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Compound D from *Zingiber cassumunar* Roxb. attenuated type 2 inflammatory cytokine-induced tight junction disruption in airway epithelial cells

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Abstract

Background: Barrier disruption in the airway mucosae has been implicated in allergic type 2 inflammatory diseases such as allergic rhinitis and asthma. *Zingiber cassumunar* Roxb. has long been used in traditional medicine to treat allergic diseases. The active compound, namely compound D, has proven anti-inflammatory benefits. However, the effect of compound D on allergic inflammation remains unclear.

Objective: This study aimed to investigate the protective effects of compound D on allergic inflammation-induced barrier disruption.

Methods: Type 2 cytokine (IL-4 and IL-13)-exposed 16HBE human bronchial epithelial cells were treated with compound D. After 24, 48, and 72 h, cytotoxicity, epithelial integrity, and tight junction (TJ) disruption were determined by viability assays, transepithelial electrical resistance measurement, and immunofluorescence staining, respectively. Moreover, the mechanism of action of compound D was investigated by western blotting.

Results: Compound D (100 and 200 μM) prevented IL-4/IL-13–induced barrier disruption at 24 and 48 h with no effect on cell viability. Compound D rescued the localization of ZO-1 to pericellular areas, and the barrier-protective effect of compound D was mediated by inhibiting STAT6 signaling.

Conclusions: Compound D can suppress IL-4/IL-13–induced epithelial inflammation and TJ disruption through STAT6 inhibition. The agent is a promising candidate for therapeutic or adjunctive treatment of type 2 inflammation–associated diseases, including asthma.

Key words: *Zingiber cassumunar* Roxb, asthma, allergic inflammation, tight junction, airway epithelial cells

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Introduction

Asthma is one of the most common atopic respiratory diseases worldwide. Recent data from the Global Burden of Disease Study found that asthma affected 262 million people in 2019, equating to an age-standardized rate of 3,416 cases per 100,000 population, and caused 461,000 deaths.[1] Allergic asthma is considered the major type of asthma, accounting for 50–70% of all cases.[2,3] It typically manifests with high levels of type 2 inflammatory cytokines, including IL-4, IL-5, and IL-13, which are predominantly from T helper 2 cells, mast cells, eosinophils, and type 2 innate lymphoid cells. These cytokines exhibit pathogenic hallmarks, including mast cell degranulation, mucus overproduction, airway hyper-responsiveness, and mucosal inflammation.[4] A multicenter study in Thailand found that most patients with asthma have the type 2 high endotype with eosinophilia and allergic sensitization.[5]

The airway epithelium is an essential physical barrier that prevents allergens and pollutants from entering lung tissue, and it is crucial in mucosal immune responses. Many studies have identified correlations between tight junction (TJ) disruption and the pathogenesis of asthma.[6,7] Because TJs participate in regulating paracellular permeability, damage to TJ induced by allergens, pollutants, or cytokines such as TNFα, IL-1β, IL-4, and IL-13 result in epithelial barrier disruption.[8,9,10,11,12,13] For example, recent studies using 16HBE human bronchial epithelial cells found that IL-4 and IL-13 induced epithelial barrier leakage through a mechanism involving the STAT6 and AKT pathways.[10,11] Other studies reported that IL-4 and IL-13 induced intracellular aggregation of TJ proteins via the JAK/STAT pathway.[12,13]

Corticosteroids are the mainstay treatment for asthma.[14] They suppress inflammatory cells and provide a protective effect by enhancing airway epithelial barrier integrity.[15,16] However, high doses of corticosteroids cause multiple adverse effects. Although more specific and effective treatments such as dupilumab, the monoclonal antibody specifically targeting IL-4 and IL-13 signaling, have been developed,[17,18] their high costs and administration difficulty remain significant concerns. Thus, alternative biological treatments that attenuate cytokine signaling are necessary.

