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The Effects of PI3K/Akt/mTOR Signaling Pathway Inhibitors on the Expression of Immune Checkpoint Ligands in Acute Myeloid Leukemia Cell Line

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ABSTRACT

Up-regulation of immune checkpoint ligands is considered as one of the most important immune escape mechanisms in acute myeloid leukemia (AML). Herein, we investigate a relationship between the inhibition of PI3K/Akt/mTOR signaling pathways and the regulation of immune checkpoint ligands in AML cells.

The HL-60 cell line was treated with idelalisib as PI3K inhibitor, MK-2206 as Akt inhibitor, and everolimus as mTOR inhibitor either in a single or combined format. Cell viability and apoptosis were evaluated using MTT and flow cytometry assays, respectively. The relative expression of PD-L1, galectin-9, and CD155 was determined by real-time PCR.

Our findings demonstrated decreased proliferation and increased apoptosis of HL-60 cells after treatment with idelalisib, MK-2206, and everolimus. As expected, the combined treatment showed a more inhibiting effect than the single treatment. Interestingly, our results elucidated that the expression of PD-L1 and Gal-9 but not MK-2206 decreased after treatment with idelalisib and everolimus. Regarding CD155, the expression of this molecule was downregulated after treatment with everolimus, but not idelalisib and MK-2206. However, combined treatment of HL-60 cells with two or three inhibitors decreased the expression levels of PD-L1, Gal-9, and CD155 checkpoint ligands.

We showed that PI3K/Akt/mTOR pathway inhibitors not only serve as cytotoxic drugs but also regulate the expression of immune checkpoint ligands and interfere with the immune evasion mechanisms of AML leukemic cells. Combinational treatment approaches to block these pathways might be a promising and novel therapeutic strategy for AML patients via interfering in immune escape mechanisms.

Keywords: Acute myeloid leukemia; Everolimus; Idelalisib; Immune evasion; MK 2206

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INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous hematopoietic malignancy defined by abnormal

proliferation and differentiation of hematopoietic myeloid precursor cells, leading to accumulation of blast cells in bone marrow, blood, and other tissues.[1,2] Among hematological malignancies, AML is the most common disorder in adult, and the prevalence increase with the age of the population. AML consists of various subtypes that are biologically, molecularly, clinically different, and complex.[1,3] Most AML patients react well to treatment with earlier therapy, but, nearly 15–30% of them are predominantly resistant to common chemotherapy.[4,5] Furthermore, several patients who achieve a complete remission are associated with increased levels of clinical relapse.[4-6] So, to find the new targeted therapies with improved cure rates and lower toxicity, the development of specific small molecules which target the main signaling pathways is an exciting alternative therapeutic strategy.[5,7]

Abnormal intracellular signaling is frequently observed in AML patients, including the phosphatidylinositol-3-kinase-Akt-mechanistic/ mammalian target of the rapamycin (PI3K-Akt-mTOR) pathway, which appears to be essential in both normal and leukemic hematopoiesis.[8,9] This pathway is involved in several cellular activities, such as cell cycle progression, cell survival, proliferation, apoptosis, differentiation, protein synthesis, metabolism, migration, autophagy, angiogenesis, and resistance to chemotherapy.[7,10,11] Prior studies have indicated that the PI3K/Akt/mTOR pathway is over-activated and correlated with reduced overall survival in AML, and then continued proliferation of leukemic cells in nearly 50-70% of cases.[7,12] The discovery of small molecule inhibitors as selective inhibitors for this pathway with a high therapeutic index, provides a new strategy for the treatment of AML patients.[9,12] During the last decades, the efficacy of PI3K/Akt/mTOR inhibitors are discussed in the preclinical and clinical studies on AML.[7] In this regard, there are some ongoing clinical trial studies in phases 1 and 2 applying idelalisib, MK-2206, and everolimus in AML either as monotherapy or in combination with other therapeutic approaches (clinicaltrials.gov).

