In Vivo Efficacy of Sivelestat in Combination with Pazufloxacin against *Legionella* Pneumonia

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Abstract

It is important to regulate excessive inflammation when we treat patients with severe infectious disease. Sivelestat sodium hydrate (sivelestat), a neutrophil elastase inhibitor, is used in the treatment of lung injury but its effect on pneumonia is unknown. We examined the efficacy of sivelestat in combination with a fluoroquinolone, in a *Legionella pneumophila* pneumonia mouse model. The combination therapy did not show a significant survival improvement compared to the treatment with fluoroquinolone alone, but reduced bacteria number and inflammatory cells in the early phase. The combination therapy can contribute to treatment of *L. pneumophila* pneumonia with protecting lungs.

Key words

Introduction

Neutrophils play important roles in the host's defense against pathogens by collaborating with other immune cells. The infiltration of activated neutrophils into the lungs and their migration into the airways are major features in response to pulmonary infection and inflammation [1]. These activated neutrophils produce a variety of pro-inflammatory cytokines, such as interleukin-8 (IL-8), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [2], and secrete numerous proteases, in particular neutrophil elastase (NE) [3]. Neutrophil elastase is a nonspecific serine protease that has bactericidal and pro-inflammatory activities [4-5], but is also cytotoxic and can cause tissue damage under conditions that induce high expression, including acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [6]. Neutrophil elastase also plays a pathogenic role in pulmonary emphysema and chronic inflammatory airway diseases [7-8].

The specific NE inhibitor, sivelestat sodium hydrate (sivelestat), has been shown to decrease neutrophil counts in bronchoalveolar lavage fluid (BALF) and lung damage in acute lung injury animal models [9-10]. In Japan, sivelestat is used in the treatment of ALI and ARDS patients with systemic inflammatory response syndrome and its clinical effectiveness has been documented [11-12].

Legionella pneumophila, which is a Gram-negative intracellular bacterium, causes serious pneumonia in humans. *L. pneumophila* pneumonia often progresses rapidly with severe systematic inflammation and is sometimes life-threatening [13], despite the strong activity fluoroquinolone and macrolide antibiotics have against *Legionella* species [14]. A variety of host cells, including macrophages and neutrophils, are involved in the immune response to *L. pneumophila* [15-16]. However, the accumulation of activated neutrophils can lead to the

production of excessive NE, which causes lung tissue damage. Thus excessive inflammation may play a role in part in the pathogenesis of severe pneumonia. We previously reported the effectiveness of sivelestat in a mouse model of severe pneumococcal pneumonia [17] and its effective case with Legionnaire's disease was reported [18], however, its efficacy against severe pneumonia is still unclear.

Pazufloxacin is a parenteral fluoroquinolone antibiotic available in Japan that has potent activity against *Legionella* species [19]. However, treating patients with severe pneumonia using antibiotics alone is difficult and thus additional therapies aimed at the various aspects of *Legionella* pathology are required. To confirm whether sivelestat improves the acute inflammation and/or survival, we examined the efficacy of sivelestat in combination with the fluoroquinolone pazufloxacin, in a murine model of severe *L. pneumophila* pneumonia.

Materials and methods

Reagent*s*

Sivelestat was kindly provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Pazufloxacin was kindly provided by Taisho Toyama Pharmaceutical Co., Ltd. (Tokyo, Japan). Both reagents were dissolved in physiological saline just before use.

Animals

Male 8-week-old A/J mice were purchased from Japan SLC, Inc. (Sizuoka, Japan). All animals were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center for Biomedical Science at Nagasaki University. Animal care and experimental procedures were performed in accordance with the Guidelines for Animal

Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

Bacterial strains

In this study we used the *L. pneumophila* NUL1 strain, which was clinically isolated from the sputum of a patient at Nagasaki University hospital. The bacteria were stored at -80°C in a Microbank system (Pro-Lab Diagnostics, Ontario, Canada) until use.

Antibiotic susceptibility testing

The MIC of pazufloxacin and sivelestat against NUL1 was determined using the microdilution method with ACES-buffered yeast extract supplemented with α-ketoglutarate broth [(19)]. Microtiter plates containing 5.0×10^5 cfu/well were incubated with pazufloxacin or sivelestat at 37°C for 72 h, and the lowest concentration of the drug that prevented visible growth was considered the MIC.

Experimental model of *Legionella pneumophila* pneumonia

To prepare the inoculum, NUL1 was cultured on a buffered charcoal yeast extract α (BCYEα) agar plate for 72 h, then the bacteria suspended in saline, harvested by centrifugation ($3,000 \times g$, 4°C, 10 min), resuspended in sterile saline and adjusted approximately to 2×10^9 cfu/ml, as estimated by turbidimetry. Anaesthetized mice were inoculated with the bacteria at 50 μl/mouse (1×10^8 cfu/mouse) intratracheally [20].

