(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 September 2021 (23.09.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/185746 A1

(51) International Patent Classification:
A61K 31/165 (2006.01) *A61P 11/00* (2006.01)
A61K 31/415 (2006.01) *A61P 31/14* (2006.01)

(21) International Application Number:
PCT/EP2021/056504

(22) International Filing Date:
15 March 2021 (15.03.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
20163464.9 16 March 2020 (16.03.2020) EP
20215629.5 18 December 2020 (18.12.2020) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

(54) **Title:** COMPOUNDS FOR USE IN TREATMENT OF RESPIRATORY DISEASE

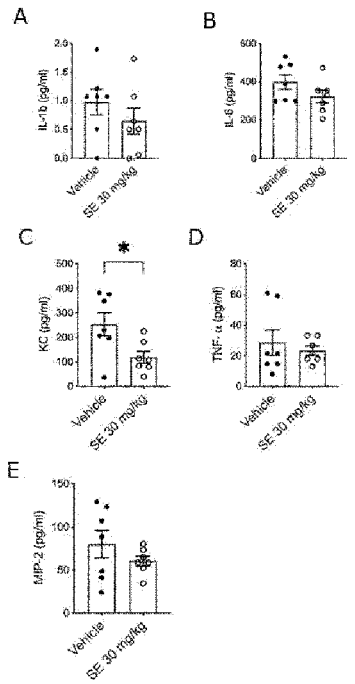

Fig. 10

(57) **Abstract:** The present invention relates to an inhibitor of the intermediate conductance calcium-activated potassium (KCa3.1) channels for use in the treatment of acute respiratory disease in a subject in need thereof, wherein said acute respiratory disease is caused by an infection with a coronavirus (CoV). The inhibitors may particularly be selected from senicapoc or derivatives thereof and TRAM-34. The coronavirus may particularly be SARS-CoV-2 also termed COVID-19.

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COMPOUNDS FOR USE IN TREATMENT OF RESPIRATORY DISEASE

Technical field of the invention

The present invention relates to compounds for use in the treatment of acute respiratory disease in relation to a viral infection in the respiratory tract. In particular the present invention relates to inhibitors of the intermediate conductance calcium-activated potassium (KCa3.1) channels for use in the treatment of acute respiratory disease wherein the respiratory disease is caused by an infection with a coronavirus (CoV).

Background of the invention

The severe acute respiratory syndrome coronavirus (SARS-CoV) strains are associated with a high number of fatalities. The current infection with SARS-CoV2 is becoming pandemic, and it results on average of 1-6% deaths in general infected population with worse outcomes (up to 15%) in patients suffering from respiratory disease, cardiovascular disease, hypertension, and diabetes. Key symptoms in the disease progression, towards a fatal outcome, is acute respiratory distress syndrome (ARDS) and pronounced dyspnea associated with hypoxia and on CT scanning as radiological changes in the lung with an ARDS-like disease. Despite current symptomatic treatment, including intensive care and respiratory support, the leading causes of death from SARS-CoV2 are ARDS-like disease and multi-organ failure. Apart from symptomatic treatment, there is currently no pharmacological treatment with an effect on the condition, and the 28-day mortality is approximately 40-50% in patients in intensive care for ARDS.

The current approaches for the development of treatment of COVID-19 infection and ARDS-like disease range from the development of vaccines and small molecules targeting the virus receptor or intracellular replication of the SARS-CoV virus, including testing of drugs for inhibition of human immunodeficiency virus (HIV).

Thus, for example in Gordon CJ, Tchesnokov EP, Feng JY, Porter DP, Gotte M. J. Biol. Chem. 2020, Feb 24 (ePub ahead of print), discloses that remdesivir potently inhibits RNA-dependent RNA polymerase from MERS-CoV.

Wandall-Frostholm, C. et al., *Br. J. Pharmacol.*, 2015, 172, pp. 4493–4505, discloses that a genetic deficiency of KCa3.1 channels in mice prevented fatal pulmonary circulatory collapse and reduced lung damage caused by pharmacological activation of calcium-permeable TRPV4 channels. Therefore, inhibition of KCa3.1 channels may have therapeutic potential in conditions characterized by abnormal high endothelial calcium signalling, barrier disruption, lung oedema and pulmonary circulatory collapse.

Simonsen, U. et al., *Acta Physiol. Scand*., 2017, 219, pp. 176–187, further discloses that certain inhibitors or blockers of the intermediate conductance calcium-activated potassium (KCa3.1) channels. It is found that a deficiency in KCa3.1 channels protects against TRPV4-induced pulmonary arterial relaxation, fluid extravasation, haemorrhage, pulmonary circulatory collapse and cardiac arrest in vivo. These data identify KCa3.1 channels as crucial molecular components in downstream TRPV4 signal transduction and as a potential target for the prevention of undesired fluid extravasation, vasodilatation and pulmonary circulatory collapse. The small molecule blockers of KCa3.1 channels include TRAM-34, senicapoc (ICA-17043) and others.

However, these studies did not address some of the cardinal changes in ARDS, hypoxia, and inflammation, and more importantly they did not address the potential treatment of ARDS and other pulmonary injuries specifically related to a viral infection with a coronavirus.

Hence, an improved treatment coronavirus related pulmonary injuries including ARDS would be advantageous, and in particular compounds for the effective treatment of ARDS and lung injuries/oedema in relation to coronavirus, which could treat both the ARDS and inhibit the spread and replication of the coronavirus in the treated subject would be advantageous.

Summary of the invention

Thus, an object of the present invention relates to providing an improved treatment of respiratory diseases which are related to, or a consequence of, viral infections, particularly respiratory issues in connection with an infection of a coronavirus such as for example MERS-CoV and particularly SARS-CoV.

In particular, it is an object of the present invention to provide compounds for treatment of respiratory disease in connection with subjects infected with coronaviruses, that solves the above mentioned problems of the prior art with providing compounds that treat both the pulmonary/respiratory disease and inhibits the replication of the virus causing the underlying infection.

Thus, a first aspect of the invention relates to an inhibitor of the intermediate conductance calcium-activated potassium (KCa3.1) channels for use in the treatment of acute respiratory disease in a subject in need thereof, wherein said acute respiratory disease is caused by an infection with a coronavirus (CoV).

Another aspect of the present invention relates to a pharmaceutical composition comprising the inhibitor of the intermediate conductance calcium-activated potassium (KCa3.1) channels for use in the treatment of acute respiratory disease in a subject in need thereof, wherein said acute respiratory disease is caused by an infection with a coronavirus (CoV) and a pharmaceutically acceptable carrier and/or excipient.

Brief description of the figures

Figure 1 shows, contractile response in pulmonary arteries obtained from mice to increasing concentrations of KCa3.1 channel blockers senicapoc and TRAM-34 following relaxation with the KCa3.1/KCa2.3 channel opener, NS309. It is observed that Senicapoc is more potent compared to TRAM-34.

Figure 2 shows, schematic diagram of the study protocol. Animals were randomly assigned in a blinded manner to one of five groups (n = 16): control, vehicle, 10 mg/kg, 30 mg/kg or 70 mg/kg BW senicapoc. Administration of drugs were given 30 minutes prior to ventilator-induced lung injury (VILI). Animals were subjected

to either control ventilation or VILI for two hours. Hereafter, animals were sacrificed and randomly selected for either histology or bronchoalveolar lavage and wet-to-dry weight analysis (n = 8 for each group).

Figure 3 shows the results of a permeability assay showing the protecting effect of senicapoc (SE) against cytokine (TNF-α)-induced permeability in the barrier function of the lung.

Figure 4A-E show, the effect of KCa3.1 knockout for the amount (pg/ml) of key proinflammatory cytokines in bronchoalveolar lavage fluid (BALF) obtained from wild type (WT) and KCa3.1$^{-/-}$ (KO) mice following ventilator-induced lung injury (VILI).

Figure 5 shows, that KCa3.1 channel inhibition by senicapoc (SE) protects against ventilator-induced lung injury observed by the differences in the PaO_2/FiO_2 ratio after 2 hr of VILI.

Figure 6 shows, that KCa3.1 channel by senicapoc (SE) inhibition protects against ventilator-induced lung injury indicated by the differences observed in the wet-to-dry lung weight ratio after 2 hr of VILI.

Figure 7 shows, that KCa3.1 channel inhibition by senicapoc (SE) protects against ventilator-induced lung injury as evidenced by the differences in protein concentration of the obtained bronchoalveolar lavage fluid after 2 hr of VILI.

Figure 8 shows, that KCa3.1 channel inhibition by senicapoc (SE) protects against ventilator-induced lung injury as indicated by the observed differences in lung quasistatic compliance after 2 hr of VILI.

Figure 9A-E show the effect of senicapoc (SE) on the amount (pg/ml) of cytokines in BALF obtained from mice injected with sterile saline (control), mice injected with DMSO (vehicle) and mice injected with solutions comprising senicapoc (SE) in concentrations of 10, 30, and 70 mg/kg before VILI. The figure shows that pre-treatment with senicapoc suppresses the key proinflammatory cytokines to a near control-group level.

Figure 10A-E show the effect of senicapoc (SE) on the amount (pg/ml) of cytokines in BALF obtained from mice injected with DMSO (vehicle) and mice injected with a solution comprising 30 mg/kg senicapoc (SE) following VILI. The figure shows that post-treatment with senicapoc suppresses the key proinflammatory cytokines to a near control-group level.

Figure 11A-E show, that KCa3.1 channel inhibition reduces lung tissue injury by depicting histological micrographs obtained during the VILI procedure. Arrows in 11B designates the presence in infiltrating neutrophils and stars indicates proteinaceous debris inside the alveolar space.

Figure 12 shows, that KCa3.1 channel inhibition by senicapoc (SE) reduces lung tissue injury by histopathological scoring of lung sections from mice that received similar vehicle or senicapoc treatments.

Figure 13A-E show, that KCa3.1 channel inhibition diminishes the degree of haemorrhage evidenced by the depicted representative histological micrographs of haemorrhage in the lung.

Figure 14 shows, that KCa3.1 channel inhibition by senicapoc (SE) diminishes the degree of haemorrhage by the summarised haemorrhage data shown as percentage area of total lung.

Figure 15 shows , that administration of senicapoc reduces (A) the level levels of BALF neutrophils and (B) neutrophils in the alveolar space by histological evaluations in a porcine model of ARDS. The data are presented as mean ± SD. *P<0.05 for senicapoc against vehicle. Vehicle, n = 9 and senicapoc, n = 8.

Figure 16 shows that senicapoc reduces histological pulmonary hemorrhage as a summary of interstitial and alveolar hemorrhage in a porcine model of ARDS. Data are presented as mean ± SD. *P<0.05 for senicapoc against vehicle. Vehicle, n = 9 and senicapoc, n = 8.

Figure 17 shows changes in PaO_2/FiO_2 ratio over time in rats that were subjected to cardiac arrest. Groups developed similarly over time. Data presented as mean ± SD. *=significant difference between the vehicle and senicapoc group at endpoint ($p<0.05$)

The present invention will now be described in more detail in the following.

Detailed description of the invention

Definitions

Prior to discussing the present invention in further details, the following terms and conventions will first be defined:

Inhibitor

In the present context, the term "Inhibitor" is interchangeable with the term "blocker" and refers to a molecule capable of inhibiting the function of a target, such as an intermediate conductance calcium-activated potassium channel protein. A blocker of such a channel will block the passage of potassium ions through a channel.

Intermediate conductance calcium-activated potassium channels

In the present context, intermediate conductance calcium-activated potassium channels are proteins that forms potassium channels activated by intracellular calcium. The proteins may be one of human or mouse intermediate conductance calcium-activated potassium channel protein 4 (KCa3.1) that are encoded by the human KCNN4 gene (UniProt number: O15554) or mouse Kcnn4 gene (UniProt number: O89109), respectively. The human channel is of most significance in the present context.

Acute respiratory disease

In the present context, acute respiratory disease refers to diseases correlated with acute respiratory disease syndrome (ARDS) which is a type of respiratory failure often associated with complications, such as but not limited to, widespread inflammation in the lungs, lung injury, hypoxaemia, and increased amounts of fluids within the lungs. Neutrophilic infiltration is considered a hallmark of human

ARDS and a central driver for the inflammatory response. Neutrophils has been found to constitute 68% of recovered lavage cells in ARDS patients. Without being bound to theory, mortality from ARDS correlates with the extent of neutrophilia in the lung. Furthermore, the neutrophil level in the lung is thought to correlate directly to the severity of abnormalities in gas exchange and the alveolar-capillary barrier.

Angiotensin-converting enzyme 2 (ACE2)

In the present context, angiotensin-converting enzyme 2 is an enzyme which acts as a receptor for coronaviruses and which is encoded by the gene ACE2 (UniProt number: Q9BYF1).

Subject

In the present context, the term "subject" refers to a patient, typically a human patient.

Coronavirus (CoV)

In the present context, the term "coronavirus" refers to a virus belonging to the coronavirus family, including all lineages thereof.

SARS-CoV

In the present context, the term "SARS-CoV" refers to a human SARS coronavirus, also known as severe acute respiratory syndrome coronavirus.

SARS-CoV-2

In the present context, the term "SARS-CoV-2" refers to severe acute respiratory syndrome coronavirus 2, which causes the disease COVID-19.

MERS-CoV

In the present context, the term "MERS-CoV" refers to Middle East respiratory syndrome coronavirus.

The present inventors have found that the intermediate conductance calcium-activated potassium (KCa3.1) channels play a pivotal role in the development of ARDS-like diseases. They have shown that mice treated with inhibitors or blockers

of these channels will not develop hypoxia and lung damage measured as accumulation of protein in bronchoalvoelar lavage fluid, or decreased lung compliance compared to untreated wildtype mice. The inventors further find that these channel blockers will inhibit the entry and/or replication of coronaviruses, either at the entry and/or downstream of the virus entry receptor angiotensin-converting enzyme 2 (ACE2). This makes these compounds particularly promising leads in the treatment of respiratory disease in connection with infections caused by coronaviruses.

Infection by SARS-Cov-2 virus induces damage to the alveolar epithelial cells whereby infiltration of immune cells increases rapidly leading to ARDS-like conditions and a severe inflammation of the lungs. The infiltrating immune cells produces a range of inflammatory biomarkers, e.g. different cytokines, that are associated with the coronavirus infection. The immune cells also release toxic mediators that further increases epithelial permeability and causes influx of protein-rich oedematous fluid followed by hypoxaemia.

