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Antiviral and Anti-Inflammatory Activities of Pochonin D, a Heat Shock Protein 90 Inhibitor, against Rhinovirus Infection

Jae-Hyoung Song[1,†], Aeri Shim[1,†], Yeon-Jeong Kim[2], Jae-Hee Ahn[1], Bo-Eun Kwon[1], Thuy Trang Pham[1], Jongkook Lee[1], Sun-Young Chang[3] and Hyun-Jeong Ko[1,*]

[1]*College of Pharmacy, Kangwon National University, Chuncheon 24341,*
[2]*College of Pharmacy, Inje University, Gimhae 50834,*
[3]*College of Pharmacy, Ajou University, Suwon 16499, Republic of Korea*

Abstract

Human rhinoviruses (HRV) are one of the major causes of common cold in humans and are also associated with acute asthma and bronchial illness. Heat-shock protein 90 (Hsp90), a molecular chaperone, is an important host factor for the replication of single-strand RNA viruses. In the current study, we examined the effect of the Hsp90 inhibitor pochonin D, *in vitro* and *in vivo*, using a murine model of human rhinovirus type 1B (HRV1B) infection. Our data suggested that Hsp90 inhibition significantly reduced the inflammatory cytokine production and lung damage caused by HRV1B infection. The viral titer was significantly lowered in HRV1B-infected lungs and in Hela cells upon treatment with pochonin D. Infiltration of innate immune cells including granulocytes and monocytes was also reduced in the bronchoalveolar lavage (BAL) by pochonin D treatment after HRV1B infection. Histological analysis of the lung and respiratory tract showed that pochonin D protected the mice from HRV1B infection. Collectively, our results suggest that the Hsp90 inhibitor, pochonin D, could be an attractive antiviral therapeutic for treating HRV infection.

Key Words: Rrhinovirus, Antiviral activity, Pochonin D, Heat-shock protein 90, Anti-inflammatory

INTRODUCTION

Human rhinoviruses (HRV) are positive single-stranded RNA viruses belonging to the family *Piconaviridae*. HRV infection in humans usually causes common cold and mild illnesses, but is sometimes associated with asthma exacerbation and viral upper respiratory tract infection (Bartlett *et al*., 2008). HRVs are divided into three distinct species including type A, type B, and type C, with over 100 immunologically non-cross reactive HRV serotypes (Park *et al*., 2012). Among the HRV serotypes, the majority accounting for 90%, use human intercellular adhesion molecule-1 (ICAM-1) as their cellular receptor and do not bind mouse ICAM-1, whereas the remaining 10% of the minor serotypes including HRV1B use a member of the low-density lipoprotein (LDL-1) receptor family and can bind the mouse counterpart (Bartlett *et al*., 2008). Thus, HRV1B is thought to be a suitable model for murine infection.

Despite showing the highest incidence and occasional complications of chronic bronchitis as well as reactive airway disease exacerbation, there is no approved medication for the treatment of rhinovirus infection. Many efforts have been made to produce vaccines to prevent rhinovirus infection, but it is very difficult to produce appropriate ones because of the existence of more than 100 immunologically non-cross-reactive rhinovirus serotypes (Ledford *et al*., 2005). Therefore, studies concentrating on the development of effective antiviral agents for treating rhinovirus infections are now underway (al-Nakib and Tyrrell, 1992).

Hsp90, a 90 kDa heat shock protein, is a highly abundant, essential, and evolutionarily conserved molecular chaperone at the center of a large protein-folding network (Geller *et al*., 2013). There are two cytoplasmic isoforms of Hsp90 in mammals. Hsp90α is an inducible isoform, whereas Hsp90β is expressed constitutively. Hsp90 function is regulated by a cohort of co-chaperones that modulate its ATPase cycle, enabling it to acquire and select client proteins, and to provide a link with other chaperone systems including proteasome degradation systems for protein degradation (Geller *et al*., 2012).

Hsp90 inhibitors are known to have broad-spectrum anti-

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***Corresponding Author**
E-mail: hjko@kangwon.ac.kr
Tel: +82-33-250-6923, Fax: +82-33-255-7865
[†]The first two authors contributed equally to this work.

cancer effects via blocking diverse pathways in cancer cells, and they also inhibit the growth and survival of cancer stem cells (Li *et al*., 2009). In addition, a recent study has shown that Hsp90 is practically required for viral protein homeostasis and is critical for viral replication, folding, and assembly (Nagy *et al*., 2011). Hsp90 has been shown to play an important role in the replication of various viruses including DNA and RNA viruses, with both positive- and negative-sense genomes, and also double-stranded RNA viruses, suggesting that Hsp90 inhibitors may have broad-spectrum antiviral effects (Geller *et al*., 2012).

In this regard, several Hsp90 inhibitors have been studied for the development of antivirals, *in vitro*, against influenza, SARS-CoV, HCV, HIV (Li *et al*., 2004), and herpes viruses (HSV1/2, CMV, VZV) (Sun *et al*., 2013), as well as against picornaviruses including poliovirus, coxsackievirus, and rhinovirus (Geller *et al*., 2012). Hsp90 inhibitors are very attractive antiviral agents for infections lacking antiviral therapies and for an urgent response to the outbreak of novel viral diseases. In addition, application of Hsp90 inhibitors to several animal models of infectious diseases was demonstrated to decrease viral replication in case of Poliovirus and HCV infections (Geller *et al*., 2007; Nakagawa *et al*., 2007). These experiments emphasize the possibility of using these inhibitors as human therapeutic agents.

