Effects of a synthetic protease inhibitor (gabexate mesilate) and a neutrophil elastase inhibitor (sivelestat sodium) on acid-induced lung injury in rats.

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Abstract

The present study was designed to examine the combined effects of a synthetic protease inhibitor (gabexate mesilate) with a specific neutrophil elastase inhibitor (sivelestat sodium) on acid-induced lung injury. Adult male Sprague-Daley rats weighing 300-350 g were anesthetized intraperitoneally with pentobarbital sodium and the right jugular vein was cannulated. Following tracheostomy, rats were ventilated mechanically and underwent intratracheal instillation of hydrochloric acid (HCl, 0.1N 1.5 ml/kg) or normal saline. Thirty minutes before HCl instillation, gabexate mesilate (10 mg/kg, i.p.) and/or sivelestat sodium (10mg/kg/h, i.v.) were administered. Bronchoalveolar lavage fluid samples were obtained 5 h after HCl instillation. In bronchoalveolar lavage fluid, the HCl-induced increases in total nucleated cell counts, neutrophil counts, optical density at 412 nm, as an index of pulmonary hemorrhage, concentrations of albumin and cytokine-induced neutrophil chemoattractant (CINC) were significantly attenuated by either gabexate mesilate or sivelestat sodium treatment. Gabexate mesilate or sivelestat sodium treatment also significantly attenuated the wet to dry weight ratio induced by HCl. However, combined treatment with both gabexate mesilate and sivelestat sodium did not exhibit additive effects on HCl-induced lung injury, compared with the single treatment. These findings suggest that gabexate mesilate and sivelestat sodium each exhibited protective effects on acid-induced lung injury, respectively, but that synergistic effects of both agents are limited in this acid-induced lung injury model.

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Key words: hydrochloric acid, acute lung injury, acute respiratory distress syndrome, neutrophil elastase, protease inhibitor, neutrophil

1. Introduction

Aspiration of gastric contents is one of the most important direct triggers for the development of acute lung injury/acute respiratory distress syndrome (ARDS) (Rubenfeld et al, 2005). Acid aspiration-induced lung injury in animals is an experimental model of acute lung injury/ARDS as a result of aspiration pneumonia. Numerous experimental studies have shown that aspiration-induced lung injury is characterized by neutrophil accumulation and neutrophil-activating pro-inflammatory cytokine production in the lungs (Davidson et al., 1999; Folkesson et al, 1995; Kennedy et al., 1989; Hagio et al., 2004; Jian et al., 2004, 2005). Sivelestat sodium, a neutrophil elastase inhibitor, is able to attenuate acid-induced lung injury by preventing neutrophil accumulation into the lung and the production of cytokine-induced neutrophil chemoattractant (CINC) and neutrophil elastase in rat (Jian et al, 2004) and hamster lungs (Hagio et al, 2004).

On the other hand, a synthetic serine protease inhibitor, gabexate mesilate, exhibits various degrees of biological activity against the inflammatory process and the coagulation system (Ohno et al., 1981; Taenaka et al., 1983). The pharmacological activity of this agent is as an anticoagulant via the blockade of thrombin and active coagulation factors (Ohno et al, 1981) and has been shown to be clinically effective in treating disseminated intravascular coagulation (Taenaka et al, 1983). However, gabexate mesilate directly prevented the activation of NF-κB in lipopolysaccharide (LPS) -stimulated murine macrophages and human monocytes resulting in reduced production of proinflammatory cytokines, including tumor necrosis factor (TNF), interleukin (IL)-6, high mobility group box 1 (HMGB1), etc (Hidaka et al., 2009; Murakami et al., 1996; Yuksel et al., 2003; Uchiba et al., 2003). Thus, the effects of gabexate mesilate are also considered to be due to its anti-inflammatory properties. In fact, gabexate mesilate has been reported to be protective for various models of acute lung injury including endotoxin (Murakami et al, 1996), ischemia/reperfusion (Harada et al, 1999), smoke inhalation (Niehaus et al, 1990) and cerulein-induced pancreatitis (Chen et al, 1997). However, there have been no previous reports about the effects of gabexate mesilate on acid-induced lung injury in animal models.

Hagiwara *et al.* (2009) recently demonstrated that sivelestat sodium could reduce various inflammatory mediators by inhibiting the NF-κB pathway in LPS-stimulated murine macrophages, which was similar to the inhibitory mechanism of gabexate mesilate via NF-κB (Aosasa, et al., 2001; Hidaka et al.,2009; Uchiba et al., 2003). In addition, gabexate mesilate was unable to inactivate neutrophil elastase (Nakatani et al, 2001) and the blockade of NF-κB by gabexate mesilate also inhibited the expression of endothelial leukocyte adhesion molecules during exposure to LPS, which was not observed with sivelestat sodium (Nakatani et al, 2001; Uchiba et al, 2003). These findings suggested that additive or synergistic therapeutic effects of gabexate mesilate and sivelestat sodium could be expected in activated neutrophil-mediated lung injury.

Accordingly, we examined the effects of gabexate mesilate on direct injury to the lung, in an acid-aspiration model of lung injury in rats. Furthermore, the present study was designed to investigate the synergistic effects of gabexate mesilate combined with sivelestat sodium on acid-induced lung injury, focusing on neutrophil accumulation and the production of neutrophil related cytokines.

2. **Materials and Methods**

2.1 Ethical considerations

The study protocol was approved by the Institutional Review Board for the care of animals at Shinshu University. The care and handling of animals was conducted in accordance with the guidelines of the National Institutes of Health. The animals had free access to commercial rodent food and were given free access to drinking water.

2.2 Animals and drugs

Adult Sprague-Dawley male rats (300-350 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan). Sivelestat sodium hydrate (sodium *N*-[2-[4-(2, 2-dimethylpropionyloxy) phenylsulfonyl amino]benzoyl] aminoacetate tetrahydrate and gabexate mesilate ([ethyl p-(6-guanidinohexanoyloxy) benzoate] methanesulfonate) were provided by Ono Pharmaceutical Co. Ltd. (Osaka, Japan).

