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Anti-histamine Effects of Dipotassium Glycyrrhizinate on Airway Smooth Muscle Cell, Implicating Its Therapeutic Mechanism for Airway Hyperresponsiveness

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Abstract: *Asthma is a common respiratory disease, with chronic inflammatory associated with airway hyperresponsiveness (AHR). Airway smooth muscle (ASM) is the critical tissue modulating bronchial tension. Abnormal activation and proliferation of airway smooth muscle cells (ASMCs) are key process during airway problem development. Histamine, a mast-cell mediator, involves in allergy and air-way inflammation, and stimulates cells via its receptors. Dipotassium Glycyrrhizinate (DG) is a derivative of Glycyrrhizic acid, with anti-inflammation effects, but the effect of DG on histamine-induced ASMC has not discussed yet. Primary rats ASMCs were extracted to establish a histamine/cell-models to investigate the effects of DG on cells, and to explore the potential pharmaceutical mechanism of DG in the treatment of AHR. The data showed that DG inhibited histamine-induced cell proliferation, migration by suppressing P38/ERK and JAK/STAT signal pathway. The data also showed that DG up-regulated Bax expression and down-regulated Bcl-2 expression, leading to the apoptosis of activated ASMCs. Molecular docking analysis illustrated the high binding affinity of DG with histamine receptor, which is verified the antagonist function of DG on histamine by competing over histamine receptor. The present study suggests the anti-histamine function of DG and the underlying mechanism that DG can relieve airway hyper-responsiveness by inhibiting histamine-induced over-activation/proliferation and regulating airway smooth muscle cell intracellular signaling pathways.*

Keywords: *airway smooth muscle cells, histamine, dipotassium glycyrrhizinate, airway hyperresponsiveness*

1. Introduction

Asthma is a common respiratory disease and the one of the four persistent diseases recognized by the world medical community [1]. The pathophysiological characteristics of asthma are airway hyperresponsiveness, airway inflammation and airway remodeling, including fibrosis, increased mucogenesis, thickening and contraction of smooth muscle, and increased vascular distribution [2].

Airway smooth muscle (ASM) plays an important role in maintaining bronchoconstriction and regulating ventilation [3]. The contraction, proliferation, and apoptosis of airway smooth muscle cells (ASMC) are related to the pathological process of many clinical diseases, such as asthma chronic bronchitis and emphysema [4],etc. The abnormal proliferation and migration of airway smooth muscle cells play an important role in the pathogenesis of asthma, which can thicken the airway wall, restrict airflow, and participate in airway remodeling, which is the main reason for airway hyperresponsiveness (AHR) [5].

Histamine (His) is one of the critical inflammatory mediators released by mast cell activation/degranulation and combined with histamine receptor to produce allergic reaction and inflammatory reaction [6]. Histamine is an important inflammatory mediator in the pathogenesis of asthma. It is closely related to the spasm of airway smooth muscle, microvascular leakage, and hypersecretion of mucus in asthma. Histamine acts on the H1 receptor on smooth muscle, causing smooth muscle contraction [7]. Asthma or airway inflammation elbow, airway epithelium damage, stimulate receptor exposure, thus easily lead to histamine (H1R mediated) stimulation, and increase

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airway reactivity [8]. Therefore, we hypothesized that the anti-histamine effects might be having the potential for relieve asthma.

Glycyrrhizic acid (GA) is one of the most important active ingredients extracted from the root of licorice, and has strong anti-viral, anti-inflammatory capacity [9]. Glycyrrhizic acid may improve symptoms of asthma [10], attenuate airway inflammation, inhibit viruses [11]. Glycyrrhizic acid was found to be one of the compounds active against SARS-CoV in vitro and was used to relieve airway symptoms of SARS-CoV [12]. Dipotassium glycyrrhizate (DG, CAS68797-35-3) is a Glycyrrhizic acid Dipotassium salt, a derivative of glycyrrhizic acid with stable chemical properties and high solubility [13]. So far, no literature has reported the anti-histamine effects of glycyrrhizic acid derivatives on airway smooth muscle cells yet.

