

Combined effects of a neutrophil elastase inhibitor (sivelestat sodium) and a free radical scavenger (edaravone) on lipopolysaccharide-induced acute lung injury in rats

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Abstract

Objective and design The present study aimed to investigate the combined effects of a neutrophil elastase inhibitor, sivelestat sodium, with a free radical scavenger, edaravone, on lipopolysaccharide (LPS)-induced acute lung injury (ALI).

Materials and methods Adult male Sprague–Dawley rats were anesthetized and instilled intratracheally with 2 mg/kg LPS. Sivelestat sodium (10 mg/kg, i.p.) and/or edaravone (8 mg/kg, i.p.) were administered 1 h after LPS instillation. The severity of pulmonary injuries was evaluated 12 h after inducing acute lung injury.

Results In lung tissues, either sivelestat or edaravone treatment alone showed significant protective effects against neutrophil infiltration and tissue injury, as demonstrated by myeloperoxidase activity and histopathological analysis. Sivelestat or edaravone treatment also attenuated the LPS-induced production of pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor alpha (TNF- α) in rat lungs. However, the LPS-induced elevation of malondialdehyde levels in rat lungs was reduced only by edaravone, but not by sivelestat. In addition, combined treatment with both sivelestat and edaravone demonstrated additive protective effects on LPS-induced lung injury, compared with single treatments.

Conclusions Combination of sivelestat and edaravone shows promise as a new treatment option for ALI/acute respiratory distress syndrome patients.

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Introduction

Acute lung injury (ALI) and its most severe manifestation, the acute respiratory distress syndrome (ARDS), remain significant causes of morbidity and mortality of patients in intensive care units throughout the world [1, 2]. This clinical disorder is well defined by acute hypoxemic respiratory failure with severe alveolar and interstitial edema and neutrophil infiltration, which results in increased vascular permeability and gas exchange abnormalities [3–7].

The contribution of infiltrated neutrophils to ALI has been widely accepted [5, 6]. Activated neutrophils release various cytotoxic mediators, including reactive oxygen species (ROS) and granule enzymes. Among them,

neutrophil elastase, a serine protease localized primarily in the azurophilic granules of neutrophils, has been implicated in causing or aggravating acute lung injury including ARDS [9, 10]. Thus, specific inhibitors of neutrophil elastase are proposed as potential therapeutic drugs against ALI. For example, sivelestat, a small molecular weight neutrophil elastase inhibitor, has been shown to protect against ALI in various animal models [9–11].

On the other hand, oxidative stress resulting from an imbalance between the production of pro-oxidants and the biological scavenger system also plays a central role in the pathogenesis of ALI [12, 13]. Infiltrated neutrophils and alveolar macrophages have been shown to produce large quantities of ROS, which directly cause tissue injury during the course of ALI. Furthermore, excessive release of ROS can oxidize and even inactivate endogenous elastase inhibitors, thus enhancing the toxicity of neutrophil elastase [10, 14]. Recently, Matsumoto et al. [15] reported that the clinical efficacy of sivelestat was significantly lower in ALI patients with severe oxidative stress levels.

As a potent free radical scavenger, edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one) has been used clinically in patients with acute ischemic stroke, and improves functional outcomes [16, 17]. We and others have previously demonstrated that edaravone successfully ameliorates lung injury in various animal models of ALI [18–20]. However, it remains to be determined whether edaravone improves the therapeutic efficacy of sivelestat through inhibition of oxidative stress. Thus, the present study aimed to compare the effects of individual versus combined treatment of sivelestat and/or edaravone on lipopolysaccharide (LPS)-induced lung injury in rats.

Materials and methods

Chemicals

Sodium taurocholate, 1,1,3,3-tetramethoxypropane, thiobarbituric acid, sodium dodecyl sulfate, hexadecyltrimethylammonium bromide, *o*-dianisidine, LPS (*E. coli* 011:B4), sivelestat sodium hydrate and hydrogen peroxide were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Edaravone was supplied by Simcere Doyea Pharmaceutical Co. (Nanking, China).

