1, a multiple myeloma cell line. Our data demonstrate that the proliferation of U266B1 cells was reduced following combinational treatment with Masitinib, Perifosine, and Bortezomib as inhibitors of c-Kit receptor, AKT, and NF-κB respectively. Additionally, co-treatment with Masitinib and Perifosine resulted in increased apoptosis. Moreover, our findings revealed that the expression of PD-L1, PVR, and IL-6 was diminished after combinational treatment with Masitinib, Perifosine, and Bortezomib.

Among immune checkpoints, various studies have reported that PD-L1 is expressed on multiple myeloma cells but not on normal plasma cells.[21] The increased expression of PD-L1 contributes to immune cells exhaustion, providing biological advantages to multiple myeloma cells, such as increased proliferation and upregulation of proteins that prevent apoptosis and drug resistance.[16]

Studies have shown that JAK2-STAT3 and MEK 1/2 induce PD-L1 expression on the surface of malignant plasma cells through IL-6 signaling.[22] Additionally, stimulation of plasma cells from multiple myeloma patients with IFN-γ and TLR ligands resulted in the upregulation of PD-L1 via MEK/ERK and MyD88 pathways, respectively.[23] Furthermore, PD-L1 expression in multiple myeloma cells was induced through MEK/ERK pathway following stimulation with APRIL.[24] Our results showed that the single or combined use of inhibitors targeting c-Kit receptor, AKT, and NF-κB led to downregulation of PD-L1 expression in the U266B1 cell line without prior stimulation with cytokines. Moreover, other studies have indicated that aberrant signaling pathways,

including PI3K/AKT/mTOR, BTK and NF-κB pathways result in upregulated PD-L1 expression.[25-27] In the same direction, PVR on the surface of cancer cells can bind to various molecules, such as TIGIT on immune cells in the tumor microenvironment, especially $CD8^+$ T and NK cells.[28] The presence of ITIM and inhibitory motifs in TIGIT leads to the activation of PI3K and NF-κB signaling pathways, which in turn reduces the immune response.[29] Multiple myeloma cells also express PVR through multiple signaling pathways. A study demonstrated that treating myeloma cell lines with DNA-toxic and DNA-damaging substances led to increased PVR expression, while the use of PI3K inhibitor reduced PVR expression.[17,30] In another study, stimulation of fibroblasts with fibroblast growth factor or the presence of mutations in RAS ultimately led to the activation of MEK/ERK messengers, eventually increasing PVR expression.[31] Additionally, other reports indicate that stimulation of dendritic cells, neutrophils, and macrophages with TLR4 agonists such as LPS increased the expression of PVR, which can be accomplished through the activation of NF-κB.[32,33] Furthermore, PVR overexpression was observed in treating melanoma cell lines with IFN-γ.[34]

Our findings showed that inhibition of c-Kit receptor and AKT alone led to a decreased PVR expression. Moreover, simultaneous inhibition of AKT and NF-κB, as well as the combined use of c-Kit receptor, AKT, and NF-κB inhibitors downregulated PVR expression. It demonstrated the role of the signaling pathways in PVR expression. However, we did not observe a significant reduction in PVR levels with the sole use of the NF-κB inhibitor or the combination of c-Kit receptor and AKT or NF-κB inhibitors. This lack of effectiveness may be attributed to the interconnected nature of these signaling networks, which are subject to complex cross-talk and feedback loops. Further studies are warranted to elucidate the cross-talk and feedback mechanisms following treatment with these signaling pathway inhibitors.

