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Diammonium glycyrrhizinate inhibits histamine-induced airway smooth muscle cell activation and proliferation by regulating MAPK/FAK signal pathway and MMP-2 activity

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Abstract: *Excessive proliferation of airway smooth muscle cells can cause airway problems. Mast cell-derived histamine induces bronchoconstriction. Glycyrrhizic acid is used to relieve COVID-19 airway symptoms from 2020 to now, but the mechanism of action is unknown. Then diammonium glycyrrhizinate is one of the derivatives of glycyrrhizic acid, the effects of diammonium glycyrrhizinate on histamine-induced airway smooth muscle cell proliferation is not discussed yet. This study was to establish a histamine/primary rat airway smooth muscle cell model, to investigate the effects of diammonium glycyrrhizinate on these cells, and to explore the potential mechanism of diammonium glycyrrhizinate in inhibiting histamine-induced airway constriction and deep airway injury. Cell viability and proliferation were assessed; matrix metalloproteinases-2 (MMP-2) activity was examined by gelatin zymography; cell cycle was detected by flow-cytometry, protein expressions of FAK were evaluated by fluorescence-immunocytochemistry; p38, Bcl-2, PCNA and GAPDH were evaluated by western blotting. Data show that diammonium glycyrrhizinate inhibited histamine–induced airway smooth muscle cell proliferation and MMP-2 activity; recovers histamine-induced airway smooth muscle cell cycle disorders, and restores the histamine-regulated intracellular signals. The present study suggests an underlying mechanism that diammonium glycyrrhizinate can relieve the airway symptom of COVID-19 patients by inhibiting histamine-induced over-activation/proliferation and regulating airway smooth muscle cell intracellular signaling pathways. The pharmaceutical potential of diammonium glycyrrhizinate requires attention.*

Keywords: *airway smooth muscle cell, histamine, diammonium glycyrrhizinate, airway constriction*

Abbreviations:

ASM	airway smooth muscle
ASMC	airway smooth muscle cell
His	histamine
DG	diammonium glycyrrhizinate
MMP-2	matrix metalloproteinase-2
PI	pyridine iodide
BSA	bovine serum albumin
P38	P38 mitogen-activated protein kinase
Bcl-2	B-cell lymphoma-2
PCNA	proliferating cell nuclear antigen
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
FAK	focal adhesion kinase
His-induced	histamine-induced

1. Introduction

Airway wall smooth muscle (ASM) functions to keep the flexibility of airway wall and maintain bronchial muscle contraction. The pathological changes of ASM, such as airway wall thickening, airflow obstruction and basic airway resistance are associated with many lung diseases[1], including allergic asthma, cystic fibrosis, and chronic obstructive pulmonary diseases[2], also the novel coronavirus

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pneumonia [3, 4]. The combination of hypertrophy (increase in cell size) and hyperplasia (increase in cell count) influences the airway wall, which is related to excessive proliferation, migration and contraction of airway smooth muscle cells (ASMC) [5-8].

Histamine (His) is a well-known chemical mediator and released from mast cell activation.[9, 10]. The His evoked a contraction of human bronchoconstriction was recognized first as one of the biological actions of His [11]. His selectively recruits the major effecter cells into tissue sites and affects their maturation, activation, polarization, and other functions leading to chronic inflammation.

Glycyrrhizic acid, a triterpene isolated from the roots and rhizomes of licorice [12], named Glycyrrhiza glabra, is the principal bioactive ingredient of anti-viral, anti-inflammatory and hepatoprotective effects [13, 14]. Glycyrrhizic acid has been used in the clinical treatment of hepatitis, bronchitis, gastric ulcer, AIDS (acquired immunodeficiency syndrome), certain cancers and skin diseases[15]. Glycyrrhizic acid is used to relieve SAS airway symptoms in 2003[16], and relieve COVID-19 airway symptoms from 2020 to now [17], but the mechanism of action is unknown. Then diammonium glycyrrhizinate (DG) is one of the derivatives of glycyrrhizic acid and has a variety of pharmacological effects, including significant anti-inflammatory, antiviral and liver protective effects [18, 19].

The present study was established a His/ASMC cell model to investigate the effects of DG on His-induced cell proliferation of ASMC and the intracellular signal pathway involved in P38 mitogen-activated protein kinase.

