Allergy and Clinical Immunology

Compared to doxorubicin, might inhibit lymphocyte immunoglobulin autocrine loop and inflammation in the acute myeloid leukaemia cell line.

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ABSTRACT

In acute myeloid leukaemia (AML) cells, the T-cell immunoglobulin and mucin-3 (TIM-3)/galectin-9 (Gal-9) autocrine loop is a crucial signalling route that triggers the cells' self-renewal by triggering the nuclear factor-kappa b (NF-kB) and β-catenin pathways. We assessed the effects of doxorubicin and oridonin on the TIM-3/Gal-9 autocrine loop in this work. Additionally, we compared the anti-inflammatory and anti-cancer effects of oridonin on U937 cells, an AML cell line, to those of doxorubicin, a common anthracycline medication used to treat AML.

The application of Cell Counting Kit-8 (CCK-8) was utilised to assess the cytotoxicity of both doxorubicin and oridonin on U937 cells, as well as the effect of galectin-9 (Gal-9) on their proliferation. Oridonin and doxorubicin's effects on interleukin-1β (IL-1β), TIM-3, and Gal-9 Real-time polymerase chain reaction was used to measure gene expression (RT-PCR). The Gal-9 The enzyme-linked immunosorbent test (ELISA) was used to determine the amount of secretion, and activation of Western blotting was used to evaluate the NF-kB pathway.

Oridonin and doxorubicin were able to completely eradicate U937 cells in a dose-dependent manner, whereas Gal-9 caused them to proliferate. Oridonin treatment of U937 cells resulted in down-regulated expression of the TIM-3, IL-1β, and Gal-9 genes, as well as NF-kB and Gal-9 secretion. phosphorylation were reduced, while all of these variables were elevated by doxorubicin. A frequent treatment for AML is doxorubicin, yet it may also increase the risk of illness by inducing inflammation and upregulating the TIM3/Gal-9 autocrine loop.return to previous behaviour. On the other hand, oridonin has the ability to block crucial signalling pathways in AML cells.and delay the recurrence of AML by reducing inflammation and tumour cell growth.

INTRODUCTION

In a leukemic condition known as acute myeloid leukaemia (AML), a clonal population of myeloid stem cells starts to proliferate and differentiate independently of cellular autonomous mechanisms. As a result, bone marrow, peripheral blood, and even other tissues will be full of cancerous cells. AML is thought to be the most common form of acute leukaemia in adults, and it has a dismal prognosis. The main cause of AML development is mutations in critical regulatory genes such fms-like tyrosine kinase 3 (FLT3), c-KIT, RAS, and p53, which are linked to both cell proliferation and apoptosis and may also be prognostic markers. Targeting these genetic changes may be helpful in treating AML, however none of the attempts have been successful thus far because of the variety of these cells. Cytarabine and anthracyclines, like doxorubicin, are currently used in combination as the primary chemotherapy regimen for the treatment of acute myeloid leukaemia (AML);

Nonetheless, the treatment's success rate under this regimen is minimal and at least 50% of cases come back fully relapse after remission.3,4 The cause of the inadequate effectiveness of current chemotherapy medications may be brought on by the activation of the tumor's inflammatory pathways small-scale setting.

Leukemic cells may become resistant to chemotherapy due to the activation of the nuclear factor kappa B (NF-κB) pathway and the production of inflammatory cytokines including inter-

Allergy and Clinical Immunology

leukin (IL)-1, IL-6, and tumour necrosis factor-alpha (TNF-α), which may also increase the chance of relapse. By causing the expression of B-cell lymphoma 2 (BCL-2) and X-linked inhibitor of apoptosis protein (XIAP) as well as TNF receptor-associated factor (TRAF)1, TRAF2, a cellular inhibitor of apoptosis protein (c-IAP)1, c-IAP2, and caspase-8 inactivation, NF-κB inhibits the release of cytochrome-c.7,8 NF-κB controls several genes that are pro-inflammatory and has a major role in inflammatory responses. Growth factors, chemokines, and inflammatory cytokines (including IL-8 and monocyte chemoattractant protein-1 (MCP-1)) are released when it is activated.