Zingiber cassumunar Roxb. has been used as a traditional herbal medicine for various conditions, including asthma.[19] Its efficacy as an anti-inflammatory and antihistamine treatment has been reported. Its extract inhibited the phosphorylation of components of the MAPK pathway in LPS-stimulated human gingival fibroblast cells and PMA-induced elevation of mucin production in human airway epithelial cells.[20,21] A previous study also revealed that the crude extract of *Z. cassumunar* (0.25-2.0 mg/ml) and its active ingredient (compound D) (0.5-4.0 mg/ml, equivalent to 2.4-19.2 mM) inhibited MMP-9 expression in PMA-stimulated human airway epithelial cells.[22] Clinical studies have indicated that the antihistamine effect of *Z. cassumunar* suppressed wheal and flare and improved the total nasal symptom score in patients with allergic rhinitis comparable to 10 mg of loratadine.[23,24] Interestingly, a randomized, double-blind, placebo-controlled study in patients with asthma found that the group orally administered *Z. cassumunar* capsule (16 mg of compound D, equivalent to 76.9 μM) had a higher asthma control test score than that of the placebo group.[25] Although the anti-asthmatic effects of *Z. cassumunar* have been increasingly studied, the mechanisms and active compounds producing these beneficial effects have not been confirmed.

Compound D ((*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol), the active constituent of *Z. cassumunar*, comprises up to 4% w/w of its extract. A previous study found that compound D could inhibit prostaglandin production and alleviate pneumonia in mice with carrageenin-induced pleurisy.[26] We hypothesized that compound D could attenuate epithelial barrier disruption induced by the inflammatory cytokines IL-4 and IL-13. Thus, this study investigated the effects of compound D on IL-4/IL-13–induced TJ disruption. We also sought to understand the mechanism of the barrier-protective effect of compound D in normal human bronchial epithelial 16HBE cells, which establish an intact barrier.

Material and Methods

Chemicals and materials

Cell culture reagents and human recombinant IL-4 and IL-13 were obtained from Invitrogen (Waltham, MA, USA). Compound D was isolated from *Z. cassumunar* rhizome extract using column chromatography and further purified by preparative layer chromatography, as reported in a previous study.[27] The purity of compound D was > 97%, using ^{1}H NMR spectroscopy methods.

Cell culture

Human bronchial epithelial cell line 16HBE (passage number 25-36) was kindly provided by Dr. Nawiya Huipao (Prince of Songkla University, Hat Yai, Thailand) and cultured as previously described.[10,11] Specifically, cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 1% nonessential amino acids, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (catalog number A5256701 Life Technologies, Waltham, MA, USA). Cells were maintained in a humidified 95% O_2/5% CO_2 atmosphere at 37°C.

Viability assay

The viability of 16HBE cells treated with various concentrations of compound D was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, catalog number 475989 Sigma-Aldrich, St. Louis, MO, USA) colorimetric assay. Briefly, the cells were seeded into 96-well culture plates at 5 × 10^5 cells/mL and grown overnight. Cells were then cultured in serum-free medium containing IL-4 and IL-13 (catalog number A42602, catalog number A42526 Thermo Fisher Scientific, Waltham, MA, USA), with or without various concentrations of compound D, for 72 h in triplicate. Based on our previous studies, compound D concentrations less than 200 μg/ml (equivalent to 961.5 μM) did not show toxicity in NCI-H292 cells.[22]

We chose compound D concentrations 50-500 μM for experiments of 16HBE cell toxicity. As for the time point used in this study, we refer to the data from our previous studies in 16HBE cells.[10,11] The medium in each well was aspirated and replaced with 200 μL of MTT solution to obtain a final concentration of 0.5 mg/mL, followed by incubation for 4 h at 37°C. After incubation, the medium was aspirated, and dimethyl sulfoxide (DMSO, catalog number D4540 Sigma-Aldrich, St. Louis, MO, USA) was added to dissolve the formazan crystals formed in the cells. The culture plates were shaken for 5 min, and absorbance was measured at 570 nm using a microplate reader (Victor 2V, PerkinElmer, Waltham, MA, USA).

The relative viability of treated cells compared with that of control cells was expressed as percentage viability. Conditions were considered toxic if the percentage viability of treated cells was significantly lower than that of control cells.

