JOURNAL OF NATURAL PRODUCTS

ARTICLE

pubs.acs.org/jnp

Protective Role of 14-Deoxy-11,12-didehydroandrographolide, a Noncytotoxic Analogue of Andrographolide, in Allergic Airway Inflammation

Shou-Ping Guan,[†] Li-Ren Kong,[†] Chang Cheng,[†] Jonathan C. W. Lim,[†,‡] and W. S. Fred Wong*[,†,§]

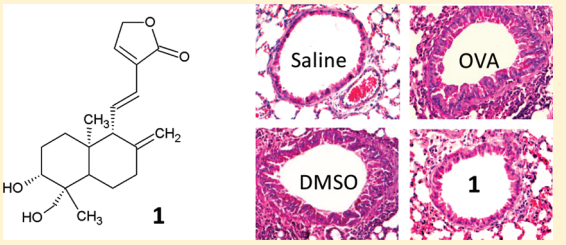
[†]Department of Pharmacology, Yong Loo Lin School of Medicine, National University Health System, Singapore

[‡]Department of Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Malaysia

[§]Immunology Program, Life Science Institute, National University of Singapore, Singapore

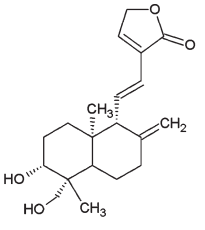
Supporting Information

ABSTRACT: Our group recently reported novel anti-inflammatory effects of andrographolide (**2**), a bioactive molecule isolated from *Andrographis paniculata*, in a mouse asthma model. However, **2** has been shown to possess cytotoxic activity. 14-Deoxy-11,12-didehydroandrographolide (**1**) is an analogue of **2** that can be isolated from *A. paniculata*. We hypothesized that **1** retains the anti-inflammatory effects for asthma but is devoid of cytotoxicity. In contrast to **2**, **1** did not elicit any cytotoxic activity in A549 and BEAS-2B human lung epithelial cells and rat basophilic leukemia (RBL)-2H3 cells using a MTS assay. Compound **1** dose-dependently inhibited ovalbumin (OVA)-induced increases in total and eosinophil counts, IL-4, IL-5, and IL-13 levels in lavage fluid, and serum OVA-specific IgE level in a mouse asthma model. Compound **1** attenuated OVA-induced airway eosinophilia, mucus production, mast cell degranulation, pro-inflammatory biomarker expression in lung tissues, and airway hyperresponsiveness. This substance also blocked p65 nuclear translocation and DNA-binding activity in the OVA-challenged lung and in TNF-α-stimulated human lung epithelial cells. The present findings reveal for the first time that **1** retains the anti-inflammatory activities of **2** for asthma probably through the inhibition of NF-κB. 14-Deoxy-11,12-didehydroandrographolide (**1**) may be considered as a safer analogue of **2** for the potential treatment of asthma.

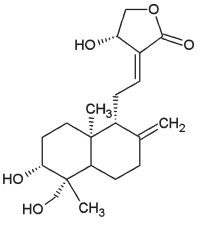


Allergic asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR).[1] Cumulative evidence has revealed that these inflammatory responses are mediated by Th2 cells together with mast cells, B-cells, and eosinophils, as well as a number of inflammatory cytokines and chemokines.[2] IL-4 is imperative for B-cell isotype switching for the synthesis of IgE. Allergen-induced cross-linking of IgE-bound FcεRI on the surface of mast cells leads to degranulation and activation, and immediate bronchoconstriction.[2,3] IL-5 is vital for the growth, differentiation, recruitment, and survival of eosinophils that contribute to inflammation and even airway remodeling in asthma.[4] IL-13 plays a pivotal role in the effector phase of Th2 responses such as eosinophilic inflammation, mucus hypersecretion, AHR, and airway remodeling.[5] In addition, chemokines such as RANTES and eotaxin are crucial to the delivery of eosinophils to the airways.[6] Airway eosinophilia, together with Th2 cytokines IL-4, IL-5, and IL-13, may ultimately contribute to AHR in asthma.[7]

Andrographolide (**2**), 14-deoxy-11,12-didehydroandrographolide (**1**), and neoandrographolide are the three major labdane diterpene lactones isolated from the leaves of the *Andrographis paniculata* (Burm.f.) Wall. ex Nees (Acanthaceae) plant.[8,9] *A. paniculata* has been used for the prevention and treatment of upper respiratory tract infection in Asian countries and also in Scandinavia.[10,11] We have reported recently a broad spectrum of anti-inflammatory effects for **2** in a mouse asthma model.[12] The potential protective roles of **1** and neoandrographolide in allergic asthma have yet to be determined.



14-Deoxy-11,12-Didehydroandrographolide (**1**)



Andrographolide (**2**)

Nonetheless, andrographolide **2** has been reported to possess cytotoxicity and to induce cell cycle arrest and apoptosis, and is also regarded as a potential antineoplastic agent.[13,14] For the treatment of asthma, it is critical to identify an analogue of **2** that

