



Suppressive effects of a pyrazole derivative of curcumin on airway inflammation and remodeling

Osamu Narumoto^{a,b}, Yukiko Matsuo^a, Masahiro Sakaguchi^c, Shunsuke Shoji^d, Naohide Yamashita^e, David Schubert^f, Kazuho Abe^g, Kazuhide Horiguchi^h, Takahide Nagase^b, Naomi Yamashita^{a,*}

^a Department of Pharmacotherapy, Research Institute of Pharmaceutical Sciences Musashino University, Tokyo, Japan

^b Department of Pulmonary Medicine, Faculty of Medicine University of Tokyo, Tokyo, Japan

^c Department of Veterinary Microbiology, School of Veterinary Medicine, Azabu University, Kanagawa, Japan

^d Division of Clinical Research, National Hospital Organization Tokyo National Hospital, Tokyo, Japan

^e Department of Advanced Medical Science, Institute of Medical Science University of Tokyo, Tokyo, Japan

^f The Salk Institute, Laboratories for Cellular Neurobiology, La Jolla, CA, USA

^g Department of Pharmacology, Research Institute of Pharmaceutical Sciences Musashino University, Tokyo, Japan

^h Department of Anatomy, Faculty of Medical Science, University of Fukui, Fukui, Japan

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ABSTRACT

To advance the control of airway epithelial cell function and asthma, we investigated the effects of a new curcumin derivative, CNB001, which possesses improved pharmacological properties. Normal human bronchial epithelial (NHBE) cells were stimulated with synthetic double-stranded RNA, Poly(I:C). CNB001 significantly suppressed IL-6, TNF- α , and GM-CSF production by NHBE cells, and did so more effectively than did curcumin or dexamethasone (DEX). CNB001 significantly inhibited the decrease of E-cadherin mRNA expression and increase of vimentin mRNA expression observed in NHBE cells induced by a combination of TGF- β 1 and TNF- α , which are markers of airway remodeling. In NHBE cells stimulated by TGF- β 1, CNB001 significantly downregulated the level of active serine peptidase inhibitor clade E member (SERPINE) 1, which is also reported to be related to airway remodeling. Whereas DEX alone significantly increased the active SERPINE1 level, the combination of DEX and CNB001 significantly suppressed active SERPINE1. In addition, CNB001 significantly suppressed neutrophil infiltration, IL-6, TNF- α , IL-13 and active SERPINE1 production in bronchoalveolar lavage fluid of the murine asthma model, which was not observed in the case of DEX. In conclusion, the curcumin derivative, CNB001, is a promising candidate to treat asthma associated with neutrophilic airway inflammation and remodeling.

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Introduction

Although various effector cells reside within asthmatic airways, airway epithelial cells actively involved in airway inflammation and remodeling, by producing inflammatory cytokines and influencing other cells (Holgate, 2010; Holgate et al., 2000; Lee et al., 2009). Airway epithelial cells are the first cells that encounter various stimulants in the airway and produce cytokines such as IL-6 and TNF- α ,

which are closely related to the pathophysiology of asthma (Ather et al., 2011; Takizawa et al., 1996; Vieira et al., 2011; Yadav et al., 2011). Viral infection of the respiratory tract is the most frequent factor involved in asthmatic exacerbations and may trigger neutrophilic inflammation in asthmatic airways (Fahy et al., 1995; Wark et al., 2002).

Airway remodeling, which is known to induce irreversible air-flow limitation, as in the case of chronic obstructive pulmonary disease is another problem to be solved (Gizycki et al., 1997). Airway remodeling is characterized by structural changes within the airway wall, including smooth muscle hypertrophy, basement membrane thickening, submucosal fibrosis, mucus cell metaplasia, epithelial shedding, and angiogenesis (Gizycki et al., 1997). Among the various factors involved in the pathogenesis of remodeling, we focused on serine peptidase inhibitor clade E member 1 (SERPINE1, also known as plasminogen activator inhibitor-1, PAI-1) and the process of epithelial-mesenchymal transition (EMT) in epithelial cells (Hackett et al., 2009; Kowal et al., 2008; Kucharewicz et al., 2003; Kuramoto et al., 2009; Miyamoto et al., 2011). SERPINE1 regulates

Abbreviations: NHBE cells, normal human bronchial epithelial cells; SERPINE1, serine peptidase inhibitor clade E member 1; EMT, epithelial-mesenchymal transitions; ECM, extracellular matrix; TNF, tumor necrosis factor; DEX, dexamethasone; Poly(I:C), polyinosinic:polycytidylic acid; GM-CSF, granulocyte-macrophage colony stimulating factor; BALF, bronchoalveolar lavage fluid; TLR3, Toll-like receptor 3; MAPK, mitogen-activated protein kinases; CUR, curcumin; OPM, OVA + Poly(I:C) + house dust mite.

* Corresponding author at: Department of Pharmacotherapy, Research Institute of Pharmaceutical Sciences, Musashino University, 1-1-20 Shinmachi Nishitokyo-shi, Tokyo 202-8585, Japan. Fax: +81 424 68 8647.

