LJ-2698, an Adenosine A₃ Receptor Antagonist, Alleviates Elastase-Induced Pulmonary Emphysema in Mice

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Abstract
Emphysema, a major component of chronic obstructive pulmonary disease (COPD), is a leading cause of human death worldwide. The progressive deterioration of lung function that occurs in the disease is caused by chronic inflammation of the airway and destruction of the lung parenchyma. Despite the main impact of inflammation on the pathogenesis of emphysema, current therapeutic regimens mainly offer symptomatic relief and preservation of lung function with little therapeutic impact. In the present study, we aimed to discover novel therapeutics that suppress the pathogenesis of emphysema. Here, we show that LJ-2698, a novel and highly selective antagonist of the adenosine A₃ receptor, a G protein-coupled receptor involved in various inflammatory diseases, significantly reversed the elastase-induced destructive changes in murine lungs. We found that LJ-2698 significantly prevented elastase-induced airspace enlargement, resulting in restoration of pulmonary function without causing any obvious changes in body weight in mice. LJ-2698 was found to inhibit matrix metalloproteinase activity and pulmonary cell apoptosis in the murine lung. LJ-2698 treatment induced increases in anti-inflammatory cytokines in macrophages at doses that displayed no significant cytotoxicity in normal cell lines derived from various organs. Treatment with LJ-2698 significantly increased the number of anti-inflammatory M2 macrophages in the lungs. These results implicate the adenosine A₃ receptor in the pathogenesis of emphysema. Our findings also demonstrate the potential of LJ-2698 as a novel therapeutic/preventive agent in suppressing disease development with limited toxicity.

Key Words: Adenosine A₃ receptor, LJ-2698, Emphysema
for patients with COPD (Celli, 2018). However, in most cases, these regimens offer only symptomatic relief and preservation of lung function. To date, there are no therapeutic options to completely reverse disease progression and cure emphysema. Therefore, it is necessary to develop novel therapeutics for the treatment or prevention of emphysema.

Previous reports have suggested the role of adenosine-mediated signaling in the maintenance of homeostasis in the lungs by regulating defense against injury and regeneration, indicating the association of adenosine-mediated signaling with chronic pulmonary disorders such as asthma and COPD (Zhou et al., 2009). Adenosine transduces signals through one of four G protein-coupled adenosine receptors (A1AR, A2AAR, A2BAR, and A3AR). Of these, the adenosine A3 receptor (A3AR) is the only adenosine receptor subtype to be overexpressed in inflammatory and cancer cells and is considered a target for the development of therapeutic agents for inflammation and cancer (Borea et al., 2015). Previous reports have demonstrated the involvement of A3AR in several pathophysiological conditions, and depending on the experimental system, A3AR has displayed conflicting roles in the regulation of ischemic conditions, inflammation, and tumor growth (Borea et al., 2015). The affinity and expression density of A3AR were significantly altered in lung tissues from patients with COPD, which were correlated with pulmonary function in matched patients (Varani et al., 2006). In addition, experimental models have demonstrated that A3AR signaling upregulates inflammation, eosinophil trafficking, degranulation of mast cells, and mucus secretion, suggesting that blockade of A3AR might be beneficial for relieving airway inflammation (Polosa and Blackburn, 2009). Considering the association of chronic inflammation with the pathogenesis of COPD, it is possible that blockade of A3AR can alleviate emphysema/COPD; however, the precise roles of A3AR signaling in emphysema remain to be elucidated, and the potential of adenosine A3 receptor agonists as therapeutic or preventive agents in emphysema/COPD has not been investigated thoroughly.

Based on these findings, we investigated the effect of the highly selective A3AR antagonist LJ-2698 (Jeong et al., 2007) in an in vivo experimental model of emphysema. LJ-2698 significantly suppressed elastase-mediated pulmonary dysfunction and lung regeneration in mice, as indicated by restoration of lung function, reduction in emphysematous lesions, and decreases in matrix metalloproteinase activity and apoptosis in the lungs. LJ-2698 significantly upregulated several cytokines associated with the repair of injured tissues. Moreover, LJ-2698 exhibited minimal toxicity in vitro and in vivo. These findings highlight the potential of LJ-2698 as a novel agent for the treatment or prevention of emphysema.