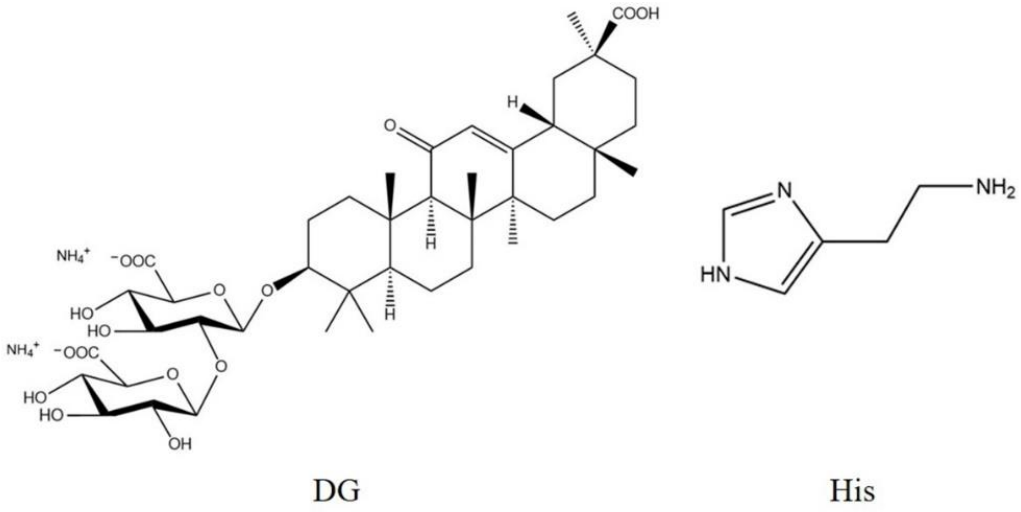

Figure 1: The molecular structures of DG and His.

2. Materials and methods

2.1. Rat primary ASMCs extraction and culture

Rat primary ASMCs were prepared as previously described [20]. Briefly, the tracheae were separated from lung tissue of rats, the connective tissues and fat were removed. The tracheae tube was then cut into about 1 mm^3 pieces and placed in Trypsin-EDTA solution(0.25% Trypsin)for 2 hours, and then placed in completed DMEM containing 10% fetal bovine serum with 100 units/mL penicillin and 100 mg/mL streptomycin in a 5% CO_2 incubator at 37℃ until cells came out from tracheae tube pieces. ASMCs were isolated by different attachment times and cultured in completed DMEM that was changed every three days, and identified by α-SMA staining. The primary 3-6 passages of cells were for following experiments.

2.2. Cell viability assay (MTT method)

As mentioned in previous study[20], cells were seeded (1.3×104 cells/well) in a 96-well plate in completed DMEM and incubated overnight at 37℃ in 5% CO2. Cells were treated when 75% confluence with the various amounts of His (0, 0.1, 0.2, 0.4, 0.6 mM), DG (0, 0.2, 0.4, 0.6, 0.8 mg/mL). Then the mixture of His (0.2 mM) with DG (0.4 mg/mL) was to interfere with the cells. The supernatants

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were collected for gelatin zymography assay (n=3-6, mean ± SEM).

2.3. Gelatin zymography analysis

The expressions of MMP-2 in cell supernatant were evaluated using gelatin zymography analysis as previously described [20]. The images were recorded by camera (Gel Doc XR+, Bio-Rad). The intensities of the bands in gels were determined and semi-quantified using the Image Lab software (version 7.0, Bio-Rad Laboratories).

2.4. Cell cycle analysis

As mentioned in previous study [21], the cells were seeded (15×104 cells/well) in a 24-well plate and cultured overnight. Cells were treated when 80% confluence with His (0.2mM), DG (0.4mg/mL) or the mixture of His (0.2 mM) with DG (0.4mg/mL) for 24h. The cells were digested with Trypsin (without EDTA), collected in a centrifuge tube and centrifuged. Then cells were washed with PBS, and stain with pyridine iodide (PI) (50μg/mL) and RNAS A (100mg/mL) at 4°C for 30 min in the dark. Flow cytometer (BD FACSCalibur™) and ModFit LT 3.1 software package are used for cycle analysis.