Several inhibitors of Hsp90 are known and their potential as therapeutic drugs have been tested at both preclinical and clinical stages (Neckers and Workman, 2012). Radicicol is a natural product and is reported as one of the most potent Hsp90 inhibitors (Moulin *et al*., 2005). It can selectively block the function of ATPase and the chaperone function of Hsp90 (Roe *et al*., 1999; Zhou *et al*., 2010). Despite its potent activity, radicicol has labile moieties that are rapidly inactivated *in vivo*, leading to poor efficacy in practice (Zhou *et al*., 2010). Pochonin D was originally isolated from *Pochonia chlamydosporia* and is a radicicol analog with potential Hsp90 inhibitory ability (Moulin *et al*., 2005; Wang *et al*., 2016; Choe *et al*., 2017). It has been shown to inhibit the replication of Herpes Simplex Virus 1 (HSV1) and the parasitic protozoan *Eimeria tenella* (Hellwig *et al*., 2003).

Collectively, we assessed the anti-rhinoviral activity of pochonin D both *in vitro* and *in vivo*. We found that pochonin D has a significant antiviral effect against HRV1B by inhibiting virus replication and attenuating HRV1B infection-associated lung inflammation.

MATERIALS AND METHODS

Reagents

Pochonin D was synthesized and purified as recently reported (Choe *et al*., 2017). Ribavirin and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Viruses and cell lines

HRV1B, HRV14, and HRV15 viruses were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA), and were cultured at 32°C in Hela cells, a human cervical cancer cell line (Song *et al*., 2013). Hela cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. MEM, FBS, trypsin-EDTA, and antibiotic-antimycotic solution were purchased from Gibco BRL (Thermo Fisher Scientific, Waltham, MA, USA).

Mice and virus infection

Wild type (WT) BALB/c mice were purchased from SPL laboratory animal company (Koatech, Pyeong-taek, Korea). All mice used in these experiments were between 4 and 5 weeks of age. Mice were intranasally infected with 1×10^8 pfu/30 μl of HRV1B. Mice were maintained in an experimental facility at the Kangwon National University. The animal experiments were approved by the Institutional Animal Care and Use Committees of Kangwon National University.

Antiviral activity assay

Antiviral activity was assessed by the SRB method using cytopathic effect (CPE) reduction as reported previously (Song *et al*., 2014). Briefly, one day prior to infection, Hela cells (2×10^4 cells/well) were seeded onto a 96-well culture plate (BD biosciences, San Jose, CA, USA). On the next day, medium was replaced with medium containing 30 mM of $MgCl_2$, 1% FBS, diluted virus suspension containing a 50% cell culture infective dose ($CCID_{50}$) of the virus, and an appropriate concentration of the test compounds. The culture plates were incubated at 32°C in 5% CO_2 for 2 days until the appropriate CPE was achieved. After incubation in ice-cold 70% acetone for 30 min, cells were stained with 0.4% (w/v) SRB in 1% acetic acid solution. Cell morphology was observed using an Axiovert microscope (Axiovert 10; Carl Zeiss, Oberkochen, Germany) to examine the effect of the compounds on HRV-induced CPE. Bound SRB was then solubilized with 10 mM unbuffered Tris-based solution, and absorbance was read at 562 nm using a VERSAmax microplate reader (Molecular Devices, Palo Alto, CA, USA) with reference absorbance measured at 620 nm. The percentage of cell viability was calculated for comparison based on the measured absorbance. In addition, the cell morphology was observed under a microscope at 32×10 magnification (St Ernst-Leitz, Wetzlar, Germany), and images were recorded.

Cytokine and chemokine assay

We performed ELISA for assessing the cytokine and chemokine levels. ELISA kits for TNF-α, IL-1β, and CCL2 (MCP1) were purchased from e-Bioscience, and the ELISA kit for CXCL1 was purchased from R&D Systems. Bronchoalveolar lavage fluid (BALF) was obtained as described previously (Seo *et al*., 2010). Lungs from mice infected with HRV1B were dissected and homogenized. The levels of cytokines and chemokines in the lung homogenates were evaluated according to the manufacturer's instructions (Seo *et al*., 2011). Absorbance was then read at 450 nm using SPECTRA MAX 340 (Molecular Devices).

Real time PCR

Total RNA was isolated from each group using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using RNase Inhibitor, M-MLV RT 5× Buffer, M-MLV Reverse Transcriptase, Oligo(dT)15 primer, and dNTP mixture manufactured by Promega (Madison, WI, USA), and quantitative real-time PCR was performed using the THUNDERBIRD™ SYBR qPCR Mix (Toyobo, Osaka, Japan) on the CFX96™Optics Module (Bio-Rad, Hercules, CA,

USA), with the following primers: HRV5′-NCR-up: 5′-TCC TCC GGC CCC TGA ATG-3′, HRV5′-NCR-down: 5′-GAA ACA CGG ACA CCC AAA G-3′, CCL2-up: 5′-TTA AAA ACC TGG ATC GGA ACC AA-3′, CCL2-down: 5′- GCA TTA GCT TCA GAT TTA CGG GT-3′, IL-6 up: 5′-CTG GAG TCA CAG AAG GAG TGG-3′, IL-6 down: 5′- GGT TTG CCG AGT AGA TCT CAA-3′, CXCL1 up: 5′-TGA GCT GCG CTG TCA GTG CC-3′, CXCL1 down: 5′-GCG TTC ACC AGA CGG TGC CA-3′, TNF-α up: 5′-TGG GAG TAG ACA AGG TAC AAC CC-3′, and TNF-α down: 5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′, IL-1β up: 5′-GTT GAC GGA CCC CAA-3′, IL-1β down: 5′-AAG ATA AGG TCC ACG GGA AAG ACA C-3′, β-actin up: 5′-CCA TCA TGA AGT GTG ACG TGG-3′, and β-actin down: 5′-GTC GCC TAG AAG CAT TTG CG-3.