Clinical reports on COVID-19 patients have shown a systemic hyper-inflammation triggered by SARS-CoV-2 infection in the lungs, thus leading to a "cytokine storm". In this condition, a patient has increased levels of inflammatory biomarkers, including: various interleukins, such as IL-6, macrophage inflammatory proteins (MIP), tumor necrosis factor-α (TNF-α), interferon-γ inducible protein (IP), C-reactive protein (CRP), ferritin and procalcitonin (PCT). The elevated cytokine production has been correlated with repolarizing potassium (K^+) channels following diverging intracellular Ca^{2+} regulations. Thus, the "cytokine storm" is thought to play an important role in the main mechanism of COVID-19 disease and, without being bound to theory, probably somehow affected or even controlled by working potassium channels. Reducing damage to the alveolar epithelial cells caused by virus attack and/or inhibition of transport of immune cells to cell tissue of the lungs (i.e. less production of cytokines) such as e.g. inhibition of potassium channels, are considered interesting methods that may be able to prevent and/or alleviate/treat the complications caused by infection with SARS-CoV-2 virus (Cardiac inflammation in COVID-19: Lessons from heart failure, Life Sciences, 260, 2020).

Thus a first aspect of the present invention is an inhibitor of the intermediate conductance calcium-activated potassium (KCa3.1) channels for use in the treatment of acute respiratory disease in a subject in need thereof, wherein said acute respiratory disease is caused by an infection with a coronavirus (CoV).

An alternative aspect is an inhibitor of the intermediate conductance calcium-activated potassium (KCa3.1) channels for use in the treatment of acute respiratory disease in a subject in need thereof, wherein said subject is also infected with a coronavirus (CoV).

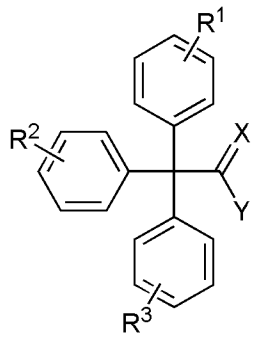
Yet another alternative aspect is a method of treating acute respiratory disease by administering an inhibitor of the intermediate conductance calcium-activated potassium (KCa3.1) channels to a subject in need thereof, wherein said acute respiratory disease is caused by an infection with a coronavirus (CoV).

Finally, another alternative aspect of the invention is the use of an inhibitor of the intermediate conductance calcium-activated potassium (KCa3.1) channels for the preparation of a medicament for use in the treatment of acute respiratory disease in a subject in need thereof, wherein said acute respiratory disease is caused by an infection with a coronavirus (CoV).

As indicated above the inhibitors of the KCa3.1 channels may preferable also inhibit the replication or entry of the coronavirus, and thus in a preferred embodiment of the invention the inhibitor of calcium-activated potassium (KCa3.1) channels is also an inhibitor of entry and/or replication of coronavirus (CoV). Preferably the inhibition of replication of coronavirus (CoV) is at the entry and/or downstream of the virus entry receptor angiotensin-converting enzyme 2 (ACE2).

Such dual inhibitors or blockers may preferably be selective and potent blocker of KCa3.1 channels selected from the group consisting of senicapoc (CAS No.: 289656-45-7) and TRAM-34 (CAS No.: 289905-88-0). These compounds are commercially available, and known blockers of KCa3.1 channels.

Senicapoc and derivatives thereof are particularly promising blockers of the KCa3.1 channels, while also inhibiting the replication of coronaviruses. Thus is one embodiment the inhibitor is a compound of formula (I):



(I)

wherein

R^1, R^2, and R^3 are independently selected from the group consisting of hydrogen and halogen,

X is selected the group consisting of S, O, and NR^4,

R^4 is selected the group consisting of H, methyl, and ethyl,

Y is selected from $N(R^5)R^6$, OR^5, and SR^5,

R^5 and R^6 are independently selected from the group consisting of hydrogen, methyl, ethyl, and propyl.

R^1, R^2, and R^3 may independently be in the *meta* or *para* position of the phenyl ring, preferably the *para* position of the phenyl ring.

In one preferred embodiment X is O. In another preferred embodiment Y is $N(R^5)R^6$. R^5 and R^6 are preferably hydrogen. The halogen is preferably selected from the group consisting of Cl, Br, and F, preferably F.

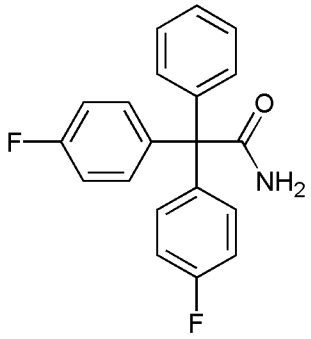
In a particularly preferred embodiment the inhibitor is a compound of formula (I), wherein:

R^1, R^2, and R^3 are independently selected from the group consisting of hydrogen, Cl, Br and F,

X is O, and

Y is NH_2.

More preferably the inhibitor is a compound of Formula (II):



(II).

Most preferably the inhibitor is senicapoc.

Viral infections in the respiratory tract may cause a number of acute respiratory diseases that may be treated with the compounds of the present invention. Thus, in one embodiment of the present invention the acute respiratory disease is selected from the group consisting of acute respiratory distress syndrome (ARDS), acute lung injury, lung oedema, pulmonary circulatory collapse, and virus pneumonia.

Hypoxia and inflammation are among the conditions and/or symptoms arising from infections, which are also treated by the present compounds.

There are a number of known coronaviruses. In a preferred embodiment the coronavirus (CoV) is selected from the group consisting of lineage B coronavirus and Lineage C coronavirus. More preferably the coronavirus (CoV) is selected from the group consisting of SARS-CoV and MERS-CoV. The SARS-CoV may particularly be selected from the group consisting of SARS-CoV-1, and SARS-CoV-2, such as preferably SARS-CoV-2. SARS-CoV-2 is a particular coronavirus also termed "coronavirus disease 2019" or COVID-19.

The inhibitors of the present invention may be administered in a therapeutically effective amount. They may be administered by any conventional route, in particular enterally, e.g. orally, e.g. in the form of tablets, capsules, drink solutions or parenterally, e.g. in the form of injectable solutions or suspensions. They may be administered as the sole active ingredient or they may be

administered in combination with at least one further active. The further active may preferably be an antiviral agent.

The one or more further active(s) may preferably be selected from the group consisting of remdesivir, lopinavir, ritonavir, ribavirin, alpha-interferon (IFN-α), or any combination thereof. Remdesivir is preferred. Particularly, lopinavir and ritonavir are provided as a combined antiviral treatment.

The one or more actives of the present invention may further be combined with administration of chloroquine.

The one or more actives of the present invention may further be combined with administration of dexamethasone.

Combinations of actives may be administered simultaneously or one after the other, i.e. in a staggered regimen.

A further aspect of the present invention is a pharmaceutical composition comprising the inhibitor of the intermediate conductance calcium-activated potassium (KCa3.1) channels for use in the treatment of acute respiratory disease in a subject in need thereof, wherein said acute respiratory disease is caused by an infection with a coronavirus (CoV) and a pharmaceutically acceptable carrier and/or excipient.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting examples.

Examples

Example 1 *– Materials and methods*

Materials

PSS was composed of (in mM): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.18 KH_2PO_4, 5.5 glucose, 1.6 $CaCl_2$ and 0.026 ethylenediaminetetraacetic acid (EDTA). KPSS, 60 mM Na^+ was replaced with 60 mM K^+. Apamin, phenylephrine, NS309 and TRAM-34 were purchased from Sigma-Aldrich (St. Louis, USA). Senicapoc was purchased from MedChemExpress (Sollentuna, Sweden). Stock solutions of NS309, TRAM-34, and senicapoc were prepared with dimethyl sulfoxide (DMSO). Before use, the stocks were diluted with PSS. The total concentration of DMSO in the organ baths never exceeded 0.2%, thus having no unspecific effects on the channels investigated. Phosphate buffered saline (PBS), formalin, haematoxylin, eosin, giemsa stain, midazolam, propofol, Ringer's acetate (sodium lactate solution), hematoxylin, rocuronium, adrenaline and other solutions and reagents were purchased from Sigma-Aldrich.

Human epithelial Calu-3 cells (ATCC, HTB-55™) are of a lung epithelial cell line obtained from ATCC, Teddington, Middlesex, United Kingdom.

Preparation of mice

KCa3.1 knockout animals were bought from Jacksons Laboratories (Maine, USA) and eight-to-12-week-old C57BL/6-backcrossed $KCa3.1^{-/-}$ male and female mice (20-30 g body weight [BW]) were derived from an established breeding colony and compared to their corresponding controls (n = 16) (Wandall-Frostholm, C. et al., *Br. J. Pharmacol.*, 2015, 172, p. 4495). Gene expression were checked using qPCR. Eight-to-12-week-old C57BL/6NTac male mice (20-30 g BW) were bought from Taconic Biosciences (Denmark) and used for myograph studies (n = 7), and for the testing of senicapoc in ventilator-induced lung injury (n = 94). All animals were housed according to local guidelines in a temperature and humidity-controlled SPF environment, kept on a 12-hour light/day cycle and allowed access to standard chow and water ad libitum. All protocols were approved by the Danish Animal Care Committee (permission no. 2017-15-0201-01351). Animal experiments were performed in adherence to the ARRIVE and BJP guidelines.

The animals were anaesthetised by subcutaneous injection of a 1:1:2 mixture consisting of hypnorm (fentanyl 315 µg/ml, fluanisone 10 mg/ml), midazolam (5 mg/ml) and sterile water (0.01 ml/g BW for induction; 0.003 ml/g BW for maintenance). The animals were kept on a 37 °C heated pad. The left carotid artery was isolated and cannulated for blood pressure monitoring and blood gas analysis. Tracheotomy was performed in a supine position, and the tracheal tube (18G cannula) was connected to a computer-controlled rodent ventilator system (Flexivent, FX1 module, SCIREQ, Canada). All groups were then ventilated with a tidal volume of 9-10 ml/kg BW, positive end-expiratory pressure (PEEP) of 3 cmH_2O, inspiratory-expiratory (I:E) ratio of 1:2 and respiratory rate (RR) of 135-140 breaths/min.

Preparation of pigs

Female crossbred Landrace/Yorkshire/Duroc pigs (42–49 kg) were fasted overnight with free access to water. In the morning, the animal was premedicated with an intramuscular injection of midazolam (1 mg/kg). Before placement of the intravenous (IV) access, an additional dose midazolam (0.625 mg/kg), s-ketamine (6.25 mg/kg), and atropine (0.5 mg) was given intramuscularly. Anesthesia was induced with intravenous midazolam (25 mg) and s-ketamine (250 mg) and maintained with a continuous infusion of fentanyl (60 µg/kg/hr) and propofol (5 mg/kg/hr). No neuromuscular blocking agent was used. The animals were placed in a supine position, intubated (8.0 mm, Portex tube, Smiths Medical International, Hoersholm, Denmark) and volume-controlled ventilated (Evita XL, Dräger, Germany) with a tidal volume of 10 mL/kg, a positive end-expiratory pressure (PEEP) of 5 cmH_2O, a fraction of inspired oxygen (FiO_2) of 0.4, and an inspiration:expiration (I:E) ratio of 1:2. The ventilation rate was adjusted to maintain $PaCO_2$ between 5.5 and 6 kPa. For fluid therapy, a bolus of Ringer's acetate (20 mL/kg) was given to the animals after placement of the intravenous access, followed by a maintenance rate of 15 mL/kg/hr for the remainder of the experimental protocol. Low blood glucose (< 3.5 mmol/L) was treated with doses of glucose intravenous (500 mg/L) until normoglycemia was achieved.

Preparation of rats

Sprague Dawley rats (Taconic, Silkeborg, Denmark) were housed pairwise at standard room temperature (22-23 °C), humidity (45%), 12-hour light/dark cycle

and with free access to food and water. Cages were enriched with bedding material, hide and toys. Data collection took place in September 2019 - December 2019. All experiments were started in the morning between 8.00-10.00 am.

Anesthesia was induced by a subcutaneous injection of FFM mixture (fentanyl 0.0788 mg/ml, fluanisone 2.5 mg/ml, and midazolam 1.25 mg/ml) (2.4 ml/kg). A single dose of ketamine (100 mg/kg) was administered intraperitoneally to prevent pharyngeal reflexes 1 minute before intubation. Rats were orally intubated using a 17-gauge venous catheter and ventilated with a tidal volume of 8 ml/kg. Supplemental oxygen and ventilation rate were adjusted to maintain PaO_2 within 80-110 mmHg and $PaCO_2$ within 35-45 mmHg. The temperature was kept at 36.5-37.5 °C by a feedback-controlled heating pad. The left femoral artery was catheterized for invasive blood pressure measurement and arterial sampling, using a PE90 tube connected to a 22-gauge venous catheter. The femoral vein was catheterized using a PE50 tube for fluid and drug administration. Saline (2 ml/h/kg) was administered intravenously following catheter placement, and anesthesia was maintained with regular subcutaneous injections of a lower FFM dose (0.6 ml/kg) every 30 minutes. Data on blood pressure, ECG, end-tidal CO_2, hind paw saturation, and rectal temperature were continuously collected using PowerLab (AD instruments, Oxford, United Kingdom). Arterial blood samples were analyzed and evaluated continuously during the study (ABL90 Flex, Radiometer, Brønshøj, Denmark).

Example 2 *– Senicapoc is superior to TRAM-34 in counteracting KCa3.1 channel-activated relaxations in mouse pulmonary small arteries*

Pulmonary arteries (PA) from C57BL/6NTac mice were dissected and mounted on isometric wire myographs. The pulmonary arteries were then incubated with the KCa2 channel-blocker apamin (0.5 µM) for 30 minutes and then contracted with phenylephrine (0.01 µM). At stable contraction, vascular relaxation was produced with the KCa3.1/KCa2.3 channel opener, NS309 (1 µM). At stable relaxation, cumulative concentrations (half log steps from 0.01 µM to 5 µM) of KCa3.1 ion channel blockers (senicapoc, n = 7; TRAM-34, n = 5) were added to separate PA preparations. Vehicle alone (PSS-diluted DMSO, n = 7) was added to separate PA segment, thus serving as control. At the end of all experiments, PAs were

stimulated once again with KPSS in order to assure vessel viability throughout the experiment. Mean diameters and length of the PA segments did not differ between groups.

Conclusion

Figure 1 shows that cumulative addition of senicapoc concentration-dependently reverses the relaxation induced by NS309 and, thus illustrating that senicapoc is a more potent blocker of the KCa3.1 channel in comparison to TRAM-34. The data in Figure 1 is represented as means ± SEM. #P<0.05 for senicapoc versus TRAM-34. *P<0.05 for senicapoc and TRAM-34 versus vehicle.

Example 3 *- Ventilator-induced lung injury of mice and analysis methods*

A mouse model of ventilator-induced lung injury (VILI) was established to assess the effect of treatment with senicapoc on key indicators related to acute respiratory diseases, such as hypoxaemia, inflammatory response, and tissue injury.

A first experimental protocol, which was used for experiments examine KCa3.1$^{-/-}$ animals and their wild type controls, was similar to the protocol depicted in Figure 2, except that no drug was administered before start of VILI. Animals were randomized to the ventilator-induced lung injury protocol. Then the VILI protocol was initiated. This consisted of the following ventilator settings: a peak inspiratory pressure (PIP) of 41 cmH_2O, PEEP of 1 cmH_2O, I:E of 1:1 and RR of 80 breaths/min for 2 h. As for all protocols, air was supplemented with 2.5% CO_2 due to the increased minute ventilation.