Received: March 25, 2011
Published: May 20, 2011

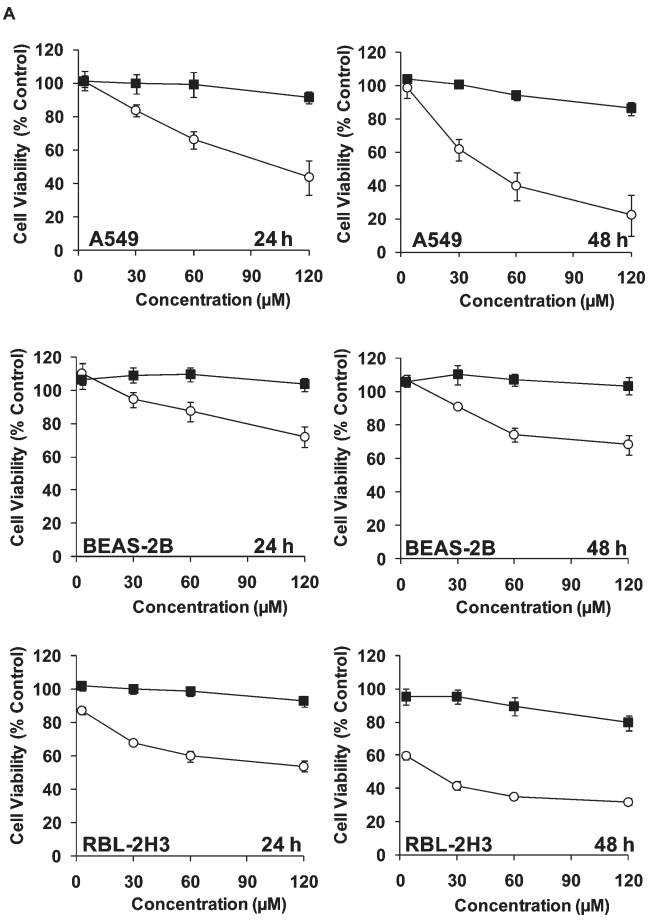


Figure 1. Effects of andrographolide (**2**, open circles) and 14-deoxy-11,12-didehydroandrographolide (**1**, filled squares) on cell viability of A549, BEAS-2B, and RBL-2H3 cells at 24 and 48 h time intervals. Compound **2** dose-dependently decreased the cell viability of all three cultured cells. In contrast, **1** had no effect on the viability of any of the cultured cells at all concentrations used and both time intervals. Values shown are the means ± SEM of six separate experiments. *Significant difference between **2** and **1**, $p < 0.05$.

retains the broad anti-inflammatory activities but with less or even no cytotoxic properties. Compound **1** is a major naturally occurring analogue isolated from *A. paniculata* and has also been identified as a metabolite of **2** when given systemically to rats.[15] This substance has been reported to be less cytotoxic to a variety of cell lines as compared to **2**.[13,16,17] In addition, **1** has been shown to inhibit cytokine and NO production from the Raw 264.7 macrophage cell line stimulated with lipopolysaccharide and IFN-γ and to attenuate high glucose-induced fibrosis and apoptosis in a renal mesangeal cell line.[18,19] The molecular mechanisms mediating the anti-inflammatory actions of **1** have not been determined unequivocally. Nevertheless, there is evidence pointing to the inhibition of NF-κB transcriptional activity by **2** and its analogues.[19,20]

Asthma is associated with persistent NF-κB activation.[21−23] In the present study, we investigated the potential cytotoxicity of **1** in lung epithelial and mast cell lines and the potential anti-inflammatory effects of **1** in a mouse asthma model. The results obtained confirm that **1** is a noncytotoxic analogue of **2** and indicate for the first time that **1** can attenuate allergic airway inflammation probably via inhibition of NF-κB activity.

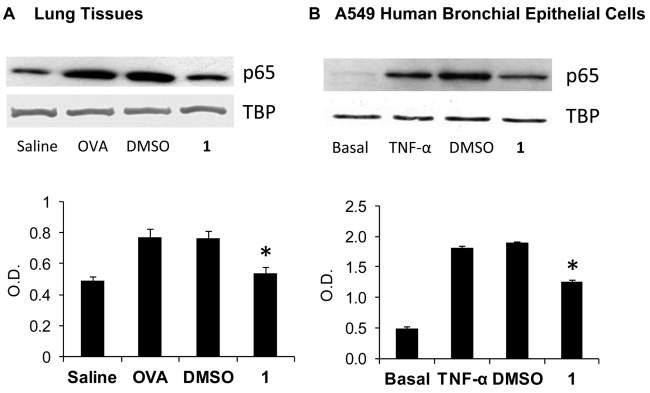


Figure 2. Effects of 14-deoxy-11,12-didehydroandrographolide (**1**) on NF-κB activity in OVA-challenged lungs (A) and in TNF-α-stimulated A549 human lung epithelial cells (B). Immunoblotting of p65 NF-κB in nuclear extract of lung tissues isolated from mice 24 h after the last OVA challenge and of A549 cells stimulated with 10 ng/mL TNF-α for 5 min, in the presence and absence of **1** (30 μM). TBP nuclear protein was used as an internal control. The experiments were repeated three times with a similar pattern of results. Nuclear p65 DNA-binding activity was determined using a TransAM p65 transcription factor ELISA kit. Values shown are the mean ± SEM of three separate experiments. *Significant difference from DMSO control, $p < 0.05$.

RESULTS AND DISCUSSION

14-Deoxy-11,12-didehydroandrographolide (1) Is Less Cytotoxic Than Andrographolide (2). Besides andrographolide (**2**), **1** is the next most abundant of the naturally occurring labdane diterpene lactones in *A. paniculata*.[8,9] Compound **1** has been shown to be a metabolite of **2** and can also be synthesized chemically from the parent compound.[15,16] Nevertheless, the present knowledge of the pharmacological actions of **1** is much less than for **2**. Indeed, a clinical study of **2** in upper respiratory tract infection has been reported,[24] whereas investigations of **1** are still at the early preclinical stage. Despite their structurally similarity, there is a notable difference between these two diterpenoids with regard to cytotoxicity. Our present findings reveal that andrographolide (**2**) dose-dependently and time-dependently reduced the viability of A549 and BEAS-2B human lung epithelial cells and RBL-2H3 mast cells (Figure 1). In contrast, **1** did not reduce cell viability of these cultured cells at all concentrations tested and at both 24 and 48 h time points. These data are consistent with observations reported by other laboratories on other cell lines[16,17] and strongly support the notion that **1** is a noncytotoxic analogue of **2**.