(such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF), matrix metalloproteinases (such as MMP2 and MMP9), adhesion molecules (such as vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule 1 (ICAM-1)), and all of them support chemotherapy resistance, metastasis, and tumour survival. According to recent research, leukaemia stem cells in all AML phenotypes—aside from M3—express T-cell immunoglobulin and mucin-3 (TIM-3), a surface marker, whereas normal hematopoietic stem cells do not.10 It has been demonstrated that in primary AML cells, TIM-3 and galectin-9 (Gal-9) as one of its ligands form an autocrine loop that triggers AML cell self-renewal by activating the NF-κB and β-catenin pathways.

Additionally, it was demonstrated that in immunodeficient mice, neutralising Gal-9 with a particular antibody can prevent human AML reconstitution.11 Based on the aforementioned data, it appears that certain chemotherapy medicines may trigger downstream signalling pathways of the autocrine loop TIM-3/Gal-9, which may lead to a recurrence of AML.

Anthracycline chemotherapy drugs like doxorubicin are used to treat a variety of malignancies, including multiple myeloma, lung, breast, non-Hodgkin's and Hodgkin's lymphomas, and acute myeloid leukaemia (AML). Its primary mechanism involves the inhibition of topoisomerase-II and the generation of free radicals, which harm various cell components and interfere with DNA repair.12 It is important to note that doxorubicin may cause inflammation by activating the NF-κB pathway and p38 mitogen-activated protein kinases (MAPK). Furthermore, by activating the inflammasome, doxorubicin increases the secretion of interleukin-1β (IL-1β). It also triggers the production of other inflammatory factors, including TNFα, IL-6, granulocyte colony-stimulating factor (GCSF), C-x-C motif ligand (CXCL)10, CXCL1, and C-C motif chemokine ligand 2 (CCL2).One diterpenoid component with certain special properties is oridonin, which has antibacterial and anti-inflammatory properties.

attributes. Additionally, this agent has some anti-tumor proper-

ties; it inhibits the migration and angiogenesis of tumour cells and induces cell cycle arrest, apoptosis, and autophagy. Additionally, by producing reactive oxygen species (ROS) and controlling telomerase activity, oridonin can slow the growth and spread of cancer cells.14–16 By preventing NF-κB from binding to chromosomal DNA and preventing NF-κB from entering the nucleolus from the cytoplasm, oridonin exerts its anti-inflammatory effects by lowering the expression of nucleoporins 88 and 214 on the nuclear membrane.17, 18 It's interesting to note that a phase I clinical trial has been using HAO472—a derivative of oridonin—to treat AML since 2015.14 Drawing from the aforementioned observations, our aim was to assess the impact of both doxorubicin and oridonin.

on the U937 cell line, a prototype for the AML cell line, and the TIM-3/Gal-9 autocrine loop. We examined in detail how they affected the production of Gal-9, the activation of the NF-κB pathway, and the expression of the genes TIM3, IL-1β, and Gal-9.

MATERIALS AND METHODS

Human AML Cell Line and Determination of Cell Viability

The Iran University of Medical Science was the site of all applied experiments, and the study proposal (Code # IR.IUMS.FMD.REC.1396.9411127008) was authorised by the research ethics committees.

The U937 cell line was procured from the Pasteur Institute located in Tehran, Iran. It was cultivated in RPMI 1640 medium that contained 10% FBS, 100 U/mL Penicillin, and 100 μg/mL Streptomycin. The medium was kept at 37˚C in an incubator with 5% CO2 humidity.

The Cell Counting Kit (CCK-8, Sigma-Aldrich, Taufkirchen, Germany) was used to assess the cytotoxicity of different concentrations of doxorubicin (A-4866, Unterach, Austria) and oridonin (O9639, Sigma-Aldrich, Taufkirchen, Germany) on U937 cells in accordance with the manual. The CCK-8 kit was utilised to ascertain the proliferation level of U937 cells after they were treated with Gal-9. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt] is utilised in this procedure to create a formazan dye that is soluble in water when bioreduced with 1-Methoxy PMS acting as an electron carrier. A 96-well plate was seeded with roughly 2×104 cells/well, and then treated with 0-160 μM of oridonin, 0-500 μM of doxorubicin, and 72-288 ng/mL of galectin 9. 10 μL of CCK-8 solution was added to each well after an overnight incubation period, and the amount of formazan was determined by measuring the absorbance at 460 nm, which is directly correlated with the quantity of living cells. Ultimately, we decided

Allergy and Clinical Immunology

on three doxorubicin and oridonin doses for the last round of trials.

