

Inhibition of c-Jun N-terminal Kinase Signaling Pathway Alleviates Lipopolysaccharide-induced Acute Respiratory Distress Syndrome in Rats

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Abstract

Background: An acute respiratory distress syndrome (ARDS) is still one of the major challenges in critically ill patients. This study aimed to investigate the effect of inhibiting c-Jun N-terminal kinase (JNK) on ARDS in a lipopolysaccharide (LPS)-induced ARDS rat model. **Methods:** Thirty-six rats were randomized into three groups: control, LPS, and LPS + JNK inhibitor. Rats were sacrificed 8 h after LPS treatment. The lung edema was observed by measuring the wet-to-dry weight (W/D) ratio of the lung. The severity of pulmonary inflammation was observed by measuring myeloperoxidase (MPO) activity of lung tissue. Moreover, the neutrophils in bronchoalveolar lavage fluid (BALF) were counted to observe the airway inflammation. In addition, lung collagen accumulation was quantified by Sircol Collagen Assay. At the same time, the pulmonary histologic examination was performed, and lung injury score was achieved in all three groups.

Results: MPO activity in lung tissue was found increased in rats treated with LPS comparing with that in control (1.26 ± 0.15 U in LPS vs. 0.77 ± 0.27 U in control, $P < 0.05$). Inhibiting JNK attenuated LPS-induced MPO activity upregulation (0.52 ± 0.12 U in LPS + JNK inhibitor vs. 1.26 ± 0.15 U in LPS, $P < 0.05$). Neutrophils in BALF were also found to be increased with LPS treatment, and inhibiting JNK attenuated LPS-induced neutrophils increase in BALF (255.0 ± 164.4 in LPS vs. 53 (44.5-103) in control vs. 127.0 ± 44.3 in LPS + JNK inhibitor, $P < 0.05$). At the same time, the lung injury score showed a reduction in LPS + JNK inhibitor group comparing with that in LPS group (13.42 ± 4.82 vs. 7.00 ± 1.83 , $P = 0.001$). However, the lung W/D ratio and the collagen in BALF did not show any differences between LPS and LPS + JNK inhibitor group.

Conclusions: Inhibiting JNK alleviated LPS-induced acute lung inflammation and had no effects on pulmonary edema and fibrosis. JNK inhibitor might be a potential therapeutic medication in ARDS, in the context of reducing lung inflammatory.

Key words: Acute Respiratory Distress Syndrome; c-Jun N-terminal Kinase Inhibitor; Lung Inflammation $C_{14}H_{18}N_2O$

INTRODUCTION

The incidence of acute respiratory distress syndrome (ARDS) has reached up to 5.0–33.8/100,000 population per year according to the recent epidemiological studies.^[1-4] The overall mortality of ARDS remains at 40–50%. Therefore, it is still a big challenge for both critically ill patients and clinicians.^[5-7] New strategies are required urgently in ARDS management.

The uncontrolled inflammatory cascade has been considered to be the main cause of the ARDS and leads to multiple organ dysfunction syndrome.^[8-10] Earlier studies have tried to block some of the inflammatory mediators but failed to generate a significant therapeutic effect in ARDS.^[11-14] During the last

decade, the inflammatory cascade was found to be activated through a rapid responding intracellular signaling system in the plasma.^[15] c-Jun N-terminal kinase (JNK) was first found to be one of the intracellular signaling pathways related with stress and inflammation; therefore, it was originally named

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as a stress-activated protein kinase.^[16] Now, JNK was known to be a member of mitogen-activated protein kinase (MAPK) family responsible for the cytokine production in the stress progress.^[15] Recently, studies confirmed that blocking JNK with a selectively JNK inhibitor SP600125 was a promising therapeutic strategy in inhibiting inflammatory process in brain injury, senile dementia, and Parkinson's disease.^[17]

Inhibiting JNK might also be beneficial in acute lung injury.^[16,18,19] We therefore examined whether JNK inhibition could act as a new therapeutic strategy in preventing ARDS progression, particularly in pulmonary inflammation, edema, and fibrosis.

