TITLE: METHODS AND MEANS FOR MODIFYING HEMODYNAMICS IN INFECTIONS

TECHNICAL FIELD

The application relates to methods and means for alleviating certain effects resulting from infection, in particular hemodynamic effects. More specifically the invention relates to peptide preparations used in the treatment of viral infections that affect the permeability of the vascular system.

BACKGROUND

Recently, the world has been hit hard by a pandemic caused by a virus called SARS-Cov-2, a coronavirus causing COVID-19. The new coronavirus mainly seems to kill by flooding and clogging the tiny air sacs in the lungs with fluid, choking off the body's oxygen supply until it shuts down the organs essential for life. Such suffocation with one's own fluid seems a model of respiratory disease that more coronaviruses may be capable of inducing. A large reservoir of such viruses in various exotic animals may cause a similar pandemic with similar suffocation as SARS-Cov-2, considering our lack of pre-existing immunity. As virus-specific vaccines and/or antiviral agents will typically become available only after the infection has already spread throughout a large part of the population, other means and methods for treatment are dearly needed, as we may expect more of these types of viral infections, typically through zoonotic occurrences, where vaccines or anti-viral agents would not be specific enough or developed too late. Therefore there is a persisting and continuous need to at least be able to combat deleterious effects that these infections will have in common. The present invention provides means and methods to do just that.

BRIEF SUMMARY OF THE INVENTION

In a first embodiment, the invention provides a method for reducing the permeability of an endothelial layer of a blood vessel in a subject, the method comprising providing to the endothelial layer a substance that reduces the ratio of Angiopoietin-2 to Angiopoietin-1 at the site of increased permeability as a result of an infection, such as with a virus. In a preferred embodiment, the invention provides said method for reducing the gas diffusion distance between lung-alveoli and the vascular network surrounding alveoli in a human subject suffering from a respiratory infection, allowing reduction of fluid in alveoli and/or allowing improved oxygen supply to the subject's body. In a preferred embodiment, said substance comprises an AQGV-peptide, an LQGV-peptide, or a functional analogue of either. In a preferred embodiment, the invention provides said method for reducing the gas diffusion distance between lung-alveoli and the vascular network surrounding alveoli in a human subject suffering from an infection with a respiratory virus. It is moreover preferred that said viral infection is caused by a virus requiring a specific receptor and a more ubiquitous binding partner present on at least a percentage of lung alveolar cells. In one preferred embodiment, it is preferred that said specific receptor is ACE-2. In another preferred embodiment, it is preferred that the more ubiquitous binding partner is a glycoprotein comprising a sialic acid residue. It is in particular preferred that said ubiquitous binding partner binds with a fMLF-like amino acid sequence, for example wherein said sequence at least comprises a membrane-proximal-external-region (MPER, herein also identified as fusogenic sequence). It is in particular preferred that said virus is a coronavirus, in particular a coronavirus with an MPER as identified in figure 11, more in particular at least comprising a fusogenic sequence as identified in figure 12. It is most preferred that said MPER at least comprises amino acid sequence KWPWIWL (amino acids identified herein by one-letter code). In a further preferred embodiment, the invention provides a method for reducing the permeability of an endothelial layer of a blood vessel in a subject, the method comprising providing to the endothelial layer a substance that reduces the ratio of Angiopoietin-2 to Angiopoietin-1 at the site of increased permeability as a result of an infection, such as with a virus, wherein the coronavirus is the COVID-19 virus (SARS-COV-2) or a mutant thereof.

The invention also provides a method for reducing the permeability of an endothelial layer of a blood vessel in a subject, the method comprising providing to the endothelial layer a substance that reduces the ratio of Angiopoietin-2 to Angiopoietin-1 at the site of increased permeability as a result of an infection, wherein said substance is administered intravenously to said subject, preferably at a rate of at least 75mg/kg/hr, or more preferably at least 90 mg/kg/hr. It is moreover preferred that the substance is administered intermittently. It is moreover preferred that during treatment the subject is monitored for haemodynamic stability. The invention also provides a method for reducing the permeability of an endothelial layer of a blood vessel in a subject, the method comprising providing to the endothelial layer a substance that reduces the ratio of Angiopoietin-2 to Angiopoietin-1 at the site of increased permeability as a result of an infection, wherein said substance is administered intravenously to said subject, said method further comprising administering an antiviral agent, such as remdesivir (GS-5734), an inhibitor of the viral RNA-dependent, RNA polymerase, to the subject. The invention also provides a method for reducing the permeability of an endothelial layer of a blood vessel in a subject, the method comprising providing to the endothelial layer a substance that reduces the ratio of Angiopoietin-2 to Angiopoietin-1 at the site of increased permeability as a result of an infection, wherein said substance is administered intravenously to said subject, said method further comprising administering an anti-inflammatory agent, such as dexamethasone or an interleukin-6 signaling inhibitors such as tocilizumab, to the subject.

The invention also provides a pharmaceutical formulation for use in a method for reducing the permeability of an endothelial layer of a blood vessel in a subject, the method comprising providing to the endothelial layer a substance that reduces the ratio of Angiopoietin-2 to Angiopoietin-1 at the site of increased permeability as a result of an infection, wherein said substance is administered intravenously to said subject, preferably at a rate of at least 75mg/kg/hr, or more preferably at least 90 mg/kg/hr. It is moreover preferred that the substance is administered intermittently. It is moreover preferred that during treatment the subject is monitored for haemodynamic stability. The invention also provides a pharmaceutical formulation for use in reducing the permeability of an endothelial layer of a blood vessel in a subject, the method comprising providing to the endothelial layer a substance that reduces the ratio of

Angiopoietin-2 to Angiopoietin-1 at the site of increased permeability as a result of an infection, wherein said substance is administered intravenously to said subject, said method further comprising administering an antiviral agent, such as remdesivir (GS-5734), an inhibitor of the viral RNA-dependent, RNA polymerase, to the subject. The invention also provides a pharmaceutical formulation for use in reducing the permeability of an endothelial layer of a blood vessel in a subject, the method comprising providing to the endothelial layer a substance that reduces the ratio of Angiopoietin-2 to Angiopoietin-1 at the site of increased permeability as a result of an infection, wherein said substance is administered intravenously to said subject, said method further comprising administering an anti-inflammatory agent, such as dexamethasone or an interleukin-6 signaling inhibitors such as tocilizumab, to the subject.

The invention also provides a pharmaceutical formulation for use according to the invention, comprising an AQGV-peptide, an LQGV-peptide, or a functional analogue of either and an excipient suitable for parenteral administration.

When a human subject is suffering from an infection, in particular a viral infection, more in particular a viral respiratory infection, many effects are seen that influence hemodynamic stability. One of the effects seen is increased permeability of blood vessels leading to fluid leakage from blood vessels into the intercellular spaces, and vice versa, resulting in aggravated and traumatic damage to the lung, and other organs. Typical signs of such suffocating damage induced by leakage from vessels include increased extra-cellular fluid in lungs with fluid overflow into alveoli. Also, thrombosis may be seen, in particular leading to (deep) venous thrombosis ((D)VT) and pulmonary embolism (PE). Particularly in respiratory infections, this all may lead to an increased diffusion distance for gasses such as oxygen and carbon dioxide to traverse the distance between alveoli and blood, and to hypoxemia. Oxygen and carbon dioxide both need to pass through a thin layer in the lungs called the alveolar-capillary membrane. This is the thin layer between the small air sacks in the lung (the alveoli) and the smallest blood vessels that travel through the lungs (lung-capillaries). How well oxygen is inhaled and can pass (diffuse) from the alveoli into the blood, and how well carbon dioxide can pass from the blood capillaries back into the alveoli to be exhaled, depends on how thick (swollen) this membrane is, and how much surface area is available for the transfer to take place.

This problem is aggravated in people already suffering from limited oxygen availability through underlying disease. Diffusing capacity may be low if there is less surface area available for the transfer of oxygen and carbon dioxide, for example with emphysema, or if a lung or part of it is removed for lung cancer, or PE and or pre-existing cardiovascular and metabolic issues and obesity.

Also, diffusing capacity may be low if lung disease is present that causes the membrane to be thicker, for example in chronic lung disease such as pulmonary fibrosis, as for example seen with COPD, and with sarcoidosis. The present invention is particularly useful for such patients having only partial lung capacity.

Acute disease can also result in low diffusing capacity, for example in aggravated viral respiratory infections with injury to the lung, often the permeability of lung-capillaries is increased generating a flux of fluid from the capillaries into the thin layer of extra-cellular-matrix separating alveoli from capillaries, with intercellular fluid retention therewith thickening (swelling) the membrane through accumulation of fluid in the extra-cellular-matrix (interstitium) separating alveolar cells from vascular cells. In such lung injury patients and patients with aggravated infectious airway infections, plasma levels of biomarkers of endothelial activation, as may be measured by ELISA, are often predictive of mortality and morbidity. In particular, the concentration of angiopoietin-2 relative to angiopoietin-1 (Ang-2/Ang-1) may be a useful biologic marker of mortality in acute lung injury (ALI) patients. Ang-2/Ang-1 is found significantly higher in patients who died of lung injury [p=0.01; Crit Care Med. 2010 Sep; 38(9): 1845–1851.]. In a multivariable analysis stratified by dead space fraction, Ang-2/Ang-1 was an independent predictor of death with an adjusted odds ratio of 4.3 (95% CI 1.3–13.5, p=0.01) in those with an elevated pulmonary dead space fraction (p=0.03 for interaction between pulmonary dead space fraction and Ang-2/Ang-1).

Similarly, D-dimer plasma levels may be used to follow a patient's health status in aggravated viral respiratory infections with injury to the lung and endothelial activation. D-dimer, the lysis product of cross-linked fibrin indicates fibrinolysis in response to clotting activation and fibrin formation (doi.org/10.1111/jth.12075). D-dimer levels are evident in febrile and convalescent phases typically following viral infections that affect vascular endothelial cells and associate with endothelial activation and plasma leakage. D-dimer assays can vary in sensitivity depending on the

lab-specific type drawn, and not all labs report the same units providing various acceptable ranges for the results. There are many things that can cause elevated D-dimer beyond venous thromboembolism (VTE), such as age or pregnancy. The D-dimer half-life of 8 hours results in elevated levels for approximately 3 days after the inciting event. Quantitative D-dimer holds a sensitivity of 94% to 98%, yet only specificity of 50% to 60%. This allows to utilize it as a screening tool but requires clinical evidence from the history and physical examination, preferably with intermittent repeated testing to confirm the diagnosis or follow a patient's health status.

Thus, the invention in one aspect provides a method for reducing the permeability of an endothelial layer of a blood vessel comprising providing to the layer a substance reducing the ratio of angiopoietin-2 to angiopoietin-1 at the site of increased permeability as a result of an infection. In one embodiment this method serves to reduce the gas diffusion distance (or at least to prevent increasing the diffusion distance) between lung-alveoli and the vascular network surrounding alveoli in a human subject suffering from a respiratory infection, in particular patients with underlying disease causing limited oxygen availability. Reduced vascular permeability in patients suffering vascular leakage is generally associated with reduced D-dimer levels. According to the invention, in one embodiment the substances to be used in the methods according to the invention include peptides that influence hemodynamics, particularly by influencing gap junctions between the cells. Such peptides include AQGV and functional analogues thereof. A functional analogue is defined as a substance that provides the same or a similar function (in kind, not necessarily in amount). Basically, any substance that decreases permeability of the vascular system may be used according to the present invention. For one, tetrapeptide AQGV (herein also referred to as EA-230) has surprisingly been found to modulate vascular permeability to the good. In particular, EA-230 significantly improves hemodynamic stability in humans, even in the absence of inflammatory activity of the patient. Permeability governs the amount of fluid leaking from blood vessels. Administration of fluid therapy generally increases leakage. Based on Phase II trial patient observations, we found a significant reduction of adverse fluid retention (fluid leakage) in patients treated with EA-230 ($p = 0.03$). Throughout clinical trials, EA-230 was shown to be safe and well tolerated. EA-230 shows significant improvements in patient recovery, over placebo patient. EA-230 treated patients are released faster from intensive care ($p=0.0232$) and hospital ($p=0.0015$).

EA-230 improves hemodynamic stability (p=0.006) and kidney function (p=0.003). Long-term patient recovery was significantly improved by EA-230. By improving vascular permeability, EA-230 can be used to reduce the infection-associated occurrence of adverse fluid in the lungs, reduce hypoxemia, reduce PE, and therewith also reduce ventilator use with its detrimental systemic effects, in particular in viral respiratory infections such as caused by influenza viruses and in particularly by coronaviruses. Thus, provided is in particular a method according to the invention wherein the active substance to control hemodynamic stability comprises an AQGV peptide. A functional and/or structural AQGV analogue according to the invention may be selected from the group consisting of peptides comprising a tetrapeptide selected from the group of AQLP, PLQA, LQGV, LAGV, PQVG, PQVA, PQVR, VGQL, LQPL, RQGV, LQVG, LQGA, LQGR, AQGA, QPLA, PQVP, VGQA, QVGQ, VGQG or other permutations of peptides of 4-12 amino acids constituted of particularly the amino acids of the above tetrapeptides. The invention further provides a method wherein the viral infection is caused by a virus requiring a specific receptor and a more ubiquitous binding partner present on at least a percentage of lung alveolar cells. In one preferred embodiment, the viral infection is caused by a coronavirus wherein the specific receptor is ACE-2, in particular wherein the coronavirus is SARS-Cov-2 or a mutant or analogue thereof. Other coronavirus infections which may be treated according to the invention carry specific receptor DPP4 (such as with MERS corona virus) or APN (aminopeptidase N). Also preferred is a method wherein the more ubiquitous binding partner is a glycoprotein comprising a sialic acid residue. It is in particular preferred that said ubiquitous binding partner binds with a fMLF-like amino acid sequence, for example wherein said sequence at least comprises a membrane-proximal-external-region (MPER, herein also identified as fusogenic sequence). Such a method according to the invention is in particular provided wherein the virus is a coronavirus. It is in particular preferred that said virus is a coronavirus, in particular a coronavirus with an MPER as identified in figure 11, more in particular at least comprising a fusogenic sequence as identified in figure 12. Alternatively, the more ubiquitous binding partner is a glycoprotein comprising a sialic acid residue as recognized by influenza virus. The combination of a specific and a more ubiquitous binding/infection site on a cell is typical for coronaviruses, with their typical effect on the hemodynamics as disclosed herein. In a further preferred embodiment, provided is a method wherein the AQGV peptide or related

substance is administered intravenously, preferably at a rate of at least 75mg/kg/hr., more preferably at least 90 mg/kg/hr. It is in particular useful to administer the AQGV peptide or related substance intermittently. Preferred use is dosing for 2-4 hours at at least 90mg/kg/hour, then reducing to 30mg/kg/hour for 2-4 hours, or for as long as it takes to monitor the patients response to treatment by clinical or laboratory diagnosis, or stop administering the substance for 1-2 hours until diagnostic studies such as point-of-care testing have completed, and then resume treatment with 2-4 hours at at least 90mg/kg/hour. It is preferred that the monitoring comprises studying the subject for hemodynamic stability and/or fibrinolysis. Treatment with AQGV peptide according to the invention may further comprise administering an antiviral agent. The invention also provides a pharmaceutical formulation comprising an AQGV peptide or related substance (preferably a functional analogue) for use in a method according to the invention, or a pharmaceutical formulation for use according to the invention, comprising an AQGV peptide, or a functional analogue thereof and an excipient suitable for parenteral administration.