In a second experimental protocol, wherein the effect of the KCa3.1 channel inhibitor, senicapoc, was tested, the first step in the model, as depicted in Figure 2, is administration of the drug (senicapoc). After stabilisation, the mice received a single intraperitoneal injection (i.p.) of senicapoc or vehicle thirty minutes before start of VILI. The animals were injected with the drug in pure DMSO, whereas the control group received a single intraperitoneal injection of sterile saline (70 mg/kg BW) at the start of the experiment, such that each subject received approximately 40-50 µL of the solvent, regardless of the dose. All stocks

had been kept at -20°C until use. Wildtype animals (n = 8 per group) were randomly assigned into one of five groups (n = 16): control (only control ventilation e.g. no VILI), vehicle (DMSO), 10 mg/kg BW senicapoc, 30 mg/kg BW senicapoc or 70 mg/kg BW senicapoc by using an online random generator.

A third experimental protocol was used to test the effect of senicapoc in a chronic setting, thus a longer experimental protocol was established. Following a 30-minute stabilization period, the animals were ventilated with a PIP of 28 cmH_2O, PEEP of 0 cmH_2O, I:E of 1:1 and RR of 70 breaths/min for 5 hours. One hour into the lung injury, animals were randomly assigned to one of two groups: vehicle (DMSO) and 30 mg/kg senicapoc. Administration were subsequently performed by a single i.p. injection.

The operator was blinded to the animal randomization. During the injurious ventilation, air was supplemented with 2.5% CO_2 due to the increased minute ventilation. All measurements described below were performed in the three animal experiments, except histological analysis which was only done in the senicapoc experiments.

The 2- and 5-hour observation periods were based on a number of pilot studies where the chosen ventilator settings prevented survival in untreated animals for longer than the given times. This allowed us to capture several features (rapid onset, impaired gas-exchange and increased alveolar-capillary barrier permeability and histological evidence of injury to the lung) of human ARDS. Of the total 16 animals in the $KCa3.1^{-/-}$ experiment, seven wild types and eight $KCa3.1^{-/-}$ animals survived and were included in the following analyses. One wild type mouse was randomised, but died before the 2-hour mark and were not included into the data analysis. In the pre-exposure senicapoc experiment, 80 mice (16 in each group, eight for wet-to-dry ratios and lavages and eight for histology) were randomized to the 2-hour ventilator-induced lung injury protocol. The number of animals from each group that were included in the following analyses were: control, n = 16; vehicle, n = 14; 10 mg/kg, n = 15; 30 mg/kg, n = 14; 70 mg/kg, n = 15. Six animals died before the 2-hour mark and were not included into the data analysis. Sixteen mice were included into the 5-hour

chronic study. Two animals died before the 5-hour mark. The remaining 14 animals were all included into the data analysis.

Blood gas analysis

Blood gas analysis was performed at prior to VILI and at end of the experiments (ABL90 Flex, Radiometer, Copenhagen, Denmark). From this, the PaO_2/FiO_2 ratio was calculated for both time points.

Respiratory mechanics

Respiratory mechanics were measured at the start of VILI and every 30 min thereafter for the entire duration of the experiment (Flexivent, SCIREQ, Montreal, Canada). A pressure-volume curve was performed by inflating and then deflating the lungs in incremental steps. From this, the quasistatic lung compliance was automatically calculated as the slope of the expiratory phase at 5 cmH_2O. In the $KCa3.1^{-/-}$ and pre-exposure experiments, measurements were performed at the start of ventilator-induced lung injury and every 30 min thereafter for the entire duration of the experiment. Lung mechanic scans were performed hourly in the post-exposure protocol.

Bronchoalveolar lavage (BAL)

To evaluate the leakage of the blood-lung barrier at the end of a protocol, the animals were exsanguinated by cutting the caudal vena. Bronchoalveolar lavage fluid (BALF) was obtained by isolating the left lung at the left lung hilus. The right lung was then exposed to lavage three times with 0.5 ml of PBS containing 0.5 mM EDTA. Total cell count in the recovered BALF was determined using an automated cell counter (NucleoCounter NC-3000, Chemometec, Denmark). Next, the recovered BALF was centrifuged (200 g, 10 min, 4 °C). The supernatant was isolated, and total supernatant protein concentration was determined spectrophotometrically (NanoDrop OneC, Thermo Scientific, USA). In order to assure the reliability of single values, protein concentration was measured as triplicates. The cell pellet was resuspended, cytospun onto slides, and stained with Giemsa. Differential cell counts were obtained by microscopic counting of a minimum of 300 cells/slide using standard morphological and staining criteria. The counting was performed in a blinded fashion.

BALF cytokine measurements

Cytokine levels (IL-6, IL-1β KC, TNF-α and MIP-2) were measured in BALF by using a multiplex assay system according to the manufacturer's instructions (Bio-Rad, USA). Prior to analysis, all samples were spun at 10.000 g at 4 °C for 10 min to avoid unwanted cellular debris. For the determination of analyt concentrations in the experimental samples, a standard curve was generated for each cytokine and a five-parameter logistic regression was derived from this. Detection limits (pg/ml) were IL-1β (1.6), KC (25), IL-6 (0.4), MIP-2 (2.7) and TNF-α (3.2).

Wet-to-dry weight analysis

Wet-to-dry weight analysis were performed. To measure the amount of accumulated lung water, a lung sample was dissected and weighed. The tissue was then dried at 65 °C until no further weight loss. The lung wet-to-dry weight ratio was calculated as a marker of the degree of lung oedema.

Histological analysis and the degree of pulmonary haemorrhage

To evaluate the lung injury, histological analysis was performed in the mice from the senicapoc experiment. The lungs were fixated *in situ*. In brief, the left lung was inflated by gentle infusion of 10% formalin through the tracheal tube. The thorax was opened where after the lungs were tied off, dissected and placed in 10% formalin for at least 24 hours. The fixed lungs were embedded in paraffin and sectioned at 5-µm thickness. The sections were stained with haematoxylin and eosin. Lung injury was assessed using a predefined scoring system based on five histological findings (neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces and alveolar septal thickening). The five independent variables were graded by using three-tiered schema where after the sum was weighted according to the relevance and then normalized to the number of fields evaluated. The resulting injury score was a value between zero and one. Twenty random high-power fields (400x total magnification) were scored per animal in a blinded fashion for each condition. In addition, the degree of haemorrhage was done by measuring areas containing aggregation of red blood cells in alveolar space using an image analysis software (ImageJ, NIH, Bethesda, MD, USA). The results were expressed as percentages of total lung area.

Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. Statistical comparison between two groups was analysed using Student´s t test. One-way analysis of variance (ANOVA) followed by pairwise comparisons using Tukey post-test was used to analyse group differences and mean levels when three or more groups were analysed. For the functional vessel data and compliance measurements, a repeated-measurements ANOVA was used to analyse data for time/concentration-dependent and between-group differences. The assumptions of the models were investigated by inspecting Q-Q plots and data were logarithmically transformed when necessary in order to generate a Gaussian-distributed data set. A $P<0.05$ was considered statistically significant. Post-tests were only done if F was significant and there was no variance inhomogeneity. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, CA). Eight animals were included in each group. This number was based on sample size calculations from a separate pilot study (Wet-to-dry ratios: senicapoc 70 mg/kg: 5.27 ± 0.80, vehicle: 7.22 ± 0.34, n = 3 for both groups). A significance level of 5% and a power of 80% was assumed.

Example 4 *- Senicapoc reduces TNF-α induced permeability changes in lung epithelial cells*

For this experiment, the human epithelial Calu-3 cells were used. This cell line is known to express KCa3.1 channels. Cells were seeded on semi-permeable membranes (3 µm pore size, 12 well, Thermo Scientific) separating an upper and a lower chamber. Cells were incubated at 37 °C, 5% CO_2 until a monolayer was formed. Next, cells were treated with senicapoc (0.1 µM, 0.3 µM or 1 µM) and 20 ng/ml TNF-α for 24 hours. For controls, cells were treated with medium or DMSO. Passage of FITC-labelled dextran (Fluorescein isothiocyanate-dextran average mw 70.000 kDa) was used to determine epithelial permeability. This was added to the upper chamber and 20 minutes after, fluorescence was evaluated in the liquid from lower chamber using a plate reader (Varioskan Lux, Thermo Scientific). Filters appropriate for 485 nm and 535 nm excitation and emission, respectively, were used for measurement.

Conclusion

The results of the permeability assay are shown in Figure 3. The two lowest doses of senicapoc (0.1 and 0.3 µM) significantly reduced the permeability changes induced by TNF-α. DMSO by itself did not affect permeability. This suggests that senicapoc can help counteract the TNF-α induced lung damage during SARS-CoV-2 infection and further supported by the observation that in rats having increased TNF-α levels induced by the cardiac arrest model as described in Example 9, senicapoc also improves the PaO_2/FIO_2 ratio 4 hours after cardiac arrest (see Figure 17).

Example 5 - *KCa3.1$^{-/-}$ diminishes pulmonary inflammation evoked by ventilator-induced lung injury in mice*

The methods and means of conducting the experiments are described in Example 3. Analysis of inflammatory cytokines in BALF of KCa3.1$^{-/-}$ (KO) and wild type (WT) mice revealed that KCa3.1 deficiency suppressed the ventilator-induced increases in IL-1ß, KC, TNF-α and MIP-2 as shown in Figure 4A and 4C-E. Figure 4B shows that IL-6 was found to be unaffected by the genetic knockout of KCa3.1.

Conclusion

The results show that KCa3.1 deficiency markedly reduced release of proinflammatory cytokines induced by the ventilator-induced lung injury. Thus, the KCa3.1 channels play a pivotal role in the development of pulmonary inflammatory responses like those observed in patients after SARS-CoV-2 infection.

Example 6 *– senicapoc inhibits development of ARDS-like conditions e.g. hypoxaemia and pulmonary inflammation in mice*

Senicapoc attenuates ventilator-induced hypoxaemia.

Senicapoc was chosen for the animal experiments and single intraperitoneal injections (10, 30 or 70 mg/kg) were administered 30 minutes before VILI (second experimental protocol). Figure 5 shows the development of hypoxaemia in terms of the inhaled oxygen over blood saturation (PaO_2/FiO_2) ratio. Addition of senicapoc (SE) completely counteracts formation of hypoxaemia compared to

vehicle-injected mice and remained at a level equivalent to the control animals. Moreover, the lung barrier function remained intact as demonstrated by the lowered wet-to-dry weight ratio (Figure 6) and decreased amount of broncholalveolar lavage (BAL) fluid protein content (Figure 7) as compared to the vehicle-injected mice. Senicapoc also protects against any dramatic changes in quasistatic lung compliance as depicted in Figure 8.

Senicapoc diminishes VILI-induced pulmonary inflammation.

Total cell count and differential cytology in the BALF obtained from the senicapoc-groups are summarised in Table 1. The majority of cells were macrophages and the total amount of cells was reduced in the vehicle group. Neutrophil infiltration in the alveolar space was observed for the vehicle-treated animals. This inflammatory response was significantly diminished in animals injected with the two highest dosages of senicapoc. No distinct changes were seen in lymphocyte count between the five groups.

Table 1

	Control	Vehicle	10 mg/kg	30 mg/kg	70 mg/kg
Total cells	179.7 ±28*	66.5 ±18	110.0 ±19	110.0 ±10	137.2 ±23*
Macrophages	172.0 ±27*	56.0 ±16	96.7 ±17	95.2 ±9	121.1 ±21*
Neutrophils	0.6 ±0.1*	2.0 ±0.4	1.1 ±0.5	0.6 ±0.2*	0.7 ±0.2*
Lymphocytes	3.1 ±0.5	5.1 ±1.3	5.9 ±0.8	5.5 ±1.3	6.3 ±2.1

Values are means ± SEM.

n = 8 for control; n = 7 for vehicle, 10, 30 and 70 mg/kg senicapoc.

*P<0.05 against vehicle group.

Figure 9A-E shows that in the BALF of vehicle-treated animals, the ventilator-induced lung injury caused a significant increase in key proinflammatory cytokines such as KC, MIP-2, IL-6 and TNF-α, whereas pre-treatment with all three senicapoc doses (10, 30, 70 mg/kg as according to the second experimental protocol) strongly suppressed these responses to a near control-group level.

Post-exposure treatment with senicapoc, as according to the third experimental protocol, diminished the production of KC while the other four cytokines remained indifferent from the vehicle group as shown in Figure 10A-E.

Senicapoc reduces tissue injury.

Evidence of histological tissue injury was evaluated in the hematoxylin and eosin-stained lung sections as shown in Figure 11A-E. Vehicle-treated animals had a high accumulation of neutrophils inside the alveolar walls. This included cells that had entered the interstitium as well as those circulating or adhering to the alveolar capillaries. The level of neutrophils inside the alveolar space was minor. Alveolar septal thickening and proteinaceous debris filling in the airspaces was observed. Formation of hyaline membranes did not occur. When quantified, this resulted in a significant increase in lung injury score compared to the senicapoc dose of 30 and 70 mg/kg, see Figure 12. These two doses together with the 10 mg/kg dose were found to be insignificant from the group receiving control ventilation. Internal haemorrhage was also assessed. Image analysis showed that mechanical ventilation in general produced minor localized haemorrhages in the lungs as depicted in Figure 13. This was, however, significantly higher in the vehicle group when compared to control, 10 mg/kg and 70 mg/kg. Figure 14 shows the summarized haemorrhage data as percentage area of total lung.

Conclusion

The results show that KCa3.1 inhibition by treatment with senicapoc reduced release of proinflammatory cytokines and recruitment of neutrophils into the lung induced by the ventilator-induced lung injury. Thus, the KCa3.1 channel plays a pivotal role in the development of a pulmonary inflammatory response and senicapoc can potentially impair hyper-inflammatory as seen in COVID-19 patients.

Example 7 *- Ventilator-induced lung injury of pigs and analysis methods*

Experimental procedure

The following procedure was used. A urinary catheter was placed by surgery in order to measure urine output. Next, sheaths were inserted by ultrasound into the external jugular vein and femoral artery (10F and 8F, respectively). A Swan-Ganz

catheter (7.5F, Edwards Lifesciences, CA, USA) was inserted through the external jugular vein to monitor cardiac output, mean pulmonary arterial pressure (MPAP), central venous pressure, mixed venous oxygen saturation, and core temperature (Vigilance Monitor, Edwards Lifesciences, CA, USA). The following parameters were also monitored throughout the experiment: ECG, heart rate, mean arterial pressure (MAP), and arterial oxygen saturation. All animals were allowed to stabilize for 30 minutes between the surgical preparation and experimental protocol. A priori determined exclusion criteria at baseline were: MAP < 45 mmHg, MPAP > 25 mmHg, PaO_2 < 18 kPa, lactate concentration > 3 mmol/L.