On the other hand, it has been extensively understood that the immune system can identify and control tumor cells described as the process called tumor immune surveillance.[13] In addition, leukemic cells use a variety of strategies to evade immune responses, resulting in tumor progression and relapse.[14] There are several mechanisms for tumor immune escape, such as the modulation of tumor surface antigen, low immunogenicity of tumor cells, recognition of tumor-specific antigens as autoantigens, and tumor-induced immunosuppression, latter of which has been the most widely studied mechanism to date.[14-16] Immunosuppression mechanisms involve the expression of the immunosuppressive receptors or their ligands, such as programmed death- 1/programmed death-ligand 1 (PD-1/PD- L1), T-cell immunoglobulin, and mucin domain-3/galectin-9 (Tim-3/Gal-9), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T cell immunoreceptor with Ig and ITIM domains (TIGIT), and lymphocyte-activation gene 3 (LAG-3) which are known as the immune checkpoint molecules.[15,17,18] The interaction of immune checkpoint receptors with their ligands causes immune cell exhaustion, which is associated with the impaired function of these cells and tumor progression.[16,19] Nevertheless, there is still no detailed information on the intracellular mechanisms and signaling pathways of these processes in AML patients.

To explore the association of the signaling pathways with the tumorigenesis and immune evasion mechanisms, we have investigated the relationship between the blockade of the PI3K/Akt/mTOR signaling pathways and the expression of the immune checkpoint ligands on AML cells as one of the crucial immune escape mechanisms.

MATERIALS AND METHODS

Reagents

Idelalisib, MK-2206, and everolimus were obtained from Cayman chemical company (Michigan, United States) as PI3K inhibitor, Akt inhibitor, and an mTOR inhibitor, respectively. These chemical compounds were dissolved in dimethyl sulfoxide (DMSO) and stored frozen in aliquots. This study was found to be ethically acceptable by the Ethical Committee of Mazandaran University of Medical Sciences (IR.MAZUMS.IMAMHOSPITAL.REC.1398.075).

Cell Line and Culture

HL-60, a human AML FAB-M2 cell line, was obtained from the Pasteur Institute of Iran (Tehran, Iran). Cells were maintained in RPMI-1640 (Biowest, Nuaille, France) medium supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Nuaille,

France), 100 units/mL of penicillin, and 100 µg/mL of streptomycin. For experimental procedures, 15000 cells/well were seeded into culture flasks and grown in a humidified atmosphere of 5% CO2 at 37°C. To define the optimal concentration of drugs, cells were treated at concentrations up to 64 µM of idelalisib, MK-2206, and everolimus for 24 and 48 hours in 96-well culture plates. Optimal concentration or half-maximal inhibitory concentration (IC50) values were calculated for all drugs using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA) curve-fitting software.

Cell Viability Assay

Cell viability was evaluated by MTT assay. Briefly, leukemic cells were seeded in 96-well plates at 15000 cells/well, treated with an optimal concentration of signaling inhibitors including 5 µM idelalisib, 5 µM MK-2206, and 2 µM everolimus, and then incubated at 37°C for 48 hours. All assays were done in triplicate. After incubation time, the MTT reagent was added to each well at a final concentration of 0.5 mg/ml. Following incubation for 4 hours at 37°C, the microplates were centrifuged at 300 g for 10 min, the supernatants were discarded and 150 µL of DMSO was added to each well. Crystals of formazan were dissolved by shaking of microplates, and absorbance was measured using a microplate spectrophotometer (Synergy H1 BioTek, Winooski, USA) at 570 nm in a 630 nm. Determination of cell viability was calculated as follows: percentage of cell viability=(absorbance $_{Sample}$− absorbance $_{blank}$)/(absorbance $_{control}$−absorbance $_{blank}$)× 100%. Culture medium without cells was considered as blank and HL-60 cells cultured without any drugs (untreated cells) were defined as control.

Cell Apoptosis Assay

HL-60 cells were seeded in 6-well plates at 2.4* 10^5 cells/well and treated with an optimal concentration of signaling inhibitors including 5 µM idelalisib, 5 µM MK-2206, and 2 µM everolimus for 48 hours. After that, apoptosis-mediated cell death was examined by a dual-color FITC-labeled Annexin V/propidium iodide (PI) apoptosis detection kit (Thermo Fisher Scientific, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, 250000 treated and untreated cells were collected after incubation time, and then washed in phosphate-buffered saline (PBS) twice and incubated with binding buffer containing 5 µL of FITC-conjugated Annexin V for 10 min at room temperature. Following incubation, cells were washed in PBS twice, then incubated with a binding buffer containing 10 µL of PI (20 µg/mL). Finally, the externalization of phosphatidylserine and the permeability to PI were evaluated using a Partec PAS flow cytometer system (Partec GmBH, Munster, Germany). Cells in the early stages of apoptosis were positively stained with Annexin V, whereas cells in the late apoptosis or necrosis were positively stained with both Annexin V and PI.