Often, the human subject or patient experiencing reduced diffusion, may be admitted into an intensive care unit (ICU) where vital signs are monitored. The patient receives medical treatment to allow the patient to recover and when vital signs are within acceptable boundaries, the patient can be released from ICU and admitted into standard hospital care. When the patient has shown to be stable at standard care, the patient can be released from the hospital and returns home. Subsequently, a patient can be readmitted into the hospital should the need arise because e.g. the condition or infection status of the patient worsens. Any improvement on the health and recovery of a patient affecting the length of stay of a patient in the ICU, the length of stay in standard care at the hospital and/or patient re-admittance, provides for a significant benefit to patients. Hence, any means and methods according to the invention that improve the health and in particular speed of recovery of a patient through use of the peptide compounds disclosed herein are of interest.

Upon testing in a clinical trial aimed at assessing the safety and tolerability of an AQGV peptide (also referred to as EA-230 herein) and its immunomodulatory effects, the peptide was found to be safe but, unexpectedly, no immunomodulatory effects were observed under the test circumstances when comparing treated patients as compared with control subjects. Instead of

observing immunomodulatory effects, the current inventors surprisingly found that upon analysis of the data obtained in the clinical trial, new and highly advantageous properties could be attributed to the AQGV peptide, which have not been observed before. These properties are apparently independent from known and observed immunomodulatory effects.

Hence, the current invention relates to the use of an AQGV peptide, and analogues thereof, for improving the clinical parameters of human patients admitted into hospital and/or intensive care such that the time period between admittance and release from hospital and/or intensive care can be shortened. In one embodiment, the use of an AQGV peptide, and analogues thereof, is for use in a medical treatment for modifying hemodynamics in human subjects. In a further embodiment, the use in human subjects for modifying hemodynamics, involves a reduction of reducing undesired fluid retention and/or a reduced use of vasopressive agents in the human subject. In another embodiment, the use of an AQGV peptide, and analogues thereof, is for use in human subjects having impaired lung function.

In one embodiment, an AQGV peptide, or a functional analogue thereof, is provided for use in a method of treatment of a human subject, wherein the use comprises a treatment for modifying hemodynamics in the human subject. Hemodynamics involves the dynamics of blood flow, i.e. the physical factors that govern blood flow through the human body. Hemodynamics in human patients can be monitored by measuring e.g. blood pressure and/or the fluid balance. When blood pressure is low and/or the fluid balance disturbed in a human patient, vasopressors, or inotropes may be used and/or fluid administered, e.g. intravenously. Inotropes and vasopressors are biologically and clinically important vasoactive medications that originate from different pharmacological groups and act at some of the most fundamental receptor and signal transduction systems in the body. More than 20 such agents are in common clinical use, yet few reviews of their pharmacology exist outside of physiology and pharmacology textbooks. Despite widespread use in critically ill patients, understanding of the clinical effects of these drugs in pathological states is poor. Adverse effects of vasopressors and inotropes depend on the mechanism of action. For the medications that have beta stimulation, arrhythmias are one of the most common adverse effects that one would like to reduce.

The current inventors have found that by using an AQGV peptide, or a functional analogue thereof, the hemodynamics in human patients post-trauma (e.g. viral infection) was significantly improved as shown by e.g. a reduced use of vasopressors and/or an improved fluid balance in human patients. The use of an AQGV peptide, or a functional analogue thereof, as described herein thus improves the hemodynamic stability in human patients. Modifying or optimizing hemodynamics in human subjects is of importance post-injury, when e.g. human subjects have suffered infection, trauma and/or blood loss. Hence, the AQGV peptide, or an analogue thereof, can advantageously be used in hemodynamic therapy. Hemodynamic therapy comprises the optimization of hemodynamics in patients in goal-directed hemodynamic therapy. Such therapies can include therapeutic interventions such as fluid management in patients and/or the use of vasopressors.

AQGV functional analogues are defined herein as peptides exerting analogous effect or function as the AQGV peptide as described herein, in kind not necessarily in amount. The AQGV peptide has a length of 4 amino acids. An AQGV functional analogue may have sequence identity, i.e. comprising at least part or the whole of the AQGV peptide. Preferably, such an AQGV functional analogue is a structural analogue of the AQGV peptide. A preferred structural analogue may be an LQGV peptide. Structural analogues of the AQGV peptide may be selected from peptides comprising amino acids selected from the group of amino acids alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), proline (P) and arginine (R). In a preferred embodiment, provided is for a AQGV structural analogue, that comprises at least 50%, more preferably at least 75%, most preferably at least 100% amino acids selected from the group of autophagy inhibiting amino acids alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), proline (P), and arginine (R). Preferably, a structural analogue of the AQGV peptide has a length in the range of 4-12 amino acids. Preferably, such a structural analogue is a linear peptide. Suitable structural analogues of AQGV may have a length less than 4, e.g. of 3, however such lengths may require higher doses of such peptides because the half-life of such peptides will be shorter and thus less preferred. Longer structural analogues, e.g. longer than 12 residues, are less preferred because of potential immunogenicity of such longer peptides. A structural AQGV analogue according to the invention may be selected from the group consisting of peptides comprising a tetrapeptide

selected from the group of AQLP, PLQA, LQGV, LAGV, PQVG, PQVA, PQVR, VGQL, LQPL, RQGV, LQVG, LQGA, LQGR, AQGA, QPLA, PQVP, VGQA, QVGQ, VGQG.

Vasopressors are a class of drugs that can elevate low blood pressure. Some vasopressors act as vasoconstrictors, other vasopressor sensitize adrenoreceptors to catecholamines - glucocorticoids, and another class of vasopressors can increase cardiac output. Whichever vasopressor is used, the current invention allows for a reduction in the use of vasopressors. A reduction in the use of vasopressors involves a reduction of the duration of vasopressor use and/or a reduction of the dosage of the vasopressor. Examples of vasopressors are e.g. epinephrine, noradrenaline, phenylephrine, dobutamine, dopamine, and vasopressin. Fluid management in patients involves monitoring e.g. oral, enteral, and/or intravenous intake of fluids and fluid output (e.g. urine) and subsequently managing fluid intake e.g. in case of an observed fluid retention (i.e. the fluid intake exceeds fluid output). Strikingly, the use of the AQGV peptide, or an analogue thereof, can reduce fluid retention. Hence, the AQGV peptide, or analogue thereof, can be used in addition to known interventions that are to improve the hemodynamics in human patients, thereby resulting in a faster improvement in hemodynamics as compared with not using an AQGV peptide, or an analogue thereof.

In another embodiment, an AQGV peptide, or a functional analogue thereof, is provided for use in the treatment of a human subject having impaired lung function. In a further embodiment, the impaired lung function is acute lung injury. In one embodiment, an AQGV peptide, or a functional analogue thereof, is provided for use in the treatment of a human subject for improving lung function. Lung function can also be assessed by measuring hypoxemia, or by determining the alveolar–arterial gradient (A-aO2, or A–a gradient). Assessing A-a gradient to assess lung function in humans is standard clinical practice (e.g. by determining the difference between the alveolar concentration (A) of oxygen and the arterial (a) concentration of oxygen. It is used in diagnosing the source and degree of hypoxemia. The A–a gradient helps to assess the integrity of the alveolar capillary unit. Improvements in lung function as compared with not receiving the AQGV peptide can include progressing to a lung function stage to a less severe stage (e.g. a patient progressing from having lung injury to being at risk of lung injury or having no lung injury). Irrespective of what assessment is made, the use of the AQGV peptide, or analogue thereof, can

improve lung function in humans having lung injury and/or an impairment of lung function in subjects absent of immunomodulatory effects.

The use of the AQGV peptide allows for improving lung function, however, it can also prevent a reduction and/or an impairment of lung function. Accordingly, lung injury with hypoxemia may be prevented. Hence, in one embodiment, the use of the AQGV peptide, or analogue thereof, allows to maintain lung function in human patients. Hence, the use of the AGQV peptide, or analogue thereof allows to protect lung function in human patients. In another embodiment, the use of the AQGV peptide, or analogue thereof, allows to prevent a reduction and/or impairment of lung function in human patients. For example, a human patient that can be classified as having no lung injury, or being at risk of having lung injury (such as with COVID-19), may receive treatment with the AQGV peptide, whereby such a patient may maintain its status instead of progressing to a (more severely) reduced lung function. Hence, human patients that are at risk of developing lung injury, e.g. due to (induced) trauma, such as infection, may, as a result of receiving treatment with the AQGV peptide, or analogue thereof, maintain their lung function status.

In another embodiment, an AQGV peptide, or a functional analogue thereof, is provided for use in the treatment of a human subject having impaired lung function whereby the use comprises modifying hemodynamics in the human subject. As treatment of lung function and treatment of hemodynamic stability can now be linked, the use of an AQGV peptide, or a functional analogue thereof, in accordance with the invention can advantageously be used to protect lung function and/or improve lung function, and modifying hemodynamics. Such combined use resulting e.g. in improved and/or maintained lung function and a reduction in the use of vasopressors and/or improved fluid management in human subjects.

In a further embodiment, the current invention provides for a reduced use of vasopressive agents. The use of vasopressive agents can be reduced by reducing the duration of the use of vasopressive agents. The use of vasopressive agents can be reduced by reducing the amount of vasopressive agents (e.g. reducing amount per dosage and/or increasing time interval between administrations). The use of vasopressive agents can be reduced by reducing the amount of vasopressive agents and the duration of the use of vasopressive agents. By reducing the use of

vasopressive agents, human subjects advantageously recover more quickly as compared with human subjects not receiving AQGV, or an analogue thereof.

In another embodiment, the use of an AQGV peptide, or a functional analogue thereof, reduces adverse fluid retention in the human subject. Fluid retention can occur in human subjects, symptoms of which e.g. include weight gain and edema. Fluid retention can be the result of reduced lung function and/or impaired hemodynamics. Hence, because the use of AQGV can affect lung function and/or hemodynamic stability in human subjects, the use of AQGV can affect fluid retention as well. Fluid retention can be the result of leaky capillaries. Hence, the use of AQGV, and analogues thereof, may have an effect on the leakiness (permeability) of capillaries, reducing leakage of plasma from the blood to peripheral tissue and/or organs. Most preferably edema can be reduced and/or avoided by the use of AQGV. Such may also be referred to as adverse fluid retention as it has an adverse effect on the patient. Whichever is the cause of fluid retention, the use of an AQGV peptide, or a functional analogue thereof can improve fluid retention dynamics in human subjects thereby alleviating symptoms associated with fluid retention such as weight gain and edema, which subsequently can reduce the use of diuretics.

In another embodiment, the use of the AQGV peptide, or a functional analogue thereof, in accordance with the invention, is not restricted to patients having lung injury and/or requiring hemodynamic therapy. The use of an AQGV peptide, or a functional analogue thereof, in accordance with the invention, includes the treatment of human patients that are believed to be at risk of having lung injury and/or anticipated to require hemodynamic therapy. Such human patients include patients that are to be admitted, or are expected to be admitted, into intensive care. Hence, the use of the AQGV peptide, or a functional analogue thereof, includes a use for trauma, such as infection, as exemplified e.g. in the examples. The use of the AQGV peptide for trauma, such as infection, may be before, but is typically during and/or after infection. It may be preferred that the use of the AQGV peptide, or an analogue thereof, is during infection with a virus. The use of AQGV peptide as is provided herein is in particular useful in patients subjected to a long duration of mechanical ventilation, i.e. longer than 2.5 hours. Hence, in a further embodiment, the use of the AQGV peptide, or an analogue thereof, is during a mechanical ventilation of longer than 2.5 hours and wherein the AQGV peptide or analogue thereof is

administered during the mechanical ventilation. In another, or further, embodiment, the use of an AQGV peptide, or a functional analogue thereof, for use in accordance with the invention is for use is in a human subject having COVID-19. It is well known that shortening the duration of mechanical ventilation is highly correlated with recovery and prevention of re-admittance of patients.

Preferably, the use of the AQGV peptide, or a functional analogue thereof, in accordance with the invention and as described above, involves the administration of the peptide into the bloodstream. It is understood that administration into the bloodstream comprises e.g. intravenous administration or intra-arterial administration. A constant supply of AQGV peptide, or an analogue thereof, is preferred, e.g. via an infusion wherein the AQGV peptide, or analogue thereof, is comprised in a physiological acceptable solution. Suitable physiological acceptable solutions may comprise physiological salt solutions (e.g. 0.9% NaCl) or any other suitable solution for injection and/or infusion. Such physiological solutions may comprise further compounds (e.g. glucose etc.) that may further benefit the human subject, and may also include other pharmaceutical compounds (e.g. vasopressors).

Preferably, the AQGV peptide is administered at a rate which is at least 50 mg/kg patient weight per hour (mg/kg/hr). Preferably the administration rate is at least 60 mg, at least 70, at least 80 or, most preferably, at least 90 mg/kg/hr. Preferably, the AQGV peptide is administered for at least 1 hour, more preferably at least 1.5 hours, most preferably at least 2 hours. Preferably, the administration of the AQGV peptide is at a rate of at least 70 mg/kg/hr. and administered for at least 1 hour, more preferably at least 1.5 hours, most preferably at least 2 hours. Preferably, the administration is during infection. More preferably, the administration is during essentially the entire duration of disease resulting from infection. Typically treatment will start after determination of a level of severity justifying the treatment. Thus the treatment may typically last from its detection until the absence of detectable infection or sufficient recovery to allow for end of treatment.

As shown in the example section, the mean arterial maximum concentrations (mean Cmax) as determined in vivo in humans for EA-230 in the Phase II clinical trial was 30500 ng/mL, in the range of 12500 to 57500 ng/mL. The mean venous Cmax found was 68400 ng/mL, in the range of 19600 to 113000 ng/mL. Hence, whichever means and methods are used for administration of EA-230 (or

AQGV), preferably, means and methods that allow to obtain an arterial Cmax in the range of 10,000 to 60,000 ng/mL and/or a venous Cmax in the range of 15000 to 120000 ng/ml can be contemplated. Thus, the route of administration may not be necessarily be restricted to intravenous administration, but may include other routes of administration resulting in similar venous and/or arterial Cmax concentrations.