After baseline physiological measurements, a two-hit lung injury encompassing repeated lung lavages and injurious mechanical ventilation was initiated. FiO_2 was increased to 1.0 and repeated lung lavages were applied. During each lavage, the lungs were filled with warm isotonic saline (37°C, 30 ml/kg). Fluid was drained passively after three minutes of apnea or until MAP fell below 60 mmHg. Hereafter, the animal was reconnected to the ventilator and allowed to compensate (increase in MAP and oxygen saturation). Time between re-lavages did not exceed five minutes. Lung lavages were repeated until a PaO_2/FiO_2 < 100 mmHg was achieved. Then injurious mechanical ventilation using a peak inspiratory pressure of 45 cmH_2O, PEEP of 4 cmH_2O and I:E of 1:1 were commenced. The respiratory rate was adjusted to maintain normocapnia. The injurious ventilation was continued until a PaO_2/FiO_2 of < 100 mmHg was achieved again. Animals requiring more than four hours of injurious mechanical ventilation were excluded from the study.

Animals were randomized into two groups: 1) 10 mg senicapoc or 2) vehicle. The primary investigators assessing all outcomes were blinded to group assignments. Thus, a neutral person from the technical staff drew a random number from a sealed non-opaque envelope. Solutions were prepared in a room away from the experimental site and containers of identical appearance were used. The total volume of the bolus was 12 ml and treatments were given intravenous over 15 minutes. A 0.1 M stock solution of senicapoc (MedChemExpress, Sweden) was prepared with DMSO and separated into aliquots. These were stored at −80°C. On each day of experimentation, one aliquot was diluted to a final concentration of 2.5% DMSO, 5% Cremophor EL and 92.5% saline. The same solution without

senicapoc was used as vehicle. Concurrently with the start of infusion, a lung protective ventilatory protocol using BiPAP mode was initiated in order to achieve a defined oxygenation goal of a PaO_2 between 8-10 kPa. Incremental FiO_2/PEEP combinations were used for this purpose and the initial setting was a peak inspiratory pressure of 35 cmH_2O, PEEP of 18 cmH_2O, and I:E of 1:2. Respiratory rate was adjusted in an effort to maintain normocapnia. The driving pressure did not exceed 17 cmH_2O above PEEP. The lung protective ventilation was maintained and adjusted for six hours. In this time period, blood gas analysis was performed every half-hour (ABL90 Flex Plus, Radiometer Medical, Denmark). Arterial blood samples were collected at every hour in the lung protective ventilatory period. Serum and plasma were isolated and stored at −80°C. Leucocyte count in the blood was obtained using an automated cell counter (Procyte Dx, Idexx, USA). At the end of the study, bronchoalveolar lavage (BAL) was performed and representative tissue samples were collected.

A total of 31 animals were included into the study. Thirteen animals were excluded according to the predefined protocol: four due to a high MPAP at baseline, four developed pneumothorax during injurious mechanical ventilation, and five exceeded the maximum 4 hours of injurious mechanical ventilation. The remaining 18 animals were distributed as follows: Senicapoc (n = 9), vehicle (n = 9). A single animal treated with senicapoc died from hypoxemia 15 minutes into the lung protective ventilation protocol. No data from this animal were included in the analysis below. No differences were observed in body weight, number of lavages, duration of lavages and duration of injurious mechanical ventilation.

Measurement of BAL

Tracheostomy was performed and by visual guidance, through which a fiberoptic bronchoscope (Olympus Medical Systems Corp, Japan) was advanced into the right lower lobe. Here, 60 ml of 37°C saline was instilled over 15 seconds and immediately withdrawn. To calculate recovery, the total volume of the BAL fluid (BALF) was summed and the last 5 ml was analyzed on a cell counter. The remaining volume of the 5 ml was spun at 1000g for 15 min at 4°C and the supernatant was stored at −80°C. Total supernatant protein concentration was determined spectrophotometrically (NanoDrop OneC, Thermo Scientific, USA). A

commercially available albumin assay kit was used to measured albumin content (Sigma-Aldrich, USA).

Histological analysis

After finishing the BAL procedure, the thorax was opened and representative samples of the left lung lobes (top, middle and lower) were collected for histological analysis. The tissue specimens were immersed in 10% formalin for 48 hours. Fixed lungs were embedded in paraffin, sectioned at 5-µm thickness, and stained with hematoxylin and eosin. At least 20 images per slide were obtained (EVOS M5000, Invitrogen), and histological assessment of the lung injury was performed on these. For scoring, the following parameters were considered: neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces and alveolar septal thickening. The lung sections were examined for perivascular cuffs as a measure of liquid accumulation. Measurement of hemorrhage in histological lung sections was assessed separately and performed as previously described. In brief, areas containing aggregation of red blood cells in interstitial and alveolar space were measured using the image analysis software (ImageJ, NIH, Bethesda, MD, USA). All histological assessments were performed in a blinded fashion.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Normality of data were investigated by inspecting Q-Q plots and data were logarithmically transformed when necessary in order to generate a Gaussian-distributed data set. Pairwise comparisons were done using Student's t-tests. For continuous variables, a repeated-measurementsANOVA was used to analyze data for time/drug-dependent and between-group differences. A $P<0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, CA). Nine animals were included in each group. This number was based on sample size calculations on the primary endpoint (PaO_2/FiO_2) from a separate pilot study (PaO_2/FiO_2: senicapoc: 140 ± 17, vehicle: 102 ± 17, n = 2 for both groups). A significance level of 5% and a power of 80% was assumed.

Example 8 – *senicapoc reduces neutrophilic infiltration and pulmonary hemorrhage in pigs*

In BALF a significantly lower number of neutrophils ($P<0.05$) were found in animals treated with senicapoc compared to vehicle as shown in Figure 15A. Also, the histological analysis revealed heavy infiltration of neutrophils into the alveolar space and the score was significantly lower for the senicapoc group ($P<0.05$) as shown in Figure 15B. Interstitial and alveolar hemorrhage was observed in both groups but to a lesser extent in animals treated with senicapoc ($P<0.05$) as shown in Figure 16.

Conclusion

Senicapoc reduces pulmonary hemorrhage and the influx of neutrophils into the lung. These results show that the KCa3.1 channels are important for neutrophil migration, thus blocking the KCa3.1 channels with senicapoc is a potential treatment to reduce alveolar neutrophil accumulation. This may improve long-term outcome in ARDS like conditions as seen in severe cases of SARS-CoV-2 infection.

Example 9 *- senicapoc improves the PaO_2/FiO_2 ratio in rats subject to cardiac arrest*

Drug preparation and randomization

Senicapoc (MedChemExpress, Monmouth Junction, NJ, USA) was initially dissolved at a concentration of 32.3 mg/ml in 100% PEG-400. The stock solution was prepared shortly before the beginning of the study and stored at -18 °C. Ten minutes before administration, the stock solution was defrosted and further diluted in cremophor and saline. The final solution consisted of 75% saline, 20% PEG-400 and 5% cremophor with a senicapoc concentration of 6.5 mg/ml. The vehicle solution had the same ratio of solvents, but did not contain any senicapoc. Randomization was performed by drawing from an opague envelope at the time of ROSC. Rats were randomized 1:1 into two groups (vehicle; n = 11 or senicapoc; n = 11) by an independent third person, who also prepared and administered senicapoc (10 mg/kg) or vehicle. The solution was infused intravenously at a rate of 0.2 ml/min. The primary investigator was blinded to group assignment.

Cardiac arrest and resuscitation

Cardiac arrest was induced by asphyxia by turning off the ventilator. Rocuronium (2.4 mg/kg) was administered 30 seconds before turning off the ventilator in order to prevent spontaneous respiration during the induction of cardiac arrest. Cardiac arrest was defined as a MAP below 20 mmHg. After 8 minutes of cardiac arrest, adrenaline 0.01mg/kg was administered and chest compressions were initiated. Chest compressions were delivered in a standardized fashion by a custom-made thumper at a rate of 200 min^{-1}, a depth of 1/3 of anterior-posterior chest diameter, and with equal 1:1 compression-/relaxation ratio. Adrenaline boluses (0.01 mg/kg) were administered every two minutes until ROSC (defined as a self-maintained MAP>40mmHg). Resuscitation was discontinued if ROSC did not occur within 8 minutes. Rats were ventilated with 100% O_2 at a rate of 100 min^{-1} during resuscitation. Oxygen administration and ventilation were adjusted according to the arterial blood samples that were regularly collected after ROSC. If MAP dropped below 50 mmHg, norepinephrine (0.3 mg/ml) infusion was initiated at 0.24 ml/h and titrated to secure a MAP of 50-60 mmHg during the observation period. Norepinephrine infusion was discontinued if MAP stabilized >60 mmHg. Saline infusion was adjusted according to the norepinephrine infusion in order to secure equal saline administration across groups. Rats were decapitated at the end of the observation period. Immediately after decapitation, cerebrum and lungs were extracted to measure wet weight. The corresponding dry weight was measured 7 days later.

Thirty-two male Sprague Dawley rats, aged 10-13 weeks, were utilized in the cardiac arrest study. Ten rats did not attain ROSC; hence twenty-two rats were randomized to receive either vehicle or senicapoc. Weight was comparable between groups (vehicle: 389±26 g and senicapoc: 396±26 g; p=0.53).

Cardiac arrest parameters, time from asphyxia to cardiac arrest and time from CPR to ROSC were comparable between groups (asphyxia to cardiac arrest: vehicle: 74±13 s and senicapoc: 73±15 s; p=0.9. CPR to ROSC: vehicle: 126±56 s and senicapoc: 118±34 s; p=0.67). Repeated measurement did not reveal any difference over time in any physiological parameters between the two groups. At 4

hours, however, the PaO_2/FiO_2 ratio was significantly higher in the senicapoc group when compared to the vehicle group, as shown in Figure 17.

Example 10 *- Investigation of clinical benefit of senicapoc as monotherapy in patients with COVID-19 infection and respiratory insufficiency*

Part 1

Aim of the study: To investigate the effect of senicapoc on patients with SARS-CoV infection in the stationary wards referred for intensive therapy due to respiratory insufficiency.

Primary aim: Number of ventilator-free days at 10 and 28 days. and.

Secondary aims: Oxygen requirement (PaO_2/FiO_2 ratio) at 48 hours and 10 days. Assess effect on 10 and 28-day mortality and safety of said compound when given alone in patients with COVID-19 infection and respiratory insufficiency, pulmonary changes observed with CT-scan, SOFA score, viral load in blood.

Design: Exploratory phase IIa, randomized, non-blinded clinical study. In the study, the patients referred to intensive therapy will be randomized to treatment with senicapoc or nothing. The treatment with senicapoc (75 mg each day for two days) will be administered as two doses over two days.

Patients´main selection criteria: Adult patients (>18 years) with COVID-19 infection requiring intensive therapy due to respiratory insufficiency. [Patients with previous known heart and renal failure will be excluded.]

Main variables for evaluation: Number of ventilator-free days, oxygen requirement (PaO_2/FiO_2 ratio), survival, pulmonary changes observed with CT-scan, SOFA score, viral load in blood, safety (adverse effects), standard serum biochemistry, hematology, and cytokines, blood levels of the compound to be tested.

Part 2

Aim of the study: To investigate the preventive effect of senicapoc on patients with SARS-CoV and dyspnea in the stationary ward for requirement of intensive care.

Primary aim: Reduce the number of patients from the stationary ward for requirement of intensive care due to respiratory insufficiency.

Secondary aims: viral load in blood, safety (adverse effects), 28-day survival, days of hospitalization.

Design: Randomized double-blinded placebo-controlled phase II clinical study with three arms. In the study, the patients are allocated to one of two dose regimens of senicapoc (peroral administration of 75 mg with 4-day interval or 150 mg once) or placebo.

Patients´ main selection criteria: Patients with SARS-CoV2 infection entering the stationary ward.

Main variables for evaluation: Number of patients with requirement for intensive care due to respiratory insufficiency, viral load in blood, safety (adverse effects), standard serum biochemistry and hematology, cytokines, blood levels of the compound to be tested.

Example 11 *- Combined treatment*

Several treatments have been proposed for reducing viral load in patients with infection caused by coronavirus species. Remdesivir inhibits RNA-dependent RNA polymerase and lower viral load in animals infected with coronavirus. Other approaches to decrease viral load includes the protease inhibitors (lopinavir, indinavir, ritonavir), ribavirin, and alpha-interferon or combinations thereof with remdesivir. However, an important observation is that the viral load of coronaviruses during the progress of the disease fall, and despite that leads to ARDS-like disease. Since we in animal studies observe effect of senicapoc on ARDS-like disease, an effect is expected by the administration of one or a combination of e.g. remdesivir, lopinavir, ritonavir, ribavirin, alpha-interferon (IFN-α) with senicapoc.

The combination can be of senicapoc with remdesivir following the designs described in Example 10 part 1 and part 2.
The combination can be of senicapoc with a combination of e.g. remdesivir, lopinavir, ritonavir, ribavirin, alpha-interferon (IFN-α) following the designs described in Example 10 part 1 and part 2.

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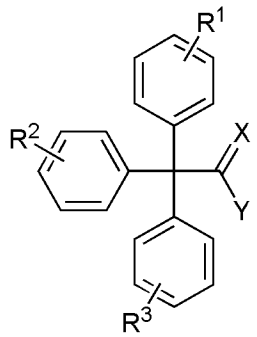
Claims

1. An inhibitor of the intermediate conductance calcium-activated potassium (KCa3.1) channels for use in the treatment of acute respiratory disease in a subject in need thereof,
wherein said acute respiratory disease is caused by an infection with a coronavirus (CoV).

2. The inhibitor for use in accordance with claim 1, wherein the inhibitor of calcium-activated potassium (KCa3.1) channels is also an inhibitor of entry and/or replication of coronavirus (CoV).

3. The inhibitor for use in accordance with any one of claims 1-2, wherein the inhibitor is selected from the group consisting of Senicapoc and TRAM-34.

4. The inhibitor for use in accordance with any one of claims 1-2, wherein the inhibitor is a compound of formula (I):



(I)

wherein
R^1, R^2, and R^3 are independently selected from the group consisting of hydrogen and halogen,
X is selected the group consisting of S, O, and NR^4,
R^4 is selected the group consisting of H, methyl, and ethyl,
Y is selected from $N(R^5)R^6$, OR^5, and SR^5,
R^5 and R^6 are independently selected from the group consisting of hydrogen, methyl, ethyl, and propyl.

5. The inhibitor for use in accordance with claim 4, wherein R^1, R^2, and R^3 are in the *para* position of the phenyl ring.

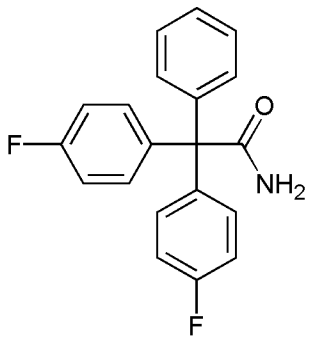
6. The inhibitor for use in accordance with any one of claims 4-5, wherein X is O.