**MATERIALS AND METHODS**

**Reagents**

Fluorescein-conjugated DQ-gelatin was purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Porcine pancreatic elastase (PPE), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. LJ-2698 was synthesized as described previously (Jeong et al., 2007).

**Cell culture**

Human retinal pigment epithelial (RPE) cells were kindly provided by Dr. Jeong Hun Kim (College of Medicine, Seoul National University, Seoul, Korea). The murine hippocampal neuronal cell line HT-22 was kindly provided by Dr. Dong Gyu Jo (College of Pharmacy, Sungkyunkwan University, Suwon, Korea). The murine macrophage cell line RAW 264.7 was kindly provided by Dr. Sang Kook Lee (Seoul National University). Murine alveolar epithelial MLE-12 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPE, HT-22, and RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (all from Welgene, Inc., Gyeongssan, Korea). MLE12 cells were cultured in HITES medium [DMEM-F12 media (Welgene) containing 1x insulin-transferrin-selenium solution (Thermo Fisher Scientific), 10 nM hydrocortisone, 10 nM β-estradiol, 10 mM HEPES, and 2 mM L-glutamine (Welgene)] supplemented with 2% FBS and antibiotics. Cells were incubated at 37°C with 5% CO2 in a humidified atmosphere.

**Cell viability assay**

Cells (2×10^5 cells/well in 96-well plates) were treated with various concentrations of LJ-2698 for three days. The cells were incubated with an MTT solution for 4 h at 37°C. The formazan products were dissolved in DMSO, and the absorbance was measured at 570 nm. The data are presented as a percentage of the control group.

**Real-time polymerase chain reaction (PCR)**

Total RNA was isolated from cultured cells or frozen lung tissues using a phenol-chloroform extraction method, reverse-transcribed using a first-strand cDNA synthesis kit (TransGen Biotech, Beijing, China), and analyzed with real-time PCR on the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific) using a SYBR Green-based qPCR master mix solution (Enzymics, Daejeon, Korea) and gene-specific primers. The primer sequences used for the real-time PCR are as follows: Mouse Il4 forward, CCT CAC AGC AAC GAA GAA CA; mouse Il4 reverse, ATC GAA AAG CCC GAA AGA GT; mouse Il10 forward, TAA GGC TGG CCA CAC TTG AG; mouse Il10 reverse, GTT TTC AGG GAT GAA GCG GC; mouse Arg1 forward, GAA CAC AGT GGC ATT TAC; mouse Arg1 reverse, TGC TTA CTT CCT TCT GCT TTC; mouse Mrc1 forward, TGA TTA CGA GCA GTG GAA GC; mouse Mrc1 reverse, GTT CAC CAG GGT CCG CTT AAC; mouse Actb forward, TGG CTA GCT CTT TCT GCT CTC; mouse Actb reverse, TGG CTA GCG ACT ACC CCC AAT TT; mouse Actb forward, TGT CCA CCT TGC AGA AGA TG; mouse Actb reverse, AGC TCA GTA ACA GTC CGC CTA G; and mouse Rn18s forward, GAAGTGCTGAGCCAC; mouse Rn18s reverse, CCA TCC AAT CGG TAG TAG CG. The thermocycler conditions were as follows: preincubation at 95°C for 15 min; 50 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 30 s; and melting curve analysis to determine re-action specificity. The quantification of relative mRNA expression was performed using the comparative cycle threshold (CT) method as described previously (Livak and Schmittgen, 2001).