In another embodiment, an AQGV peptide, or a functional analogue thereof, is provided for any use in accordance with the invention as described above, wherein the human subject is admitted to intensive care, and wherein the use improves parameters measured of the human subject, the parameters of the human subject typically those being determined to assess whether the patient needs to remain in intensive care or not. As shown above, parameters that are assessed when a human patient is in intensive care include parameters related to lung function and hemodynamics. In any case, the use of the AQGV peptide, or analogue thereof, is to improve such parameters to thereby reduce the length of stay in the intensive care unit. Not only does the use of the AQGV peptide, or analogue thereof reduce the length of stay in the intensive care, the effect of the use of the AQGV peptide, or analogue thereof, also reduces the length of stay in the hospital and reduces re-admittance into the hospital.

In any case, the use of the AQGV peptide, or a functional analogue thereof has a profound effect on lung function and/or hemodynamics in human subjects thereby advantageously benefiting human subjects when e.g. suffering from induced trauma, e.g. when undergoing mechanical ventilation. Hence, in one embodiment, the use of the AQGV peptide, or a functional analogue thereof, is for use in patients subjected to mechanical ventilation. In another embodiment, the use of the AQGV peptide, or a functional analogue thereof, is for use in human patients experiencing or thought to be experiencing COVID-19 or a similar infection.

The invention relates to a distinct and new class of drugs: autophagy inhibiting compounds that comprise peptides and/or amino acids that target the nutrient sensing system of the mechanistic target of rapamycine, mTOR and inhibit autophagy. Upon testing formyl-peptide related signaling effects of an autophagy inhibiting AQGV peptide the peptide was found to unexpectedly attenuate p38/ p38-MK2-HSP27 and/or PI3K/AKT/mTOR pathways that govern signal cytoskeleton contraction in modulating vascular permeability. Hence, the current invention relates to the use of

an autophagy inhibiting peptide herein also referred to as an AQGV peptide, and analogues (functional equivalents) thereof, for improving vascular permeability.

Without being bound by theory, the effect of the AQGV peptide, or a functional analogue thereof, may have an effect on vasoconstriction. Vasoconstriction involves the narrowing of the blood vessels resulting from contraction of the muscular wall of the vessel. Hence, in one embodiment, the use of an AQGV peptide, or a functional analogue thereof, in accordance with the invention, involves inducing vasoconstriction.

The invention also provides a method for identifying a peptide capable of reducing p38 MAPK kinase activity, comprising providing cells with a peptide comprising amino acids, said amino acids for at least 50%, preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably 100%, amino acids selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R), providing said cells with fMLP and detecting phosphorylation of p38 MAPK in the absence and presence of said peptide at an appropriate time interval, preferably in the order of minutes, most preferably from about half a minute to about 5 minutes e.g. 30 to 600 seconds after provision of fMLP, and comparing the results to determine said peptide's effect on said phosphorylation. Having tested the autophagy inhibiting AQGV peptide, we detect FPR-activation of FPR-expressing cells with prototype FPR-ligand fMLP to cause rapidly induced and significant ($p < 0.05$; p38 from 60 to 600 sec, PKB at 600 sec) changes in phosphorylation status of PKB (also known as AKT) (figure 10a) and p38 MAPK kinases (figure 10c), but not (or not detected) in STAT3, JNK (figure 10b) and P42/p44MAPK/ERK1,2 (figure 10d) kinases.

Therewith the invention also provides a method for identifying a peptide capable of reducing PI3K/AKT/mTOR activity, comprising providing cells with a peptide consisting of amino acids, said amino acids for at least 50%, preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably 100%, amino acids selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R), providing said cells with fMLP and detecting phosphorylation of PKB (AKT) in the absence and presence of said peptide at an appropriate time interval, preferably in the order of minutes, most preferably from about half a minute to about 5

minutes, e.g.30 to 600. AQGV peptide effects on p38 MAPK (figure 10c) are already detected at 30 seconds after FPR-stimulation, AQGV peptide effects on PKB(AKT) follow (figure 10a) in a bi-phasic pattern at 300 sec. Both AQGV peptide effects on p38 and PKB-mediated signalling last for the full 600 seconds tested whereas the other kinases tested were not affected throughout. As this acute and specific response to treatment shows specific and rapid effects of autophagy-inhibiting-AQGV peptide on p38 signaling in the context of regulation of the PI3K/AKT/mTOR pathway, said pathway is governing the balance between proteolysis and proteogenesis regulating cytoskeleton changes affecting vascular permeability. Such activities are not detected in STAT3, JNK (figure 10b) and P42/p44MAPK/ERK1,2 (figure 10d) kinases tested with AQGV peptide. It is shown that AQGV peptide reduces p38 MAPK kinase activated changes as well as reduces PI3K/AKT/mTOR activated induced changes in cell cytoskeleton reorganization affecting endothelial cell contraction and adverse vascular permeability.

The invention also provides a method for identifying a peptide capable of reducing PI3K/AKT/mTOR activity, comprising providing cells with a peptide consisting of amino acids, said amino acids for at least 50%, preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably 100%, amino acids selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R), providing said cells with fMLP and detecting phosphorylation of PKB (AKT) in the absence and presence of said peptide at an appropriate time interval, preferably in the order of minutes, most preferably from about half a minute to about 5 minutes, e.g.30 to 600 seconds after provision of fMLP, and comparing the results to determine said peptide's effect on said phosphorylation. Identified AQGV peptide is useful and capable of addressing adverse vascular permeability, such as manifested by edema with vascular leakage, adverse leukocyte extravasation and hypotension in human subjects.

The invention also provides a method for identifying a peptide capable of reducing PI3K/AKT/mTOR activity, comprising providing cells with a peptide consisting of amino acids, said amino acids for at least 50% selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R), providing said cells with fMLP and detecting phosphorylation of PKB (AKT) in the absence and presence of said

peptide at an appropriate time interval, preferably in the order of minutes, most preferably from about half a minute to about 5 minutes, e.g.30 to 600 seconds after provision of fMLP, and comparing the results to determine said peptide's effect on said phosphorylation. The invention therewith also provides method for identifying a peptide capable of reducing cytoskeleton reorganization, comprising providing cells with a peptide consisting of amino acids, said amino acids for at least 50% selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R), and providing said cells with fMLP and detecting phosphorylation of p38 MAPK and/or PKB (AKT) in the absence and presence of said peptide at an appropriate time interval, preferably in the order of minutes, most preferably from about half a minute to about 5 minutes after provision of fMLP, and comparing the results to determine said peptide's effect on said phosphorylation.

Typically, as the invention herein provides a molecular mode-of-action (MoA) of the group of autophagy inhibiting peptides its effects do not necessarily depend on their exact sequence. Instead, their constituent amino acids provide common household, "no-danger or tissue-repair" signals to the nutrient-sensing system of mTOR; leading to inhibition of autophagy and resulting in resolve of disease. These tissue-repair signal molecules change the balance of proteogenesis versus proteolysis in a cell of and lead to resolve of disease in three steps:

Administered peptide or amino acid fragments thereof are for taken up by amino acid transport, PEPT1/2 transport, by common endocytosis, in the case of vascular cells by elastin receptor mediated endocytosis or phagocytosis.

Internalized peptide is hydrolyzed and its amino acids are presented to the nutrient-sensing system of mTOR.

Particular amino acids inhibit autophagy, therewith inhibiting proteolysis and leading to proteogenic resolve and pharmaceutical effect.

Various peptides, either derived from breakdown of peptide hormones or assembled as novel synthetic peptide essentially comprising amino acids selected from the group of autophagy inhibiting amino acids, meeting one or more of the characteristics of the above description have been shown in various animal models in mice or rats to provide potent resolve of excess or adverse – local or systemic- vascular permeability through effects on endothelial cells lining our

vasculature. Several have or are being rationally developed further in various stages of human clinical trials. Exploiting the autophagy inhibiting mechanism involved through future clinical application of these autophagy inhibiting compounds and related peptide drugs provides an exciting novel avenue for the rational treatment of disease. However, with several autophagy inhibiting peptide formulations for intravenous application, peptide solubility difficulties have been experienced that decrease availability of autophagy-inhibiting amino acids, necessitating providing stock solutions of peptide in cumbersome large volumes to avoid aggregation of peptide and loss of pharmaceutical effect.

It is a purpose of this disclosure to provide said autophagy-inhibiting amino acids in a most expedient way to a subject deemed in need thereof. Therewith, the invention provides a tartrate or a citrate of a, preferably recombinant or synthetic, autophagy inhibiting peptide, said peptide having an amino acid sequence comprising at least 50%, more preferably at least 75%, most preferably at 100% amino acids selected from the group of autophagy inhibiting amino acids alanine (A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I) and proline (P). More preferably, the invention provides a stock solution, preferably aqueous, comprising a peptide-tartrate or a peptide citrate of a, preferably recombinant or synthetic, autophagy inhibiting peptide, said peptide having an amino acid sequence comprising at least 50%, more preferably at least 75%, most preferably at 100% amino acids selected from the group of autophagy inhibiting amino acids alanine (A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I) and proline (P).

The invention provides a suitable solution for several autophagy inhibiting peptides to mitigate aggregation of said peptides and identifies tartrate (from tartaric acid, preferably from (+)-tartaric acid) and more preferably citrate (from citric acid) as a suitable counter-ion, pharmaceutical excipient or anion of choice for preparing a salt of an autophagy inhibiting peptide that is a neutral peptide as defined herein above. A variety of salts were screened herein to determine their influence on aggregation of neutral peptide according to the invention, indeed revealing that neutral peptide “salts out” of solution in an anion-specific and concentration-dependent manner. Aggregation points of such salts (the point of concentration below which aggregated peptide-salt tends to resolve) Peptide-sulfate, peptide-maleate, peptide-adenosine monophosphate and

peptide-adenosine in aqueous solution were found to show aggravated aggregation in relation to peptide-acetate aggregation, whereas surprisingly tartrate, and more surprisingly peptide-citrate, showed (strongly) reduced aggregation in aqueous solution in comparison to peptide-acetate.

It is preferred that said autophagy inhibiting peptide-salt according to the invention comprises <25% charged residues selected from the group K, H and R. It is more preferred that said autophagy inhibiting peptide comprises <25% charged residues selected from the group D, K, R, H, and E. It is most preferred that said autophagy inhibiting peptide-salt does not comprise residues selected from the group D, K, R, H, and E. It is furthermore preferred that said solution is an aqueous solution. In a most preferred embodiment, the solution is a so-called stock solution, preferably an aqueous stock solution. A stock solution generally is a concentrated solution of an active substance, herein autophagy inhibiting peptide-salt, that will be diluted to some lower concentration for actual use of said substance, a so-called working solution.

Such lower concentration working solutions are for example infusion fluids, e.g. for intravenous or intra-abdominal use to which the peptide is added from the stock solution for administrating therapy to a patient, as often seen in critically ill patients, for example at the intensive care of an hospital or at the battlefield. Under such conditions it is useful, and often considered a requisite, to have the active (peptide) drug available in a small (stock) volume for dilution into the infusion fluid. So-called stock solutions are generally provided and used to save solubilisation and preparation time, conserve materials, reduce storage space, and improve the accuracy with which lower concentrated solutions are prepared to work with. Stock solutions of drugs are often prepared and then provided or stored for imminent intravenous use, for example in critically ill patients. However, due to its by default higher peptide concentration, a stock solution with an autophagy inhibiting peptide invariably runs higher risks on peptide drug aggregation than a final working solution. Stock solutions are generally prepared at a concentration well below an aggregation concentration of the salt in question (e.g. 40-50%) to prevent salt-out events under possibly prolonged storage at various ambient conditions. Risk of peptide aggregation (salting-out) is a phenomenon that the invention provides to avoid or mitigate herein with a stock solution according to the invention. Such stock solutions generally are diluted 10- to 100-fold, or more, to provide a suitable working solution. It is however also an object of the present invention to

provide working solutions of the peptide-salts according to the invention. Particularly because in the application of the peptides of the invention relatively high amounts/concentrations of the peptide salts must be given, it is a prerequisite that the working solutions are far away from salting out points and yet are presented in a relatively small volume.

A variety of salts were screened herein to determine their influence on aggregation of neutral peptide, indeed revealing that neutral peptide "salts out" of solution in an anion-specific and concentration-dependent manner. Peptide-sulfate and peptide-maleate were found to show aggravated aggregation in relation to peptide-acetate aggregation, whereas surprisingly tartrate, and even more surprisingly peptide-citrate, showed (strongly) reduced aggregation in comparison to peptide-acetate.

The invention therewith contributes to improved solubility of this distinct and new class of drugs that is emerging: small autophagy inhibiting peptides comprising amino acids that preferentially inhibit autophagy and target the nutrient sensing system of the mechanistic target of rapamycin, mTOR. Typically, peptides are defined as having 50 or less amino acids, for the purpose of this disclosure, proteins are defined as having >50 amino acids. A autophagy inhibiting peptide herein is defined as a linear, branched or circular string of no longer than 50 amino acids that comprises a peptide sequence with at least 50%, more preferably at least 75%, most preferably 100% amino acids selected from the group of autophagy inhibiting amino acids alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), proline (P), isoleucine (I) and arginine (R). Molecular mode-of-action (MoA) of this group of peptides does not depend on their exact sequence. Instead, their constituent amino acids provide common household, "no-danger or tissue-repair" signals to the nutrient-sensing system of mTOR; leading to inhibition of autophagy and resulting in resolve of disease

In another embodiment, the invention provides a peptide, preferably a salt of an organic acid, such as a maleate, more preferably an acetate, more preferably a tartrate, most preferably a citrate of a, preferably recombinant or synthetic, autophagy inhibiting peptide, said peptide having an amino acid sequence comprising 50%, more preferably at least 75%, most preferably at 100% amino acids selected from the group of autophagy inhibiting amino acids alanine (A), glutamine (Q), leucine (L), valine (V) glycine (G) and proline (P). More preferably, the invention provides a

stock solution, preferably aqueous, comprising a peptide-tartrate or a peptide citrate of a, preferably recombinant or synthetic, autophagy inhibiting peptide, said peptide having an amino acid sequence comprising at least 50%, more preferably at least 75%, most preferably at 100% amino acids selected from the group of autophagy inhibiting amino acids alanine (A), glutamine (Q), glycine (G), valine (V), leucine (L), and proline (P).