On the other hand, cancer plasma cells communicate with the stromal cells of the bone marrow to create a network of cytokines, chemokines, and other soluble molecules that promote growth, survival, and the immune system suppression.[35] IL-6 plays a vital role in the bone marrow microenvironment, acting as a growth factor for activated B-cells and differentiation towards the plasma cells.[19] Some studies have demonstrated an increased production of IL-6 in many cancers, including multiple myeloma.[36,37] Previous studies have shown that NF-κB activation in cancerous plasma cells can lead to the overexpression of IL-6.[38,39] Furthermore, AKT causes the phosphorylation and degradation of IκBα and activation of NF-κB as an IL-6 transcription factor.[40] Additionally, inhibiting ERK with in the RAS/MAPK/ERK pathway has been shown to downregulate IL-6 expression.[41] Our data showed a significant reduction in in both the expression and secretion of IL-6 by U266B1 cells with either the single-use or combination of c-Kit receptor, AKT, and NF-κB inhibitors. However, we did not observe a significant reduction in IL-6 expression with a sole use of the c-Kit receptor inhibitor. This suggests that the downstream signaling pathways leading to the expression of IL-6 may be activated through the MAPK and AKT pathways, thereby diminishing the impact of c-Kit receptor inhibition at an upstream point in these signaling cascades.

The main advantage of our study was to evaluate the effects of three approved signaling pathway inhibitors including Masitinib as a c-Kit receptor inhibitor, Perifosine as an AKT inhibitor, and Bortezomib as an NF-κB inhibitor, on the expression of PDL-1, PVR and IL-6 which are key inhibitory molecules applied by plasma cells. However, it should be noted that investigation on primary tumoral plasma cells isolated from a number of patients with multiple myeloma could bring better and more comprehensive results. Moreover, it would be informative to apply immunoassay techniques such as immunoblot and immunofluorescence methods to monitor the activation of the down-stream adaptor molecules and to explore the protein expression of the checkpoint molecules and confirm the mRNA expression results. Unfortunately, due to our limitations, we were unable to perform these complementary experiments.

In conclusion, our findings indicate that the expression of PD-L1, PVR, and IL-6 is significantly downregulated following either single or co-treatment with signaling pathways inhibitors, including Masitinib (c-Kit receptor inhibitor), Perifosine (AKT inhibitor), and Bortezomib (NF-κB inhibitor). These pathway inhibitors demonstrated not only cytotoxic effects but also the potential to disrupt the mechanisms through which multiple myeloma cells evade the immune system. However, further investigation is necessary using different multiple myeloma cell lines to comprehend the role of signaling pathways in the immune evasion. Combination treatment approaches to block these pathways might be a promising therapeutic

approach for multiple myeloma patients via interfering with immune evasion mechanisms.

STATEMENT OF ETHICS

This study was found to be ethically acceptable by the Ethical Committee of Mazandaran University of Medical Sciences (IR.MAZUMS.IMAMHOSPITAL.REC.1399.051).

FUNDING

This study was supported by Mazandaran University of Medical Sciences. Project number 8118.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

The authors thank the investigators and study teams involved in this project.

Data Availability

Upon reasonable request (specify contact method)

AI Assistance Disclosure

If applicable, list any artificial intelligence (AI) tools used in preparing this manuscript.