LPS-induced acute lung injury

This study was performed in accordance with the Guidelines for Animal Experiments of the Second Military Medical University, China. Male Sprague–Dawley rats (250–300 g) were housed with free access to food and water under a natural day/night cycle. All rats were acclimatized

for a week before any experimental procedures. To perform LPS-induced lung injury, animals were anesthetized with 10% chloralhydrate (0.4 ml/100 g body weight, i.p.), and LPS (2 mg/kg at 0.5 ml/kg in saline) was instilled via the trachea. Rats were then allowed to recover consciousness after the treatment.

Drug treatment

Animals were randomly divided into the following groups: (a) sham group; (b) LPS-induced ALI group; (c) LPS–sivelestat group; (d) LPS–edaravone group; (e) LPS–sivelestat plus edaravone–ALI group. One hour after inducing acute lung injury, rats were administered intraperitoneally with saline (sham and LPS-induced ALI groups), sivelestat sodium hydrate (10 mg/kg) and/or edaravone (8 mg/kg). Each experimental group consisted of nine rats. All the rats were killed by an overdose of chloralhydrate 12 h after the induction of ALI. The left lower lung tissues were fixed in 4% paraformaldehyde for histopathological analysis. Other parts of lung tissues were removed and then stored at -80°C until use.

Histopathological analysis

For histopathological analysis, the left lower lung tissues were fixed as described above. The samples were dehydrated and embedded in paraffin. Sections (4 μm thickness) were cut and stained with hematoxylin and eosin (H&E). The total surface of the slides was scored by two blinded pathologists with expertise in lung pathology. Briefly, the criteria for scoring lung inflammation was as follows [21]: 0, normal tissue; 1, minimal inflammatory change; 2, no obvious damage to the lung architecture; 3, thickening of the alveolar septae; 4, formation of nodules or areas of pneumonitis that distorted the normal architecture; 5, total obliteration of the field.

Myeloperoxidase determination

Activity of myeloperoxidase (MPO), an enzyme present in neutrophils, was used as a marker of neutrophil infiltration. MPO activity was determined in lung as described [22]. Lung tissues (100 mg of tissue) were homogenized in 2 ml of 20 mM potassium phosphate buffer (pH 7.4). After centrifuging at 15,000g for 20 min, the pellet was resuspended in 2 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide and sonicated for 30 s. After being heated at 60°C for 2 h, the samples were centrifuged at 10,000g for 10 min. The supernatant (25 μl) was added to 725 μl of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml *o*-dianisidine and $5 \times 10^{-4}\%$ hydrogen peroxide. MPO activity was

measured spectrophotometrically as the change in absorbance at 460 nm at 37°C, using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Results are expressed as units of myeloperoxidase activity per g wet tissue (1 unit defined as change in absorbance of 1/min).

Malondialdehyde level determination

Levels of malondialdehyde (MDA), as an index of membrane lipid peroxidation, were determined as previously described [19]. Lung tissues were homogenized (100 mg/ml) in 10 vol of 1.15% KCl solution containing 0.85% NaCl and then centrifuged at 1,500g for 15 min. 200 µl of the homogenates were then added to a reaction mixture consisting of 1.5 ml 0.8% thiobarbituric acid, 200 µl 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (adjusted to pH 3.5 with NaOH) and 600 µl distilled H₂O. The mixture was then heated at 95°C for 40 min. After cooling to room temperature, the samples were cleared by centrifugation (10,000g, 10 min) and their absorbance measured at 532 nm, using 1,1,3,3-tetramethoxypropane as an external standard. The level of lipid peroxides was expressed as nmol MDA/mg protein (Bradford assay).

Measurement of cytokine levels in lung tissues

Lung tissues (100 mg) were homogenized and sonicated in 1 ml PBS containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 1 µg/ml each antipain, leupeptin, and pepstatin A). Lung homogenates were then centrifuged at 1,000g for 10 min. The supernatants were filtered through 0.45 µm pore-size sterile filters, and frozen at -80°C until use for the measurement of the levels of cytokines in lung tissues. The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instruction.

Statistical analysis

Data were expressed as mean ± SEM. Statistical significance was estimated by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. $p < 0.05$ was considered statistically significant.

Results

Tissue MDA levels

The therapeutic efficacy of sivelestat and/or edaravone was assessed at 1 h after intratracheal LPS instillation. As

shown in Fig. 1, a significant increase in lung MDA level was observed in LPS-treated animals compared with saline-treated sham animals, indicating membrane lipid peroxidation in lung tissues as a result of the ALI. Administration of edaravone, but not sivelestat, significantly attenuated lung MDA levels in LPS-treated rats. In addition, the lung MDA levels of the sivelestat plus edaravone–ALI group was significantly lower than those of the sivelestat–ALI group, whereas there was no difference in lung MDA levels between the sivelestat plus edaravone–ALI group and the edaravone–ALI group.