Measurement of TJ integrity

Transepithelial electrical resistance (TEER) was measured to analyze TJ integrity. Cells were seeded onto Transwell permeable inserts with a 0.4-μm-pore-size membrane (2.5×10^5 cells/well, Corning, Corning, NY, USA) and cultured for 14 days (TEER ≥ 900 Ω·cm^2). Baseline TEER was initially measured, and the cells were pretreated with various concentrations of compound D for 1 h before exposure to IL-4 and IL-13 (10 ng/mL each). TEER was measured after 24, 48, and 72 h of treatment using an EVOM2 volt/ohm meter (World Precision Instruments, Inc., Sarasota, FL, USA). The results are expressed as a percentage of the baseline values.

Immunocytochemistry

After treatment for 48 h, 16HBE monolayers were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min. Cells were permeabilized in 0.1% Triton-X 100 for 10 min. The cells were blocked in PBS with 5% FBS for 30 min at room temperature and incubated at 4°C overnight with a mixture of 1:200 occludin antibody (catalog number 40-4700, Invitrogen) and 1:200 ZO-1 antibody (catalog number 339100, Invitrogen). The cells were subsequently incubated with a fluorophore-conjugated secondary antibody (1:1000, catalog number A11008, A32727, Invitrogen) at room temperature for 1 h and then counterstained with Hoechst to stain nuclei. Images were captured using confocal-integrated Opera Phenix™ Plus High-Content Screening System (PerkinElmer).

To quantify the colocalization, the areas of ZO-1 and occludin were segregated by Opera Phenix software. The localization of cytoplasmic ZO-1 in the area of transmembrane tight junction protein-occludin represented 'ZO in OCC area,' and vice versa. The average percentage of the overlapping signal between ZO-1 and OCC called %colocalization, was calculated from 9 visual images per experiment using Opera Phenix software.

Western blot analysis

Cells were seeded into 12-well plates at a density of 5×10^5 cells/mL. After treatment, cell lysates were harvested using RIPA lysis buffer. 20 μg of loaded proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Pre- Running 60 volts for 30 minutes and Running 100 volts for 100 minutes) and then transferred (220 milliamperes for 120 minutes) to a nitrocellulose membrane. The membrane was blocked for 1 h with 5% nonfat dried milk (catalog number M530 Bio-Rad, Hercules, CA, USA) and incubated overnight with anti-rabbit antibodies (all from Cell Signaling Technology, Danvers, MA, USA) against p44/42 ERK (catalog number 91025, 91015), STAT6 (catalog number 53975), pSTAT6 (catalog number 565545), AKT (catalog number 92725), pAKT (catalog number 92715), and β-actin (catalog number 49705). Subsequently, the membrane was washed four times with tris-buffered saline containing Tween–20 and incubated for 1 h at room temperature with 1:5000 horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Abcam, Cambridge, MA, USA). The signal was captured using ChemiDoc (Bio-Rad) to determine the densit ometric ratio.

Statistical analysis

The results are presented as mean ± standard error of the mean. One-way ANOVA with Bonferroni's post-hoc test was used to compare among groups of different concentrations and to compare between groups of IL-4/IL-3 with or without compound D. Two-way ANOVA with Tukey's post-hoc test was applied for testing the effect of compound D concentrations on TEER results with the interval factor. Statistical tests are performed in GraphPad Prism 9 (GraphPad, La Jolla, CA, USA). A p-value < 0.05 was considered statistically significant.

