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Treatment by PI3K/mTOR Inhibitor BEZ235 Combined with TLR-7/8 Agonist Interfere with Immune Evasion Mechanisms of WEHI-3 Mouse Leukemia Cells

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ABSTRACT

Background: Several PI3K/Akt/mTOR pathway inhibitors and TLR agonists induce tumor cell death. However, the mechanisms of these therapeutic approaches in acute myeloid leukemia (AML) cells are still unknown.

Objectives: To investigate the effects of BEZ235, as a dual inhibitor of PI3K and mTOR pathways, and TLR7/8 agonist R848 on the expression and regulation of the immune inhibitory molecules in myeloid leukemia cells.

Methods: WEHI-3 leukemia cells were incubated with dual PI3K and mTOR inhibitor BEZ235 and TLR7/8 agonist R848 for 48 hrs. Firstly, cell viability was assessed by MTT method. The semi-quantitative relative mRNA expression of Galectin-9 (Gal-9), PD-L1, PVR, and STAT3 was assessed according to HPRT as a housekeeping gene. Finally, the protein expression of phosphorylated STAT3 was evaluated by western blotting analysis.

Results: WEHI-3 cells showed growth inhibition following treatment with BEZ235 and R848 whose combination exerted more proliferation arrest. The mRNA expression of Gal-9, PD-L1 and PVR immune checkpoint molecules significantly reduced in treated cells with BEZ235 and R848. Combined treatment indicated more reduction compared with the single treatment. Finally, the expression and phosphorylation of STAT3 were down-regulated after a single or dual treatment with BEZ235 and R848.

Conclusion: Our results conclude that treatment with the combination of BEZ235 and R848 interferes with immune evasion mechanisms through STAT3-signaling pathway in WEHI-3 leukemia cells.

Keywords: Immune evasion, TLR7/8 agonist, PI3K/mTOR, AML, BEZ235, R848

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INTRODUCTION

Acute myeloid leukemia (AML) is defined as a clonal disorder of malignant myeloid progenitor cell proliferation resulting in impairments in normal cell differentiation, causing abnormal accumulation of immature myeloid precursor cells in the peripheral blood and bone marrow (1). The disease is highly heterogeneous in terms of clinical presentation, genetics, and phenotypic characteristics, which influence the therapeutic effects, prognosis, and eventually, overall survival (2). Although most AML patients respond well to initial therapy, a remarkable number of patients are correlated with a high risk of relapse (3). For decades, AML had a poor prognosis, and was lethal due to the lack of an effective therapeutic strategy (1). Nowadays, the standard therapeutic strategies for AML are hematopoietic stem cell transplantation accompanied with chemotherapy using a combination of cytotoxic drugs like cytarabine and anthracyclines for killing the highly proliferating tumor cells (4, 5). However, the side effects including infection, bleeding, and anemia are still the major post-therapeutic complications in AML patients (6). Many investigations have been conducted in an attempt to discover novel medicinal medications, principally from natural products or synthesized compounds that prevent the development of malignant cells (1, 7).

The host immune system plays a dynamic role in the detection, eradication, as well as progression of tumors (8). In AML, leukemic blasts could escape the host immune responses through numerous immune escape mechanisms, like up-regulation of immune checkpoint ligands such as programmed death-ligand 1 (PD-L1), PD-L2, poliovirus receptor (PVR; CD155), and Galectin-9 (Gal-9) (8, 9). The exhaustion process of infiltrated T cells is caused by the cross-linking of the checkpoint receptors with their ligands which is linked to diminished T cells function and tumor development (10). New immunotherapy approaches for AML patients have widely concentrated on tumor escape from immune responses (11). However, the precise mechanisms and signaling pathways involved in the up-regulation of immune checkpoint molecules are unclear and need more investigation. In this regard, the constitutive activation of signal transduction pathways that lead to leukemic cell proliferation, survival, and drug resistance is often associated with an inferior prognosis because of multiple genetic aberrations like chromosomal alterations or gene mutations (12, 13). Among different pathways, two of the most common dysregulated signaling pathways in leukemia are PI3K/Akt/mTOR and STAT3 pathways (13, 14). Previous reports have shown the constitutive activation of the PI3K/AKT/mTOR axis in nearly 50–80% of AML patients, suggesting it as a potential therapeutic target (15). Accordingly, numerous inhibitors of these pathways have been discovered, some of them are now being tested in clinical trials for AML and some other tumors (16). In addition, continuously phosphorylated STAT3 (pSTAT3) is reported in approximately 70% of both human solid and hematologic malignancies (17). For activation and dimerization of STAT3, phosphorylation of the tyrosine705 (Tyr_{705}) and serine727 (Ser_{727}) residues are required (17). Accordingly, the STAT3 protein plays a vital role in proliferation, cell differentiation, inflammatory response, immunity, and survival. Persistent STAT3 activation induces progression of the cell cycle, metastasis, and tumor invasion (18, 19). On the other hand, previous studies suggest that Toll-like receptors (TLRs) are expressed by various hematological malignant cells (20). In this context, TLR-2, TLR-4, TLR-7, TLR-8, and TLR-9 are expressed in leukemic cells obtained from AML patients and different myeloid cell lines, which are correlated with both resistance to chemotherapy and monocytic differentiation (21). Accordingly, agonists for TLR-3, TLR-4, TLR-7/8, and TLR-9 are among the most promising