Molecular docking is widely used in drug discovery. Molecular docking can predict ligand target interactions at the molecular level [14]. However, the possible interaction structurally between DG and Histamine receptor was unknown.

The purpose of molecular docking is to analyze the effect of DG on histamine receptor protein, and verify the possible interaction between DG and histamine receptor through cell experiments and molecular docking simulation. The present study established a His/ASMC cell model to investigate the effects of Dipotassium glycyrrhizate on His-induced cell proliferation of ASMC, to explore the cellular mechanism of Dipotassium glycyrrhizate and the therapeutic potential on asthma, to examine its anti-histamine function, aiming to provide a theoretical and therapeutic basis for its alleviating asthma.

2. Materials and methods

2.1. Chemicals and materials

Histamine, loratadine (LTD, a histamine receptor antagonist) were purchased from Aladdin (Shanghai, China). Dipotassium glycyrrhizinate was purchased from Xi'an Biological Company (Xi'an, China). All cell culture materials were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA) or Aladdin Industrial (Shanghai, China). Otherwise, it would be stated.

2.2. Rat primary ASMC extraction and culture

Rat primary ASMCs were prepared as previously described[15]. Briefly, the tracheae tissue was separated and cut into about 1 mm3 pieces, then digested with 0.1% collagenase (Sigma, USA) for 2 h at 37°C, then placed in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin and 100 mg/mL streptomycin, incubated at 5% CO2 and 37°C. ASMCs were purified by varying adhesion times and cultured in completed DMEM which was changed every day. The extracted ASMCs were verified by Western blot using α-SMA specific antibody. The primary 3-6 passages of cells were for following experiments.

2.3. Molecular Docking

Computational Molecular Docking was performed to elucidate binding modes between DG and histamine receptor using Discovery Studio (DS) 4.5 (Molecular Operating Environment). The crystallographic structures of proteins Histamine receptor (PDB ID: 3RZE) were retrieved from protein database website (www.pdb.org). To initiate docking study in DS 4.5, water molecules and heteroatoms were manually removed out from the protein structures. The compound DG is connected with the target Histamine receptor respectively for the Libdock molecule using the Libdock method in Dock Ligands module. The docking parameter is the default, the output indicator Libdock Score is collected. Then, the molecular interactions were analyzed and visualized by based on DS consensus scoring function. Hydrogen bond interactions between the ligands and active site residues were also assessed.

2.4. Cell proliferation assay (MTT method)

Cells were seeded (1.4 × 104cells/well) in a 96-well plate in completed DMEM and incubated overnight at 37 °C in 5% CO2. Then cells were treated with His(0, 0.001, 0.01, 0.1, 0.2, 1, 10 mM), DG(0, 4, 6, 8, 10 mM), or DG(7 mM) mixed with histamine (0.1 mM), or LTD(0, 0.01, 0.02, 0.03, 0.04 mg/mL) or LTD(0.03 mg/mL) mixed with His (0.1 mM) in triplicates separately for 24 h in serum free

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(SF) condition. After incubation with MTT for 4 hours, DMSO was added, and optical density (OD) was read at 570nm with Bio Rad microplate reader. The total cell number/mL at each condition was calculated by an ASMC standard curve (cell numbers vs OD570 nm), n = 3–6. The inhibitory rates of ASMCs treated with DG relative to untreated cells were calculated (n = 3–6): inhibitory rate (%) relative to untreated cells = [1–(mean of treated cells)/(mean of untreated cell control)] × 100%.

2.5. Cell morphology and time-lapse photography

ASMCs in 24-well plate were treated with His(0.1 mM), DG(7 mM), His(0.1 mM)+DG(7 mM), LTD(0.03 mg/mL) and His(0.1 mM)+LTD(0.03 mg/mL) and cultured continuingly up to 48 hours; the images were taken at time points 0, 8, 12, 24, 48 hours at the same position within well by a Leica X10 objective lens and the computer imaging software (Leica Application Suite).