Flow cytometry

Cells from the bronchial alveolar lavage fluid (BALF) in the lungs were collected and stained with the following antibodies: fluorescein isothiocyanate conjugated anti-CD11b, phycoerythrin: Cy-7 conjugated anti-Ly6G, allophycocyanin conjugated anti-Ly6C, and phycoerythrin conjugated anti-F4/80. All antibodies used for flow cytometric analysis were purchased from BD Biosciences. The data were acquired on a FACS Verse system (BD Bioscience) and analyzed using the BD FACSuite software.

Histological analysis

To evaluate the effect of pochonin D on lung histology in HRV1B-infected mice, we dissected the lung tissue and performed histological analysis as reported previously (Ahn *et al*., 2008). Briefly, lung tissues were fixed in 4% formaldehyde (Masked Formalin, DANA Korea, Incheon, Korea), dehydrated in graded concentrations of ethanol, washed in xylene, and embedded in paraffin. Paraffin blocks were sliced to obtain 10-μm-thick sections, which were then stained with hematoxylin and eosin (H&E). The sections were observed by a pathologist in a blind test under a light microscope. Each sample was scored based on the extent of edema, hemorrhage, and cell infiltration.

Scanning electron microscopy

To analyze the mouse respiratory tract, we excised the mouse respiratory tract after HRV1B infection with or without pochonin D treatment. The tissues were washed with PBS, and then fixed in 4% glutaraldehyde (Ted Pella, Redding, CA, USA) and 1% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 M cacodylate buffer (pH 7.4) for 4 h. After fixation, the tissues were rinsed thrice in 0.1 M cacodylate buffer (Sigma-Aldrich) (pH 7.4), for 10 min. Samples were then sequentially immersed in 60, 70, 80, 90, and 100% ethanol (Merck, Kenilworth, NJ, USA) for 20 min each. Samples were then immersed in ethanol and isoamyl acetate (Sigma-Aldrich) buffer, and dried. The dried samples were observed using a SUPRA55V VP-FESEM (Carl Zeiss) at the Korean Basic Science Institute, Chuncheon.

Statistical analysis

We used Student's *t*-test to compare differences between two groups. To compare multiple groups, we carried out one-way ANOVA followed by the Newman-Keuls test. Values of $p<0.05$ were considered significant at a 95% confidence interval.