14-Deoxy-11,12-didehydroandrographolide (1) Inhibits NF-κB Activation. Persistent NF-κB activation has been observed in allergic airway inflammation in humans.[21−23] Antigen receptor activation in T and B lymphocytes and mast cells culminates in NF-κB activation.[2,25,26] In addition, TNF-α stimulation of airway epithelial cells triggers NF-κB activation.[27] To determine if **1**, like andrographolide (**2**), could inhibit NF-κB in ovalbumin (OVA)-challenged mice,[12] the nuclear translocation of the p65 subunit and p65 DNA-binding activity in lung tissues from mice treated with **1** were examined. OVA challenge markedly raised the level of p65 subunit in the nuclear extract of lung tissues and promoted nuclear p65 DNA-binding activity (Figure 2A). Compound **1** (1 mg/kg) significantly ($p < 0.05$) reduced both nuclear p65 translocation and DNA-binding activity to the basal levels, showing for the first time that **1**, like **2**,

dx.doi.org/10.1021/np2002572 |J. Nat. Prod. 2011, 74, 1484–1490

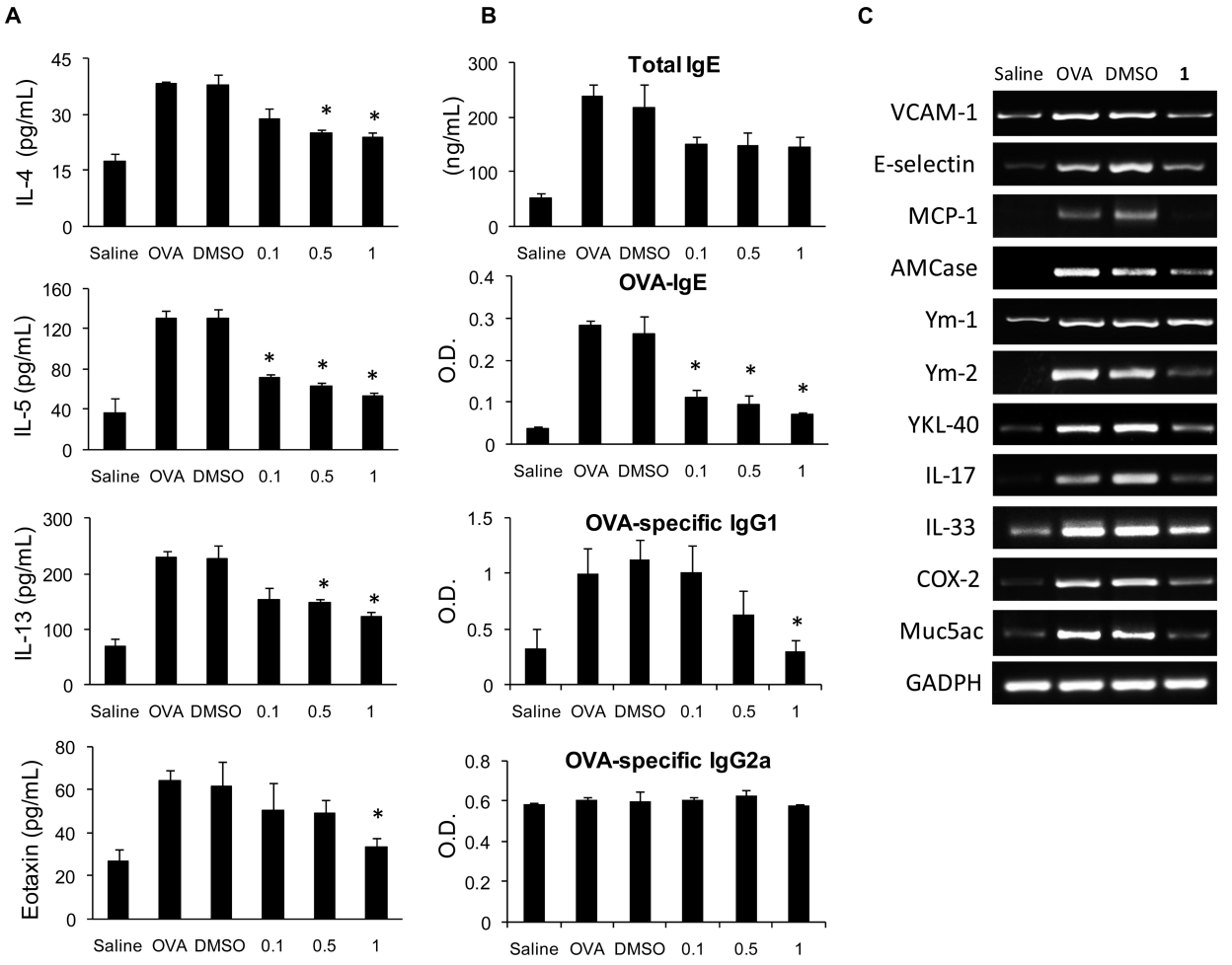


Figure 3. Effects of 14-deoxy-11,12-didehydroandrographolide (**1**) on BAL fluid Th2 cytokines, serum immunoglobulins, and lung inflammatory biomarkers. (A) BAL fluids were collected 24 h after the last OVA aerosol challenge. Levels of IL-4, IL-5, IL-13, and eotaxin were analyzed using ELISA ($n = 6-9$ mice). Lower limits of detection were as follows: IL-4 and IL-5 at 4 pg/mL, IL-13 at 15.6 pg/mL, and eotaxin at 2 pg/mL. (B) Mouse serum was obtained 24 h after the last OVA aerosol challenge. The levels of total IgE, OVA-specific IgE, OVA-specific IgG1, and OVA-specific IgG2a were analyzed using ELISA ($n = 6-9$ mice). Values shown are the means ± SEM. *Significant difference from DMSO control, $p < 0.05$. (C) Lung tissues were collected 24 h after the last OVA aerosol challenge. Total mRNA was extracted using TriZol reagent, and the PCR products were separated in a 2% agarose gel visualized under UV light. GADPH was used as an internal control. The experiments were repeated three times ($n = 3$ mice) with a similar pattern of results.

may exert anti-inflammatory actions via inhibition of NF-κB activity in vivo.