RNA Isolation and Semi-quantitative Real-time PCR

HL-60 cells were treated in 6-well plates at 2.4* 10^5 cells/well, with 5 µM idelalisib, 5 µM MK-2206, and 2 µM everolimus for 48 hours. After that, to measure the gene expression of *PD-L1*, *Gal-9*, and *CD155*, total RNA was extracted using the Denazist Asia kit (Mashhad, Iran) based on the manufacturer's instructions. The RNAs were then reverse transcribed to cDNA by Yekta-tajhiz cDNA synthesis kit (Tehran, Iran), in brief by Moloney murine leukemia virus reverse transcriptase, coupled with random hexamers under standard conditions. Semi-quantitative Real-Time PCR (qRT-PCR) was carried out in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR green as the detection dye (Ampliqon, Copenhagen, Denmark). The specific primer pairs were shown in Table S1, and the primer efficiencies were confirmed by standard curves following the reaction. Finally, the transcript levels of *PD-L1*, *Gal-9*, and *CD155* were normalized to β-actin and the relative expression of each molecule was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 6 and SPSS20 software. Quantitative data are expressed as mean±SD. Analysis was performed using the Kolmogorov-Smirnov test to determine the normality distribution of the obtained data, and one-way analysis of variance (ANOVA) followed by the Dunnett test for multiple comparisons. Statistical correlations were done by the Spearman correlation test. *P-values* less than 0.05 were considered statistically significant.

RESULTS

PI3K/Akt/mTOR Inhibitors Cause Growth Inhibition in HL-60 Cells

The effects of PI3K/Akt/mTOR inhibitors on growth inhibition were examined in the HL-60 cell line. Cell viability was measured via MTT assay following 48 hours of exposure to various concentrations of idelalisib, MK-2206, and everolimus. As shown in Figure 1, after a single exposure to idelalisib, MK-2206, and everolimus at concentrations up to 64 μM, HL-60 cells indicated a dose-dependent reduction in viability, with an IC_{50} value of 5 μM, 5 μM, and 2 μM, respectively. In the next step, we measured the anti-proliferative activity of single or combined treatment by MTT assay using the calculation of the relative cell proliferation index for all samples. In this experiment, we found that the proliferation of HL-60 cells was significantly reduced by treatment with idelalisib, MK-2206, and everolimus (p<0.0001, Figure 2). As expected, co-treatment with these drugs led to an enhanced cell growth inhibition when compared to a single treatment (Figure 2).

PI3K/Akt/mTOR Inhibitors Induce Apoptosis in HL-60 Cells

Since apoptosis may contribute to a decrease in cell viability, we also examined apoptosis induction of idelalisib, MK-2206, and everolimus in HL-60 cells. Flow cytometric analysis was carried out with Annexin V-FITC/PI staining of HL-60 cells. As indicated in Figures 3A and 3B, the apoptotic level of HL-60 was increased after treatment with inhibitors when compared with untreated cells, but a single treatment with any of the three drugs could not induce a significant difference in apoptosis of HL-60 cells. Combined treatment with all three drugs led to increased cell apoptosis compared to either single treatment or co-treatment with two drugs (Figures 3A and 3B, p=0.01). Notably, the apoptotic cells were mainly in the late stage of apoptosis.