immunotherapeutic agents for AML therapy (22). TLR agonists exert anti-tumor indirect effects by stimulating immune response to suppress tumor growth and direct pro-apoptotic and killing activities against various tumors, including AML cells (23).

Although several reports have shown that PI3K/Akt/mTOR pathway inhibitors and several TLR agonists have caused tumor cell death by inducing apoptosis in a variety of solid and hematopoietic malignancies, the alternative mechanisms of these therapeutic strategies in leukemia especially AML are still unknown. The current study has investigated the anti-leukemic effects of BEZ235, as a double inhibitor of PI3K and mTOR, and TLR7/8 agonist on the expression of the immune checkpoint molecules as one of the most important mechanisms of immune evasion in WEHI-3 mouse leukemic cells.

MATERIALS AND METHODS

Chemical Compounds and Reagents

Dual PI3K/mTOR inhibitor BEZ235 (Dactolisib) and TLR-7/8 agonist R848 (Resiquimod) were purchased from InvivoChem (Illinois, United States). These drugs were dissolved in cell culture grade DMSO (Sigma-Aldrich, Missouri, USA) to a stock concentration of 2.1 mmol/L, and 15.9 mmol/L, respectively, and stored frozen in aliquots. Cytarabine (Alexan 100mg/5ml) was purchased from EBEWE Pharma Company (Unterach am Attersee, Austria) as a conventional chemotherapy medication treatment of AML, with a stock concentration of 82.23 mmol/L.

Mouse Myeloid Cell Line and Culture

The mouse WEHI-3 leukemic cell line was first identified in 1969 and exhibited acute myelomonocytic leukemia features (24). The WEHI-3 cell was obtained from the Biological Resource Center of Iran (Tehran, Iran). The cells were maintained in a complete growing DMEM medium (Biowest, Nuaille, France), containing 10% heat-inactivated fetal bovine serum (Biowest, Nuaille, France), 100 units/ml penicillin, and 100 µg/ml streptomycin (Biowest, Nuaille, France) in 75 cm^2 culture flasks (SPL Life Sciences, South Korea). Culture flasks were incubated at 37°C with 5% CO_2 in a humidified incubator (Binder, Tuttingen, Germany).

Colorimetric Cytotoxic Assay

The cytotoxic effect of BEZ235, R848, and cytarabine was evaluated using the colorimetric MTT assay. Briefly, 15000 cells of WEHI-3 leukemic cells were seeded into a 96-well flat-bottom culture microplate. The cells were treated with various concentrations of BEZ235, cytarabine (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 and 2048 nM), and R848 (5, 10, 20, 40, 80, 160, 320, 640, and 1280 µM) for 24 and 48 hours. Subsequently, 20 µl of MTT reagent (5 mg/mL) was added to each well and incubated for 4 hours at 37°C. The microplates were centrifuged for 10 min at 300 g, and formazan crystals were dissolved in 150 µL of DMSO by shaking microplates. The optical density (OD) of each well was measured at 570 and 630 nm using ELISA plate reader (Synergy H1 BioTek, Winooski, USA). The potency of cell growth inhibition for all agents was expressed as the half-maximal inhibitory concentration (IC50) values. Then, the proliferation of WEHI-3 cells was evaluated via MTT assay following treatment with BEZ235 and R848. In addition, cytarabine, a conventional chemotherapy medication and 0.1% DMSO were applied as positive and negative controls, respectively. All experiments were conducted in triplicates. Finally, the relative cell proliferation index was calculated by dividing the mean ratio of OD values of each group to OD values obtained from the control group.