Tissue MPO levels

The LPS-induced ALI was also manifested by an increase of lung MPO activity (an indicator of neutrophil infiltration; Fig. 2). Both sivelestat and edaravone significantly reduced the lung MPO activity compared with the LPS-treated group. In addition, the reduction of lung MPO activity was also observed in the combined sivelestat and edaravone treatment group, and showed significant differences from those in the LPS-induced ALI groups treated with sivelestat or edaravone alone.

Histological changes

As shown in Fig. 3a, light microscopy results showed the normal histology of lungs. Administration of LPS resulted in diffuse edematous changes in alveolar interstitium, alveolar thickening, extensive leukocyte infiltration, marked decreases in alveolar air space, and with scores of 3.4 ± 0.3

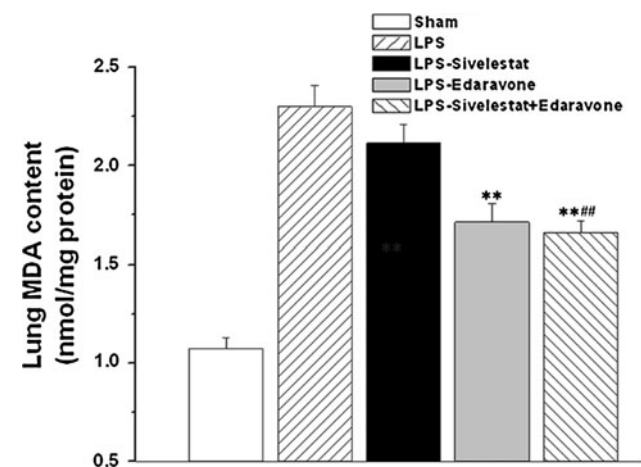


Fig. 1 Effect of therapeutic treatment with sivelestat and/or edaravone on lung MDA level in LPS-induced acute lung injury. Rats were instilled intratracheally with LPS (2 mg/kg) to develop acute lung injury for 12 h, not treated, or subjected to the following treatment: sivelestat (10 mg/kg) and/or edaravone (8 mg/kg) given 1 h after administration of LPS. Data are mean ± SEM of nine rats for each group. ** $p < 0.01$ versus LPS; *** $p < 0.01$ versus LPS–sivelestat

(Fig. 3b). In LPS-induced ALI rats treated with sivelestat or edaravone alone, these histological changes in lungs were significantly reduced to scores of 2.3 ± 0.3 (Fig. 3c) or

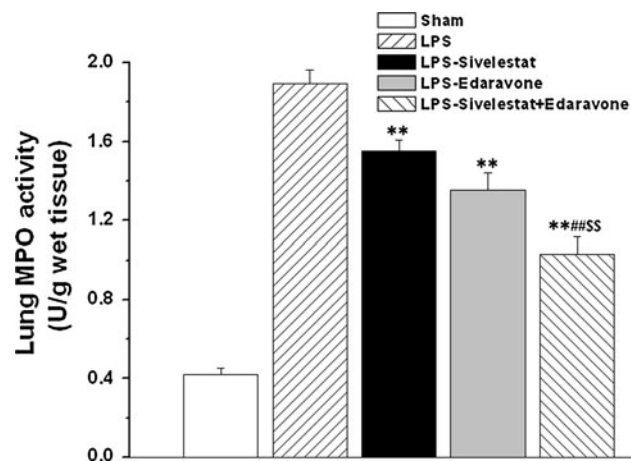


Fig. 2 Effect of therapeutic treatment with sivelestat and/or edaravone on lung MPO activity in LPS-induced acute lung injury. Rats were instilled intratracheally with LPS (2 mg/kg) to develop acute lung injury for 12 h, not treated, or subjected to the following treatment: sivelestat (10 mg/kg) and/or edaravone (8 mg/kg) given 1 h after administration of LPS. Data are mean \pm SEM of nine rats for each group. ** $p < 0.01$ versus LPS; ## $p < 0.01$ versus LPS-sivelestat; SS $p < 0.01$ versus LPS-edaravone

1.8 ± 0.2 (Fig. 3d), respectively. In addition, sivelestat and edaravone together had an additive effect and reduced the histological changes to scores of 1.0 ± 0.2 (Fig. 3e).