2.6. In-vitro cell-gap closure assessment (cell migration and proliferation)

ASMCs were seeded into 24-well plates and cultured overnight. The cell-gaps were made with fine pipette-tips after cell-monolayer confluence, and then washed with PBS to remove the debris; cell-monolayers with gaps were treated with His(0.1 mM), DG(7 mM), His(0.1 mM) + DG(7 mM), LTD(0.03 mg/mL) and His(0.1 mM) + LTD(0.03 mg/mL) in serum free condition(n = 3–6). The widths of cell-gaps were measured before treatment(0 h) and after treatments at time-points (8, 24, 48 and 72 h); the cell-gap closure rate (%) relative to the gap at 0 h was calculated = [1–(width of treated cell-gaps at 8, 24, 48 or 72 h)/(width of cell-gaps at 0 h)] × 100%. The photos were taken at each time points under a fluorescence microscope (63x) (Carl Zeiss, Germany).

2.7. Western blot assay

The total protein contents of ASMCs were determined using the BCA kit (Sigma). Equal amount of protein (20 μg/lane) of the cell lysates collected from each treatment conditions was loaded, and separated by 4-15% gradient SDS-PAGE(Bio-Red); then transfer to the PVDF membrane (Sigma). The membranes were blocked with 2% BSA in PBS containing 0.1% Tween 20 (TBST) at room temperature for 1h and incubated overnight at 4 °C with corresponding primary antibodies: P38, Bcl-2, Bax (Ab-Cam) ERK, JAK1, STAT5 (Santa Cruz). The membranes were then washed, and incubated with horseradish peroxidase-linked secondary antibody for 1 h at room temperature. After washing again, the signals were amplified by enhanced chemiluminescent reagents and detected and analyzed with Bio-Rad Gel Doc/Chemi Doc Imaging System.

2.8. Statistical analysis

GraphPad Prism 7.0 software was used for statistical analysis. Data have been presented as the means ± standard errors of the mean (SEM). Statistically significant differences were analyzed using a two-tailed Student's t-test;*P< 0.05 was taken as significant;**P< 0.01 very significant;***P< 0.001 very very significant.

3. Results

3.1. Molecular Docking Results

To gain better understanding of the correlation between DG and histamine receptor, a docking study was performed by fitting DG into the active site of histamine. As shown that histamine receptor was docked with DG. DG had a good interaction with histamine receptor with high binding score (170.125). The binding model of DG with histamine receptor was depicted in Figure 1A. In the Figure 1C, DG formed a hydrogen bond with amino acid TRY108, ILE115, TRP428, PHE432, PHE435 and a strong hydrophobic interaction with TRY431, ASP107. It could be more intuitive to see from Figure 1D. Overall, DG occupied within histamine receptor, exhibiting stabilizing binding conformation.