Western Blotting Analysis

To measure the effects of BEZ235 and R848 on protein levels of pSTAT3-Ser727, WEHI-3 cells were incubated with the optimal concentration of these drugs for 48

hours. Subsequently, after the incubation, cells were harvested and lysed to prepare the whole protein extracts. Briefly, cells were homogenized with 100 µL of RIPA buffer (Santa Cruz Biotechnology Inc., California, USA) containing a protease inhibitor, PMSF, sodium orthovanadate, and phosphatase inhibitor cocktail (Sigma-Aldrich, Missouri, USA) based on the manufacturer's instruction. Lysates were centrifuged at 10,000 g for 10 min at 4 °C, and the protein samples were collected and the supernatants were kept at -80°C. The protein amount was measured applying the BCA protein assay kit (Kalazist, Tehran, Iran). Equal quantities of total protein (40 µg) were separated by electrophoresis in 10% SDS-PAGE gel electrophoresis based on their molecular weight (Bio-Rad, California, USA). The separated proteins were transferred and blotted to a PVDF paper membrane (Merck Millipore, Darmstadt, Germany) in a tank transfer system (Bio-Rad, California, USA), and were blocked in Tris-buffered saline containing 4% BSA and 0.1% tween 20 (TBST buffer) for 1 hour at room temperature. The blocked membranes were washed with TBST, and subsequently incubated with primary antibodies including rabbit anti-phospho-STAT3 (Ser727) antibody (Cell Signaling Technology, Massachusetts, USA), and mouse anti-β-actin antibody as the internal control (Santa Cruz Biotechnology Inc., California, USA) at 4 °C overnight. The following day, the membranes were washed 5 times with TBST, and then were incubated with the suitable secondary antibodies: HRP-labeled goat anti-rabbit IgG antibody and goat anti-mouse IgG antibody (both from Sigma-Aldrich, Missouri, USA) for 2 hours at room temperature. The membranes were then washed with TBST and the protein bands were developed and detected employing a chemiluminescence blotting substrate kit (ECL kit; CMG, Isfahan, Iran). A chemiluminescence image analyzer system (G:BOX instrument; Syngene, Cambridge, UK) was applied to view the developed membranes. The obtained results were expressed in standard units and the Gene Tools software (Syngene, Cambridge, UK) was used to measure the bands' intensity.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from WEHI-3 cells using the DenaZist Asia RNA extraction kit (Mashhad, Iran) and quantified using a nano-spectrophotometer (WPA, Cambridge, England). Complementary DNA (cDNA) was reverse-transcribed from 1 µg of total RNA in a 20µl reaction mixture containing 4µl of 5X first-strand buffer, 1µl random hexamer primer, 1µl dNTP 10mM, 0.5µl RNasein 40u/µl, 1µl M-MLV RT enzyme, and appropriate RNase/DNase water. The mixture was then incubated at 70°C for 5 min, 37°C for 1 hour, and 70°C for 5 min using the Yekta-Tajhiz cDNA synthesis kit (Tehran, Iran). Primers were obtained from metabion international AG (Planegg, Germany) and their sequences are shown in Table 1. Quantitative Real-Time PCR assays for STAT3, PD-L1, Galectin-9, PVR, as well as the reference gene hypoxanthine-guanine phosphoribosyl transferase (HPRT), were run for all groups using StepOne Real-Time PCR System (Applied Biosystems, California, USA) using SYBR green detection dye (Yekta-Tajhiz, Tehran, Iran). The cycles were set at 1 cycle of 94°C for 5 min, 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Each run was completed with a melting curve analysis to confirm the absence of the primer dimers and the amplification specificity. In addition, the primer efficiencies were confirmed by serial dilution standard curves. The Ct values were determined for each sample by comparing the values of the target gene with those of the HPRT constitutive gene products. Finally, the relative mRNA expression of STAT3, PD-L1, Galectin-9, and PVR were calculated via the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Experimental data are represented

Table 1. Primers used for Real-Time PCR quantification.

Gene	Primers (5'-3')	Product Size (bp)
PD-L1	F: CGGACTACAAGCGAATCA R: GTTGGTGGTCACTGTTTG	156 bp
Galectin-9	F: CCAGCCTATACCATACCTT R: GGACAACAGCATTCTCATT	175 bp
PVR	F: TGACGGCAACTGGTATGT R: TACGGTGGAGATTAGAAGCATA	160 bp
STAT3	F: GAGTCAAGATTGGGCATA R: TTGGCTTCTCAAGATACC	179 bp
HPRT	F: GGGATTTGAATCACGTTTGTG R: TTACTGGCAACATCAACAGG	114 bp

F, forward primer; R, reverse primer

as mean±SD. Statistical analysis was accomplished using GraphPad Prism 6 (San Diego, CA, USA). Data were analyzed using the Kolmogorov-Smirnov test for determining the normality distribution. One-way ANOVA was used for the comparisons between the treated and untreated groups and the post hoc Tukey's *b*-test for multiple comparisons. P-values of less than 0.05 ($P<0.05$) were considered statistically significant as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$.