**Animal experiment**

The animal experiment was performed according to a protocol approved by the Seoul National University Institutional Animal Care and Use Committee. Mice were given standard mouse chow and water ad libitum and housed in a tempera-
ture- and humidity-controlled facility with a 12-h light/12-h dark cycle. Eight-week-old FVB mice were administered vehicle [20% DMSO dissolved in sterile distilled water containing 20% polyethylene glycol (PEG)] or LJ-2698 (50 μg/kg) by oral gavage 6 times per week for 5 weeks. One week after drug treatment, 0.25 units of PPE was intratracheally instilled into the lungs of mice. Body weight changes were monitored during the treatment. Changes in pulmonary function in vehicle- or LJ-2698-treated mice were analyzed using the FlexiVent (Scireq, EMKA Technologies, Montreal, Canada) (Vanoirbeek et al., 2010) according to the protocol recommended by the manufacturer. Mice were euthanized by inhalation of an overdose of isoflurane, and lungs were excised after perfusion with ice-cold PBS and then embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA, USA). Frozen tissue blocks were used for further analyses. H&E-stained tissues were used to determine structural changes in the lungs. Structural changes were quantified by calculating the mean linear intercept (MLI) as previously described (Dunnill, 1962; Chen et al., 2010).

**In situ zymography**

Dried cryosections of the lungs were incubated with fluorescein-conjugated DQ-gelatin diluted in low gelling temperature agarose for 3 h at room temperature. Fluorescein isothiocyanate (FITC) fluorescence was detected at an excitation wavelength of 460-500 nm and an emission wavelength of 512-542 nm under a fluorescence microscope and photographed.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining**

Analysis of apoptotic DNA fragmentation was performed by using a TUNEL assay kit (Millipore, MA, USA) according to the manufacturer’s instructions. Briefly, cryosections (8 μm) were fixed in 1% paraformaldehyde for 10 min at room temperature. After washing, sections were postfixed in precooled ethanol:acetic acid (2:1) for 5 min at −20°C. Then, the sections were incubated with working strength TdT enzyme for 1 h at 37°C. The sections were incubated with working strength stop/wash buffer for 10 min at room temperature. Anti-digoxigenin conjugate was then applied to the sections for 30 min at room temperature. The slides were counterstained with DAPI.

**Immunofluorescence staining**

Cryosections (8 μm) were prepared for immunofluorescence analysis. The sections were fixed in 4% paraformaldehyde (PFA) for 30 min followed by permeabilization in 0.2% Triton X-100 for 15 min at room temperature. Next, the slides were incubated with anti-CD206 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight. After washing, sections were incubated with Alexa Fluor-conjugated secondary antibody for 1 h at room temperature. The nuclei were stained with DAPI and analyzed by confocal microscopy (LSM 700; Carl Zeiss Microscopy, Jena, Germany).

**Statistical analysis**

The data are presented as the mean ± SD. The statistical significance was determined with a two-tailed Student’s t-test.

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**Fig. 1.** Suppression of elastase-induced emphysema by oral administration of LJ-2698 with minimal toxicity. (A) Schematic diagram of the experimental schedule. (B) Inhibition of elastase-induced pulmonary dysfunction by treatment with LJ-2698 (50 μg/kg). Changes in lung function were monitored using FlexiVent. (C) Alleviation of elastase-mediated pulmonary destruction in lungs from LJ-2698-treated mice. Right. Quantification of structural changes in the airspace in each treatment group. (D) Body weight changes in each treatment group during the animal experiment. The bars represent the mean ± SD; *p<0.05, **p<0.01, and ***p<0.001 compared with the PPE-treated group. PPE, porcine pancreatic elastase; LJ, LJ-2698; MLI, mean linear intercept.
using Microsoft Excel software (Microsoft Corp., Redmond, MA, USA) or GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). A p-value less than 0.05 was considered statistically significant.