The mechanism of action of **1** in TNF-α-stimulated A549 human lung epithelial cells in vitro was verified. TNF-α plays a critical role in asthma[28,29] and is a potent stimulator of human airway epithelial cells.[27] A sharp increase in total nuclear p65 level and p65 DNA-binding activity was observed upon TNF-α treatment, and **1** markedly abated these TNF-α-mediated responses (Figure 2B).

14-Deoxy-11,12-didehydroandrographolide (1) Reduces Bronchoalveolar Lavage Fluid Th2 Cytokines, Serum Immunoglobulins, and Lung Inflammatory Biomarkers. OVA challenge caused a notable increase in IL-4, IL-5, IL-13 and eotaxin levels in bronchoalveolar lavage (BAL) fluid. Compound **1** significantly ($p < 0.05$) reduced IL-4, IL-5, and IL-13 and, to a lesser extent, eotaxin levels in BAL fluid, in a dose-dependent manner (Figure 3A). Similar findings were observed in OVA-challenged mice with disrupted NF-κB function via conditional knockout of IKKβ in the airway epithelium.[30] In addition, repression of the NF-κB signaling pathway has been shown to block IL-13-induced eotaxin production in cultured human airway smooth muscle cells.[31] Therefore, the observed reduction of IL-4, IL-5, IL-13, and eotaxin levels in BAL fluid from **1**-treated mice may be due to inhibition of NF-κB activation in the inflammatory and airway resident cells.

Elevated serum IgE levels are a hallmark of the Th2 immune response. NF-κB plays a crucial role in B-cell proliferation and development,[25,32] and IL-4 and IL-13 are important in directing B-cell growth, differentiation, and secretion of IgE.[3,5] A marked elevation in serum total IgE, OVA-specific IgE, and OVA-specific IgG1 levels was observed, with no changes in OVA-specific IgG2a levels, in OVA-challenged mice. Compound **1** significantly ($p < 0.05$) suppressed OVA-specific IgE levels and, to a lesser extent, the serum levels of total IgE and OVA-specific IgG1 (Figure 3B). This substance had no effects on the serum level of OVA-specific IgG2a. This may be attributed to the inhibition of NF-κB during B-cell activation and of IL-4- and IL-13-mediated class switching to IgE. These findings imply that **1** is able

dx.doi.org/10.1021/np2002572 |J. Nat. Prod. 2011, 74, 1484–1490

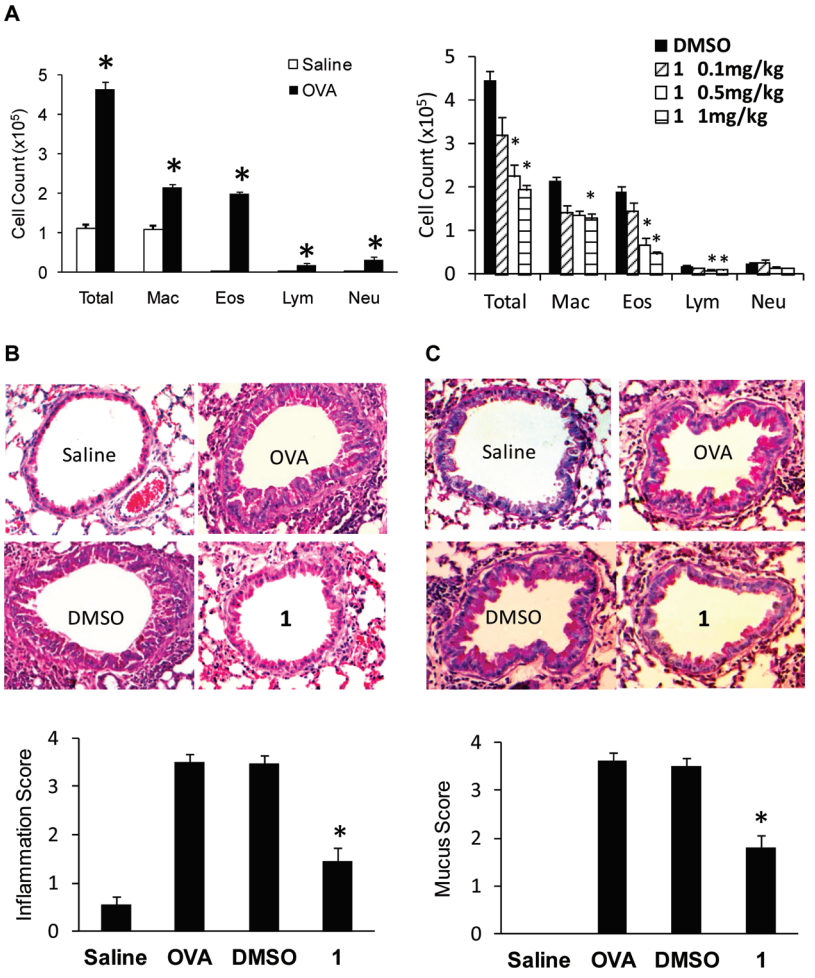


Figure 4. Effects of 14-deoxy-11,12-didehydroandrographolide (**1**) on OVA-induced inflammatory cell recruitment and mucus hypersecretion. (A) Inflammatory cell counts in BAL fluid obtained from sensitized mice 24 h after the last saline aerosol ($n = 7$ mice) or OVA aerosol ($n = 7$ mice) challenge. **1** dose-dependently reduced OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 h after the last OVA aerosol challenge (DMSO, $n = 7$; 0.1 mg/kg, $n = 8$; 0.5 mg/kg, $n = 7$; and 1 mg/kg, $n = 10$). Differential cell counts were performed on a minimum of 500 cells to identify eosinophils (Eos), macrophages (Mac), neutrophils (Neu), and lymphocytes (Lym). Histological slides showing lung tissue eosinophilia (B, magnification 200×) and mucus secretion (C, magnification 200×) 24 h after the last challenge of saline aerosol, OVA aerosol, OVA aerosol plus DMSO, or OVA aerosol plus 1 mg/kg **1** are displayed. Quantitative analyses of inflammatory cell infiltration and mucus production in lung sections were performed as previously described.[12] Scoring of inflammatory cells and goblet cells was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from DMSO control, $p < 0.05$.

to modify the Th2 immune activity in the OVA mouse asthma model.