Results

Effect of compound D on IL4/IL-13–induced barrier disruption

To evaluate the barrier-protective effect of compound D, TEER was measured in 16HBE cells exposed to IL-4 and IL-13. After 48 and 72 h of IL-4 and IL-13 (10 ng/mL each) treatment, TEER was significantly decreased by 23.11% ($p < 0.01$) and 33.44% ($p < 0.0001$), respectively, when compared with the DMSO, which was used as a negative control, indicating that these cytokines could impair the airway epithelial barrier. However, there was no significant reduction in TEER at 24 h (**Figure 1**). Since the previous study showed that the efficacy of *Z. cassumunar* capsule on asthmatic patients was 16 mg (equivalent to 76.9 μM).[25] In this study, we opted for the lower and upper concentrations of compound D. Cells, which were then pretreated with various concentrations of compound D (0.1, 1, 100, and 200 μM) for 1 h and then cotreated with cytokines. The results showed that compound D at 100 μM

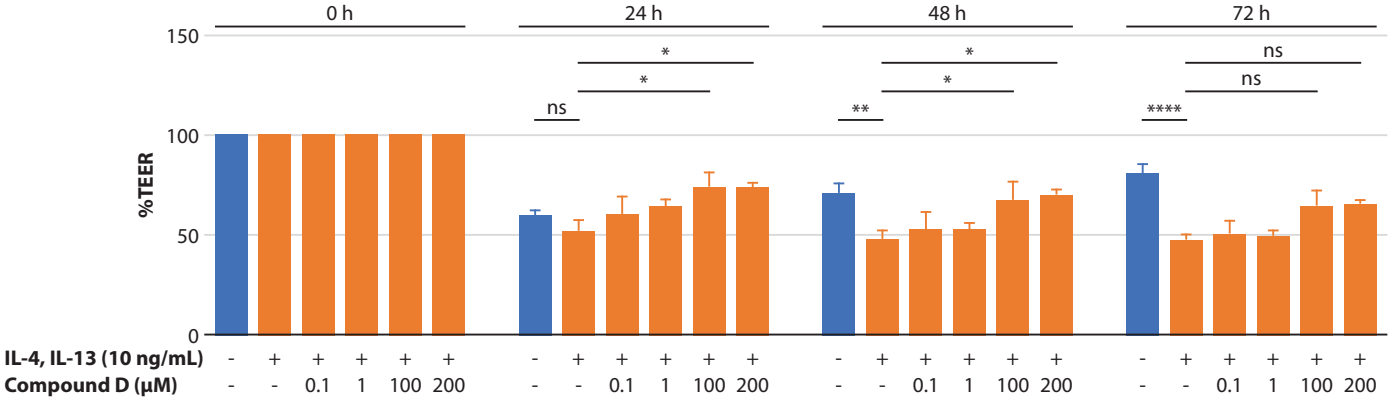

Figure 1. Effects of compound D on type-2 cytokine-induced barrier disruption. TEER measurement of 16HBE cells that were pretreated with or without compound D at indicated concentrations (0.1, 1, 100, and 200 μM) at different time points (24, 48, and 72 h). 100 μM, and 200 μM of compound D showed significant suppression of IL-4 and IL-13 induced TJ disruption at 24 and 48 h. Data were shown as %TEER compared with baseline. ns: non-significant different, *$p < 0.05$, **$p < 0.01$, ****$p < 0.001$; Two-way ANOVA with Tukey multiple comparison test (n = 6).

and 200 μM dramatically attenuated the ability of cytokines to disrupt barrier function. After 24 h of the treatment of compound D at 100 μM and 200 μM, TEER was significantly increased by 22.14% (p = 0.01) and 21.95% (p = 0.01), respectively. TEER was also increased by 19.55% (p = 0.03) and 22.23% (p = 0.01) after 48 h of 100 μM and 200 μM of compound D treatment, respectively. However, no effect was observed at 72 h (**Figure 1**). These results indicated that compound D can improve airway epithelial barrier function by preventing epithelial permeability caused by type 2 cytokines.

Cytotoxicity of cytokines and compound D

To clarify whether the effects of cytokines on TEER were attributable to cytotoxicity or the regulation of TJs, a viability assay was performed to determine whether IL-4, IL-13, and compound D exerted any toxic effects. As analyzed by one-way ANOVA with Bonferroni's post-hoc test, the viability of cytokine-treated cells was 96.01% ± 2.72% (**Figure 2A**), whereas the values after treatment with 50, 100, and 500-μM compound D were 81.27% ± 2.90% (p = 0.05), 81.73% ± 4.09% (p = 0.10), and 82.92% ± 2.60% (p = 0.05), respectively (**Figure 2B**). These results indicated that compound D up to 500 μM did not induce significant cytotoxicity in epithelial cells, suggesting that its effects on barrier disruption involved the regulation of TJ permeability.

