

affect their future career. It remains very difficult to screen all early-career elite athletes yearly for EIB. Therefore, one of the goals of this research was to define risk factors and/or biomarkers that might predict EIB with high sensitivity and to screen early-career athletes from different sport disciplines who might be more susceptible to have EIB. Therefore, we looked at the atopic state (determined via skin prick test) and the AQUA score⁹ of our athletes and correlated these with maximal fall in FEV₁ and EIB status. In our cohort of early-career elite athletes, being atopic or having an AQUA score of 6 or more on its own does not indicate the presence of EIB. Second, sputum cytokine mRNA levels and serum damage-associated molecular patterns were investigated to predict EIB. Using receiver-operating characteristic (ROC) analysis, cutoff values were determined, which were able to predict, with a high sensitivity, the presence of EIB in early-career elite athletes (see Fig E4 in this article's Online Repository at www.jacionline.org). Athletes with sputum IL-8 mRNA levels lower than 190 or serum uric acid levels below 92 μ M have a very low risk of developing EIB ($P = .003$ and $P = .044$, respectively; see Table E1 in this article's Online Repository at www.jacionline.org). However, athletes with sputum IL-1 β mRNA levels higher than 300 are more prone to have or develop EIB ($P = .0331$; positive predictive value = 0.85; Table E1).

To conclude, EIB and markers of potential epithelial damage are already present in 12- to 13-year-old early-career elite athletes, in absence of overt cellular inflammation. We suggest that in early-career elite athletes, EIB can be screened by searching for "wheezing during exercise" and using the AQUA questionnaire on a first level to look for athletes who are at risk for EIB development. However, this would lead to a substantial number of nonatopic yet EIB+ athletes who would be missed in screening. On a second level, potential biomarkers such as serum CC16, serum uric acid, sputum IL-8, and IL-1 β mRNA levels might be of use to identify athletes who are more prone to develop EIB, independent of their atopy status. This however needs to be validated in a larger cohort. This selected group of athletes should then be referred to specialized centers to undergo an exercise challenge test or an EVH test to confirm the presence of EIB.

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Novel pendrin inhibitor attenuates airway hyperresponsiveness and mucin expression in experimental murine asthma



To the Editor:

The transmembrane anion exchanger pendrin (*SCL26A4*) exchanges Cl⁻ with bases, such as HCO₃⁻, I⁻, and SCN⁻, and is the most highly upregulated gene in endobronchial biopsies from patients with asthma.^{1,2} Interestingly, patients with mutant pendrin have a low prevalence of asthma, and pendrin-null mice show reduced allergic airway inflammation.³⁻⁵ These observations indicate that pendrin is a novel target for allergic asthma. In this study, we identified a novel pendrin inhibitor, YS-01 (2-(4-(tert-butyl)phenyl)-4-(thiophen-2-ylmethylene)oxazol-5(4H)-one), with strong therapeutic effects on allergic inflammation in a mouse model of ovalbumin (OVA)-induced asthma.

YS-01 was identified by the high-throughput screening of 54,400 synthetic compounds (Fig 1, A). Detailed methodology is provided in the Methods section in the Online Repository at www.jacionline.org. YS-01 potently inhibited pendrin-mediated Cl⁻/SCN⁻, Cl⁻/I⁻, Cl⁻/HCO₃⁻, and Cl⁻/OH⁻ exchange activity in a dose-dependent manner (Fig 1, B and C; see Fig E1, A-C, in this article's Online Repository at www.jacionline.org). YS-01 showed no cytotoxicity up to 30 μ M in NIH3T3 and CHO-K1 cells and more potently inhibited pendrin

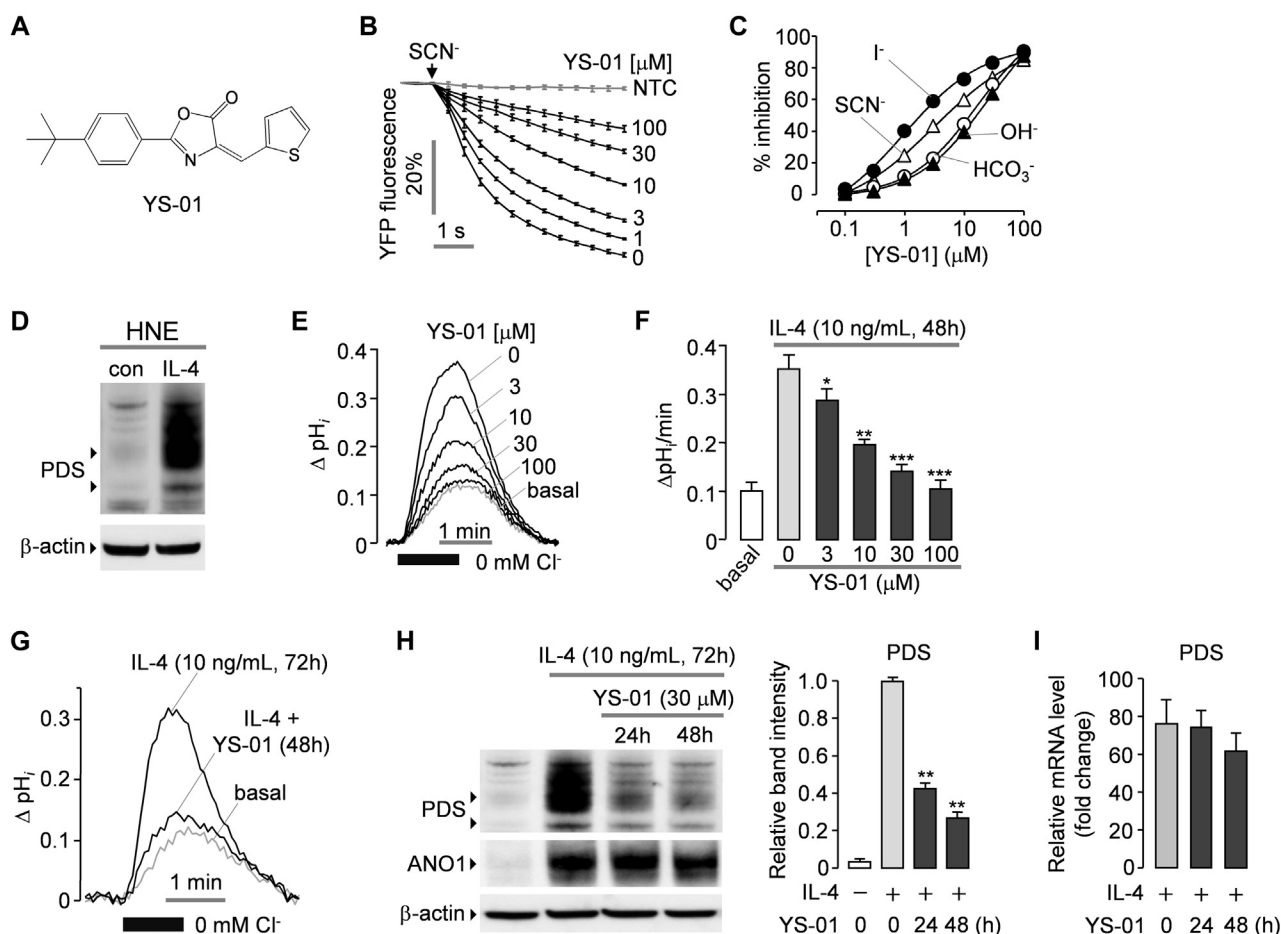


FIG 1. YS-01, a novel pendrin (PDS) inhibitor, blocks the pendrin-mediated cellular response in IL-4-stimulated HNE cells. **A**, Chemical structures of YS-01. **B**, Inhibitory effect of YS-01 on PDS-mediated Cl⁻/SCN⁻ exchange activity (mean ± SE, n = 5). **C**, YS-01 dose-response for the inhibition of PDS-mediated Cl⁻/I⁻, Cl⁻/SCN⁻, Cl⁻/HCO₃⁻, and Cl⁻/OH⁻ exchange activity (mean ± SE, n = 4–5). **D**, Representative immunoblot of PDS in untreated and IL-4-treated HNE cells. **E**, PDS-mediated Cl⁻/HCO₃⁻ exchange in IL-4-treated HNE cells. **F**, Initial rates of change in ΔpH_i/min (mean ± SE, n = 3–4). **G**, Cl⁻/HCO₃⁻ exchange activities after exposure to YS-01 in IL-4-treated HNE cells. **H**, Representative immunoblot results for PDS and ANO1 in IL-4-treated HNE cells (mean ± SE, n = 5). **I**, PDS mRNA expression levels were determined by real-time PCR in IL-4-treated HNE cells (mean ± SE, n = 5). ANO1, Anoctamin-1; NTC, nontransfected cells. **P* < .05, ***P* < .01, and ****P* < .001.

activity than the recently identified pendrin inhibitors, PDS_{inh}-A01 and PDS_{inh}-C01 (see Fig E1, D–I).⁶ YS-01 inhibited both human and mouse pendrin-mediated Cl⁻/I⁻ exchange with nearly identical potency (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). YS-01 weakly inhibited Cl⁻/HCO₃⁻ exchange activities of SCL26A3 and SLC26A6 with IC₅₀ greater than 100 μM and did not affect SCL26A7 and SLC26A9. Cystic fibrosis transmembrane conductance regulator, anoctamin-1, human ether-a-go-go-related gene channel, and 5-HT_{2A} activities were not affected by YS-01 (see Fig E2, C–J).