E-mail address: naoyama@musashino-u.ac.jp (N. Yamashita).

extracellular matrix (ECM) proteolysis (Kowal et al., 2008; Kucharewicz et al., 2003), and the attenuation of SERPINE1 has been reported to exhibit therapeutic properties for airway remodeling (Kuramoto et al., 2009; Miyamoto et al., 2011). Although EMT is a complex process involving various cells, the airway epithelial cells are one of the main targets related to airway remodeling (Hackett et al., 2009; Holgate et al., 2000; Lee et al., 2009).

Here, we explored the effects of a new curcumin derivative on epithelial cell function. Curcumin is a polyphenol derived from turmeric (*Curcuma longa*), a plant that has been reported to possess anticancer, antioxidant, and anti-inflammatory properties (Aggarwal and Harikumar, 2009; Fang et al., 2005; Ray et al., 2003; Santel et al., 2008; Weber et al., 2005; Wong et al., 2010). Although curcumin possesses good pharmacological properties, the utility of curcumin as a potential therapeutic drug is limited because of its poor bioavailability (Anand et al., 2007). Curcumin blocks multiple sites of transforming growth factor beta (TGF- β) signaling (Gaedeke et al., 2004), which plays a key role in the production of SERPINE1 (Dennler et al., 1998) and induction of EMT (Zavadiil and Bottinger, 2005). Therefore, we hypothesized that a new curcumin derivative might control not only airway inflammation but also EMT in asthmatic conditions that currently cannot be satisfactorily controlled by any other drugs, including steroids.

We used a curcumin derivative called CNB001, which is more stable and shows stronger cell-protecting effects than curcumin (Liu et al., 2008; Maher et al., 2010). We first compared the anti-inflammatory effect of CNB001 with that of dexamethasone (DEX) and curcumin. We then evaluated the effects of CNB001 on pathways that are thought to be involved in remodeling.

Materials and methods

Chemicals

Curcumin, dexamethasone (DEX), polyinosinic:polycystidylic acid (Poly(I:C)), human recombinant tumor necrosis factor (TNF)- α , and human TGF- β 1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). House dust mite allergen (HDM) derived from *Dermatophagoides farinae* (Derf) was provided by Dr. Shirai (Itea Inc., Tokyo, Japan), and contained 8 μ g/mg major allergen Derf1. Ovalbumin (OVA) was purchased from Sigma (St. Louis, MO, USA).

CNB001

CNB001 was synthesized as a hybrid molecule between cyclohexyl bisphenol A and curcumin, both of which have a proven neuroprotective effect (Liu et al., 2008). The purity of CNB001 was more than 98%. CNB001 is reported to be superior to curcumin in neuroprotective effects in cell-culture assays for trophic factor withdrawal, oxidative stress, and glucose starvation, as well as amyloid-induced toxicity (Liu et al., 2008).

Cell culture and treatment

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Walkersville, MD, USA). The cells were cultured in bronchial epithelial growth medium (BEGM) supplemented with BulletKit (Lonza Walkersville, Inc. MD, USA) on collagen type I-coated dishes at 37 °C in a 5% CO₂/95% air incubator. BulletKit contains human recombinant epidermal growth factor, hydrocortisone, transferrin, insulin, bovine pituitary extract, retinoic acid, triiodothyronine, gentamicin, and amphotericin (concentrations not stated). Cells were used on passage 3 for experiments pertaining to EMT, and on passages 4 to 6 for other experiments.

After the NHBE cells reached 80% to 100% confluency, the cells were incubated overnight in BEGM without BulletKit. Stock solutions

of CNB001, curcumin, and DEX (10 mmol/L) were prepared in ethanol and diluted to the appropriate concentration with culture medium. CNB001, curcumin, or DEX was added to the medium at the concentrations described in each experiment. One hour later, Poly(I:C) was added at a concentration of 10 μ mol/L. Cells and supernatants were collected 18 h after treatment of Poly(I:C). Preliminary experiments showed that CNB001 was not toxic at the dose of 10 μ mol/L. A trypan-blue exclusion test of viability showed no significant difference between the control and the CNB001 group (trypan-blue positive cells, 4.6% $\pm$ 0.6%, 3.2% $\pm$ 1.7%, respectively, P > 0.05 NS, n = 6). Initial control experiments included solvent (ethanol) at doses of up to 1 μ L/mL, which did not significantly affect cell viability or production of cytokines.

To induce EMT, cells were grown on 10-cm dishes until 80% to 100% confluency. They were then incubated with TGF- β 1 (5 ng/mL) with or without TNF- α (10 ng/mL).

Real-time PCR and immunoassay

Total RNA was extracted by using Trizol Reagent (Invitrogen Corporation, Carlsbad, CA, USA) and then reverse transcribed by using a cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). A Light Cycler-Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) was used for real-time PCR. Primers are listed in Table 1.

Interleukin-6 (IL-6), granulocyte-macrophage colony stimulating factor (GM-CSF), and TNF- α levels were measured by using a Quantikine Immunoassay kit (R&D systems, Minneapolis, MN, USA). Active human SERPINE1 (PAI-1) was measured by using a specific ELISA Quantifying kit (Molecular Innovations, MI, USA).