2.5. Fluorescence immunocytochemistry

As mentioned in previous study [22], cells cultured with His (0.2 mM), DG (0.4 mg/mL) or the mixture of His (0.2 mM) with DG (0.4 mg/mL) in a 24-well plate, after 24 - hour treatment (SF), were fixed with precooled methanol 4℃ for 30 minutes and permeated with 0.05% Triton X - 100 (Sigma) in PBS for 15 minutes at room temperature and then washed and blocked with 3% bovine serum albumin (BSA) in PBS. All primary antibodies against focal adhesion kinase (FAK) were diluted in 3% BSA and incubated for 120 minutes at room temperature, and fluorescent conjugated secondary antibodies were applied, cell nuclei staining with propidium iodide (100μg/mL). Combine images using immunochemical microscope (X40/X100) (Carl Zeiss, ZEN-3-0-blue-Hotfix-4).

2.6. Western blot assay

As mentioned in previous study [20], the protein contents of ASMC lysis were determined using BCA method. The equal proteins were subjected to separation by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with the corresponding antibody (P38, Bcl-2, PCNA or GAPDH as a house-keeping protein) for 90 minutes at room temperature; then incubated with horseradish peroxidase-linked secondary antibody for 45 minutes. Enhanced chemiluminescence (Perkin Elmer, Waltham, USA) was used to develop the signals. The images were captured using a ChemiDoc™ CRS+Molecular Imager (Bio-Rad Laboratories, USA) and quantified by Image Lab software (version 7.0, Bio-Rad Laboratories).

2.7. Statistical analyses

GraphPad Prism 8.0 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. His or DG influenced viability of ASMCs

The results from MTT assay (Figure 2) demonstrated that ASMC proliferation was induced by His (0, 0.1, 0.2, 0.4, 0.6 mM) dose-dependently (Figure 2A), and inhibited by DG (0, 0.2, 0.4, 0.6, 0.8 mg/mL) (Figure 2B). When cell treated with His (0.2 mM), DG (0.4 mg/mL) and the mixture of His (0.2 mM) with DG (0.4 mg/mL), the cell viabilities (%) relative to untreated 100 ±0.12) were 111.2 ±2.84, 75.2 ±4.23 and 99.4 ±1.27 (mean ± SEM) respectively (Figure 2C).

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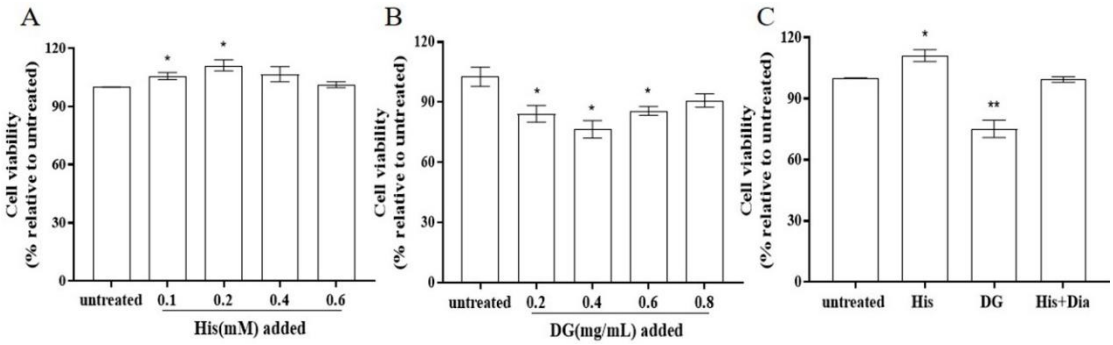

*Figure 2: His or DG influenced cell viability and proliferation of ASMCs. (A) A range of concentrations of His; (B) A range of concentrations of DG; (C) His (0.2 mM), DG (0.4 mg/mL), the mixture of His with DG (His+DG, 0.2 mM+0.4 mg/mL). Cells were treated with different concentrations of Intervention for 24h. The cell viability (%) relative to untreated cells were calculated. Data represented as the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01.*

3.2. His or DG influenced MMP-2 activity of ASMCs

The date from zymography analysis showed that His or DG influenced pro-MMP-2 and active-MMP-2 secretions/activities of ASMCs in culture supernatants(Figure 3A).When ASMCs were treated with His (0.2 mM), DG (0.4mg/mL) and the mixture of His (0.2 mM) with DG (0.4 mg/mL) respectively, the relative expression rates for pro-MMP-2 of ASMCs to untreated cells were 217.3±39.62, 356.0±53.91, 153.6±30.51 and 230.4±38.8 (%, mean ± SEM) (Figure 3B) and for active-MMP-2 were 479.2±39.96, 657.4±40.52, 431.3±43.55 and 554.5±21.47 (%, mean ± SEM)(Figure 3C).

