

Oridonin attenuates apoptosis and NLRP3 inflammasome activation in IL-4-stimulated human bronchial epithelial cells in an in vitro pediatric asthma model

Weiwei Wang^{A,B,D,F}, Dan Ming^{A–D}

Department of Pediatrics, Tianjin Hospital, China

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Address for correspondence

Weiwei Wang
E-mail: tjyywww@sina.com

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Abstract

Background. Asthma is a chronic illness that causes recurrent inflammation and airway constriction. The primary risk factors for asthma development are exposure to environmental allergens and house dust mites, which can trigger deoxyribonucleic acid (DNA) damage. Oxidative stress can also cause DNA impairments and plays a crucial role in the progression of human immunological disorders.

Objectives. The aim of the study was to evaluate the effects of oridonin (ORD) on proliferation, inflammation and apoptosis in interleukin 4 (IL-4)-stimulated human bronchial epithelial (16HBE) cells.

Materials and methods. Proliferation was assessed using a 5-Bromo-2-deoxyuridine (BrdU) assay, while acridine orange (AO), ethidium bromide (EB), propidium iodide, and 4',6-diamidino-2-phenylindole (DAPI) measured apoptosis. The protein expression levels of apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), cleaved caspase-1, and nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3) were detected with western blot.

Results. The results established that IL-4 stimulation markedly decreased ($p < 0.05$) the proliferation of 16HBE cells, while the administration of ORD increased their proliferation. Apoptosis and DNA damage were enhanced in the IL-4-stimulated group, whereas ORD exhibited anti-apoptotic activity. Moreover, the treatment with ORD significantly reduced ($p < 0.05$) the IL-4-induced expression of cleaved caspase-1, ASC and NLRP3 proteins.

Conclusions. The findings suggest that NLRP3 is a direct target for ORD-mediated anti-inflammatory actions in injured 16HBE cells. Therefore, ORD may be a novel therapy against NLRP3-related disorders, including pediatric asthma (PA).

Key words: apoptosis, oridonin, NLRP3 inflammasome, inflammation, pediatric asthma

Cite as

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Background

Asthma is a chronic inflammatory syndrome that causes recurrent inflammation and narrowing of the airways.¹ Pediatric asthma (PA) is a persistent inflammatory respiratory disease characterized by inflammation and airway hyper-responsiveness and alterations.² Despite substantial developments in treatment strategies, the increasing occurrence of PA has become a burden to human mental and physical health due to its substantial prevalence and related juvenile death.^{3,4} The elevated incidence of asthma has been ascribed to numerous risk factors, including genetics, house dust mites, allergens, environmental pollution, and childhood obesity.^{5,6}

Reports have shown that chronic airway inflammation stimulates airway restoration, which is accompanied by generation of excessive extracellular matrix (ECM).⁷ Furthermore, it is well documented that a central pathological feature of asthma involves stimulated inflammatory cells that produce several pro-inflammatory cytokines to induce hypersecretion of mucus in the affected airway.⁸ Accordingly, potential drugs that can prevent mucus discharge, ECM production and airway inflammation may be effective in PA treatment. Although effective, enduring corticosteroid usage in youngsters leads to hormone resistance and toxic effects.⁹ Therefore, it is vital to understand the multifaceted molecular mechanisms of PA to improve future therapies.

Oridonin (ORD) is a bioactive ent-kaurane diterpenoid isolated from the aromatic plant *Rabdosia rubescens*, which has been comprehensively employed in the Chinese traditional medicine.¹⁰ Accumulating evidence propose that ORD exerts apoptotic and anti-proliferative activities in numerous malignant cells, including mammary, colorectal, and liver cancer cells.^{11–13} Studies unveiled that ORD triggered apoptosis in several tumor cells such as acute gliomas, leukemia, melanoma cells, and prostate, and these in vitro trials have exposed that ORD prompts cell death, recovers the phagocytosis, and prevents cell cycle progress.^{14,15} In ancient times, ORD was used as a remedy for many inflammatory disorders.¹⁶ Previously, ORD has been documented to constrain MAPK/NF- κ B stimulation to suppress the generation of pro-inflammatory cytokines.¹⁷ Furthermore, ORD has demonstrated protective anti-inflammatory action in neuroinflammation, colitis and sepsis.^{18–20}

However, the principal mechanisms through which ORD interacts with its targets remain unidentified. Recently, it has been shown that nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3) is a direct target for ORD-mediated anti-inflammatory action.²¹ The NLRP3 inflammasome is a complex of multiple proteins including NLRP3, caspase-1 and apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC).²² The activation of the complex stimulates caspase-1 and initiates the cleavage and production

of cytokines, exhibiting profound action in inflammation and immunity. However, the protective role and the underlying mechanisms of ORD against cell apoptosis in bronchial epithelial cells remain unknown.