In primary cultures of human nasal epithelial (HNE) and human bronchial epithelial cells, IL-4 treatment strongly upregulated pendrin expression and pendrin-mediated Cl⁻/HCO₃⁻ exchange activity, and YS-01 potently inhibited pendrin-mediated Cl⁻/HCO₃⁻ exchange activity (Fig 1, D–F, and see Fig E3, A–C, in this article's Online Repository at www.jacionline.org). We observed the effect of long-term

treatment with YS-01 on the functional expression of pendrin and other ion channels involved in airway surface liquid (ASL) regulation. Interestingly, the IL-4-induced upregulation of Cl⁻/HCO₃⁻ exchange activity and pendrin protein expression levels were strongly decreased by long-term treatment with YS-01, without changes in mRNA expression levels, but the IL-4-induced upregulation of anoctamin-1 was not affected (Fig 1, G–I). The mRNA expression levels and ion channel activities of anoctamin-1, cystic fibrosis transmembrane conductance regulator, and epithelial sodium channels (ENaC) were not altered by long-term treatment with YS-01 (see Fig E3, D–F).

Pretreatment with YS-01 significantly attenuated OVA-induced airway hyperresponsiveness (Fig 2, A) and reduced the numbers of eosinophils and neutrophils in the bronchoalveolar lavage fluid of OVA-sensitized mice. A histological analysis showed that the increased inflammatory infiltration and epithelial thickness and the increased number of goblet cells in the central

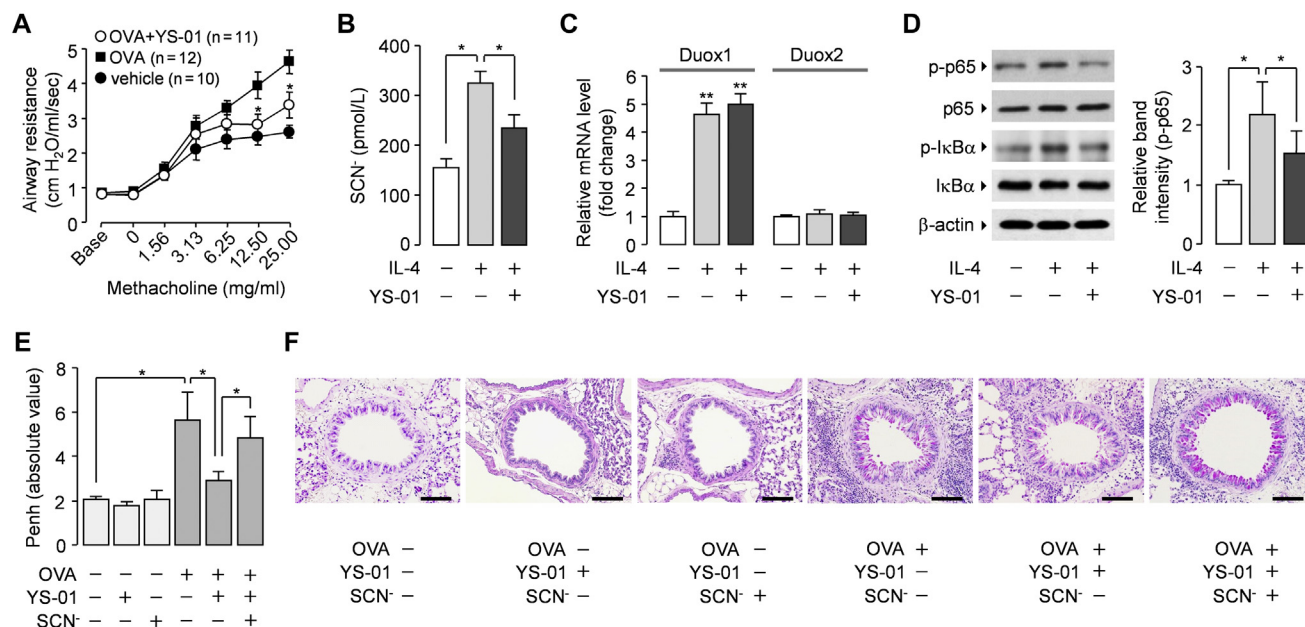


FIG 2. YS-01 ameliorates airway inflammation via the inhibition of the pendrin/SCN⁻/NF-κB pathway in a mouse model of OVA-induced allergic asthma. **A**, Airway resistance in animals sensitized and challenged with OVA. **B**, Effect of IL-4 and YS-01 on SCN⁻ transport in HNE cells (mean ± SE, n = 5). **C**, *Duox1* and *Duox2* levels, determined by real-time PCR after YS-01 treatment in IL-4-treated HNE cells (mean ± SE, n = 3). **D**, Immunoblotting results for HNE cells treated with IL-4 with or without YS-01. (right) Phospho-p65 band intensity (mean ± SE, n = 3). **E**, Airway resistance in response to methacholine challenge in OVA-sensitized mice (mean ± SE, n = 3-4). **F**, Representative PAS staining of airway tissues. Scale bar, 100 μm. PAS, Periodic acid-Schiff. **P* < .05 and ***P* < .01.

airways were attenuated by treatment with YS-01 in OVA-challenged mice. The average inflammation score of OVA-challenged mice was significantly lower in the YS-01-treated group than in the untreated group, but the serum levels of OVA-specific IgE were not altered by YS-01, indicating that YS-01 does not act on the general allergic response mechanism (see Fig E4 in this article's Online Repository at www.jacionline.org).

Recent studies have shown that nuclear factor kappa B (NF-κB) activation by increased hypothiocyanite (OSCN⁻) production via the upregulation of pendrin, peroxidases, and dual oxidase (Duox1/Duox2) in the airway epithelium is involved in allergic airway inflammation.^{7,8} The SCN⁻ concentration at the apical surface was significantly increased by IL-4 treatment and inhibited by YS-01 (Fig 2, B). Real-time PCR analyses showed that IL-4 treatment significantly increased the mRNA expression of *Duox1* but did not affect *Duox2*, and YS-01 did not affect the mRNA expression of *Duox1* or *Duox2* (Fig 2, C). Pretreatment with YS-01 significantly inhibited the IL-4-induced activation of NF-κB in HNE cells (Fig 2, D). Notably, the intranasal administration of NaSCN significantly blocked the inhibitory effect of YS-01 on airway hyperresponsiveness and abolished the protective effect of YS-01 on lung injury in OVA-challenged asthmatic mice (Fig 2, E and F). In addition, YS-01 significantly blocked the NF-κB activation in the lungs of OVA-treated mice, and treatment of SCN⁻ inhibited the protective effect of YS-01 in a transgenic NF-κB reporter mice (see Fig E4, G). Application of YS-01 to an established model of allergic asthma significantly decreased OVA-induced airway hyperresponsiveness and periodic acid-Schiff-positive cells in airway epithelium (see Fig E5 in

this article's Online Repository at www.jacionline.org). These results suggest that YS-01 may be useful in both preventing and treating allergic asthma.

Pendrin is associated with the airway inflammation-mediated upregulation of *MUC5AC* in the airway epithelium.^{4,9} Notably, our results showed that treatment with YS-01 significantly decreased the IL-4-induced *MUC5AC* transcript (see Fig E6, A, in this article's Online Repository at www.jacionline.org). In addition, pretreatment with YS-01 attenuated IL-4- and IL-13-induced goblet cell hyperplasia in HNE cells (see Fig E6, B). ASL thickness in IL-13-treated primary cultures of airway epithelial cells is significantly higher in pendrin-null mice and deaf patients carrying a mutant *SLC26A4* gene compared with controls.^{3,9} In HNE cells expressing wild-type pendrin, IL-4 and IL-13 treatment strongly increased pendrin protein expression and decreased the total volume of ASL and fluid meniscus compared with those of controls, and YS-01 treatment almost completely rescued the IL-4- and IL-13-induced decreases in the total volume of ASL and fluid meniscus. However, the total volume was not altered by IL-4 and YS-01 in HNE cells expressing mutant pendrin (see Fig E6, C-F). This result is consistent with that of Haggie et al,⁶ who found that PDS_{inh}-A01 significantly increased ASL depth in IL-13-treated human bronchial epithelial cells. *MUC5AC* inhibition and an increase in ASL thickness by YS-01 might provide additional beneficial effects on airway inflammatory diseases, such as chronic obstructive pulmonary disease, cystic fibrosis, and asthma.

There is a possibility that alterations in hearing or thyroid hormone level may be induced by pendrin inhibition because the patients carrying *SLC26A4* gene mutations are associated with

prelingual deafness and goiter.¹ However, hearing threshold and plasma levels of T3 and T4 were not changed after treatment of YS-01 (10 mg/kg/d) for 1 week (see Fig E7, A–C, in this article's Online Repository at www.jacionline.org). In addition, YS-01 did not affect tracheal smooth muscle contraction (see Fig E7, D).

In summary, YS-01 reduced airway hyperresponsiveness and airway inflammation via the inhibition of the SCN[−]/NF-κB pathway. In addition, YS-01 showed protective effects against IL-4- and IL-13-induced goblet cell hyperplasia and ASL depletion (see Fig E8 in this article's Online Repository at www.jacionline.org). These results indicate that pendrin inhibitors are promising candidates for the treatment of allergic asthma.

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Physiologic response to chronic house dust mite exposure in mice is dependent on lot characteristics



To the Editor:

Multiple models of allergic airway inflammation using house dust mite (HDM) extract have been developed to study experimental asthma in mice. Acute exposure models develop airway hyperresponsiveness (AHR), T_H2-mediated airway inflammation, and mucus secretion,^{1,2} but airway remodeling with peribronchial collagen deposition requires chronic exposures.³⁻⁵ Presently there are no standard exposure methodologies, because each model varies with respect to the species, duration, and/or dose of HDM used and different exposure protocols affect phenotypic outcomes.⁴ In addition, phenotypic outcomes depend on the HDM extract source.⁶ Lack of reproducible physiological responses to allergen extracts has long been known and is well documented in mice and humans after cutaneous exposure,^{7,8} but has been less well detailed in models of allergen inhalation.