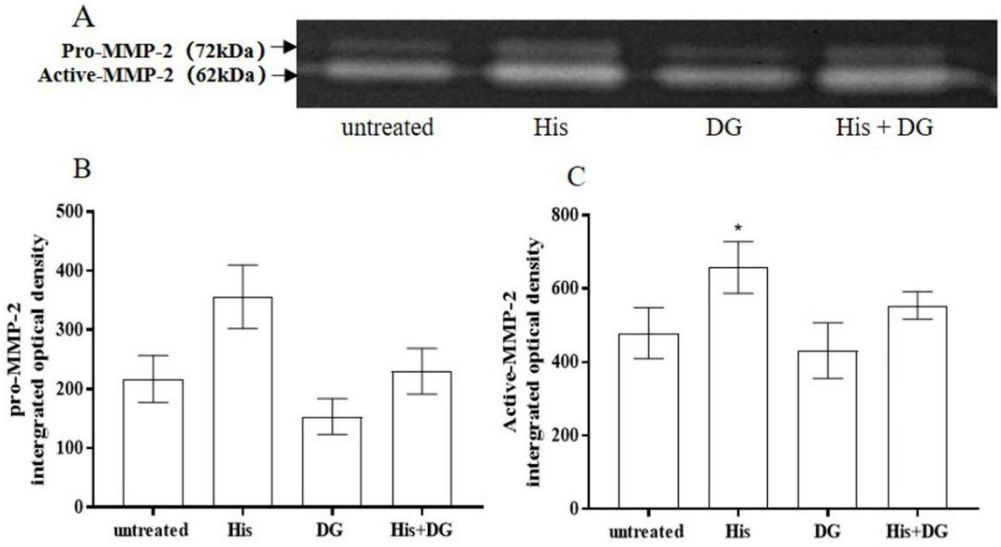

*Figure 3: Effects of His (0.2 mM), DG (0.4 mg/mL) and the mixture of His with DG (His + DG, 0.2mM+0.4mg/mL) on MMP-2 activity secreted by ASMCs. (A) gelatin zymography images ; (B) band intensity semi-quantization of pro-MMP-2; (C) band intensity semi-quantization of active-MMP-2. Data are shown as mean±SEM. *P <0.05,**P <0.01 (n = 3).*

3.3. The His-induced dys-regulation of cell cycle was recovered by DG

Flow cytometry cell cycle analysis showed that compared with the untreated cells, the G0/G1 phase of the His-treated cells was decreased by 1.37%, and the S phase increased by 1.26%, G2/M phase decreased by 0.12% (Figure 4B). G0/G1 phase of treatment of DG-treated cells was increased by 2.53%, S phase decreased by 1.77%, G2/M phase decreased by 0.74% (Figure 4C). After treatment with the mixture of His with DG, the G0/G1 phase recovered by 1.48%, and the S phase decreased by 1.46%, but the G2/M phase decreased by 0.01% respectively (Figure 4D). The differences in the cell percentage of G2/M phase of the four groups were small, and the mixture of His with DG promoted the differentiation of ASMCs in the S phase to a great extent.