Objectives

The current study explored the mechanisms underlying interleukin 4 (IL-4)-induced human bronchial epithelial (16HBE) cell injury and assessed the protective actions of ORD. Specifically, the study investigated the regulatory effects of ORD on apoptosis and IL-4-induced deoxyribonucleic acid (DNA) damage to identify an innovative approach to PA treatment.

Materials and methods

Chemicals

Oridonin (empirical formula: $C_{20}H_{28}O_6$ and molecular weight (MW): 364.43 g/mol; Thermo Fisher Scientific, Waltham, USA), with a purity $\geq 98\%$, confirmed with high-performance liquid chromatography (HPLC), was initially dissolved in dimethyl sulfoxide (DMSO) to prepare a 20-mM stock solution, and stored at -20°C for further use. Other chemicals, including RPMI-1640 media, fetal bovine serum (FBS), antibiotics, phosphate-buffered saline (PBS), 5-Bromo-2-deoxyuridine (BrdU), acridine orange (AO), ethidium bromide (EB), 4',6-diamidino-2-phenylindole (DAPI), DMSO, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and sodium dodecyl-sulfate (SDS), were acquired from Thermo Fisher Scientific. The antibodies for western blot examination were procured from Beyotime Biotechnology (Haimen, China).

Cell culture

The 16HBE cells (Shanghai Aiyuan Biotechnology Co., Ltd., Shanghai, China) were cultured in RPMI-1640 medium containing FBS (10%) and 1% of antibiotics, and incubated at 37°C and 5% CO_2 in a moistened chamber. The 16HBE cells were arbitrarily stratified into 4 groups: 16HBE control cells, 16HBE cells + ORD (20 $\mu\text{M}/\mu\text{L}$), 16HBE cells + IL-4 (100 ng/ μL), and 16HBE cells + IL-4 (100 ng/ μL) + ORD (20 $\mu\text{M}/\mu\text{L}$).

Cell proliferation assay

Proliferation was estimated in 16HBE cells using the BrdU technique. Concisely, cells were seeded onto 96-well plates and grown in a culture medium containing IL-4 in the presence or absence of ORD. The BrdU (10 μL) was added separately to all wells after 48 h, and the plates were incubated for 4 h. Afterward, treated

cells were incubated with BrdU for 60 min. Wells were washed 3 times with PBS, a substrate solution (200 μ L) was added to each well for 25 min, and sulfuric acid was added to the wells in the final step. The optical density (OD) was determined for each well at 490 nm, and the relative rate of cell proliferation was calculated as a percentage of the control. Three independent replicates were performed in each group.

Analysis of apoptosis with acridine orange and ethidium bromide double staining

Morphological and nuclear changes were assessed in treated 16HBE cells with AO and EB double staining.²³ The 16HBE cells were grown in a medium spiked with IL-4, with or without ORD, and kept for 1 day. Each group of cells had a mixture of AO and EB added, and were incubated at room temperature in the dark for 20 min. Cells were then washed using PBS to remove unbound AO and EB, and viewed under a fluorescence microscope (Nikon Eclipse TS100; Nikon Corp., Tokyo, Japan).

Cell apoptosis assay

The 16HBE cells were grown in a medium supplemented with IL-4 in the presence or absence of ORD for 24 h and then treated with 5% cigarette smoke extract (CSE) for 1 h. The cells were fixed with cold methanol and acetone (1:1) for 5 min, then incubated with DAPI and propidium iodide (PI) solution for 10 min. The apoptotic morphology of the cells was observed using fluorescence microscope (Nikon Eclipse TS100). For quantification, the 16HBE cells were washed with cold PBS buffer and harvested, and the number of stained cells was counted and expressed as a percentage of apoptotic cells. Three independent trials were performed to determine the mean apoptosis rate.

Western blot study

The 16HBE cells were cultured for 1 day in a medium spiked with IL-4 in the presence or absence of ORD (20 μ M). The cells were then lysed in an ice-cold lysis

buffer containing protease inhibitors and used for a western blot assay. Cellular protein content was measured using a Pierce™ BCA Protein Assay Kit (Pierce Chemical, Dallas, USA). Briefly, the proteins were electrophoretically dispersed and transferred to a polyvinylidene difluoride (PVDF) film. Afterward, the film was blocked overnight at 4°C and then incubated overnight at 4°C with primary antibodies (1:100 dilutions); secondary antibodies (1:1000) were added subsequently. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Protein visualization employed an Odyssey imaging system (LI-COR Biosciences, Lincoln, USA).