RESULTS

Cell Viability of WEHI-3 Leukemic Cells Following Treatment with BEZ235 and R848

WEHI-3 cells were treated via increasing concentrations of BEZ235 and R848 for 24 and 48 hours. Obtained results from the cell viability experiments indicated that the 48 hours treatment is better than the 24 hours which was selected for further experiments. Our result showed that WEHI-3 cells indicate a dose-dependent decrease in viability after single treatment with increasing concentrations of BEZ235, cytarabine, and R848, with IC50 values of 132 nM, 124 nM, and 63 µM, respectively (Figure 1). Therefore, the anti-proliferative activity of BEZ235 and R848 evaluated via MTT assay, and then the relative cell proliferation index was calculated for all conditions. We have shown that the proliferation of WEHI-3 cells significantly decreased via treatment with BEZ235, R848, and cytarabine ($P<0.0001$, Figure 2). Combinational treatment with BEZ235, and R848 indicated more growth inhibition compared with single treatment (Figure 2).

The Expression of Immune Checkpoint Ligands on WEHI-3 Leukemia Cells Following Treatment with BEZ235 and R848

To more precisely elucidate the immune evasion mechanisms underlying the blocking of PI3K/mTOR pathway and TLR stimulation on WEHI-3 leukemia cells, gene transcription expression of some immune checkpoint molecules including PD-L1, Gal-9, and PVR on WEHI-3 cells was determined after treatment with dual PI3K/mTOR inhibitor BEZ235 and TLR-7/8 agonist R848. The relative expression of these molecules was investigated via a qRT-PCR assay using HPRT as a housekeeping internal control. It was found that the mRNA expression of PD-L1 gene transcript significantly down-regulated after treatment with both BEZ235, and R848 ($P<0.0001$), but not cytarabine ($P>0.05$), compared with untreated cells. Remarkably, PD-L1 expression reduced more after combinational treatment with BEZ235 and R848 ($P<0.0001$, Figure 3a). Similarly, our results showed that the expression of Gal-9 significantly decreased in WEHI-3 cells treated with BEZ235 ($P<0.0001$), R848 ($P=0.0007$), and more vigorously after combined treatment ($P<0.0001$, Figure 3b). Finally, regarding PVR, the relative gene

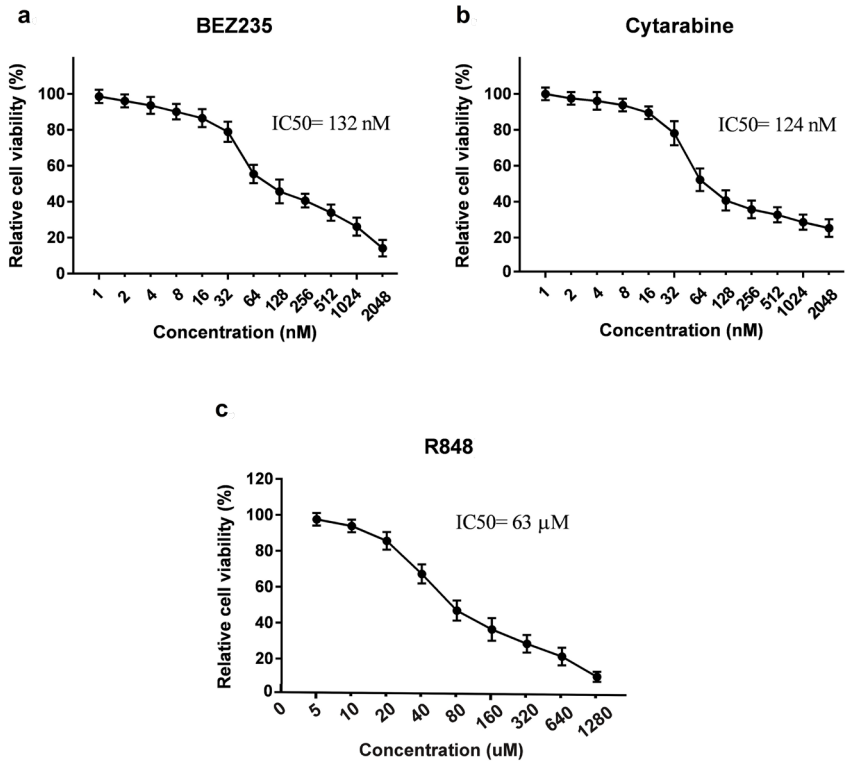

Figure 1. Determining the IC50 values of BEZ235, R848, and cytarabine. WEHI-3 leukemic cells were treated with increasing concentrations of BEZ235 (a), cytarabine (b), and R848 (c) for 48 hours. Cell viability was determined by measuring the absorbance at 570 nm after the addition of MTT reagent. IC50 value of BEZ235, cytarabine, and R848 were calculated to be 132 nM, 124 nM, and 63 µM, respectively. Data are presented as mean±SD.