Animals

Specific-pathogen-free female BALB/c mice (8 weeks old) were purchased from SLC (Shizuoka, Japan). The animals were housed in a specific pathogen-free environment and allowed free access to food and water. All experimental protocols were approved by the Musashino University Animal Care Committee and were in accordance with the NIH Guide for the care and use of laboratory animals. Mice were initially intraperitoneally immunized with 10 μ g OVA and 2 mg aluminum hydroxide gel on days 0 and 14, as previously reported (Matsuo et al., 2009; Yamashita et al., 2006). From day 16 onwards, the animals were treated every other day for 2 weeks as follows. Mice were intraperitoneally injected with 25 mg/kg CNB001, 25 mg/kg curcumin, 1 mg/kg DEX, or solvent alone. CNB001 and curcumin were dissolved in 100% ethanol (40 μ L for 1 mg) and then diluted 10 fold with 1% (v/v) Tween80/Saline (Sigma). DEX was dissolved in 100% ethanol to a concentration of 0.1% w/v and then diluted 10 fold with 1% Tween80/Saline. Thirty minutes after intraperitoneal injection, the mice were subjected to inhalation of OVA, followed by intranasal administration of Poly(I:C) (500 μ g/kg) and HDM (8 μ g/kg) dissolved in 80 μ L of saline. Saline alone was used for control mice. Inhalation of OVA (20 mg/mL) was conducted at a

Table 1
Primers used for real-time PCR.

Target gene	Primer sequences	Amplicon size (bp)
GAPDH	Forward 5'-TGACCACCAACTGCTTAGC-3'	87
	Reverse 5'-GGCATGGACTGTGGTCATGAG-3'	
E-cadherin	Forward 5'-TGAAGGTGACAGAGCCTCTGGAT-3'	151
	Reverse 5'-TGGGTGAATTCGGGCTTGTT-3'	
Vimentin	Forward 5'-CCTTGAACGCAAAGTGAATC-3'	106
	Reverse 5'-CTGAGTTTCCGAATAGCCTG-3'	

flow rate of 0.3 mL/min for 10 min by using a micromist nebulizer (Devilbiss, Somerset, PA, USA).

Twenty-four hours after the last treatment, the mice were sacrificed and bronchoalveolar lavage fluid (BALF) was obtained by instilling and aspirating 3 times with 1 mL aliquots of PBS (recovery >85%). BALF was centrifuged at 540 g for 10 min (at 4 °C) and the supernatants were stored at –80 °C for ELISA. The cell pellets were resuspended in 1 mL PBS. After total BALF cell counts were obtained, the resuspended cells were adhered onto glass slides at 640 rpm for 2 min by using a Shandon Cytospin 4 (Thermo Electron Corp., Waltham, MA, USA). The BALF cells on glass slides were stained with Diff-Quik (International Reagents Corp, Osaka, Japan) and were differentially counted at 200 cells per slide (one slide per animal).

For histological examination, mice were perfused transcardially with PBS and then 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4). The lungs were removed and then immersed in 4% paraformaldehyde. After washing with PBS, the specimens were soaked overnight in 30% sucrose in PBS, embedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, IN, USA), and then quickly frozen. Cryostat sections were cut and thaw-mounted on poly-L-lysine-coated glass slides (Matsunami Glass, Osaka, Japan). The samples were analyzed by haematoxylin–eosin (HE) staining.

Concentrations of IL-6, TNF- α , IL-13, and active murine SERPINE1 (PAI-1) in BALF were measured by using a specific ELISA Quantifying kit (R&D systems, MN, USA and Molecular Innovations, MI, USA, respectively) according to the manufacturers' instructions.

Statistical analysis

The significance of the difference between groups was assessed by using the Wilcoxon/Kruskal–Wallis nonparametric test as implemented in JMP8.0.2 (SAS Institute, Cary, NC, USA). The test was conducted as two sided. *P* values less than 0.05 were considered to be significant. The data were expressed as mean $\pm$ SEM.

Results

Comparing the effects of CNB001, curcumin, and DEX on Poly(I:C)-induced expression of inflammatory cytokines

Poly(I:C), a synthetic analog of viral double-stranded RNA and a Toll-like receptor (TLR) 3 ligand, acts as a potent proinflammatory stimulus for lung epithelial cells by inducing the secretion of cytokines, which are important for allergic airway inflammation (Bachar et al., 2004; Groskreutz et al., 2006). As shown in Fig. 1A, and later in Fig. 2, stimulation of NHBE cells with 10 μ g/mL Poly(I:C) caused a significant increase in expression of inflammatory cytokines, IL-6, GM-CSF, and TNF- α . We first tested whether CNB001, DEX, or curcumin could suppress the expression of inflammatory cytokines. Pretreatment with either CNB001 or curcumin for 1 h significantly blocked the expression of IL-6 in a dose-dependent manner (Figs. 1A, B). DEX significantly inhibited the expression of IL-6 in a dose-dependent manner up to a concentration of 0.1 μ mol/L; however, higher concentrations of DEX did not further reduce the expression of IL-6 (Fig. 1C). The maximum suppression was 94% at the dose of 10 μ mol/L for CNB001, 88% at the dose of 10 μ mol/L for curcumin, and 73% at the dose of 0.1 μ mol/L for DEX. In all further experiments, we used CNB001 and curcumin at a concentration of 10 μ mol/L, and DEX at a concentration of 0.1 μ mol/L.