Bioinformatics Study

To investigate the potential effects of oridonin and doxorubicin on the TIM-3/Gal-9 autocrine loop and identify associated interactions, Pathway Studio was utilised as a bioinformatics database.

Real-time PCR

The levels of TIM-3(HAVCR2), Gal-9, and IL-1β mRNA were measured by real-time PCR. Six-well plates were seeded with roughly 3×106 cells/well, treated with specific dosages of lipopolysaccharide (L2262, SigmaAldrich, Taufkirchen, Germany), and left to incubate overnight. A commercial kit (Thermo Scientific, Massachusetts, USA) was used for cDNA synthesis after the cell supernatant was collected after 24 hours. Total RNA was extracted using TRIZOL reagent (DNA biotech, Tehran, Iran) by the guanidinium thiocyanatephenol-chloroform extraction procedure. Lastly, real-time PCR was used to assess the target genes' expression levels. Specific primers (Table 1) and SYBR Green I MasterMix (Takara, Shiga, Japan) were used, and GAPDH was used as the house-keeping gene to normalise the data.

Enzyme-linked Immunosorbent Assay (ELISA)

The amount of Gal-9 secreted into the tissue culture media by U937 cells was measured using ELISA. About 3×106 cells/mL were planted in 6-well tissue culture plates and given specific dosages of doxorubicin and oridonin. Following a 24-hour incubation period, the cell culture supernatant was collected and utilised to quantify human Gal-9, following the manufacturer's instructions and a commercial ELISA kit (Biorbyt, Cambridge, UK).

Western Blotting

Immunoblotting was performed on the lysed treated cells to evaluate total NF-κB and its phosphorylated form. To put it briefly, 6 well plates were seeded with 3×106 cells/mL and treated with doxorubicin and oridonin. Following a 24-hour incubation period, the cells were extracted, rinsed with cold PBS, and lysed in 15 minutes on ice using RIPA buffer (Santa Cruz, Texas, USA) that contained protease and phosphatase inhibitors. The lysate was centrifuged for 15 minutes at 4˚C at 10,000 g. The Bicinchoninic Acid (BCA) Protein Assay Kit (Parstous, Mashad, Iran) was utilised to evaluate the protein content. The components were separated using polyvinylidene difluoride (PVDF) membrane electrotransfer, and the proteins were resolved us-

ing 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 0.05% Tween 20 was added to Tris-buffered saline, which was used to rinse the membrane.
(TBST), blocked with 5% skim milk, then incubated with rabbit anti-beta actin antibody (ab8227, Abcam, Cambridge, UK) in conjunction with optimised doses of primary mouse monoclonal antibodies against NF-κB p65 and phospho-NF-κB p65 (sc-514451 and sc-136548, Santa Cruz, Texas, USA). Following three thorough washes with TBST (five minutes each), the membrane was incubated for one hour with polyclonal antibodies conjugated with horseradish peroxidase (HRP) against either mouse or rabbit IgG or IgG. The immunoblots were visualised using electrochemiluminescence (ECL) substrate (Millipore, Massachusetts, USA) following an additional thorough washing.

Statistical Analysis

The data were displayed using GraphPad Prism version 7 (San Diego, CA, USA), and the statistical analysis was carried out using STATA/SE version 12.0 software (STATA Corp, TX, USA). First, the normal distribution of the variables was assessed using the Kolmogorov-Smirnov test. The continuous variables were then compared between more than two independent groups using One-way ANOVA and Bonferroni after being represented as mean±standard deviation (SD). We ran nonparametric testing at the protein level. Tests like the Dunn and Kruskal-Wallis were used to compare data between more than two independent groups. Furthermore, in multiple testing studies, Benjamini–Hochberg was used to control the false discovery rate (FDR).

RESULTS

The Effect of Oridonin and Doxorubicin on Signaling Pathways

Based on Pathway Studio's bioinformatics research, oridonin has the potential to inhibit the NF-κB, β-catenin, and WNT signalling pathways. This means that by upsetting this crucial loop, it may be able to diminish the growth of AML cells, whereas doxorubicin functions in the opposite direction (Figure 1).