The National Institutes of Health stresses the importance of scientific rigor and transparency for enhancing reproducibility in research. Among several key areas, they highlight authentication of biological and/or chemical resources. Although most recently published articles (PubMed; 2016-2018) using murine chronic HDM exposure cite the HDM source, fewer than 11% provide the extract lot number or describe its components in detail and almost 40% of articles did not cite the species of HDM extract (see Table E1 in this article's Online Repository at www.jacionline.org). Furthermore, HDM dose normalization in these studies can be based on protein content, antigen content, or dry extract weight. Because HDM extracts are prepared from live mites, and the allergen and endotoxin content can differ between preparations, we hypothesized that the response to extracts may differ among lots even when prepared from the same commercial supplier and used in the same exposure protocol.

As proof of concept, we started with 2 lots of HDM (HDM0 and HDM1) with similar concentrations of total protein, Der P1, and endotoxin units (Greer Laboratories, Lenoir, NC). Respiratory mechanics were evaluated after either acute or chronic administration of HDM. Mechanics parameters were then fit to 2 different mathematical models of the lung—the single-compartment model and the constant-phase model—to determine total respiratory system and compartmentalized responses, respectively (see this article's [Methods](#) section in the Online Repository at www.jacionline.org). When administered acutely (intranasal 100 µg 1×/wk for 3 weeks),

METHODS

Cell-based screening

CHO-K1 cells expressing human wild-type pendrin and YFP-F46L/H148Q/I152L were plated in 96-well microplates at a density of 2×10^4 cells per well and incubated for 48 hours. Each well of the 96-well plate was washed 2 times with 200 μ L of PBS, and it was filled with 50 μ L of HEPES-buffered solution. Test compounds were added at 50 μ M. After 10 minutes of incubation at 37°C, the 96-well plate was placed on the FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) for a fluorescence assay. The compound collection used for screening (55,000 synthetic small molecules) was purchased from ChemDiv (San Diego, Calif). Each well was assayed individually for pendrin-mediated I^- influx by recording fluorescence continuously (400 ms per point) for 1 second (baseline). Then, 50 μ L of NaI-substituted HEPES-buffered solution (NaI replacing NaCl) was added using a liquid injector at 1 second and YFP fluorescence was recorded for 5 seconds. The initial iodide influx rate was determined from the initial slope of fluorescence by nonlinear regression, after the infusion of iodide.

Synthesis of YS-01

Glycine (150 mg, 2 mmol) was dissolved in 1.5 mL of 10% sodium hydroxide solution. Then, 4-*tert*-butylbenzoyl chloride (0.34 mL, 2.4 mmol) was added at room temperature under stirring for 6 hours. After acidification until pH 2 by HCl, the produced mixture was filtered with eluting water. Then, the precipitate was filtered and washed once more with methylene chloride to obtain (4-*tert*-butylbenzoyl)glycine (450 mg, 95.7%, white solid).

^1H NMR (400 MHz, DMSO-*d*₆) δ 12.54 (s, 1H), 8.72 (t, 1H, J = 5.8 Hz), 7.77 (d, 2H, J = 8.4 Hz), 7.46 (d, 2H, J = 8.8 Hz), 3.88 (d, 2H, J = 6.0 Hz), 1.26 (s, 9H); ESI (m/z) 236 (MH^+).

(4-*tert*-butylbenzoyl)glycine (450 mg, 1.91 mmol) and 1-ethyl-3-(3-dimethylamino) propyl carbodiimide hydrochloride (550 mg, 2.87 mmol) were sequentially added to 0.1 mol methylene chloride (19.1 mL) at room temperature under stirring for 12 hours. Then, furfural (0.17 mL, 2.10 mmol) and triethylamine (0.53 mL, 3.83 mmol) were added at room temperature under stirring for 12 hours. The mixture was distilled off under reduced pressure. After dissolving with methanol, the precipitate was filtered to obtain YS-01 (480 mg, 85%, yellow solid).

^{13}C NMR (400 MHz, DMSO-*d*₆) δ 166.7, 162.1, 157.2, 137.6, 136.9, 136.6, 130.7, 128.6, 128.1(2), 126.6(2), 124.9, 122.8, 35.5, 31.2(3).

^1H NMR (400 MHz, DMSO-*d*₆) δ 8.02 (d, 1H, J = 5.2 Hz), 7.96 (d, 2H, J = 7.6 Hz), 7.80 (d, 1H, J = 3.6 Hz), 7.66 (s, 1H), 7.61 (d, 2H, J = 7.6 Hz), 7.22 (t, 1H, J = 4.4 Hz), 1.29 (s, 9H); ESI (m/z) 296 (MH^+).

Short-circuit current

Snapwell inserts containing anoctamin-1 (ANO1)- and cystic fibrosis transmembrane conductance regulator (CFTR)-expressing Fischer rat thyroid (FRT) and primary cultures of HNE cells were mounted on Ussing chambers (Physiologic Instruments, San Diego, Calif). HNE cells were obtained as previously described.^{E1} For the measurement of short-circuit current in HNE cells, the apical and basolateral bath were filled with symmetrical HCO_3^- -buffered solution containing (in mM) 120 NaCl, 5 KCl, 1 MgCl_2 , 1 CaCl_2 , 10 D-glucose, 5 HEPES, and 25 NaHCO_3 (pH 7.4). For ANO1- and CFTR-expressing FRT, the apical bath was filled with half- Cl^- solution containing (in mM) 60 NaCl, 60 Na-gluconate, 5 KCl, 1 MgCl_2 , 1 CaCl_2 , 10 D-glucose, 5 HEPES, and 25 NaHCO_3 (pH 7.4) and the basolateral bath was filled with the HCO_3^- -buffered solution. The basolateral membrane was permeabilized with 250 $\mu\text{g/mL}$ of amphotericin B to measure the apical membrane currents of ANO1 and CFTR. All cells were bathed for a 20-minute stabilization period and aerated with 95% O_2 /5% CO_2 at 37°C. Short-circuit currents and apical membrane currents were measured with an EVC4000 Multi-Channel V/I Clamp (World Precision Instruments, Sarasota, Fla) and PowerLab 4/35 (AD Instruments, Castle Hill, Australia). The data were recorded and analyzed using Labchart Pro 7 (AD Instruments). The sampling rate was 4 Hz.

Measurement of anion exchange

For $\text{Cl}^-/\text{HCO}_3^-$ and Cl^-/OH^- measurements, intracellular pH (pH_i) was measured in wild-type pendrin-expressing CHO-K1, HNE, and human bronchial epithelial cells using the pH sensor SNARF5-AM (Molecular Probes, Eugene, Ore). The cells were loaded with 5 μM SNARF5-AM for 30 minutes, and then mounted in a perfusion chamber on the stage of an inverted fluorescence microscope (Nikon, Tokyo, Japan) equipped with a cooled charge-coupled device camera (Zyla sCMOS; Andor Technology, Belfast, United Kingdom) and image acquisition and analysis software (Meta Imaging Series 7.7; Molecular Devices, Biberach, Germany). The cells were perfused with the HCO_3^- -buffered solution. To measure $\text{Cl}^-/\text{HCO}_3^-$ exchange activity, the HCO_3^- -buffered solution was changed to Cl^- -free HCO_3^- -buffered solution containing (in mM) 120 Na-gluconate, 5 K-gluconate, 1 MgCl_2 , 1 CaCl_2 , 10 D-glucose, 5 HEPES, and 25 NaHCO_3 (pH 7.4). Applying external Cl^- -free HCO_3^- -buffered solution increases the efflux of Cl^- and the influx of HCO_3^- via pendrin. To maintain the pH of HCO_3^- -buffered solutions, the solutions were continuously gassed with 95% O_2 and 5% CO_2 . For Cl^-/OH^- exchange measurement, the HEPES-buffered solution was replaced with a Cl^- -free HEPES-buffered solution for the generation of a Cl^- gradient driving cytosolic Cl^- efflux and OH^- influx through pendrin. SNARF5 fluorescence was recorded at an excitation wavelength of 515 ± 10 nm and emission wavelength of 640 ± 10 nm, and intracellular pH calibration was performed with solutions containing 145 mM KCl, 10 mM HEPES, and 5 μM nigericin, with the pH adjusted to 6.2 to 7.6.

For Cl^-/I^- and Cl^-/SCN^- measurements, YFP fluorescence was measured in pendrin and YFP (a halide sensor)-expressing CHO-K1 cells. Cl^-/I^- exchange activity was measured as described earlier for cell-based screening. To measure Cl^-/SCN^- exchange activity, HEPES-buffered solution was replaced with NaSCN-substituted HEPES-buffered solution (with NaSCN replacing NaCl) for the generation of an SCN^- gradient driving SCN^- influx through pendrin. YFP fluorescence changes by SCN^- influx were monitored using the FLUOstar Omega Microplate Reader (BMG Labtech) and MARS Data Analysis Software (BMG Labtech).

Thallium flux assay

HEK-293T cells were stably transfected with human ether-a-go-go-related Gene (hERG) and seeded at a density of 7×10^4 cells per well in poly-L-lysine-coated 96-well plate, and the cells were incubated for 48 hours. Four hours before the assay, the cells were switched from 37°C to 28°C for enhanced membrane expression of hERG channel. After 4 hours, the medium was replaced with 80 μL /well of the FluxOR (Invitrogen, Carlsbad, Calif) loading buffer and incubated for 1 hour at 37°C in the dark. The loading buffer was removed and 100 μL of assay buffer was added to each well. To measure the effect of YS-01 on hERG channels, the cells were pretreated with YS-01 for 10 minutes. FluxOR fluorescence (excitation/emission: 490/525 nm) was recorded for 4 seconds before addition of 20 μL of the stimulus buffer containing thallium ions, and the fluorescence was monitored for an additional 56 seconds. FluxOR fluorescence was recorded and analyzed using FLUOstar Omega Microplate Reader (BMG Labtech) and MARS Data Analysis Software (BMG Labtech).