7. The inhibitor for use in accordance with any one of claims 4-6, wherein Y is $N(R^5)R^6$.

8. The inhibitor for use in accordance with any one of claims 4-7, wherein R^5 and R^6 are hydrogen.

9. The inhibitor for use in accordance with any one of claims 4-8, wherein the halogen is selected from the group consisting of Cl, Br, and F, preferably F.

10. The inhibitor for use in accordance with any one of claims 1-9, wherein the inhibitor is a compound of Formula (II):



(II).

11. The inhibitor for use in accordance with any one of claims 1-10, wherein the acute respiratory disease is selected from the group consisting of acute respiratory
distress syndrome (ARDS), acute lung injury, lung oedema, pulmonary circulatory collapse, and virus pneumonia.

12. The inhibitor for use in accordance with any one of claims 1-11, wherein the coronavirus (CoV) is selected from the group consisting of SARS-CoV and MERS-CoV.

13. The inhibitor for use in accordance with claim 12, wherein the SARS-CoV virus is SARS-CoV-2.

14. The inhibitor for use in accordance with any one of claims 1-13, wherein the inhibitor is administered in combination with at least one further active, wherein the further active is selected from the group consisting of remdesivir, lopinavir, ritonavir, ribavirin, alpha-interferon, or any combination thereof.

15. A pharmaceutical composition comprising the inhibitor for use in accordance with any of claims 1-14 and a pharmaceutically acceptable carrier and/or excipient.

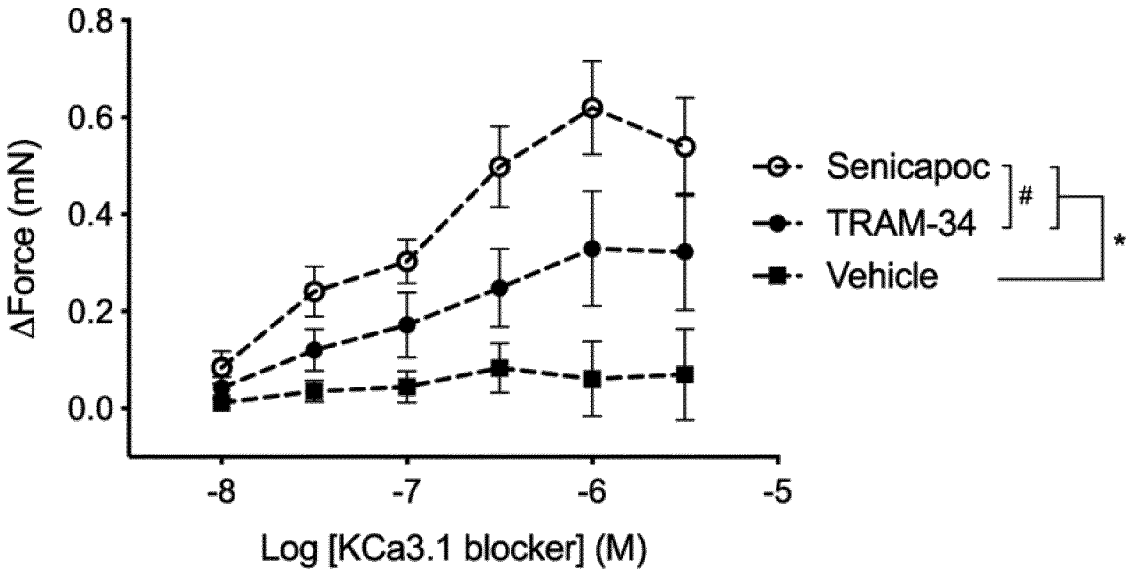

0.8
0.6
0.4
0.2
0.0
ΔForce (mN)
-8
-7
-6
-5
Log [KCa3.1 blocker] (M)
Senicapoc
TRAM-34
Vehicle
#
*

Fig. 1

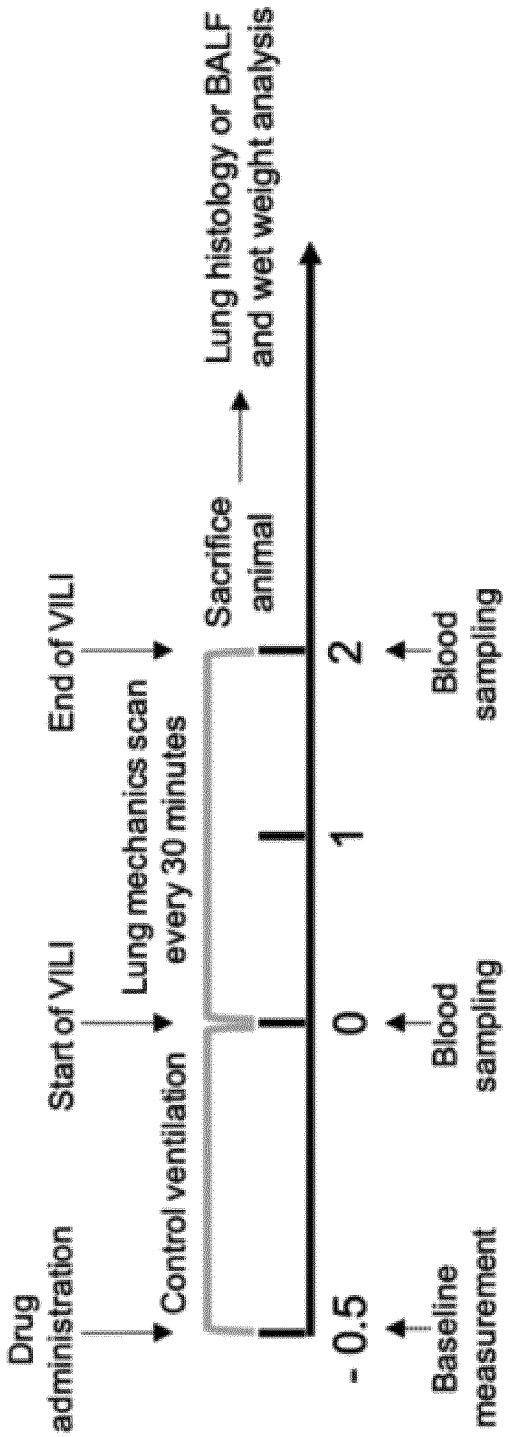

Drug administration
Control ventilation
Start of VILI
Lung mechanics scan every 30 minutes
End of VILI
Sacrifice animal
Lung histology or BALF and wet weight analysis
- 0.5
0
1
2
Baseline measurement
Blood sampling
Blood sampling
Blood sampling

Fig. 2

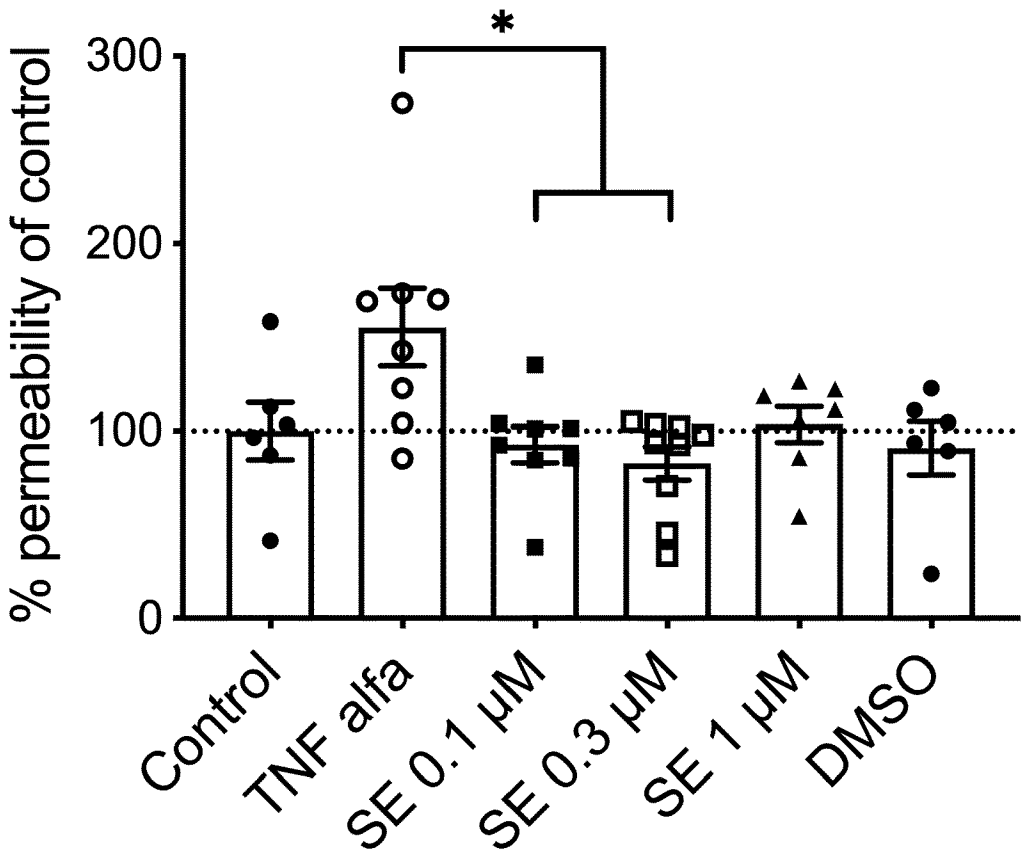

*
300
200
100
0
% permeability of control
Control
TNF alfa
SE 0.1 µM
SE 0.3 µM
SE 1 µM
DMSO

Fig. 3

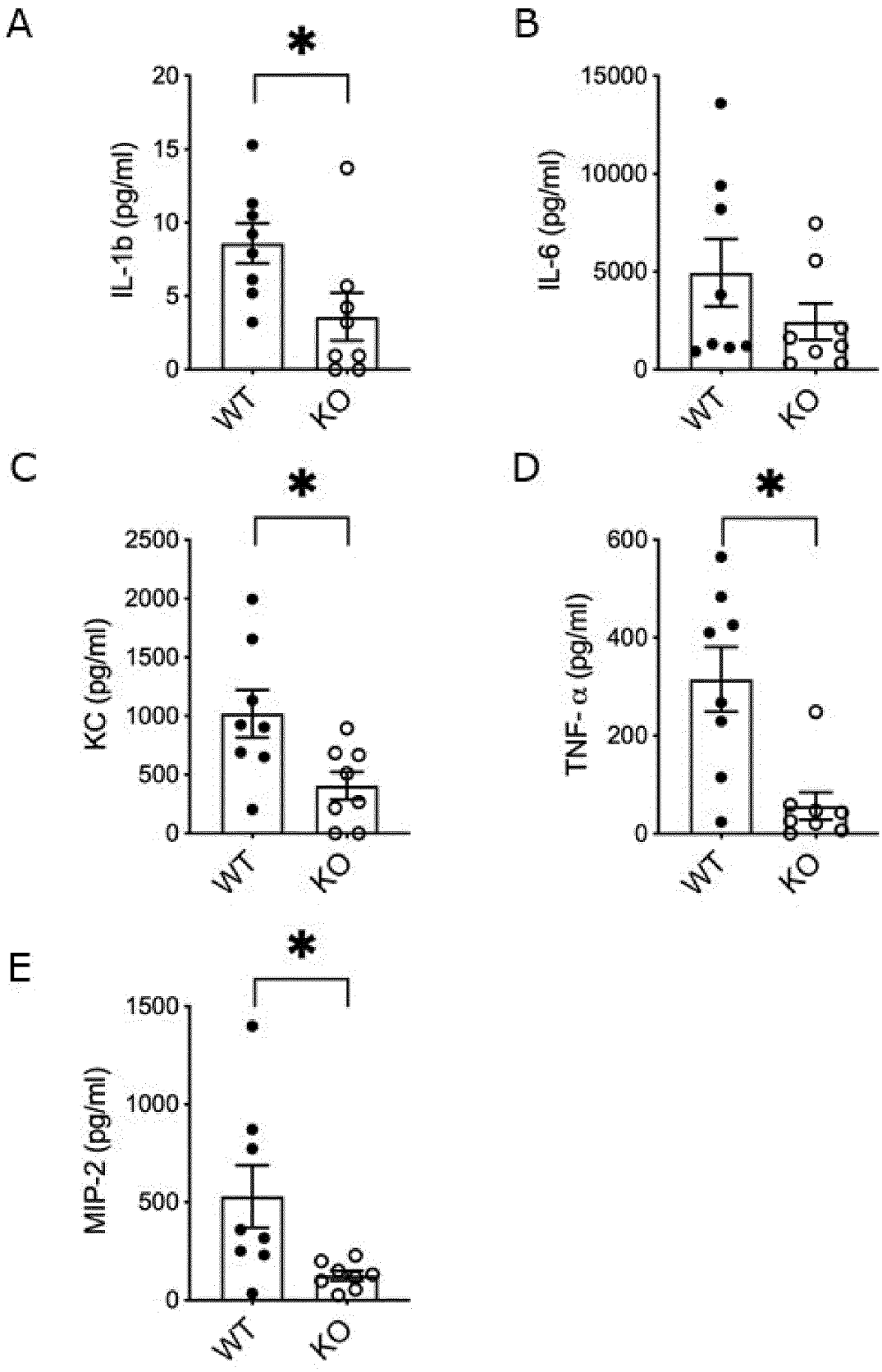

A
IL-1b (pg/ml)
20
15
10
5
0
WT
KO
*
B
IL-6 (pg/ml)
15000
10000
5000
0
WT
KO
*
C
KC (pg/ml)
2500
2000
1500
1000
500
0
WT
KO
*
D
TNF- α (pg/ml)
600
400
200
0
WT
KO
*
E
MIP-2 (pg/ml)
1500
1000
500
0
WT
KO
*