Treatment protocol

Treatment commenced 24 h after inoculation. Sivelestat alone (4.8 mg/kg; SIV mice),

pazufloxacin alone (5.0 mg/kg; PZFX mice), or a combination of sivelestat and pazufloxacin (the same dose of each drug as used alone; SIV+PZFX mice) were injected intraperitoneally into the mice twice a day. In the control group (CTRL), saline was injected into the mice instead of sivelestat or pazufloxacin. Viable bacteria counts and cytokines in the lungs, cell counts in BALF and histopathological examinations were analyzed in each group on day 2 (12 h after the 2nd treatment). The survival rates of mice were analyzed after 7-day treatment.

Bacteriological examinations

The mice were sacrificed by cervical dislocation on day 2 (12 h after the 2nd treatment). The lungs were dissected under aseptic conditions and suspended in 1 ml saline. The organs were homogenized with a homogenizer (AS One Co., Osaka, Japan), quantitatively inoculated onto BCYEα agar plates using serial dilutions and incubated at 37°C for 72 h to count viable bacteria.

Histological examinations

The mice were sacrificed by cervical dislocation on day 2. Lung tissue was fixed in 10% buffered formalin and stained with hematoxylin-eosin.

Cytokine examination

Concentrations of MIP-2, TNF-α and IL-1β in the lung were assayed using mouse cytokine enzyme-linked immunosorbent assay (ELISA) test kits (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Bronchoalveolar lavage (BAL) fluid examination

BAL was performed as described previously [21]. Briefly, mice were sacrificed on day 2 after inoculation. The chest was opened to expose the lungs and a disposable sterile plastic cut-down intravenous catheter was inserted into the trachea. BAL was performed three times sequentially using 1 ml saline each time and the recovered fluid fractions were pooled for each animal. Fluid recovery was routinely ≥90%. Leukocytes in BALF samples obtained from each mouse were washed and counted with a hemocytometer. For differential cell counts, cells were centrifuged at $1,000 \times g$ for 1 min and then fixed onto slides, which were then stained with Diff-Quik stain. Differential cell counts were performed by counting 100 cells.

Statistical analysis

Data represented the mean ± standard error of the mean (SEM) and statistical analyses were performed using Scheffe's test following the Kruskal-Wallis non-parametric test. Statistical significance was defined as $p < 0.05$. Survival analysis was performed by Kaplan-Maier method.

Results

MICs

The MICs of pazufloxacin and sivelestat against the NUL1 strain were 0.063 and >128 mg/L, respectively.

Bacteriological examination

The number of viable bacteria in the lungs was analyzed on day 2. The mean number of viable bacteria in the lungs of CTRL, SIV, PZFX, and SIV+PZFX mice was 6.49 ± 0.19, 6.01 ± 0.24, 5.93 ± 0.37 and 4.96 ± 0.44 $\log_{10}$ cfu/lung (mean $\pm$ SEM, n = 4 to 6), respectively (Fig. 1). There were no sterile mice after treatment in any group. The number of viable bacteria in the lungs of the SIV+PZFX mice was significantly fewer than that in lungs of the CTRL mice ($p = 0.045$).

Cytokines in the lung

The concentrations of MIP-2 (Fig. 2a), IL-1β (Fig. 2b) and TNF-α (Fig. 2c) in the lungs were analyzed on day 2. Although the cytokine levels of the PZFX and SIV+PZFX mice appeared to be less than those of the CTRL mice, there were no significant differences.

BALF examination

The total cell counts in BALF on day 2 of the CTRL, SIV, PZFX, and SIV+PZFX mice were 2.78 ± 0.45, 2.02 ± 0.37, 2.32 ± 0.29 and $1.01 \pm 0.25 \times 10^5$ cells/mL (mean $\pm$ SEM, n = 2 to 6), respectively (Fig. 3a). The percentage of neutrophils in BALF of the CTRL, SIV, PZFX and SIV+PZFX mice were 79.5 ± 0.5, 75.8 ± 5.8, 88.0 ± 0.6 and $58.0 \pm 8.5\%$ (mean $\pm$ SEM, n = 2 to 6), respectively (Fig. 3b). There was a statistical difference between the percentage of neutrophils in BALF between PZFX and SIV+PZFX mice ($p = 0.039$).

Histopathological examination

The histopathological examinations of the lung specimens from mice sacrificed on day 2 are shown in Fig. 4. In the CTRL mice, numerous neutrophils infiltrated into the alveolar spaces

and some alveoli were occupied by neutrophils (Fig. 4a). In the SIV (Fig 4b) and the PZFX (Fig 4c) mice, many neutrophils were also observed but slightly fewer than in the CTRL mice. Furthermore, in the SIV+PZFX mice we found noticeably fewer inflammatory cells compared with the CTRL mice (Fig. 4d).