Effect of compound D on TJ localization

We used immunocytochemistry to detect the paracellular localization of ZO-1 and occludin to evaluate the regulation of TJ permeability. The results illustrated that 10 ng/mL IL-4 and IL-13 drastically affected the colocalization of ZO-1 and occludin by increasing the cytoplasmic accumulation of ZO-1 and inducing abnormal clump cytoplasmic localization of ZO-1, indicating that compound D reversed the abnormal localization of TJ proteins induced by type 2 cytokines (**Figure 3**).

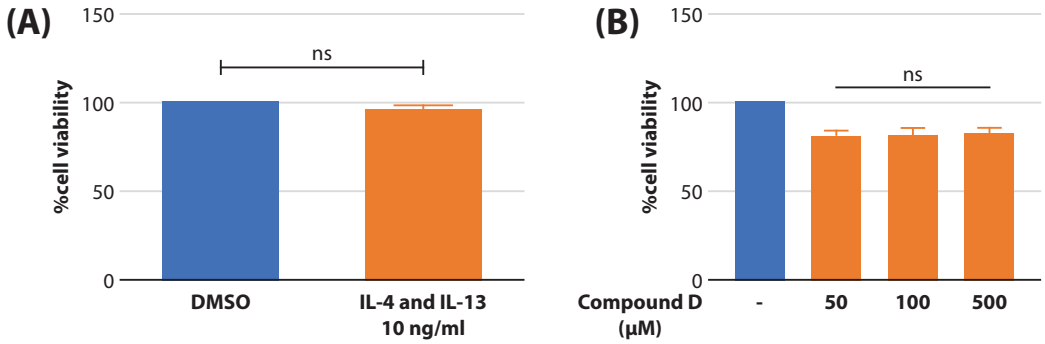

Figure 2. Effects of IL-4 and IL-13 (10 ng/mL) (A) and varied concentrations of compound D (B) on cell viability of 16HBE cells determined by MTT assay. Viable cell numbers are shown as a percentage of untreated cells. Data were mean ± S.E.M. ns: non-significant difference; One-way ANOVA with Bonferroni multiple comparison test (n = 4).