2.3 Animal models and study design (Fig.1)

Animals were anesthetized with pentobarbital sodium (40mg/kg) intraperitoneally and maintained with injections every two hours. The right jugular venous line (a 3-Fr silicon tube) was prepared for pretreatment with the drugs and fluid maintenance (normal saline 1 ml/hour). Following tracheostomy, the animals were ventilated mechanically (Respirator Model SN-480-7, Rodent Ventilator; Shinano, Tokyo, Japan) for 5 h at a tidal volume (Vt.) of 10 ml/kg, a frequency of 50 breaths/min and a fraction of inspired O_2 (FiO_2) of 1.0. The rats were placed horizontally on a board inclined at 45 degrees, and hydrochloric acid (HCl, 0.1N 1.5 ml/kg) or normal saline was instilled into the trachea via the tracheostomy with 0.5ml of air, followed immediately by 5 seconds of chest percussion to facilitate the uniform spread of the acid.

Animals were randomly divided into the following five experimental groups (Fig.1): 1) Control (Cont) group: normal saline instillation, normal saline administration and normal saline infusion, 2) HCl group: HCl instillation, normal saline administration and normal saline infusion, 3) Gabexate mesilate group: HCl instillation, gabexate mesilate administration and normal saline infusion, 4) sivelestat sodium group: HCl instillation, normal saline administration and sivelestat sodium infusion, 5) gabexate mesilate plus sivelestat sodium combination group: HCl instillation, gabexate mesilate administration and sivelestat sodium infusion. The agents were administered thirty minutes before HCl or normal saline instillation. Gabexate mesilate (10 mg/kg) or normal saline (same volume as gabexate mesilate, about 1ml/body) were administered once intraperitoneally. Sivelestat sodium (10mg/kg/h) or normal saline (1ml/kg/h, same volume of sivelestat sodium) were infused continuously into the right jugular vein with an infusion pump (TE-312, Terumo, Tokyo, Japan). In addition, gabexate mesilate or sivelestat sodium treatment alone was also performed independently in rats undergoing normal saline administration and normal saline infusion.

In the present study, the following two experiments were performed separately. Experiment 1; 5 h after the instillation of the HCl or normal saline, animals were sacrificed ad a blood sample was drawn from the left ventricle of the heart at the end of the experiment and bronchoalveolar lavage was performed to collect bronchoalveolar lavage fluid. The lungs of each animal were lavaged with 20 ml of normal saline (5ml of normal saline x 4 times, n=8 in each group). Experiment 2: 5 h after HCl or normal saline instillation, lung tissue samples were isolated to evaluate the histopathological

changes and assess wet to dry weight ratios (*n*=8 in each group).

2.4 Measurements

2.4.1 Blood sample:

White blood cells were counted and the differential counts of neutrophils were performed on blood smears stained with May-Giemsa stain. Platelet counts were performed on ethylendiaminetetraacetic acid (EDTA) anticoagulated blood using an automated counting device (Sysmex KX-21, SYSMEX, Japan).

2.4.2 Bronchoalveolar lavage fluid

Distilled water (0.9 ml) was added to 0.1 ml of the bronchoalveolar lavage fluid sample in a test tube. The test tube was gently inverted and centrifuged at 3000 *g* for 10 min. The optical density (OD) of the sample was read at 412 nm with a Double Beam Spectrophotometer (UVIDEC-610A, Japan Spectroscopic Co., Ltd, Tokyo, Japan) to assess the pulmonary hemorrhage.

The remaining bronchoalveolar lavage fluid was then centrifuged at 3000 *g* for 10 minutes. The supernatant was removed and stored at –70 °C for subsequent experiments. The cell pellet was mixed with 1 mL of distilled water and the total cells were counted. Cell monolayers were prepared by cytocentrifugation for neutrophil counts. Differential counts were performed on 200 cells from smears stained with May-Giemsa stain. The albumin concentration in the bronchoalveolar lavage fluid was determined by nephelometric immunoassay. CINC-1 (rat IL-8) concentration in the bronchoalveolar lavage fluid was measured by enzymed-linked immunosorbent assay (ELISA) kit (TFB Co., Ltd, Tokyo, Japan).

2.4.3 Assessment of pulmonary edema and histapathological findings:

The right lung was weighed (wet weight) and then heated at 80°C in a convection oven (Programmable incubator IC-300P, Iuchi Osaka, Japan) for 72 h until the tissue reached a constant dry weight. The wet to dry weight ratio was calculated to assess pulmonary edema.

The left lung was perfused with 10% formaldehyde and then inflated to a pressure of 25 cm H_2O. The samples were fixed in 10% formalin solution and then embedded in paraffin. Sections (5 μm) were stained with hematoxylin-eosin for light microscopic analysis. Neutrophil accumulation in lungs was quantitated by counting neutrophils at the alveolar septal walls. Microscopic fields containing other structures, such as airways, large vessels, and pleura, were excluded. Neutrophil entrapment was expressed as the mean number of neutrophils per 10 high-power fields at 1,000 magnifications (Neut/10HPF).

2.5 Statistical analysis

Data are presented as the mean ± standard deviation (S.D.) for each experimental group. Comparisons among the experimental groups were conducted with the analysis of variance (Tukey-Kramer test). A *p*-value of 0.05 was used as the cut-off point for significance.

3 **Results**

Neither sivelestat sodium nor gabexate mesilate alone treatment affected any of the parameters in control rats, compared with those in both baseline and control groups (data not shown).

3.1 Blood white blood cells and neutrophil counts

White blood cells and neutrophil counts are shown in Fig.2. HCl instillation significantly reduced the peripheral white blood cell counts and increased neutrophil counts. Likewise, gabexate mesilate and sivelestat sodium also both significantly reduced the white blood cells and increased neutrophil counts compared with controls. However, the reduction in white blood cells after HCl administration was less with the combined treatment of sivelestat sodium and gabexate mesilate, and neutrophil counts in peripheral blood increased significantly compared with that in HCl group. There were no significant differences in platelet counts before and after HCl instillation and among the experimental groups (data not shown).