METHODS

Experimental animals

Thirty-six male Sprague-Dawley rats aged 7–8 weeks (180–280 g) were purchased from the Medical Experimental Center of Southern Medical University, Guangzhou, China (License No.: SCXK[Guangdong]-2011-0015). The experimental protocol was approved by the Animal Care Committee of the Ethical Committee on Animal Research at Sun Yat-Sen University. All procedures were performed strictly according to the *National Institute of Health Guide for the Care and Use of Laboratory Animals*.

Reagents

SP600125, the JNK inhibitor (C₁₄H₈N₂O, powder, 50 mg/bottle) was purchased from the Biomol Co., (Exeter, UK). Lipopolysaccharide (LPS) was purchased from *Escherichia coli* (Sigma-Aldrich, St. Louis, USA). Masson trichrome staining solution was purchased from Sigma Chemical Co., (St. Louis, USA). The myeloperoxidase (MPO) determination kit was purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). The mouse radioimmunoassay kit was purchased from Radioimmunoassay Institute of the General Hospital of Chinese People's Liberation Army (Beijing, China). Soluble collagen kit was purchased from Biocolor Ltd., (Antrim, UK).

Experimental protocols

Thirty-six rats were divided randomly into three groups: control, LPS, and LPS + JNK inhibitor (SP600125). All rats were anesthetized with 10% chloral hydrate (300 mg/kg body weight) before any procedures. Sodium chloride (0.9%, 0.5 ml) was given intratracheally in the control group. LPS (10 mg/kg, 0.5 ml) was given intratracheally in the LPS group. JNK inhibitor (SP600125, 10 mg/kg) was administered intravenously through caudal vein following LPS injection (10 mg/kg, 0.5 ml) intratracheally in the LPS + JNK inhibitor group. Moreover, an equal volume of the solvent of SP600125 (glycol, 20% polypropylene glycol, 15% polyoxyethylated castor oil, 5% ethanol, and 30% normal saline) was injected intravenously in both control group and LPS group.

Bronchoalveolar lavage and lung tissue harvesting

All rats were sacrificed at 8 h after LPS administration. The bronchoalveolar lavage was performed with intratracheal

instillation of 6 ml normal saline into the right lower lobe. BALF was collected for neutrophils counting. The right upper lobe was also used for Masson trichrome collagen staining. The right middle lobe was used to determine MPO activity. The left lower lobe was harvested for the measurement of wet-to-dry weight (W/D) ratio of the lung. The left upper lobe was fixed with 5% formaldehyde for hematoxylin and eosin (H and E) staining.

Pathologic observation of lung tissue

The lung sections were fixed in 5% formaldehyde solution and stained with H and E. Pathological changes of lung tissue were evaluated under a light microscope (Olympus BX51, Tokyo, Japan). A previously described scoring system was used for quantification of lung injury severity.^[20] The pathological features were determined by the following changes: focal thickening of alveolar membrane, capillary congestion, intra-alveolar hemorrhage, pulmonary interstitial neutrophil infiltration, and intra-alveolar neutrophil infiltration. Each feature was scored from 0 to 3 based on absence (0), presence or mild (1), moderate (2), and severe (3). A total histology score (THS) was then calculated.

Neutrophils count in the bronchoalveolar lavage fluid

The neutrophils in bronchoalveolar lavage fluid (BALF) were counted under a light microscope (Olympus BX51, Tokyo, Japan).

Myeloperoxidase activity of the lung tissue

Lung tissue (100 mg) was homogenized in 2 ml extraction buffer. MPO activity was then measured according to the instruction provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The optical density of the microplates was read at 460 nm in a plate reader (Thermo Multiskan MK3, Philadelphia, PA, USA).

Lung wet-to-dry weight ratio

The wet weight of lung tissue was measured once the lung was harvested. The dry weight was obtained by placing the lung tissue into an 80°C incubator (LW Scientific Incubator-30L/1 Cubic ft, Lawrenceville, GA, USA) for 48–72 h till the weight getting stable.

Collagen measurement in lung tissue

The lung tissue was homogenized in 1 ml of Sircol reagent for 30 min and then centrifuged at 5000 ×g for 10 min. The pellet was then transferred into 1 ml of soda reagent. Collagen was measured with Sircol™ Collagen Assay kit according to the manufacturer's instruction (Biocolor Ltd., Carrickfergus, County Antrim, UK).