Heeding aggregation risk, a vial with a stock solution of AQGV peptide as defined here above for use in a clinical trial hitherto contained no more than (0.8 mol/L) active substrate in solution. Based on the current invention such a stock solution of an AQGV-salt of an organic acid, in particular of AQGV peptide-maleate, AQGV peptide-acetate AQGV peptide-tartrate or AQGV peptide-citrate (but not of adenosine or adenosine monophosphate) now is provided having an amino acid sequence comprising at least 50%, more preferably at least 75%, most preferably at 100% amino acids selected from the group of autophagy inhibiting amino acids alanine (A), glutamine (Q), glycine (G), valine (V), leucine (L) and proline (P), to contain at least 0.85 mol/L, more preferably at least 0.9 mol/L, more preferably at least 1 mol/L, more preferably at least 1.2 mol/L, more preferably at least 1.4 mol/L, more preferably at least 1.6 mol/L, most preferably at least 1.8 mol/L, of said AQGV peptide-acetate, AQGV peptide tartrate or AQGV peptide-citrate. In a more preferred embodiment, the invention provides a stock-solution of said AQGV peptide-tartrate or said AQGV peptide-citrate wherein the concentration of said AQGV peptide is in the range of 2 mol/L () to 2.5 mol/L. In a more preferred embodiment, the invention provides a stock-solution of said AQGV-peptide-citrate wherein the concentration of said peptide-citrate is in the range of 2.5 mol/L to 3 mol/L. In a more preferred embodiment, the invention provides a stock-solution of said peptide-citrate wherein the concentration of said peptide-citrate is in the range of 3 mol/L to 3.5 mol/L. In a more preferred embodiment, the invention provides a stock-solution of said peptide-citrate wherein the concentration of said peptide-citrate is in the range of 3.5 mol/L to 4.5 mol/L. In a more preferred embodiment, the invention provides a stock-solution of said peptide-citrate wherein the concentration of said peptide-citrate is in the range of 4.5 mol/L to 5.5 mol/L. In a more preferred embodiment, the invention provides a stock-solution of said peptide-citrate wherein the concentration of said peptide citrate is equal to or larger than 5.5 mol/L. It is preferred that said stock solution is an aqueous solution.

It is preferred that said stock solution is an aqueous solution of autophagy inhibiting amino acids comprising a dipeptide AQ, QQ, LQ, GQ, PQ, VQ, AL, LL, QL, GL, PL, VL, QA, QL, QG, QP, QV, LA, LG, LP, LV, a tripeptide AQG, QQG, LQG, GQG, PQG, VQG, ALG, LLG, QLG, GLG, PLG, VLG, QAG, QLG, QGG, QPG, QVG, LAG, LGG, LPG, LVG or a tetrapeptide AQGV, QQGV, LQGV, GQGV, PQGV, VQGV, ALGV, LLGV, QLGV, GLGV, PLGV, VLGV, QAGV, QLGV, QGGV, QPGV, QVGV, LAGV, LGGV, LPGV, LVGV, or a mixture thereof.

It is preferred that a peptide according to the invention has a peptide sequence with length of 2-40 amino acids, preferably 3-30 amino acids, preferably 4-20 amino acids. It is most preferred that said peptide according to the invention has a peptide sequence that comprises at least 6 amino acids, in particular when at least 4 of those inhibit autophagy. A maximum length of a peptide-tartrate or peptide citrate according to the invention preferably comprises at most 50 amino acids, more preferably at most 40 amino acids, more preferably at most 30 amino acids , more preferably at most 20 amino acids, more preferably at most 15 amino acids, more preferably at most 12 amino acids, most preferably at most 9 amino acids.

The invention provides a method for reducing p38 MAPK kinase activity leading to cytoskeleton reorganization, comprising providing cells, preferably having a formyl-peptide-receptor associated with their surface, with a source of autophagy inhibiting amino acids, preferably wherein said source is an AQGV peptide as provided herein, said amino acids for least 50% selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R).

The invention provides a method for reducing formyl-peptide-receptor (FPR) mediated p38 MAPK kinase activity, comprising providing cells, preferably having a formyl-peptide-receptor associated with their surface, with a source of autophagy inhibiting amino acids, preferably wherein said source is an AQGV peptide as provided herein, said amino acids for least 50% selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R).

The invention provides a method for reducing PI3K/AKT/mTOR activity leading to cytoskeleton reorganization, comprising providing cells, preferably having a formyl-peptide-receptor associated with their surface, with a source of autophagy inhibiting amino acids, preferably wherein said

source is an AQGV peptide as provided herein, said amino acids for least 50% selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R).

The invention provides a method for reducing formyl-peptide-receptor (FPR) mediated PI3K/AKT/mTOR activity, comprising providing cells, preferably having a formyl-peptide-receptor associated with their surface, with a source of autophagy inhibiting amino acids, preferably wherein said source is an AQGV peptide as provided herein, said amino acids for least 50% selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R).

The invention provides a method for reducing cytoskeleton reorganization, comprising providing cells, preferably having a formyl-peptide-receptor associated with their surface, with a source of autophagy inhibiting amino acids, preferably wherein said source is an AQGV peptide as provided herein, said amino acids for least 50% selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R).

The invention provides a method for reducing formyl-peptide-receptor (FPR) mediated cytoskeleton reorganization, comprising providing cells, preferably having a formyl-peptide-receptor associated with their surface, with a source of autophagy inhibiting amino acids, preferably wherein said source is an AQGV peptide as provided herein, said amino acids for least 50% selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R).

The invention provides a method for modifying vascular permeability comprising providing cells, preferably having a formyl-peptide-receptor associated with their surface, with a source of autophagy inhibiting amino acids, preferably wherein said source is an AQGV peptide as provided herein, selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R).

The invention provides a method for improving tissue repair comprising providing cells, preferably having a formyl-peptide-receptor associated with their surface, with a source of autophagy inhibiting amino acids, preferably wherein said source is an AQGV peptide as provided herein,

selected from the group of alanine (in one letter code: A), glutamine (Q), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P) and arginine (R).

The invention provides a method according to the invention, wherein said peptide comprising said autophagy inhibiting amino acids comprises a dipeptide AQ, QQ, LQ, GQ, PQ, VQ, AL, LL, QL, GL, PL, VL, QA, QL, QG, QP, QV, LA, LG, LP, LV, a tripeptide AQG, QQG, LQG, GQG, PQG, VQG, ALG, LLG, QLG, GLG, PLG, VLG, QAG, Q0LG, QGG, QPG, QVG, LAG, LGG, LPG, LVG or a tetrapeptide AQGV, QQGV, LQGV, GQGV, PQGV, VQGV, ALGV, LLGV, QLGV, GLGV, PLGV, VLGV, QAGV, QLGV, QGGV, QPGV, QVGV, LAGV, LGGV, LPGV, LVGV, or a mixture thereof.

In another embodiment, this application finds the SARS-COV-2 spike protein (see also figures 11-13) to carry a distinct peptide-motif sequence KWPWYIWL or variant KWPWYVWL in a membrane proximal external region (MPER) capable of binding to a receptor of the FPR-family of receptors. COVID-19 is activating FPR-mediated pathways, in particular through interaction of vascular cells with its spike protein, therewith leading to vascular leakage, thrombotic events and modulating Ang1/Ang2 ratio. Said MPER region may as well be involved in sparsely reported and incidental thrombotic events after vaccination with distinct coronavirus-based-vaccines that express said spike protein not fixated in a prefusion state. As AQGV-peptide inhibits formyl peptide-activated FPR-mediated pathways (p38-MK2-HSP27 and PI3K-AKT-mTOR, see figure 10) involved in disrupting vascular integrity, AQGV-peptide improves vascular leakage and thrombotic events by inhibiting thrombus formation and modulating Ang1/Ang2 ratio after events causing expression of at least said fusogenic region with motif KWPWYIWL or variant KWPWYVWL in a subject. Surprisingly, said fusogenic region with motif KWPWYIWL or variant KWPWYVWL also comprises an FPR-binding site participating in inducing vascular leakage in a subject. Therewith, the present application also provides alternative treatment or use A method of treatment of a subject deemed to express a peptide or protein comprising a fusogenic region derived from a virus, said method comprising adoptive cell therapy using at least one cell provided with a receptor recognizing said fusogenic site. It is preferred that said fusogenic region at least comprises peptide motif KWPWYIWL or at least comprises peptide motif KWPWYVWL, in particular wherein said cell is a transformed T-cell, such as a CAR-T or TCR-T cell, preferably wherein said cell is directed against a (preferably CD8+) T-cell epitope comprising or overlapping said fusogenic region. Such adoptive

cellular therapy (see for example June et al, Adoptive cellular therapy: A race to the finish line *Science Translational Medicine* 25 Mar 2015: Vol. 7, Issue 280, pp. 280ps7) as here provided uses of at least one cell provided with a receptor recognizing a fusogenic region derived from a virus in method of treatment of a subject deemed to express a peptide or protein comprising said fusogenic region, wherein said fusogenic region at least comprises peptide motif KWPWYIWL or at least comprises peptide motif KWPWYVWL, and wherein said cell is a transformed T-cell, such as a CAR-T or TCR-T cell preferably directed against a (preferably CD8+) T-cell epitope comprising or overlapping said fusogenic region.

FURTHER EMBODIMENTS

1. An AQGV peptide, or a functional analogue thereof, for use in the treatment of a human subject, the use comprising modifying hemodynamics in the human subject.

2. An AQGV peptide, or a functional analogue thereof, for use in the treatment of a human subject, wherein the human subject is suffering from a viral infection and wherein the use comprises modifying hemodynamics in the human subject.

3. An AQGV peptide, or a functional analogue thereof, for use in the treatment of a human subject having impaired lung function, the use comprising modifying hemodynamics and improving hypoxemia in the human subject.

4. An AQGV peptide, or a functional analogue thereof, for use as in accordance with any one of further embodiments 1-3, wherein the use reduces fluid retention in the human subject.

5. An AQGV peptide, or a functional analogue thereof, for use in accordance with any of further embodiments 1-4 wherein the use comprises a reduced use of vasopressive agents.

6. An AQGV peptide, or a functional analogue thereof, for use in accordance with any one of further embodiments 1-5 wherein the use comprises a reduced fluid intake.

7. An AQGV peptide, or a functional analogue thereof, for use in accordance with further embodiment 5, wherein the reduced use of vasopressive agents comprises a reduced duration of vasopressive agent use.

8. An AQGV peptide, or a functional analogue thereof, for use in accordance with any one of further embodiments 3-6, wherein the subject is suffering from a respiratory viral infection.

9. An AQGV peptide, or a functional analogue thereof, for use in accordance with any one of further embodiments 3-8 wherein the use improves lung function in the human subject.

10. An AQGV peptide, or a functional analogue thereof, for use in accordance with further embodiment 9, wherein the improved lung function involves an improved oxygen saturation of blood.

11. An AQGV peptide, or a functional analogue thereof, for use in accordance with any one of further embodiments 3-10, wherein the human subject has impaired lung function the impaired lung function being ARDS.

12. An AQGV peptide, or a functional analogue thereof, for use as in accordance with any one of further embodiments 1-11, wherein the use reduces leakage of plasma from the blood to peripheral tissue and/or organs.

13. An AQGV peptide, or a functional analogue thereof, for use in accordance with any one of further embodiments 1-12, wherein the use is in a human subject suffering from or at risk of suffering from detrimental effects of mechanical ventilation.

14. An AQGV peptide, or a functional analogue thereof, for use in accordance with any one of further embodiments 1-13, wherein the use is in a human subject at risk of having edema.

15. An AQGV peptide, or a functional analogue thereof, for use in accordance with any one of further embodiments 2-14, wherein the human subject is suffering or thought to be suffering from to corona virus infection.

16. An AQGV peptide, or a functional analogue thereof, for use in accordance with further embodiment 15, wherein the infection is SARS-Cov-2 infection.

17. An AQGV peptide, or a functional analogue thereof, for use in accordance with any one of further embodiments 1-16, wherein the peptide is administered into the bloodstream.

18. An AQGV peptide, or a functional analogue thereof, for use in accordance with further embodiment 17, wherein the peptide is administered at a rate of at least 70 mg/ kg body weight / hour.

19. An AQGV peptide, a functional analogue thereof, for use in accordance with further embodiment 15 or further embodiment 16, wherein the peptide is administered for at least 1 hour.

20. An AQGV peptide, a functional analogue thereof, for use in accordance with any one of further embodiments 15-19, wherein the administration is intermittent.

21. An AQGV peptide, or a functional analogue thereof, for use in accordance with any one of further embodiments 1-20, wherein the human subject is admitted into intensive care, and wherein the use improves parameters measured of the human subject, the parameters of the human subject determined to assess remaining in intensive care.

22. An AQGV peptide, or a functional analogue thereof, for use in accordance with further embodiment 21, wherein the improvement in parameters results in a reduced length of stay at intensive care.

23. An AQGV peptide, or a functional analogue thereof, for use as in accordance with any one of further embodiments 1-22, wherein the uses induces vasoconstriction.

24. An AQGV peptide, or a functional analogue thereof, for use as in accordance with any one of further embodiments 1-23, wherein the subject is deemed at risk of VALI or VILI.

25. An AQGV peptide, or a functional analogue thereof, for use as in accordance with any one of further embodiments 1-23, wherein the subject is deemed to express a peptide or protein comprising a fusogenic region derived from a virus.

26. An AQGV peptide, or a functional analogue thereof, for use as in accordance with further embodiment 25, wherein said fusogenic region at least comprises peptide motif KWPWYIWL or variant KWPWYVWL.

27. An AQGV peptide, or a functional analogue thereof, for use as in accordance with further embodiment 25 or 26 , wherein said fusogenic region at least comprises an FPR-binding site.

28. A method of treatment comprising administering an AQGV peptide, or a functional analogue thereof, to a human subject, the human subject being in need of maintaining hemodynamic stability.

29. A method of treatment comprising administering an AQGV peptide, or a functional analogue thereof, to a human subject, the human subject being in need of improving hemodynamic stability.

30. A method of treatment comprising administering an AQGV peptide, or a functional analogue thereof, to a human subject, the human subject having impaired lung function, wherein

the treatment of administering an AQGV peptide comprises maintaining or improving hemodynamic stability in the human subject.

31. A method of treatment comprising administering an AQGV peptide, or a functional analogue thereof, intermittently to a human subject, the human subject having impaired lung function, wherein the treatment of administering an AQGV peptide comprises maintaining or improving hemodynamic stability in the human subject.

31. A method of treatment of a subject deemed to express a peptide or protein comprising a fusogenic region derived from a virus, said method comprising adoptive cell therapy using at least one cell provided with a receptor recognizing said fusogenic site.