3.2 Total nucleated cell and neutrophil counts in bronchoalveolar lavage fluid

Recovery rates of bronchoalveolar lavage fluid samples were greater than 90% in all groups and there were no significant differences in the recovery rates between the groups. Total nucleated cell count and the neutrophil counts in bronchoalveolar lavage fluid are summarized in Fig. 3. The total nucleated cell counts and neutrophil differential increased significantly after HCl instillation. Gabexate mesilate and sivelestat sodium significantly reduced the neutrophil counts compared with the HCl group. The reduction in total nucleated cell and neutrophil counts was also observed with the combined treatment of sivelestat sodium and gabexate mesilate, and showed a significant difference from those in the gabexate mesilate group (Fig.3).

3.3 Albumin concentration and pulmonary hemorrhage index in bronchoalveolar lavage fluid

Albumin concentrations in bronchoalveolar lavage fluid were significantly higher in the HCl group compared with the control group, suggesting increased lung permeability due to HCl instillation. Gabexate mesilate and sivelestat sodium significantly reduced the albumin concentration, compared with the HCl group. The combined treatment significantly improved lung permeability compared with HCl alone and gabexate mesilate treatment (Fig.4). Likewise, the absorbance (OD_{412}), an index of pulmonary hemorrhage, was significantly higher in the HCl group than that in the other groups, suggesting marked hemorrhage in lung tissue after exposure to HCl. Both sivelestat sodium and gabexate mesilate significantly improved the absorbance in bronchoalveolar lavage fluid. However, the effect of combined treatment of gabexate mesilate plus sivelestat sodium on reduced pulmonary hemorrhage was not statistically significant compared with gabexate mesilate or sivelestat sodium, suggesting that there was no synergistic effect on the protection against pulmonary hemorrhage.

3.4 Cytokines in bronchoalveolar lavage fluid

CINC-1 in bronchoalveolar lavage fluid increased significantly in the HCl group compared with that in the control group. Both sivelestat sodium and gabexate mesilate were effective in reducing the production of this proinflammatory cytokine compared with the HCl group (Fig. 5). However, there was no significant effect of combined treatment of gabexate mesilate plus sivelestat sodium in further reducing CINC-1, compared with gabexate mesilate or sivelestat sodium.

Results described above were obtained by experiment 1. Following two results were

done in separated experiment 2.

3.5 Lung wet to dry weight ratios

Wet to dry weight ratios were significantly higher in the HCl group compared with the control group, suggesting an increase in lung edema after HCl instillation. Sivelestat sodium significantly decreased the wet to dry weight ratio. However, there was no significant improvement in the wet to dry weight ratio between the single agent treatments and the combined gabexate mesilate and sivelestat sodium treatments (Fig. 6).

3.6 Lung histopathological findings

Neutrophil entrapment in the alveolar wall was significantly increased in the HCl group. Gabexate mesilate significantly reduced the neutrophil counts (Neut/10HPF) in lung tissue samples, as did sivelestat sodium, and a synergistic effect was achieved with the combination treatment of these agents (Fig.7). Microscopic examination of the lungs revealed that the alveolar wall was thickened and damaged by HCl instillation. Neutrophil infiltration and hyaline membrane formation were also observed in the alveolar spaces. These findings were attenuated in the treatment groups, especially in the combined treatment group (Fig.8).

4 Discussion

In the present study, we have demonstrated the protective effects of gabexate mesilate and sivelestat sodium on acid-induced lung injury in rats, respectively. In addition, the combined treatment with gabexate mesilate and sivelestat sodium exhibited further significant reductions in neutrophil accumulation and albumin concentrations in bronchoalveolar lavage fluid, compared with gabexate mesilate treatment alone. However, the combined treatment did not achieve a significant reduction in CINC-1, lung edema (wet to dry weight ratio) or lung hemorrhage, suggesting that the additive or synergistic effect was limited in acid-induced lung injury in rats.

The results following sivelestat sodium treatment in the present study were mainly consistent with our previous (Jian et al., 2004, 2005) and other studies (Folkesson et al, 1995; Hagio et al, 2004) in acid aspiration-induced lung injury. Protective effects of sivelestat sodium against other types of acute lung injury have also been associated with a reduction of neutrophil infiltration (Sakamaki et al., 1996; Yamamoto et al., 2000; Iba et al., 2006). Sivelestat sodium reduced neutrophil counts in bronchoalveolar lavage fluid and attenuated LPS-induced (Sakamaki et al, 1996) or hyperoxic lung injury (Yamamoto et al, 2000). Furthermore, it has been shown that neutrophil elastase can potentially be pro-inflammatory. For example, neutrophil elastase can induce the secretion of IL-6 and IL-8 from epithelial cells and enhance neutrophil migration (Bedard et al., 1993; Walsh et al., 2001). On the other hand, various cytokines, including TNF-α and IL-8, promote the release of neutrophil elastase from neutrophils (Rainger et al, 1998). We have previously shown that sivelestat sodium reduced CINC-1, TNF-α and IL-6 concentrations in bronchoalveolar lavage fluid from acid-aspirated rats, and that these effects were associated with reduced neutrophil accumulation and neutrophil elastase production in the lung (Jian et al., 2004, 2005). Yamaguchi et al. (1997) demonstrated that NE-induced *in vitro* CINC-1 production by Kupffer cells isolated from normal rats was inhibited by sivelestat sodium. Taken together, these findings suggest a possible interaction among neutrophils, neutrophil elastase and the inflammatory cytokines. Thus, activated neutrophils recruited to the lung and the release of neutrophil elastase could mainly contribute to the development of acid-induced lung injury.