We next compared the anti-inflammatory effects of CNB001 with those of DEX and curcumin. CNB001 inhibited Poly(I:C)-induced production of IL-6, GM-CSF, and TNF- α to a significantly greater extent than did DEX or curcumin (Fig. 2). Addition of CNB001 to DEX suppressed inflammatory cytokines significantly more effectively than did DEX alone; however, the levels of inflammatory cytokines observed were almost the same as those obtained with CNB001 alone.

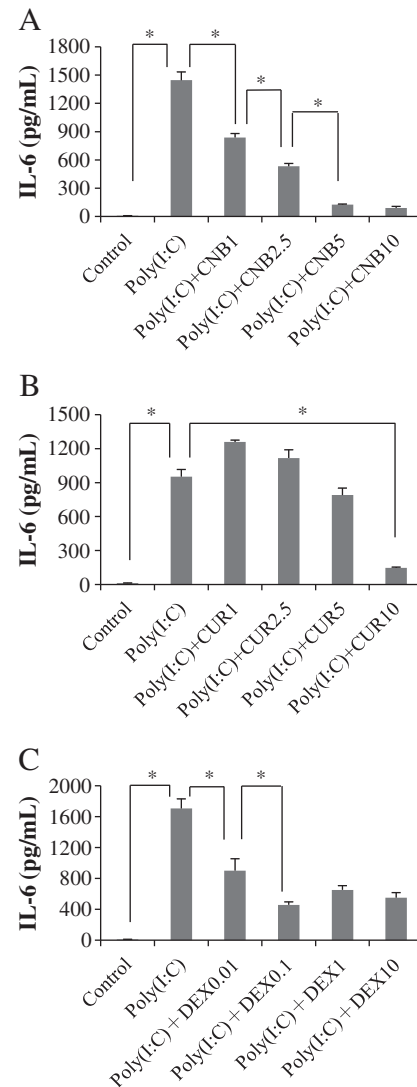


Fig. 1. Dose-dependent effects of CNB001, dexamethasone, and curcumin on Poly(I:C)-induced expression of IL-6. NHBE cells were treated with CNB001 (panel A), curcumin (panel B), or dexamethasone (DEX) (panel C) at the concentrations indicated for 1 h prior to stimulation by Poly(I:C). Cell supernatants and cells were harvested 18 h after the addition of Poly(I:C). Definition of abbreviations: CNB1, 1 μ mol/L CNB001; CNB2.5, 2.5 μ mol/L CNB001; CNB5, 5 μ mol/L CNB001; CNB10, 10 μ mol/L CNB001; CUR1, 1 μ mol/L curcumin; CUR2.5, 2.5 μ mol/L curcumin; CUR5, 5 μ mol/L curcumin; CUR10, 10 μ mol/L curcumin; DEX0.01, 0.01 μ mol/L DEX; DEX0.1, 0.1 μ mol/L DEX; DEX1, 1 μ mol/L DEX; DEX10, 10 μ mol/L DEX. Data represent means $\pm$ SD, **P* < 0.05, *n* = 3 for each group. Similar experiments were conducted 3 times, and one representative experiment is shown.

CNB001 reduces EMT induced by TGF- β 1 plus TNF- α

Next, we examined the effect of CNB001 on EMT, which is related to airway remodeling. TGF- β 1 is the best-known inducer of EMT in airway epithelial cells, and induction of EMT is enhanced by combining TGF- β 1 with TNF- α (Doerner and Zuraw, 2009; Takahashi et al., 2010). To examine the suppressive effects of CNB001, we used TGF- β 1 and TNF- α together as stimulants to induce EMT in NHBE cells. We examined the expression of E-cadherin and vimentin, which are markers of EMT (Zavadil and Bottinger, 2005). After 72 h, the cells were harvested, and the expression levels of E-cadherin and vimentin were analyzed by performing real-time PCR. Co-stimulation by TGF- β 1 and TNF- α significantly decreased the expression of E-cadherin and significantly increased the expression of vimentin (Fig. 3). Treatment with CNB001 significantly suppressed the decrease in E-cadherin expression and the increase of vimentin