Statistical analyses

The data from each group were statistically analyzed using GraphPad Prism v. 8.0.1 (GraphPad Software, San Diego, USA) and Statistical Package for Social Sciences (SPSS) v. 25 software (IBM Corp., Armonk, USA). The Shapiro–Wilk test determined the normality of data distribution. Comparisons between groups (control, $n = 6$; ORD, $n = 6$; IL-4-induced, $n = 6$; and IL-4+ORD, $n = 6$) utilized one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The Kruskal–Wallis test generated p -values because the sample size was small. The continuous data are presented as mean \pm standard deviation ($M \pm SD$), and differences were considered statistically significant for $p < 0.05$.

Results

All variables had a normal distribution. Table 1 presents the comparison of the variables between the groups.

The influence of oridonin on IL-4-induced 16HBE cell proliferation

To examine the effects of ORD on PA, we evaluated the proliferation of IL-4-treated 16HBE cells using an in vitro model (Fig. 1), with BrdU used as a marker. Proliferation was marginally elevated in the ORD group, whereas

Table 1. Comparison of the groups

Variables	Control ($n = 6$)	ORD ($n = 6$)	IL-4 inducer ($n = 6$)	IL-4+ORD ($n = 6$)	p -value*	Welch's p -value
AO/EB	5.23 \pm 0.39	6.90 \pm 0.52	80.18 \pm 6.13	51.10 \pm 3.9	<0.001	<0.001
ASC	1.00 \pm 0.07	0.74 \pm 0.05	1.73 \pm 0.13	0.89 \pm 0.06	<0.001	<0.001
Cleaved caspase-5	1.00 \pm 0.07	0.83 \pm 0.06	1.60 \pm 0.12	0.99 \pm 0.076	<0.001	<0.001
DAPI	9.35 \pm 0.71	9.40 \pm 0.71	83.28 \pm 6.37	53.52 \pm 4.09	<0.001	<0.001
MTT	100.01 \pm 7.61	99.97 \pm 7.61	49.12 \pm 3.76	77.26 \pm 5.91	<0.001	<0.001
NLRP3	1.00 \pm 0.076	0.41 \pm 0.031	1.49 \pm 0.111	0.67 \pm 0.049	<0.001	<0.001
PI	6.20 \pm 0.47	7.03 \pm 0.53	91.10 \pm 6.97	49.73 \pm 3.80	<0.001	<0.001

AO/EB – acridine orange/ethidium bromide; ASC – C-terminal caspase recruitment domain; DAPI – 4',6-diamidino-2-phenylindole; MLRP3 – nucleotide-binding domain and leucine-rich repeat protein 3; PI – propidium iodide; ORD – oridonin; IL-4 – interleukin 4; * based on Kruskal–Wallis test. Data are presented as mean \pm standard deviation ($M \pm SD$).

it declined considerably in the IL-4-treated cells compared to the control cells ($p < 0.05$, Fig. 1). Moreover, the supplementation of the IL-4-treated 16HBE cells with ORD markedly enhanced proliferation compared to the IL-4-treated 16HBE cells without ORD ($p < 0.05$).

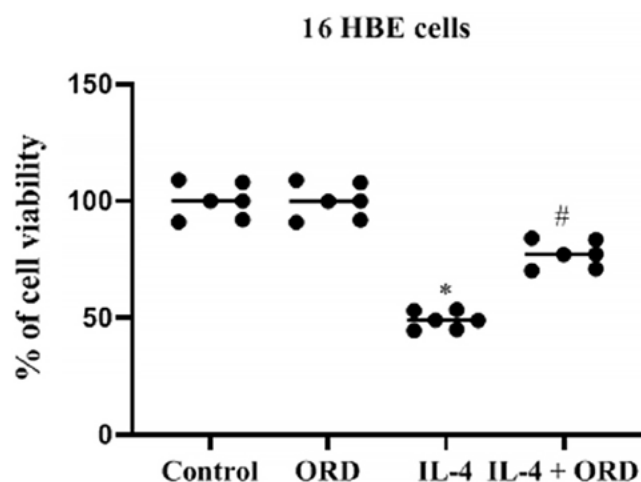


Fig. 1. Oridonin (ORD) ameliorates human bronchial epithelial (16HBE) cell proliferation. 16HBE cell relative proliferation was determined using the 5-Bromo-2-deoxyuridine (BrdU) method. The figure presents spectrofluorometric quantification of apoptosis. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's test for post hoc analysis

* $p < 0.05$, # $p < 0.01$ compared to the control cells; ORD – oridonin; IL-4 – interleukin 4.