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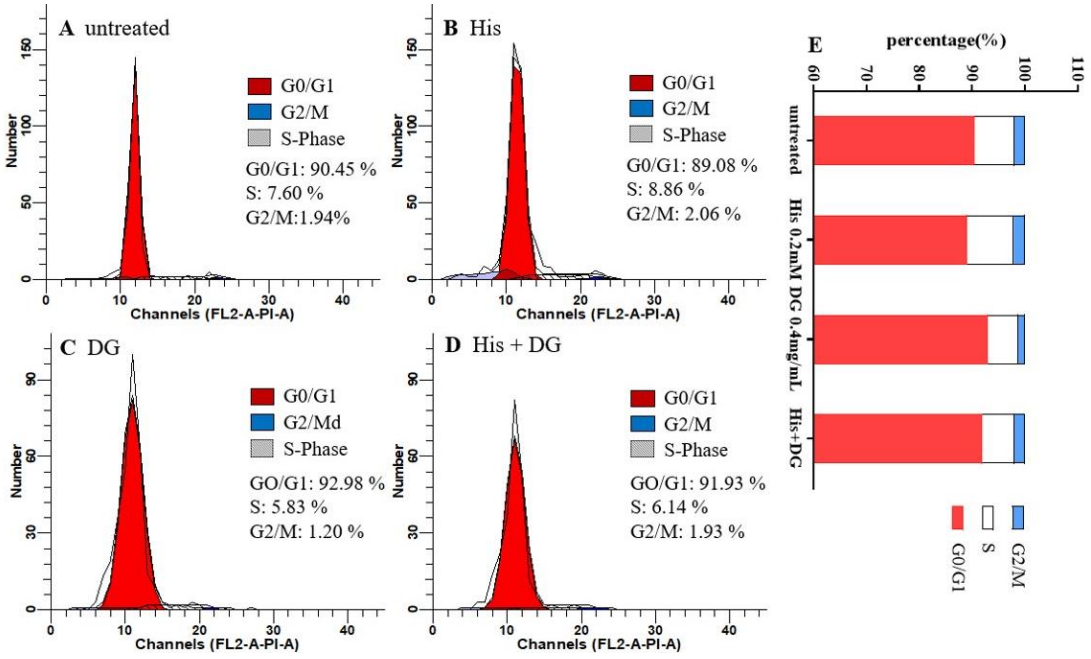

Figure 4: DG regulated cell cycles (Flow-cytometry analysis). The ASMCs were cultured with (A) untreated; (B) His (0.2mM); (C) DG (0.4mg/mL) and (D) the mixture of His with DG (His + DG, 0.2 mM+0.4 mg/mL) for 24h; after 20h PI staining and Flow-cytometry analysis were carried out. ModFit LT 3.1 software package were used to examine the cell cycle. (E) Summary of the change in percentage.

3.4. The His-disrupted FAK expression in ASMC was adjusted by DG

The data from immunocytochemistry illustrated that after His treatment, the expression of FAK in ASMCs were increased. Compared with untreated cells, FAK expressions were decreased by DG treatment. Then DG adjusted the expression of FAK destroyed by His when His was mixed with DG.

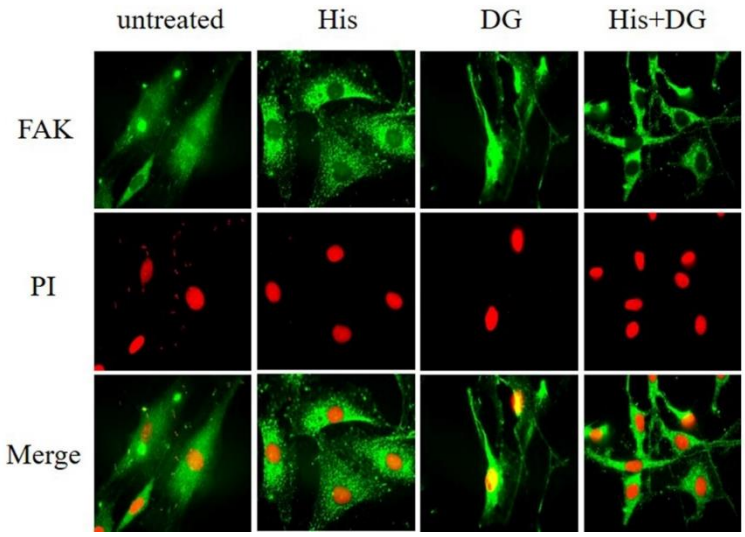

Figure 5: DG adjusted the expression of focal adhesion FAK destroyed by His of ASMCs with His (0.2mM), DG (0.4mg/mL) and the mixture of His with DG (His+DG, 0.2mM+0.4mg/mL) for 24h. The green fluorescence is for target proteins and the red fluorescence is cell nucleus stain with propidium iodide (PI). Images were taken and combined using immunochemical microscope (Carl Zeiss, ZEN-3-0-blue-Hotfix-4, X100).

3.5. DG decreased the expression of P38, Bcl-2 and PCNA increased by His

The images from Western blotting (Figure 6) demonstrated that His-treated cells increased P38, Bcl-2 and PCNA expressions of ASMCs, but DG-treated cells decreased P38, Bcl-2 and PCNA expressions

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of ASMCs. Then DG decreased the protein expression of P38, Bcl-2 and PCNA increased by His of ASMCs when His was mixed with DG.

