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Oridonin Could Inhibit Inflammation and T-cell Immunoglobulin and Mucin-3/Galectin-9 (TIM-3/Gal-9) Autocrine Loop in the Acute Myeloid Leukemia Cell Line (U937) as Compared to Doxorubicin

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ABSTRACT

The T-cell immunoglobulin and mucin-3 (TIM-3)/galectin-9 (Gal-9) autocrine loop is an indispensable signaling in acute myeloid leukemia (AML) cells, which induces their self-renewal through activation of nuclear factor-kappa b (NF-kB) and β-catenin pathways. In this study, we evaluated the effects of oridonin and doxorubicin on the TIM-3/Gal-9 autocrine loop. We also evaluated oridonin anti-inflammatory and anti-cancer properties on U937 cells, as an AML cell line in comparison to doxorubicin as a common anthracycline drug for AML treatment.

Cell counting kit-8 (CCK-8) was applied to evaluate the cytotoxicity of oridonin and doxorubicin on U937 cells and also to determine the impact of galectin-9 (Gal-9) on their proliferation. The effects of oridonin and doxorubicin on *Gal-9*, *TIM-3*, and *interleukin-1β (IL-1β)* gene expression were determined by real-time polymerase chain reaction (RT-PCR). The Gal-9 secretion level was measured by enzyme-linked immunosorbent assay (ELISA) and activation of NF-kB pathway was assessed by western blotting.

In a dose-dependent manner, oridonin and doxorubicin were capable to eradicate U937 cells while Gal-9 expanded them. Following the treatment of U937 cells with oridonin, the expression of *Gal-9*, *TIM-3*, and *IL-1β* genes was down-regulated, and the Gal-9 secretion and NF-kB phosphorylation were diminished, whereas doxorubicin increased all of these factors.

Doxorubicin is a common treatment agent in AML, but it may induce inflammation and up-regulate the TIM3/Gal-9 autocrine loop, consequently can enhance the possibility of disease relapse. Meanwhile, oridonin is capable to inhibit the essential signaling pathways in AML cells and reduce the inflammation and expansion of tumor cells and postpone AML recurrence.

Keywords: Acute myeloid leukemia; Doxorubicin; Galectin9; NF-kappa B; Oridonin

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INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disorder of leukocytes in which, a clonal population of myeloid stem cells begins to grow and differentiate out of cellular autonomous controls. Thus, there will be lots of malignant cells in bone marrow, peripheral blood, and even other tissues. Meanwhile, AML is regarded as the most prevalent type of acute leukemia in adults with a poor prognosis.[1] Development of AML mainly points out to some mutations in essential regulatory genes associated with cell proliferation and apoptosis such as *fms-like tyrosine kinase* 3 *(FLT3)*, *c-KIT*, *RAS*, and *p53* which might be considered as prognostic factors, too. Although targeting these genetic alterations could be beneficial in AML treatment, none of them were successful so far, due to the heterogeneity of these cells.[1,2]

Currently, a combination of cytarabine and anthracyclines such as doxorubicin is applied as the main chemotherapy regime for the treatment of AML; however, the success rate of treatment in this regime is low and at least 50% of cases return with complete remission relapse.[3,4] The reason for the low efficiency of current chemotherapy drugs might be due to the induction of inflammatory pathways in the tumor microenvironment. It seems that the activation of the nuclear factor kappa B (NF-κB) pathway and production of inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha (TNF-α) may result in resistance of leukemic cells to chemotherapy and may boost the risk of relapse.[5,6]

NF-κB inhibits cytochrome-c release by provoking B-cell lymphoma 2 (BCL-2) and X-linked inhibitor of apoptosis protein (XIAP) expression, as well as inducing the expression of TNF receptor-associated factor (TRAF)1, TRAF2, a cellular inhibitor of apoptosis protein (c-IAP)1, c-IAP2, and caspase-8 inactivation as a result.[7,8] NF-κB has a key role in the inflammatory responses and regulates lots of pro-inflammatory genes. Its activation triggers the production of inflammatory cytokines (e.g. IL-1, IL-6, and TNF-α), chemokines (e.g. IL-8 and monocyte chemoattractant protein-1(MCP-1)), growth factors (e.g. vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF)), matrix metalloproteinases (e.g. matrix metallopeptidases (MMP)2 and MMP9), and adhesion molecules (e.g. intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM)), which all of them promote tumor survival, metastasis, and chemotherapy-resistance.[9]