OVA challenge markedly up-regulated lung mRNA levels of the adhesion molecules VCAM-1 and E-selectin, and chemokine MCP-1, which are pivotal for pulmonary recruitment of inflammatory cells.[6,33] Chitinases including acidic mammalian chitinase (AMCase), Ym1, Ym2, and YKL-40 have been shown recently to play critical roles in airway inflammation and remodeling.[34−36] IL-17 and IL-33 are two effector cytokines that have been shown to be essential for airway inflammation and remodeling.[37,38] COX-2 is known to be essential for PGD_2-mediated airway eosinophilia and hyper-responsiveness,[39] and Muc5ac, essential for mucus hypersecretion.[40] Pretreatment with **1** (1 mg/kg) strongly suppressed VCAM-1, E-selectin, MCP-1, AMCase, Ym-2, YKL-40, Muc5ac, COX2, IL-17, and IL-33 levels in the allergic airways (Figure 3C). These findings are likely to be due to **1**-mediated NF-κB inhibition, as the genes encoding for these proteins contain the κB site for NF-κB within their promoters.[41]

14-Deoxy-11,12-didehydroandrographolide (1) Suppresses Allergic Airway Inflammation. We have reported recently major anti-inflammatory roles for andrographolide (**2**) in a mouse asthma model.[12] It was considered imperative to investigate if **1**, a noncytotoxic analogue of **2**, retains the same anti-inflammatory profile for asthma. OVA challenge markedly increased total cell and eosinophil counts. Compound **1** (0.1, 0.5, and 1 mg/kg) significantly decreased total cell and eosinophil counts in BAL fluid in a dose-dependent manner (Figure 4A). At 1 mg/kg, **1** also reduced macrophage and lymphocyte counts. A complete blood count of peripheral blood obtained from **1**-treated mice was conducted. There was no difference in white blood count, red blood count, and hematocrit and hemoglobin concentrations in mice with or without **1** treatment (data not shown). Hence, **1**-induced reduction in eosinophil pulmonary recruitment is unlikely to be due to any nonspecific cytotoxic effects of the drug.

Leukocyte transmigration into the airways is orchestrated by cytokines such as IL-4, IL-5, and IL-13 and coordinated by

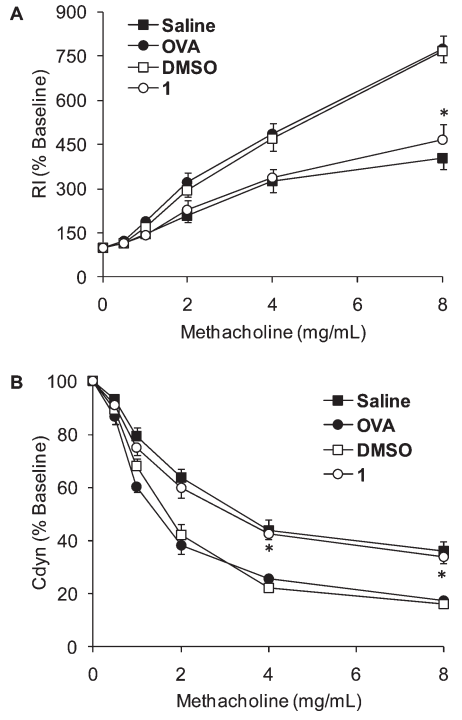


Figure 5. Effects of 14-deoxy-11,12-didehydroandrographolide (**1**) on OVA-induced AHR. Airway responsiveness of mechanically ventilated mice in response to aerosolized methacholine was measured. AHR is expressed as the percent change from the baseline level of (A) lung resistance (Rl, $n = 7-9$ mice) and (B) dynamic compliance (Cdyn, $n = 7-9$ mice). Rl is defined as the pressure driving respiration divided by flow. Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. *Significant difference from DMSO control, $p < 0.05$.

specific chemokines such as eotaxin and RANTES in combination with adhesion molecules as exemplified by VCAM-1 and E-selectin.[6,33] IL-13 is by far the most potent inducer of eotaxin expression in airway epithelial cells.[31] IL-17 has been shown to induce eotaxin production from airway smooth muscle cells,[42] whereas IL-33 can promote eotaxin release from macrophages.[43] OVA challenge induced marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared with saline aerosol challenge. Compound **1** (1 mg/kg) markedly diminished the eosinophil-rich leukocyte infiltration (Figure 4A and B), likely due to suppression of E-selectin, VCAM-1, MCP-1, IL-17, and IL-33 expression and eotaxin production in OVA-challenged lungs.

IL-4, IL-5, IL-13, and IL-17 play a critical role in goblet cell hyperplasia and mucin Muc5ac gene and protein expression in mice.[40,44,45] OVA-challenged mice developed goblet cell hyperplasia and mucus hypersecretion in the bronchi, which were markedly suppressed by **1** (1 mg/kg) (Figure 4C). Selective ablation of NF-κB function in the airway epithelium has been shown to reduce OVA-induced mucus production in mice.[46] As such, the reduction in mucus production in the lungs of **1**-treated mice may be attributable to a significant drop in IL-4, IL-5, IL-13, and IL-17 levels and an inhibitory action on NF-κB in the airway epithelium.

14-Deoxy-11,12-didehydroandrographolide (1) Prevents Lung Mast Cell Degranulation. OVA aerosol challenge markedly decreased intact mast cell number and increased degranulating mast cells in the lungs. Compound **1** (1 mg/kg) reduced mast cell degranulation and restored intact mast cell number similar to saline aerosol control (Figure S1, Supporting Information). In a preliminary study, **1** failed to prevent IgE-stimulated RBL-2H3 cell release of β-hexosaminidase (data not shown), indicating that this compound does not block mast cell degranulation directly. As such, the observed reduction in mast cell degranulation by **1** is likely due to the reduction in serum OVA-specific IgE level induced by this compound.