Measurement of 5-HT_{2A} activity

FRT cells expressing human 5-HT_{2A}, ANO1(abc), and YFP-F46L/H148Q/I152L were plated in a 96-well plate at a density of 2×10^4 cells per well and incubated for 48 hours. Each well of the 96-well plate was washed 2 times with 200 μL of PBS, and it was filled with 100 μL of PBS. To measure the effect of YS-01 on 5-HT_{2A} channels, cells were pretreated with YS-01. After 10 minutes of incubation at 37°C, the 96-well plate was placed on the FLUOstar Omega Microplate Reader for YFP fluorescence measurement. Each well was assayed individually for 5-HT_{2A}-mediated I^- influx by recording YFP fluorescence continuously (800 ms per point) for 2 seconds (baseline). Then, 100 μL of 140 mM I^- solution containing 20 μM 5-HT (Sigma-Aldrich, St Louis, Mo) was added at 2 seconds and then YFP fluorescence was recorded for 10 seconds.

Immunoblot analyses

CHO-K1, HNE cells were lysed with cell lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4 , and protease inhibitor mixture). Whole-cell lysates were centrifuged at 15,000g for 10 minutes at 4°C to remove cell debris, and equal amounts (50 μg of protein or 5 μg protein/lane) of supernatant protein were separated on a 4% to 12% Tris-glycine precast gel (KOMA BIOTECH, Seoul, Korea) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, Mass). The membrane was blocked with 5% nonfat skim milk in TBS including 0.1% Tween 20 (TBST) or 5% BSA in TBST for 1 hour at room temperature. The membrane was then incubated overnight with a primary antibody for pendrin (sc-50346; Santa Cruz Biotechnology, Santa Cruz, Calif), NF- κB p65 (4764S; Cell Signaling Technology, Danvers, Mass), p-NF- κB p65 (3033S; Cell Signaling Technology), I $\kappa\text{B}\alpha$ (9242S; Cell Signaling Technology), p-I $\kappa\text{B}\alpha$ (9141S; Cell Signaling Technology), β -actin (sc-47778; Santa Cruz Biotechnology), or ANO1 (ab64085; Abcam, Cambridge, Mass) at 4°C. After washing with TBST, the membrane was incubated with secondary antibody for 60 minutes at room temperature, and then washed 3 times with TBST for 5 minutes. The membrane was visualized using the ECL Plus western blotting detection system (GE Healthcare Amersham, Piscataway, NJ).

Real-time RT-PCR analysis

Total mRNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed using random hexamer primers, an oligo (dT) primer, and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCRs were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, Calif) and Thunderbird SYBR qPCR mix (Toyobo, Osaka, Japan). The thermal cycling conditions included an initial step of 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds, 55°C for 20 seconds, and 72°C for 10 seconds in a 96-well reaction plate. The primer sequences are listed in [Table E1](#).

Measurement of ASL and fluid meniscus volumes

Total volume of ASL and fluid meniscus, a fluid along with the cell culture insert wall, was measured by fluid absorption through filter paper. Whatman filter paper (10 mm diameter circle; GE Healthcare) was placed on the apical side of a 12-well Transwell insert (Costar, Cambridge, Mass) for 10 seconds. The absorption of ASL and fluid meniscus was determined from the weight changes in the filter paper. The weight of the filter paper was measured using an analytical balance (Sartorius BP61S; Sartorius AG, Göttingen, Germany).

Sensitization and challenge with OVA

The experiments were performed using 8-week-old BALB/c mice. The study protocols were approved by the Institutional Animal Ethics Committee of Yonsei University. Mice were bred and maintained under standard laboratory conditions (12/12-hour light/dark cycle, controlled temperature [21°C $\pm$ 2°C] and humidity [55% $\pm$ 5%], and ad libitum access to food and water) in the Animal Facility. All experiments were performed according to the guidelines of the Yonsei University Committee on Animal Research. Mice were divided into 3 groups: a vehicle group (PBS-sensitized and -challenged mice treated with vehicle), the OVA group (OVA-sensitized and -challenged mice treated with vehicle), and the OVA + YS-01 group (OVA-sensitized and -challenged mice treated with YS-01). Age- and sex-matched 8-week-old mice were sensitized by intraperitoneal injection of OVA (Sigma-Aldrich) on days 0 and 14. The sensitizing emulsion consisted of 50 μg of OVA and 2 mg of aluminum potassium sulfate in 200 μL of saline. On days 21, 22, and 23, the sensitized mice were lightly anesthetized by isoflurane inhalation and challenged with 100 μg of OVA in 30 μL of saline administered intranasally. Control mice were treated in the same way with PBS. YS-01 (10 mg/kg) was administered 12 hours before each intranasal OVA challenge by intraperitoneal injection.

Assessment of airway reactivity to methacholine challenge

Airway responses to methacholine were measured 24 hours after the last OVA exposure using the FlexiVent system (Scireq, Montreal, QC, Canada). Mice were anesthetized by the intraperitoneal injection of a mixture of Zoletil (30 mg/kg; Virbac Laboratories, Carros, France) and Rompun (10 mg/kg; Bayer, Leverkusen, Germany), then tracheostomized and connected to the FlexiVent. Baseline airway resistance was measured after the nebulization of saline for 10 seconds using an Aeroneb ultrasonic nebulizer (SCIREQ, Montreal, Quebec, Canada). After baseline measurements, mice were exposed to increasing concentrations of nebulized methacholine (0, 1.56, 3.13, 6.25, 12.5, and 25 mg/mL). Whole-body plethysmography (Buxco, Miami, Fla) was also used to measure bronchial airway responsiveness. Enhanced pause (P_{enh}) was used as the main index of airway responsiveness. P_{enh} was measured for 2 minutes in baseline conditions. Mice were then exposed to the inhalation of PBS or methacholine (25 mg/mL) for 2 minutes. P_{enh} results are expressed as absolute values.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed after the assessment of airway responses. Cells were pelleted by centrifugation and resuspended in PBS to obtain cell counts. Cytospins were prepared with a cytocentrifuge (Shandon Cytospin 4 cytocentrifuge; Thermo Scientific, Waltham, Mass) and were stained with the Diff-Quik Stain Set (Dade Behring, Newark, Del) to assess inflammation.

Histologic assessment of inflammation

For the assessment of airway inflammation, the left lungs were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Histological slides were prepared from 5- μm sections and stained with hematoxylin and eosin. The inflammatory score indicating the severity of peribronchial inflammation was evaluated by 3 blinded observers using a previously reported method.^{E2} To assess goblet cell hyperplasia, sections were stained with periodic acid-Schiff using a periodic acid-Schiff Staining Kit (Sigma-Aldrich) according to the manufacturer's protocol. The harvested nasal mucosa and HNE cells on the Transwell inserts were gently washed with PBS and fixed with 4% paraformaldehyde. After deparaffinization and hydration, the slides were immersed in periodic acid solution for 5 minutes at room temperature. After being rinsed in distilled water, the slides were immersed in Schiff's reagent for 15 minutes at room temperature and then washed in running tap water for 5 minutes.

OVA-specific IgE measurements

OVA-specific IgE in the serum was measured using the Anti-Ovalbumin IgE (mouse) ELISA Kit (Cayman Chemical, Ann Arbor, Mich) according to the manufacturer's instructions. To measure OVA-specific IgE levels in the serum, diluted serum was added to an anti-IgE antibody–precoated 96-well plate, followed by incubation with the OVA-biotin conjugate. The bound biotinylated OVA was detected with streptavidin-horseradish peroxidase using 3,3',5,5'-tetramethylbenzidine as a substrate.

Transepithelial SCN^- transport

Nasal epithelial cells were plated on Transwell permeable supports and cultured at the air-liquid interface for 14 days. Complete differentiated epithelial cells were incubated with IL-4 (Invitrogen, 10 ng/mL) for 48 hours, and then YS-01 (30 μM) or vehicle was added for 30 minutes. The Transwell inserts with cells were washed with PBS and the basolateral side was treated with 1 mL of PBS containing 10 mM of glucose and 5 μCi of S^{14}CN (total concentration of SCN^- : 86 μM). The apical side was filled with 0.5 mL of PBS containing 5 μM of amiloride to block epithelial sodium. Subsequently, the apical fluid was collected every 5 minutes and placed in scintillation vials for the evaluation of radioactivity.

Measurements of T3 and T4

Female balb/c mice were dosed intraperitoneally with 10 mg/kg of YS-01 every 24 hours over a 7-day period to test the negative effects of YS-01. Twenty-four hours after the last administration, the hearing of the mice was measured and the plasma was obtained. Total plasma levels of triiodothyronine (T3) or thyroxine (T4) were assayed using a mouse ELISA kit (Calbiotech, San Diego, Calif; T3043T-100 or T4044T-100) according to the manufacturer's instructions.

Auditory brainstem response

Hearing levels were determined in each mouse by measuring the auditory brainstem response (ABR) thresholds using an auditory-evoked potential workstation and the BioSig software (Tucker-Davis Technologies, Alachua, Fla). The output from the speakers was calibrated by using a PCB 377C10 microphone (PCB Piezotronics, Inc, New York, NY) and was found to be within ± 4 dB for the frequency range tested. Mice were anesthetized, following which each ear was stimulated with an ear probe sealed inside the ear canal. Body temperature of the mice was maintained at 38°C with an isothermic heating water-pad. The intensity of clicking sounds was decreased from 70 dB SPL to 10 dB SPL in 5-dB decrements. The average values of ABR were calculated and the hearing threshold was defined as the lowest recognizable ABR response until wave I of the ABR could no longer be visually discerned.