The influence of oridonin on IL-4-induced 16HBE cell apoptosis

We explored the apoptotic activity of ORD on 16HBE cells using AO and EB (Fig. 2A), DAPI (Fig. 3A), and PI (Fig. 4A) assays. Control 16HBE cells and ORD-treated cells showed no substantial variation in apoptosis or DNA damage. Conversely, the administration of ORD attenuated IL-4-induced apoptosis and DNA damage in 16HBE cells (Fig. 2B, 3B, 4B).

Oridonin inhibited NLRP3 inflammasome activation in IL-4-stimulated 16HBE cells

The western blot analysis was used to assess NLRP3 inflammasome activation. The results demonstrated that ORD did not markedly alter protein expression levels of ASC, cleaved caspase-1, or NLRP3 in control 16HBE cells (Fig. 5A). However, IL-4 stimulation significantly increased ($p < 0.05$) the cleaved caspase-1 (Fig. 5B), ASC (Fig. 5C) and NLRP3 protein levels (Fig. 5D), which were suppressed by ORD (Fig. 5A–D).

Discussion

Asthma is recognized as a highly prevalent lethal clinical condition that causes a substantial health and economic burden.¹ The characteristic hallmarks of bronchial

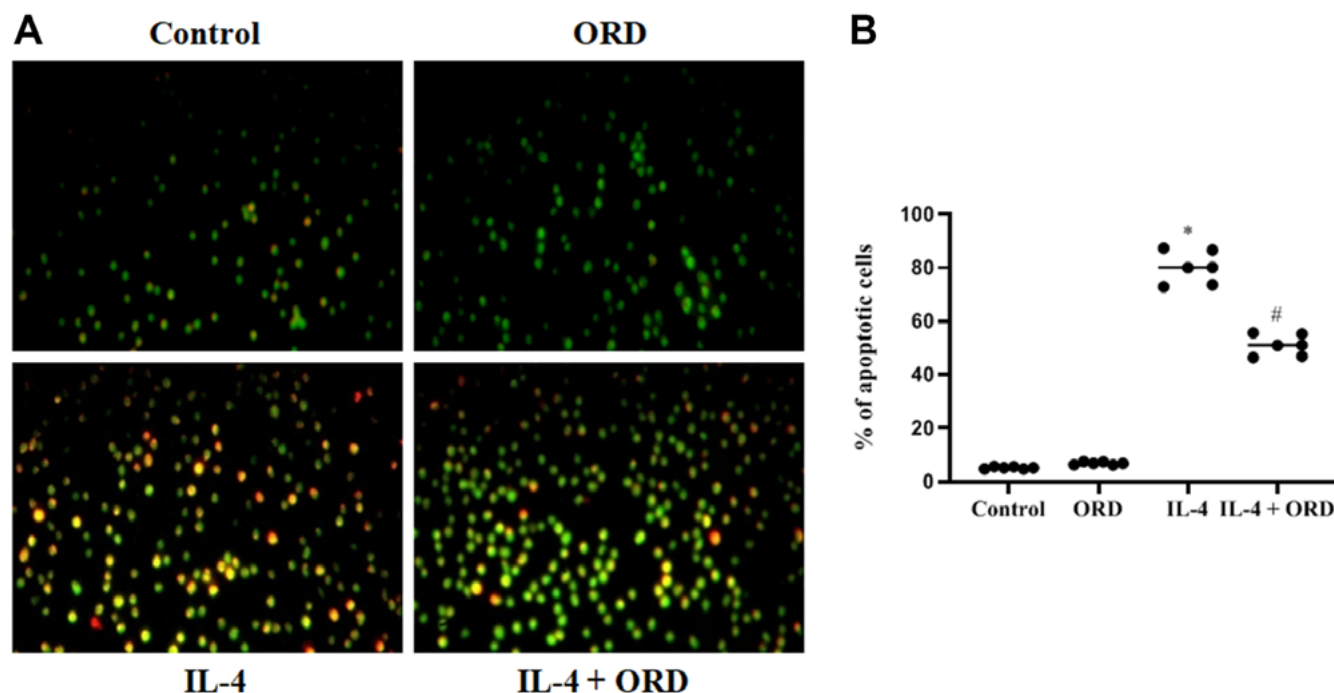


Fig. 2. Oridonin (ORD) inhibits apoptosis on human bronchial epithelial (16HBE) cells, as evidenced by acridine orange/ethidium bromide (AO/EB) staining. A. The apoptosis was evaluated using dual AO/EB staining and viewed with fluorescence microscopy. Effects of ORD on the percentage of cells display apoptotic morphology; B. Quantification of apoptosis in the spectrofluorometry. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's test for post hoc analysis

* $p < 0.05$, # $p < 0.01$ compared to the control cells; IL-4 – interleukin 4.