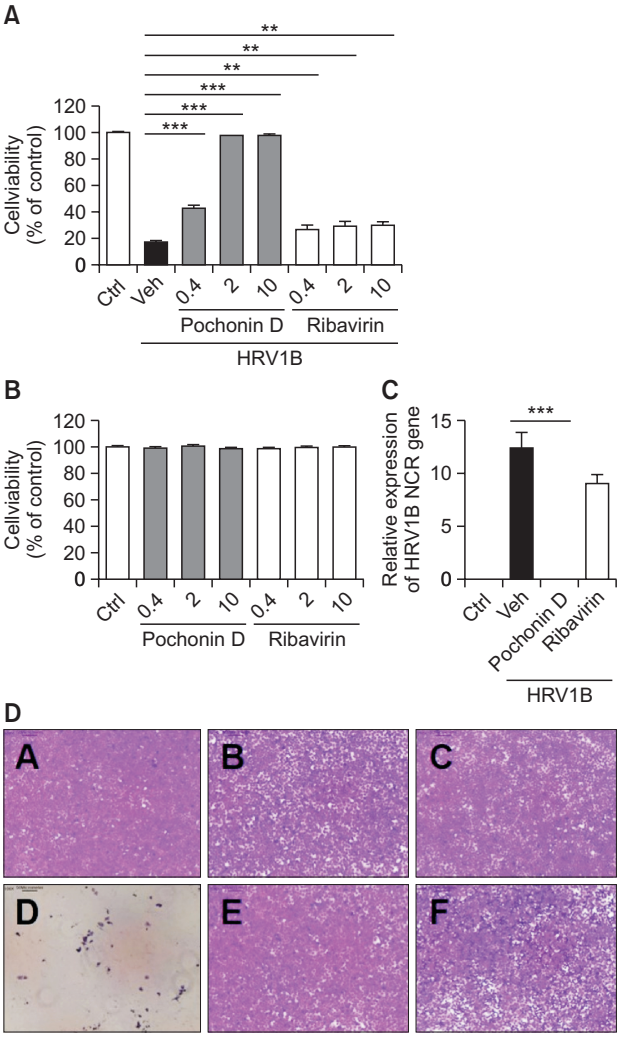

Fig. 1. The effect of pochonin D on HRV1B-induced CPE. Hela cells were infected with the diluted HRV1B suspension containing a 50% cell culture infective dose ($CCID_{50}$), and were treated with the indicated concentration of pochonin D or ribavirin. CPE on virus-infected Hela cells were analyzed at 2 days after infection. (A) SRB assay was performed as described in the Materials and Methods section. Absorbance at 520 nm was determined. (B) Cell viability was evaluated from results of the SRB assay. (C) Relative HRV gene expression in HRV1B-infected cells was assessed by real time PCR. Antiviral activity of pochonin D against HRV1B in virus-infected Hela cells. The virus-infected Hela cells were treated with pochonin D. After 2 days incubation at 32°C in 5% CO_2, the cell morphology was observed at 32×10 total magnification by microscopy. (D-A) Uninfected cells, (D-B) Uninfected cells treated with 10 μM pochonin D, (D-C) Uninfected cells treated with 2 μM pochonin D, (D-D) HRV1B-infected cells, (D-E) HRV1B-infected cells treated with 10 μM pochonin D, (D-F) HRV1B-infected cells treated with 2 μM pochonin D. Bar graphs indicate the average ± SEM. $^{**}p<0.01$ and $^{***}p<0.001$, Newman-Keuls Multiple Comparison Test (ANOVA).

RESULTS

Antiviral activity of pochonin D against human rhinoviruses *in vitro*

To identify the anti-HRV1B effect of pochonin D, we per-

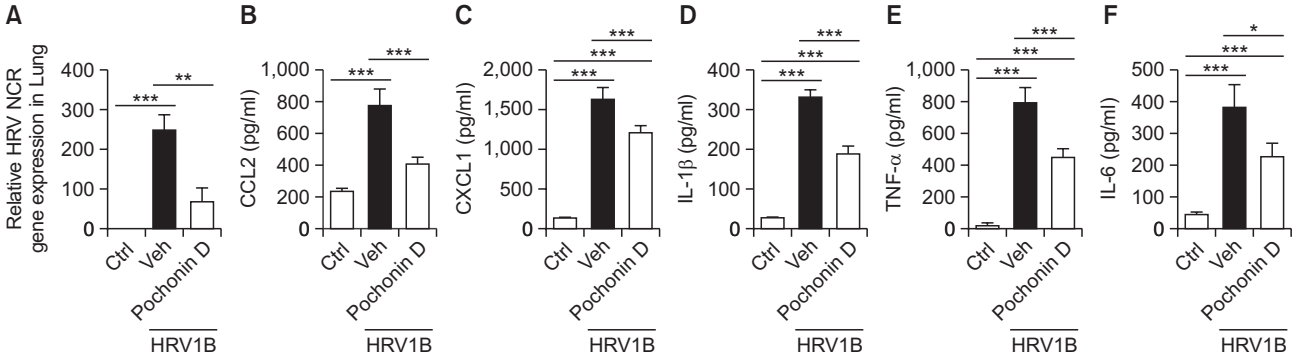

Fig. 2. Pochonin D prevents HRV infection in mice. BALB/c mice were intranasally infected with 1×10^8 pfu/30 μl of HRV1B. They were administered with 200 μg/kg of pochonin D 1 h before infection and 4 h after infection. After 8 h of infection, the virus titer and the level of chemokines and cytokines was analyzed by Real time PCR and ELISA. (A) Viral titer and levels of (B) CCL2, (C) CXCL1, (D) IL-1β, (E) TNF-α, (F) and IL-6 in lungs isolated from control mice, vehicle-treated HRV1B-infected mice, or pochonin D-treated HRV1B-infected mice. Data are means of 8 mice per group. **p*<0.05, ***p*<0.01, and ****p*<0.001, Newman-Keuls Multiple Comparison Test (ANOVA).

formed an antiviral activity assay with pochonin D in Hela cells. The antiviral SRB assay demonstrated that pochonin D possessed strong antiviral activity of about 90% against HRV1B at a concentration of 2 μM and 10 μM, and marginal antiviral activity of about 16% at a concentration of 0.4 μM (Fig. 1A, 1B). We also confirmed the antiviral activity of pochonin D against HRV14 and HRV15 infection in Hela cells (Supplementary Fig. 1). Pochonin D was not toxic to Hela cells with about 100% cell viability at a concentration of 10 μM (Fig. 1A, 1B). Further evidence of the inhibitory effects of pochonin D on HRV1B infection in Hela cells was obtained by real-time PCR analysis (Fig. 1C). RNA extraction was performed 48 h after HRV1B infection. Consistent with the results of the SRB assay, pochonin D treatment (10 μM) significantly decreased the viral 5′NCR mRNA of HRV1B 48 h after infection.

To evaluate the effect of pochonin D on HRV1B-induced CPE, we observed the morphology of HRV1B-infected cells. Two days after infection of Hela cells with HRV1B, mock cells (Fig. 1D-A) and cells treated with 10 μM pochonin D (Fig. 1D-B) or 2 μM pochonin D (Fig. 1D-C) showed typical spread-out shape and normal morphology. At the tested concentration, no signs of cytotoxicity with pochonin D were observed (Fig. 1D-B, 1D-C). Infection with HRV1B in the absence of pochonin D resulted in severe CPE (Fig. 1D-D). However, addition of 10 μM pochonin D or 2 μM pochonin D to infected Hela cells inhibited formation of visible CPE (Fig. 1D-E, 1D-F). Thus, the CPE induced by virus infection is prevented in the presence of pochonin D.