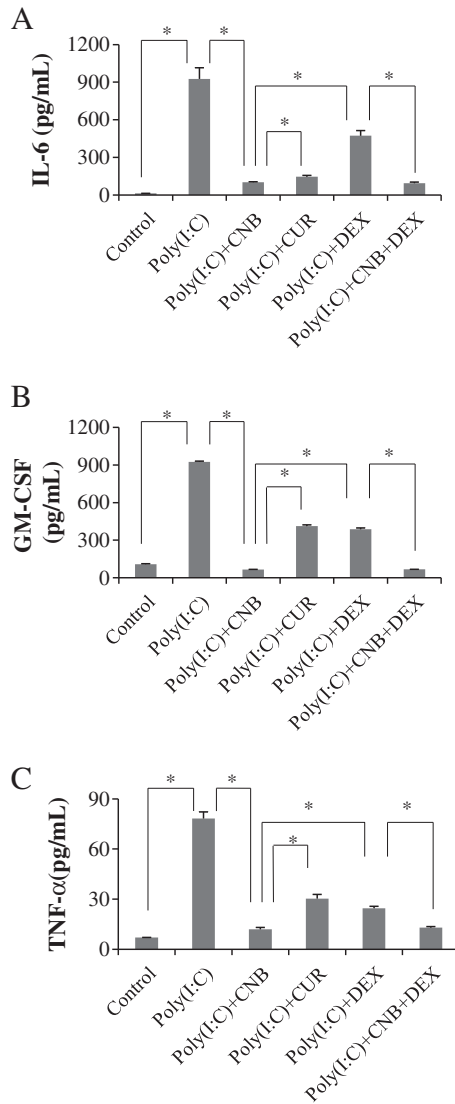


Fig. 2. Anti-inflammatory effects of CNB001, curcumin, and dexamethasone. NHBE cells were treated with CNB001, curcumin, or dexamethasone (DEX) for 1 h prior to stimulation by Poly(I:C). Control cells were not treated or stimulated. Eighteen hours after the addition of Poly(I:C), cell supernatants and cells were harvested. Protein expression of IL-6 (panel A), GM-CSF (panel B), and TNF-α (panel C) was compared between cell supernatants obtained from control cells, Poly(I:C)-stimulated cells, and Poly(I:C)-stimulated cells treated with CNB001, curcumin, DEX, or CNB plus DEX. Definition of abbreviations: CNB, 10 μmol/L CNB001; DEX, 0.1 μmol/L dexamethasone; CUR, 10 μmol/L curcumin. Data represent means ± SD, **P* < 0.05, *n* = 3 for each group. Similar experiments were conducted 3 times, and one representative experiment is shown.

expression, suggesting that CNB001 efficiently suppresses EMT. In contrast, DEX caused no significant change either in E-cadherin or vimentin expression.

CNB001 inhibits active SERPINE1 synthesis

We next evaluated active SERPINE1 (PAI-1), which plays an important role in airway remodeling by regulating ECM proteolysis. SERPINE1 acts directly through plasmin formation and indirectly through plasmin-mediated activation of matrix metalloproteinases (Kowal et al., 2008; Kucharewicz et al., 2003). For the stimulant, we used TGF-β1 (2 ng/mL), which is an inducer of SERPINE1 and is related to airway remodeling (Dennler et al., 1998; Kowal et al., 2008; Miyamoto et al., 2011). Both CNB001 and curcumin suppressed TGF-β1-induced active SERPINE1 (Fig. 4). CNB001 showed a significantly stronger effect than that of curcumin. In contrast to CNB001, DEX significantly augmented

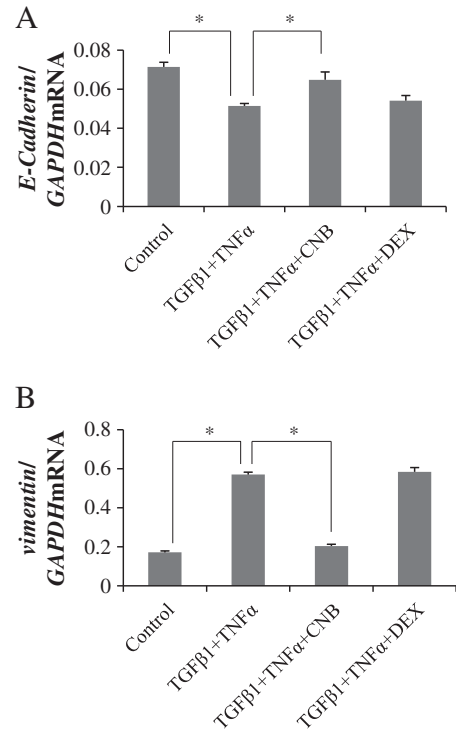


Fig. 3. Effects of CNB001 and dexamethasone on the expression of E-cadherin and vimentin. EMT was induced in NHBE cells by 72-h treatment with TGF-β1 (2 ng/mL) plus TNF-α (4 ng/mL). The cells were either treated with TGF-β1 plus TNF-α, or TGF-β1 plus TNF-α in combination with CNB001 (10 μmol/L) or dexamethasone (DEX; 0.1 μmol/L). At the end of the induction period, the E-cadherin (panel A) and vimentin (panel B) mRNA expression levels in the cells were measured and normalized to levels of GAPDH mRNA. Data represent means ± SD, **P* < 0.05, *n* = 3 for each group. Similar experiments were conducted 3 times, and one representative experiment is shown.

the production of active SERPINE1. When CNB001 was added to the DEX, the induction of active SERPINE1 was significantly and greatly suppressed (Fig. 4).