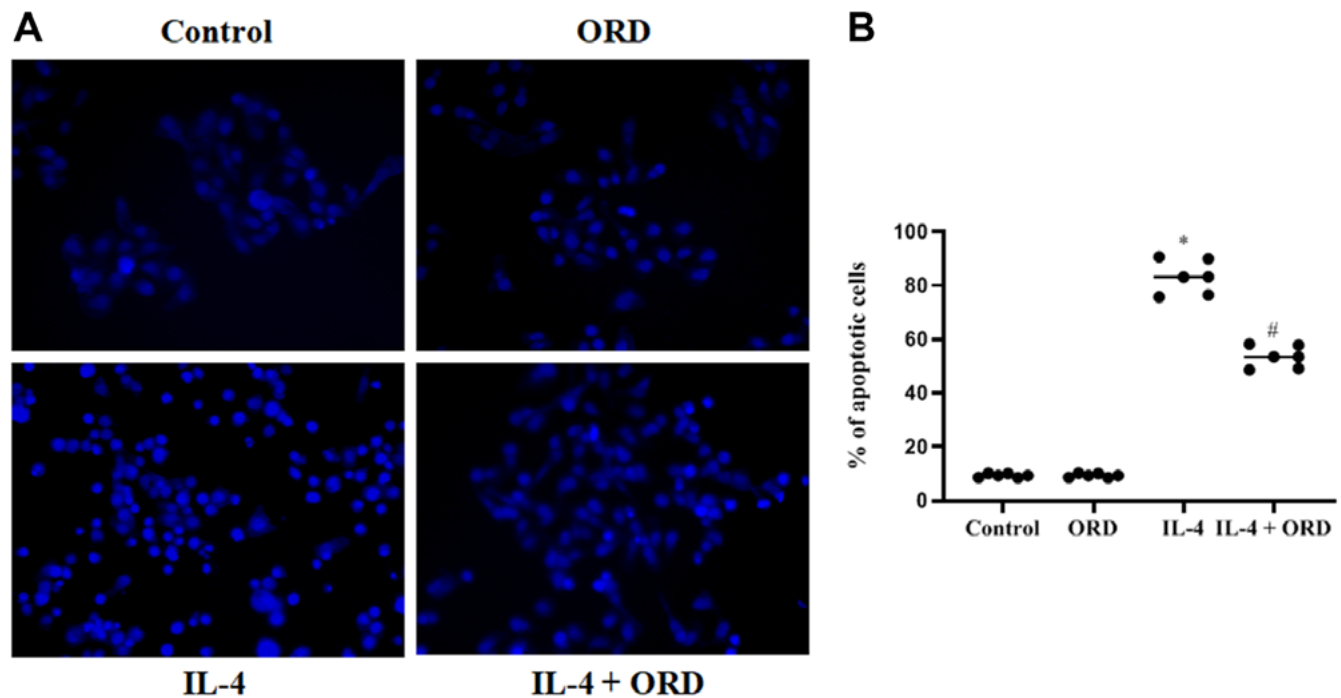


Fig. 3. Oridonin (ORD) mitigates apoptosis on human bronchial epithelial (16HBE) cells, as visualized using 4',6-diamidino-2-phenylindole (DAPI) staining. A. The apoptosis was evaluated using staining with DAPI and viewed with fluorescence microscopy. Effects of ORD on the percentage of cells display apoptotic morphology; B. Quantification of apoptosis in the spectrofluorometry. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's test for post hoc analysis

* $p < 0.05$, # $p < 0.01$ compared to the control cells; IL-4 – interleukin 4.