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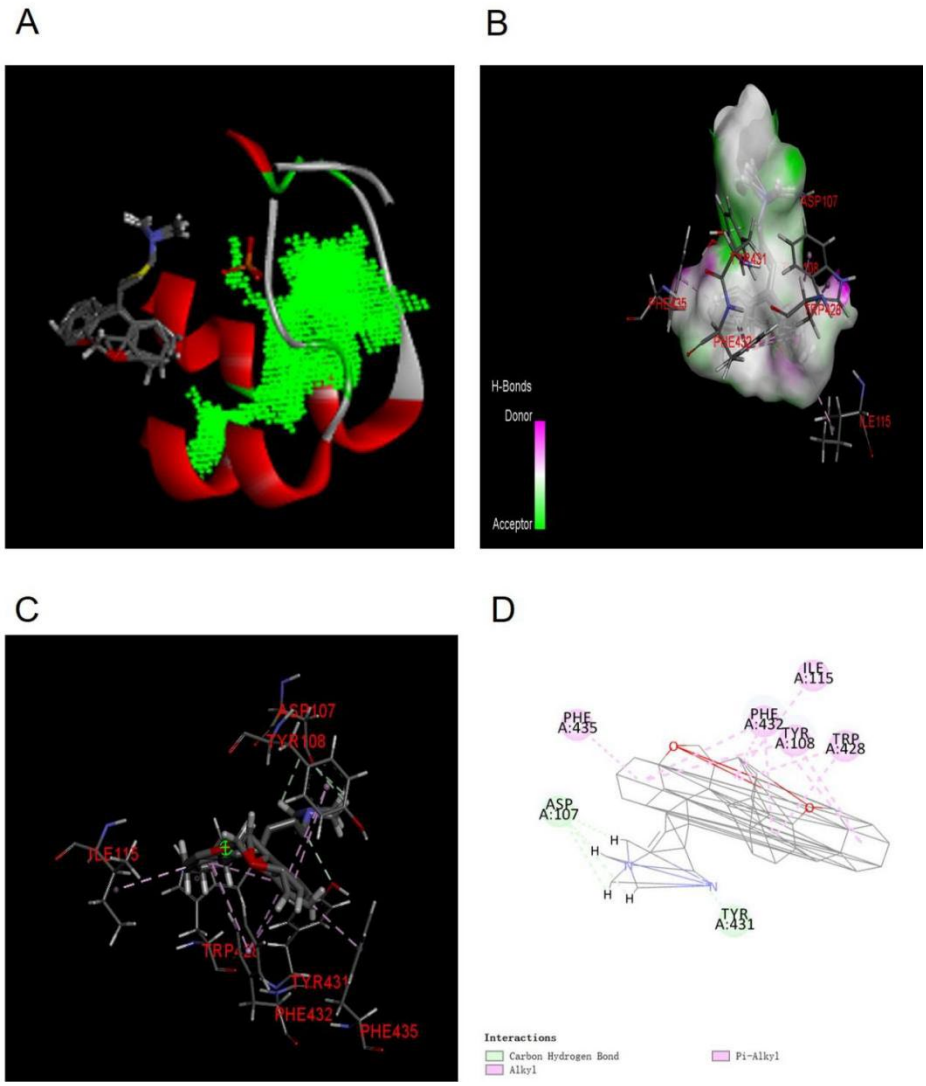

Figure 1: Molecular Docking tests of DG target histamine receptor. (A) The binding mode of DG in complex with histamine receptor (PDB code 3RZE). (B) Molcad surface H-acceptor/donor density. (C) Interactions between DG and residues. (D) The 2D diagram of complex

3.2. DG inhibits the His-induced ASMCs over-activation

The cell treated with His(0.1 mM), DG(7 mM), His(0.1 mM)+DG(7 mM), LTD(0.03 mg/mL) and His(0.1 mM)+LTD(0.03 mg/mL) viability (%) relative to untreated cells well were 114.8 ± 5.312, 57.04 ± 7.575, 72.13 ±10.77, 79.44 ± 1.006, 92.23 ± 4.478 (mean ± SEM). The results from MTT experiment demonstrated that His increased (Figure 2A) and DG inhibited (Figure 2B) the viability of ASMCs and cell grown respectively. When DG and His co-cultured with ASMCs, DG showed a competitive effect over His by inhibiting the His-caused excessive activation of cells (Figure 2D).

The changes in cell morphology were observed during the culture period with His(0.1mM), DG(7 mM), His(0.1 mM) + DG(7 mM), LTD(0.03 mg/mL) and His(0.1 mM) + LTD(0.03 mg/mL). DG inhibited the cell growth compared with untreated cells, also decreased the His-induced cell fast-grow. However, similar to LTD (histamine receptor antagonist), DG showed the similar antagonistic effect on cells (Figure 2E).