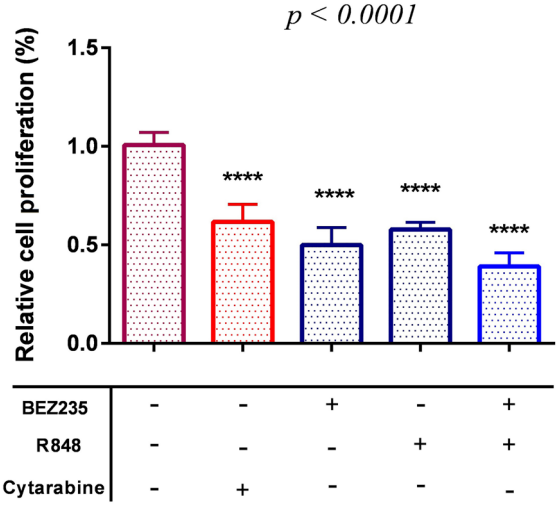

Figure 2. Effects of BEZ235, R848, and cytarabine on cell proliferation. WEHI-3 leukemic cells were treated with BEZ235 and R848 for 48 hours either in single or combination format. Cytarabine was also applied as a conventional chemotherapy drug. Cell proliferation capacity was evaluated by MTT assay. Data are presented as mean±SD. One-way ANOVA with Tukey's post hoc test was used for analyses. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

transcript expression significantly decreased after treatment with BEZ235 (P=0.003) and R848 (P=0.004), as well as with combined treatment compared with untreated cells (P<0.0001, Figure 3c). However, similar to PD-L1, the expression of Gal-9 and PVR was not different, following treatment with cytarabine (P>0.05, Figure 3b and 3c).

STAT3 Expression and Phosphorylation on WEHI-3 Leukemia Cells Treated with BEZ235 and R848

To better recognize the influence of STAT3 pathway on immune checkpoint ligands expression in WEHI-3 leukemia cells after blockade of PI3K/mTOR pathway and stimulation of TLR-7/8, we analyzed the effects of the BEZ235 and R848 on protein expression of Ser727-phosphorylated STAT3 by western blot method. As shown in Figure 4a, our data indicated that the single treatment with BEZ235 and R848

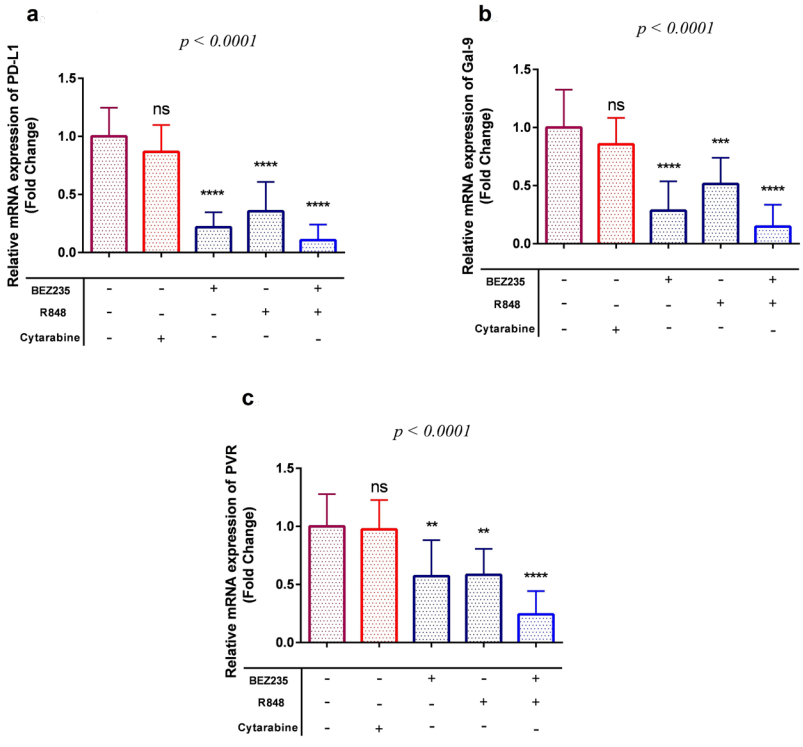

Figure 3. Effects of BEZ235, R848, and cytarabine on the expression of PD-L1, Galectin-9, and PVR. WEHI-3 cells were incubated with BEZ235 and R848 for 48 hours either in single or combination format. Cytarabine was also applied as a positive control. Total RNA was extracted and cDNA was synthesized. Real-Time PCR was done with specific primers for PD-L1, Galectin-9, PVR, and HPRT. Relative transcript levels of PD-L1 (a) Galectin-9 (b) and PVR (c) are shown. Gene expression results are represented as mean±SD of $2^{-\Delta\Delta Ct}$ after normalization with HPRT as an internal control. One-way ANOVA with Tukey's post hoc test was used for analyses. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