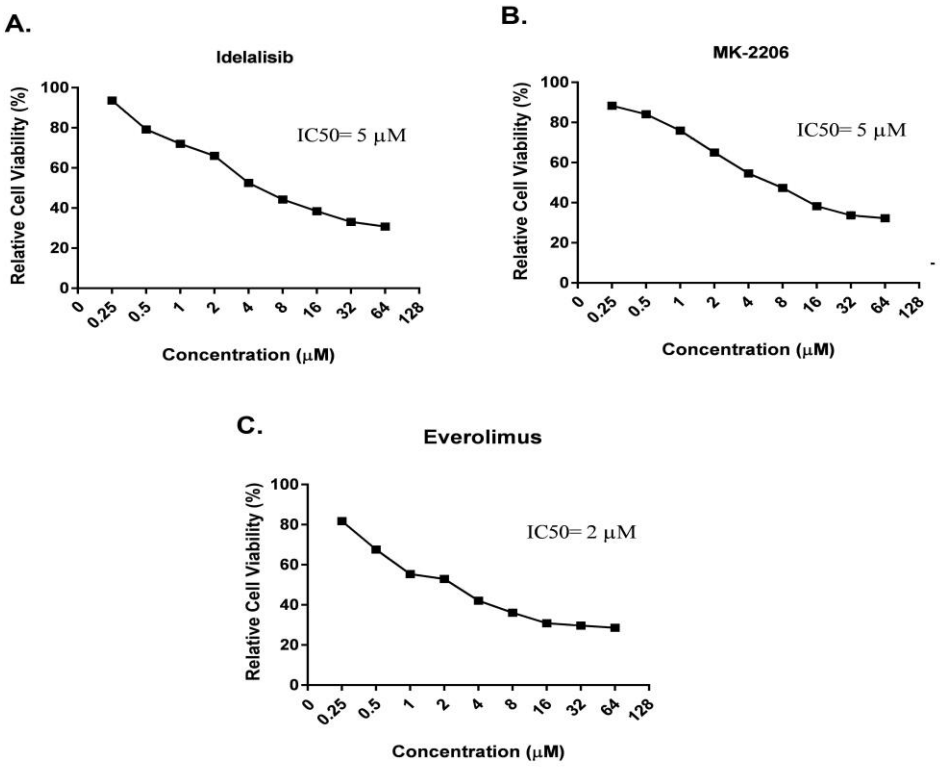


Figure 1. Determination of IC_{50} value for idelalisib, MK-2206, and everolimus. HL-60 cells were treated with various concentrations (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 μM) of idelalisib (A), MK-2206 (B), and everolimus (C) for 48 hours. Cell viability was measured by MTT assay. The IC_{50} values of idelalisib, MK-2206, and everolimus were calculated to be 5 μM, 5 μM, and 2 μM, respectively.

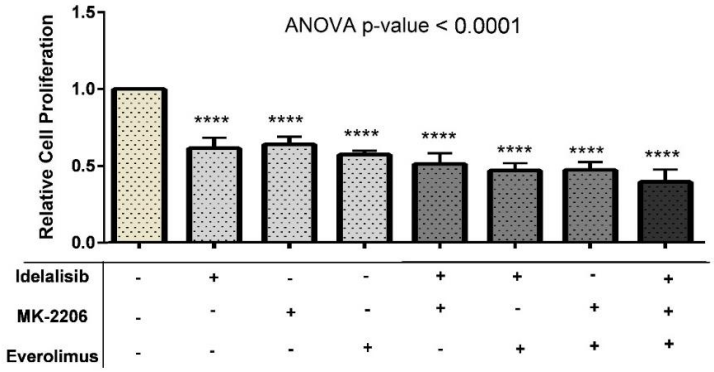
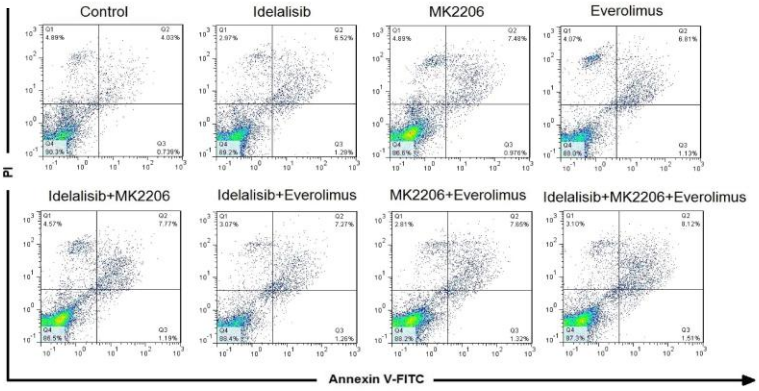


Figure 2. Effects of PI3K/Akt/mTOR inhibitors on proliferation of HL-60 cells. HL-60 cells were cultured in the absence or presence of idelalisib, MK-2206, and everolimus for 48 hours either in single or combined treatment. After that, cell proliferation was determined by MTT assay. The results are expressed as the mean ± SD of three independent experiments. One-way ANOVA with Dunnett post hoc test was used for analyses. ****p<0.0001;

A.