RESULTS

Inhibitory effect of LJ-2698 on elastase-induced pulmonary emphysema

Based on the possible association of A3AR signaling with the development of emphysema/COPD and to discover a novel active compound to suppress emphysema, we examined the inhibitory effect of LJ-2698 on elastase-induced emphysema in mice, a widely used animal model for pulmonary emphysema (Mahadeva and Shapiro, 2002). LJ-2698 was orally administered to mice for 5 weeks. One week after LJ-2698 treatment, PPE was instilled into the murine lungs (Fig. 1A). Because deregulation in lung function is a main characteristic of emphysema (Barnes et al., 2015), we examined whether treatment with LJ-2698 attenuates PPE-mediated pulmonary dysfunction. Murine lung function was determined as compliance, with the static value indicating the change in lung volume per unit change in the transpulmonary pressure (Papantrinopoulou et al., 2012), and tissue elasticity. As shown in Fig. 1B, increased lung compliance as a result of reduced lung tissue elasticity, a typical pulmonary dysfunction found in patients with emphysema, was observed in PPE-instilled mice compared with the control group, and treatment with LJ-2698 significantly alleviated PPE-induced pulmonary dysfunction in mice. Microscopic observation of H&E-stained tissues clearly demonstrated that LJ-2698 markedly suppressed PPE-induced airspace enlargement (Fig. 1C). These results suggest that LJ-2698 significantly attenuates elastase-induced pulmonary emphysema. Importantly, during the animal experiment, the body weight of mice in the LJ-2698-treated group was not significantly different compared with that of the vehicle- or the PPE-treated groups (Fig. 1D). Therefore, although additional in vivo investigation is needed for a precise determination of the toxicity of LJ-2698, these findings indicate the minimal toxicity of LJ-2698.

The protease-antiprotease imbalance plays an important role in the development of emphysema (Sharafkhaneh et al., 2008). Because elastase further activates matrix metalloproteinases (Ferry et al., 1997), we examined the suppressive effect of LJ-2698 on elastase-induced gelatinase activity by in situ zymography using fluorochrome-conjugated DQ-gelatin. As expected, upregulation of matrix metalloproteinase activity was observed in the lungs of PPE-instilled mice, and the PPE-mediated elevation in matrix metalloproteinase activity in the lungs was markedly suppressed by treatment with LJ-2698 (Fig. 2A, 2B). In addition, the increase in the number of TUNEL-positive cells in the lungs as a consequence of PPE-mediated lung damage was also significantly downregulated in the lungs of LJ-2698-treated mice (Fig. 2C, 2D). These results confirmed the suppressive effect of LJ-2698 on the formation of pulmonary emphysema.

Anti-inflammatory action of LJ-2698

Macrophages play important roles in inflammation and tissue repair. Upon stimulation by various pathogenic foreign elements, macrophages produce a number of cytokines, chemokines, and growth factors, resulting in the induction of inflammatory responses (Abdulkhaleq et al., 2018). In addition
to inducing inflammation and acute lung injury in response to causative factors, macrophages also mediate tissue damage repair following polarization into M2 macrophages and the secretion of several anti-inflammatory cytokines such as IL4 and IL10 (Krzyszczyk et al., 2018). Because LJ-2698 markedly suppressed elastase-induced formation of emphysematous lesions in the lungs, we next investigated whether LJ-2698 can modulate the expression of several cytokines associated with the inhibition of inflammation and the resolution of lung injury by M2 macrophages. We first examined the effect of LJ-2698 in vitro using RAW 264.7 murine macrophage cells. Previous reports demonstrated secretion of elastase by activated macrophages (Web and Gordon, 1975), activation of macrophages by stimulation with lipopolysaccharide (LPS) (Meng and Lowell, 1997), and induction of elastase production in macrophages by LPS stimulation (Duc Dodon and Vogel, 1985). Therefore, for consistency with the in vivo experimental conditions, we examined the effect of LJ-2698 on the regulation of the expression of anti-inflammatory cytokines in LPS-stimulated macrophages. Stimulation of macrophages with LPS (100 ng/mL) for 24 h reduced the expression of anti-inflammatory cytokines such as IL4 and IL10 in mouse macrophages, whereas treatment with LJ-2698 (0.1 μM) significantly ameliorated LPS-mediated downregulation of these anti-inflammatory cytokines (Fig. 3A). The mRNA expression of M2 macrophage-associated markers, including Arg1 [encoding arginase 1, an M2 macrophage-specific marker (Yang and Ming, 2014)] and Mrc1 [encoding mannose receptor C-type 1 (also known as CD206), a receptor highly expressed in M2 macrophages (Zhang et al., 2017)], was also significantly downregulated in LPS-stimulated macrophages, which was significantly restored by treatment with LJ-2698 (Fig. 3B). Moreover, consistent with the in vivo results, LJ-2698 had minimal effects on the viability of three normal cell lines derived from alveolar epithelium (MLE-12), RPE, and the hippocampus (HT-22); the viability was over 50% even at the highest concentration of LJ-2698 (1 μM), a 10-fold higher concentration than that regulating the expression of several anti-inflammatory cytokines in mouse macrophages (Fig. 3C), indicating minimal toxic effects of LJ-2698 in vitro.