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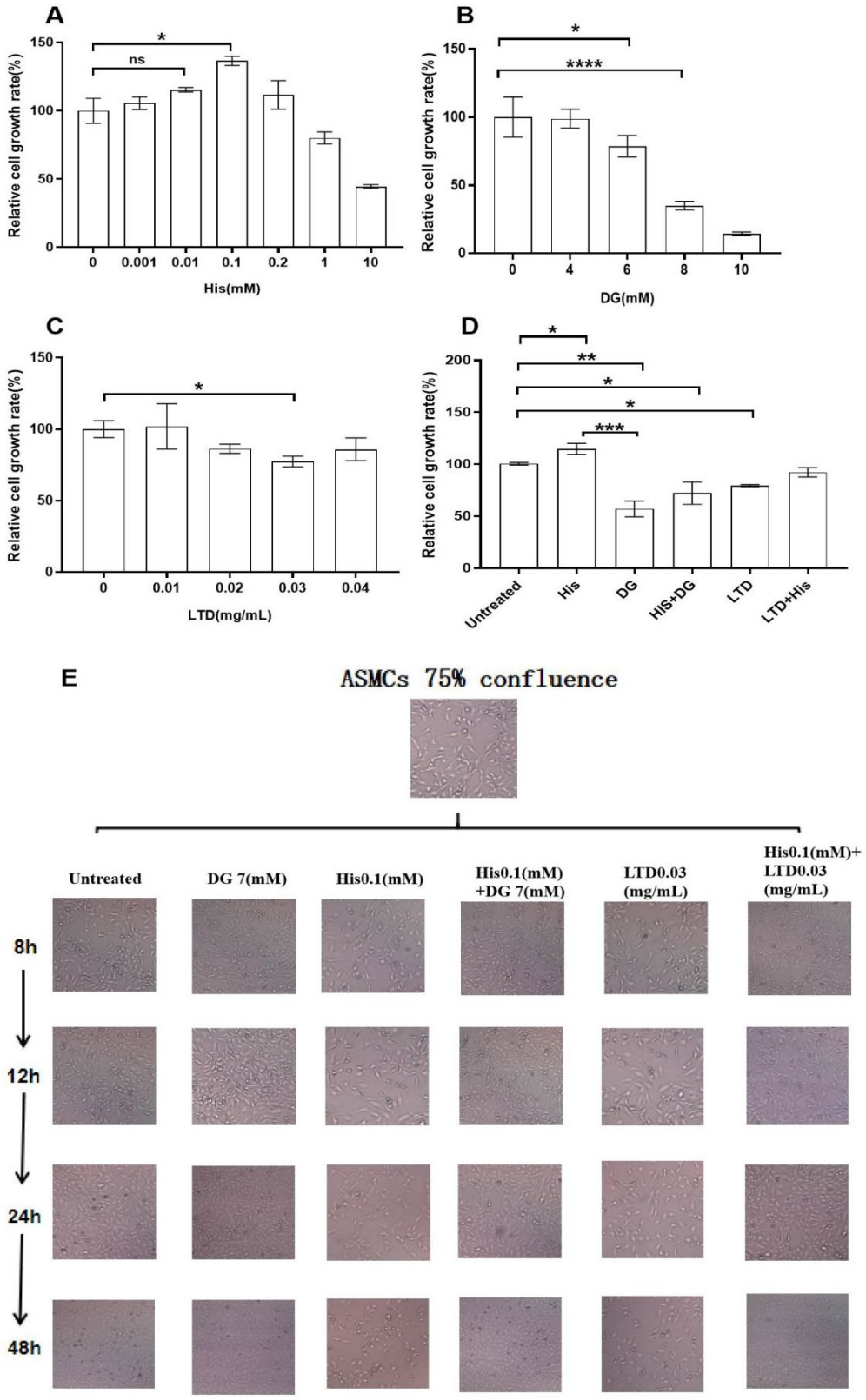