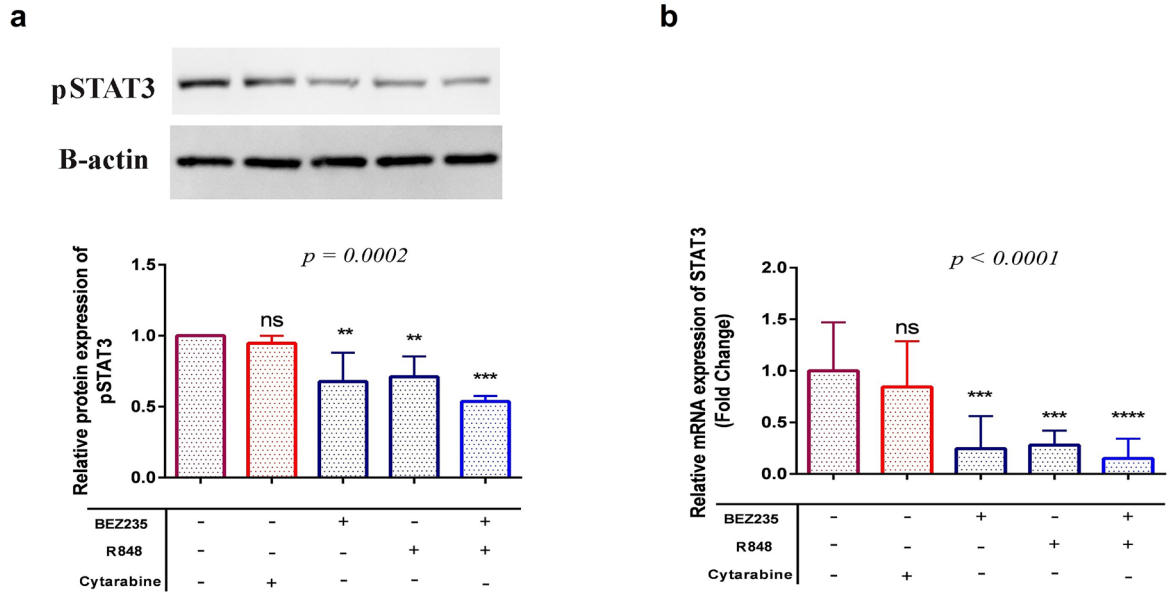

Figure 4. Effects of BEZ235, R848, and cytarabine on the expression and phosphorylation of STAT3. WEHI-3 cells were treated with BEZ235 and R848 for 48 hours either in single or combination format. Cytarabine was also applied as a positive control. a. Total protein was extracted and the p-STAT3 protein level was measured by western blot using β-actin as an internal control. b. Relative transcript mRNA level of STAT3 was quantified by Real-Time PCR assay. Data are presented as mean±SD. One-way ANOVA with Tukey's post hoc test was used for analyses. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

induced the down-regulation of pSTAT3 level (P=0.004, P=0.0100, respectively); however, combinational treatment induced a notable reduction in pSTAT3 level (P=0.0002). The expression of pSTAT3 protein did not significantly change after treatment with cytarabine (P>0.05, Figure 4a). We also determined the mRNA expression of STAT3 via qRT-PCR method. Our data showed that the STAT3 mRNA expression significantly reduced following treatment with BEZ235, and R848 as compared with untreated cells (P=0.0001 and P=0.0002, respectively). Interestingly, the down-regulation of STAT3 mRNA level was more remarkable in combinational treatment with BEZ235 and R848 (P<0.0001, Figure 4b). However, after treatment with cytarabine, the expression level of STAT3 reduced slowly but was not different (P>0.05, Figure 4b).