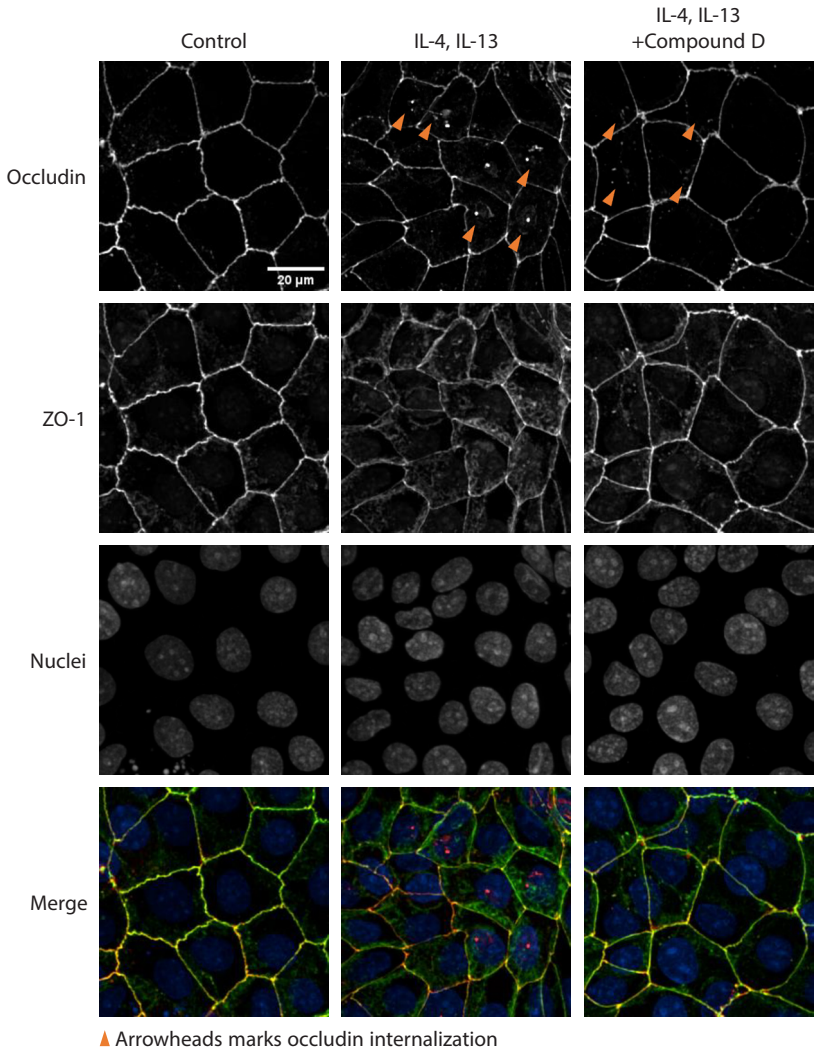

Figure 3. Effect of compound D on ZO-1 and occludin mislocalization induced by IL-4 and IL-13. Arrowhead: abnormal localization of occludin. Scale bars represent 25 µm. The regions of occludin and ZO-1 were marked and segmented, and intensities of ZO-1 that localized in the occludin region, as well as the occludin in the ZO-1 region, were calculated. The box plot shows the mean percentage of change in the aforementioned intensities. Data were shown as a % change of colocalization intensity compared with untreated cells. $^{*}p < 0.05$, $^{**}p < 0.01$; One-way ANOVA with Bonferroni multiple comparison test (n = 6).