Figure 2: DG or His affected cell growth rate (RGR %) of ASMCs. The RGR % for the cells treated with: (A) His(0, 0.001, 0.01, 0.1, 0.2, 1, 10 mM), (B) DG(0, 4, 6, 8, 10 mM), (C) LTD(0, 0.01, 0.02, 0.03, 0.04 mg/mL), (D) His(0.1 mM), DG(7 mM), LTD(0.03 mg/mL) His(0.1 mM) + DG(7 mM) and His(0.1 mM) + LTD(0.03 mg/mL) for 24 h (MTT, 96-well plate). (E) Time-lapse photography: the images were taken at different time points in the same area (mid-zone) in the cell culture wells, ASMCs were treated with His(0.1 mM), DG(7 mM), LTD(0.03 mg/mL) His(0.1 mM) + DG(7 mM) and His(0.1 mM) + LTD(0.03 mg/mL)

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3.3. DG inhibits the migration of ASMCs induced by Histamine

The data of the cell migration scratch experiment proves that his promotes cell migration, and DG significantly reduces the migration ability of ASMCs. DG can inhibit the effect of His in promoting the migration of ASMCs (Figure 3). The cell migration rate at 48h of His(0.1 mM), His(0.1 mM) + LTD(0.03 mg/mL), LTD(0.03 mg/mL), His(0.1mM) + DG(7mM) and DG(7mM) were 81.17±3.04, 59.02±7.35, 52.245±4.625, 23.15±3.73, 16.815±4.655(%, mean ± SEM). Again, DG has similar effect of LTD (histamine receptor antagonist), on cells (Figure 3). The cell gap closure rate (%), relative to the gap at 0 h (before treatment) was calculated and presented as the means ± SEM.*P < 0.05.