Gabexate mesilate, a synthetic protease inhibitor, inhibits various kinds of proteases, such as trypsin, plasmin kallikrein and thrombin (Ohno et al., 1981; Taenaka et al., 1983). In the present study, gabexate mesilate also significantly decreased neutrophil counts, albumin, pulmonary hemorrhage and the concentrations of CINC-1 in bronchoalveolar lavage fluid, resulting in attenuation of acid-induced lung injury. Several studies have demonstrated the anti-inflammatory properties of gabexate mesilate in various experimental models. Gabexate mesilate prevented the endotoxin-induced pulmonary vascular injury, primarily by inhibiting the production of TNF-α in endotoxin-stimulated monocytes (Murakami et al,1996). Yuksel et al. (2003) reported that gabexate mesilate suppressed TNF-α production in human monocytes by inhibiting the activation of both NF-κB and activator protein-1. In a rat model of ischemia/reperfusion injury, gabexate mesilate attenuated the hepatic injury and suppressed hepatic levels of TNF-α and IL-8 (Harada et al, 1999). Consistent with these early findings, we also found in this study that gabexate mesilate diminished CINC concentrations in bronchoalveolar lavage fluid and prevented the lung injury induced by acid aspiration. Based on the previous findings of the mechanism of gabexate mesilate,

the effect of gabexate mesilate on the reduced production of CINC might be due to inactivation of NF-κB pathways, although the precise mechanism was not examined in the present study. Our results suggested that gabexate mesilate could improve acid-induced lung injury, mainly through its anti-inflammatory properties. Thus, this agent may have therapeutic potential in patients with ARDS caused by acid-aspiration pneumonia.

However, the additive or synergistic effect of gabexate mesilate plus sivelestat sodium was limited against acid aspiration in the present study, although the combined treatment showed significant protection in certain parameters in bronchoalveolar lavage fluid. While the reason for this remains undetermined in the present study, we have two possible explanations. It is well known that this lung injury model exhibits a biphasic injury pattern, with an "early phase" (0-1h after aspiration) that is associated with the direct chemical effects of the acid, and an additional "late phase" (4-6 h after aspiration) that is associated with neutrophil-mediated inflammation (Kennedy et al, 1989). It has shown that although the production of neutrophil elastase in this model was almost abolished by sivelestat sodium, the parameters in lung injury were not fully inhibited (Hagio et al., 2004; Jian et al., 2004). Thus, direct chemical injury by the acid was partially responsible for acid-induced lung injury. In other words, the prevention of the lung injury and edema formation after acid instillation by sivelestat sodium might be adequate, although the cytokine production or inflammatory process were fully prevented. Alternatively, we suggest that the injurious interplay between the NF-κB pathway mediated cytokine and neutrophil elastase seemed to be minimally or unrelated to the development of acid-induced lung injury in rats. Since there is no information about a direct relationship or pharmacological interaction between sivelestat sodium and gabexate mesilate, it will be necessary to further examine the pharmacological interaction of both agents.

In the LPS-exposed pulmonary circulation, microscopic examination has demonstrated narrowing or obstruction of the capillary lumen due mainly to adhesion and plugging of neutrophils and sticking of platelets (Kiefmann et al.,2004; Iba et al., 2006). In these experiments, platelet counts were decreased in peripheral blood. Thus, the coagulation system might contribute in part to the development of or further damage in certain experimental models of acute lung injury. Initially, we anticipated that gabexate mesilate would exhibit anti-coagulation effects as well as the anti-inflammatory effects in the present model. We measured platelet counts in the peripheral blood and found that there were no significant differences between normal controls and acid-treated rat. Lian et al. (2005) also reported that platelet counts and fibrin degradation product (FDP) in peripheral blood did not significantly change after acid aspiration in rats. Thus, we believe that the coagulation system is unlikely to contribute to the development of acid-aspiration lung injury in rats, although platelet and FDP representative only a portion of the coagulation system. Accordingly, the main protective effect of gabexate mesilate in acid-aspiration lung injury could be due to its anti-inflammatory properties.

In summary, a synthetic protease inhibitor, gabexate mesilate, and a neutrophil elastase inhibitor, sivelestat sodium, are useful to protect against acid-induced lung injury in rats. However, combined treatment with gabexate mesilate and sivelestat sodium, did not show an additive protection against the inflammation induced by acid-induced lung injury in rats. We conclude that the synergistic effects of gabexate

mesilate combined with sivelestat sodium may be limited in the acid-induced rats lung injury model. There might be interaction between the anti-inflammatory pathways affected by gabexate mesilate and sivelestat sodium in this experimental model in rats.

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Figure Legends

Figure 1. Experimental protocol. There were five experimental groups. Experiment 1) Control (Cont) group: normal saline instillation, normal saline administration and continuous normal saline infusion, 2) HCl group: HCl instillation, normal saline administration and continuous normal saline infusion, 3) gabexate mesilate group: HCl instillation, gabexate mesilate administration (intraperitoneally) and continuous normal saline infusion, 4) sivelestat sodium group: HCl instillation, normal saline administration and continuous sivelestat sodium infusion, 5) gabexate mesilate plus sivelestat sodium combination group: HCl instillation, gabexate mesilate administration (intravenously) and continuous sivelestat sodium infusion.

Figure 2. Total white blood cell and neutrophil counts in peripheral blood from rats treated with normal saline (cont, n=8), HCl (n=8), gabexate mesilate (n=8), sivelestat sodium (n=8), and gabexate mesilate puls sivelestat sodium (n=8). Values are expressed as the mean ± S.D. + P< 0.05 vs. the control group; * P< 0.05 vs. the HCl group.

Figure 3. Total nucleated cell counts and neutrophil counts in bronchoalveolar lavage fluid from rats treated with normal saline (cont, n=8), HCl (n=8), gabexate mesilate (n=8), sivelestat sodium (n=8), and gabexate mesilate puls sivelestat sodium (n=8). Values are expressed as the mean ± S.D. + P< 0.05 vs. the control group; * P< 0.05 vs. the HCl group. $ P <0.05 vs. the gabexate mesilate group.