Tissue IL-6 and TNF- α levels

We also determined the effects of sivelestat and/or edaravone on protein levels of pro-inflammatory cytokines IL-6 and TNF- α in lung tissues using an ELISA method (Fig. 4). It was found that IL-6 and TNF- α levels were significantly elevated in the LPS-induced ALI group, while individual and combined treatment with sivelestat and edaravone effectively prevented this elevation. The additive effect of sivelestat and edaravone on IL-6 and TNF- α reduction was also observed in lung tissues.

Discussion

The results of the present study demonstrated the protective effects of sivelestat and edaravone on LPS-induced lung injury in rats. In addition, combined treatment of both drugs exhibited further significant reductions in neutrophil infiltration and pro-inflammatory cytokine levels, and

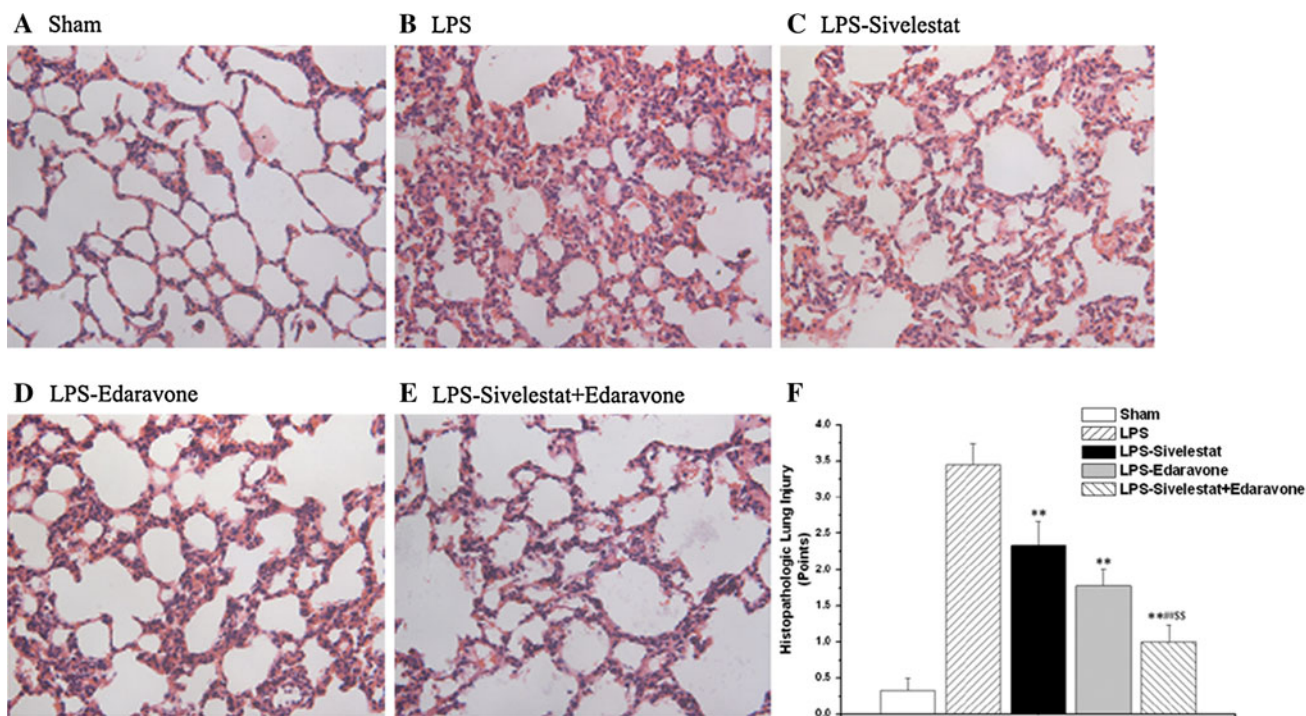


Fig. 3 Effect of therapeutic treatment with sivelestat and/or edaravone on morphological changes in the lung after induction of LPS-induced acute lung injury. Twelve hours after induction of acute lung injury, the left lower lung was removed for histopathological examination using hematoxylin and eosin staining. Original magnification $\times 100$. Representative images from nine animals per group are shown. **a** Sham, saline only; **b** LPS-induced acute lung injury rats;