Comparing the *in vivo* effects of CNB001, DEX, and curcumin by using a murine model of asthma

We next evaluated the effect of CNB001 on the prevention of airway inflammation *in vivo*. An OVA-specific IgE induced mouse model has been commonly used to examine allergic inflammation, which can be efficiently suppressed by treatment of the model with DEX alone (Brusselle et al., 1995; Chi et al., 2011; Wills-Karp et al., 1998). Intra-tracheal administration of HDM, which is a common allergen in humans, is recently shown to be a new asthmatic model

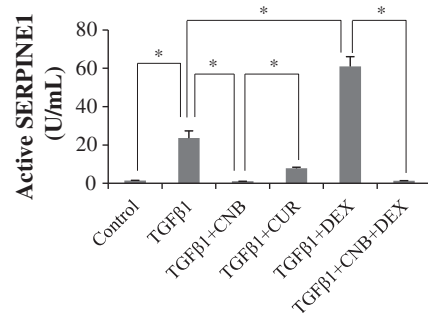


Fig. 4. Effect of CNB001 and dexamethasone on levels of active human SERPINE1. NHBE cells were treated with CNB001 (10 μmol/L) or dexamethasone (DEX; 0.1 μmol/L) for 1 h prior to stimulation by TGF-β1 (2 ng/mL). **P* < 0.05, *n* = 3 for each group. Similar experiments were conducted 3 times, and one representative experiment is shown.

without systemic immunization (Fattouh et al., 2005; Gough et al., 2003; Phipps et al., 2009; Trompette et al., 2009). In addition, combining Poly(I:C) stimulation with the OVA-specific IgE induced model represents exacerbation of asthma induced by respiratory viral infections and induced neutrophil accumulation in the BALF (Kim et al., 2007; Takayama et al., 2011). To examine the effects of various treatments on the control of severe airway inflammation, we used the mouse model exposed to OVA plus HDM plus Poly(I:C).

In these mice, the total cell counts in BALF, especially neutrophil counts, were significantly suppressed by CNB001 treatment (Figs. 5A, B), but not by curcumin treatment. In addition, adding CNB001 to DEX significantly increased the suppressive effects compared with those of DEX alone. Eosinophil counts were not significantly suppressed by CNB001 alone, but were significantly suppressed when CNB001 was used in combination with DEX (Fig. 5C). Histological

analysis also demonstrated that CNB001 suppressed airway inflammation beneath airway epithelial cells (Fig. 6C).

We then measured cytokine production in the BALF of the mouse model exposed to OVA plus HDM plus Poly(I:C). CNB001 significantly suppressed IL-6, TNF- α , and IL-13 expression in these mice (Figs. 7A–C). We chose IL-6 and TNF- α in order to compare effects of CNB001 *in vivo* with that *in vitro*. We also examined IL-13 to analyze the effects of CNB001 on Th2 allergic inflammation *in vivo*. Furthermore, the concentration of active murine SERPINE1 in BALF was significantly suppressed by CNB001, and significantly enhanced by DEX (Fig. 7D). Addition of CNB001 plus DEX tended to suppress the level of active SERPINE1 when compared with that observed after addition of DEX alone, though the difference was not statistically significant (Fig. 7D).

Discussion

We studied the effects of a new curcumin derivative, CNB001, as a therapeutic agent for airway inflammation and remodeling. By using epithelial cells stimulated by Poly(I:C) *in vitro*, we showed that CNB001 suppressed the cytokines IL-6, TNF- α , and GM-CSF, which are produced by epithelial cells during the inflammation and play important roles in the pathogenesis of asthma (Doherty and Croft, 2011; Morjaria et al., 2011; Saha et al., 2009). Moreover, CNB001 suppressed these cytokines more effectively than did DEX or curcumin. Our *in vivo* study of a mouse model of asthma showed that CNB001 suppressed IL-6, TNF- α , and IL-13 production, and reduced the number of inflammatory cells, especially neutrophils, in BALF. Furthermore, CNB001 prevented EMT *in vitro*, and suppressed the activity of SERPINE1, a protein that is associated with airway remodeling both *in vitro* and *in vivo*. In addition, combining CNB001 with DEX suppressed the activity of SERPINE1, which was augmented by DEX alone. Our results showed the promising effects of CNB001, in combination with DEX, on airway inflammation and remodeling.

The anti-inflammatory effects of curcumin are mediated through inhibition of the activation of NF- κ B and p38MAPK (mitogen-activated protein kinases) (Camacho-Barquero et al., 2007; Jang et al., 2007; Song et al., 2010; Sugimoto et al., 2002; Venugopal et al., 2007). Curcumin inhibits the phosphorylation of I- κ B (inhibitor of NF- κ B) and prevents translocation of NF- κ B into the nucleus (Oh et al., 2011; Song et al., 2010). NF- κ B is activated by Poly(I:C), which we used here for stimulation. NF- κ B plays a central role in the production of inflammatory cytokines such as TNF- α , IL-6, and IL-8 (Bruewer et al., 2003; Wong and Tergaonkar, 2009). In our *in vitro* and *in vivo* study, CNB001 suppressed the synthesis of inflammatory cytokines to a significantly greater extent than did curcumin. By using the *in vivo* model, we also showed significant inhibition of IL-13, which is a critical cytokine for asthma pathogenesis (Wills-Karp et al., 1998).