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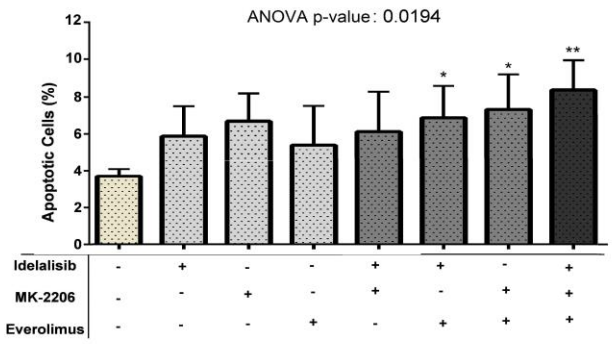

Figure 3. Effects of PI3K/Akt/mTOR pathway inhibitors on apoptosis of HL-60 cells. HL-60 cells were cultured in the absence or presence of idelalisib, MK-2206, and everolimus for 48 hours either in single or combined treatment. After that, apoptotic cells were detected by Annexin-V/PI staining assay via flow cytometry. A. Representative flow cytometric dot plot is shown. B. The percentage of apoptotic HL-60 cells is represented. The results are expressed as the mean±SD of three independent experiments. One-way ANOVA with Dunnett post hoc test was used for analyses. *p<0.05; ** p<0.01.

The Expression of Immune Checkpoint Ligands Was Reduced after Treatment with PI3K/Akt/mTOR Pathways Inhibitors

To further understand the molecular events underlying the immune escape mechanisms of AML, the effects of PI3K/Akt/mTOR inhibitors on the expression of key immune checkpoint molecules including PD-L1 as PD-1 ligand, Gal-9 as Tim-3 ligand, and CD155 as TIGIT ligand were studied on HL-60 cell line. The relative expression of PD-L1, Gal-9, and CD155 mRNA was evaluated in all samples by a semi-quantitative Real-Time PCR method using β-actin as an internal control. Our results demonstrated that the expression of PD-L1 was significantly reduced on HL-60 cells after treatment with idelalisib, and everolimus alone when compared to the untreated group (p=0.003 and p<0.0001, respectively), but the difference was not significant for the MK-2206 group (Figure 4A). Interestingly, the expression of PD-L1 was decreased after co-treatment with two or three drugs (p<0.0001, Figure 4A). Similar to PD-L1, our results showed the down-regulation of Gal-9 immune checkpoint molecule on HL-60 cells after treatment with idelalisib, and everolimus alone (p<0.0001, Figure 4B), but not for MK-2206 (Figure 4B). However, after combined treatment of HL-60 with two or three inhibitors, Gal-9 expression was strongly reduced (p<0.0001, Figure 4B). Our data indicated that the expression of CD155 was significantly decreased on HL-60 treated with everolimus (p=0.02, Figure 4C), but not with idelalisib and MK-2206 (Figure 4C).