In recent studies, T-cell immunoglobulin and mucin-3 (TIM-3) have been reported as a surface marker on leukemia stem cells in all AML phenotypes, except for M3, while normal hematopoietic stem cells do not express this molecule.[10] It has been shown that there is an autocrine loop between TIM-3 and galectin-9 (Gal-9) as one of its ligands in primary AML cells, which induces the self-renewal of AML cells through activation of NF-κB and β-catenin pathways. It was also shown that the neutralization of Gal-9 with a specific antibody can halt human AML reconstitution in immunodeficient mice.[11] According to the mentioned observations, it seems that some of the chemotherapy drugs may induce downstream signaling pathways of the TIM-3/Gal-9 autocrine loop, which can potentially result in AML recurrence.

Doxorubicin is an anthracycline chemotherapy drug that is used for the treatment of various cancers such as lung, breast, non-Hodgkin's and Hodgkin's lymphoma, multiple myeloma, and AML. Its mechanism is mainly based on inhibition of topoisomerase-II and production of free radicals which cause damage to different parts of cells and disrupt DNA repair.[12] It is essential to mention that doxorubicin may induce inflammation by activation of p38 mitogen-activated protein kinases (MAPK) and NF-κB pathway. In addition, doxorubicin increases interleukin-1β (IL-1β) secretion through activation of the inflammasome and also induces the generation of other inflammatory factors such as TNF-α, IL-6, granulocyte colony-stimulating factor (GCSF), C-x-C motif ligand (CXCL)10, CXCL1, C-C motif chemokine ligand 2 (CCL2).[5,13]

Oridonin is a diterpenoid component with some unique characteristics, such as anti-bacterial and anti-inflammatory properties. Moreover, this agent has some anti-tumor activities, which induces cell cycle arrest, apoptosis, and autophagy, as well as inhibiting the angiogenesis and migration of tumor cells. In addition, oridonin can hesitate the growth and expansion of cancer cells by generating reactive oxygen species (ROS) and regulation of telomerase activity.[14-16] Oridonin inserts its anti-inflammatory properties by hindering the binding of NF-κB to chromosomal DNA and also inhibiting NF-κB entrance from the cytoplasm to the nucleolus by reducing the

expression of nucleoporins 88 and 214 on the nuclear membrane.[17,18] Interestingly, the application of HAO472 as an oridonin derivative is under phase I of a clinical trial for AML treatment since 2015.[14]

Based on the mentioned observations, we intended to evaluate the effects of oridonin and doxorubicin on the TIM-3/Gal-9 autocrine loop in the U937 cell line as a prototype of the AML cell line. In detail, we evaluated their effects on the expression of *Gal-9*, *TIM-3*, and *IL-1β* genes, production of Gal-9, and also activation of the NF-κB pathway.

MATERIALS AND METHODS

Human AML Cell Line and Determination of Cell Viability

All applied experiments were performed at the Iran University of Medical Science and the Research Ethics Committees approved the study proposal (Code # IR.IUMS.FMD.REC.1396.9411127008).

The U937 cell line was obtained from Pasteur Institute (Tehran, Iran) and propagated in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 100 U/mL Penicillin and 100 µg/mL Streptomycin and maintained at 37˚C in a humidified 5% CO_2 incubator. The cytotoxicity of various concentrations of oridonin (O9639, Sigma-Aldrich, Taufkirchen, Germany) and doxorubicin (A-4866, Unterach, Austria) on U937 cells was evaluated by Cell Counting Kit (CCK-8, Sigma-Aldrich, Taufkirchen, Germany) according to the manual. We also treated U937 cells with Gal-9 and their proliferation level was determined with the CCK-8 kit. In this method, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] is used to produce a water-soluble formazan dye upon bioreduction in the presence of 1-Methoxy PMS as an electron carrier. Approximately, 2×10^4 cells/well were seeded in a 96-well plate and treated with 0-160 µM of oridonin, 0-500 µM of doxorubicin, and 72-288 ng/mL of galectin-9. After overnight incubation, 10 µL of CCK-8 solution was added to each well and the amount of formazan was measured by reading the absorbance at 460 nm which is directly proportional to the number of living cells. In the end, we selected three doses of oridonin and doxorubicin for the final experiments.