Airway remodeling, which is a cause of decreased respiratory function in asthma and cannot be effectively treated by standard therapies, has become one of the major targets of therapies for asthma. In airway remodeling, epithelial cells produce inflammatory cytokines that induce smooth muscle cell hyperplasia and an increase in activated fibroblasts and myofibroblasts that deposit excessive extracellular matrix (Skold, 2010; Westergren-Thorsson et al., 2010). Recent evidence suggests that epithelial cells play an active role in initiating this process (Hackett et al., 2009; Holgate et al., 2000; Willis et al., 2006). Because there is no generally accepted methods to define airway remodeling yet, the evidence of reduced EMT is acceptable to explain the prevent airway remodeling, we examined the effect of CNB001 on EMT. Loss of E-cadherin defines epithelial de-differentiation, and an increase in vimentin represents differentiation to mesenchymal cells, which represents EMT (Willis et al., 2006; Zavadil and Bottinger, 2005). EMT can be induced by TGF- β 1 and enhanced by TNF- α , IL-1 β , and fibronectins (Dennler et al., 1998; Takahashi et al., 2010). Here, we confirmed the loss of E-cadherin expression and the increase in vimentin expression in epithelial cells

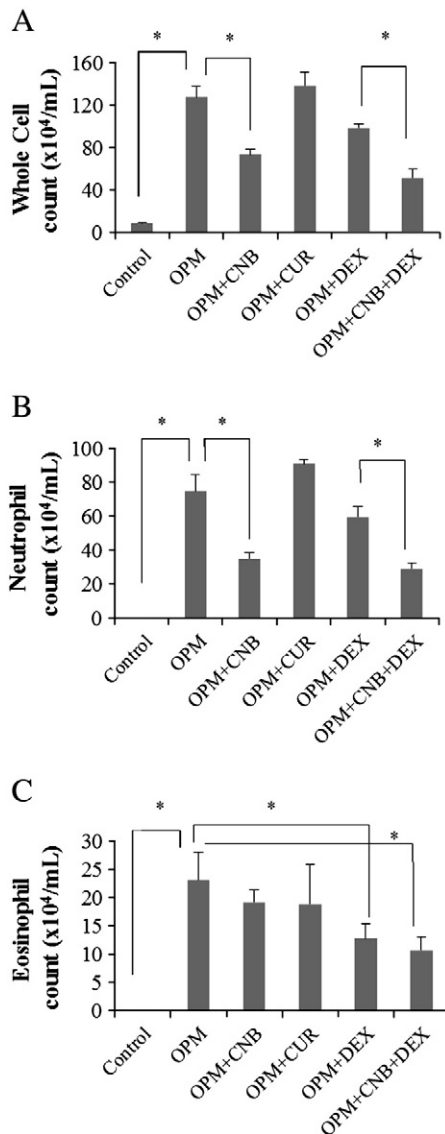


Fig. 5. *In vivo* effect of CNB001, curcumin, and dexamethasone on blood cell counts in bronchoalveolar lavage fluid of a murine asthmatic model. OVA-sensitized BALB/c mice were intraperitoneally injected with 25 mg/kg CNB001 (CNB), 25 mg/kg curcumin (CUR), 1 mg/kg dexamethasone (DEX), or solvent alone. Thirty minutes after, the mice were intranasally administered house dust mite and Poly(I:C), and OVA-immunized by inhalation of ovalbumin (OVA) (indicated as OPM in the figure). Control mice were treated with saline alone. Total cell numbers (A), neutrophil (B) and eosinophil numbers (C) in bronchoalveolar lavage fluid are shown. Data represent means $\pm$ SD, * P < 0.05, n = 7–8 for each group.

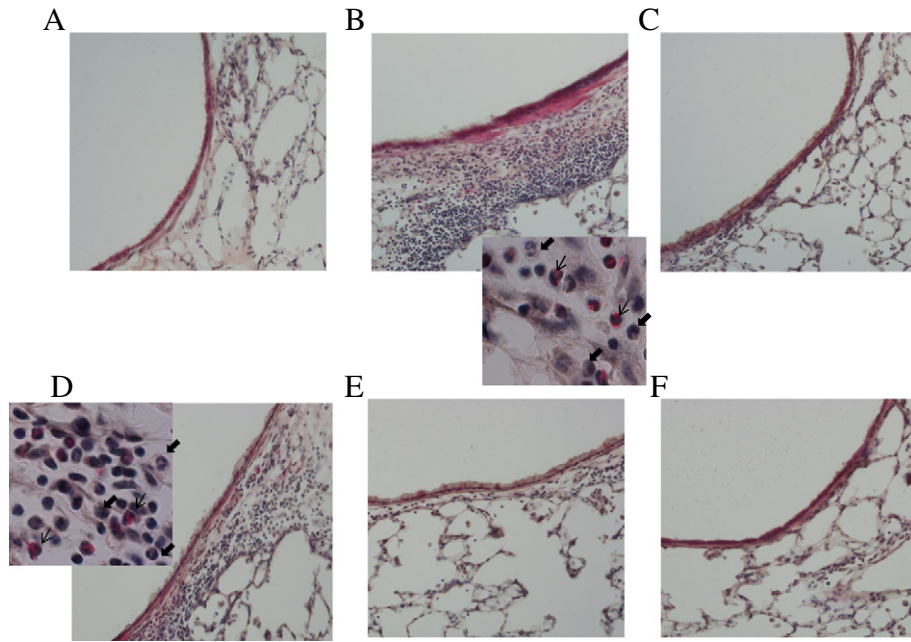


Fig. 6. Histological analysis by hematoxylin and eosin staining. OVA-sensitized BALB/c mice, treated as described in the legend of Fig. 5, were subjected to histological examination. The mice were perfused transcardially with PBS and then 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4). A, control; B, OVA + Poly(I:C) + house dust mite (OPM); C, OPM plus CNB001; D, OPM plus curcumin; E, OPM plus dexamethasone (DEX); F, OPM plus CNB001 plus DEX. → eosinophils ◆ neutrophils (arrows indicated some of typical cells, original magnification $\times 1000$).

treated with TGF- β 1 and TNF- α *in vitro*, suggesting the occurrence of EMT.