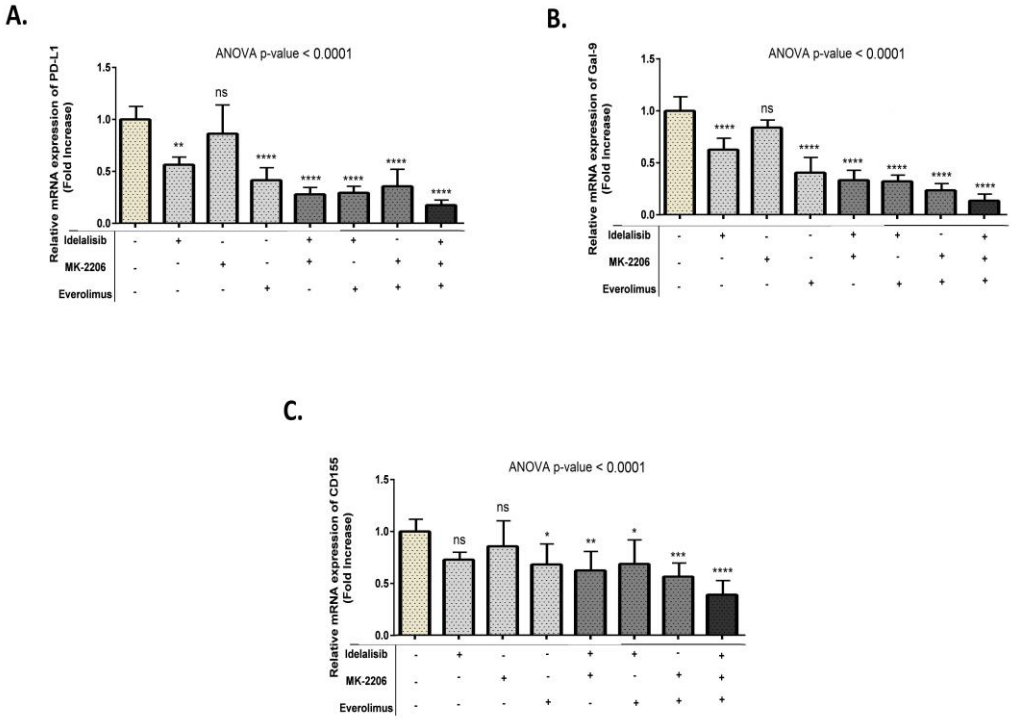

Figure 4. Effects of PI3K/Akt/mTOR pathway inhibitors on the expression of PD-L1, Gal-9, and CD155. HL-60 cells were cultured in the absence or presence of idelalisib, MK-2206, and everolimus for 48 hours either in single or combined treatment. After that, total RNA was extracted from all combinations and single-stranded cDNA was synthesized. Real-time PCR was performed with specific primers for PD-L1, Gal-9, CD155, and β-actin. (A) Relative mRNA transcript levels of PD-L1. (B) Relative mRNA transcript levels of Gal-9. (C) Relative mRNA transcript levels of CD155. Gene expression results are represented as mean±SD of $2^{-\Delta\Delta Ct}$ after normalization with β-actin as an internal control. One-way ANOVA with Dunnett post hoc test was used for analyses. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Nevertheless, CD155 expression was vigorously decreased after combined treatment with two or three inhibitors (p<0.0001, Figure 4C). Finally, the current results were analyzed to find any correlations between the expressions of these immune checkpoint ligands. Our results indicated positive correlations between the expression of PD-L1, Gal-9, and CD155 on HL-60 after treatment with each inhibitor (Figure 5).