Bioinformatics Study

Pathway Studio was used as a Bioinformatics database to study the possible effects of oridonin and doxorubicin on the TIM-3/Gal-9 autocrine loop and to determine the related interactions.

Real-time PCR

Real-time PCR was used to determine the TIM-3 (HAVCR2), Gal-9, and IL-1β mRNA levels. Approximately, 3×10^6 cells/well were seeded in 6-well plates, treated with selected doses of oridonin, doxorubicin, and lipopolysaccharide (L2262, Sigma-Aldrich, Taufkirchen, Germany), and incubated overnight. After 24 h, cell supernatant was removed and total RNA was extracted; using TRIZOL reagent (DNA biotech, Tehran, Iran) through guanidinium thiocyanate-phenol-chloroform extraction method and applied for cDNA synthesis; using a commercial kit (Thermo Scientific, Massachusetts, USA). Finally, the expression level of target genes was evaluated by real-time PCR; using specific primers (Table 1) and SYBR Green I MasterMix (Takara, Shiga, Japan) and the results were normalized; using GAPDH as the house-keeping gene.

Table 1. The details of primers used in real-time PCR

Gene Name	Primer Sequences (5′ to 3′)	Primer Length (bps)	Amplified Fragment Length (bps)
Galectin-9	F-GATGAGAATGCTGTGGTCCG	20	260
	R- GAAGCCGCCTATGTCTGCA	19	
TIM-3 (HAVCR2)	F- TCTTCCCTTTGACTGTGTCCT	21	175
	R- TTCAAACACAGGACAGGCTC	20	
IL-1β	F- AGCTTGGTGATGTCTGGTCC	20	167
	R- ACGCAGGACAGGTACAGATT	20	
GAPDH	F- GCACCGTCAAGGCTGAGAAC	20	138
	R- TGGTGAAGACGCCAGTGGA	19	

F: Forward primer, R: Reverse primer, TIM-3: T-cell immunoglobulin and mucin-domain containing-3, IL-1β: Interleukin 1-Beta.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was carried out to measure the amount of Gal-9 secretion from U937 cells to the tissue culture medium. Approximately, 3×10^6 cells/mL seeded in 6-well tissue culture plates and treated with selected doses of oridonin and doxorubicin. After 24 h incubation, the cell culture supernatant was harvested and used to measure human Gal-9; using a commercial ELISA kit (Biorbyt, Cambridge, UK) according to the manufacturer's recommendations.

Western Blotting

For assessment of total NF-κB and its phosphorylated form, the treated cells were lysed and applied for immunoblotting. In brief, 3×10^6 cells/mL were seeded in 6-well plates and treated with oridonin and doxorubicin. After 24 h incubation, cells were harvested and washed with cold PBS and lysed using RIPA buffer (Santa Cruz, Texas, USA) containing phosphatase and protease inhibitors within 15 minutes on ice. The lysate underwent centrifugation at 10,000 g for 15 min at 4˚C. Protein concentration was determined by the Bicinchoninic acid (BCA) protein assay kit (Parstous, Mashad, Iran). The ingredients were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were electrotransferred on polyvinylidene difluoride (PVDF) membrane. The membrane was rinsed with Tris-buffered saline containing 0.05% Tween 20 (TBST) and blocked by 5% skimmed milk and incubated with optimized concentrations of primary mouse monoclonal antibodies against NF-κB p65 and phospho-NF-κB p65 (sc-514451 and sc-136548, Santa Cruz, Texas, USA) in parallel with rabbit anti-beta actin antibody (ab8227, Abcam, Cambridge, UK). After an extensive wash (three times, each 5 min) with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated polyclonal antibodies against rabbit IgG or mouse IgG for 1 h. After another extensive washing, the immunoblots were visualized using electrochemiluminescence (ECL) substrate (Millipore, Massachusetts, USA).