In vitro optical imaging

Eight-week-old NF- κ B luciferase-dTomato reporter mice were sensitized by intraperitoneal injection of OVA (50 μ g, Sigma-Aldrich) and 2 mg of aluminum potassium sulfate on days 0 and 14. On days 21, 22, and 23, the sensitized mice were anesthetized by isoflurane inhalation and challenged with 150 μ g of OVA in 30 μ L of saline administered intranasally. Control mice were treated in the same way with PBS. YS-01 (10 mg/kg) was administered 12 hours before each intranasal administration. NF- κ B expression in the lung of NF- κ B reporter mice was compared using the IVIS system. The lungs were isolated from the mice and fluorescently imaged (Ex 570, Em 620) using the

IVIS spectrum. The average fluorescent density of images was analyzed using Living Image 4.3.1 software (PerkinElmer, Waltham, Mass).

Measurement of tracheal contraction

The experiments were performed using 4-week-old SD male rat. The study protocols were approved by the Institutional Animal Ethics Committee of Yonsei University. The rats were sacrificed and the trachea strips were isolated. After separation of connective tissues, the trachea was cut into rings of 3 mm length. Tracheas were mounted for tension recording under a 1 g tension and were allowed to equilibrate for 1 hour in organ bath containing 25 mL of an oxygenated physiological solution. The solutions were continuously gassed with 95% O₂ and 5% CO₂ at 37°C. After 1 hour of stabilization, 0.3 μ M of carbachol was used to induce the sustained contractile response in organ bath. Once the sustained tension was established, YS-01 (30 μ M), forskolin (10 μ M), and IBMX (100 μ M) were applied to the bath. Tracheal contraction was measured with an FORT 10G transducer (World Precision Instruments, Sarasota, Fla) and PowerLab 4/35 (AD Instruments, Castle Hill, Australia). The data were recorded and analyzed using Labchart Pro 7 (AD Instruments).

Statistical analysis

The results of multiple experiments are presented as means $\pm$ SE. Statistical analyses were performed with Student *t* tests or ANOVAs, as appropriate. A value of *P* less than .05 was considered statistically significant.

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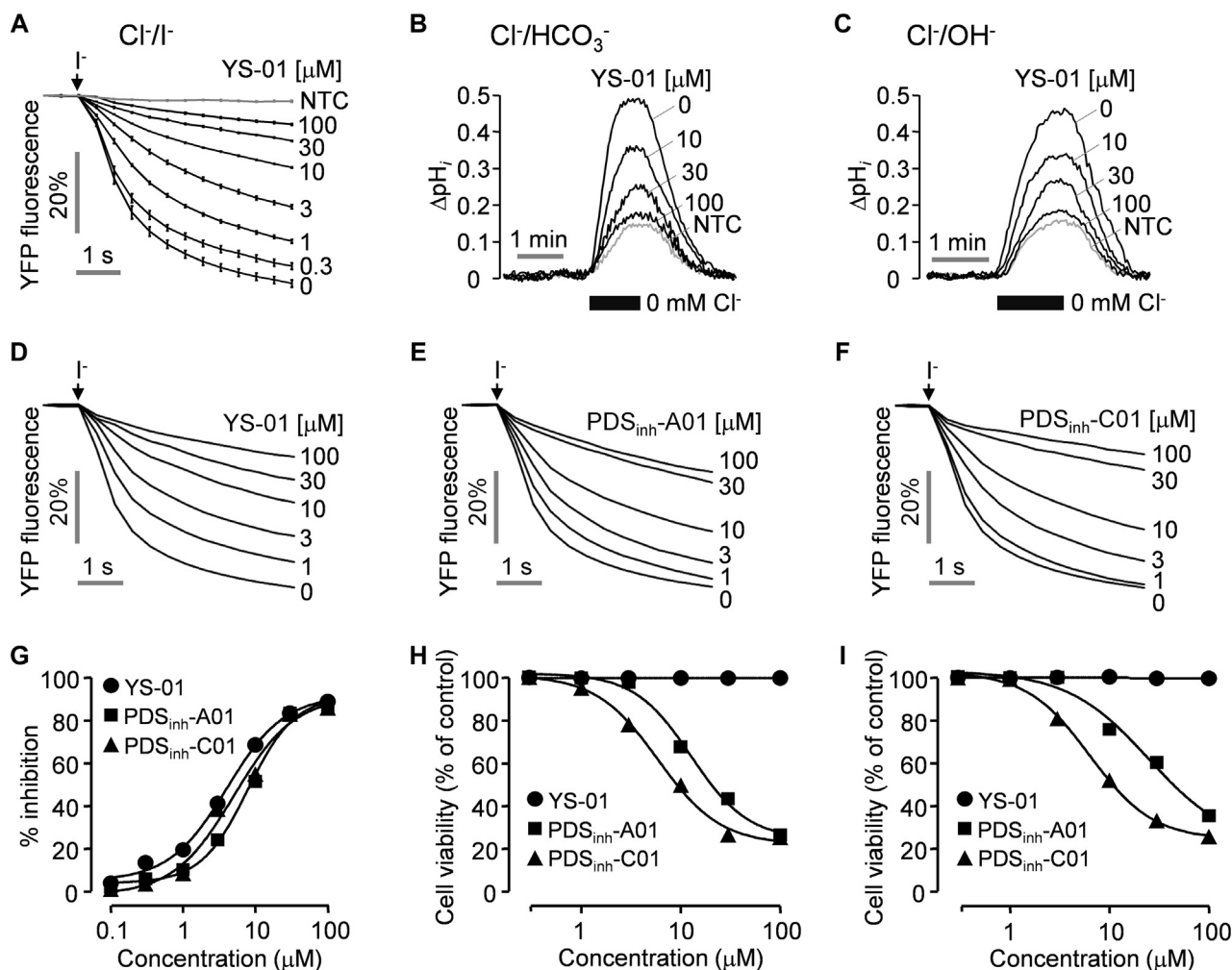


FIG E1. Pendrin-mediated Cl⁻/base exchange activity in CHO-K1 cells expressing human pendrin. **A**, Pendrin-mediated Cl⁻/I⁻ exchange activity (mean ± SE, n = 5). Indicated concentrations of YS-01 were pretreated for 10 minutes. **B** and **C**, Representative traces of intracellular pH. Application of Cl⁻-free solution caused intracellular alkalization via pendrin-mediated Cl⁻/HCO₃⁻ and Cl⁻/OH⁻ exchange activity. Indicated concentrations of YS-01 were pretreated for 10 minutes. **D-F**, Pendrin-mediated Cl⁻/I⁻ exchange activity was measured in CHO-K1 cells expressing human pendrin. Indicated concentrations of YS-01, PDS_{inh}-A01, and PDS_{inh}-C01 were pretreated for 10 minutes. **G**, Summary of dose-responses of YS-01, PDS_{inh}-A01, and PDS_{inh}-C01 (mean ± SE, n = 3). **H** and **I**, Effect of YS-01, PDS_{inh}-A01, and PDS_{inh}-C01 on cell viability in NIH3T3 (**H**) and CHO-K1 (**I**) cells. The cells were treated with YS-01, PDS_{inh}-A01, and PDS_{inh}-C01 for 24 hours. The cell viability was determined by MTS colorimetric assay (mean ± SE, n = 3). NTC, Nontransfected cells; PDS, pendrin.