c Sivelestat (10 mg/kg) therapeutically injected 1 h after LPS instillation; **d** Edaravone (8 mg/kg) therapeutically injected 1 h after LPS instillation; **e** Sivelestat (10 mg/kg) and edaravone (8 mg/kg) therapeutically injected 1 h after LPS instillation. **f** The severity of lung injury was scored as described in “Materials and methods”. Data are mean \pm SEM of nine rats for each group. ** $p < 0.01$ versus LPS; ## $p < 0.01$ versus LPS-sivelestat; SS $p < 0.01$ versus LPS-edaravone

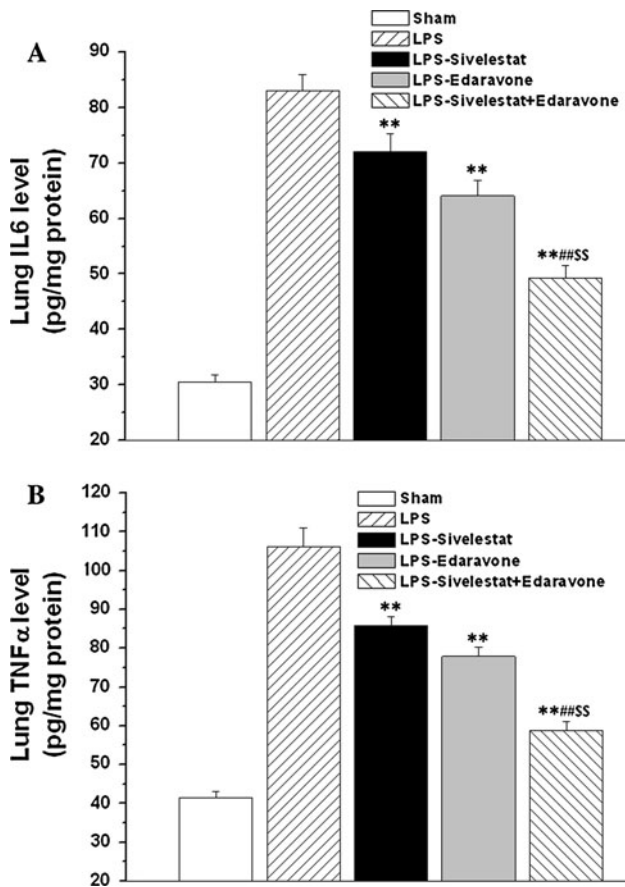


Fig. 4 Effect of therapeutic treatment with sivelestat and/or edaravone on IL-6 and TNF- α protein levels in lung tissues. Rats were instilled intratracheally with LPS (2 mg/kg) to develop acute lung injury for 12 h, not treated, or subjected to the following treatment: sivelestat (10 mg/kg) and/or edaravone (8 mg/kg) given 1 h after administration of LPS. IL-6 (a) and TNF- α (b) concentration in lung tissues were determined as described in “Materials and methods”. Data are mean \pm SEM of nine rats for each group. ** p < 0.01 versus LPS; *** p < 0.01 versus LPS-sivelestat; ^{SS} p < 0.01 versus LPS-edaravone

improvement of histological changes, indicating the additive effects of sivelestat and edaravone on LPS-induced lung injury.

Neutrophil elastase is a member of the chymotrypsin superfamily of serine proteases [23]. Under physiological conditions, the main function of neutrophil elastase is to degrade foreign organic molecules phagocytosed by neutrophils, thus playing a central role in host defense [23]. However, in inflammatory tissues, neutrophil elastase is rapidly released from activated neutrophils, then degrades numerous extracellular matrix components including collagen, elastin, fibrin, fibronectin, and laminin, and finally leads to tissue destruction [10, 23]. In addition to its proteolytic activity, neutrophil elastase also exhibits pro-inflammatory effects. It can induce the production of IL-6, IL-8, and TNF- α , and enhance neutrophil migration [24, 25].