DISCUSSION

The immune system is commonly compromised in most patients with leukemia (8). In this context, in response to anti-tumor reactions, malignant cells utilize various mechanisms to evade immune elimination (9, 10). Accordingly, several immune checkpoint receptor/ligand systems such as PD-1/PD-L1, 2, CTLA-4/CD80, 86, Tim-3/Galectin-9, and TIGIT/PVR have been reported to associate with evasion from immune surveillance, immune cell exhaustion, disease progression, poor survival in hematological malignancies and several pathological situations (25-31). Therefore, the blockade of these pathways has been established as a potential strategy for cancer immunotherapy in the last few years; however, most of the treated patients had a poor response, suggesting the need for additional approaches (8, 9, 30). According to mounting evidence, effective reversal of tumor immune evasion requires approaches that combine potent immunostimulation, inhibition of immune checkpoints, and signaling pathway inhibitors (8, 9). PI3K/Akt/mTOR signaling pathway inhibitors and TLR agonists have recently become important for cancer therapy, and several previous studies on these approaches have mainly focused on the inhibition of tumor cell proliferation and disease progression. In the present investigation, we have evaluated the alternative therapeutic potential of PI3K/mTOR pathway inhibitor BEZ235 and TLR7/8 agonist R848 that control the expression of immune checkpoint ligands in WEHI-3 leukemia cell line. The data presented here clearly show that the combination treatment with BEZ235 and R848 could be a possible way for controlling the expression of immune checkpoint ligands. Furthermore, the obtained results have demonstrated that reduced STAT3 phosphorylation may contribute to modulating the expression of immune checkpoint molecules.

The PI3K/Akt/mTOR pathways over-activation involves AML pathogenesis, treatment resistance, and disease progression providing strong support for the therapeutic anti-cancer application of inhibitors for these pathways (13, 15, 32). Previous findings have highlighted that the specific inhibition of these pathways has anti-proliferative effects and induces apoptosis in AML (32, 33). Previous results together with our unpublished data have shown that the combinational blockade of these pathways, dual or triple, are more effective than single blockade (15, 33). In this regard, preclinical studies have shown that BEZ235 has beneficial pharmaceutical properties as an effective anti-leukemic agent in AML (15, 32, 33). However, other mechanisms may be attributed to the PI3K/Akt/mTOR pathway inhibitors. Our data have indicated down-regulation of Gal-9, PD-L1, and PVR in BEZ235 treated WEHI-3 cells. These results are in line with our unpublished study, showing that the expression of these immune checkpoint molecules decreases in the human AML cell line following treatment with PI3K/Akt/mTOR inhibitors including everolimus, idelalisib, and MK-2206. Based on these results, it could be speculated that

the PI3K/Akt/mTOR pathway inhibitors not only serve as cytotoxic agents but also control the immune checkpoint ligands expression and interfere with the immune escape mechanisms of AML cells. On the other hand, some studies have indicated that the PI3K/Akt/mTOR signaling pathway can positively activate the STAT3 kinase activity (34, 35). Surprisingly, the blockade of PI3K/Akt/mTOR signaling pathway is a potential therapeutic target in cancer therapy, but its inhibitors can cause resistance by activating STAT3, which provides scientific evidence for combining both PI3K/Akt/mTOR and STAT3 inhibitors (34).

Aberrant activation of STAT3 is extensively found in tumors, which is correlated with therapeutic resistance, poor prognosis, and short disease-free survival (36-39). STAT3 activation usually requires phosphorylation of tyrosine705 (Tyr705) and serine727 (Ser727) residues which is abnormally over-expressed in AML blast cells as well as the WEHI-3 cells (14). The mechanisms of this activation are unknown; however, it has been suggested that mutations in kinase receptors and autocrine IL-6 secretion can induce constitutive activation of STAT3 in AML cells (14, 40). As recently shown, STAT3 appears to play a role in AML cell proliferation and survival, but whether or not it contributes to immune evasion has not been definitely confirmed (40, 41). Targeting STAT3 has revealed their outstanding anti-tumor properties via decreasing the invasion activity of tumor cells (38, 39). Therefore, it is an attractive but challenging target for cancer therapy, because pharmacologic inhibition of non-enzymatic proteins has proved to be difficult (19, 42). Hence, blocking upstream kinase activity via small molecule inhibitors has provided an alternative approach instead of direct inhibition of STAT3 for AML therapy (43). Moreover, it has been assumed that inhibiting STAT3 activation may induce antitumor immunity due to the restoration of T cell responsiveness and loss of immune tolerance (39). In support of this concept, our data have demonstrated that BEZ235 reduces the expression of PD-L1, Gal-9, and PVR as a possible immune evasion mechanism on WEHI-3 cells through down-regulation of STAT3 expression and phosphorylation. In agreement with our findings, previous studies have also shown that STAT3 binds to PD-L1 promoter and increases PD-L1 transcription in various tumor cells (44). Taken together, these evidences to date, suggest that direct or indirect targeting of STAT3 might pave a new way for the prevention of tumor immune evasion and cancer therapy.