Antiviral activity of pochonin D against HRV1B *in vivo*

To assess and verify the *in vivo* antiviral activity of pochonin D against HRV1B, we first determined the pathological phenotype of mice after intranasal HRV1B infection. BALB/c mice were intranasally infected with 1×10^8 pfu/30 μl of HRV1B. Mice were killed at 8 h, 1 day, 3 days, and 5 days after virus inoculation, and the levels of pro-inflammatory cytokines including CCL2, CXCL1, IL-1β, TNF-α, and IL-6, and virus titers in the lungs were assessed. Mice infected with HRV1B produced significantly higher levels of CCL2, CXCL1, IL-1β, TNF-α, and IL-6 (Supplementary Fig. 2A-2E) with elevated virus titers (Supplementary Fig. 2F) at 8 h and 1 day after infection than the uninfected control mice. The levels of pro-inflammatory cytokines and virus titers peaked at 8 h after infection, and were reduced by day 5 to those observed in uninfected mice as reported previously (Bartlett *et al*., 2008). We therefore decided to monitor the lung cytokine levels and virus titers at 8 h after HRV1B infection for evaluating the antiviral activity of pochonin D in mice.

To assess the antiviral activity of pochonin D against HRV1B, mice were intraperitoneally administered 200 μg/kg of pochonin D, at 1 h prior and 4 h after intranasal HRV1B infection. We performed placebo infection in control mice, and administered vehicle intraperitoneally in HRV1B-infected mice as a negative control. After 8 h of infection, we sacrificed the mice and obtained lung samples. Real-time PCR analysis of viral mRNA in lung tissues revealed that the virus titer was significantly reduced in pochonin D-treated mice compared to that of vehicle-treated mice after HRV1B infection (Fig. 2A). We thus confirmed that pochonin D has an anti-HRV activity *in vitro* as well as *in vivo* when administered systemically via the intraperitoneal route.

Pochonin D treatment reduced pulmonary cytokine/chemokine production in rhinovirus-infected mice

Virus-induced cytokines and chemokines were secreted in the alveolar spaces and BALF at 8 h after HRV1B infection. Virus infection increases the secretion of CXCL1, CCL2, IL-6, IL-1β, and TNF-α from leukocytes and activates innate immune responses to induce inflammation (Subauste *et al*., 1995; Saklatvala *et al*., 1996; Bartlett *et al*., 2008). To evaluate the effect of pochonin D on pulmonary cytokines and chemokines induced by HRV infection, mice were infected and treated with 200 μg/kg of pochonin D twice, 1 h before and 4 h after intranasal HRV1B infection. At 8 h after HRV1B infection, lungs and BALF were obtained from the mice, and subjected to ELISA for measuring cytokines and chemokines. In the lungs, the levels of CCL2, IL-1β, TNF-α, IL-6, and CXCL1 were significantly increases by HRV1B infection, and these were decreased by pochonin D treatment (Fig. 2B-2F). Similarly, the levels of IL-1β, TNF-α, IL-6 and CXCL in the BALF were significantly increased by HRV1B infection and reduced by pochonin D treatment; however, the level of CCL2 in BALF was not significantly altered after HRV1B infection (Fig. 3). These data indicate that increased secretion of pro-inflammatory cytokines and chemokines induced by rhinovirus infection was mitigated by pochonin D treatment.

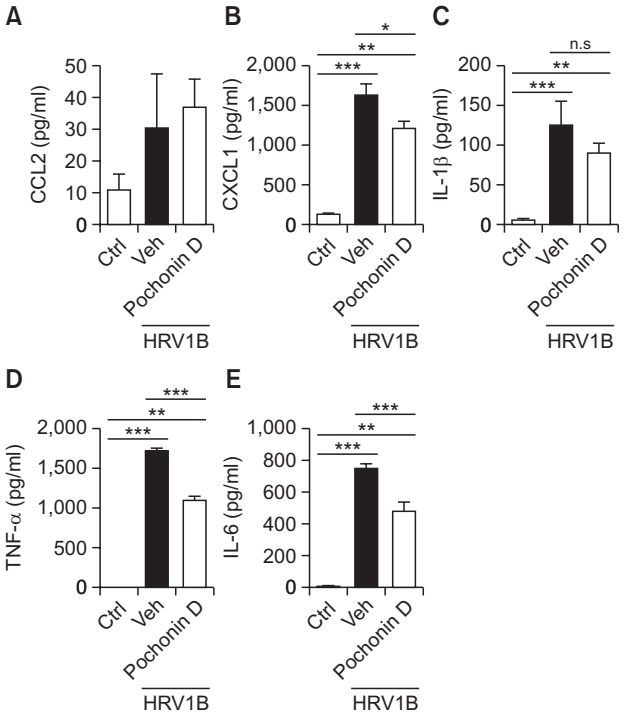

Fig. 3. Pochonin D reduced pro-inflammatory cytokines and neutrophil chemoattractant chemokines in the BALF of HRV1B-infected mice. Mice were infected with 1×10^8 pfu/30 μl of HRV1B. Treatment with pochonin D (200 μg/kg) was performed twice, at 1 h before infection and at 4 h after infection. After 8 h of infection, BALF was collected from the control mice and virus-infected mice treated with pochonin D or vehicle. The levels of CCL2 (MCP-1), CXCL1, IL-1β, TNF-α, and IL-6 in BALF were analyzed by ELISA. Data are means for 8 mice per group. Concentrations of (A) CCL2, (B) CXCL1, (C) IL-1β, (D) TNF-α, and (E) IL-6. Bar graphs depict the mean ± SEM. *p<0.05, **p<0.01, and ***p<0.001, Newman-Keuls Multiple Comparison Test (ANOVA).