Figure 4. Albumin concentrations and pulmonary hemorrhage index in bronchoalveolar lavage fluid samples from rats treated with normal saline (cont, n=8), HCl (n=8), gabexate mesilate (n=8), sivelestat sodium (n=8), and gabexate mesilate puls sivelestat sodium (n=8). Values are expressed as the mean ± S.D. + P< 0.05 vs. the control group; * P< 0.05 vs. the HCl group. $ P <0.05 vs. the gabexate mesilate group.

Figure 5. Cytokine-induced neutrophil chemoattractant-1(CINC-1) levels in bronchoalveolar lavage fluid from rats treated with normal saline (cont, n=8), HCl (n=8), gabexate mesilate (n=8), sivelestat sodium (n=8), and gabexate mesilate puls sivelestat sodium (n=8). Values are expressed as the mean ± S.D. + P< 0.05 vs. the NS group; * P< 0.05 vs. the HCl group.

Figure 6. Wet to dry weight ratios of lungs from rats treated with normal saline (cont, n=8), HCl (n=8), gabexate mesilate (n=8), sivelestat sodium (n=8), and gabexate mesilate puls sivelestat sodium (n=8). Values are expressed as the mean ± S.D. + P< 0.05 vs. the control group; * P< 0.05 vs. the HCl group.

Figure 7. Neutrophil entrapment in the lungs of rats treated with normal saline (cont, n=8), HCl (n=8), gabexate mesilate (n=8), sivelestat sodium (n=8), and gabexate mesilate puls sivelestat sodium (n=8). The values are expressed as the mean number of neutrophils per 10 high-power fields (X 1000) (Neut/10HPF) ± S.D. + P< 0.05 vs. the control group; * P< 0.05 vs. the HCl group, $ P <0.05 vs. the gabexate mesilate group

Figure 8. Photomicrographs of the light microscopic appearance of lung tissue from

the HCl, sivelestat sodium, gabexate mesilate, and gabexate mesilate puls sivelestat sodium at x 200 magnification. Sections were stained with hematoxylin and eosin. In the HCl group, congested alveolar walls, alveolar edema and hyaline membranes with disruption of the alveolar wall were observed. Numerous neutrophils and red blood cells are present in the alveolar spaces. In the sivelestat sodium or gabexate mesilate groups, few neutrophils and red blood cells were seen in the airspaces. In gabexate mesilate puls sivelestat sodium group, little alveolar edema or hyaline membranes was evident. The integrity of the alveolar walls was better preserved in this treatment groups.

Fig.1

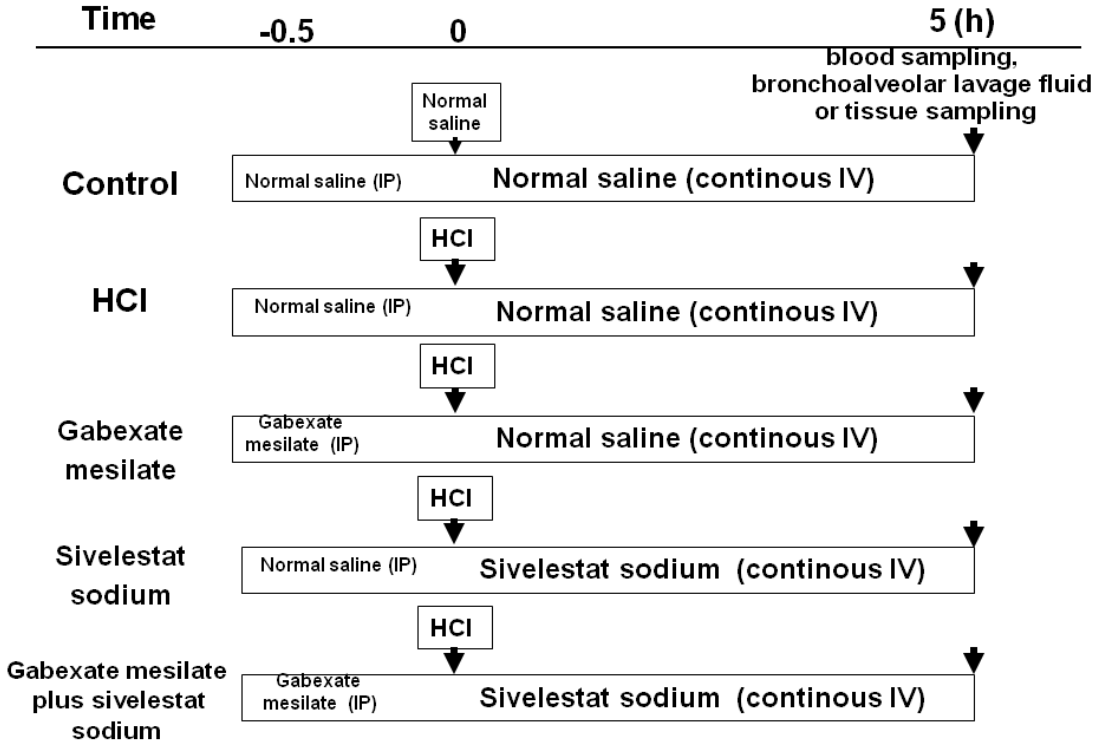

Time
-0.5
0
5 (h)
blood sampling,
bronchoalveolar lavage fluid
or tissue sampling
Normal saline
Control
Normal saline (IP)
Normal saline (continous IV)
HCl
HCl
Normal saline (IP)
Normal saline (continous IV)
HCl
Gabexate mesilate
Gabexate mesilate (IP)
Normal saline (continous IV)
HCl
Sivelestat sodium
Normal saline (IP)
Sivelestat sodium (continous IV)
HCl
Gabexate mesilate plus sivelestat sodium
Gabexate mesilate (IP)
Sivelestat sodium (continous IV)