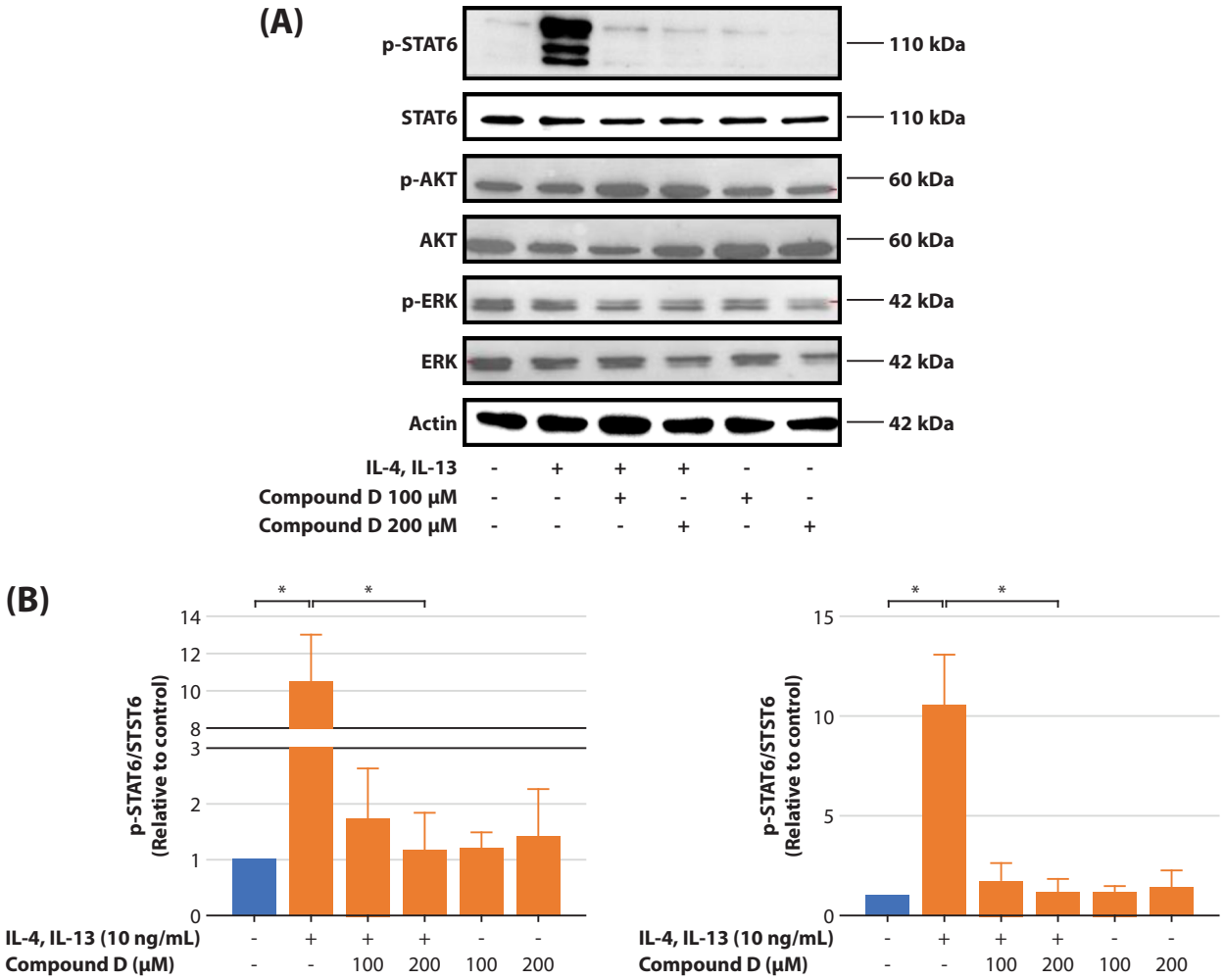

Figure 4. IL-4/IL-13-induced epithelial breakage and the barrier-protective effect of compound D were mediated by STAT6 phosphorylation. There is no correlation of IL-4/ IL-13 and compound D with either ERK1/2 or AKT. (A) Representative blots of indicated proteins. (B) The calculated densitometry of indicated proteins normalized by that of β-actin. *$p < 0.05$ (n = 4-6).

The epithelial barrier-protective effect of compound D involved STAT6 inhibition

The STAT6, ERK1/2, and AKT pathways are characterized by IL-4 and IL-13 receptor signaling.[28,29,30] *Z. cassumunar* extract has been demonstrated to exert its effect by activating the ERK1/2–MAPK pathway.[20,21] Meanwhile, our recent studies in 16HBE cells have shown that STAT6 was required for IL-4/IL-13–induced TJ disruption.[10,11] To investigate the protective mechanism of compound D on TJ function, the activation of STAT6, ERK1/2, and AKT, as indicated by their phosphorylation, was determined by western blotting following cytokine and compound D treatment.

As presented in **Figure 4**, treatment with IL-4/IL-13 increased STAT6 protein expression. This effect was attenuated by treatment with 200-μM compound D, whereas lower concentration (100 μM) had no such effect (**Figure 4B**). ERK1/2 and AKT protein expression was not altered by treatment with IL-4/IL-13 or compound D alone or in combination. Taken together, these results showed that compound D suppresses the type 2 cytokine-induced STAT6.

Discussion

Many cytokines, allergens, pollutants, and infectious agents induce TJ disruption, thereby participating in the pathogenesis of asthma. Our results supported previous findings regarding the roles of IL-4 and IL-13 in inducing epithelial barrier disruption.[10,11,12,13] In this study, we demonstrated that pretreatment with 100 or 200 μM compound D significantly induced TJ assembly, whereas lower concentrations (0.1 and 1 μM) did not exhibit such significant efficacy. TJs and adherens junctions, which comprise claudin, occludin, zonula occludens, and E-cadherin, are important for determining epithelial permeability.[7] Immunocytochemistry also demonstrated the same suppressive effect of compound D on abnormal ZO-1 localization induced by IL-4 and IL-13, indicating the increased TJ assembly. However, it is difficult to clarify the exact mechanism of action of compound D on TJ. Experiments using western blot analysis to investigate the change of tight junction proteins after cotreatment and compound D alone are required in future studies to confirm the mode of the compound D effect, whether inducing assembly, building new tight junctions, or preventing degradation.