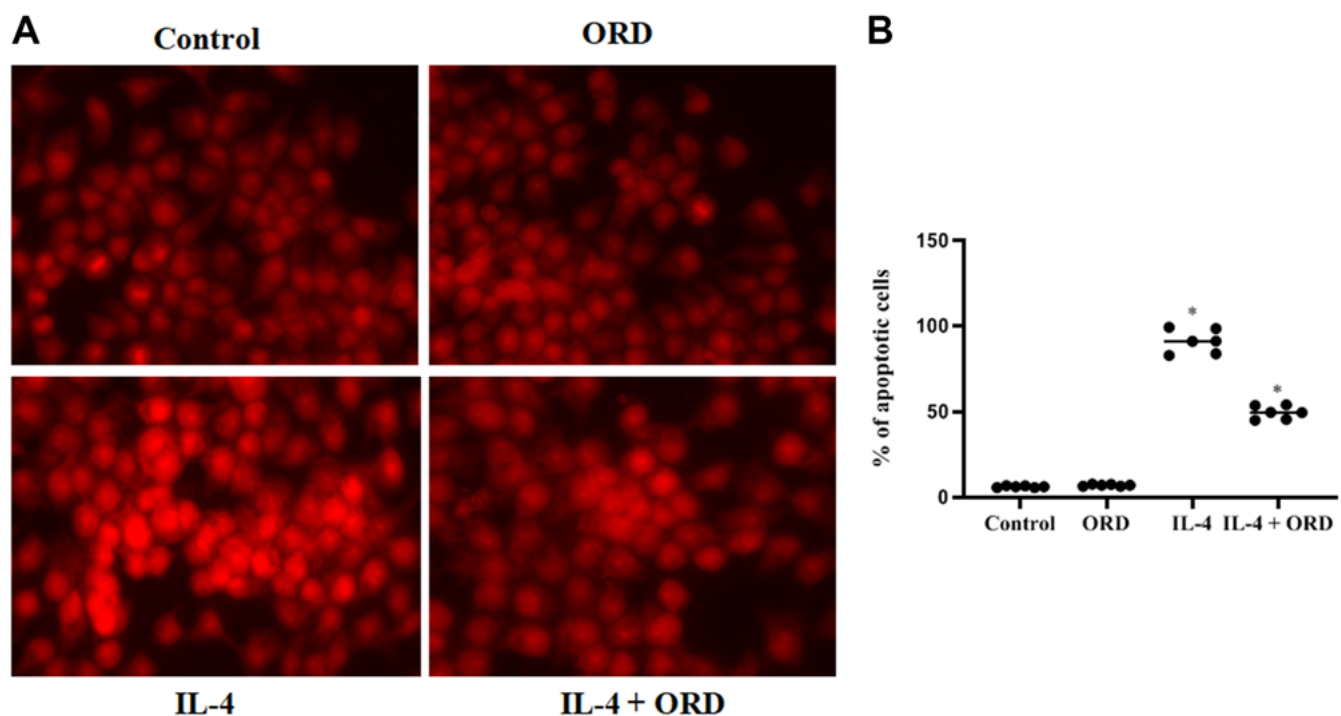


Fig. 4. Oridonin (ORD) reduces apoptosis on human bronchial epithelial (16HBE) cells, as confirmed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. A. The apoptosis was assessed using a TUNEL assay and observed with a fluorescence microscope. Effects of ORD on the percentage of cells display apoptotic cell morphology; B. Quantification of apoptosis in the spectrofluorometry. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's test for post hoc analysis

* $p < 0.05$ compared to the control cells; IL-4 – interleukin 4.