Masson's trichrome staining of the collagen in lung tissue

The lung sections were fixed in 5% formaldehyde solution and stained with Masson trichrome staining solution according to the instruction provided by the manufacturer (Sigma Chemical Co., St. Louis, USA). Collagen deposition was evaluated under a light microscope (Olympus BX51, Tokyo, Japan).

Statistical analysis

The SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for the data analysis. The normal distribution of quantitative data is tested by Kolmogorov-Smirnov and Shapiro-Wilk tests. The quantitative data were expressed as a mean \pm standard deviation (SD) for normal distribution and median (interquartile range) for non-normal distribution. These data were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test for normal distribution data and Dunnett's *post hoc* test for non-normal distribution data. A value of $P < 0.05$ was considered to denote a significant difference for all analyses.

RESULTS

Pathological observation of lung tissue

The structure of the alveoli in the control group was complete and there was no obvious pathological change under a light microscope [Figure 1a]. The structure of alveoli in LPS group was destroyed and the inflammatory cells, mainly neutrophils, were found widely spread in most alveoli. Red blood cells and fibrin were exuded into alveoli and spread along alveolar-capillary membrane [Figure 1b]. In LPS + JNK inhibitor group, the structure of the alveoli was partially restored compared with that in LPS group. The neutrophils and red blood cells infiltration were reduced comparing with that in LPS group. The alveolar-capillary interval was not as thick as that in LPS group [Figure 1c].

THS of lung injury was significantly higher in the LPS group comparing with that in control group (13.42 ± 4.82 vs. 3.60 ± 0.55 , $P = 0.001$). Meanwhile, THS decreased significantly in the LPS + JNK inhibitor group comparing with that in LPS group (7.00 ± 1.83 , $P = 0.001$) [Figure 2].

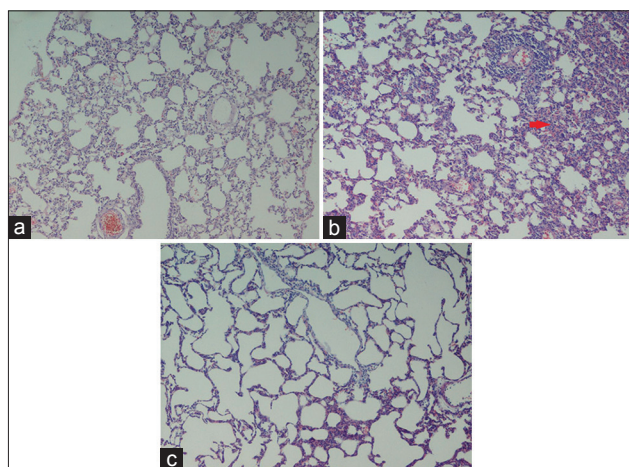


Figure 1: Effects of the JNK inhibitor on lung injury in LPS-treated rats. Representative lung specimens obtained from the control group (a: Control group; b: LPS group, and c: LPS + JNK inhibitor group, original magnification $\times 100$, Hematoxylin and Eosin Staining). The neutrophil counts in large and small airways, and hemorrhage markedly decreased on JNK inhibitor treated group. The red arrow points to the neutrophils and red blood cells infiltration. JNK: c-Jun N-terminal kinase; LPS: Lipopolysaccharide.

Neutrophils in bronchoalveolar lavage fluid

The neutrophils in BALF increased significantly in LPS group compared with that in control group (255 ± 164.4 /ml vs. 53 (58.5) ml, $P < 0.05$). It was significantly reduced in LPS + JNK inhibitor group comparing with that in LPS group (255.0 ± 164.4 /ml vs. 127.0 ± 44.3 /ml, $P < 0.05$) [Figure 3].

Myeloperoxidase activity in the lung tissue

MPO activity in the lung tissue increased significantly in the rats treated with LPS comparing with that in control group (1.26 ± 0.15 U/mg in LPS group vs. 0.77 ± 0.27 U/mg in control group, $P < 0.05$). Moreover, this increase was reduced significantly in rats treated with SP600125 (1.26 ± 0.15 U/mg in LPS group vs. 0.52 ± 0.12 U/mg in LPS + JNK inhibitor group, $P < 0.05$) [Figure 4].