Survival examination

The survival rates of each group were analyzed after a 7-day-treatment (Fig. 5). All CTRL mice had died by day 6, but over 60% of mice survived at least until day 8 in the other groups. The SIV+PZFX mice demonstrated the best survival, with a significant difference compared to CTRL mice ($p = 0.010$, Kaplan-Meier).

Discussions

It is important for the treatment of severe infectious diseases to regulate the excessive inflammation. We studied the efficacy of the combination therapy of sivelestat and a fluoroquinolone antibiotic in a mouse model with severe *L. pneumophila* pneumonia. This study demonstrated that the combination therapy can contribute to treatment of *L. pneumophila* pneumonia with attenuating of lung inflammation derived from excessive NE in the acute phase.

L. pneumophila is a pathogen that causes pneumonia with rapid worsening and is sometimes life threatening. *L. pneumophila* infects macrophages initially and the infected macrophages produce a variety of mediators, which subsequently stimulate inflammatory cells, including neutrophils, T cells, natural killer cells and B cells [16]. A previous study demonstrated that NE levels are elevated and the mean anti-elastase capacity decreased in pneumonic lobes of

patients with community-acquired pneumonia [22]. Although activated neutrophils are considered to play a role in the host's defense against invading microbial pathogens, paradoxically they can produce excessive NE that damages lung tissue in severe pneumonia.

L. pneumophila is very susceptible to several antibiotics, such as the fluoroquinolones, ketolides and macrolides [14]. Pazufloxacin, a fluoroquinolone antibiotic available in Japan, is also effective against *Legionella* [19, 23]. In this study, pazufloxacin showed slight decrease of the number of viable bacteria, however, the combination therapy showed more effective for decreasing of bacteria with a significant difference in spite of the short duration of treatment. This suggests that the sivelestat supports the host's defense against *Legionella*, because sivelestat does not have antibacterial activity against NUL1.

MIP-2, IL-1β and TNF-α are produced mainly by macrophages in *L. pneumophila* infection [16] and mediate neutrophil recruitment and migration into lung tissue and alveolar spaces. In particular, TNF-α also enhances the bactericidal activity of macrophages and neutrophils and stimulates the production of NE from neutrophils [16]. Furthermore, these cytokines are also produced by activated neutrophils [2]. In results of cytokine concentration, we could not observe the effectiveness of sivelestat and/or pazufloxacin. These results indicate that the production of these cytokines is independent of sivelestat at least in the acute phase.

The marked number of neutrophils in BALF is a major finding in patients with *Legionella* pneumonia [15]. As shown in Fig. 3b, *Legionella* pneumonia mouse model also showed an elevated percentage of neutrophils. The percentage of neutrophils was not suppressed in the PZFX mice but slightly reduced in the SIV+PZFX mice. This finding implies that the migration of neutrophils into the airway lumen is induced not only by bacteria but also by active NE. The paradoxical results between leukocyte chemoattractant cytokines and cell fraction in BALF may derive from the time lag. In addition, the histopathological findings

agreed well with BALF examination. The findings in the SIV+PZFX mice seemed to be the most effectively improved. Because NE can degrade proteoglycans in the glycocalyx and components of the endothelial basement membrane [24], the combination therapy may inhibiting the vicious cycle. Thus, NE can partly play a role in the pathogenesis in *L. pneumophila* pneumonia.

The prolonged survival of the SIV+PZFX mice were most likely due to the improved bacteriological and inflammatory findings in the SIV+PZFX mice after 2 treatments but there was no significant difference compared with PZFX mice. Surprisingly, SIV mice also survived in this study. Although we cannot clearly explain the reason of this survival improvement, the reduced inflammatory cells in histopathological findings may be a sign of this effect. We need further analyses about this.

The continuous infusion of sivelestat has previously been shown to decrease NE activity and lung hemorrhage in lung injury animal models induced by acid- or endotoxin-inhalation [9, 25]. Thus, the lower effectiveness in this study may be due to the difference in drug administration.

Finally, the A/J mouse used in this study is known as a susceptible animal to *Legionella* because of *Naip5* gene mutation concerning permissiveness to intracellular *Legionella* replication, whereas other mice are resistant to *Legionella* [26]. Therefore, alternative approaches may be needed to explain the effectiveness of the combination therapy of sivelestat and antibiotics against *L. pneumophila* pneumonia.

In conclusion, the combination therapy of sivelestat and pazufloxacin showed similar survival rate of the treatment with pazufloxacin alone, but decreased the number of viable bacteria in the acute phase in a mouse model of *L. pneumophila* pneumonia. We found a significant difference in the percentage of neutrophils in BALF between the combination

therapy and the treatment with pazufloxacin alone. These findings imply that sivelestat can participate in the regulation of infection and inflammation. Treatment with sivelestat in addition to antibiotics can be expected to be effective against *L. pneumophila* pneumonia with reducing lung damage due to activated NE.