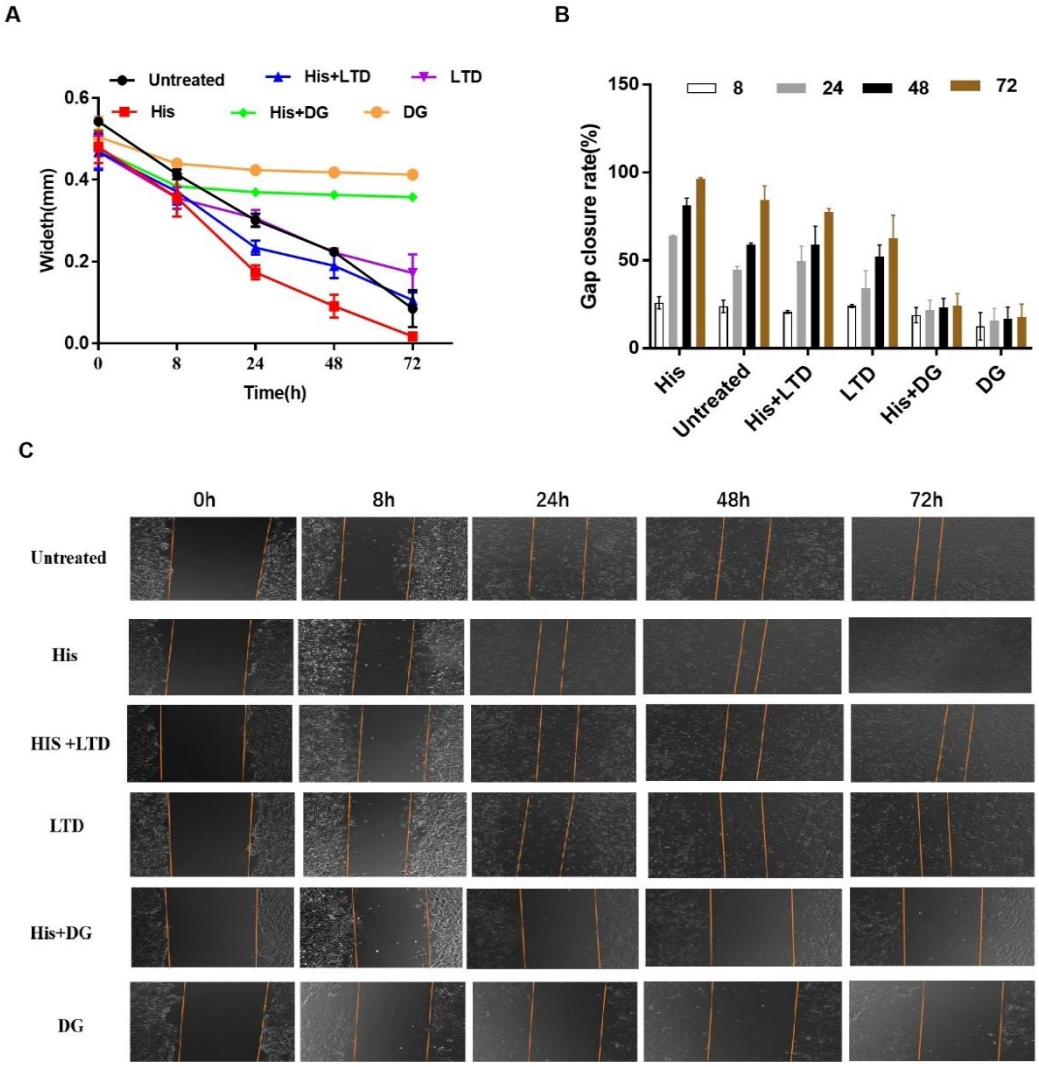

Figure 3: DG inhibits His-induced cell migration of ASMCs. The cell-scratch of ASMCs mono-layers were made (24 well plate) and treated with His(0.1 mM), DG(7 mM), His(0.1 mM) + DG(7 mM), LTD(0.03 mg/mL) and His(0.1 mM)+LTD(0.03 mg/mL) in SF condition (n = 3). At 0 h (before treatment), and at 8, 24, 48 and 72 h, the widths of cell-gaps were measured and the photos were taken with Carl Zeiss, Germany. (A) Time-Width, (B) The gap-closure rate (%) relative to the gap at 0h, (C) Picture of ASMCs after scratches

3.4. DG regulated the expressions of P38, ERK, JAK1, STAT5, Bax, Bcl-2 in ASMCs

The images from Western blotting (Figure 4) demonstrated that His-treated cells increased and DG-treated cells decreased the protein expressions of P38, ERK, JAK1, STAT5 and Bcl-2 expressions of ASMCs, but His decreased and DG increased Bax expression of ASMC. DG and LTD have similar effects on protein expressions of ASMCs.

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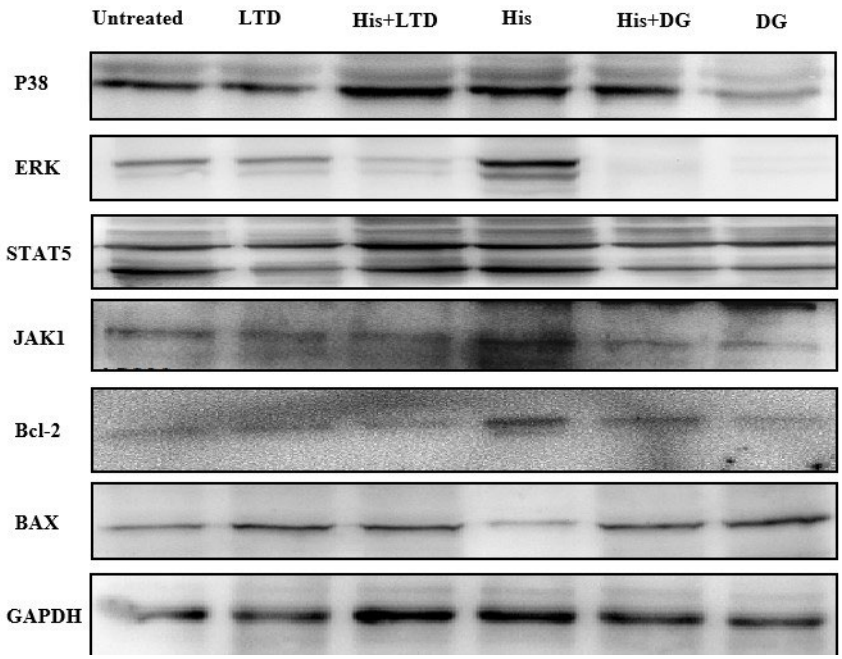