Considering the effective results obtained from combination therapies, and the importance of treatment with TLR agonists in cancer therapy, in this study we have decided to examine the combinational targeting of PI3K/mTOR and TLR7/8 pathways in AML cell line. The anti-tumor effects of TLR agonists are mediated by either direct effects on tumor cells or activation of immune responses to suppress tumors growth (4). Recent results indicated that TLR agonists induce tumor-selective apoptosis, cell cycle arrest, and employ growth-inhibitory effects in different types of tumor cells including AML (4, 45). Our results are in agreement with previous reports showing that TLR7/8 agonist R848 has a direct anti-leukemia activity especially the growth inhibition of WEHI-3 leukemic cells. Consistent with our results, Ignatz-Hoover et al. could also show that the TLR7/8 activation induces growth inhibition and AML differentiation, and considerably interferes with the growth of the human AML blasts in immunodeficient mice (46). Interestingly, these data suggest that TLR7/8 activation has direct anti-leukemic effects independent of its immune-modulating properties that are presently under examination for tumor treatment (46). However, some researchers have suggested that the anti-tumor effects of TLR agonists, as mentioned above, could be TLR-dependent or independent mechanisms (45). Notably, a recent report has revealed that treated tumor cells with TLR7/8 agonists show inhibition in cell proliferation by cell-

cycle arrest in G_1/S phase via opioid growth factor receptor pathway (47). Correspondingly, our data have shown that the STAT3 pathway is one of the mechanisms involved in this process. A recent report showed that impairment of STAT3 signaling in the tumor microenvironment (TME), together with the activation of TLR7/8 signaling, could produce efficient anti-tumor immunity. Therefore, simultaneous stimulation of TLR7/8 receptors with blockade of STAT3 pathway can be used as a potential therapeutic approach for cancer immunotherapy (48). On the other hand, TLR agonists are furthermore regarded as possible drug targets in AML due to their indirect anti-leukemic effects caused by the innate immune activation in the TME and breaking the immunosuppression process (49, 50). Following this concept, we have also indicated here that R848 disrupts the mechanisms of tumor escape, which contributes to the down-regulation of Gal-9, PD-L1, and PVR expression on WEHI-3 cells. Controversial reports have also indicated that TLR signaling may mediate immune suppressive effects via up-regulation of PD-L1 on tumor cells and then inhibition of CTLs (51, 52). The contributing factors of these inconsistencies are unidentified, although they might be attributable to the use of various experimental conditions. Nevertheless, our data have suggested that the down-regulation of PD-L1, Gal-9, and PVR is due to the R848 effect on the STAT3 expression and phosphorylation. However, the exact molecular interactions behind the TLR agonist effects are not known and deserve further studies. Our work together with previous studies suggests that the treatment based on TLR agonists alone might not always be efficient as a monotherapy, and hence comes the need for introducing combinatorial treatment (49, 53). Indeed, although the constitutive activation events for TLR and PI3K/Akt/mTOR signaling are independent, these signaling networks are closely related and are subject to complex cross-talk and feedback interactions (54). The combinational treatment with R848 and BEZ235 can further reduce the growth inhibition, and the expression of immune checkpoint ligands via the down-regulation of STAT3 expression and phosphorylation in WEHI-3 cells.

CONCLUSION

In conclusion, our data indicated the therapeutic potential of dual PI3K/mTOR inhibitors BEZ235 and TLR7/8 agonist R848 for the treatment of AML mediated by modulation of PD-L1, Gal-9, and PVR expression as immune-escape molecules and decreasing the STAT3 expression and phosphorylation. Finally, our results reinforce the idea of combinational-targeted PI3K/mTOR and TLR-7/8 pathways as a novel therapeutic strategy for AML. Nonetheless, more research is required to expand on our findings, and explore the therapeutic potential of this alternative.

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CONFLICT OF INTEREST

The authors state that no commercial or financial relationships influenced their research. We shall immediately declare that none of the authors listed on the manuscript work for a state agency that serves a core aim other than research and/or education. We furthermore assert that none of the authors are functioning as an official representative or on state government in posting this manuscript.

AVAILABILITY OF DATA AND MATERIAL

The datasets that support the findings of this

study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

HA-O conceived the original idea and designed the experiments. ST performed the experiments and wrote the manuscript. RA-N performed statistical analyses. AA and EZ conceived the study. HA-O edited the manuscript and approved the final draft. The ultimate manuscript was reviewed and confirmed by all authors.

ETHICAL APPROVAL

The Ethical Committee of Mazandaran University of Medical Sciences deemed this study to be morally permissible (IR.MAZUMS.IMAMHOSPITAL.REC.1398.143).

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