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Figure legends

Figure 1

The number of viable bacteria in the lungs after 2 treatments (day 2) in the *Legionella* pneumonia mouse model. The mean number of viable bacteria in the lungs of CTRL, SIV, PZFX, and SIV+PZFX mice was 6.49 ± 0.19, 6.01 ± 0.24, 5.93 ± 0.37 and 4.96 ± 0.44 $\log_{10}$ cfu/lung (mean $\pm$ SEM, n = 4 to 6), respectively. $*p < 0.05$ vs. CTRL.
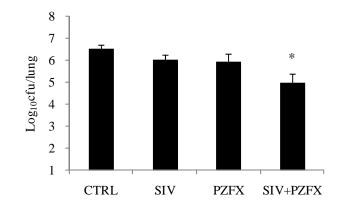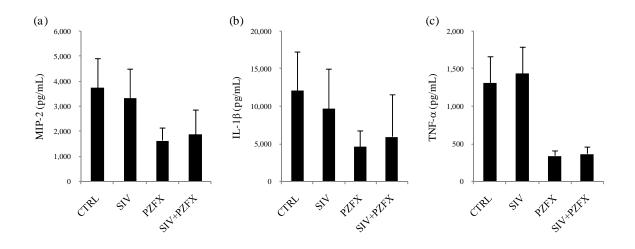
Figure 2

The cytokine concentrations in the lung after 2 treatments in the *Legionella* pneumonia mouse model. MIP-2 (a), IL-1β (b) and TNF-α (c) were measured by ELISA.

Figure 3

Bronchoalveolar lavage fluid examinations after 2 treatments in the *Legionella* pneumonia

mouse model. (a) The total cell counts in the BALF on day 2 of the CTRL, SIV, PZFX, and

SIV+PZFX mice were 2.78 ± 0.45, 2.02 ± 0.37, 2.32 ± 0.29 and $1.01 \pm 0.25 \times 10^5$ cells/ml

(mean $\pm$ SEM, n = 2 to 6). (b) The rate of neutrophils in BALF of the CTRL, SIV, PZFX and

SIV+PZFX mice were 79.5 ± 0.5, 75.8 ± 5.8, 88.0 ± 0.6 and $58.0 \pm 8.5\%$ (mean $\pm$ SEM, n =

2 to 6). *$p < 0.05$.
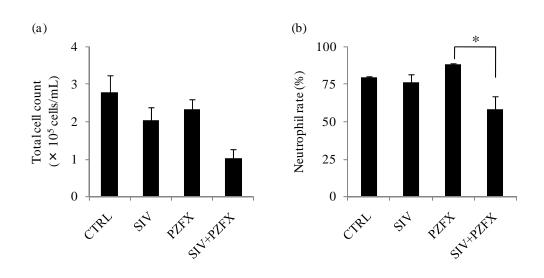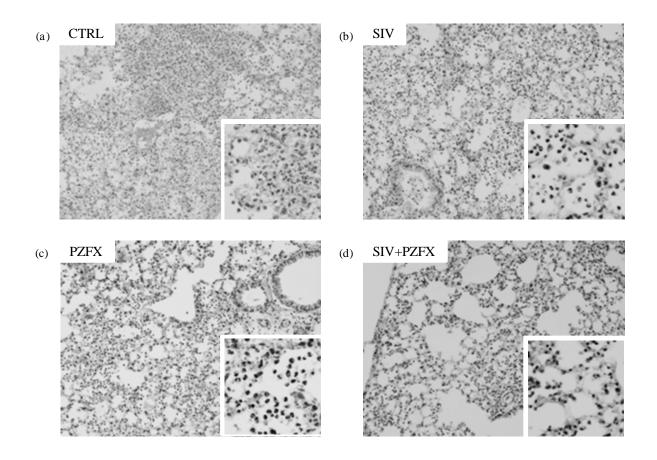
Figure 4

Histopathological findings of the lung after 2 treatments. Representative data of each group is shown (n ≥ 5). Enlarged image was in a square. A number of neutrophils infiltrated in the lung and occupied partially the alveolar space in CTRL mice (a). Slightly fewer cells were observed in PZFX (b) or SIV (c) mice, and noticeably fewer in SIV+PZFX mice (d).

Figure 5

The survival rates within the observation period. Mice with severe *Legionella* pneumonia were treated with each drug or saline twice a day for 7 days (n = 7 or 8). All CTRL mice died by day 6, however, over 60% of mice survived in the other groups. The survival of SIV, PZFX and SIV+PZFX mice was significantly better than the CTRL mice (p = 0.048, 0.037 and 0.010, respectively, Kaplan-Meier).