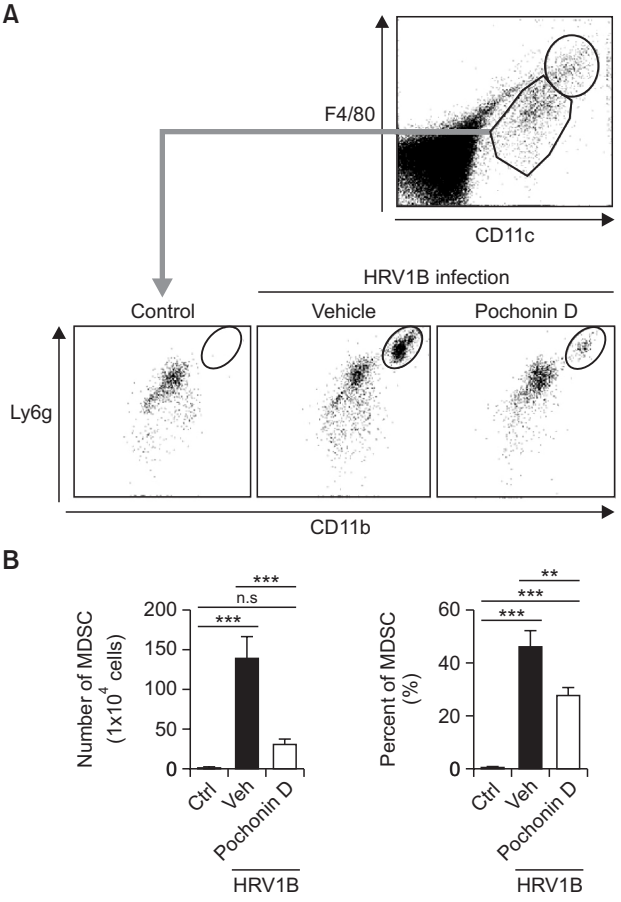

Fig. 4. Neutrophil infiltration into BALF was reduced by pochonin D treatment in HRV1B-infected mice. Mice were infected with HRV1B. Pochonin D was administered intraperitoneally 1 h before infection and 4 h after infection. After 8 h of infection, cells were collected from the BALF in lungs and stained with fluorescent dye-conjugated anti-CD11b, anti-Ly6G, anti-Ly6C, and anti-F4/80 antibodies. The cells were then analyzed by flow cytometry. (A) Dot plots of $CD11b^+Ly6G^+$ cells in BALF cells, (B) The number of MDSCs in BALF cells (left), and the percentage of MDSCs in BALF cells (right) isolated from control mice, virus-infected mice, and virus-infected mice treated with pochonin D (n=4/group). Results are shown as means ± SEM. ND, not detected, ns, not significant, **p<0.01, ***p<0.001, Newman-Keuls Multiple Comparison Test (ANOVA).

Alteration of cellular infiltrates in bronchoalveolar spaces after rhinovirus infection by pochonin D treatment

To identify the mechanisms underlying the anti-inflammatory effects of pochonin D in rhinovirus infection, we analyzed cellular infiltrates in the BALF of rhinovirus-infected mice after pochonin D or vehicle treatment. A few changes in the total cell numbers in the lung and BALF were observed after treatment of HRV1B-infected mice with pochonin D (Supplementary Fig. 3).

Recent studies suggest that myeloid-derived suppressor cells (MDSCs), which expand during cancer, inflammation, and infection, might be involved in increasing the level of cytokines and chemokines in influenza-induced pulmonary inflammation (Jeisy-Scott *et al*., 2011; Atretkhany and Drutskaya, 2016). We investigated whether the same was true for rhinovirus. We therefore assessed cellular infiltration of neutrophils into the BALF of HRV1B-infected mice treated with pochonin D or vehicle (Fig. 4). $CD11c^+$ $F4/80^+$ alveolar macrophages were excluded from the gating for neutrophils. The number of MDSCs in BALF showing the phenotype of granulocytic neutrophils was significantly higher in rhinovirus-infected mice than in non-infected mice. Moreover, the number of these cells decreased with pochonin D treatment in virus-infected mice (Fig. 4B). Similarly, the percentage of MDSC cells in BALF from rhinovirus-infected mice was higher than that in control mice, and the percentage of granulocytic neutrophils in BALF from HRV1B-infected mice was reduced by pochonin D treatment. Thus, we could confirm that inflammatory cell infiltrates in the lung were significantly increased by HRV1B infection, and could be significantly inhibited by administration of pochonin D.