Statistical Analysis

The statistical analysis was performed using STATA/SE version 12.0 software (STATA Corp, TX, USA) and the data were presented by GraphPad Prism version 7 (San Diego, CA, USA). First of all, using the Kolmogorov-Smirnov test, the normal distribution of the variables was evaluated. Then the continuous variables were expressed as mean±standard deviation (SD) and were compared among more than two independent groups by One-way ANOVA and Bonferroni. At the protein level, we performed nonparametric tests. Kruskal–Wallis, and Dunn tests were performed to compare between more than two independent groups. Moreover, Benjamini–Hochberg was done to control the false discovery rate (FDR) in multiple testing experiments.

RESULTS

The Effect of Oridonin and Doxorubicin on Signaling Pathways

Bioinformatics analysis by Pathway Studio showed that oridonin may block NF-κB, β-catenin, and WNT signaling pathways, hence, it might be capable to reduce the expansion of AML cells by disrupting this vital loop (Figure 1) while doxorubicin acts oppositely.

Cytotoxicity of Doxorubicin and Oridonin on U937 Cells

CCK-8 test showed that both oridonin and doxorubicin are capable to eliminate tumor cells in a dose-dependent manner. In this regard, the IC-50 concentration of oridonin and doxorubicin was estimated at 127 μM and 370 nM, respectively. Therefore, three non-cytotoxic levels, including 20, 40, and 80 μM for oridonin and 40, 80, and 160 nM for doxorubicin were selected for final experiments (Figure 2A and B).

The Effect of Gal-9 on Proliferation of U937 Cells

The effect of galectin-9 on U937 cells was evaluated by the CCK-8 test. The results showed that galectin-9 can enhance the proliferation of U937 cells in a dose-dependent manner (Figure 2C).

The Effect of Doxorubicin and Oridonin on *IL-1β*, *TIM-3*, and *Gal-9* Gene Expression

Real-time PCR showed that oridonin significantly down-regulates *IL-1β* gene expression in the U937 cell line. In addition, 20-40 μM doses of oridonin could diminish *TIM-3* (HAVCR2) and *Gal-9* gene expression, too (Figure 3A, B, and C). In contrast, doxorubicin increased the expression of the mentioned gens.

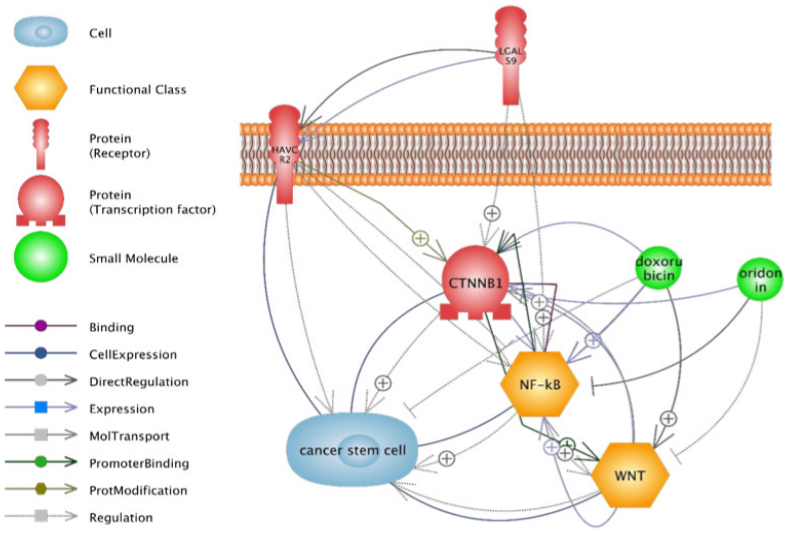

Figure 1. Evaluation of the effects of oridonin and doxorubicin on T-cell immunoglobulin mucin-3 (TIM-3)/galectin-9 autocrine loop: As it is shown oridonin in contrast to doxorubicin may interrupt this loop by inactivating β-catenin, WNT, and nuclear factor-kappa b (NF-κB) signaling.