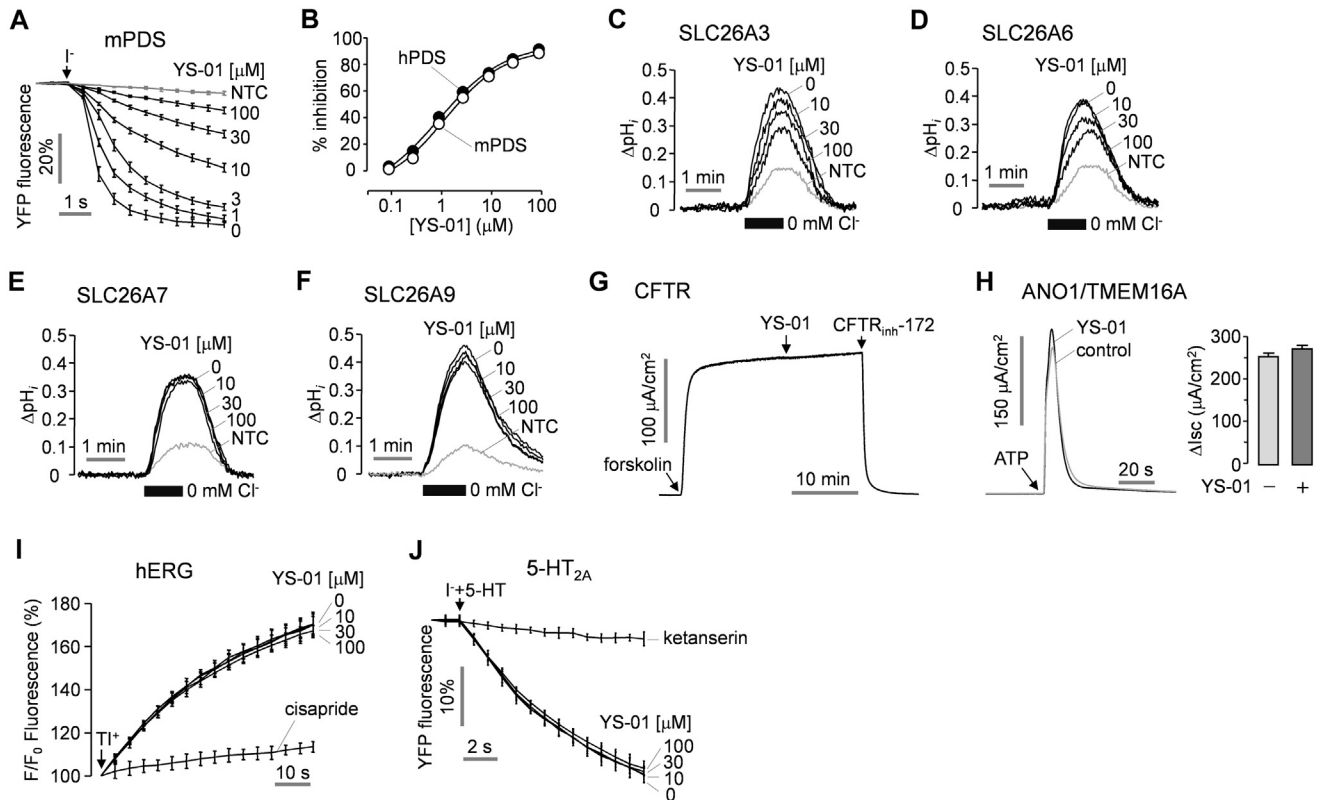


FIG E2. Characterization of YS-01. **A**, Inhibition of mouse pendrin (mPDS)-mediated Cl⁻/I⁻ exchange activity by YS-01 was measured in CHO-K1 cells expressing mPDS (mean $\pm$ SE, n = 5). Indicated concentrations of YS-01 were pretreated for 10 minutes. **B**, Summary of YS-01 dose-response for inhibition of human pendrin (hPDS)-mediated (closed circle) and mPDS-mediated (open circle) Cl⁻/I⁻ exchange activity. **C-F**, Representative traces of intracellular pH of CHO-K1 cells expressing SLC26A3, SLC26A6, SLC26A7, and SLC26A9 are shown. Application of Cl⁻-free solution caused intracellular alkalization via Cl⁻/HCO₃⁻ exchange. Indicated concentrations of YS-01 were pretreated for 10 minutes. **G**, Effect of YS-01 (100 μ M) on CFTR chloride channel activity was measured in FRT cells expressing human CFTR. CFTR current was activated by 20 μ M forskolin and inhibited by 10 μ M CFTR_{inh-172}. **H**, Effect of YS-01 (100 μ M) on ANO1 chloride channel activity was measured in FRT cells expressing human ANO1. YS-01 was added 10 minutes before ANO1 activation by 100 μ M ATP. **Right**, Summary of peak current (mean $\pm$ SE, n = 3). **I**, Effect of YS-01 on hERG (Kv11.1) potassium channel activity was measured in HEK293T cells expressing human Kv11.1 (mean $\pm$ SE, n = 4). Indicated concentrations of YS-01 were pretreated for 10 minutes. hERG channel was inhibited by 50 μ M cisapride. **J**, Effect of YS-01 on 5-HT_{2A} channel activity was measured in FRT cells expressing human 5-HT_{2A} (mean $\pm$ SE, n = 4). Indicated concentrations of YS-01 were pretreated for 10 minutes. 5-HT_{2A} channel was activated by 10 μ M 5-HT and inhibited by 10 μ M ketanserin. NTC, Nontransfected cells. ****P* < .001.

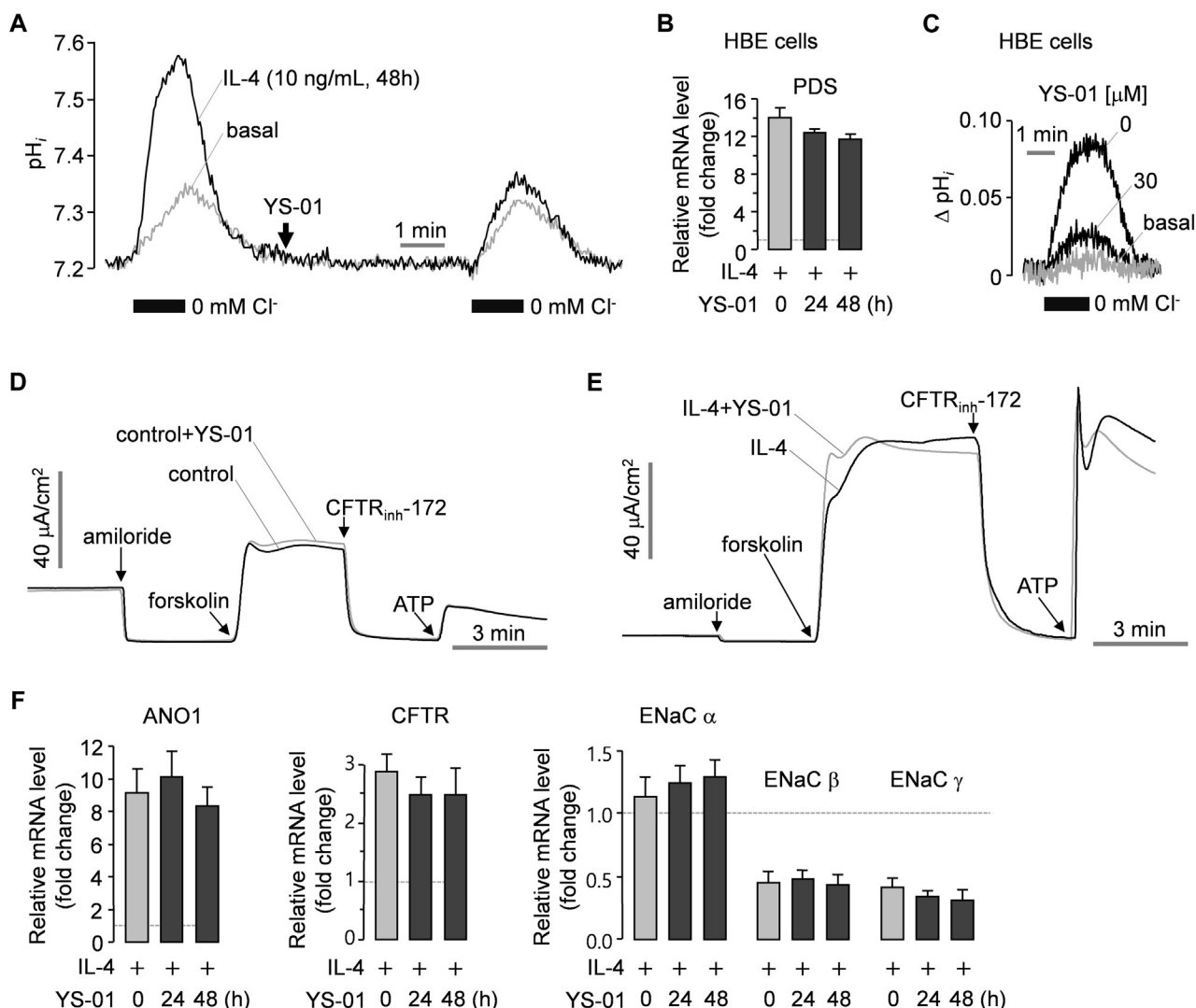


FIG E3. Effect of YS-01 on pendrin and other ion channels in HNE cells. **A**, Representative traces of intracellular pH of untreated (gray line) and IL-4-treated (10 ng/mL) HNE cells. Pendrin was inhibited by YS-01 (50 μ M). **B**, mRNA expression levels of pendrin were determined at the indicated time points after YS-01 (30 μ M) treatment by real-time quantitative PCR in IL-4-treated HBE cells (mean $\pm$ SE, $n = 3$). **C**, Cl^-/HCO_3^- exchange activities were measured in IL-4-treated HBE cells. YS-01 pretreated for 5 minutes. **D** and **E**, Short-circuit current recordings in HNE cells. Representative traces showing the effects of YS-01 on ENaC, CFTR, and CaCC channel activities. After 48 hours of exposure to YS-01 (30 μ M), the channel activities were measured in the absence or presence of IL-4. Amiloride (100 μ M), forskolin (20 μ M), CFTR_{inh}-172 (10 μ M), and ATP (100 μ M) were added to the apical bath. **F**, ANO1, CFTR, and ENaC mRNA expression levels were determined at the indicated time points after YS-01 (30 μ M) treatment by real-time quantitative PCR in IL-4-treated HNE cells (mean $\pm$ SE, $n = 5$). HBE, Human bronchial epithelial. * $P < .05$, ** $P < .01$, and *** $P < .001$.