Previous studies showed that compound D prevented MUC2, MUC5AC, COX-2 mRNA expression, and COX-2 protein expression mediated by ERK1/2.[20,21] Many studies mentioned that IL-4 and IL-13 might be regulated by the MAPK, STAT6, and AKT pathways.[10,11,28,29,30] For example, inhibition of p38 MAPK activity is directly correlated with the inhibition of IL-4–induced gene activation, and this mechanism is mediated by STAT6.[30] Other studies also found that IL-4– and IL-13–induced airway epithelial leakage was prevented by inhibiting STAT6 and AKT phosphorylation,[10,11] and the cytokine-induced barrier disruption is abolished by STAT6 inhibitor (AS1517499).[11] However, the effect of STAT6 inhibitor on tight junction localization has not yet been investigated. Our study revealed the same relationships of IL-4 and IL-13 with STAT6, but ERK1/2 or AKT phosphorylation was not involved in cytokine-induced airway barrier disruption. This might be explained by the use of different cell passages or concentrations of compound D, IL-4, and IL-13. Further studies are needed, such as measurements of epithelial-derived cytokines, including IL-25, IL-33, and TSLP, to determine their involvement in the barrier-protective effect of compound D. The effective concentration for the protective effect of compound D on the epithelium was approximately two-fold higher than that used in a previous study[25] of patients with asthma but lower than the oral no-observed-adverse-effect level in a study of the safety of compound D in rats.[32] The efficacy of lower concentrations of compound D should also be studied.

To our knowledge, this is the first study to reveal the effects of compound D on epithelial barrier integrity. We also investigated the upstream mechanism by which compound D influences epithelial assembly. Compound D elicited its barrier-protective effect by inhibiting STAT6 phosphorylation.

To induce TJ disruption, we used IL-4 and IL-13, which cause type 2 inflammation. Thus, our findings might not explain the efficacy of compound D using stimulants that induce non-type 2 inflammation. Future research using different stimulants might increase our knowledge of the effects of compound D on epithelial function.

Although corticosteroids protect the epithelium, we did not compare the effects of corticosteroids and compound D on TJs. More in vitro and in vivo studies are needed to better understand compound D and its potential clinical use. Meanwhile, our data suggested that compound D could suppress IL-4/IL-13-induced STAT6 and epithelial barrier disruption, which are also the hallmarks of other allergic diseases, such as atopic dermatitis and allergic rhinitis. Intriguingly, the therapeutic potential of compound D in these diseases warrants further investigation.

Zingiber cassumunar has already been proven safe in many studies in both mice and humans.[31,32,33,34] No gross and histological toxicity in major organs as well as changes in laboratory parameters were observed after a single or multiple doses of *Z. cassumunar* in rats.[32] A phase 3 study of Phlai capsule treatment in patients with allergic rhinitis also revealed no significant changes in laboratory tests or serious adverse effects.[34] Thus, compound D may be useful as an adjunctive treatment to reduce the adverse effects of high corticosteroid doses. However, compound D is 4% w/w of its extract with the current extraction method. Thus, high doses of the extract may be needed to show the TJ assembly effect. Studies focusing on improving the yield of extraction are crucial.

Conclusion

The present study showed that compound D attenuated airway epithelial TJ disruption via STAT6 inhibition. This finding supports the rationale that compound D could be a natural compound candidate for the therapeutic or adjunctive treatment of type 2 inflammation–associated diseases such as asthma.

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Declaration of competing interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Author's contributions

- Poachanukoon O: conceptualization, resources, funding acquisition, writing (review and editing), supervision, project administration.
- Termworasin P: conceptualization, methodology, investigation, formal analysis, writing (original draft), visualization.
- Tharabenjasin P: project administration, investigation, writing (review and editing).
- Dechatiwongse Na Ayudhya T: resources, methodology, writing (review and editing).
- Moonwiriyakit A: conceptualization, methodology, investigation validation, resources, funding acquisition, writing (review and editing), supervision.

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