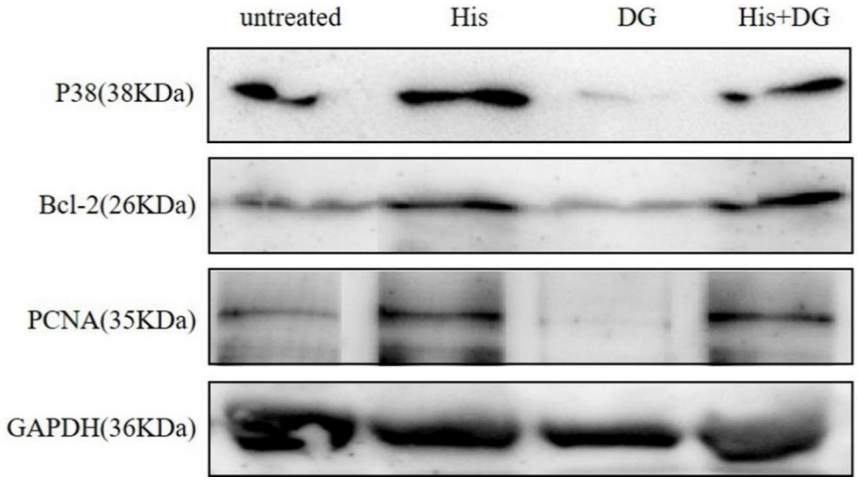

Figure 6: Effects of DG or His on P38, Bcl-2, PCNA and GAPDH protein expression of ASMCs. Protein expressions of P38, Bcl-2 and PCNA by ASMCs treated with His (0.2mM), DG (0.4mg/mL) and the mixture of His with DG (His+DG, 0.2mM+0.4mg/mL) for 24h.

4. Results

Changes in ASM, such as cell excessive proliferation, smooth muscle restructure, cause airway problems. His plays a role in airway obstruction via smooth muscle contraction, bronchial secretion, and airway mucosal edema. As shown in our data, His activated ASMC, induced ASMC proliferation and elevated MMP-2 activation, which can lead to airway pathological changes. The current in vitro study uses the His/ASMC model 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.

His induces cell growth and proliferation by up-regulating the expression of P38, Bcl-2 and PCNA in the intracellular signal pathway, while DG recover this process by converting the expression of P38, Bcl-2 and PCNA, showing the anti-histamine effects. P38 is a key cellular regulator for gene expression, cytoskeleton remodeling, cell cycle and apoptosis. Bcl-2 is an important factor together with Bax, controlling mitochondrial apoptosis pathway [23]. P38 is necessary for Bcl-2 function in regulating apoptosis, autophagy, cell cycle arrest and senescence after DNA damage [24]. PCNA is synthesized in the cell nucleus and exists in the cell nucleus. It is closely related to cell DNA synthesis, plays an important role in the initiation of cell proliferation, and is expressed in all phases of the cell cycle. Our data indicates that DG inhibited His-induced ASMC activation/proliferation through P38/Bcl-2/PCNA pathway.

FAK is a prominent kinase, assembling various signals from integrins, growth factors or mechanical stimulations and then further activating MAPK signal pathways to regulate cell growth, cell adhering and migration. Interestingly, our data clearly shows that His can promote the expression of FAK, implicating that it has an effect on the migration of ASMCs. DG inhibits the His-induced FAK expression, showing the ability of anti-histamine on ASMC.

Matrix metalloproteinases, a group of extracellular matrix proteins, are involved in extracellular matrix degradation and growth factor release. MMP-2 is a marker for ASMC activation and a key enzyme for type-IV collagen degradation [25]. The expression of MMP-2 is regulated by P38 is a regulator for cell activation and proliferation [26-28]. Again, the data demonstrated that DG inhibited the His-induced ASMC activation associated with inhibiting His-induced MMP-2 activity.

In summary, this study emphasizes the underline mechanism that DG involves in overall connection of ASMC responses from extracellular to intracellular. His acts as a mediator to stimulate a cascade of signals within the cell, involving the classic MAPK/FAK pathway, and leading to ASMC abnormally proliferation, disarrangement and causing airway constriction. However, DG seems to inhibit the airway constriction caused by His, which requires great attention in pharmacy.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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