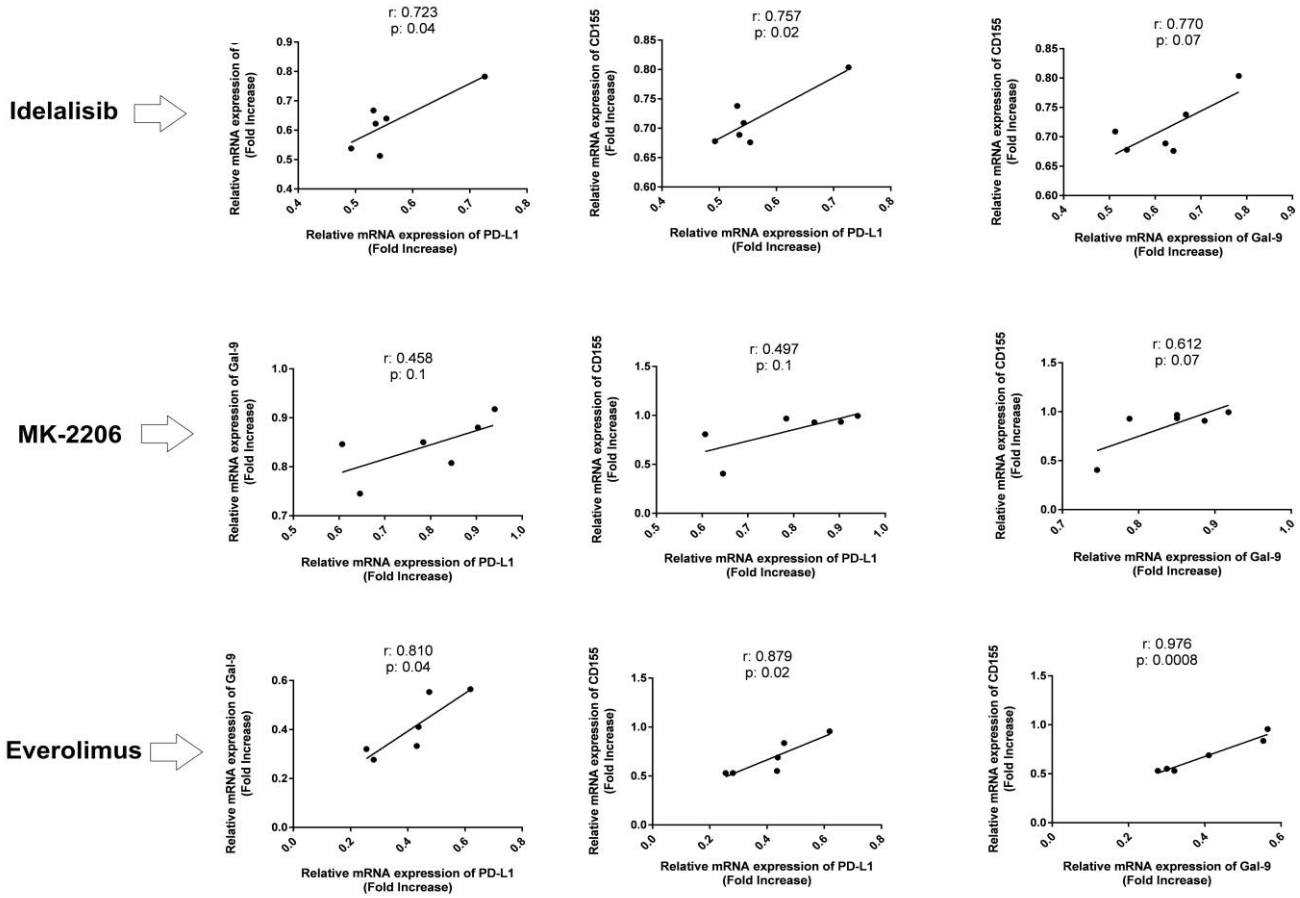

Figure 5. Correlation analysis of PD-L1, Gal-9, and CD155 expression after treatment with PI3K/Akt/mTOR pathway inhibitors. Correlation analysis of PD-L1, Gal-9, and CD155 mRNA expression with each other was performed after treatment of HL-60 cells with idelalisib, MK-2206, and everolimus. Statistical correlations were done by the Spearman correlation test.

DISCUSSION

Understanding the immune escape mechanisms of cancerous cells in the tumor microenvironment is crucial to developing and improving new approaches for tumor-targeted therapy.[17,20] There are numerous mechanisms for tumor cells to escape from the host immune system, one of the most important of which is the expression of immune checkpoint molecules.[20-22] This mechanism includes the over-expression of immune checkpoint receptors on immune cells and their ligands on tumor cells, such as PD-1/PD-L1, Tim-3/Gal-9, and TIGIT/CD155.[20,21] These interactions play a pivotal role in the immune-suppressing behavior of the tumor microenvironment, which may also inhibit the antitumor immunity and eventually lead to the immune escape of tumor cells.[15,23,24] Targeting these pathways is an appealing approach for cancer immunotherapy, but the clinical efficacy remains poor in some tumors. These conditions need a greater comprehension of the complex and diverse molecular mechanisms and the intracellular signaling pathways in