Cytotoxicity of Doxorubicin and Oridonin on U937 Cells

The CCK-8 test demonstrated the dose-dependent tumour cell elimination capabilities of both doxorubicin and oridonin. Regarding this, it was estimated that the IC-50 concentrations of doxorubicin and oridonin were 370 nM and 127 μM, respectively. Thus, for the final studies, three non-cytotoxic levels—20, 40, and 80 μM for oridonin and 40, 80, and 160 nM for doxorubicin—were chosen (Figure 2A and B).

Allergy and Clinical Immunology

The Effect of Gal-9 on Proliferation of U937 Cells

Using the CCK-8 assay, the impact of galectin-9 on U937 cells was assessed. The outcomes demonstrated that galectin-9 can, in a dose-dependent way, increase U937 cell proliferation (Figure 2C).

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

In the U937 cell line, oridonin dramatically reduces the expression of the IL-1β gene, according to real-time PCR results. Furthermore, the expression of the Gal-9 gene and TIM-3 (HAVCR2) may be diminished by oridonin dosages of 20–40 μM (Figure 3A, B, and C). In contrast, doxorubicin enhanced the aforementioned genes' expression.

The Effect of Doxorubicin and Oridonin on Gal-9 Secretion

Gal-9 secretion increased in the presence of LPS, an agent that induces inflammation, and in the presence of LPS plus doxorubicin; in contrast, low doses (e.g., 20 μM) of oridonin, an anti-inflammatory agent, did not increase Gal-9 secretion (Figure 3D).

The Effect of Doxorubicin and Oridonin on NF-κb Expression

Using Western blotting, oridonin was able to lower both the phosphorylated and activated forms of NF-κB, with the reduction of the activated form being more substantial than the downregulation of the total NF-κB. Figure 4 shows that doxorubicin did not alter the overall form of NF-κB, but it did enhance its phosphorylated form.

DISCUSSION

One of the worst leukaemias is AML, for which there is now no effective treatment. Recurrence occurs in about 50% of patients following taking chemotherapy medications as the primary therapeutic approach. According to epidemiologic research, AML has a increased occurrence in elderly people. In the interim, we understand that there are more inflammatory mediators produced in older adults as opposed to younger ones; as a result, it appears that there could be a connection between inflammation and the likelihood of an AML relapse.[19] One one of the primary chemical changes that come with ageing is the improvement of NF-κB signalling that progressively leads to the excessive expression of many inflammatory genes and the enhancement of various inflammatory intermediaries.[20] To sum up, NF-κB is a significant transcription factor that controls a number of Both natural and modified immunity and is thought to be a crucial inflammatory mediator.[21]Moreover, the advancement of cancer may be an additional aspect of NF-κB activation. In studies indicate that in this particular scenario, activation of this transcription factor accelerates the development of cancer via preventing cancer cells from entering apoptosis in addition to causing metastasis and angiogenesis.[22] A recent investigation demonstrated that TIM-3/Gal-9 is likewise impacted by NF-κB. signalling and may have the ability to improve survival percentage of AML cells.[11] Consequently, NF-κB inhibition could be efficient in the autocrine loop of TIM-3/Gal-9 suppression. Our research demonstrated that oridonin reduced the overall concentration of NF-kB and its rate of phosphorylation following therapy, however, Doxorubicin accelerated the rate of phosphorylation, yet the The overall sum did not change. decrease in the active or NF-κB's phosphorylated form after oridonin therapy demonstrated that it has a restraint on particular pathway, which may also lessen inflammation but could potentially obstruct AML advancement in the opposite way to Doxorubicin.