32. A method according to further embodiment 31 wherein said fusogenic region at least comprises peptide motif KWPWYIWL

33. A method according to further embodiment 31 wherein said fusogenic region at least comprises peptide motif KWPWYVWL.

33. A method according to any of further embodiments 31 to 33 wherein said cell is a transformed T-cell, such as a CAR-T or TCR-T cell.

34. A method according to embodiment 33 wherein said cell is directed against a T-cell epitope comprising or overlapping said fusogenic region.

35. Use of at least one cell provided with a receptor recognizing a fusogenic region derived from a virus in method of treatment of a subject deemed to express a peptide or protein comprising said fusogenic region.

36. Use according to further embodiment 35 wherein said fusogenic region at least comprises peptide motif KWPWYIWL

37. Use according to further embodiment 35 wherein said fusogenic region at least comprises peptide motif KWPWYVWL.

38. Use according to any of further embodiments 35 to 37 wherein said cell is a transformed T-cell, such as a CAR-T or TCR-T cell.

39. Use according to embodiment 38 wherein said cell is directed against T-cell epitope comprising or overlapping said fusogenic region.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Early infections with the SARS-Cov-2 virus mostly run a mild or even uneventful course. That Stage I course is seen in >80% of people infected. This majority of patients experience an upper airway infection of nose and throat, with a dry cough, that generally passes in 2-12 days. In the remaining <20% of cases, two distinct pathological stages may develop, often starting at around the time that in mild cases the viral infection is to reduce due to an emerging immune response directed against the virus. Stage II is a viral pneumonia (pulmonary phase with lung injury) with permeability losses of the alveolar-capillary membrane (Stage IIA) that diffusely and profusely affects the deeper airways and alveoli of both lungs (Stage IIB), causing reduced uptake of oxygen due to respiratory failure. This two-sided pneumonia may be followed by stage III, a full-blown disease with general malaise, high fever and ultimately organ (kidney, liver, heart) failure at large.

Figure 2: Acute disease in Stage II increases vascular permeability and results in fluid leakage from lung capillaries into the lung tissues (see also lung injury in figure 3). This angiopoietin regulated permeability is depicted here. Angiopoietin 1 (ANG1) is constitutively secreted by perivascular mural cells. When gaps form between cells, ANG1 is released in the vascular lumen. Ligand-binding of (ANG1) to TIE2 induces sequestration of the tyrosine kinase Src and thus establishes stable expression of VE-cadherin on the surface of the endothelial cell, allowing gaps to close. ANG2 is stored in Weibel-Palade (WBP) bodies and rapidly released upon triggering signals. Its binding to TIE2 abolishes ANG1-induced sequestration of Src, resulting in the internalization of VE-cadherin.

Figure 3: Acute disease in stage II results in lung injury characterized by edematous lung tissues causing low gas (oxygen and carbon-dioxide) diffusing capacity. SARS-COV-2 infection starts in Type II cells. The permeability of lung-capillaries is increased by increased vascular cell gap formation as depicted in figure 2. This process generates a flux of fluid from the capillaries into the thin layer of extra-cellular-matrix separating alveoli from capillaries, with intercellular fluid retention therewith thickening (swelling) the membrane through accumulation of fluid in the extra-cellular-matrix (causing a widened edematous interstitium), separating alveolar cells from vascular cells, and entering the alveoli. This typically evokes local inflammatory activity of white blood cells that migrated to the lung tissue. All-in-all, gas diffusion is severely hampered causing

difficulties breathing. In such Stage II lung injury patients, plasma levels of biomarkers of endothelial activation, as may be measured by ELISA, are often predictive of mortality. In particular, the concentration of angiopoietin-2 relative to angiopoietin-1 (Ang-2/Ang-1) may be a useful biologic marker of mortality in acute lung injury (ALI) patients.

Figure 4: Schematic depiction in the infection stages of Figure 1 of intermittent dosing of AQGV-peptide in various stages of COVID-19. Shadowed bars indicate time slots wherein AQGV peptide or related substance is administered intravenously, preferably at a rate of at least 75mg/kg/hr., more preferably at least 90 mg/kg/hr. It is in particular useful to administer the AQGV peptide or related substance intermittently. Preferred use is dosing for 2-4 hours of at least 75mg/kg/hr., more preferably at least 90mg/kg/hour, then optionally reducing to 30mg/kg/hour for 2-4 hours (in between shaded bars), or for as long as it takes to monitor the patients response to treatment by clinical or laboratory diagnosis, or stop administering the substance for 1-2 hours until diagnostic studies have completed, and then resume treatment with 2-4 hours of at least 75mg/kg/hr., more preferably at least 90mg/kg/hour. Depending on the stage of disease, therapeutic effects of EA-230 may be monitored by determining hypoxia, plasma Ang2/Ang1 ratio and plasma levels of D-dimer in Stage II and III.

Figure 5: The need for treatment of hemodynamic instability by use of vasopressors (left) and by use of fluid therapy to adjust net fluid balance were considerably improved in the first 24 hours of intensive care unit (ICU) in those patients given EA-230 peptide. Therewith, EA-230 significantly improves hemodynamic recovery, providing a significant improvement of hemodynamic stability (reducing a composite measure of required fluid therapy and blood pressure medication; 2-way ANOVA; p=0.006).

Figure 6: Both the ICU (p=0.02) and hospital (p=0.001) length-of-stay was shorter in patients treated with EA-230 (AQGV) compared to the placebo group. AQGV-peptide EA-230 reduced the number of patients at the ICU at 24 hours by 48%; and reduced hospital length of stay by 20%.

Figure 7: Overview of solubility experiments with results in Table 1.

Figure 8: Based on the results depicted in figure 7 the concentration below which an aggregated peptide-salt tends to resolve of the neutral-peptides salts screened were determined (aggregation points). It can be concluded that changing the anion significantly influences the solubility

characteristics of AQGV. Higher solubility (solubility in 0.9% NaCl) and therewith higher aggregation points were observed for the AQGV-citric acid (AQGV-citrate) and -tartaric acid (AQGV-tartrate) salt, whereas maleic acid and KHSO4 salts showed lower solubility, compared to AQGV-Ac. Using adenosine-monophosphate or adenosine did not provide solubility. Citric acid seems to be a special case. Highly concentrated solution does not crystallize or aggregate but tend to form a highly viscous solution.

Figure 9: Formyl-peptide-receptor mediated vascular permeability after cell and tissue trauma. The human formyl peptide receptor (FPR) is N-glycosylated and activates cells via G(i)-proteins. Site-directed mutagenesis of extracellular Asn residues prevented FPR glycosylation but not FPR expression in cell membranes. However, in terms of high-affinity agonist binding, kinetics of GTPgammaS binding, number of G(i)-proteins activated, and constitutive activity, non-glycosylated FPR is much less active than native FPR. Mitochondrial N-formyl peptides (F-MIT) released from trauma/cell damage activate formyl peptide receptor (FPR) leading to changes in endothelial cell cytoskeleton which subsequently induces endothelial contraction and vascular permeability, leukocyte extravasation and hypotension. N-Formyl peptides are common molecular signatures of bacteria and mitochondria that activate the formyl peptide receptor (FPR). FPR activation by mitochondrial N-formyl peptides (F-MIT) elicits changes in cytoskeleton-regulating proteins in endothelial cells that lead to increased endothelial cell contractility with increased vascular leakage and extravasation of leukocytes. FPR activation via mitochondrial N-formyl peptides (F-MIT) originating from tissue damage after injury such as trauma is a key contributor to impaired barrier function following cell and tissue injury or trauma, resulting in detrimental vascular effects such as adverse vascular permeability with edema, vascular leakage, adverse leukocyte extravasation and hypotension.

Figure 10: Formyl-peptide-receptor mediated peptide effects. FPR-activation of FPR-expressing cells with prototype FPR-ligand fMLP causes rapidly induced and significant ($p < 0.05$; p38 from 60 to 600 sec, PKB at 600 sec) changes in phosphorylation status of PKB (also known as AKT) (figure 10a) and p38 MAPK kinases (figure 10c), but not (or not detected) in STAT3, JNK (figure 10b) and P42/p44MAPK/ERK1,2 (figure 10d) kinases. AQGV peptide effects on p38 MAPK (figure 10c) are already detected at 30 seconds after FPR-stimulation, AQGV peptide effects on PKB(AKT) follow

(figure 10a) in a bi-phasic pattern at 300 sec. Both AQGV peptide effects on p38 and PKB-mediated signalling last for the full 600 seconds tested whereas the other kinases tested were not affected throughout. This acute and specific response to treatment shows specific and rapid effects of autophagy-inhibiting-AQGV peptide on p38 signaling in the context of regulation of the PI3K/AKT/mTOR pathway. Said pathway is governing the balance between proteolysis and proteogenesis regulating cytoskeleton changes affecting vascular permeability. It is shown that AQGV peptide reduces p38 MAPK kinase activated changes as well as reduces PI3K/AKT/mTOR activated induced changes in cell cytoskeleton reorganization affecting endothelial cell contraction and adverse vascular permeability. AQGV peptide is useful and capable of addressing adverse vascular permeability, such as manifested by edema with vascular leakage, adverse leukocyte extravasation and hypotension in human subjects.

Figure 11: AQGV-peptide to target viral-spike-protein-induced pulmonary and vascular leakage in Coronavirus infections as seen in SARS, MERS and COVID-19. SARS-CoV-2 spike (S) glycoproteins are class I viral fusion proteins which promote viral entry into cells and are the main target of antibodies (White et al., Critical reviews in biochemistry and molecular biology. 2008 Jan 1;43(3):189-219.). The C terminal end of spike protein contains a heptad repeat (HR2), a short linker region (the membrane proximal external region or MPER), a transmembrane helix domain (TMD) and a C-terminal cytoplasmic or internal domain (CTD/IC). After binding of the ACE2 receptor on the target cell to the receptor binding domain (RBD) on S protein, the heptad repeat 1 (HR1) and heptad repeat 2 (HR2) domains form a six-helix bundle fusion core (6HB), bringing the viral with the fusogenic MPER domain and cellular membranes together for fusion and cell entry (Walls et al., Tectonic conformational changes of a coronavirus spike glycoprotein promote membrane fusion. Proceedings of the National Academy of Sciences. 2017 Oct 17;114(42):11157-62.; Xia et al., . Fusion mechanism of 2019-nCoV and fusion inhibitors targeting HR1 domain in spike protein. Cellular & molecular immunology. 2020 Feb 11:1-3.). MPER is essential for viral entry into cells as identified in figure 12. Note that at least one T cell epitope, allowing generation of CD8+ T-cell cross-reactivity against SARS-CoV-2 and other coronavirus strains (Lee et al, Front. Immunol., 05 November 2020 | https://doi.org/10.3389/fimmu.2020.579480) overlaps with the fusogenic site as identified in figure 12. In patients, virus-specific $CD4^+$ and $CD8^+$ T cell responses

are associated with milder disease, suggesting an involvement of said fusogenic region in protective immunity against COVID-19. Typically, said fusogenic site, and therewith said T-cell epitope, is strongly conserved in SARS-COV-2 (Guo E, Guo H (2020) CD8 T cell epitope generation toward the continually mutating SARS-CoV-2 spike protein in genetically diverse human population: Implications for disease control and prevention. PLOS ONE 15(12): e0239566), herein it is provided to develop adoptive cellular therapy (ACT) directed against said fusogenic region that may be used in viral or vaccine based infections such as with corona virus or vaccine.

Figure 12: The short membrane proximal external region (MPER) connects the HR2 and transmembrane domain, and contains an aromatic-amino-acid-rich fusogenic peptide sequence which destabilizes the membrane during fusion (Mahajan M, Bhattacharjya S. NMR structures and localization of the potential fusion peptides and the pre-transmembrane region of SARS-CoV: Implications in membrane fusion. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2015 Feb 1;1848(2):721-30.; Guillén J, Kinnunen PK, Villalaín J. Membrane insertion of the three main membranotropic sequences from SARS-CoV S2 glycoprotein. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2008 Dec 1;1778(12):2765-74.). This fusogenic region may sometimes be referred to as "membrane proximal ectodomain region" or "pre-transmembrane region" (PTM). The MPER peptides 1185-LGKYEQYIKWPWYVWLGF-1202 and 1193-KWPWYVWLGFIAGLIAIV-1210 from SARS-CoV-1 have been shown to intercalate into lipid membranes and to be highly surface active; the corresponding fusogenic sequences in SARS-CoV-2 and MERS-CoV are identical except for a V to I substitution at position 1216.

Figure 13: This application finds the SARS-COV-2 spike protein to carry a distinct and conserved fusogenic motif in its MPER domain (KWPWYIWL) that is capable of binding to FPR. This motif is highly homologous to related coronavirus spike protein motifs for which binding to FPR has been demonstrated. (Mills, Biochim Biophys Acta Mol Basis Dis. 2006 Jul; 1762(7): 693-704). Vascular leakage in COVID-19 is at least partly modulated by binding and/or fusing of this spike protein comprising at least the minimally essential fusogenic sequence KWPWYIWL or variant KWPWYVWL to pulmonary vascular cells carrying the formyl-peptide receptor, and therewith may cause thrombotic events in coronavirus infection or vaccination against corona with a spike protein-vaccine such as ChAdOx1-S, in particular when such a vaccine is not modified to express

the spike proteins in a prefusion state only. FPR-mediated pathways are known to be activated in thrombotic events (Salamah et al., The formyl peptide fMLF primes platelet activation and augments thrombus formation. J Thromb Haemost. 2019; 17: 1120– 1133.) as well as in in acute lung injury and acute respiratory disease syndromes (ALI/ARDS) with pulmonary vascular leakage as a major clinical symptom (Thorax. 2017;72:928–936). COVID-19 (in particular the severe) infections with SARS-COV-2 typically increase risks on ALI/ARDS with pulmonary vascular leakage, leading to major fatalities.

DETAILED DESCRIPTION

Examples

Autophagy inhibiting peptides

One letter code

In describing protein or peptide composition, structure and function herein, reference is made to amino acids. In the present specification, amino acid residues are expressed by using the following abbreviations. Also, unless explicitly otherwise indicated, the amino acid sequences of peptides and proteins are identified from N-terminal to C-terminal, left terminal to right terminal, the N-terminal being identified as a first residue. Ala: alanine residue; Asp: aspartate residue; Glu: glutamate residue; Phe: phenylalanine residue; Gly: glycine residue; His: histidine residue; Ile: isoleucine residue; Lys: lysine residue; Leu: leucine residue; Met: methionine residue; Asn: asparagine residue; Pro: proline residue; Gln: glutamine residue; Arg: arginine residue; Ser: serine residue; Thr: threonine residue; Val: valine residue; Trp: tryptophane residue; Tyr: tyrosine residue; Cys: cysteine residue. The amino acids may also be referred to by their conventional one-letter code abbreviations; A=Ala; T=Thr; V=Val; C=Cys; L=Leu; Y=Tyr; I=Ile; N=Asn; P=Pro; Q=Gln; F=Phe; D=Asp; W=Trp; E=Glu; M=Met; K=Lys; G=Gly; R=Arg; S=Ser; and H=His.