14-Deoxy-11,12-didehydroandrographolide (1) Reduces AHR. IL-4, IL-5, and IL-13 have been shown to induce AHR, in which major basic protein and cysteinyl-leukotrienes have been implicated.[4−6,47] Recently, IL-33 was found to directly trigger AHR in mice.[43] In addition, IgE-mediated mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines.[2,26] OVA-challenged mice developed AHR, which is typically reflected by high lung resistance (Rl) and low dynamic compliance (Cdyn) (Figure 5). Rl is defined as the pressure driving respiration divided by flow. Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. Compound **1** (1 mg/kg) dramatically reduced Rl and restored Cdyn in OVA-challenged mice in response to methacholine, suggesting that the immune-mediated airway pathology in vivo was modified.

We report here for the first time that 14-deoxy-11,12-didehydroandrographolide (**1**), a naturally occurring noncytotoxic analogue of andrographolide (**2**), effectively reduced OVA-induced inflammatory cell recruitment into BAL fluid, IL-4, IL-5, IL-13, and eotaxin production, serum IgE synthesis, pulmonary eosinophilia, mucus hypersecretion, mast cell degranulation, and AHR in a mouse asthma model, probably via inhibition of NF-κB activity. These findings support a potential therapeutic role for **1** in the treatment of asthma subject to further laboratory study.

EXPERIMENTAL SECTION

Cell Cultures. A549 human lung epithelial cells, BEAS-2B human transformed lung epithelial cells, and RBL-2H3 rat basophilic leukemia cells were obtained from the American Type Tissue Collection (Rockville, MD). A549 and BEAS-2B cells were cultured in RPMI 1640, while RBL-2H3 cells were grown in DMEM. Both media were supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL), and cells were grown at 37 °C in a humidified 5% CO_2 incubator. To explore the anti-inflammatory mechanism of action of 14-deoxy-11,12-didehydroandrographolide (**1**) (>98% purity, TCM Institute of Chinese Materia Medica, Nanjing, People's Republic of China), A549 cells (1×10^6 cells) were seeded in a 100 mm Petri dish, and at 90% confluence, cells were treated with 30 μM **1** or vehicle (0.1% DMSO) for 4 h before stimulation with 10 ng/mL TNF-α for 30 min and protein lysates were prepared for analysis.

Cell Viability Assay. A549 cells (3×10^3/well), BEAS-2B cells (5×10^3/well), and RBL-2H3 cells (3×10^3/well) were seeded in flat-bottomed 96-well plates overnight and then incubated with increasing concentrations (3−120 μM) of **1** or andrographolide (**2**) (>98% purity, Sigma, St. Louis, MO) for 24 and 48 h at 37 °C. Cell viability was analyzed using the CellTiter 96 AQ_{ueous} cell proliferation assay (Promega, Madison, WI) according to the manufacturer's protocol. This MTS assay is based on the ability of viable cells to convert a soluble tetrazolium salt to a colored formazan product. Absorbance was recorded at 490 nm (Tecan microplate reader Infinite F200; Mannedorf, Switzerland).

Animals. Female BALB/c mice, 6 to 8 weeks old (Animal Resources Center, Canning Vale, Western Australia, Australia), were sensitized and challenged with OVA as described.[12] Briefly, mice were sensitized by ip injections of 20 μg of OVA and 4 mg of $Al(OH)_3$ suspended in 0.1 mL of saline on days 0 and 14. On days 22, 23, and 24, mice were challenged with 1% OVA aerosol for 30 min. Compound **1** (0.1, 0.5, and 1 mg/kg) or vehicle (1% DMSO) in 0.1 mL of saline was given by ip injections 2 h before and 10 h after each OVA aerosol challenge. Saline aerosol was used as a negative control. Animal experiments were performed according to the Institutional Guidelines for Animal Care and Use Committee of the National University of Singapore (protocol no. 06/08).

Bronchoalveolar Lavage Fluid and Serum Analysis. Mice were anesthetized 24 h after the last aerosol challenge, and BAL was performed as described.[12] Briefly, tracheotomy was performed, and a cannula was inserted into the trachea. Ice-cold PBS (0.5 mL × 3) was instilled into the lungs, and BAL fluid was collected. BAL fluid total and differential cell counts and cytokine and chemokine levels were determined as described previously.[12] IL-4 and IL-5 ELISA were obtained from BD PharMingen (San Diego, CA). Eotaxin, IL-13, and IFN-γ ELISA were purchased from R&D Systems (Minneapolis, MN). Blood was collected by cardiac puncture, and serum levels of total IgE and OVA-specific IgE, IgG1, and IgG2a levels were determined as described previously.[12]

Histologic Analysis. Lungs were fixed in 10% neutral formalin, paraffinized, cut into 6 μm sections, and stained with hematoxylin and eosin (H&E) for examining cell infiltration and with periodic acid-Schiff stain (PAS) for measuring mucus production. Quantitative analysis was performed blind as described.[12] Lung tissue mast cells were stained using toluidine blue, and the number of intact and degranulating mast cells was counted in the entire lung sections, as described previously.[48]

Measurement of AHR. Mice were anesthetized and tracheotomy was performed as described.[12] The animal was intubated with a cannula that is connected to a multiport that leads to the pneumotach, ventilator, and nebulizer within the FinePointe Series RC Sites (Buxco Research System, Wilmington, NC). The mouse was ventilated at a fixed breathing rate of 140 breaths/min, and the Rl and Cdyn in response to increasing concentrations of nebulized methacholine (0.5−8.0 mg/mL) were recorded using FinePointe data acquisition and analysis software (Buxco Research System). Results are expressed as a percentage of the respective basal values in response to PBS.