REFERENCES

1. Kumar SK, Rajkumar V, Kyle RA, van Duin M, Sonneveld P, Mateos M-V, et al. Multiple myeloma. Nat Rev Dis Primers. 2017;3(1):17046.
2. Röllig C, Knop S, Bornhäuser M. Multiple myeloma. Lancet (London, England). 2015;385(9983):2197-208.
3. Rajkumar SV. Treatment of multiple myeloma. Nat Rev Clin Oncol. 2011;8(8):479-91.
4. Kazandjian D. Multiple myeloma epidemiology and survival: A unique malignancy. Sem Oncol. 2016;43(6):676-81.
5. Palumbo A, Anderson K. Multiple myeloma. New England J Med. 2011;364(11):1046-60.
6. Anderson KC, Carrasco RD. Pathogenesis of myeloma. Annual Rev Pathol. 2011;6:249-74.
7. Hideshima T, Anderson KC. Signaling Pathway Mediating Myeloma Cell Growth and Survival. Cancers. 2021;13(2).
8. Ashman LK, Griffith R. Therapeutic targeting of c-KIT in cancer. Expert Opin Investig Drugs. 2013;22(1):103-15.
9. Abbaspour Babaei M, Kamalidehghan B, Saleem M, Huri HZ, Ahmadipour F. Receptor tyrosine kinase (c-Kit) inhibitors: a potential therapeutic target in cancer cells. Drug Des Devel Ther. 2016;10:2443-59.
10. Keane NA, Glavey SV, Krawczyk J, O'Dwyer M. AKT as a therapeutic target in multiple myeloma. Expert Opin Ther Targets. 2014;18(8):897-915.
11. Matthews GM, de Matos Simoes R, Dhimolea E, Sheffer M, Gandolfi S, Dashevsky O, et al. NF-κB dysregulation in multiple myeloma. Sem Cancer Biol. 2016;39:68-76.
12. Vrábel D, Pour L, Ševčíková S. The impact of NF-κB signaling on pathogenesis and current treatment strategies in multiple myeloma. Blood Rev. 2019;34:56-66.
13. Abramson HN. The Multiple Myeloma Drug Pipeline-2018: A Review of Small Molecules and Their Therapeutic Targets. Clin Lymphoma Myeloma Leuk. 2018;18(9):611-27.
14. Kobayashi Y, Lim SO, Yamaguchi H. Oncogenic signaling pathways associated with immune evasion and resistance to immune checkpoint inhibitors in cancer. Sem Cancer Biol. 2020;65:51-64.
15. Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. Curr Opin Immunol. 2014;27:16-25.
16. Tamura H. Expression, Functions, and Treatment Target of PD-L1 (B7-H1) in Multiple Myeloma. J Immunol Sci. 2018.
17. Fionda C, Abruzzese MP, Zingoni A, Soriani A, Ricci B, Molfetta R, et al. Nitric oxide donors increase PVR/CD155 DNAM-1 ligand expression in multiple myeloma cells: role of DNA damage response activation. BMC Cancer. 2015;15(1):17.
18. Tsukamoto H, Fujieda K, Senju S, Ikeda T, Oshiumi H, Nishimura Y. Immune-suppressive effects of interleukin-6 on T-cell-mediated anti-tumor immunity. Cancer Sci. 2018;109(3):523-30.
19. Lauta VM. A review of the cytokine network in multiple myeloma. Cancer. 2003;97(10):2440-52.
20. Parrish C, Scott GB, Cook G. Immune dysfunction in multiple myeloma. Int J Hematol Oncol. 2013;2(1):49-59.
21. Tremblay-LeMay R, Rastgoo N, Chang H. Modulating PD-L1 expression in multiple myeloma: an alternative strategy to target the PD-1/PD-L1 pathway. J Hemato Oncol. 2018;11(1):46.