Fig. 4

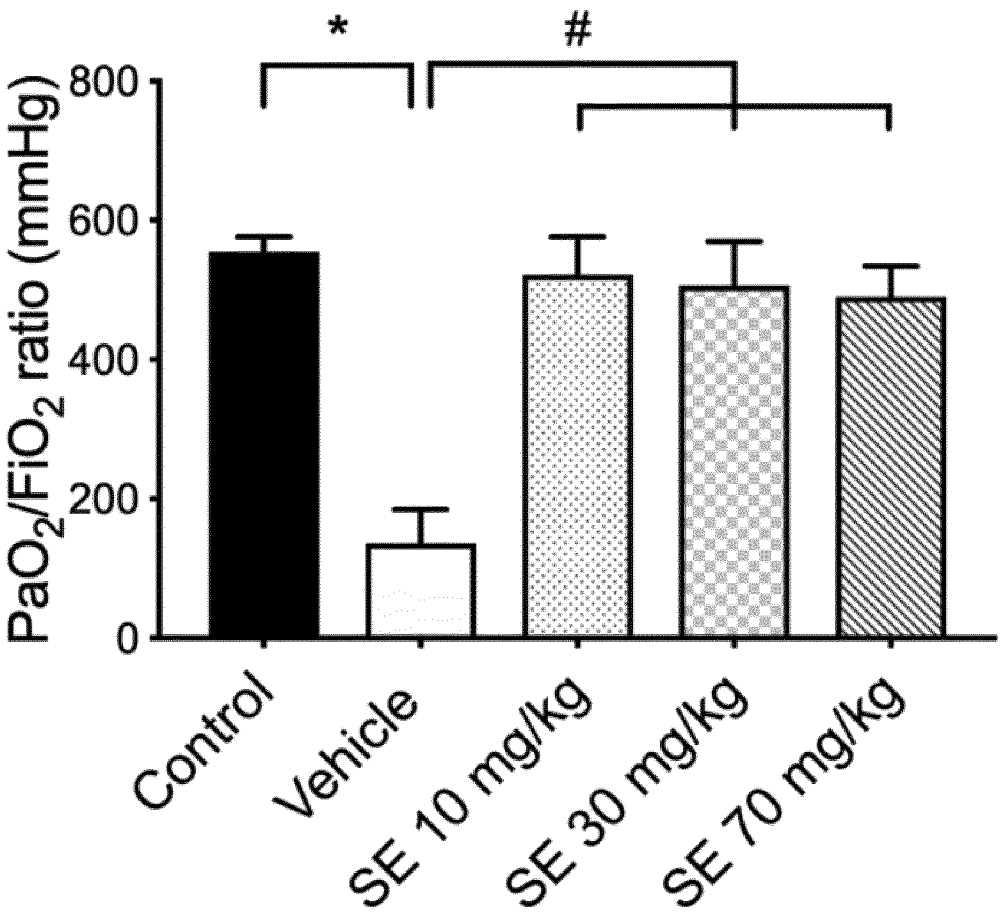

*
#
800
600
400
200
0
PaO2/FiO2 ratio (mmHg)
Control
Vehicle
SE 10 mg/kg
SE 30 mg/kg
SE 70 mg/kg

Fig. 5

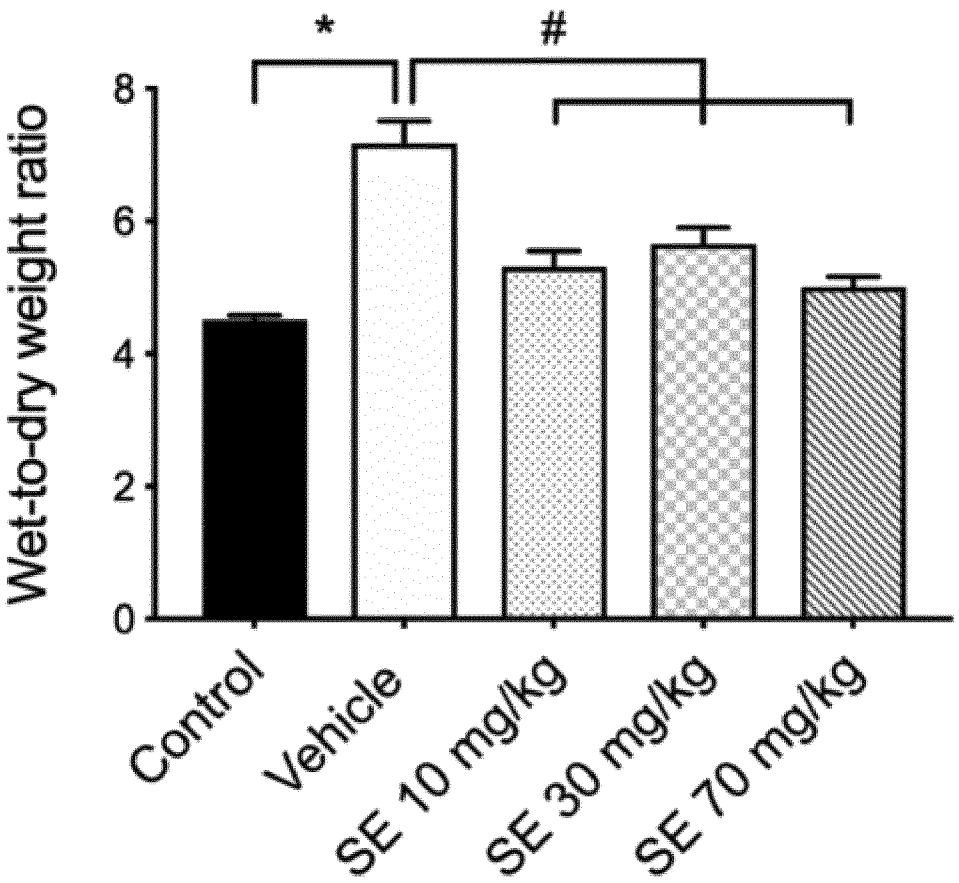

*
#
Wet-to-dry weight ratio
8
6
4
2
0
Control
Vehicle
SE 10 mg/kg
SE 30 mg/kg
SE 70 mg/kg

Fig. 6

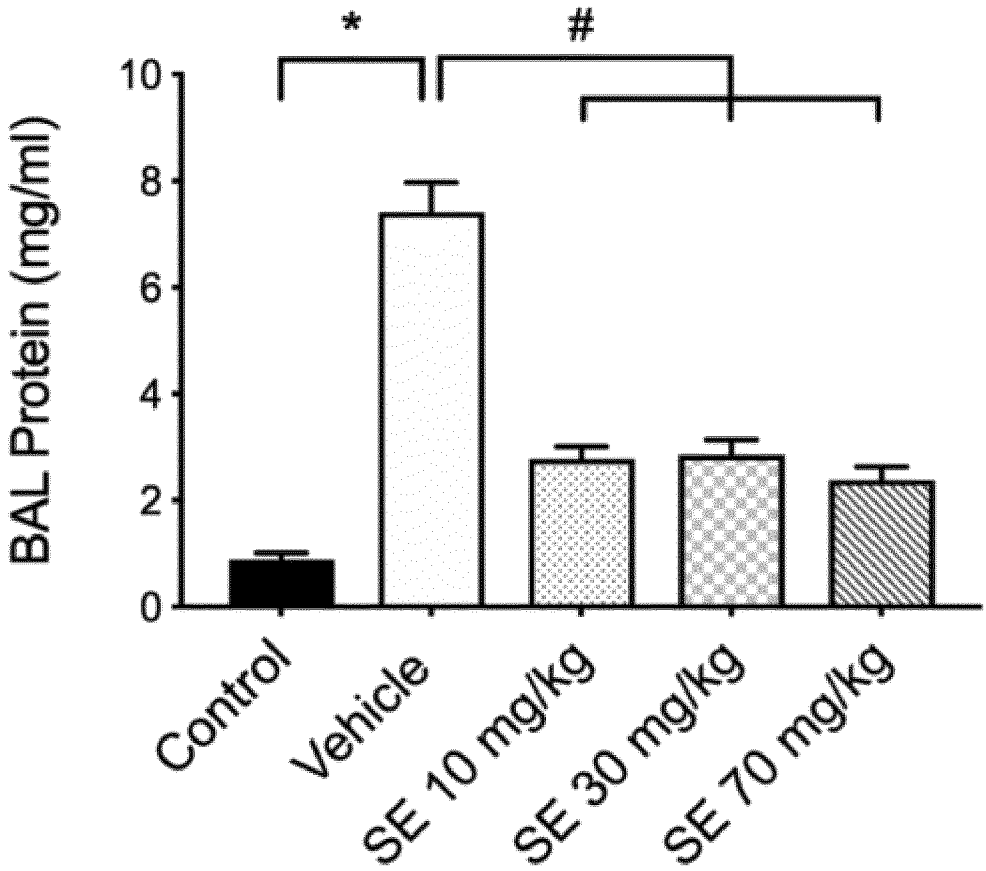

*
#
BAL Protein (mg/ml)
10
8
6
4
2
0
Control
Vehicle
SE 10 mg/kg
SE 30 mg/kg
SE 70 mg/kg

Fig. 7

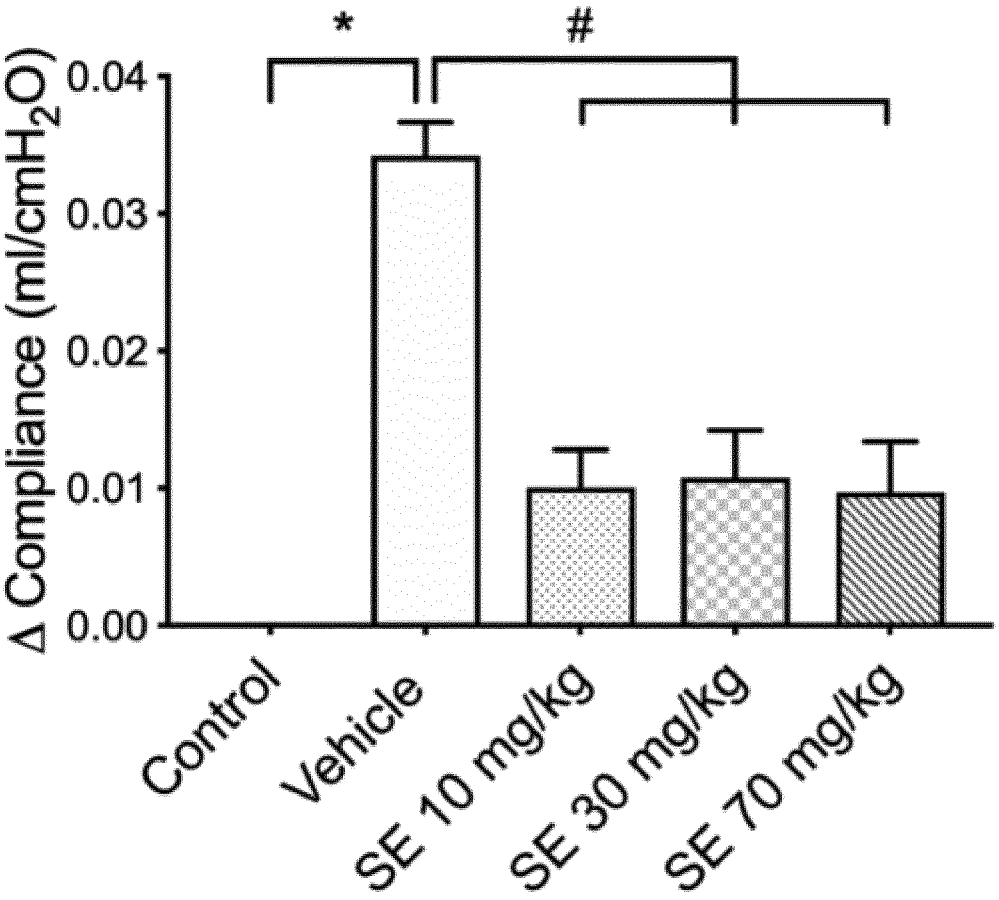

*
#
Δ Compliance (ml/cmH2O)
0.04
0.03
0.02
0.01
0.00
Control
Vehicle
SE 10 mg/kg
SE 30 mg/kg
SE 70 mg/kg

Fig. 8

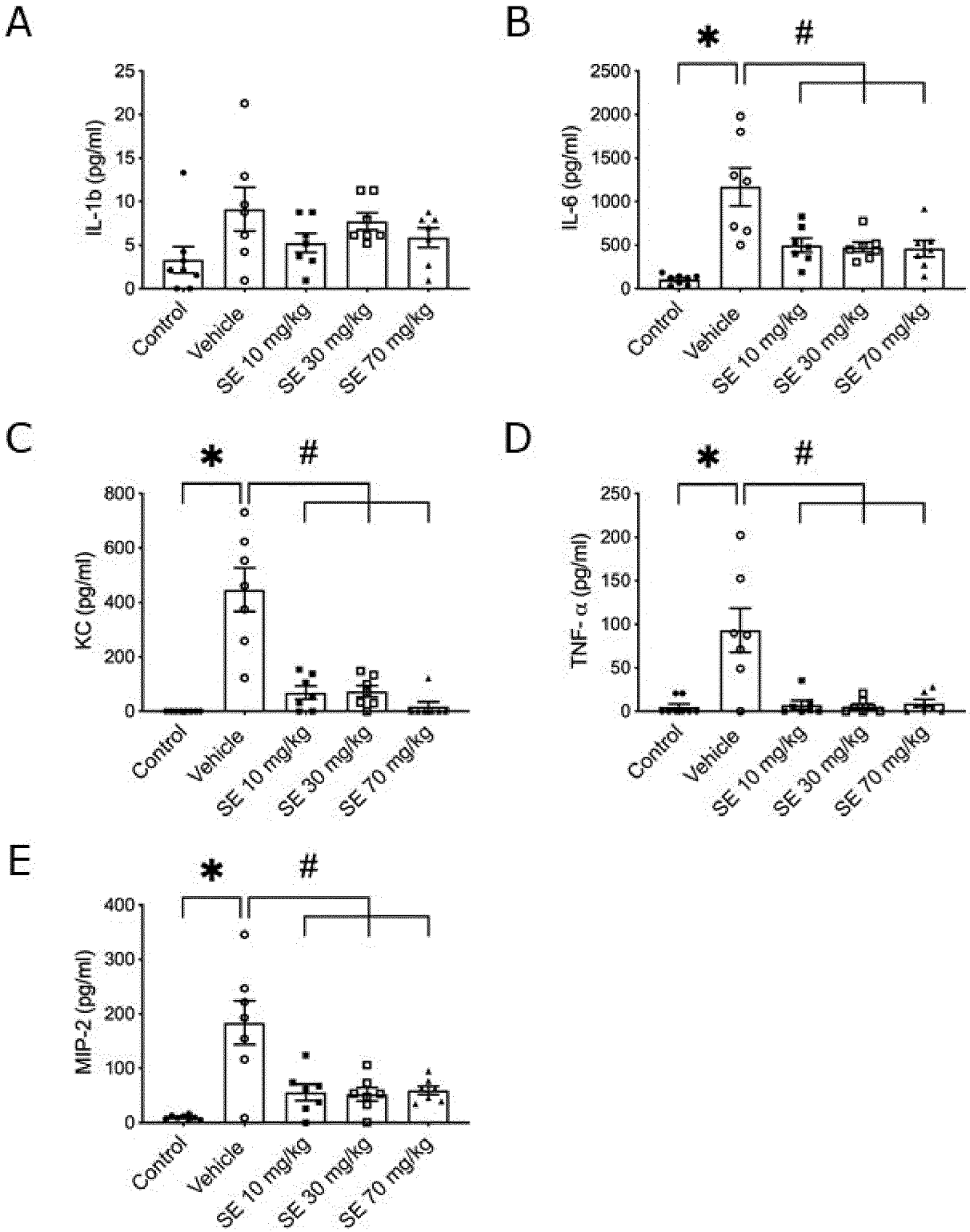

A
IL-1b (pg/ml)
25
20
15
10
5
0
Control
Vehicle
SE 10 mg/kg
SE 30 mg/kg
SE 70 mg/kg
B
*
#
IL-6 (pg/ml)
2500
2000
1500
1000
500
0
C
KC (pg/ml)
800
600
400
200
0
D
TNF- α (pg/ml)
250
200
150
100
50
0
E
MIP-2 (pg/ml)
400
300
200
100
0