Fig.2

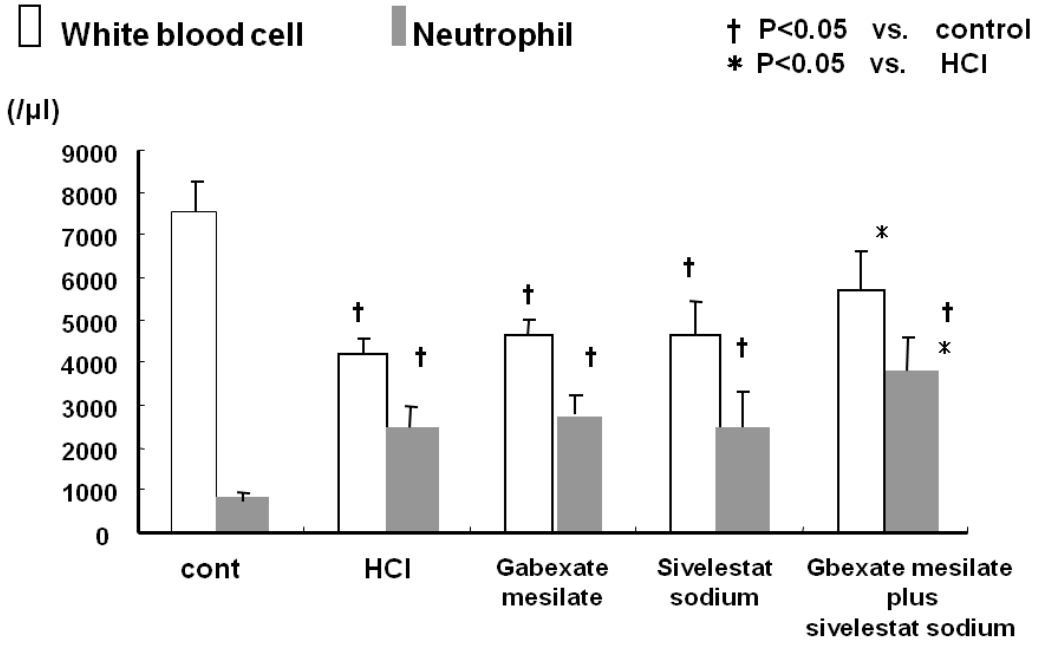
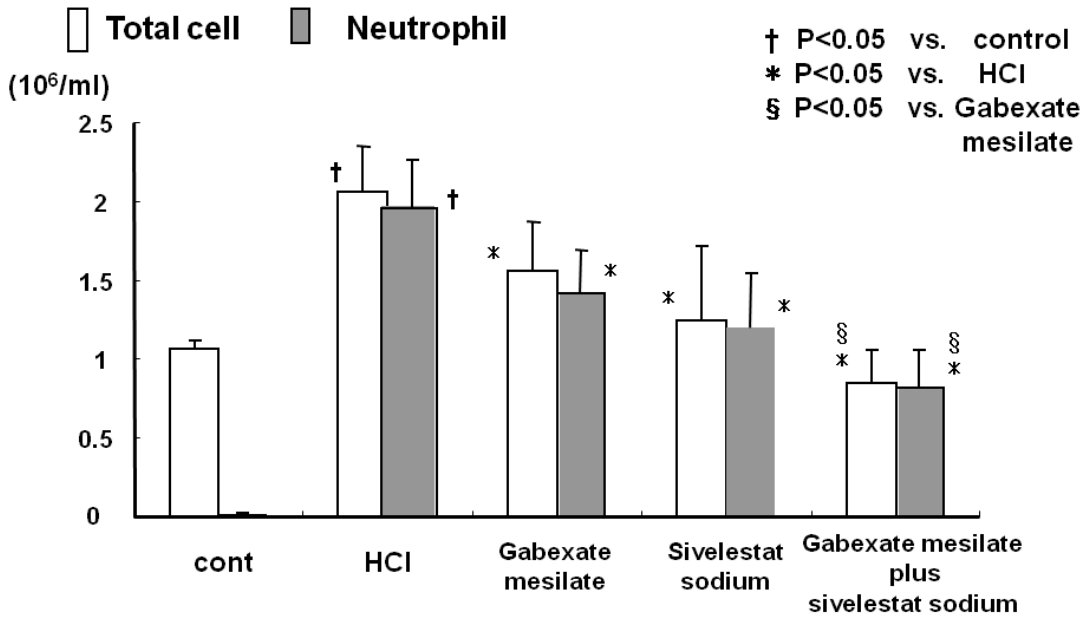

White blood cell
Neutrophil
† P<0.05 vs. control
* P<0.05 vs. HCl
(/μl)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
cont
HCl
Gabexate mesilate
Sivelestat sodium
Gbexate mesilate plus sivelestat sodium

Fig.3


Total cell
Neutrophil
(10⁶/ml)
† P<0.05 vs. control
∗ P<0.05 vs. HCl
§ P<0.05 vs. Gabexate mesilate
2.5
2
1.5
1
0.5
0
cont
HCl
Gabexate mesilate
Sivelestat sodium
Gabexate mesilate plus sivelestat sodium