22. Tamura H, Ishibashi M, Yamashita T, Tanosaki S, Okuyama N, Kondo A, et al. Marrow stromal cells induce B7-H1 expression on myeloma cells, generating aggressive characteristics in multiple myeloma. Leukemia. 2013;27(2):464-72.
23. Iwasa M, Harada T, Oda A, Bat-Erdene A, Teramachi J, Tenshin H, et al. PD-L1 upregulation in myeloma cells by panobinostat in combination with interferon-γ. Oncotarget. 2019;10(20):1903-17.
24. An G, Acharya C, Feng X, Wen K, Zhong M, Zhang L, et al. Osteoclasts promote immune suppressive microenvironment in multiple myeloma: therapeutic implication. Blood. 2016;128(12):1590-603.
25. Yi M, Niu M, Xu L, Luo S, Wu K. Regulation of PD-L1 expression in the tumor microenvironment. J Hematol Oncol. 2021;14(1):10.
26. Taghiloo S, Norozi S, Asgarian-Omran H. The effects of Pi3k/Akt/Mtor signaling pathway inhibitors on the expression of immune checkpoint ligands in acute myeloid leukemia cell line. Iran J Allergy Asthma Immunol. 2022;21(2):178-88.
27. Soltanshahi M, Taghiloo S, Asgarian-Omran H. Expression Modulation of Immune Checkpoint Molecules by Ibrutinib and Everolimus Through STAT3 in MCF-7 Breast Cancer Cells. Iran J Pharmaceutical Res. 2022;21(1).
28. Kučan Brlić P, Lenac Roviš T, Cinamon G, Tsukerman P, Mandelboim O, Jonjić S. Targeting PVR (CD155) and its receptors in anti-tumor therapy. Cell Mol Immunol. 2019;16(1):40-52.
29. Liu L, You X, Han S, Sun Y, Zhang J, Zhang Y. CD155/TIGIT, a novel immune checkpoint in human cancers (Review). Oncol Rep. 2021;45(3):835-45.
30. Soriani A, Zingoni A, Cerboni C, Iannitto ML, Ricciardi MR, Di Gialleonardo V, et al. ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. Blood. 2009;113(15):3503-11.
31. Hirota T, Irie K, Okamoto R, Ikeda W, Takai Y. Transcriptional activation of the mouse Necl-5/Tage4/PVR/CD155 gene by fibroblast growth factor or oncogenic Ras through the Raf-MEK-ERK-AP-1 pathway. Oncogene. 2005;24(13):2229-35.
32. Kamran N, Takai Y, Miyoshi J, Biswas SK, Wong JS, Gasser S. Toll-like receptor ligands induce expression of the costimulatory molecule CD155 on antigen-presenting cells. PloS one. 2013;8(1):e54406.
33. Pende D, Castriconi R, Romagnani P, Spaggiari GM, Marcenaro S, Dondero A, et al. Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: relevance for natural killer-dendritic cell interaction. Blood. 2006;107(5):2030-6.
34. Inozume T, Yaguchi T, Furuta J, Harada K, Kawakami Y, Shimada S. Melanoma Cells Control Antimelanoma CTL Responses via Interaction between TIGIT and CD155 in the Effector Phase. J Invest Dermatol. 2016;136(1):255-63.
35. Rosean TR, Tompkins VS, Tricot G, Holman CJ, Olivier AK, Zhan F, et al. Preclinical validation of interleukin 6 as a therapeutic target in multiple myeloma. Immunol Res. 2014;59(1-3):188-202.
36. Frassanito MA, Cusmai A, Iodice G, Dammacco F. Autocrine interleukin-6 production and highly malignant multiple myeloma: relation with resistance to drug-induced apoptosis. Blood. 2001;97(2):483-9.
37. Cao Y, Luetkens T, Kobold S, Hildebrandt Y, Gordic M, Lajmi N, et al. The cytokine/chemokine pattern in the bone marrow environment of multiple myeloma patients. Exp Hematol. 2010;38(10):860-7.
38. Lee C, Oh JI, Park J, Choi JH, Bae EK, Lee HJ, et al. TNF α mediated IL-6 secretion is regulated by JAK/STAT pathway but not by MEK phosphorylation and AKT phosphorylation in U266 multiple myeloma cells. Bio Med Res Int. 2013;2013:580135.
39. Xiao W, Hodge DR, Wang L, Yang X, Zhang X, Farrar WL. NF-kappaB activates IL-6 expression through cooperation with c-Jun and IL6-AP1 site, but is independent of its IL6-NFkappaB regulatory site in autocrine human multiple myeloma cells. Cancer Biol Ther. 2004;3(10):1007-17.
40. Hsu J-h, Shi Y, Hu L, Fisher M, Franke TF, Lichtenstein A. Role of the AKT kinase in expansion of multiple myeloma clones: effects on cytokine-dependent proliferative and survival responses. Oncogene. 2002;21(9):1391-400.
41. Gerlo S, Haegeman G, Vanden Berghe W. Transcriptional regulation of autocrine IL-6 expression in multiple myeloma cells. Cell Signall. 2008;20(8):1489-96.