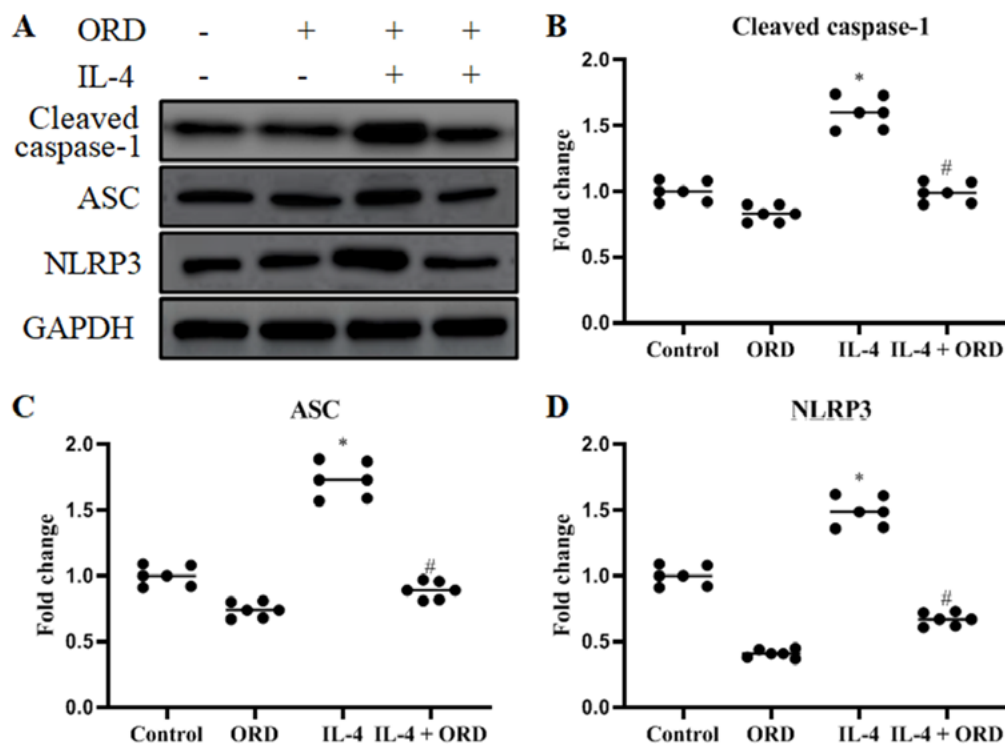


Fig. 5. Western blot analysis of vascular cleaved caspase-1, apoptosis-associated speck-like protein (ASC) and NLR family pyrin domain containing 3 (NLRP3). A. Representative bands of cleaved caspase-1, ASC and NLRP3 proteins; B. Cleaved caspase-1 protein expression measured in various cohorts; C. ASC marker expression quantified in different cohorts; D. NLRP3 protein levels quantified in various cohorts. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's test for post hoc analysis

* $p < 0.05$, # $p < 0.01$ compared to the control cells; ORD – oridonin; IL-4 – interleukin 4; GAPDH – glyceraldehyde 3-phosphate dehydrogenase.

asthma are airway stenosis, as a consequence of airway restoration, restricted airflow, inflammation, and airway hyper-responsiveness.^{1,2} Many reports have demonstrated that airway remodeling is central to PA pathogenesis.^{2–4} As such, remedies that counter the formation of airway alterations represent an opportunity to develop an innovative PA therapy. Therefore, exploring the anti-inflammatory properties of putative anti-asthmatic drugs is essential. The current study examined for the first time the anti-inflammatory activity of ORD as an anti-asthmatic natural substitute. Its molecular actions in airway remodeling pathogenesis, mostly on airway epithelial cell apoptosis, were scrutinized using IL-4-stimulated 16HBE cells in an in vitro asthma model.

The reparative effects of short-chain fatty acids (SCFAs) were studied by incubating cells with IL-4, IL-13 or house dust mite extract, and SCFA. Short-chain fatty acid affected zonular occludin-1 (ZO-1), MAPK signaling pathway expression and IL-4-induced cytokine production.²⁴ The current study demonstrated the effects of ORD on IL-4-stimulated 16HBE cells as an in vitro model of PA by evaluating proliferation using the BrdU technique. It was found that 16HBE cell proliferation was somewhat increased in the ORD group but decreased in the IL-4 treated cells. Additionally, ORD supplementation increased the proliferative growth of IL-4-stimulated 16HBE cells compared to 16HBE cells without ORD. These findings show that IL-4 stimulation significantly reduced proliferation of 16HBE cells used as an in vitro cell line model.

Immune cells release mediators such as the type 2 cytokines IL-4 and IL-13, which are associated with allergies, as well as reactive oxygen species (ROS) and proteases

that play a role in epithelial destruction.²⁵ The present study determined the effects of ORD on IL-4-stimulated 16HBE cells by exploring its apoptotic activity using AO and EB, PI, and DAPI assays. The ORD-treated cells showed no substantial variation in apoptosis and DNA damage compared to the untreated controls. However, the administration of ORD attenuated IL-4-induced apoptosis and DNA damage in 16HBE cells. These findings indicate that IL-4 stimulation considerably augmented apoptosis.

In the current study, the western blot analysis demonstrated that ORD inhibited NLRP3 inflammasome activation in IL-4-stimulated 16HBE cells. The western blot results showed that in control 16HBE cells, ORD had no appreciable impact on the levels of ASC, cleaved caspase-1 or NLRP3 protein expression. However, IL-4 stimulation increased the levels of ASC, cleaved caspase-1 and NLRP3 proteins, which ORD inhibited. From this, we concluded that IL-4 stimulation significantly augmented inflammation and apoptosis of the 16HBE cells. Furthermore, ORD treatment markedly increased cell proliferation, reduced apoptosis and inhibited IL-4-induced inflammation in 16HBE cells, indicating protection against IL-4-stimulated cellular damage. These outcomes are consistent with previous reports and confirm IL-4-stimulated inflammatory impairment in 16HBE cells.^{26,27} Moreover, the supplementation with ORD had no negative impact on the 16HBE cells relative to IL-4 stimulation, which suggests that ORD is safe for bronchial epithelial cells and safe for the treatment of asthma.