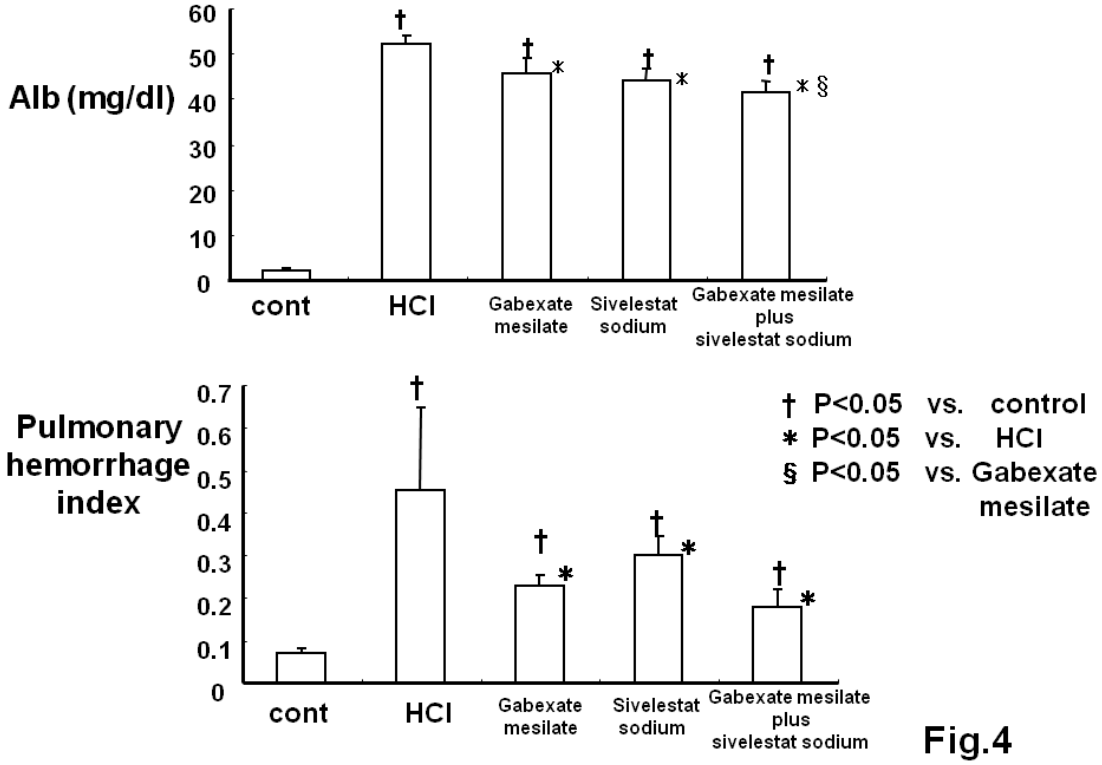

Alb (mg/dl)
60
50
40
30
20
10
0
cont
HCl
Gabexate mesilate
Sivelestat sodium
Gabexate mesilate plus sivelestat sodium
Pulmonary hemorrhage index
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
† P<0.05 vs. control
* P<0.05 vs. HCl
§ P<0.05 vs. Gabexate mesilate

Fig.4

Fig.5

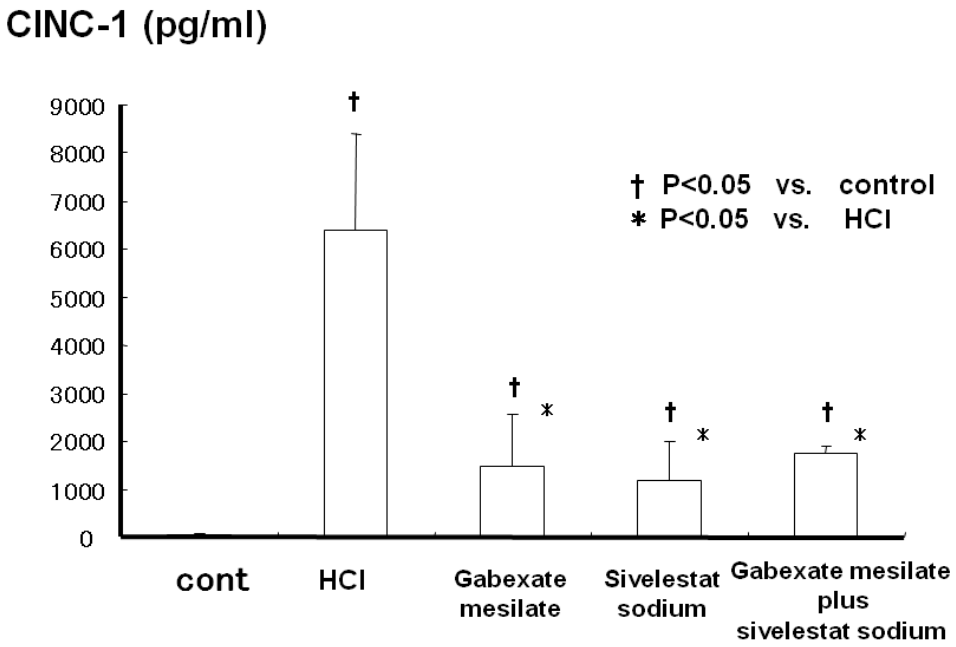

CINC-1 (pg/ml)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
† P<0.05 vs. control
∗ P<0.05 vs. HCl
cont
HCl
Gabexate mesilate
Sivelestat sodium
Gabexate mesilate plus sivelestat sodium