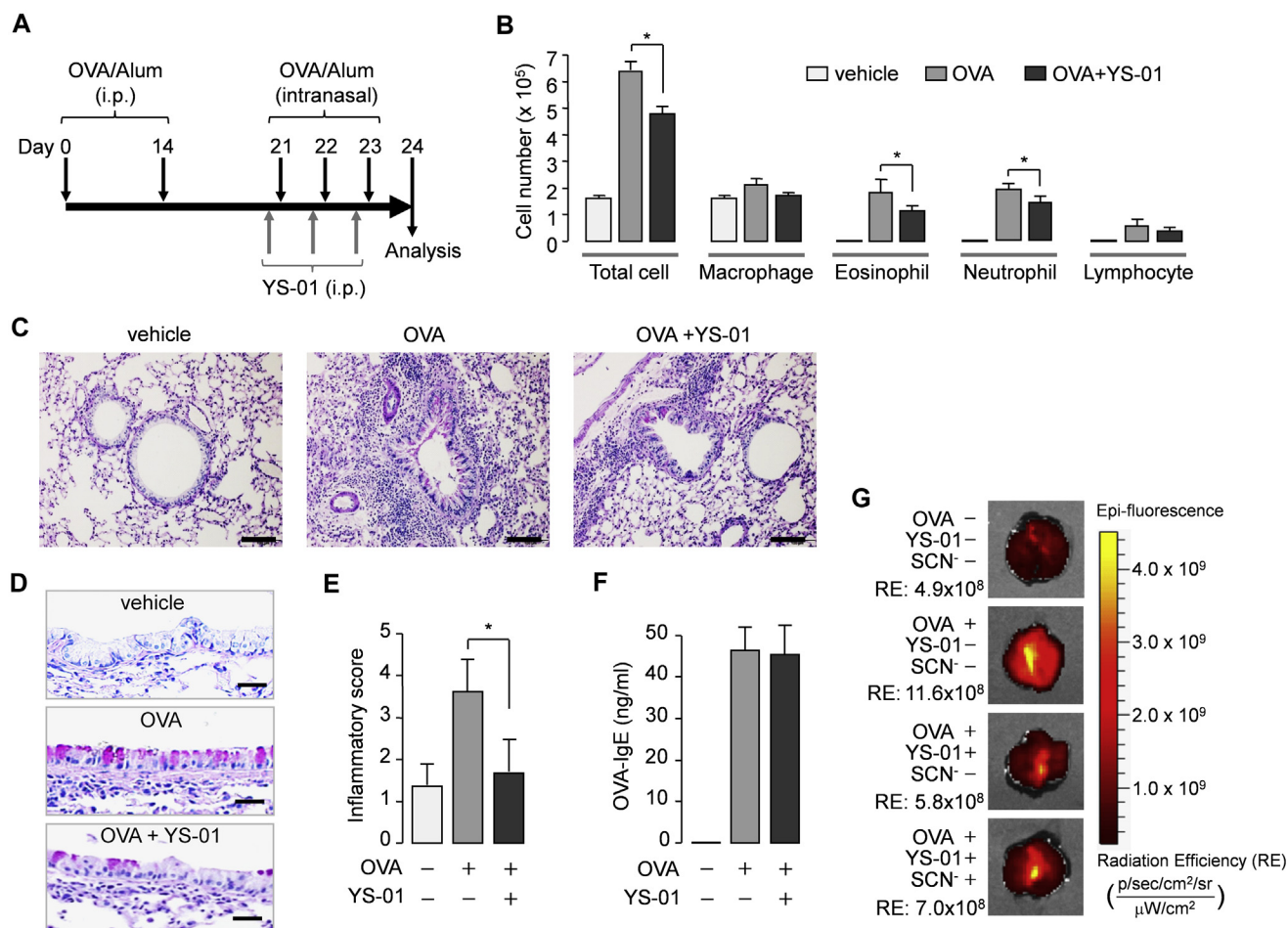


FIG E4. Amelioration of allergic airway inflammation by YS-01 in a mouse model of asthma. **A**, Protocol of induction and time-course of resolution of allergic airway inflammation after allergen challenge in wild-type mice. **B**, Inflammatory cells in the bronchoalveolar lavage fluid were isolated by centrifugation and stained with Diff-Quik staining reagent. The cell numbers were quantified under a light microscope (mean $\pm$ SE, $n = 10$). **C**, Representative histology of airways stained with hematoxylin and eosin. Scale bar, 100 μ m. **D**, Periodic acid-Schiff staining of airway sections from vehicle-treated, OVA-sensitized/challenged, and YS-01-treated (10 mg/kg) OVA-sensitized/challenged mice. Scale bar, 50 μ m. **E**, Summary of inflammatory score (mean $\pm$ SE, $n = 4$). **F**, OVA-specific IgE levels in serum (mean $\pm$ SE, $n = 4$). **G**, NF- κ B expression in the lung of NF- κ B reporter mice was compared using the IVIS system. The lungs were isolated from the mice and fluorescently imaged (Ex 570, Em 620) using the IVIS spectrum. The average fluorescent density of images was analyzed using Living Image 4.3.1 software. *i.p.*, Intraperitoneal. * $P < .05$.

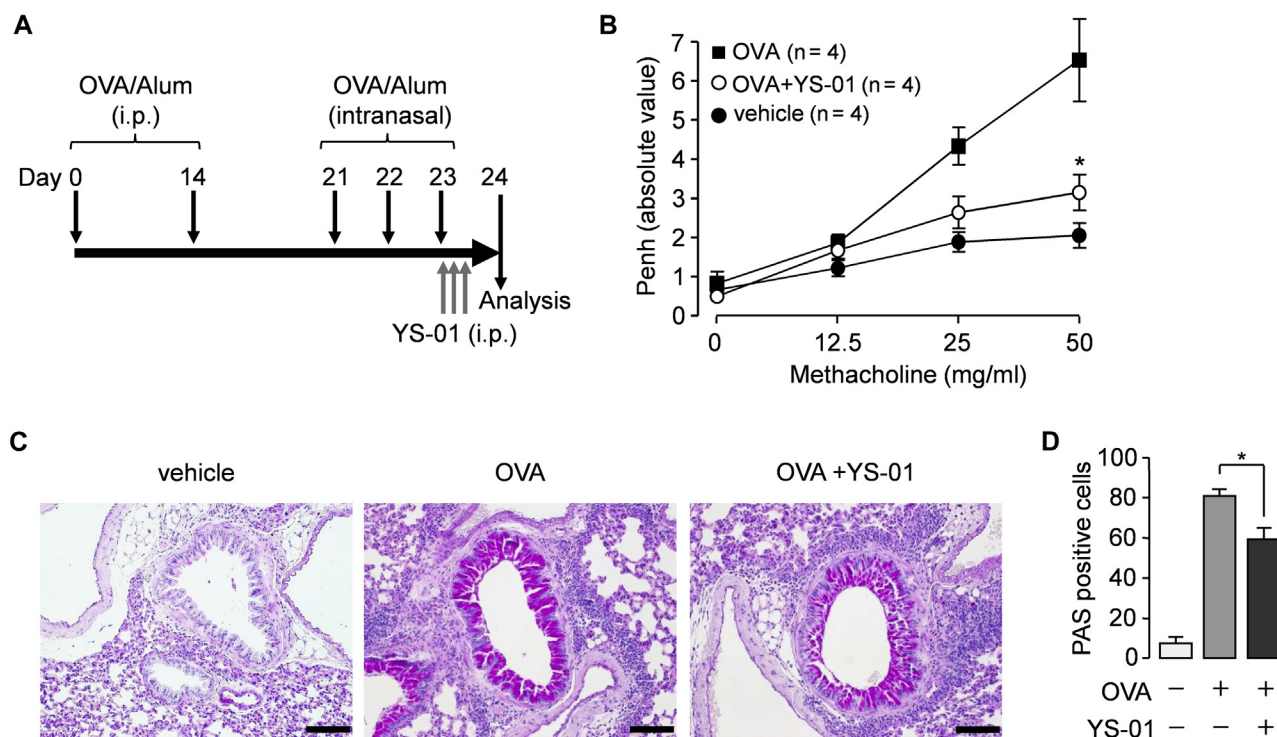


FIG E5. Amelioration of allergic asthma by YS-01 in an established model of allergic asthma. **A**, Protocol of induction and time-course of allergic airway inflammation after allergen challenge in wild-type mice. YS-01 (10 mg/kg 3 times/d) was applied after the third OVA challenge. **B**, Airway responsiveness measured by whole-body plethysmography in an OVA-induced asthma model. P_{enh} was measured in response to increasing doses of methacholine (mean $\pm$ SE, $n = 4$). **C**, Representative PAS staining of airway tissues. Scale bar, 100 μ m. **D**, Summary of PAS-positive cells (mean $\pm$ SE, $n = 4$). *i.p.*, Intraperitoneal; PAS, periodic acid-Schiff. * $P < .05$.