We also determined the effect of LJ-2698 on the expression of these anti-inflammatory factors in murine lungs in vivo. Consistent with the in vitro findings, downregulation of the transcription of IL4, IL10, Arg1, and Mrc1 by elastase treatment was significantly restored in the lungs in the LJ-2698-treated mice (Fig. 4A). Immunofluorescence (IF) analysis confirmed that the population of M2 macrophages in the lungs was also significantly elevated by the administration of LJ-2698 (Fig. 4B, 4C). Uprogulation of the recruitment of CD206-positive M2 macrophages in the lungs from elastase-instilled mice appeared to be an autonomous resolution mechanism in the host in response to elastase-induced extensive lung injuries. In addition, the enhanced recruitment of M2 macrophages by administration of LJ-2698 compared with elastase treatment may contribute to the inhibitory effect of LJ-2698 on elastase-induced pulmonary emphysema. Collectively, these results indicate that LJ-2698 exerts anti-inflammatory action by regulating cytokine expression and inducing the recruitment of M2 macrophages; these anti-inflammatory effects of LJ-2698 may be associated with the inhibitory effect of LJ-2698 on elastase-induced pulmonary emphysema. In addition, the minimal effects of LJ-2698 on the viability of various normal cells and changes in body weight of mice also indicate no overt toxicity of LJ-2698 in vitro or in vivo.

**DISCUSSION**

Although the socioeconomic burden caused by COPD/ emphysema has gradually increased, the pathophysiologic

![Fig. 3. Amelioration of lipopolysaccharide-mediated decreases in the expression of anti-inflammatory cytokines and M2 macrophage-related markers in murine macrophages by treatment with LJ-2698.](https://doi.org/10.4062/biomolther.2019.162)
A3AR antagonist LJ-2698 on the formation of emphysematous lesions in vivo and identified LJ-2698 as a potential anti-emphysema agent. Consistent with previous studies suggesting elastase-induced lung tissue damage caused by degradation of the extracellular matrix, mucins, surfactant proteins, and host defensive cytokines and the generation of bradykinin (von Bredow et al., 2003; Henke et al., 2011; Sahoo et al., 2014), instillation of elastase markedly degraded the airspace and induced apoptosis in the lungs. Our results showed that LJ-2698 significantly suppressed elastase-induced apoptosis of pulmonary epithelial cells and lung tissue damage. Previous studies have demonstrated that the elastase-induced apoptosis of pulmonary epithelial cells was mediated by activation of protease-activated receptor-1 (PAR-1) (Suzuki et al., 2005), induction of alterations in mitochondrial membrane permeability (Ginzberg et al., 2004), and placenta growth factor (PIGF)-mediated activation of c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), and protein kinase C delta (PKCδ) signaling pathways (Hou et al., 2013, 2014). Modulation of these signaling pathways might be associated with the mechanism underlying the LJ-2698-mediated inhibitory effect on elastase-induced apoptosis in the lungs.