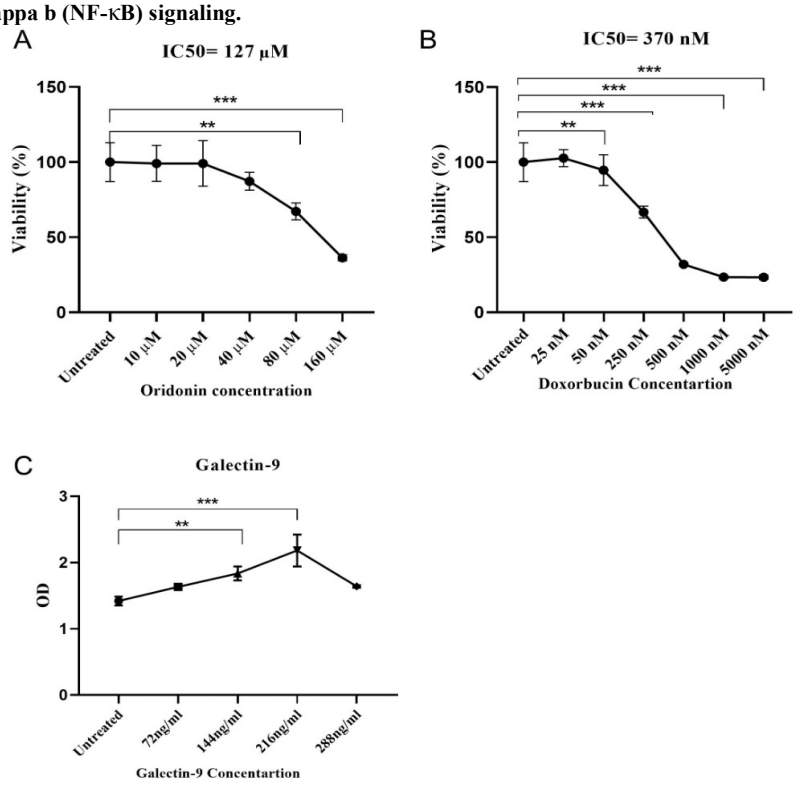

Figure 2. The effect of various concentrations of oridonin and doxorubicin for evaluation of IC 50 (A, B) and Galectin-9 (C) on the viability of U937 cells after 24 hours. A-B: Data represents the mean (± SD) of viability percentage obtained from two independent experiments, each performed in triplicate. C: Data represents the optical density (OD) obtained in cell counting kit (CCK) assay, pointing out to viable cells. Statistical analysis was performed on the percentage of viability, using One-way ANOVA and Bonferroni. Data represent mean ± SD. (*$p < 0.05$, ***$p < 0.001$).

The Effect of Doxorubicin and Oridonin on Gal-9 Secretion

In the presence of LPS as an inflammation-inducing agent, Gal-9 secretion was increased and when LPS was used in combination with doxorubicin, the secretion of Gal-9 was increased significantly, while oridonin as an anti-inflammatory agent did not increase Gal-9 secretion in low doses (e.g. 20 μM) (Figure 3D).

The Effect of Doxorubicin and Oridonin on NF-κb Expression

Western blotting showed that oridonin not only down-regulated total NF-κB but also reduced its phosphorylated form and the reduction of the activated form was more significant. While doxorubicin increased the phosphorylated form of NF-κB but did not affect its total form (Figure 4).