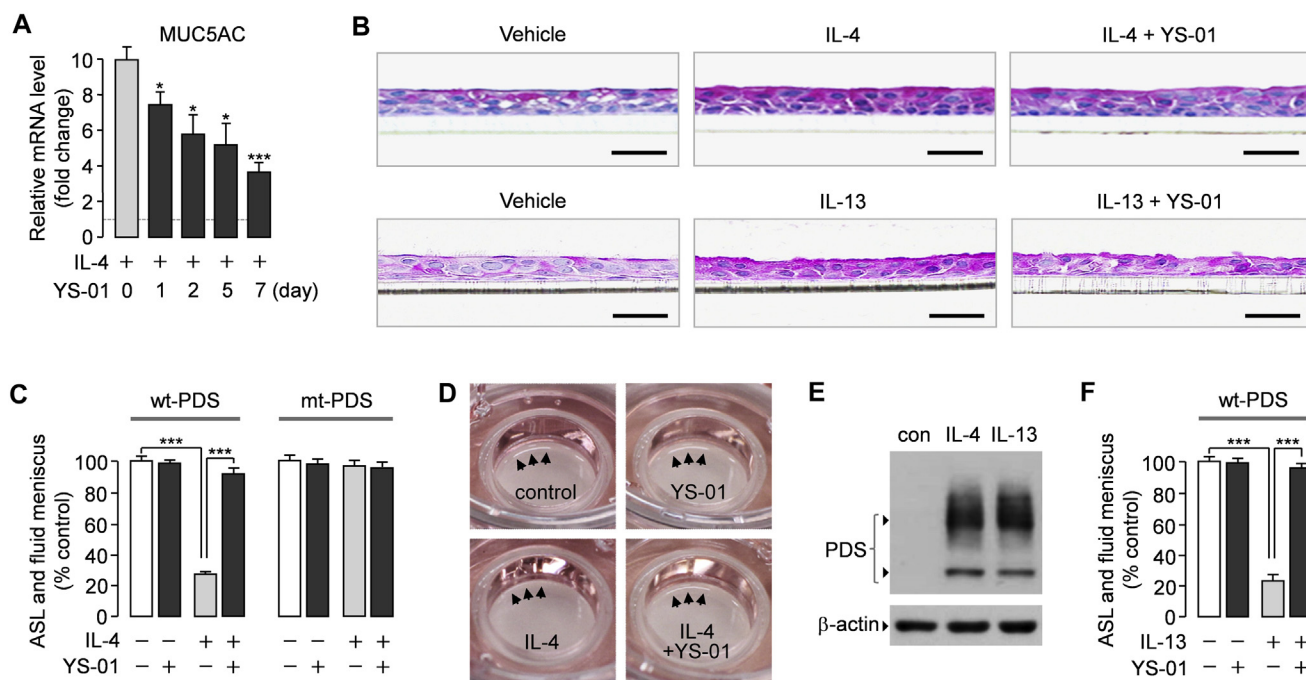


FIG E6. Effect of YS-01 on mucus production and ASL volume regulation in IL-4-treated HNE cells. **A**, MUC5AC mRNA expression levels were determined at indicated time points after YS-01 (30 μ M) treatment in IL-4-treated (10 ng/mL) HNE cells (mean $\pm$ SE, $n = 4$). **B**, Periodic acid-Schiff staining showing goblet cell hyperplasia of IL-4- and IL-13-treated (10 ng/mL) HNE cells in the absence or presence of YS-01 (30 μ M) on day 7. **C**, Measurement of total volume of ASL and fluid meniscus in HNE cells expressing wild-type (wt) or mutant (mt) pendrin (PDS). HNE cells were treated with IL-4 (10 ng/mL, 48 hours) in the presence or absence of 30 μ M YS-01 (mean $\pm$ SE, $n = 3-4$). **D**, Representative image of Transwell inserts with normal HNE cells. Arrow indicates fluid meniscus of HNE cells treated with IL-4 (10 ng/mL, 48 hours) in the presence or absence of 30 μ M YS-01. **E**, Representative immunoblot of PDS in IL-4- and IL-13-treated HNE cells. **F**, Measurement of total volume of ASL and fluid meniscus. HNE cells were treated with IL-13 (10 ng/mL, 48 hours) in the presence or absence of 30 μ M YS-01 (mean $\pm$ SE, $n = 3-4$). * $P < .05$ and *** $P < .001$. Scale bar, 30 μ m.

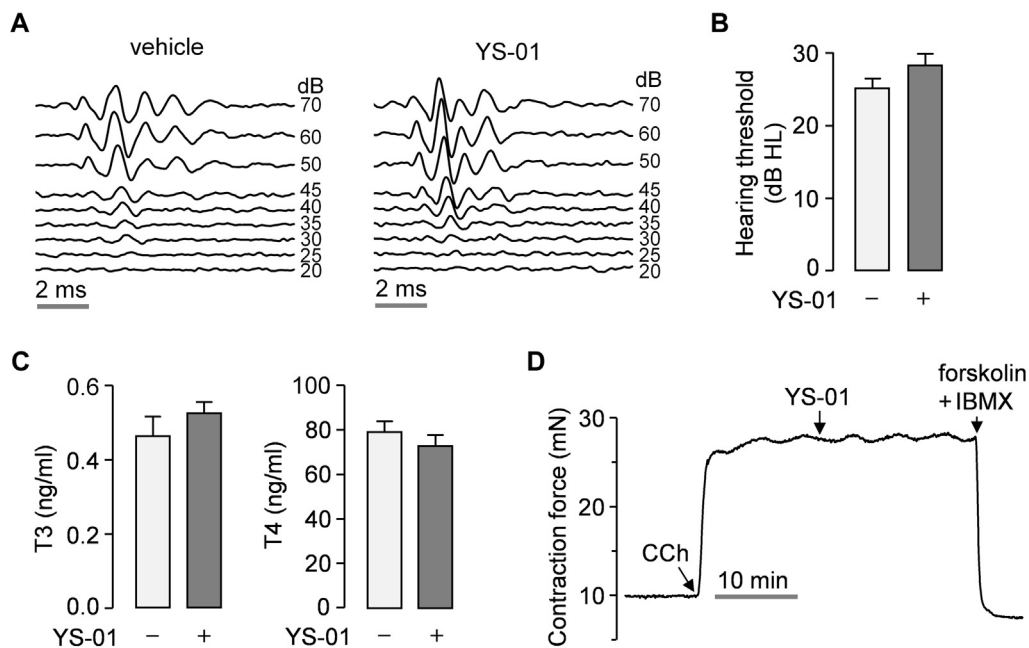


FIG E7. Effect of YS-01 on hearing threshold and plasma thyroid hormone levels. **A**, Representative examples of ABR waveforms from control and YS-01-treated (10 mg/kg/d for 7 days) mice. **B**, Summary of hearing threshold (mean $\pm$ SE, $n = 8-10$). **C**, Plasma levels of T3 and T4 were measured in YS-01-treated (10 mg/kg/d for 7 days) and untreated mice (mean $\pm$ SE, $n = 4$). **D**, Representative force trace that shows airway smooth muscle (ASM) contractile response in rat tracheal rings. YS-01 (30 μ M) was applied after induction of ASM contraction by carbachol (CCh) at a submaximal concentration (300 nM). ASM relaxation was induced by forskolin and IBMX.

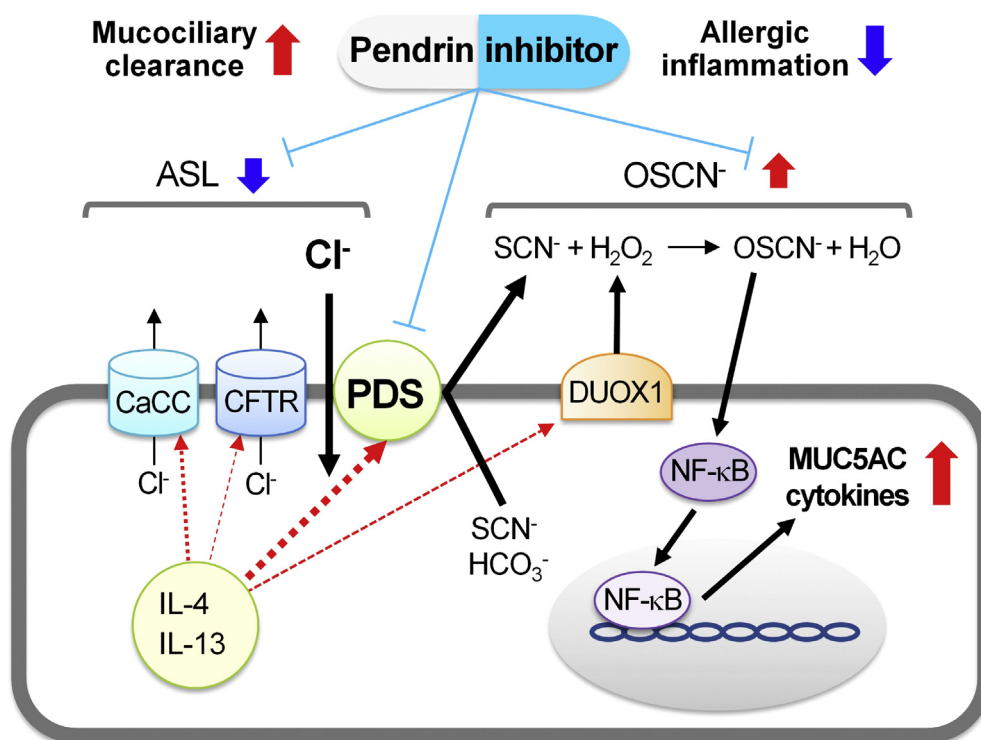


FIG E8. Schematic showing possible roles for pendrin inhibitor in airway inflammation. Pendrin inhibitor can decrease NF-κB activation by blocking SCN⁻ transport in airway epithelium and improve mucociliary clearance by inhibiting the upregulated pendrin-mediated ASL depletion in airway inflammation.

TABLE E1. Primer sequences used for quantitative RT-PCR

mRNA	Primer sequences	PCR product size
Pendrin	5'-TTC CCA AAG TGC CAA TCC ATA G-3' 5'-CCG CAG TGA TCT CAC TCC AAC-3'	83 bp
ANO1	5'-GGA GAA GCA GCA TCT ATT TG-3' 5'-GAT CTC ATA GAC AAT CGT GC-3'	82 bp
CFTR	5'-AGG AGG CAG TCT GTC CTG AA-3' 5'-CAC TGC TGG TAT GCT CTC CA-3'	237 bp
ENaC α	5'-CAG CCC ATA CCA GGT CTC AT-3' 5'-ATG GTG GTG TTG TTG CAG AA-3'	221 bp
ENaC β	5'-TCC TAC CCT CGT CCC TAC CT-3' 5'-CCA GGA AGG AGA AAA CCA CA-3'	151 bp
ENaC γ	5'-ACC ACC AGC CAT GGT CTA AG-3' 5'-GTT CAG GTC CCG GGA TTT AT-3'	211 bp
<i>Duox1</i>	5'-TTC ACG CAG CTC TGT GTC AA-3' 5'-AGG GAC AGA TCA TAT CCT GGC T-3'	96 bp
<i>Duox2</i>	5'-ACG CAG CTC TGT GTC AAA GGT-3' 5'-TGA TGA ACG AGA CTC GAC AGC-3'	90 bp
β -Actin	5'-GCA AAG ACC TGT ACG CCA ACA C-3' 5'-ATC TCC TTC TGC ATC CTG TC-3'	82 bp