Peptides

Peptide shall mean herein a natural biological or artificially manufactured (synthetic) short chain of amino acid monomers linked by peptide (amide) bonds. Glutamine peptide shall mean herein a natural biological or artificially manufactured (synthetic) short chain of amino

acid monomers linked by peptide (amide) bonds wherein one of said amino acid monomers is a glutamine. Chemically synthesized peptides generally have free N- and C-termini. N-terminal acetylation and C-terminal amidation reduce the overall charge of a peptide; therefore, its overall solubility might decrease. However, the stability of the peptide could also be increased because the terminal acetylation/amidation generates a closer mimic of the native protein. These modifications might increase the biological activity of a peptide and are herein also provided.

Peptide synthesis

In this application, peptides are either synthesized by classically known chemical synthesis on a solid support (Ansynth BV, Roosendaal, The Netherlands) or in solution (Syncom BV, Groningen, The Netherlands and Diosynth BV, Oss, The Netherlands). Pharmaceutical peptide compositions may be synthesized using trifluoroacetate as a counter-ion or salt after which trifluoroacetate is exchanged by a counter-ion such as maleate (from maliec acid), acetate (from acetic acid), tartrate (from tartaric acid) or citrate (from citric acid). The drug substance of AQGV (EA-230) for use in pre-clinical and clinical human studies has been manufactured by Organon N.V (formerly Diosynth B.V.), (Oss, The Netherlands), whereas filling and finishing of the final product has been performed by Octoplus Development, Leiden (The Netherlands). Molecular weight of EA-230 (AQGV) is 373g/mol).

FPR mediated vascular permeability and hypotension

Although the concept that active contraction of endothelial cells regulate vascular permeability was first suggested by Majno in 1961 (J Biophys Biochem ytol (1961) 11:571.10.1083/jcb.11.3.571), currently the intracellular events regulating endothelial contractile activity are still relatively unknown. N-Formyl peptides are common molecular signatures of bacteria and mitochondria that activate the formyl peptide receptor (FPR). FPR activation by mitochondrial N-formyl peptides (F-MIT) or by bacterial N-formyl peptides (F-MLP) such as N-formyl-methionyl-leucyl-phenylalanine elicits changes in cytoskeleton-regulating proteins in endothelial cells that lead to increased endothelial cell contractility with increased vascular leakage and extravasation of leukocytes. FPR activation is a key contributor to impaired barrier function in following trauma. It has been proposed that in patients, mitochondrial components from damaged tissue can initiate the genesis of vascular leakage (Wenceslau et al., Front Immunol. 2016; 7: 297). For evolutionary reasons,

mitochondria share several characteristics with bacteria, and when fragments of mitochondria are released into the circulation, they are recognized by cells carrying the formyl-peptide-receptor (FPR). Due to protein translation initiation by formyl-methionine in both bacteria and mitochondria, N-formyl peptides are common molecular signatures of bacteria and mitochondria and are known to play a role in the initiation of vascular leakage by activating the formyl peptide receptor (FPR).

The vasculature, composed of vessels of different morphology and function, distributes blood to all tissues and maintains physiological tissue homeostasis. Among others, to illustrate its central role in maintaining homeostasis, the vasculature not only serves as the main carrier in gas exchange from lung to tissues (e.g. oxygen (and vice versa e.g. carbon dioxide)) but also carries nutrients from gut to liver to tissues and toxic by-products resulting metabolism from tissues to kidney to urine for excretion.

In a range of pathologies, the vasculature is often affected by, and engaged in, the disease process. This foremost results in adverse vascular permeability with edema, adverse vascular leakage, adverse leukocyte extravasation and hypotension and may also result in excessive formation of new, unstable, and hyper permeable vessels with poor blood flow, which further promotes hypoxia and disease propagation. Chronic adverse vessel permeability may also facilitate metastatic spread of cancer. Thus, there is a strong incentive to learn more about (and be able to modulate) an important aspect of vessel biology in health and disease: the regulation of vessel permeability.

Endothelial cells in different vessels and in different organs have distinct functions and morphologies (Aird WC. Molecular heterogeneity of tumor endothelium. Cell Tissue Res. 2009;335:271–81.), but in general serve to provide a barrier between blood and tissue. In certain organs, such as the brain and in endocrine organs, endothelial cells present certain morphological features that reflect the need for communication between the organs and the circulation. In the brain, the vasculature forms a particularly strong barrier, the blood–brain barrier (BBB) to protect the brain parenchyma from detrimental edema. In hormone-producing organs, such as the endocrine pancreas, endothelial cells display specialized fenestrae on their surface.

These are diaphragm-covered 'holes' in the plasma membrane, which allow extremely rapid exocytosis of hormones. In most organs, the endothelial cells form a dynamic barrier between the blood and the tissue. In resting conditions, the vasculature continuously leaks solute and small molecules but restricts extravasation of larger molecules and cells. In many diseases, including cancer, the vascular barrier disintegrates and leakage increases and may become chronic. The leakage of larger molecules and cells may result in edema, adverse leukocyte extravasation and hypotension, and often disease progression.

It is well recognized that for example kinins such as bradykinin are involved in a series of physiological and sometimes pathological vascular responses affecting endothelial barrier function. Most of their actions are mediated by the activation of 2 G protein-coupled receptors, named B1 and B2.The activation of kinin receptors may play a key role in the modulation of atherosclerotic risk through the promotion of microangiogenesis, inhibition of vascular smooth muscle cell growth, coronary vasodilatation, increased local nitric oxide synthesis, or by exerting antithrombotic actions. The bradykinin B1 receptor (B1R) is typically absent under physiological conditions, but is highly inducible following tissue injury, stress, burns, traumatic damage, such as for example recently reported in COVID-19 disease.

Damage induced by tissue injury may cause a significant and time-dependent increase in des-Arg9–bradykinin (des-Arg9–BK) responsiveness that parallels B1R mRNA expression. It induces the activation of some members of the mitogen activated protein kinase (MAPK) family, namely, extracellular signal-regulated kinase (ERK) and p38 MAPK. The blockade of p38 MAPK but not ERK pathways with selective inhibitors, results in a significant reduction of the upregulated contractile response caused by the selective B1R agonist des-Arg9–BK, and largely prevents the induction of B1R mRNA expression enhancing tissue damage induced adverse vascular permeability.

Among other stress stimuli, exposure to hypoxia as a consequence of impaired blood flow, or as a consequence of impaired gas exchange between alveoli and the surrounding capillaries, also causes structural changes in the endothelial cell layer of blood vessels that alter its permeability and its interaction with leukocytes and platelets. These structural changes again cause impaired endothelial cell barrier function resulting in detrimental vascular effects such as adverse vascular permeability with edema, vascular leakage, adverse leukocyte extravasation and hypotension (see

also figure 1), and may further deteriorate gas exchange from lung to blood and from blood to tissue, and vice versa.

One of the well characterized cytoskeletal changes in response to stress involves the reorganization of the actin cytoskeleton and the formation of stress fibers. Kayyali et al., (J Biol Chem (2002) 277(45) : 42596–602) describe cytoskeletal changes in pulmonary microvascular endothelial cells in response to hypoxia and potential mechanisms involved in this process. The hypoxia-induced actin redistribution appears to be mediated by components downstream of MAPK p38, which is activated in pulmonary endothelial cells in response to hypoxia. Results indicate that kinase MK2, which is a substrate of p38, becomes activated by hypoxia, leading to the phosphorylation of one of its substrates, HSP27. As anoyher example F-actin rearrangement is also an early event in burn-induced endothelial barrier dysfunction, and HSP27, a target of p38 MAPK/MK2 pathway, plays an important role in actin dynamics. As HSP27 phosphorylation is known to alter actin distribution and thus contractility of cells, Kayyali et al., provide that the p38-MK2-HSP27 pathway causes changes in vascular permeability due to actin redistribution, as for example observed in hypoxia.

Taken together these results indicate that tissue damage stimulates the p38-MK2-HSP27 pathway leading to significant alteration in the actin cytoskeleton. It has previously also been shown that inhibition of the p38 MAPK pathway ameliorates vascular dysfunction by significantly reducing endothelial cell contraction (Wang et al, APMIS (2014) 122(9):832).

In recent years also another pathway, the PI3K/AKT/mTOR [phosphatidylinositol 3' kinase (PI3K), protein kinase B (PKB or AKT) and mammalian target of rapamycin (mTOR)] pathway has been identified to be essential for regulating endothelial cell contractility and Tsuji-Tamura and Ogawa indeed (Journal of Cell Science 2016 129: 1165-1178) identified inhibitors of phosphatidylinositol 3-kinase (PI3K)–Akt-pathway and inhibitors of mammalian target of rapamycin complex 1 (mTORC1) inhibitors as potent inducers of endothelial cell elongation required for restoring vascular permeability governed by vascular endothelial cells. Such elongation is required to fill the gaps that form between endothelial cells when these cells contract after p38-MK2-HSP27 and/or PI3K/AKT/mTOR signaled cytoskeleton reorganization. It is these gaps (again see figure 1) through which adverse leakage and adverse extravasation occurs that explains the resulting edema,

vascular leakage, adverse leukocyte extravasation and loss of vascular fluid with a risk for hypotension.

Closing of these gaps is in general governed by the ratio of various angiogenic factors such as angiopoietin-2 to angiopoietin-1 at the site of increased vascular permeability, whereby angiopoetin-2 in general induces endothelial cell apoptosis (there with enhancing gap-formation) and angiopoietin-1 counters gap formation by facilitating endothelial vcel elongation and gap closure. Inhibition of the p38 pathway, but not of the ERK1/2 pathway, attenuates angiopoetin-2-mediated endothelial cell apoptosis (Li et al, Exp Ther Med. 2018 Dec; 16(6): 4729–4736.Published online 2018 Oct 1)). In addition, the PI3K/AKT/mTOR pathway modulates the expression of other angiogenic factors as nitric oxide and angiopoietins (Karar and Mayti, Front. Mol. Neurosci., 02 December 2011, https://doi.org/10.3389/fnmol.2011.00051).

Thus, inhibiting signaling events in the p38/ p38-MK2-HSP27 and/or PI3K/AKT/mTOR pathways - that signal cytoskeleton contraction- reduces vascular permeability, and therewith reduces adverse permeability and adverse extravasation, with resulting edema, vascular leakage, adverse leukocyte extravasation and loss of vascular fluid with a risk of hypotension. Methods and means for such inhibition are objects of this invention.

Use of EA-230 in mitigating ventilation requirements and ventilation-associated-lung-injury in COVID-19

Infections with the SARS-Cov-2 virus that causes COVID-19 mostly run a mild or even uneventful course. That course is seen in >80% of people infected. This majority of patients experience an upper airway infection of nose and throat, with a dry cough, that generally passes in 2-12 days, after which the virus will have gone from the body. These patients may or may not experience common flu-like signs such as fever, fatigue, headache and muscle pain during the period of infection with the virus. They may not need treatment with AQGV-peptide according to the invention.

In the remaining cases distinct pathological states may develop (figures 1, 2 and 3), often starting at around the time that in mild cases the viral infection is considered bound to reduce due to an emerging immune response directed against the virus. First a viral pneumonia appears with

increased vascular permeability in the lungs (pulmonary phase) that diffusely and profusely affects the deeper airways and alveoli of both lungs, causing reduced uptake of oxygen and respiratory failure. This two-sided pneumonia may be rapidly followed by full-blown systemic disease with general malaise, high fever and ultimately organ (kidney, liver, heart) failure. Patients that show these symptoms and have difficulties breathing will typically be admitted to a hospital, may enter the intensive care unit (ICU), may be put on a ventilator and need to be put into an induced coma and, even then, in its worse course, may die. These patients may very well be helped with treatment with AQGV-peptide.

Increased vascular permeability leads to respiratory failure.

As an immediate serious complication of COVID-19, lung function is severely reduced by accumulation of fluid in the lungs (figure 3). This pulmonary edema is caused by increased permeability of blood vessels in the lung as a reaction to the virus infection. Fluid from the vascular network surrounding the alveoli leaks out into the lungs, where this fluid is destroying lung tissue and erasing cells that transport oxygen. The diffuse and profuse increase of adverse fluid and necrotic (dead) cells in both lungs acutely increases the distance that oxygen has to travel through lung and vascular tissues from air to blood, and therewith hampers its exchange from air to all body tissues at large. Vice versa, the diffusion of CO2 from the blood to the air in the lungs is also hampered. These patients typically develop an acute respiratory failure and react with intensely labored breathing, therewith trying to make up for the oxygen shortage they experience.

COVID-19 in the hospital cohort.

Roughly one-third of COVID-19 patients with the above two complications (now at around 5-10% of those infected with SARS-Cov-2) have such grave disease that they need to be treated in the hospital. In some countries, most of these patients are admitted to the intensive care unit (ICU). In other countries a smaller group is selected for treatment at the ICU and other patients are either deemed to recover without intensive care treatment or are (treated only palliatively and) left to die. The number of patients admitted to hospital or ICU at one point in time may be immense, due to the steep rise of infection rates seen in a pandemic. As of end-June, 2020, COVID-19 has been confirmed in >8 million people worldwide, now carrying a confirmed case fatality rate of close to 6%. There is an urgent need for effective treatment of this cohort of patients with grave COVID-19.

Ventilator-associated-lung-injury (VALI) and Ventilator-induced-lung-injury (VILI).

Current management of grave COVID-19 is supportive and respiratory failure is the leading cause of mortality (Ruan et al. Intensive Care Med. 2020; DOI:10.1007/s00134-020-05991-x). At the ICU, COVID-19 patients generally are hooked up to a system of mechanical ventilation to provide respiratory relief. However, mechanical ventilation in itself may induce ventilator-assisted-lung injury (VALI; www.ncbi.nlm.nih.gov/pubmed/12559881), and VILI (ventilator-induced-lung-injury with increased edema and aggravated hypoxemia). VALI and VILI (https://www.ncbi.nlm.nih.gov/pubmed/24283226) are recognized herein to play a distinct role in accelerating multiple organ failure associated with COVID-19. Patients requiring mechanical ventilation consume a disproportionately high amount of healthcare resources, both in the ICU and after hospital discharge. Their short-term and long-term mortality is high, and they suffer a very heavy symptom burden for prolonged periods. Hospital survivors have a significant degree of functional and cognitive limitations, and a high readmission rate. Some remain at high risk for death after hospital discharge. Prolonged hospitalization for PMV patients who are at high risk of death does not meet current standards of cost-effectiveness. Consequently, minimizing ventilator requirement and thus minimizing risks on VALI and VILI may paradoxically be key to reduce mortality during COVID-19. Currently no pharmacological methods to combat VALI of VILI are available that address these problems as well as AQGV peptide as provided herein.