Figure 4: Effects of DG or His on P38, ERK, JAK1, STAT5, Bax, Bcl-2 protein expression of ASMCs. Protein expressions of P38, ERK, JAK1, STAT5, Bax, Bcl-2 by ASMCs treated with His(0.1 mM), DG(7 mM), His(0.1 mM) + DG(7 mM), LTD(0.03 mg/mL) and His(0.1mM) + LTD(0.03 mg/mL) for 24h

4. Discussion

The activation, differentiation and proliferation of airway smooth muscle cells are the central process of the occurrence and development of asthma. Histamine acts on H1 receptors of smooth muscle cells, which leads to smooth muscle contraction and ASMC activation. This study established a histamine-induced cell activation/proliferation model using primary ASMC to investigate the effects of DG on His-induced ASMC, and fund that DG has anti-histamine effects against His-induced ASMC activation and proliferation in vitro (Figure 2-3), showing its pharmaceutical potential for AHR relieve and alleviate asthma symptoms.

P38 mitogen-activated protein kinase (MAPK) is a key cellular regulator for gene expression, cytoskeleton remodeling, cell cycle and apoptosis [15]. The extracellular signal-regulated kinase (ERK) signaling pathway is an important intracellular signal transduction pathway, which is involved in various pathophysiological processes such as chronic airway inflammation, AHR and airway smooth muscle proliferation in asthma [16]. JAK / STAT signaling pathway regulates a variety of biological responses, including development, differentiation, cell proliferation and survival, immune response [17]. Histamine up-regulated the expressions of P38, ERK, JAK1, STAT5, Bcl-2 in the intracellular signal pathway to induce cell growth and proliferation; while DG suppressed the expressions of P38, ERK, JAK1, STAT5, Bcl-2 and increased the expression of Bax to inhibit cell grow and induces cell apoptosis,, showing the anti-histamine effects.

Apoptosis is a form of program cell death, which related with the apoptosis-stimulating gene Bax and apoptosis-inhibiting gene Bcl-2 [18]. Over-expression of Bax can antagonize the protective effect of Bcl-2 and induce cell death; the ratio of Bax/Bcl-2 is a indicator for cell apoptosis [19]. Present data showed that histamine can down regulate Bax/Bcl-2 ratio, inducing abnormal activation and proliferation of airway smooth muscle cells, and increase cell viability. However, DG can up regulate the ratio of Bax/Bcl-2, inducing apoptosis of over-proliferative ASMCs (Figure 4).

On the basis of the targets of histamine receptor, explore the binding ability with DG by molecular docking. Molecular docking is used as a computational tool to forecast the binding power and combination pattern of proteins and ligands [20]. The data in figure 1 further proved the accuracy and reliability of the virtual computation of network pharmacology molecules and molecular docking. Based on targeting histamine receptor, DG showed the high binding ability. Putting together with the experimental verification of cell model, DG can significantly inhibit His-induced ASMC proliferation to reduce the severity of airway hyperresponsiveness.

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In summary, Histamine promotes ASMCs activation and proliferation by up-regulating intracellular expressions of P38, ERK, JAK1, STAT5 signal cascade, leading to ASMC abnormally proliferation and causing airway constriction. While LTD and DG can inhibit those histamine-induced effects, also DG can induce Bax expression, leading to cell apoptosis in-vitro, to reduced ASM constriction. However, the anti-histamine function of DG can be used for treating asthma and AHR clinically, which requires great attention in pharmacy.

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