Immunoblotting, mRNA Expression, and NF-κB DNA-Binding. Nuclear proteins were extracted from lung tissues and A549 cells using a nuclear extract kit (Active Motif, Carlsbad, CA). Lung and cell culture nuclear proteins (10 μg per lane) were separated by 10% SDS-PAGE, and immunoblots were developed as described previously.[12] Immunoblots were probed with anti-p65 (Cell Signaling Technology, Beverly, MA) and anti-TATA binding protein (TBP, Abcam, Cambridge, UK). Primers for inflammatory biomarkers are shown in Table S1 (Supporting Information), and PCR products were run in a 2% agarose gel and visualized under ultraviolet light. Nuclear proteins were also analyzed for NF-κB DNA-binding activity using the TransAM NF-κB p65 transcription factor assay kit (Active Motif).

Statistical Analysis. Data are presented as means ± SEM. One-way ANOVA followed by Dunnett's test was used to determine significant differences between treatment groups. Significance levels were set at $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information. Table S1 listing the sequences of the specific primers used for RT-PCR analysis, and Figure S1 showing the protective effects of 14-deoxy-11,12-didehydroandrographolide (**1**) on OVA-induced lung mast cell degranulation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 65-6516-3263. Fax: 65-6873-7690. E-mail: phcwongf@nus.edu.sg.

■ ACKNOWLEDGMENT

This work was partly supported by BMRC grants 06/1/21/19/443 and 09/1/21/19/595 from the BioMedical Research Council of Singapore and by grant HQ/S10-095COT0_21 from the Exploit Technology Private Limited to W.S.F.W.

■ REFERENCES

(1) Galli, S. J.; Tsai, M.; Piliponsky, A. M. *Nature* **2008**, *454*, 445–454.
(2) Galli, S. J.; Grimbaldeston, M.; Tsai, M. *Nat. Rev. Immunol.* **2008**, *8*, 478–486.
(3) Li-Weber, M.; Krammer, P. H. *Nat. Rev. Immunol.* **2003**, *3*, 534–543.
(4) Takatsu, K.; Nakajima, H. *Curr. Opin. Immunol.* **2008**, *20*, 288–294.
(5) Wills-Karp, M. *Immunol. Rev.* **2004**, *202*, 175–190.
(6) Hogan, S. P.; Rosenberg, H. F.; Moqbel, R.; Phipps, S.; Foster, P. S.; Lacy, P.; Kay, A. B.; Rothenberg, M. E. *Clin. Exp. Allergy* **2008**, *38*, 709–750.
(7) Cockcroft, D. W.; Davis, B. E. *J. Allergy Clin. Immunol.* **2006**, *118*, 551–559.
(8) Koteswara Rao, Y.; Vimalamma, G.; Rao, C. V.; Tzeng, Y. M. *Phytochemistry* **2004**, *65*, 2317–2321.
(9) Pholphana, N.; Rangkadilok, N.; Thongnest, S.; Ruchirawat, S.; Ruchirawat, M.; Satayavivad, J. *Phytochem. Anal.* **2004**, *15*, 365–371.
(10) Coon, J. T.; Ernst, E. *Planta Med.* **2004**, *70*, 293–298.
(11) Poolsup, N.; Suthisisang, C.; Prathanturarug, S.; Asawamekin, A.; Chanchareon, U. *J. Clin. Pharm. Ther.* **2004**, *29*, 37–45.
(12) Bao, Z.; Guan, S.; Cheng, C.; Wu, S.; Wong, S. H.; Kemeny, D. M.; Leung, B. P.; Wong, W. S. F. *Am. J. Respir. Crit. Care Med.* **2009**, *179*, 657–665.
(13) Kumar, R. A.; Sridevi, K.; Kumar, N. V.; Nanduri, S.; Rajagopal, S. *J. Ethnopharmacol.* **2004**, *92*, 291–295.
(14) Varma, A.; Padh, H.; Shrivastava, N. *Evid.-Based Complementary Altern. Med.* **2011**, *2011*, Article ID 815390.
(15) He, X.; Li, J.; Gao, H.; Qiu, F.; Cui, X.; Yao, X. *Chem. Pharm. Bull.* **2003**, *51*, 586–589.
(16) Nanduri, S.; Nyavanandi, V. K.; Thunuguntla, S. S.; Kasu, S.; Pallerla, M. K.; Ram, P. S.; Rajagopal, S.; Kumar, R. A.; Ramanujam, R.; Babu, J. M.; Vyas, K.; Devi, A. S.; Reddy, G. O.; Akella, V. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4711–4717.
(17) Tan, M. L.; Kuroyanagi, M.; Sulaiman, S. F.; Najimudin, N.; Muhammad, T. S. T. *Pharm. Biol.* **2005**, *43*, 501–508.
(18) Lee, M. J.; Rao, Y. K.; Chen, K.; Lee, Y. C.; Chung, Y. S.; Tseng, Y. M. *J. Ethnopharmacol.* **2010**, *132*, 497–505.
(19) Chao, W. W.; Kuo, Y. H.; Lin, B. F. *J. Agric. Food Chem.* **2010**, *58*, 2505–2512.
(20) Xia, Y. F.; Ye, B. Q.; Li, Y. D.; Wang, J. G.; He, X. J.; Lin, X.; Yao, X.; Ma, D.; Slungaard, A.; Hebbel, R. P.; Key, N. S.; Geng, J. G. *J. Immunol.* **2004**, *173*, 4207–4217.
(21) Gagliardo, R.; Chanez, P.; Mathieu, M.; Bruno, A.; Costanzo, G.; Gougat, C.; Vachier, I.; Bousquet, J.; Bonsignore, G.; Vignola, A. M. *Am. J. Respir. Crit. Care Med.* **2003**, *168*, 1190–1198.
(22) Hart, L. A.; Krishnan, V. L.; Adcock, I. M.; Barnes, P. J.; Chung, K. F. *Am. J. Respir. Crit. Care Med.* **1998**, *158*, 1585–1592.
(23) Pantano, C.; Ather, J. L.; Alcorn, J. F.; Poynter, M. E.; Brown, A. L.; Guala, A. S.; Beuschel, S. L.; Allen, G. B.; Whittaker, L. A.;