Wet-to-dry weight ratio of lung tissue

No significant difference was found in W/D ratios of lung tissues among three groups (0.20 ± 0.02 in control group vs. 0.18 ± 0.02 in LPS group vs. 0.18 ± 0.02 in LPS + JNK inhibitor group, $P > 0.05$) [Figure 5].

Collagen content in lung tissue

Collagen content in lung tissue was significantly higher in LPS group than that in control group (45.08 ± 5.97 mg/g in LPS group vs. 8.65 ± 6.74 mg/g in control group, $P < 0.05$). However, there was no significant difference between LPS + JNK inhibitor group and LPS group (52.08 ± 14.06 mg/g in LPS + JNK inhibitor vs. 45.08 ± 5.97 mg/g in LPS group, $P > 0.05$) [Figure 6].

Masson's trichrome staining of the collagen in lung tissue

No collagen deposition was observed in control group [Figure 7a]. Slight collagen deposition was found in both LPS group and LPS + JNK inhibitor group but no obvious difference was found between these two groups [Figure 7b and 7c].

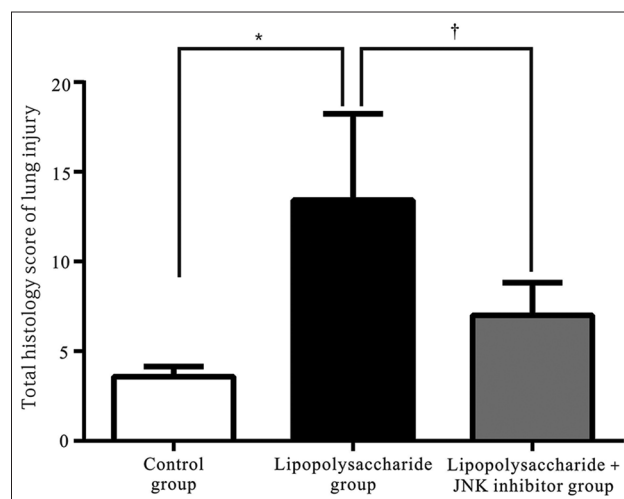


Figure 2: The total histology score was significantly lower in the LPS + JNK inhibitor group than that in the LPS group. The values presented are the mean \pm SD ($n = 12$ in each group). * $P < 0.05$ versus control. † $P < 0.05$ versus LPS group. JNK: c-Jun N-terminal kinase; LPS: Lipopolysaccharide; SD: Standard deviation.

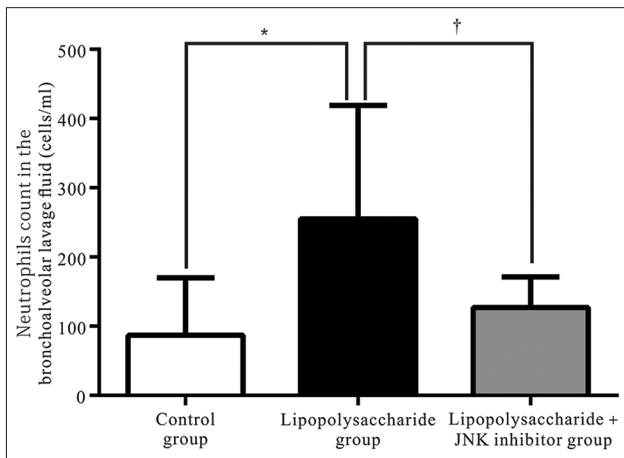


Figure 3: The amount of neutrophils in BALF was significantly lower in the LPS + JNK inhibitor group than that in the LPS group. The values presented are the mean \pm SD ($n = 12$ in each group). * $P < 0.05$ versus control. † $P < 0.05$ versus LPS group. BALF: Bronchoalveolar lavage fluid; LPS: Lipopolysaccharide; JNK: c-Jun N-terminal kinase; SD: Standard deviation.