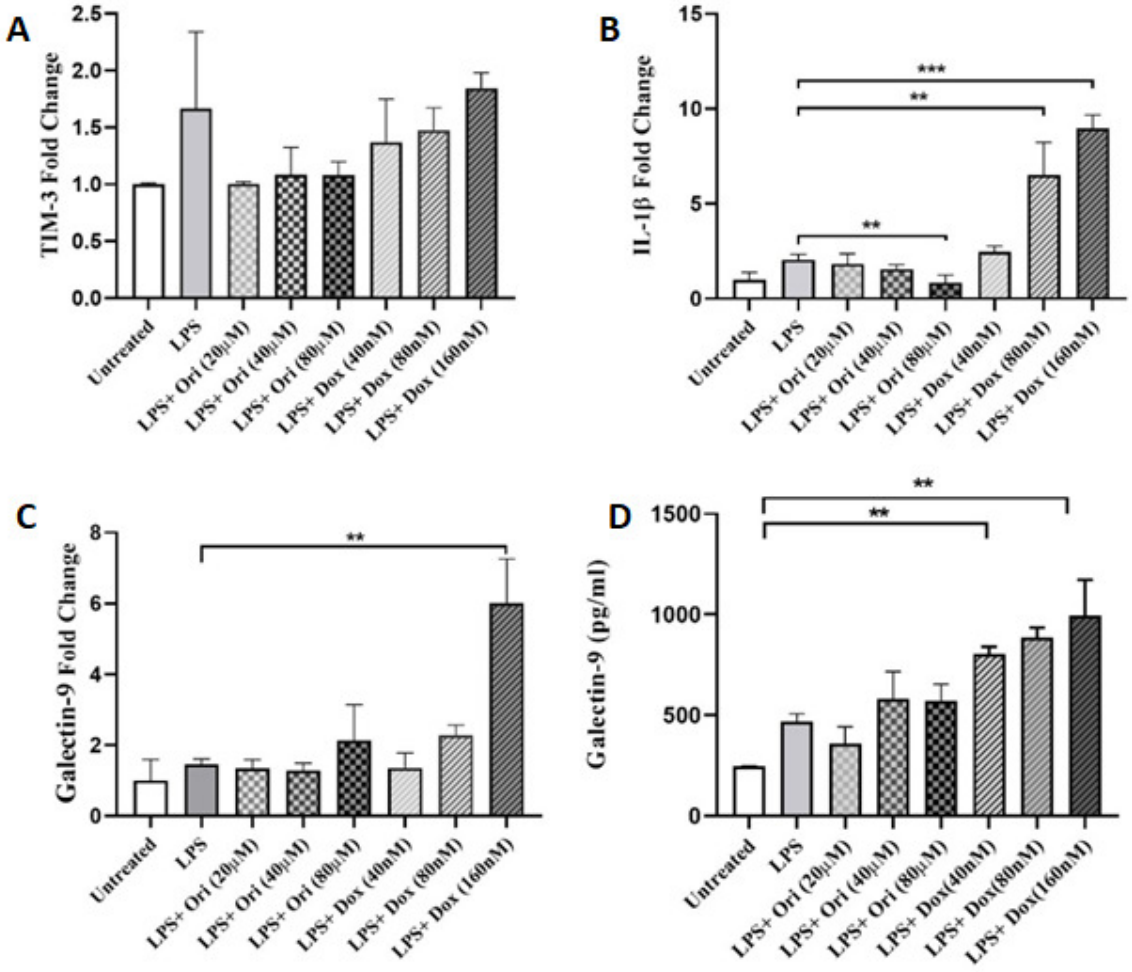

Figure 3. The effect of various concentrations of oridonin and doxorubicin in presence of lipopolysaccharides (LPS) on the gene expression of T-cell immunoglobulin mucin-3 (*TIM-3*), *galectin-9*, and *IL-1β* and the secretion of galectin-9 after 24 hours. A-C: Data represents the mean (± SD) of fold changes from two independent experiments, each performed in triplicate. Statistical analysis was performed on Log2 fold change, using One-way ANOVA and Bonferroni. Error bars indicate ± SD. (*p<0.05). D: Data represents the mean (± SD) concentration of Galactin-9 secreted following treatment with LPS and the studied medications in two independent experiments, each performed in duplicate. Statistical analysis was performed on the optical density, using One-way ANOVA and Bonferroni. Data represent mean ± SD. (*p < 0.001).**