Fig.6

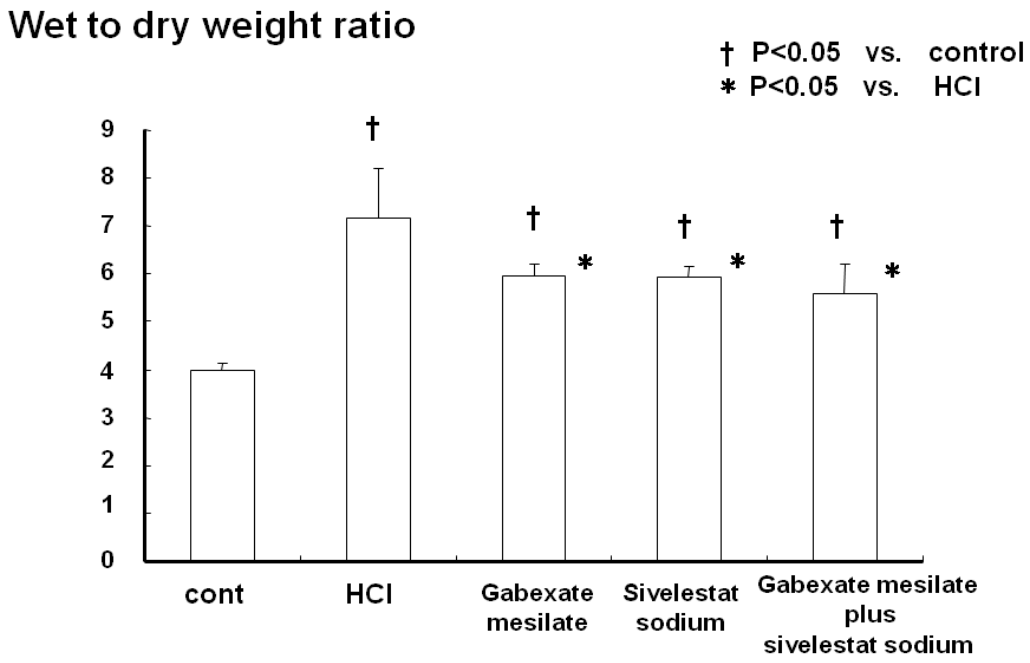
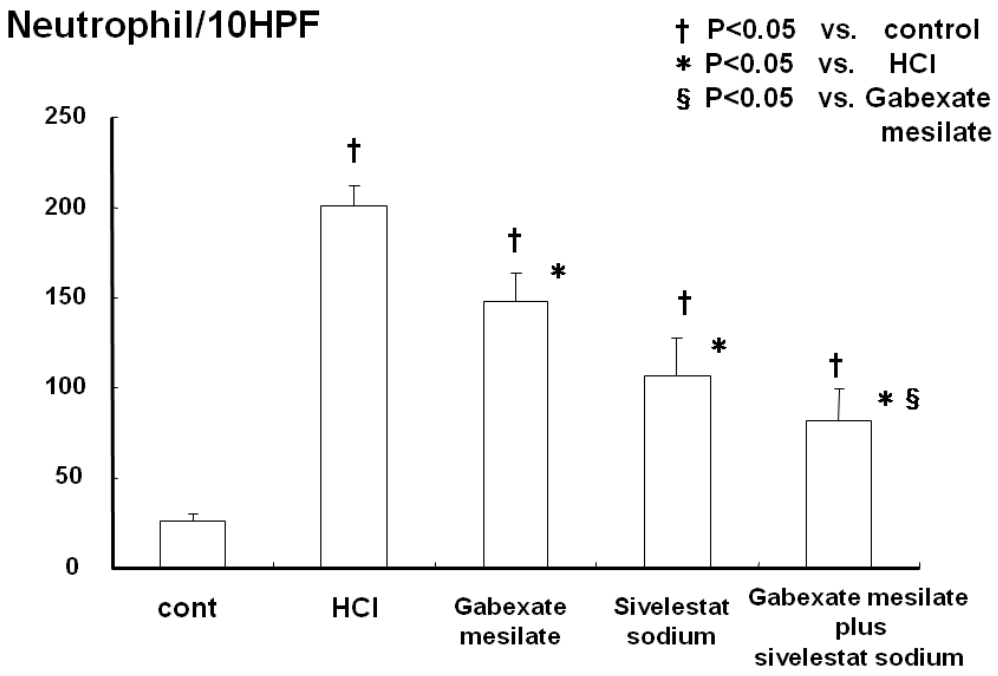

Wet to dry weight ratio
† P<0.05 vs. control
* P<0.05 vs. HCl
9
8
7
6
5
4
3
2
1
0
†
†
†
†
*
*
*
cont
HCl
Gabexate mesilate
Sivelestat sodium
Gabexate mesilate plus sivelestat sodium

Fig.7


Neutrophil/10HPF
† P<0.05 vs. control
* P<0.05 vs. HCl
§ P<0.05 vs. Gabexate mesilate
250
200
150
100
50
0
†
†
*
†
*
†
* §
cont
HCl
Gabexate mesilate
Sivelestat sodium
Gabexate mesilate plus sivelestat sodium

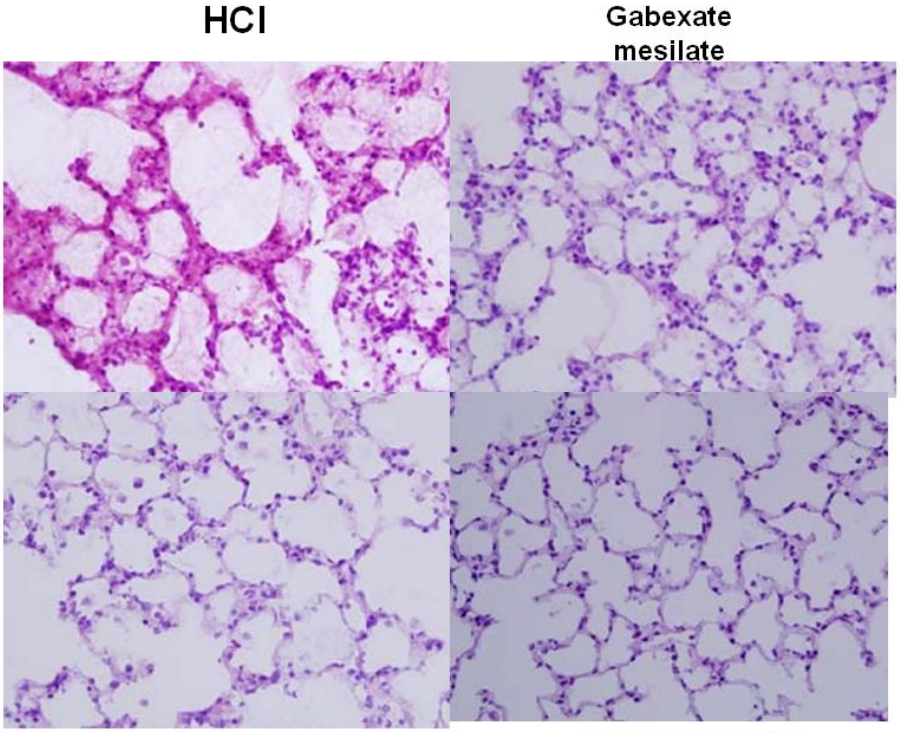

HCl
Gabexate mesilate
Sivelestat sodium
Gabexate mesilate plus sivelestat sodium

Fig.8