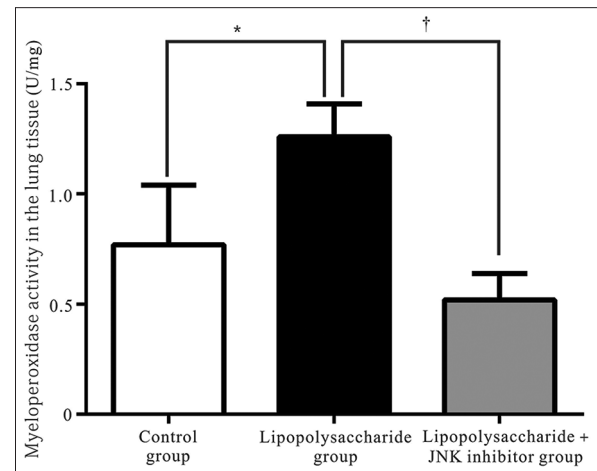


Figure 4: MPO activity. Higher MPO activity was induced after LPS injection, and MPO activity was significantly lower in animals in the LPS + JNK inhibitor group than that in the LPS group. The values presented are the mean \pm SD ($n = 12$ in each group). * $P < 0.05$ versus control. † $P < 0.05$ versus LPS group. MPO: Myeloperoxidase; LPS: Lipopolysaccharide; JNK: c-Jun N-terminal kinase; SD: Standard deviation.

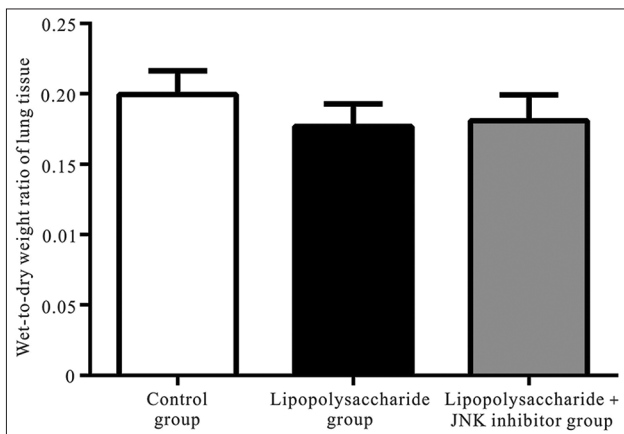


Figure 5: W/D ratio of lung tissue. No significant difference was found in W/D ratio of the lung tissue among the three groups. The values presented are the mean \pm SD ($n = 12$ in each group). SD: Standard deviation; W/D: Wet-to-dry weight.

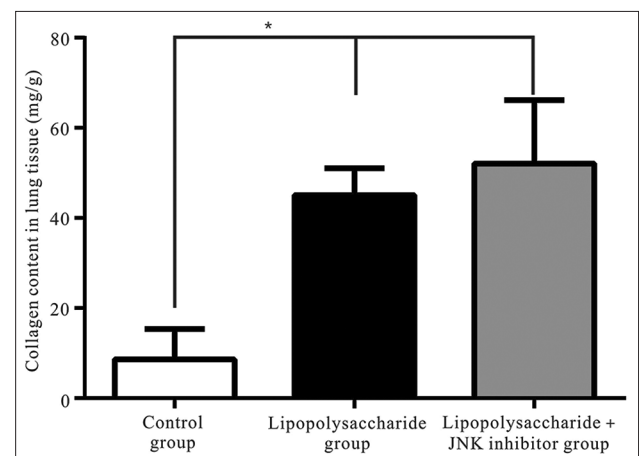


Figure 6: Collagen content in lung tissue. Collagen content in lung tissue was significantly higher in LPS group than that in control group. There was no significant change between LPS group and LPS + JNK inhibitor group. The values presented are the mean \pm SD ($n = 12$ in each group). * $P < 0.05$ versus control. LPS: Lipopolysaccharide; JNK: c-Jun N-terminal kinase; SD: Standard deviation.

DISCUSSION

In this study, we investigated the effect of a JNK selective inhibitor, SP600125, in a rat model of LPS-induced ARDS. Pulmonary inflammation, fibrosis, and edema were all studied to evaluate the effects of inhibiting JNK pathway on ARDS. Acute lung inflammation was one of the major pathological changes occurring in ARDS. In this study, we found that JNK inhibitor administration resulted in a significant attenuation of LPS-induced acute pulmonary inflammation. The alveoli structure was partially restored, the neutrophils infiltration was reduced, and the alveolar-capillary interval was normal in rats treated with JNK inhibitor. These findings were consistent with Lee *et al.*^[19] and Arndt *et al.*'s^[21] study in ARDS model, which showed JNK inhibition alleviated LPS-induced neutrophils infiltration in the lung. However, the studies by Lee *et al.* and Arndt *et al.* were only focused on inflammation but not lung fibrosis and edema. Lung collagen accumulation

and edema were found existed early in ARDS and associated with poor prognosis of ARDS patients.^[22-24] In this study, we demonstrated that JNK inhibitor alleviated lung inflammation but had no effect on lung collagen accumulation and lung edema. This implicated the efficacy of JNK inhibitor in inflammation and also the safety in edema and fibrosis as a possible therapeutic drug in ARDS in the future.