Fig. 9

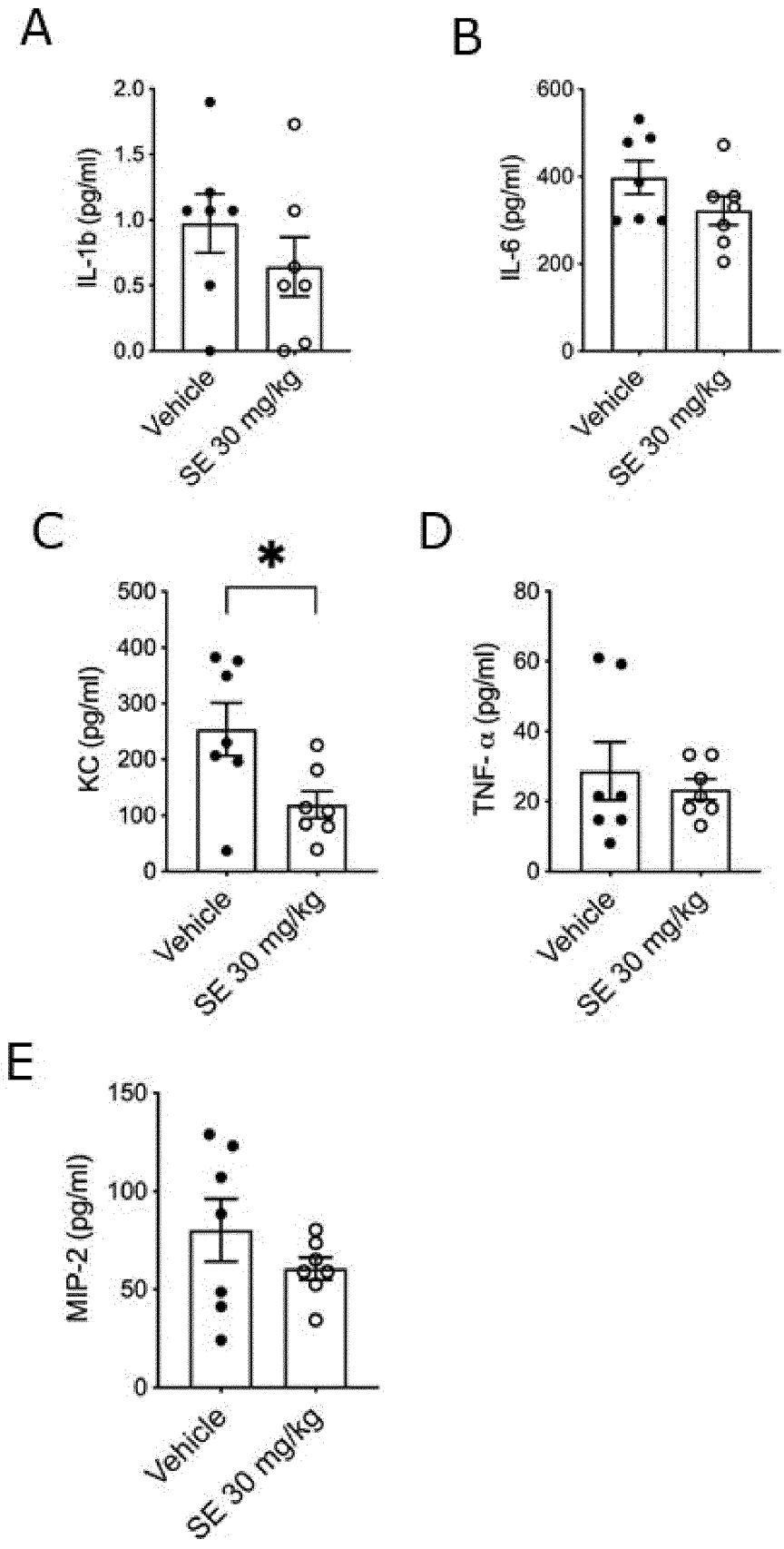

A
IL-1b (pg/ml)
2.0
1.5
1.0
0.5
0.0
Vehicle
SE 30 mg/kg
B
IL-6 (pg/ml)
600
400
200
0
Vehicle
SE 30 mg/kg
C
*
KC (pg/ml)
500
400
300
200
100
0
Vehicle
SE 30 mg/kg
D
TNF- α (pg/ml)
80
60
40
20
0
Vehicle
SE 30 mg/kg
E
MIP-2 (pg/ml)
150
100
50
0
Vehicle
SE 30 mg/kg

Fig. 10

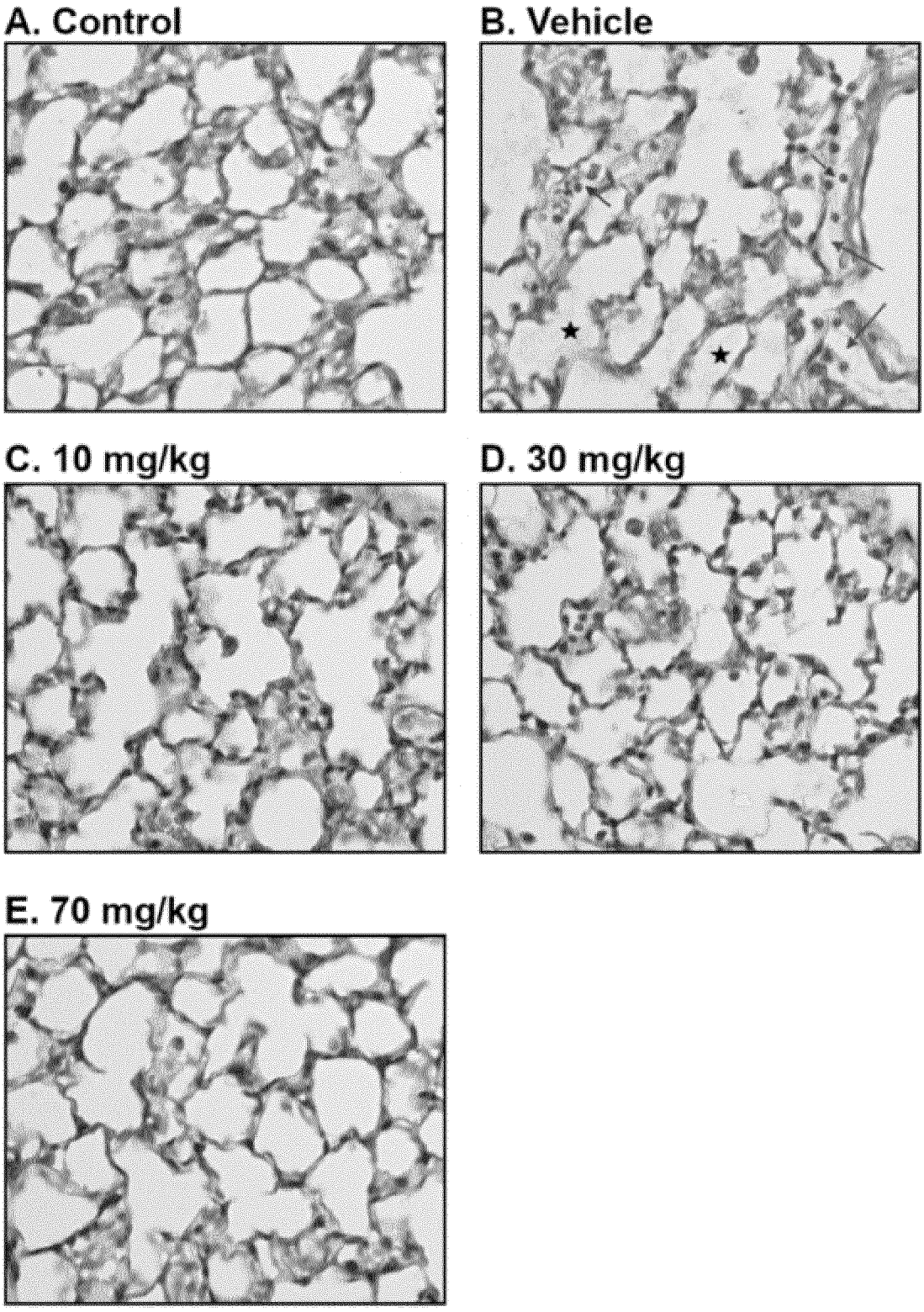


Fig. 11

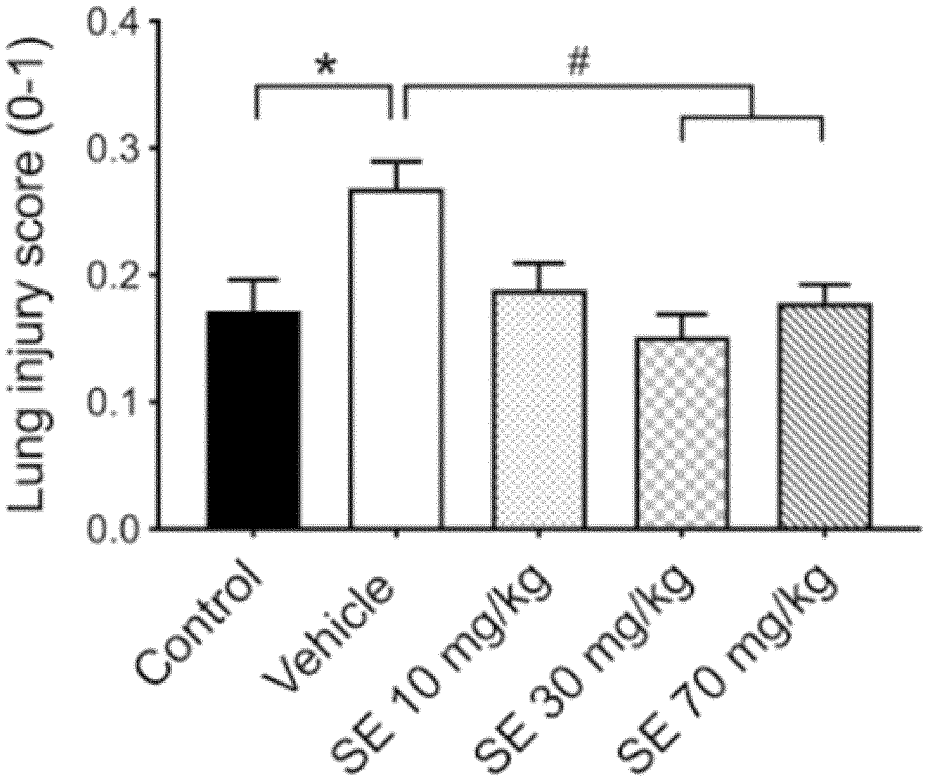

Lung injury score (0-1)
0.4
0.3
0.2
0.1
0.0
*
#
Control
Vehicle
SE 10 mg/kg
SE 30 mg/kg
SE 70 mg/kg