Several reports have documented that ORD possesses anti-cancer, anti-inflammatory and apoptosis-modulatory

activities.^{13–15} Previously, ORD has been administered in various inflammatory disorders.¹⁶ Many studies revealed that ORD exerts protective actions in numerous disorders, including sepsis, colitis and neuroinflammation.^{18–20} Also, ORD has been shown to inhibit MAPK/NF- κ B activation by suppressing pro-inflammatory cytokine production.¹⁷ Thus, the healing action of ORD on IL-4-stimulated 16HBE cells is perhaps attributed to its anti-inflammatory and anti-apoptotic effects.

In asthma, bronchial epithelial cell impairment is firmly connected to cellular inflammation. The Chinese traditional medicine *Rabdosia rubescens* is frequently consumed as a remedy for inflammatory diseases.¹⁰ However, its unknown mechanism of action restricts its medical usage. Here, we established that ORD, the main active constituent of *Rabdosia rubescens*, openly or covalently links to NLRP3 and has substantial anti-inflammatory properties in vitro, suggesting that ORD can be employed as an innovative therapeutic against NLRP3-stimulated disorders.

The current research demonstrated that IL-4 remarkably augmented NLRP3 inflammasome activation in 16HBE cells, while ORD treatment marginally reduced ASC, cleaved caspase-1 and NLRP3 protein expression. Such a marginal response may be due to stimulation of the NLRP3 inflammasome in 16HBE control cells, which negates the requirement for ORD to have a substantial influence. Conversely, ORD intensely reduced excessive activation of the NLRP3 inflammasome in IL-4-stimulated cells, indicating that ORD protects 16HBE cells by inhibiting NLRP3 inflammasome signaling.

Excessive stimulation of the NLRP3 inflammasome has a crucial role in inflammation and allergic airway disorders, including PA.²¹ Hyperexpression of ASC, cleaved caspase-1 and NLRP3 occurred in lipopolysaccharide (LPS)-stimulated HBE cells.²² Previous studies have suggested that ORD could prevent MAPK and NF- κ B stimulation and attenuate inflammasome-induced cytokine generation, including tumor necrosis factor alpha (TNF- α) and IL-6.^{17,28} The current findings indicate that ORD targets NLRP3 to exert its anti-inflammatory effects.

Limitations

A few restrictions must be taken into account when evaluating our results. By utilizing destructive techniques for particular time points, and conducting experiments using inflammatory and apoptotic markers, such as fluorescein-labeled dyes, it is possible to determine the impact of ORD on 16HBE cells and determine more mechanisms of action of ORD as an anti-asthmatic natural substitute. Even though its molecular actions in airway remodeling pathogenesis occur mostly through airway epithelial cells, apoptosis was scrutinized using IL-4-stimulated 16HBE cells as an in vitro asthma model in this study.

Conclusions

One of the signs of immune-mediated respiratory illnesses, such as asthma, is a compromised epithelium, which frequently causes increased sensitivity to inhaled substances and exacerbates these airway disorders. We demonstrated, for the first time, that ORD helped restore the barrier functions of IL-4-stimulated 16HBE cells, which may be mediated by downregulating the expression of NLRP3. In summary, ORD alleviated cellular proliferation and exhibited anti-apoptotic activity in IL-4-induced 16HBE cell impairments by suppressing NLRP3 inflammasome signaling pathways. These findings indicate that ORD may be a potential anti-asthmatic agent and contribute to its well-known anti-inflammatory activities.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.7927638>. The package contains the following files:

Supplementary Table 1. Results of normality tests as presented in Fig. 1.

Supplementary Table 2. Results of normality tests as presented in Fig. 2.


Supplementary Table 3. Results of normality tests as presented in Fig. 3.

Supplementary Table 4. Results of normality tests as presented in Fig. 4.

Supplementary Table 5. Results of normality tests as presented in Fig. 5B–D.

ORCID iDs

Weiwei Wang  <https://orcid.org/0000-0002-3919-5277>

Dan Ming  <https://orcid.org/0000-0001-8041-3566>

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