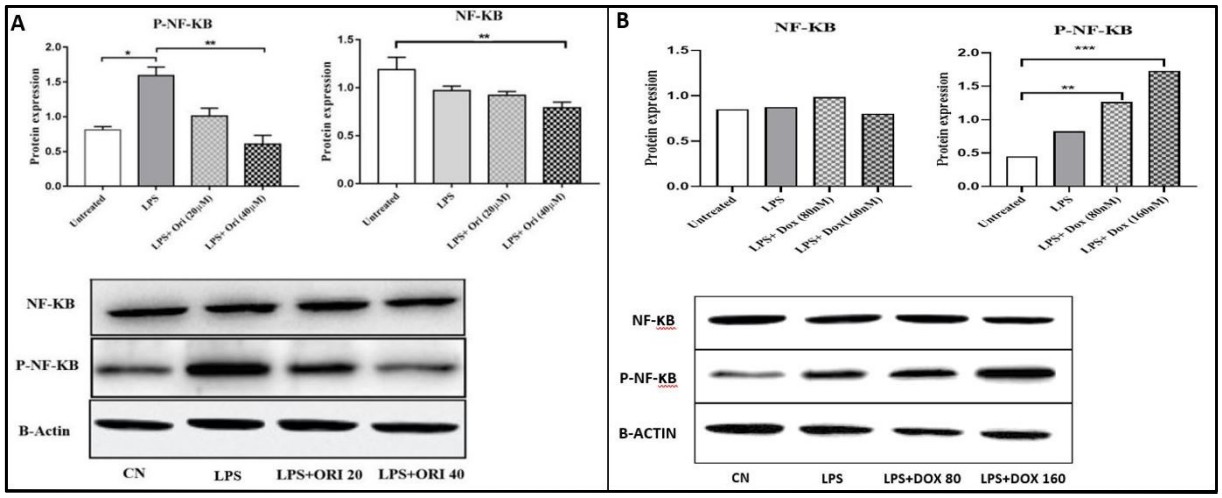

Figure 4. The effect of oridonin and doxorubicin in the presence of lipopolysaccharides on nuclear factor-kappa b (NF-κB) expression: The effect of A) oridonin at concentrations of 20 µM and 40 µM as well as B) doxorubicin at concentrations of 80 nM and 160 nM in the presence of lipopolysaccharides (LPS) on the protein level of NF-κB and phospho-nuclear factor-kappa b (p-NF-κB) in U937 cell line after 24 h treatment was measured. Statistical analysis was performed on normalized protein expression with β-actin from one experiment, performed in triplicate, using Kruskal–Wallis, Dunn test, Benjamini–Hochberg. Data represent the mean ± interquartile range (IQR). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, N/S not significant).

DISCUSSION

AML is one of the deadliest leukemias which up to now no efficient therapy has been developed against it. About 50% of the patients experience recurrence after receiving chemotherapy drugs as a main therapeutic approach. Epidemiologic studies show that AML has a higher incidence in older individuals. Meanwhile, we are aware that more inflammatory mediators are produced in elderly people than youngsters; thus it seems that there might be a linkage between inflammation and incidence of AML recurrence.[19] One of the main molecular alterations along with aging is the enhancement of NF-κB signaling which gradually results in overexpression of multiple inflammatory genes and augmentation of different inflammatory mediators.[20] In brief, NF-κB is an important transcription factor that regulates various aspects of innate and adaptive immunity and is regarded as a key mediator of inflammation.[21]Furthermore, another facet of NF-κB activation could be cancer progression. In this context, some studies show that activation of this transcription factor promotes cancer progression by inhibiting the apoptosis of cancer cells as well as inducing angiogenesis and metastasis.[22] A recent study showed that NF-κB is also involved in TIM-3/Gal-9 signaling and might be capable to increase the survival rate of AML cells.[11] Therefore, NF-κB inhibition may be effective in TIM-3/Gal-9 autocrine loop suppression. In this study, we showed that oridonin down-regulated the total amount of NF-kB and its phosphorylation rate after treatment, meanwhile, doxorubicin increased its phosphorylation rate but the total amount remained stable. Reduction of the active or phosphorylated form of NF-κB following oridonin treatment revealed that it has an inhibitory effect on this pathway and not only may diminish inflammation but also could impede AML progress in the opposite to doxorubicin.

One of the most important inflammatory cytokines which are regulated by NF-κB is IL-1β which is regarded as a major regulatory molecule in inflammation, innate immunity, and hematopoiesis. Moreover, it is an effective cytokine in various steps of cancer development including its initiation, angiogenesis, and metastasis. For instance, it may induce DNA alteration through promoting the synthesis of reactive oxygen intermediates and nitrogen active species in phagocytic cells which can pave the way for the onset of cancer in some conditions. It also promotes

the production of angiogenic factors such as IL-8, VEGF, hepatocyte growth factor (HGF), and fibroblast growth factor (FGF) and also enhances the production of elements involved in metastasis of the tumor cells such as MMPs, IL-6, TNF-α, transforming growth factor-beta (TGF-ß).[23,24]