NF-κB regulates a number of key inflammatory cytokines, including IL-1β, which is thought to be a key regulatory molecule in innate immunity, inflammation, and hematopoiesis. Furthermore, it functions as an effective cytokine during several stages of the development of cancer, such as angiogenesis, metastasis, and initiation. For example, it may cause DNA alterations by encouraging the production of nitrogen-active species and reactive oxygen intermediates in phagocytic cells, which in certain cases might lead to the development of cancer. It also increases the synthesis of components involved in the metastasis of the tumour cells, such as MMPs, IL-6, TNF-α, and transforming growth factor-beta (TGF-ß), as well as angiogenic factors like VEGF, IL-8, hepatocyte growth factor (HGF), and fibroblast growth factor (FGF).Earlier Research indicates that IL-1β plays a significant role in AML. Carey et al. were able to indirectly reduce the IL1β pathway and prevent the growth of AML cells by disrupting P38MAPK signalling.[25] Naturally, IL-1β may increase the activity of the GATA2 transcription factor, a crucial regulator of a group of inflammatory genes that affect the development of AML, via P38MAPK-dependent processes.[25] Furthermore, IL-1β promotes the production of growth factors such stem cell factor (SCF), which significantly slows the disease's spread.[26] We aimed to evaluate the degree of this cytokine's expression after treatment with doxorubicin and oridonin by taking into account the previously described notions.

Unlike doxorubicin, oridonin reduces the expression level of IL-1β, which in turn reduces inflammation, according to the research. Undoubtedly, assessing IL-1β secretion by immunoassay techniques could offer more corroborating cues.

Another important chemical in the promotion of AML is gal-9, which is released in great quantities by these cells and aids in the invasion of tumour cells by reducing the release of IL-2 by CTLs and inhibiting the cytotoxicity of NK cells.27 It was first thought to be a chemoattractant factor for eosinophils, but over time, additional roles were identified for it, including inciting inflammation in monocytes and dendritic cells. It binds to its receptor, TIM-3, which causes T helper-1 (TH1) and T helper-17 (TH17) cells to undergo apoptosis. This suggests that Gal-9 has the opposite role in the T regulatory response.28 According to a recent study, Gal-9 and its receptor on AML leukaemia stem cells form an essential autocrine loop that activates a variety of beneficial signalling pathways.

in the persistence and growth of AML cells. To determine whether there may be any potential inhibitory effects, we thus assessed the effects of doxorubicin and oridonin on the synthesis of these two essential molecules. In order to test our theory, we first looked at how Gal-9 affected AML cell proliferation as measured by CCK-8. The findings indicated that Gal-9 may cause cell proliferation. The TIM-3/Gal9 autocrine loop has been shown by Kikushige et al. to have a significant role in the proliferation of AML cells. Thus, in addition to the chemotherapy regimen, blocking the Gal-9 or TIM-3/Gal-9 autocrine loop may be a good option to prevent the growth of AML cells.

As previously noted, doxorubicin and oridonin can kill tumour cells by causing autophagy or apoptosis, as well as cell cycle arrest. We treated U937 cells with different dosages of doxorubicin and oridonin to test their cytotoxicity; the IC50 values for both compounds were found to be 127 µm and 370 nM, respectively. Ultimately, three doses that showed less than 30% cytotoxicity were selected for additional research. According to our findings, oridonin not only completely destroys cancer cells but also reduces the expression of TIM-3, which may prevent an AML recurrence. Yet, doxorubicin increased the expression of TIM3. Future research determining the TIM-3 protein modification by western blotting or flow cytometry will undoubtedly offer more beneficial suggestions.

In addition, we evaluated the effects of doxorubicin and oridonin on Gal-9 secretion and discovered that oridonin lowers Gal-9 expression levels than doxorubicin does. As previously noted, Gal-9 promotes death in immunocompetent cells like TH1 cells, which can suppress immunity against tumours and increase the growth of AML cells.

In this in vitro investigation, we demonstrated that doxorubicin enhanced the production of these molecules whereas oridonin decreased it, potentially disrupting the TIM-3/Gal-9 loop. Therefore, we deduced that orodonin may help prevent both an AML

recurrence and the cell's ability to proliferate. Doxorubicin can destroy tumour cells, however through upregulating TIM-3/Gal-9 signalling, it can also cause cancer to recur.Of course,These results ought to be applied to patient-derived AML cells and ultimately validated in a randomised controlled experiment.

In summary, it appears that blocking the TIM-3/Gal-9 autocrine loop may be beneficial in the treatment of AML, as it may be a significant mechanism for AML recurrence. Furthermore, it should be remembered that certain chemotherapy medications might increase the levels of important molecules like TIM-3 and Gal-9 and cause inflammation. Consequently, it would appear that treating AML patients with an anti-inflammatory component in addition to or instead of chemotherapy medications could lead to a more favourable outcome for the patient.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

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Allergy and Clinical Immunology

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