We also show that LJ-2698 exerts an anti-inflammatory effect by inducing the expression of anti-inflammatory cytokines and the recruitment of M2 macrophages in the lungs. The anti-inflammatory effect of LJ-2698 was also revealed in murine macrophages. Since chronic inflammation is a major pathogenic cause of the development of pulmonary emphysema, targeting lung inflammation is considered a therapeutic approach for emphysema (Yao et al., 2008; Cazzola et al., 2012). In support of this notion, the orally available anti-inflammatory phosphodiesterase 4 (PDE4) inhibitor roflumilast has been utilized for the treatment of COPD in the clinic (Wedzicha et al., 2016). Therefore, the anti-inflammatory action of LJ-2698 appears to be closely associated with its inhibitory effect on pulmonary emphysema. Moreover, the anti-inflammatory effect of LJ-2698 is consistent with a recent finding of an anti-inflammatory effect following treatment with an A3AR antagonist (Min et al., 2016). Because adenosine signaling via A3AR plays a role in airway inflammation (Tilley et al., 2003), targeting A3AR may be a reasonable approach to controlling pulmonary inflammation. However, in recent studies, A3AR agonists exhibited anti-inflammatory effects in a bleomycin model of pulmonary inflammation and fibrosis (Morschi et al., 2008) and attenuated lung ischemia-reperfusion injury (Mulloy et al., 2013). Therefore, A3AR is associated with both pro- and anti-inflammatory actions depending on the model system, and additional mechanisms might also be involved in the anti-inflammatory effect of A3AR antagonism. One possible mechanism is the involvement of peroxisome proliferator-activated receptor (PPAR). Our recent study suggests that both A3AR agonists and antagonists act as partial PPARγ agonists and PPARα antagonists (Yu et al., 2017). Considering the involve-
ment of PPARγ in the anti-inflammatory action and differentiation of monocytes into an M2 macrophage phenotype (Bouhlel et al., 2007; Kapadia et al., 2008), the effect of LJ-2698 on the promotion of anti-inflammatory cytokine production might be through activation of PPARγ in addition to A3AR blockade. In addition, elastase and other proteases activate protease-activated receptor-mediated signal transduction. Protease-activated receptors (PARs) are G protein-coupled receptors activated by proteolytical cleavage of the amino-terminus, acting as sensors for extracellular proteases (Rothmeier and Ruf, 2012). Among several PARs, PAR-2 is activated by elastase and associated with inflammation (Rothmeier and Ruf, 2012; Muley et al., 2016). Therefore, additional studies are necessary to investigate the involvement of the regulation of PPARγ and/or PAR-2 in the inhibitory effect of LJ-2698 on elastase-induced emphysema in the lungs.

Finally, consistent with a previous finding (Dorotea et al., 2018), we observed minimal toxicity of LJ-2698 in several normal cell lines derived from different organs and in mice. These results suggest the potential of LJ-2698 to be efficacious in attenuating pulmonary emphysema with limited toxicity. Importantly, these effects were achieved by oral administration of a very low dose (50 μg/kg) of LJ-2698, suggesting high potency of LJ-2698 in the suppression of emphysema. Emphysema is a chronic disease, and thus long-term therapy is required. Indeed, most agents that are effective in preclinical models of COPD/emphysema, such as anti-inflammatory agents, have not been approved for the treatment of emphysema/COPD in the clinic due to a lack of effectiveness, low potency, side effects, and/or toxicity (Wedzicha et al., 2016). Therefore, high potency, oral bioavailability and limited toxicity by LJ-2698 would strengthen its utility for the treatment or prevention of emphysema. Further investigation is required to elucidate the mechanism of action of LJ-2698 in the blockade of emphysema.

In summary, in the present study, we found that LJ-2698 suppressed elastase-induced pulmonary emphysema by inducing the production of anti-inflammatory cytokines and the recruitment of M2 macrophages. In addition, LJ-2698 displayed no overt toxicity in several normal cell lines and in mice. These results collectively suggest the potential of LJ-2698 as an efficacious therapeutic and/or preventive agent for pulmonary emphysema with limited toxicity. Further studies are warranted to investigate the detailed mechanism of action of LJ-2698 and its potential additional biological activities in advanced preclinical and clinical settings.

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