Rhinovirus-induced lung pathology was relieved by pochonin D treatment

Next, we assessed the histological changes in lungs of HRV1B-infected mice. Lungs from uninfected mice exhibited typical normal pulmonary tissue, whereas rhinovirus-infected mice demonstrated characteristic inflammatory lesions with viral infection, including necrotizing bronchiolitis and interstitial pneumonia (Fig. 5A). On the other hand, rhinovirus-infected

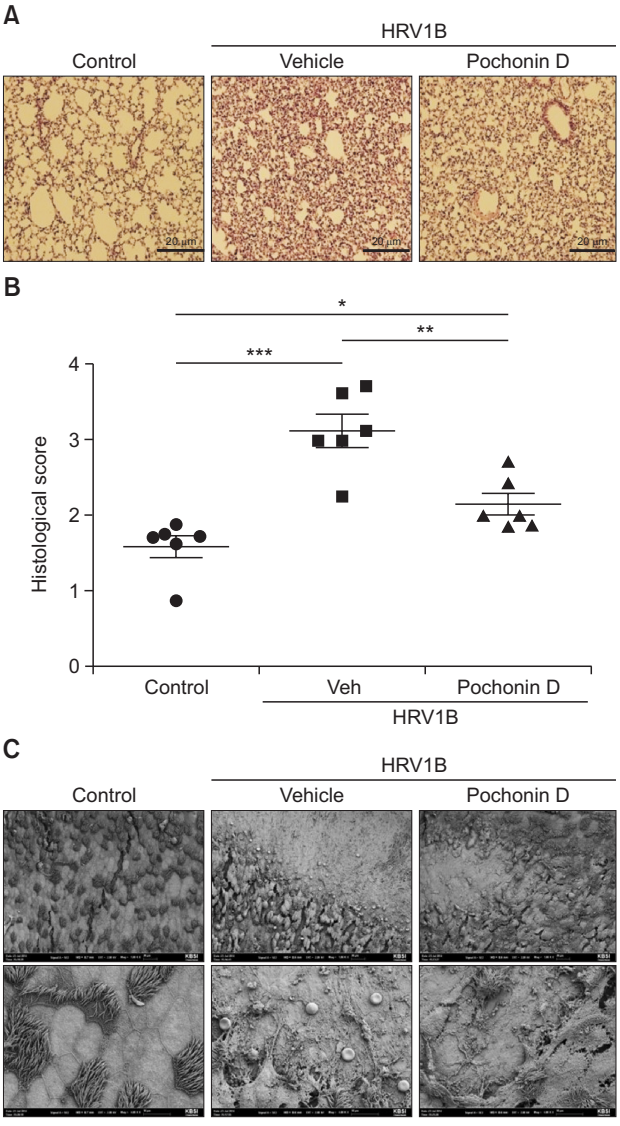

Fig. 5. Pochonin D treatment ameliorates the lung pathology induced by HRV1B infection. Mice were infected with 10^8 pfu/30 μl pfu HRV1B intranasally. Pochonin D treatment was performed twice, before and after virus infection. Eight hours after infection, the lung was isolated and histological analysis was performed as described in the Materials and Methods section. (A) Representative H&E stained sections were prepared from HRV1B-infected mice treated with pochonin D or vehicle. (B) Histological scores based on edema, hemorrhage, and cell infiltration. Scores from 1 to 5 were given based on the criteria. Bar graphs show the mean ± SEM. *p<0.05, **p<0.01, and ***p<0.001, Newman-Keuls Multiple Comparison Test (ANOVA). (C) Airway cilia in trachea isolated from HRV1B-infected mice were observed by SEM. The trachea of control, HRV1B-infected, and HRV1B- infected pochonin D-treated groups by SEM. Lower magnification ×1000 and higher magnification ×4000.

mice treated with pochonin D showed moderate inflammation with reduced necrosis, inflammatory cell infiltrates, and pulmonary edema compared to those in rhinovirus-infected mice without treatment. We scored the lung sections based on the extent of edema, hemorrhage, and cell infiltration (Fig. 5B). HRV1B-infected mice showed serious hemorrhage, edema, and cell infiltration compared to control mice, and the pochonin D treatment group showed reduced damage induced by HRV1B infection. We also observed the changes in the airway cilia of the trachea by SEM (Fig. 5C). Uninfected mice showed abundant cilia in the trachea with regular arrangement and intact septum. However, the trachea of rhinovirus-infected mice was damaged, and we could observe abnormal cilia with some phlegm. On the contrary, the condition of the trachea in HRV1B-infected mice treated with pochonin D showed amelioration of damage caused by HRV1B infection compared to that in vehicle-treated mice. Despite the presence of phlegm and decreased number of cilia, the cilia in pochonin D-treated mice after HRV1B infection were relatively intact. These results suggest that the lung and trachea were significantly damaged by rhinovirus infection, and treatment with pochonin D protected the mice from histological damage induced by HRV1B.