Lung collagen accumulation was caused by increased synthesis of procollagen I and/or imbalanced synthesis and degradation of collagen in the early stage of ARDS.^[25,26] A previous study by our colleagues showed that transforming growth factor- β (TGF- β) was a critical cytokine that regulated the synthesis of procollagen I and homeostasis

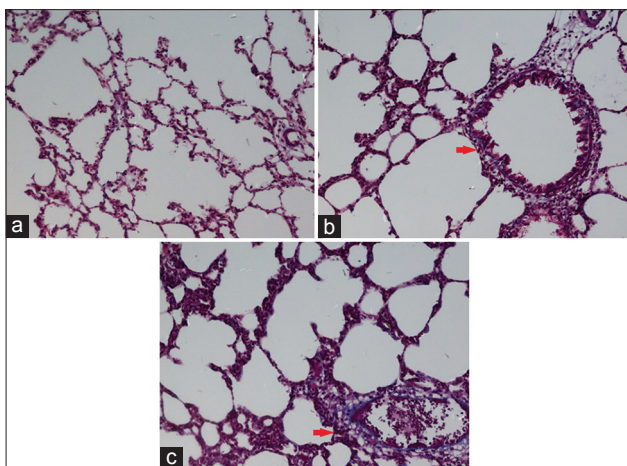


Figure 7: Masson's trichrome staining of the collagen in lung tissue specimens after LPS administration in rats. Representative lung specimens obtained from the control group (a: original magnification $\times 200$), LPS group (b: original magnification $\times 200$), and LPS + JNK inhibitor group (c: original magnification $\times 200$). Collagen deposition was obvious in lung tissue in LPS and JNK inhibitor group. There was no significant difference between LPS + JNK inhibitor group and LPS group. The red arrow points to collagen deposition. LPS: Lipopolysaccharide; JNK: c-Jun N-terminal kinase.

of collagen.^[27] TGF- β 1 was found to work through the p38 MAPK signal pathway but not JNK.^[28-35] Thus, we postulated that p38, but not JNK, was involved in the LPS-induced acute pulmonary fibroproliferation and JNK inhibitor had no obvious effect on pulmonary fibrosis in ARDS.

Pulmonary edema is another main pathological change of ARDS, which occurred due to impaired alveolar fluid clearance, increased capillary endothelial permeability, and damaged alveolar epithelial barrier.^[36] In a recent study by Zheng *et al.*,^[37] the lung edema was significantly attenuated with JNK inhibitor. However, in this study, no significant difference was found among control, LPS group, and LPS + JNK inhibitor group. Other pathways might be involved in LPS-induced lung edema. In our previous study, we found that LPS-induced dysfunction of airway epithelial barrier in ARDS and p38 was involved in the LPS-induced dysfunction of airway epithelial barrier.^[38] An earlier study by Migneault *et al.*^[39] also reported that LPS downregulates ENaC mRNA via ERK1/2 and p38 MAPK in alveolar epithelial cells. Frank *et al.*^[36] found that TGF- β 1 downregulated the expression of ENaC, an important sodium channel in the surface of alveolar epithelial cells, and affected the liquid transport in the alveoli. Whether the crosstalk existed between JNK and these factors needs more investigation.

The present study had some limitations. SP600125 is a general inhibitor of JNK family including JNK1, JNK2, and JNK3.^[18] Further research might be needed to investigate the detail underlying mechanism of JNK family.

In conclusion, our results indicated that inhibition of JNK exerted its anti-inflammatory activity without any effects on pulmonary fibrosis and edema in ARDS. Taking together, manipulation of JNK/MAPK pathway could be a potential

therapeutic target for ARDS in the context of suppressing inflammation.

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Conflicts of interest

There are no conflicts of interest.

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