We also focused on the production of active SERPINE1, which is reported to play an essential role in tissue remodeling of lung, kidney, and cardiovascular systems after inflammation (Ha et al., 2009; Huang et al., 2008; Kuramoto et al., 2009; Shetty et al., 2008; Sisson et al., 2009). Kuramoto et al. reported increased expression of SERPINE1 in

BALF of murine asthma models compared with that of healthy mice (Kuramoto et al., 2009). In addition, they showed that inhalation of urokinase-type plasminogen activator reduces the level of active SERPINE1 and suppresses airway remodeling in these mice (Kuramoto et al., 2009). Miyamoto et al. report that airway remodeling is suppressed in *Serpine1*-deficient mice compared to wild-type mice, and following intra-airway administration of *Serpine1*

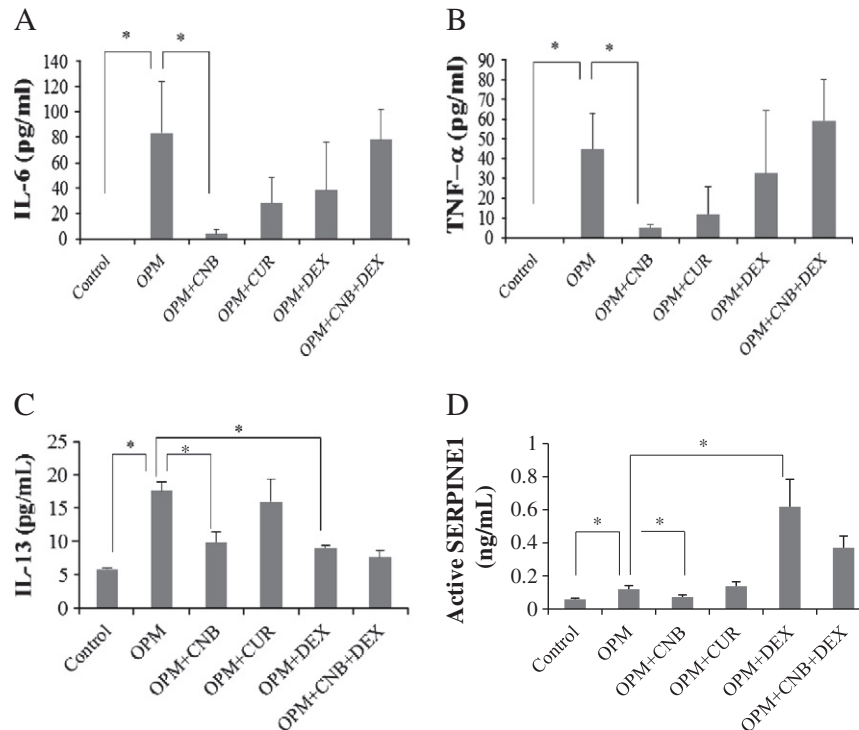


Fig. 7. *In vivo* effect of CNB001, curcumin, and dexamethasone on cytokine synthesis in a murine asthma model. IL-6 (A), TNF- α (B), IL-13 (C), and active murine SERPINE1 (D) levels were measured in bronchoalveolar lavage fluid obtained from OVA-sensitized BALB/c mice that were treated as described in the legend of Fig. 5. OPM indicates OVA + Poly(I:C) + house dust mite. Data represent means $\pm$ SD, * $P < 0.05$, $n = 5-6$ for each group.

siRNA in wild-type mice (Miyamoto et al., 2011). Although DEX possesses strong anti-inflammatory effects, DEX and its receptor complex promote the expression of *Serpine1* by affecting the promoter region of the *Serpine1* gene (Kimura et al., 2009; Wickert et al., 2007; Yamamoto et al., 2004). This may be one of the reasons that glucocorticoids cannot prevent airway remodeling (Kuramoto et al., 2009). Here, we observed that DEX increased the level of active SERPINE1 both *in vivo* and *in vitro*, but, when combined with CNB001, it suppressed the level of active SERPINE1. These results suggest that a combination of glucocorticoids and CNB001 might prevent increased expression of SERPINE1, and thereby suppress airway remodeling.

Our results suggest that a new curcumin derivative, CNB001, could potentially be used for controlling the function of airway epithelial cells by reducing inflammation and airway remodeling in asthma. We focused on epithelial cells in this study, and further studies are needed to determine the effects of this derivative on other cell types involved in EMT and airway remodeling.

Conflict of interest statement

No conflicts of interests are declared by the authors.

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