AQGV peptide EA-230 reduces adverse vascular fluid permeability.

EA-230 has surprisingly been found to modulate vascular permeability to the good. In particular, EA-230 significantly improves hemodynamic stability after open heart surgery in humans, even in the absence of inflammatory activity of the patient. Permeability governs the amount of fluid leaking from blood vessels. Administration of fluid therapy generally increases leakage. Based on Phase II trial patient observations, we found a significant reduction of adverse fluid retention (fluid leakage with fluid overload) in patients treated with EA-230 ($p = 0.03$). Throughout surgery, EA-230 was shown to be safe and well tolerated. EA-230 given during surgery shows significant improvements in patient recovery after surgery, over placebo patient. EA-230 treated patients are released faster from intensive care ($p=0.0232$) and hospital ($p=0.0015$). EA-230 improves hemodynamic stability ($p=0.006$) and kidney function ($p=0.003$). Long-term patient recovery was

significantly improved by EA-230. By improving vascular permeability, EA-230 can be used to reduce the COVID-19 associated occurrence of adverse fluid in the lungs, and therewith also reduce ventilator use with its detrimental systemic effects.

AQGV peptide EA-230 allows point-of-care determination of its effects on COVID-19 development. Moreover, EA-230 has a very short half-life, which facilitates intermittent dosing of the drug and determination of its actual effects at bedside to determine progress of the patient during treatment and make rapid decisions about continuation or discontinuation of treatment. EA230 exhibited a very short elimination half-life and a large volume of distribution (LPS-study: geometric mean and 95% confidence interval: 0.17 [0.12–0.24] hours and 2.2 [1.3–3.8] L/kg, respectively).

Respiratory failure is a common complication not only of COVID-19 and flu but of other respiratory diseases caused by coronaviruses such as SARS and MERS. The phenomenon became more widely known after the 2005 outbreak of the avian H5N1 influenza virus, also known as “bird flu”, when the high fatality rate was linked to an out-of-control systemic multi-organ failure. Now it is a SARS variant, what if next time we have to face a MERS variant? Vaccines and antivirals may differ, fighting respiratory failure stays the same. AQGV peptide can still be used.

Summary of AQGV peptide EA-230 effects

Early administration led us detect novel and truly beneficial effects of EA-230 on hemodynamics, kidney function, length of stay in ICU and hospital, that relate to improved hemodynamic stability. Treatment of patients with EA-230 during surgery significantly reduced the need for hemodynamic therapy (combined fluid therapy and blood pressure medication; p=0.006). Besides these improved hemodynamics, EA-230 significantly improved kidney function (as determined by its effects on the glomerular filtration rate) and plasma levels of kidney function biomarker creatinine (p=0.003). It also significantly shortened recovery stay at the ICU and significantly reduced length of stay in the hospital. On average, EA-230-treated patients needed about 8 days of hospital care where placebo-treated patients needed about 10 days. Also, fewer EA-230-treated patients needed re-hospitalization than placebo-treated patients did.

Effects of EA-230 in human patients

A prospective, randomized, double-blind, placebo-controlled study was performed in which 180 elective patients undergoing on-pump coronary artery bypass grafting were enrolled. Patients

were randomized in a 1:1 ratio and received either EA-230, 90 mg/kg/hour, or a placebo. These were infused at the start of the surgical procedure until the end of the use of the cardiopulmonary bypass. The main focus in this first-in-patient study was on safety and tolerability of EA-230. The primary efficacy endpoint was the modulation of the inflammatory response by EA-230. A key secondary endpoint was the effect of EA-230 on renal function.

Design and setting

The present study was a single-center, prospective, double-blind, placebo-controlled, randomized, single-dose phase II study. It has an adaptive design to evaluate the safety and immunomodulatory effects of EA-230 in patients undergoing for coronary artery bypass grafting (CABG). 180 eligible patients were included and were randomized to receive either active or placebo treatment in a 1:1 ratio. This was a first-in-patient safety and tolerability study, of which the primary efficacy objective was to assess the immunomodulatory effects of EA-230. The key-secondary efficacy endpoint was the effect of EA-230 on renal function. This study was described in accordance with the Standard Protocol Items: Recommendations for Interventional Trial (SPIRIT) guidelines, and registered at clinicaltrials.gov under number NCT03145220.

Randomization and stratification

Patients were randomized by non-blinded independent study personnel for active or placebo treatment. Study personnel used Good-Clinical-Practice-approved data management software (Castor EDC, Amsterdam, the Netherlands) in this process. The Castor system applies a stratified randomization to ensure equal distribution between active and placebo treatment of patients with known risk factors for adverse outcomes. Three strata were included: 1) a CABG procedure; 2) pre-operative renal function with an estimated GFR of ≤30, 31-90 and >90 ml/min/1.73 m2; and 3) a EuroSCORE II of <4 or ≥4 (Nashef et al. Eur J Cardiothora Surg 2012 Apr;41(4):734-44).

Blinding

Double-blind conditions were maintained for all patients, the attending physicians and the medical study team personnel involved in all blinded study procedures, data collection and/or data analyses. Non-blinded study personnel not involved in any other study procedures prepared the study medication. Infusion systems and solutions for active and placebo treatment were identical

in appearance and texture. Unblinding was authorized by the sponsor after completion of the study, performance of a blinded data review and locking of the database.

Study Intervention

Intravenous infusion of EA-230, 90 mg/kg/hour, or placebo, was initiated at the moment of first surgical incision using an automated infusion pump. Infusion rate was set at 250 mL/hour, and infusion was continued until cessation of the CPB, or after 4 hours of continuous infusion, whichever comes first.

The EA-230 formulation was packed in sterile 5 mL glass vials, containing 1500 mg/vial, dissolved in water for injection at a final concentration of 300 mg/mL with an osmolality of 800 to 1000 mOsm/kg. The placebo formulation consisted of sodium chloride diluted in water for injection in identical sterile 5 mL glass vials containing 29 mg/mL to reach a solution with an identical osmolality. EA-230 and placebo were prepared for continuous intravenous infusion with an osmolality of <400 mOsm/Kg by adding the appropriate amount of EA-230 or placebo to 1000 mL normal saline under aseptic conditions. Placebo and active treatment vials, were manufactured by HALIX BV (Leiden, the Netherlands).

Adverse events (AEs)

All AEs were judged by the investigators with regard to severity ('mild, moderate, or severe') according to Common Terminology Criteria for Adverse Events guidelines 4.030 and their perceived relation to the study drug ('definitely, probably, possibly, or unrelated/unlikely to be related'). SAEs or SUSARs include death, life-threatening disease, persistent and/or significant disability and/or incapacity, and hospitalization and/or prolongation of inpatient hospitalization.

Ethical considerations, Data quality assurance & Patient and public involvement

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki (ICH E6(R1), the Medical Research Involving Human Subjects Act, guidelines of Good Clinical Practice and European Directive (2001/20/CE). Informed consent was obtained before any study-specific procedures were performed. Data was handled confidentially and anonymously and Good-Clinical-Practice standards were applied. The handling of patient data in this study complies with the Dutch Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgegevens, WBP). Patients and the public were not involved in the design and/or the conduct of the study protocol.

Study outcome was disseminated to all study participants individually. The burden of the intervention was assessed by the independent ethics committees CMO and CCMO, which includes laymen members.

Results

When assessing the data obtained during the clinical trial, strikingly, no immunomodulatory effects were apparently observed as no significant difference of plasma levels between the EA-230 and placebo group were observed for IL-8, IL-10, IL-1RA, IL-17, MCP-1 and ICAM and other cytokines tested. This was also the case for IL-6 plasma levels, the primary endpoint of the study. Strikingly, significantly less patients suffering from fluid retention were found the EA-230 treatment group (see table 1). Various parameters were further analyzed and it was found that hemodynamic parameters (such as vasopressor use and/or fluid balance) and/or kidney parameters were advantageously affected by the use of EA-230 as compared with placebo. We conclude that timing of EA-230 dosing was too early, or at least not sufficiently done during a hyper inflammatory state of the CABG patients.

Surprisingly, however, in the absence of any observed immunomodulatory effects, it was found that the length of stay in the ICU (intensive care unit), and also in hospital, of patients treated with the AQGV peptide, was significantly reduced. Upon an in depth analysis of parameters monitored in the human subjects during the study, it was delineated/found that the use of the AQGV peptide, advantageously modulated the hemodynamics of the treated patients. It was also found that parameters related to kidney function in human patients were shown to have improved significantly, or were maintained and did not deteriorate, even despite the absence of any observed immunomodulatory effects in these patients. Parameters related to kidney function and/or hemodynamics are generally monitored in patients and determine the length of stay in either ICU or hospital. The use of the AQGV thus allows to advantageously improve parameters that are monitored in human patients to thereby reduce the length of stay in either ICU or hospital.

Table 1. Adverse events (AEs) in the EASI-study.

AEs, serious adverse events (SAE), and suspected unexpected serious adverse reaction (SUSAR with differences between treatment groups are listed here. Significantly less (Chi Square $P < 0.05$)

AEs were found in the EA-230 treatment group (217) than in the placebo treated group (283). Significantly less patients (Chi Square $P < 0.05$) suffering from fluid retention were found the EA-230 treatment group (n=2) than in the placebo treated group (n=11), $p < 0.05$.

	EA-230 (N=91)			Placebo (N=89)			Overall (N=180)		
	e	n	(%)	e	n	(%)	e	n	(%)
Any AE	217	78	(85.7)	283	81	(91)	500	159	(88.3)
Any SAE	23	12	(13.2)	19	17	(19.1)	32	29	(16.1)
Any SUSAR	0	0	(0)	1	1	(1.1)	1	1	(0.6)
AE of mild intensity	188	76	(83.5)	231	78	(87.6)	419	154	(85.6)
AE of moderate intensity	23	22	(24.2)	45	27	(30.3)	68	49	(27.2)
AE of severe intensity	6	5	(5.5)	7	4	(4.5)	13	9	(5)
Blood and lymphatic system disorders									
- Overall	6	5	(5.5)	8	8	(9)	14	13	(7.2)
Anaemia	5	5	(5.5)	8	8	(9)	13	13	(7.2)
Haemorrhagic diathesis	1	1	(1.1)	0	0	(0)	1	1	(0.6)
Gastrointestinal disorders									
- Overall	27	21	(23.1)	36	23	(25.8)	63	44	(24.4)
Nausea	15	15	(16.5)	12	12	(13.5)	27	27	(15)
Infections and infestations									
- Overall	15	13	(14.3)	22	17	(19.1)	37	30	(16.7)
Metabolism and nutrition									

disorders									
- Overall	10	10	(11)	22	14	(15.7)	32	24	(13.3)
Fluid retention	2	2	(2.2)	9	9	(10.1)	11	11	(6.1)
Psychiatric disorders									
- Overall	9	9	(9.9)	14	13	(14.6)	23	22	(12.2)
Delirium	5	5	(5.5)	9	9	(10.1)	14	14	(7.8)
Renal and urinary disorders									
- Overall	4	4	(4.4)	12	11	(12.4)	16	15	(8.3)

N = Number of patients involved

n = Number of patients experiencing the event

e = Number of events

Table 2. Average on-pump length of patients with average age of patients, split in quartiles Q1, Q2, Q3 and Q4 of pump length, and of all patients tested (Q1-Q4).

Quartiles	Treatment group	Average on-pump-length in minutes (+/- SD)	Age in years (+/- SD)
Q1	EA-230	112 +/- 12	68.5 +/- 7.3
Q1	Placebo	113 +/- 7	70.3 +/- 7.9
Q2	EA-230	137 +/- 5	66.5 +/- 9.5
Q2	Placebo	136 +/- 6	68.1 +/- 6.9
Q3	EA-230	164 +/- 8	66.3 +/- 8.7
Q3	Placebo	159 +/- 8	68.3 +/- 11.0

Q4	EA-230	211 +/- 24	65.0 +/- 7.4
Q4	Placebo	207 +/- 22	64.0 +/- 10.6
Q1-Q4	EA-230	156 +/- 37	66.5 +/- 8.3
Q1-Q4	Placebo	153 +/- 39	67.7 +/- 9.3

Hemodynamic stability in the EASI-study

In general, the use of vasopressors was reduced in the group that was treated with EA-230. Patients were divided in quartiles based on treatment duration. In Table 3, descriptive frequencies of the 2 variables: days on vasopression and nett fluid balance day 0 -2 (first 72 hours) are shown. The groups were split in patients without acute kidney injury (AKI) and with AKI, as well in patients without treatment (placebo) and with treatment with EA-230 (active). EA-230 decreased the net (netto) fluid balance in patients both with and without AKI. EA-230 decreased the need for vasopressors in patients with AKI.

Table 3.

Frequencies

Statistics

RIFLE score (incl urine output)	Treatment group			days on vasopression	Netto fluid balance day 0 - 2
no AKI	Placebo	N	Valid	42	42
			Missing	0	0
		Mean		1,38	1951,97
		Median		1,00	1957,15
		Std. Deviation		,795	1027,255
	Active	N	Valid	51	51
			Missing	0	0
		Mean		1,37	1348,47
		Median		1,00	1213,70
		Std. Deviation		1,131	1304,211
AKI	Placebo	N	Valid	47	47
			Missing	0	0
		Mean		2,23	3342,04
		Median		2,00	2965,20
		Std. Deviation		2,108	2275,286
	Active	N	Valid	39	39
			Missing	0	0
		Mean		1,97	2549,16
		Median		2,00	2328,80
		Std. Deviation		1,423	1517,623

Modulation of fluid balance and vasopressor use by treatment with EA-230

The effects of EA-230 versus placebo were tested in uni- and multivariate models (see table 4). Input/independent variable: treatment group (EA-230 or placebo). Output/dependent variables were: endpoint of fluid balance first 72 hours, days on vasopression or vasopressor score (area under the curve). Effects of EA-230 versus placebo were tested on two combined variables in model A: fluid balance first 72 hours + days on vasopression and model B: Fluid balance first 72 hours + vasopressor score AUC. The results of testing in both multivariate models showed

significant improvement of hemodynamic parameters in patients receiving EA-230. This was observed in model A (fluid balance first 72 hours + days on vasopression) p = 0.006 and in model B (fluid balance first 72 hours + vasopressor score AUC) p = 0.008. In the group of patients that showed no AKI, hemodynamic effects of EA-230 were significantly better as well, illustrating that improvement in hemodynamics can occur independent of kidney failure.