Previous studies suggest an important function for IL-1β in AML. Carey et al, through the blocking of the P38MAPK signaling, could indirectly suppress the IL-1β pathway and inhibited the expansion of AML cells.[25] Of course, IL-1β, through P38MAPK-dependent mechanisms, may enhance the activity of the GATA2 transcription factor which is a key regulator for a set of inflammatory genes that influence the progression of AML.[25] In addition, IL-1β stimulates the generation of growth factors such as stem cell factor (SCF), which have a significant effect on the spreading of the disease.[26] By considering the mentioned concepts, we intended to assess the expression level of this cytokine following oridonin and doxorubicin treatment. The experiments showed that oridonin decreases the expression level of IL-1β and consequently reduced inflammation in contrast to doxorubicin. Surely, evaluation of IL-1β secretion by immunoassay methods may provide further confirmatory hints.

Gal-9 is another fundamental molecule in AML promotion which is secreted in large amounts by these cells and is beneficial in tumor cells' invasion through suppressing the cytotoxicity of NK cells and decreasing IL-2 secretion by CTLs.[27] Initially, it was considered as an eosinophil chemoattractant factor, but gradually more functions such as provoking inflammation by monocytes and dendritic cells were discovered for it. It causes apoptosis in T helper-1 (T_H1) and T helper-17 (T_H17) cells and increases T regulatory response following ligation to its receptor named TIM-3 which means that Gal-9 has the opposite role.[28]

A recent study showed that Gal-9 and its receptor on AML leukemia stem cells comprise an indispensable autocrine loop that leads to the activation of diverse signaling pathways being useful in the survival and expansion of AML cells. So we evaluated the impacts of oridonin and doxorubicin on the production of these two pivotal molecules to make sure if there are any possible inhibitory effects. To examine our hypothesis, first, we evaluated Gal-9 effects on the proliferation of AML cells by CCK-8 and the results showed Gal-9 could induce proliferation of the cells. Previously, Kikushige et al showed that the TIM-3/Gal-9 autocrine loop has a key role in the expansion of AML cells. Thus, targeting the Gal-9 or TIM-3/Gal-9 autocrine loop could be a suitable candidate to inhibit the expansion of AML cells, along with the chemotherapy regime.

As mentioned before, oridonin and doxorubicin are capable of eradicating tumor cells; using cell cycle arrest and inducing apoptosis or autophagy. To check oridonin and doxorubicin cytotoxicity, we treated U937 cells with various doses of these chemicals, and their 127 μm and 370 nM concentration were determined as IC50 respectively, finally, three doses with less than 30% cytotoxicity were chosen for further studies. Our results showed that oridonin not only annihilate cancer cells but also decreased TIM-3 expression, thus, it may halt AML recurrence. But doxorubicin enhanced TIM-3 expression. Surely, the determination of TIM-3 protein alteration by flow cytometry or western blotting in future studies could provide more helpful hints.

Furthermore, we assessed oridonin and doxorubicin effects on Gal-9 secretion and found that oridonin reduces Gal-9 expression levels as compared to doxorubicin. As mentioned before, Gal-9 can stimulate the proliferation of AML cells and repress immunity against tumors by promoting apoptosis in immunocompetent cells such as T_H1 cells.

In this in vitro study, we showed that oridonin can reduce Gal-9 and TIM-3 expression which may disturb the TIM-3/Gal-9 loop while doxorubicin increased these molecules. Hence we concluded that not only oridonin can prevent the proliferation of AML cells but also it may avoid the relapse of the disease. Although doxorubicin can eradicate tumor cells, it may induce cancer relapse by increasing TIM-3/Gal-9 signaling. Surely, these finding should be implemented for AML cells derived from the patients and finally confirmed in a randomized controlled trial.

In conclusion, it seems that the TIM-3/Gal-9 autocrine loop can be an important mechanism for AML recurrence, thus its targeting could be helpful in AML treatment. Moreover, it should be considered that some chemotherapy drugs can induce inflammation and augment vital molecules including TIM-3 and Gal-9. Thus it seems that the application of an anti-inflammatory component besides the chemotherapy drugs or substituting them with chemotherapy medication with anti-inflammatory properties may result in more successful treatment in AML patients.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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