tumor cells.[15,24] Blockade of signaling pathways using small molecule inhibitors is a new promising strategy for cancer treatment. Various small molecule inhibitors are currently being evaluated in clinical trials for hematopoietic and non-hematopoietic malignancies. However, little is known about their possible associations with the immune evasion mechanisms. Accordingly, in this study, we aimed to find any associations between the PI3K/Akt/mTOR signaling pathways and the expression of immune checkpoint ligands PD-L1, Gal-9, and CD155 on AML cells. Our results revealed that the blockade of PI3K/Akt/mTOR pathways could be a potential target for regulating the expression of PD-L1, Gal-9, and CD155 on the HL-60 myeloproliferative cell line. The PI3K/Akt/mTOR pathway is of particular interest among the main abnormal intracellular signaling pathways engaged in hematological malignancies.[5,7,25] Over-activation of PI3K/Akt/mTOR suggests an essential role for this axis in tumor progression of several hematological malignancies, including AML, chronic myelogenous leukemia (CML), and acute lymphoblastic leukemia (ALL), as well as in lymphoproliferative disorders.[3,25,26] Recent studies have demonstrated that the PI3K/Akt/mTOR pathway is activated in 50–80% of AML patients.[12,27] Over-activation of the PI3K/Akt/mTOR pathways may be involved in several tumor immune evasion, leading to tumor progression.[27] Although high levels of mTOR signaling are displayed in all AML patients, it is assumed that dual or triple blockade of the PI3K/Akt/mTOR pathway could be more effective than single blockade in AML.[28] The limited efficacy of a single blockade strategy could be explained by the existence of signaling feedback loops. Indeed, although the constitutive activation events for PI3K, Akt, and mTOR are independent, these signaling networks are closely related and are subject to complex cross-talk and feedback interactions. The resulting rationale for dual or triple inhibition of these pathways is supported by preclinical data demonstrating enhanced anti-tumor activity[29,30] Previous reports suggested that the blockade of these pathways reduces cell viability and induces apoptosis of AML cells.[3,31,32] Similar to these findings, we showed that the combination blockade of PI3K/Akt/mTOR pathways by small molecule inhibitors reduces the cell viability and enhances the apoptosis in the HL-60 cell line. Since the PI3K/Akt/mTOR pathway is involved in the activation of some oncogenes that participated in AML oncogenesis, we postulated that this pathway might regulate the immune escape mechanisms and especially the expression of immune checkpoint ligands in AML. Our findings indicated a strong correlation between the expression of PD-L1, Gal-9, and CD155 and the over-activation of the PI3K/Akt/mTOR pathway in AML. Previous reports showed the associations between over-expression of immune checkpoint ligands and tumor immune escape.[33-35] Interaction of the PD-1/PD-L1, Tim-3/Gal-9, and TIGIT/CD155 pathways were able to regulate the host immune response and T-cell exhaustion in the tumor microenvironment.[36-39] Over-activation of PD-L1 and Gal-9 and CD155 is reported in a substantial number of solid and hematopoietic malignancies, but little is known about the expression of CD155 on leukemia cells.[24,35,39-41] Previous findings demonstrated the activation of receptor-mediated signaling pathways, such as PI3K/Akt/mTOR, JAK/STAT, MEK/ERK, and NF-κB promote the immune escape mechanisms by regulating the expression of PD-L1 in several tumors.[23,42,43] In this regard, several reports indicated that the up-regulation of PD-L1 is heavily dependent on PI3K/Akt/mTOR signaling pathway in glioma, lung cancer, and pancreatic cancer.[44-46] In contrast with these reports, PD-L1 expression is also correlated with the MEK/ERK signaling pathway in myeloma cells, but not the PI3K/Akt/mTOR signaling pathways.[47] It also should be noted that the expression regulation of these ligands is very complex and most probably depends on the status of underlying signaling pathways. Interestingly, most of the research in this area is related to PD-L1 expression and not much information is available about other checkpoint ligands like Gal-9 and CD155. To our knowledge, there is no evidence to investigate the association between the expression of immune checkpoint ligands with PI3K/Akt/mTOR signaling pathways in hematological malignancies. Here, our data showed that the combination blockade of the PI3K/Akt/mTOR pathway by small molecule inhibitors is associated with the down-regulation of PD-L1, Gal-9, and CD155 in the AML cell line. These results highlight the importance of the combination blockade of the PI3K/Akt/mTOR pathway as a promising target for potentiating anti-tumor immune responses by regulating the expression of immune checkpoint ligands on AML cells.

In conclusion, our findings demonstrated that the expression of PD-L1, Gal-9, and CD155 is significantly

decreased after co-treatment with small molecules, including idelalisib, MK-2206, and everolimus, as PI3K/Akt/mTOR pathway inhibitors. We showed that PI3K/Akt/mTOR pathway inhibitors not only serve as cytotoxic drugs but also regulate the expression of immune checkpoint ligands and interfere with the immune evasion mechanisms of AML leukemic cells. Combinational treatment approaches to block these pathways might be a promising and novel therapeutic strategy for AML patients via interfering in immune escape mechanisms.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships.

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