Table 4. Goal-directed hemodynamic therapy by EA-230.

An analysis is shown for model A for the total group and for subgroups of acute kidney injury split conform the RIFLE criteria: No AKI (placebo n = 42, EA-230 n = 50), Risk (placebo n = 31, EA-230 n = 34), and Injury (placebo n = 16, EA-230 n = 6). The corresponding p-values are listed.

Significant improvement of active over placebo		TREATMENT EFFECTS ON:		
		Univariate 1. Fluid balance first 72 hours	Univariate 2. Days on vasopression	Multivariate 1 and 2 combined
RIFLE stage	Total group	0.441	0.807	0.006
	No AKI	0.017	0.996	0.048
	Risk	0.807	0.564	0.753
	Injury	0.051	0.055	0.114
Uni- and multivariate general linear model analysis				

Combined, these results indicate that the use of EA-230 can improve and/or maintain hemodynamics in human patients, as assessed i.a. by affecting the duration of vasopressor use, amount of vasopressor administered and/or fluid balance. In particular, EA-230 improves hemodynamic stability in humans. Permeability governs the amount of fluid leaking from blood vessels. Administration of fluid therapy generally increases leakage. Based on Phase II trial patient observations, we found a significant reduction of adverse fluid retention (fluid leakage) in patients treated with EA-230 (p = 0.03). Also, contractility governs tone. It is often adjusted by administration of blood-pressure medications, which, however, may show major detrimental side

effects. Based on Phase II trial patient observations, we found a considerable reduction of required blood pressure medication use in the half of patients treated longest with EA-230 (>156 min; p=0.093). We also determined mean maximum concentrations (mean Cmax) as determined in vivo in humans for EA-230 in the Phase II clinical trial. Mean arterial Cmax found: 30500 ng/mL (range 12500 to 57500 ng/mL). Mean venous Cmax found: 68400 ng/mL (range 19600 to 113000 ng/mL)

EA-230 has an advantageous effect on kidney function

Effects of EA-230 on modulation in incidence of different stages of acute kidney injury (AKI) were determined according to the RIFLE criteria (RIFLE: risk, injury, failure, loss of kidney function, and end-stage kidney disease classification, Clin Kidney J. 2013 Feb; 6(1): 8–14). In the EA-230 group, the number of patients with no AKI increased, whereas the number of patients in the Injury category of the RIFLE criteria decreased. Furthermore, the use of EA-230 significantly improved GFR. Creatinine clearance, a biomarker of kidney function, was significantly improved in patients treated with EA-230. When kidney function was taken into account, clearance of creatinine was significantly improved when EA-230 was used, when kidney function was below 60 mL/min. When kidney function was above 60 mL/min, no differences were observed. When pre-treatment kidney function was above 60 mL/min/1.73m2, no differences were found between groups. These results indicate that the use of EA-230 can improve and/or maintain kidney function in human patients.

Length of stay in ICU, hospital and readmissions

In the study, effects on length of stay at the ICU of patients and length of stay in the hospital (inpatient care) were investigated. Treatment with EA-230 resulted in a significant reduction of the length of stay (LOS) at the ICU as well as at the hospital. LOS in the ICU and the hospital was reduced in the EA-230 group. The patients treated with EA-230 also showed a considerable (p=0.09) reduction of the number of re-admissions to the hospital up to 90 days after surgery (See table 5).

Table 5. Number of readmissions in the EASI-study (CABG-study). The number of patients that had to be re-admitted to the hospital due to clinical disease in the period post-treatment. Readmittance was scored in the period of 28 days after operation, and in the period ranging from 29-90 days after operation, and for the total period of 90 days after operation. Readmittance was reduced in patients receiving EA-230 treated group.

Table of Re-admissions	CABG-study Re-admission day 28	CABG-study Re-admission day 90	Total CABG-study Re-admission	Total patients
Placebo	5	5	10	89
EA-230	2	2	4	91
Total	7	7	14	180

Furthermore, in the patient group treated with AQGV, the number of patients suffering from AKI Injury was reduced, and when patients suffered AKI injury, these patients did not have a prolonged length-of-stay, as observed in the placebo group and length of stay was similar to patients having no AKI or patients being at risk of AKI.

Treatment with EA-230 herewith shows strong beneficial effects on recovery. EA-230-treated patients required significantly less hemodynamic therapy, regained post-surgical kidney function significantly faster and remained for a shorter period of time in the Intensive Care Unit (ICU) and in the hospital, as compared to placebo-treated patients.

These novel hemodynamic effects of EA-230 are independent of anti-inflammatory effects of EA-230. In short, significant improvements of hemodynamic stability, kidney function and recovery of EA-230 treated patients relate to novel effects of EA-230 on blood vessel-permeability and blood vessel-contractility. EA-230 shows significant improvements in patient recovery, over placebo patients. EA-230 treated patients are released faster from intensive care (p=0.0232) and hospital (p=0.0015). EA-230 improves hemodynamic stability (p=0.006) and kidney function (p=0.003). Whilst the primary endpoint – short term inflammatory cytokine (IL-6) reduction – was missed, long-term patient recovery was significantly improved by EA-230.

Significant improvement was found of hemodynamic stability (reducing fluid therapy and blood pressure medication; p=0.006), with: significant improvement of kidney function (improved glomerular filtration rate reduces plasma creatinine; p=0.003), significant reduction of patients suffering from adverse fluid retention during recovery (2 for EA-230, 9 for placebo; p=0.03), and considerable reduction of re-admissions to the hospital in the 90 days after treatment (4 for EA-230, 10 for placebo; p=0.09).

Further analysis biomarkers related to vasoconstriction and/or vasodilation.

In view of the effects observed on hemodynamics and lung function, plasma samples are further analyzed with regard to selected biomarkers. Plasma samples of control patients and patients receiving the EA-230 are analyzed with regard to biomarkers Endothelin-1, VEGF, Angiotensin II, ANG2/ANG1 ratio, and cAMP and natriuretic peptides.

In vitro effects of EA-230 and AQGV analogues.

In an in vitro transwell assay the effects of the AQGV peptide (EA-230), and analogues thereof, is tested on human endothelial cells. Briefly, endothelial cells are cultured in transwell culture dishes and culture medium is supplemented with AQGV peptides, and analogues thereof, or control compounds known to affect endothelial layer permeability, vasoconstriction and/or vasodilation. Suitable human endothelial cells are e.g. HUVECs (Park et al., Stem Cell Rev. 2 (2): 93–102, 2006; Jiménez et al., Cytotechnology 65, 1-14, 2012) and HMEC-1 (Ades EW, et al. J. Invest. Dermatol. 99(6): 683-690, 1992.). The permeability of the endothelial layer is determined by measuring the penetration of a macromolecule. Furthermore, levels of biomarkers are also determined in culture medium. Experiments are carried as outlined e.g. in Cox et al., Shock, 43(4):322-6; 2015. In HUVEC permeability tests, established human endothelial vascular cells (HUVEC), capable of lining blood vessels, are grown in cell-culture (i.e. n=5) on sieves, in multiple test formats, allowing determination of leak-through products depending on various test-concentrations of EA-230 peptide or placebo controls used, establishing pharmacological parameters of EA-230-peptide-effects on permeability in human cells, with or without effectors, such as thrombin, bradykinine, lipopolysaccharide, (LPS), spike protein of coronavirus, nucleic acid of coronavirus, high mobility group box 1 (HMGB1) protein, and reversing their effects with AQGV-peptides.

Also, Bravo et al (J Pharmacol Toxicol Methods. 2018 Jan - Feb;89:47-53) developed an impedance-based contraction assay using the xCELLigence RTCA MP system. This technology utilizes special 96-well E-plates with gold microelectrode arrays printed in individual wells to monitor cellular adhesion by recording the electrical impedance in real time. The impedance change (percentage vs. control) can be used as the readout for cellular contraction. Established human aortic smooth muscle cells (HaSMC), capable of contracting blood vessels, are grown in cell-culture (i.e. n=3) on gold-electrodes, in multiple test formats, allowing electrical-impedance-determination of

endothelin-1 induced smooth muscle cell contractions, depending on various test-concentrations of EA-230-peptide or placebo controls used, establishing pharmacological parameters of EA-230-effects on contractility in human cells. In addition, isolated aneurysmatic (n=3) / control (n=3) patient human aortic smooth muscle cells (APaSMC), differentially capable of contracting blood vessels, are grown in cell-cultures on gold-electrodes in multiple test formats, allowing electrical-impedance-determination of ionomycin-induced smooth muscle cell contractions of patient-versus-control cells, depending on various test-concentrations of EA-230-peptide or placebo controls used, detecting effects of EA-230 in patient cells, with or without effectors, such as thrombin, bradykinine, lipopolysaccharide, (LPS), spike protein of coronavirus, nucleic acid of coronavirus, high mobility group box 1 (HMGB1) protein, and reversing their effects with AQGV-peptides. Similar studies are used to various test-concentrations of EA-230-peptide or placebo controls used, detecting effects of EA-230 in human lung organoid cultures, with or without effectors, such as thrombin, bradykinine, lipopolysaccharide, (LPS), spike protein of coronavirus, nucleic acid of coronavirus, high mobility group box 1 (HMGB1) protein, and reversing their effects with AQGV-peptides. Similar studies are used to various test-concentrations of EA-230-peptide or placebo controls used, detecting effects of EA-230 in experimental mice provided with human ACE2 receptor, with or without effectors, such as thrombin, bradykinine, lipopolysaccharide, (LPS), spike protein of coronavirus, nucleic acid of coronavirus, high mobility group box 1 (HMGB1) protein, and reversing their effects with AQGV-peptides.

Examples of pharmaceutical compositions for use in method of reducing the permeability of an endothelial layer of a blood vessel in a subject, the method comprising:
providing to the endothelial layer a substance that reduces the ratio of Angiopoietin-2 to Angiopoietin-1 at the site of increased permeability as a result of an infection.

EXAMPLE 1

AQGVLPGQ -maleate

To prepare 1L of the composition, mix

AQGVLPGQ-maleate --1.8 mol

0.9% NaCl --1L

EXAMPLE 2

LQGVLPGQ-maleate

To prepare 1L of the composition, mix

LQGVLPGQ-maleate --1.8 mol

0.9% NaCl --1L

EXAMPLE 3

AQGLQPGQ-maleate

To prepare 1L of the composition, mix

AQGLQPGQ-maleate --1.8 mol

0.9% NaCl --1L

EXAMPLE 4

LQGLQPGQ-maleate

To prepare 1L of the composition, mix

LQGLQPGQ-maleate --1.8 mol

0.9% NaCl --1L

EXAMPLE 5

AQGV-maleate

To prepare 1L of the composition, mix

AQGV-maleate --1.8 mol

0.9% NaCl --1L

EXAMPLE 6

LQGVL-maleate

To prepare 1L of the composition, mix

LQGVL-maleate --1.8 mol

0.9% NaCl --1L

EXAMPLE 7

AQGLQ -maleate

To prepare 1L of the composition, mix

AQGLQPGQ-maleate --1.8 mol

0.9% NaCl --1L

EXAMPLE 8

LQGLQ-maleate

To prepare 1L of the composition, mix

LQGLQ-maleate --1.8 mol

0.9% NaCl --1L

EXAMPLE 9

AQGVLPGQ -acetate

To prepare 1L of the composition, mix

AQGVLPGQ-acetate --1.8 mol

0.9% NaCl --1L

EXAMPLE 10

LQGVLPGQ-acetate

To prepare 1L of the composition, mix

LQGVLPGQ-acetate --1.8 mol

0.9% NaCl --1L

EXAMPLE 11

AQGLQPGQ-acetate

To prepare 1L of the composition, mix

AQGLQPGQ-acetate --1.8 mol

0.9% NaCl --1L

EXAMPLE 12

LQGLQPGQ-acetate

To prepare 1L of the composition, mix

LQGLQPGQ-acetate --1.8 mol

0.9% NaCl --1L

EXAMPLE 13

AQGV-acetate

To prepare 1L of the composition, mix

AQGV-acetate --1.8 mol

0.9% NaCl --1L

EXAMPLE 14

LQGVL-acetate

To prepare 1L of the composition, mix

LQGVL-acetate --1.8 mol

0.9% NaCl --1L

EXAMPLE 15

AQGLQ -acetate

To prepare 1L of the composition, mix

AQGLQPGQ-acetate --1.8 mol

0.9% NaCl --1L

EXAMPLE 16

LQGLQ-acetate

To prepare 1L of the composition, mix

LQGLQ-acetate --1.8 mol

0.9% NaCl --1L

EXAMPLE 17

AQGVLPGQ -tartrate

To prepare 1L of the composition, mix

AQGVLPGQ-tartrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 18

LQGVLPGQ-tartrate

To prepare 1L of the composition, mix

LQGVLPGQ-tartrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 19

AQGLQPGQ-tartrate

To prepare 1L of the composition, mix

AQGLQPGQ-tartrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 20

LQGLQPGQ-tartrate

To prepare 1L of the composition, mix

LQGLQPGQ-tartrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 21

AQGV-tartrate

To prepare 1L of the composition, mix

AQGV-tartrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 22

LQGVL-tartrate

To prepare 1L of the composition, mix

LQGVL-tartrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 23

AQGLQ -tartrate

To prepare 1L of the composition, mix

AQGLQPGQ-tartrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 24

LQGLQ-tartrate

To prepare 1L of the composition, mix

LQGLQ-tartrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 25

AQGVLPGQ -citrate

To prepare 1L of the composition, mix

AQGVLPGQ-citrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 26

LQGVLPGQ-citrate

To prepare 1L of the composition, mix

LQGVLPGQ-citrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 27

AQGLQPGQ-citrate

To prepare 1L of the composition, mix

AQGLQPGQ-citrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 28

LQGLQPGQ-citrate

To prepare 1L of the composition, mix

LQGLQPGQ-citrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 29

AQGV-citrate

To prepare 1L of the composition, mix

AQGV-citrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 30

LQGVL-citrate

To prepare 1L of the composition, mix

LQGVL-citrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 31

AQGLQ -citrate

To prepare 1L of the composition, mix

AQGLQPGQ-citrate --1.8 mol

0.9% NaCl --1L

EXAMPLE 32

LQGLQ-citrate

To prepare 1L of the composition, mix

LQGLQ-citrate --1.8 mol

0.9% NaCl --1L