DISCUSSION

Generally, virus-specific proteins have drawn attention for the treatment of viral infection as targets. However, the focus of antiviral approaches has recently started to move toward targeting host factors essential to virus multiplication. Hsp90, a molecular chaperone that regulates the function, turnover, and trafficking of several proteins including signaling and regulatory proteins, is one of the important host factors that play critical roles in the viral life cycle. Hsp90 inhibitors have been reported to inhibit Ebola virus (EBOV) replication, and cause degradation of the viral polymerase (Smith *et al*., 2010). However, the exact mechanism underlying the anti-EBOV activity of Hsp90 inhibitors remains unknown. In influenza virus infection, Hsp90 is required for viral genome replication. As Hsp90 associates with subunits of the influenza virus, inhibition of Hsp90 leads to degradation of viral subunits. Besides, Hsp90 inhibitors reduce the levels of the assembled polymerase complex, resulting in decreased viral RNA levels (Momose *et al*., 2002). A recent study showed that Hsp90 is also required for the replication of beta-herpesviruses (Burch and Weller, 2005). In the human cytomegalovirus infection model, Hsp90 inhibition resulted in degradation of the viral polymerase and reduction of viral gene expression via downregulation of the PI3-kinase pathway (Basha *et al*., 2005). Similarly, in the flock house virus, Hsp90 influences RNA polymerase stability (Kampmueller and Miller, 2005). Collectively, pharmacological inhibitors of Hsp90 have potential as broad spectrum antiviral agents. In addition to their universal activity against diverse viral infections, Hsp90 inhibitors show the possibility of overcoming viral drug resistance. Most antiviral agents lead to generation of drug-resistant variants, which is one of the major issues in the development of effective antiviral therapy (zur Wiesch *et al*., 2011). Interestingly, Hsp90 inhibitors are not reported to induce viral drug resistance till date. Therefore, they might be particularly useful for antiviral therapy against viruses prone to develop drug resistance (Geller *et al*., 2012).

Hsp90 inhibitors also have potent anti-inflammatory and anti-oxidative actions in vascular tissues (Hsu *et al*., 2007). Hsp90 inhibitors were shown to extend survival, attenuate inflammation, and reduce lung injury in murine sepsis (Chatterjee *et al*., 2007). Hsp90 was also suggested to participate

in viral capsid protein folding and in the assembly of various picornaviruses including poliovirus, rhinovirus, and coxsackievirus, which renders Hsp90 an attractive candidate for the development of antiviral vaccines (Brenner and Wainberg, 1999). Hsp90 is also important for subcellular localization of specific mRNAs in regions neighboring the mitochondria, which could explain the inhibitory effect of Hsp90 inhibitors on RNA polymerase.

Human rhinoviruses cause common cold in humans, and can sometimes accelerate airway diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis (Zaheer *et al*., 2014). As an important human respiratory virus, HRV is a non-enveloped positive-sense single-strand RNA virus involved in 50-80% of upper respiratory tract infections and has also been associated with lower respiratory tract disease in high-risk populations, for example in patients with asthma or other airway inflammations (Gern and Busse, 1999). Generally, symptoms of rhinovirus in mice are not severe. However, our present data showed that the levels of pro-inflammatory cytokines such as TNF-α and IL-6 in the lung and BALF of mice were increased upon intranasal HRV1B infection, which is reported to contribute to the pathogenesis of asthma during long-term infection (Liebhart *et al*., 2002; Jartti and Korppi, 2011; Rincon and Irvin, 2012).

Ribavirin is the only antiviral drug approved by the FDA for treatment of RSV infection (Molinos-Quintana *et al*., 2013), and is also a broad-spectrum antiviral drug for RNA viruses including FLU-A, HRV 14, RSV, and CVB3 (Shi *et al*., 2007). Although ribavirin is known to have a broad-spectrum antiviral activity against several respiratory viruses, it has limitations due to its controversial efficacy and toxicity (Kneyber *et al*., 2000). Indeed, ribavirin did not show efficient antiviral activity against HRV1B infection in our experiment, and 50 μg/ml of ribavirin showed only marginal antiviral activity in Hela cells infected with HRV1B (data not shown).

In the present study, we analyzed the antiviral activity of pochonin D against HRV infection. Although pochonin D is a well-known Hsp90 inhibitor (Moulin *et al*., 2005; Wang *et al*., 2016; Choe *et al*., 2017), it is still uncertain that the inhibition of Hsp90 by pochonin D is directly associated with the antiviral activity of it. We found that treatment with pochonin D lowered the level of pro-inflammatory cytokines in the lung and BALF of mice, which were increased by rhinovirus infection. Furthermore, the virus titers of HRV-infected mice treated with pochonin D were significantly decreased to levels similar to those in naïve mice. We also examined the levels of pro-inflammatory chemokines/cytokines (CCL2, CXCL1, TNF-α, IL-6, and IL-1β) in lung lysates and lung RNA. Their concentrations were decreased by pochonin D treatment in HRV1B-infected mice, and were comparable to the chemokines/cytokines levels in naïve mice. These data suggest that pochonin D may reduce inflammatory damage in rhinovirus-infected mice. We also found that neutrophil infiltration into the inflammatory site was reduced by pochonin D treatment in HRV1B-infected mice. This reduction may be due to the mild viral infection and inflammation in pochonin D-treated group. Finally, we observed the histopathology of the lung and airway, and found that pochonin D treatment ameliorated the damage induced by rhinovirus infection in the lung and airway.

In vitro, 10 μM of pochonin D did not influence cell viability; however, slight toxicity was observed at pochonin D concentrations greater than 50 μM (data not shown). Adverse effects were also observed in mice treated with pochonin D at 1.75 mg/kg and 600 μg/kg, but not with 200 μg/kg (Data not shown). The dose of 200 μg/kg pochonin D was non-toxic to mice and was also more effective at controlling HRV infection compared to the dose of 600 μg/kg. Therefore, it is necessary to use an appropriate dose of pochonin D ensuring both safety and efficacy in antiviral therapy.

Collectively, blocking Hsp90 with pochonin D induces an antiviral effect against rhinovirus infection, and reduces the inflammatory response. As a result, treatment with pochonin D enables recovery from HRV1B virus infection in mice.

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