Fig. 12

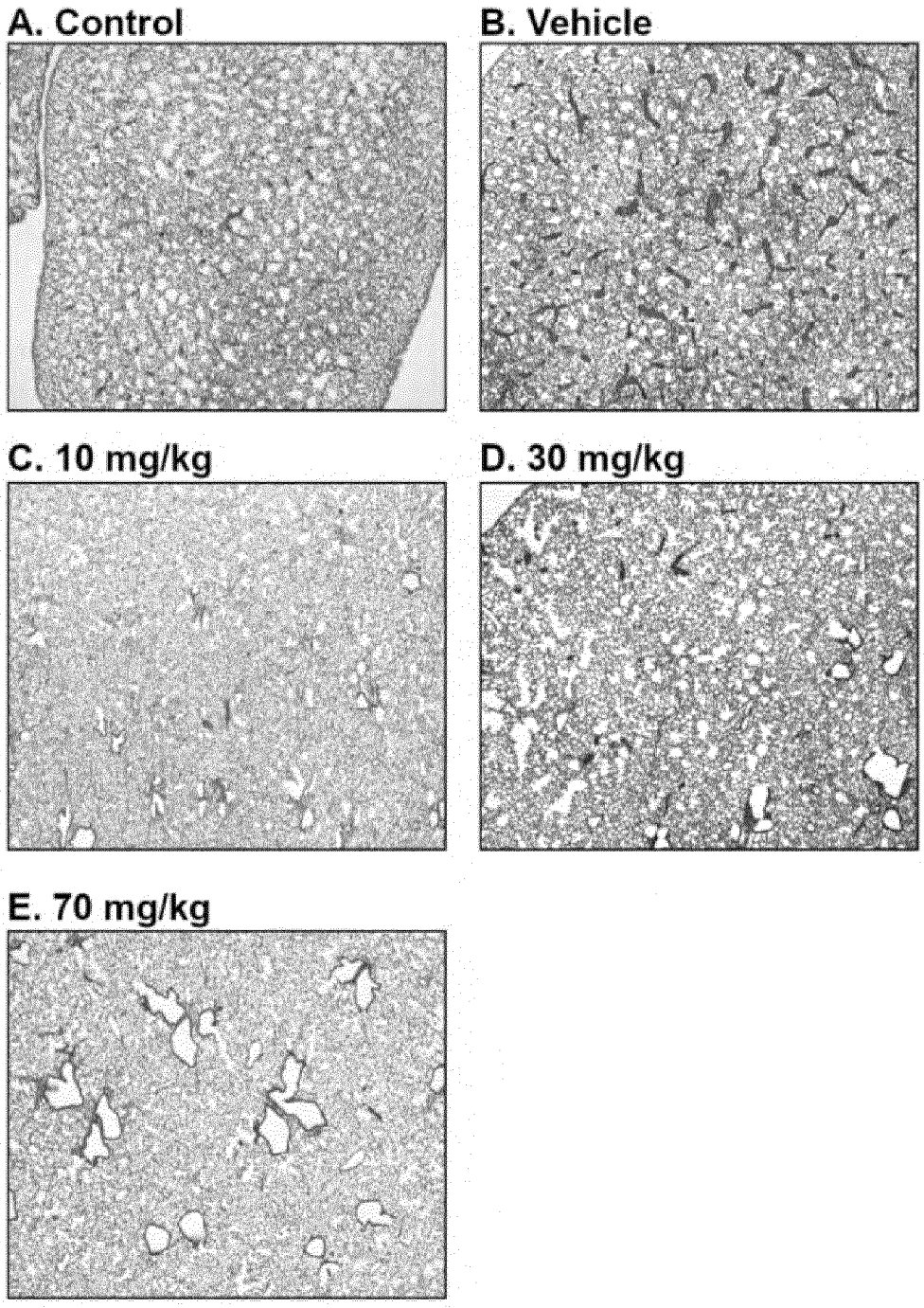


Fig. 13

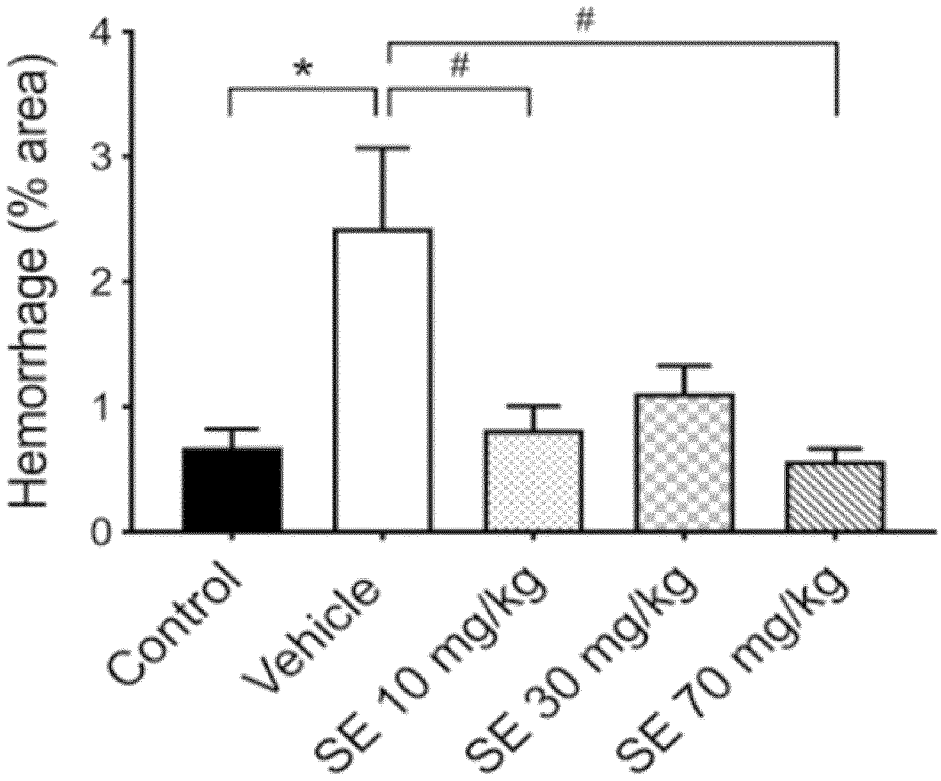
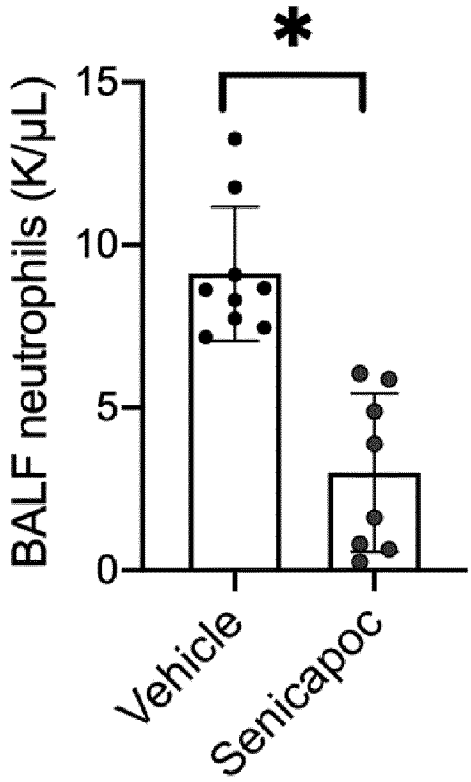

Hemorrhage (% area)
0
1
2
3
4
*
#
#
Control
Vehicle
SE 10 mg/kg
SE 30 mg/kg
SE 70 mg/kg

Fig. 14

A)


*
BALF neutrophils (K/µL)
15
10
5
0
Vehicle
Senicapoc

B)

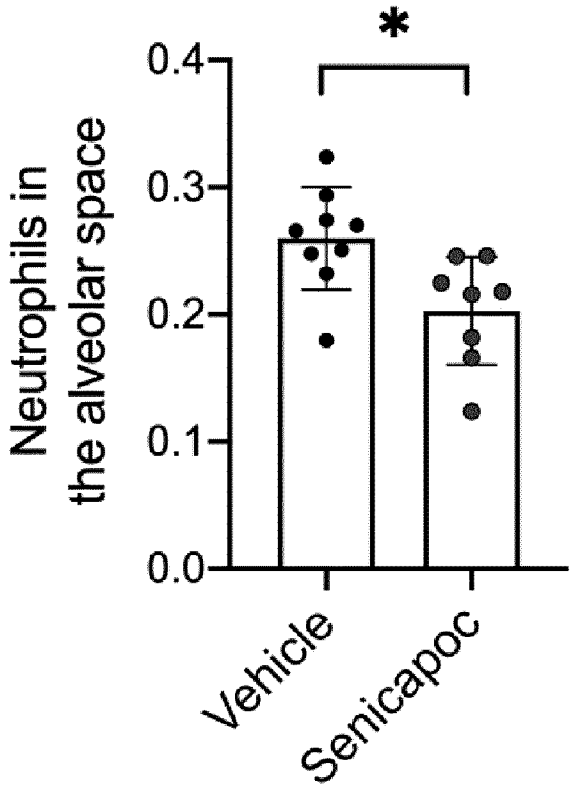

*
Neutrophils in the alveolar space
0.4
0.3
0.2
0.1
0.0
Vehicle
Senicapoc

Fig. 15

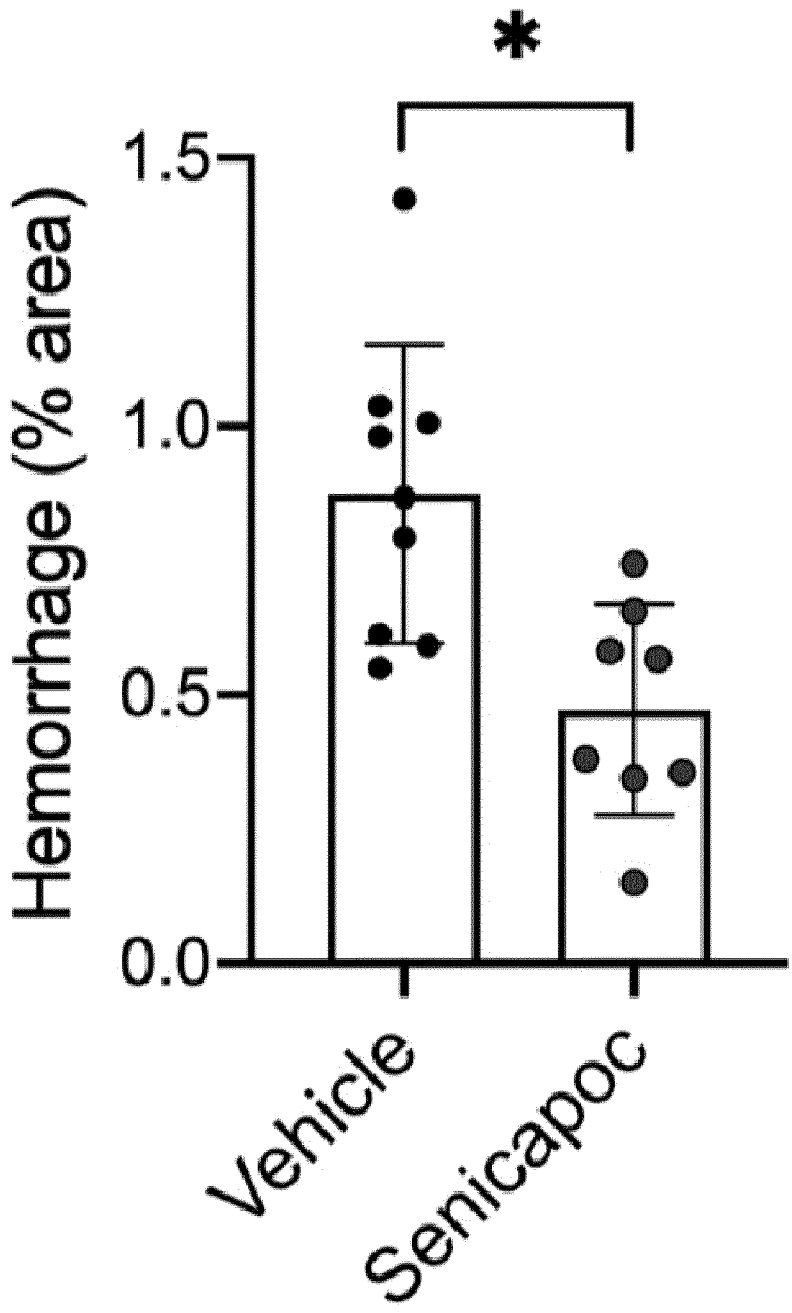


Fig. 16

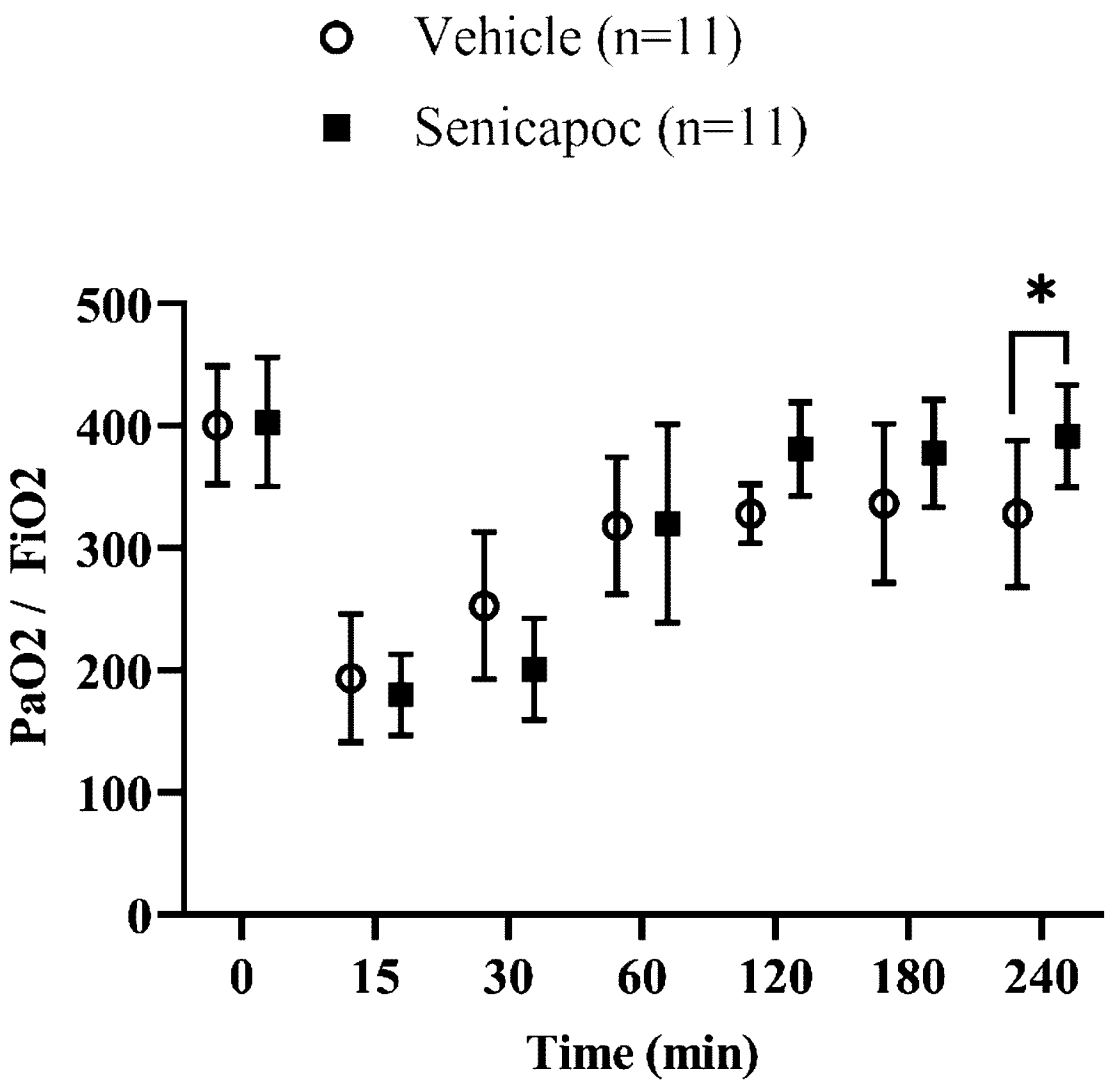

Vehicle (n=11)
Senicapoc (n=11)
PaO2 / FiO2
500
400
300
200
100
0
0
15
30
60
120
180
240
Time (min)
*

Fig. 17

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/056504

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/165 A61K31/415 A61P11/00 A61P31/14
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YING-HUI JIN ET AL: "A rapid advice guideline for the diagnosis and treatment of 2019 novel coronavirus (2019-nCoV) infected pneumonia (standard version)", MILITARY MEDICAL RESEARCH, vol. 7, no. 1, 6 February 2020 (2020-02-06), XP055725328, DOI: 10.1186/s40779-020-0233-6 page 13, right-hand column, paragraph 4 page 17, left-hand column, paragraph 4 - right-hand column, paragraph 2 ----- -/--	1-15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2021	04/06/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bazzanini, Rita

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PETERSEN A G ET AL: "The KCa3.1 channel blocker, senicapoc prevents the development of lung oedema and hypoxaemia in a ventilator-induced acute injury model", BRITISH JOURNAL OF PHARMACOLOGY - CONFERENCE ABSTRACTS FROM THE BRITISH-PHARMACOLOGY-SOCIETY MEETING (PHARMACOLOGY); LONDON, UK; DECEMBER 18 -20, 2018, vol. 176, no. 16, August 2019 (2019-08), pages 3060-3061, XP009522507, the whole document -----	1-15
A	U. SIMONSEN ET AL: "Emerging roles of calcium-activated K channels and TRPV4 channels in lung oedema and pulmonary circulatory collapse", ACTA PHYSIOLOGICA, vol. 219, no. 1, 16 September 2016 (2016-09-16), pages 176-187, XP055725246, GB ISSN: 1748-1708, DOI: 10.1111/apha.12768 cited in the application the whole document -----	1-15
A	C. HENRÍQUEZ ET AL: "The calcium-activated potassium channel KCa3.1 plays a central role in the chemotactic response of mammalian neutrophils", ACTA PHYSIOLOGICA, vol. 216, no. 1, 19 July 2015 (2015-07-19) , pages 132-145, XP055725242, GB ISSN: 1748-1708, DOI: 10.1111/apha.12548 the whole document -----	1-15
A	WO 2011/073662 A1 (ASTRAZENECA AB [SE]; ASTRAZENECA UK LTD [GB] ET AL.) 23 June 2011 (2011-06-23) page 13, line 6 page 43, lines 7-23 -----	1-15

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2021/056504

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011073662 A1	23-06-2011	NONE	