Bevelander, M.; Irvin, C. G.; Janssen-Heininger, Y. M. *Am. J. Respir. Crit. Care Med.* **2008**, *177*, 959–969.
(24) Chang, J.; Zhang, R. M.; Zhang, Y.; Chen, Z. B.; Zhang, Z. M.; Xu, Q.; Yang, Y. P.; Long, Y. Y.; Lin, L. L.; Cai, H. Y.; Gao, J.; Lu, N.; Mao, B.; Wang, L.; Li, T. Q. *Zhong Xi Yi Jie He Bao* **2008**, *6*, 1238–1245.
(25) Schulze-Luehrmann, J.; Ghosh, S. *Immunity* **2006**, *25*, 701–715.
(26) Klemm, S.; Ruland, J. *Immunobiology* **2006**, *211*, 815–820.
(27) Newton, R.; Holden, N. S.; Catley, M. C.; Oyelusi, W.; Leigh, R.; Proud, D.; Barnes, P. J. *J. Pharmacol. Exp. Ther.* **2007**, *321*, 734–742.
(28) Brightling, C.; Berry, M.; Amrani, Y. *J. Allergy Clin. Immunol.* **2008**, *121*, 5–10.
(29) Vroling, A. B.; Duinsbergen, D.; Fokkens, W. J.; van Drunen, C. M. *Allergy* **2007**, *62*, 1310–1319.
(30) Broide, D. H.; Lawrence, T.; Doherty, T.; Cho, J. Y.; Miller, M.; McElwain, K.; McElwain, S.; Karin, M. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 17723–17728.
(31) Li, L.; Xia, Y.; Nguyen, A.; Lai, Y. H.; Feng, L.; Mosmann, T. R.; Lo, D. *J. Immunol.* **1999**, *162*, 2477–2487.
(32) Siebenlist, U.; Brown, K.; Claudio, E. *Nat. Rev. Immunol.* **2005**, *5*, 435–445.
(33) Kelly, M.; Hwang, J. M.; Kubes, P. *J. Allergy Clin. Immunol.* **2007**, *120*, 3–10.
(34) Zhu, Z.; Zheng, T.; Homer, R. J.; Kim, Y. K.; Chen, N. Y.; Cohn, L.; Hamid, Q.; Elias, J. A. *Science* **2004**, *304*, 1678–1682.
(35) Zhao, J.; Zhu, H.; Wong, C. H.; Leung, K. Y.; Wong, W. S. F. *Proteomics* **2005**, *5*, 2799–2807.
(36) Chupp, G. L.; Lee, C. G.; Jarjour, N.; Shim, Y. M.; Holm, C. T.; He, S.; Dziura, J. D.; Reed, J.; Coyle, A. J.; Kiener, P.; Cullen, M.; Grandsaigne, M.; Dombret, M. C.; Aubier, M.; Pretolani, M.; Elias, J. A. *N. Engl. J. Med.* **2007**, *357*, 2016–2027.
(37) Kearley, J.; Buckland, K. F.; Mathie, S. A.; Lloyd, C. M. *Am. J. Respir. Crit. Care Med.* **2009**, *179*, 772–781.
(38) Nembrini, C.; Marsland, B. J.; Kopf, M. *J. Allergy Clin. Immunol.* **2009**, *123*, 986–994.
(39) Shiraishi, Y.; Asano, K.; Niimi, K.; Fukunaga, K.; Wakaki, M.; Kagyo, J.; Takihara, T.; Ueda, S.; Nakajima, T.; Oguma, T.; Suzuki, Y.; Shiomi, T.; Sayama, K.; Kagawa, S.; Ikeda, E.; Hirai, H.; Nagata, K.; Nakamura, M.; Miyasho, T.; Ishizaka, A. *J. Immunol.* **2008**, *180*, 541–549.
(40) Morcillo, E. J.; Cortijo, J. *Curr. Opin. Pulm. Med.* **2006**, *12*, 1–6.
(41) Kumar, A.; Takada, Y.; Boriek, A. M.; Aggarwal, B. B. *J. Mol. Med.* **2004**, *82*, 434–448.
(42) Rahman, M. S.; Yamasaki, A.; Yang, J.; Shan, L.; Halayko, A. J.; Gounni, A. S. *J. Immunol.* **2006**, *177*, 4064–4071.
(43) Liew, F. Y.; Pitman, N. I.; McInnes, I. B. *Nat. Rev. Immunol.* **2010**, *10*, 103–110.
(44) Justice, J. P.; Crosby, J.; Borchers, M. T.; Tomkinson, A.; Lee, J. J.; Lee, N. A. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2002**, *282*, L1066–L1074.
(45) Fujisawa, T.; Velichko, S.; Thai, P.; Hung, L. Y.; Huang, F.; Wu, R. *J. Immunol.* **2009**, *183*, 6236–6243.
(46) Birrell, M. A.; Hardaker, E.; Wong, S.; McCluskie, K.; Catley, M.; De Alba, J.; Newton, R.; Haj-Yahia, S.; Pun, K. T.; Watts, C. J.; Shaw, R. J.; Savage, T. J.l; Belvisi, M. G. *Am. J. Respir. Crit. Care Med.* **2005**, *172*, 962–971.
(47) Leigh, R.; Ellis, R.; Wattie, J. N.; Hirota, J. A.; Matthaei, K. I.; Foster, P. S.; O'Byrne, P. M.; Inman, M. D. *Am. J. Respir. Crit. Care Med.* **2004**, *169*, 860–867.
(48) Pushparaj, P. N.; Tay, H. K.; Ng, S. C.; Pitman, N.; Xu, D.; McKenzie, A.; Liew, F. Y.; Melendez, A. *J. Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 9773–9777.