In the case of ALI/ARDS, accumulating data have shown that neutrophil elastase activity is significantly increased in bronchoalveolar lavage fluid and/or plasma from patients with ARDS [26], or ALI/ARDS animal models [27, 28]. In the present study, we found that therapeutic treatment of sivelestat, a small molecular weight inhibitor of neutrophil elastase, exhibited protective effects against LPS-induced lung injury. In agreement with our findings, previous studies have reported the protective effects of sivelestat against ALI/ARDS caused by other stimuli such as acid aspiration [29], mechanical ventilation [30], hemorrhagic shock [31], and hyperoxia [32]. Taken together, these findings support the idea that neutrophil elastase plays an important role in the pathogenesis of ALI/ARDS.

Irrespective of the etiology of ALI/ARDS, abnormal generation of ROS occurs during the course of ALI, leading to oxidative stress. The uncontrolled generation of ROS even results in oxidative damage. The lung is especially vulnerable to ROS because it possesses the largest endothelial surface area of any organ in the body [33]. On this basis, treatment with reactive oxygen scavengers has been proposed for ALI/ARDS.

ROS include free radical intermediates, such as singlet oxygen (O), superoxide (O_2^-) and hydroxyl free radical (OH^-), as well as nonradical molecules, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [34]. Of them, the hydroxyl radical is more destructive than any other ROS, and directly causes more than 50% of the free radical-mediated molecular destruction of cells [35]. Edaravone is known to mainly trap hydroxyl radicals and inhibit OH-dependent lipid peroxidation; moreover it also scavenges other free radicals such as the peroxy radical [36]. In the present study, we demonstrated that therapeutic treatment with edaravone significantly protected rats against LPS-induced neutrophil infiltration, pro-inflammatory cytokine production, and tissue injury in lung. In addition, edaravone treatment significantly alleviated membrane lipid peroxidation levels in the lungs of rats with ALI. These data suggest that the antioxidant property of edaravone may account for its protective effects in LPS-induced lung injury.

Increasing evidence suggests that the hypoxanthine-xanthine oxidase system contributes significantly to LPS-induced ALI [37]. It has been found that both LPS and pro-inflammatory cytokines can induce a dramatic increase in lung xanthine oxidase expression and activity and prominent lung injury, which can be successfully prevented by xanthine oxidase inhibition [38–40]. These studies clearly suggest that the ROS production in LPS-mediated lung injury is at least partly dependent on the activation of the hypoxanthine-xanthine oxidase system. Besides its proteolytic and pro-inflammatory activity, neutrophil elastase is

also known to facilitate the conversion of xanthine dehydrogenase to xanthine oxidase, thus increasing the activity of an enzyme system which can produce oxidative stress [41]. Previous studies have shown that administration of sivelestat significantly decreased the production of hepatic or testicular MDA caused by ischemia–reperfusion injury [42, 43]. In contrast, the present study showed that sivelestat alone has no effect on lung MDA production induced by LPS instillation. We noticed that effective sivelestat treatment was either prophylactically administered before induction of ischemia–reperfusion injury in the study by Nakano et al. [43], or given with a higher dose of 60 mg/kg in the study by Tsounapi et al. [42], whereas it was administered 1 h after inducing ALI with a dose of 10 mg/kg in the present study. Thus, we believe that the use of different protocols of sivelestat administration, different animal models, and different injured sites may account for the discrepancy between the results of two previous studies and those of the present study.

Although the potential effectiveness of sivelestat has been proved in various animal models of ALI/ARDS, controversial results are reported in two independent clinical trials. A phase III study conducted by Tamakuma et al. [44] showed an improvement in pulmonary function and favorable trends in the mortality rate and the duration of mechanical ventilation. However, Zeiher et al. [45] later demonstrated that intravenous sivelestat had no effect on 28-day all-cause mortality or ventilator-free days in a heterogeneous acute lung injury patient population managed with low tidal volume mechanical ventilation. To clarify the mechanisms underlying these discordant results, Matsumoto et al. [15] recently observed the relationship between the clinical efficacy of sivelestat and the baseline oxidative stress levels in ALI patients. They found that sivelestat therapy was more effective in ALI patient with lower oxidative stress levels. In the present study, we showed for the first time that the efficacy of sivelestat in attenuating LPS-induced lung injury was significantly enhanced in combination with antioxidant therapy, i.e. the free radical scavenger edaravone. Taken together, these findings suggest that the combination of neutrophil elastase inhibitor and free radical scavenger may become a new treatment option for ALI/ARDS patients.

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