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

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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: Rhinovirus, Antiviral activity, Pochonin D, Heat-shock protein 90, Anti-inflammatory

INTRODUCTION

Human rhinoviruses (HRV) are positive single-stranded RNA viruses belonging to the family Picornaviridae. 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 isofrom, 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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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 chlamydospora* 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 sulfonfrodamine 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 1×10⁸ 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 cytotoxic effect (CPE) reduction as reported previously (Song et al., 2014). Briefly, one day prior to infection, Hela cells (2×10⁵ 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 MgCl₂, 1% FBS, diluted virus suspension containing a 50% cell culture infective dose (CCID₅₀) of the virus, and an appropriate concentration of the test compounds. The culture plates were incubated at 32°C in 5% CO₂ 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 Axiosvert 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-Leizit, 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™ SYB qPCR Mix (Toyobo, Osaka, Japan) on the CFX96™Optics Module (Bio-Rad, Hercules, CA).
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 FACSc Verity system (BD Biosciences) 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 immered 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.

RESULTS

Antiviral activity of pochonin D against human rhinoviruses in vitro

To identify the anti-HRV1B effect of pochonin D, we per-
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-A, 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.
Fig. 3. Pochonin D reduced pro-inflammatory cytokines and neutrophil chemoattractant chemokines in the BALF of HRV1B-infected mice. Mice were infected with $1 \times 10^5$ 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).

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 mice.
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 fowl 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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