

A novel synthetic, host-defence peptide protects against organ injury/dysfunction in a rat-model of severe hemorrhagic shock

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MINI-ABSTRACT (50 words)

This study evaluated the role of host-defence/antimicrobial peptides in trauma-associated hemorrhagic shock (HS). Trauma-associated HS resulted in the release of the host defence peptide LL-37. The synthetic host defence peptide Pep19-4LF attenuated the HS-associated organ injury/dysfunction in the rat by activating pro-survival pathways and by inhibiting local and systemic inflammation.

ABSTRACT

Objective: To evaluate (i) levels of the host-defence/antimicrobial peptide LL-37 in patients with trauma and hemorrhagic shock (HS) and (ii) the effects of a synthetic host-defence peptide; Pep19-4LF on multiple organ failure (MOF) associated with HS.

Background: HS is a common cause of death in severely injured patients. There is no specific therapy that reduces HS-associated MOF.

Methods: (i) LL-37 was measured in 47 trauma/HS patients admitted to an urban major trauma center. (ii) Male Wistar rats were submitted to HS [90min, target mean blood pressure (MAP): 27-32mmHg] or sham operation. Rats were treated with Pep19-4LF [66 (n=8) or 333µg/kg x h (n=8)] or vehicle (n=12) for 4h following resuscitation.

Results: Plasma LL-37 was 12-fold higher in patients with trauma/HS compared to healthy volunteers. HS-rats treated with Pep19-4LF (high dose) had a higher MAP at the end of the 4h resuscitation period (79±4 vs. 54±5 mmHg) and less renal dysfunction, liver injury and lung inflammation than HS-rats treated with vehicle. Pep19-4LF enhanced (kidney/liver) the phosphorylation of (i) protein kinase B and (ii) endothelial nitric oxide synthase. Pep19-4LF attenuated the HS-induced (i) translocation of p65 from cytosol to nucleus, (ii) phosphorylation of IKK on Ser^{176/180} and (iii) phosphorylation of IκBα on Ser^{32/36} resulting in inhibition of NF-κB and formation of pro-inflammatory cytokines. Pep19-4LF prevented the release of TNFα caused by heparan sulfate in human mononuclear cells by binding to this DAMP.

Conclusions: Trauma-associated HS results in release of LL-37. The synthetic host-defence/antimicrobial peptide Pep19-4LF attenuates the organ injury/dysfunction associated with HS.

Keywords

Antimicrobial peptides, hemorrhagic shock, multiple organ failure, NF- κ B pathway, LL-37

INTRODUCTION

Severe injuries account for 9% of the deaths worldwide.¹ Although guidelines for the early management of hemorrhagic shock (HS; including resuscitation and organ support strategies) have decreased the rates of immediate (on scene/within 60min) and early (emergency department and operating room/within 1-4h) deaths,² post-injury multiple organ failure (MOF) is still associated with significant morbidity and mortality². Therapeutic agents that reduce the incidence and severity of MOF following HS could, therefore, have a major global impact on patient outcomes and resource utilization. The MOF after HS is associated with excessive systemic inflammation, secondary to the release of damage-associated molecular patterns (DAMPs) from extensive tissue damage and ischemia reperfusion injury.³ To date, there are no specific pharmacological interventions used clinically to prevent MOF following/associated with HS.

Host-defence/antimicrobial peptides are known for over 100 years and form part of the innate immune system of insects, plants, and vertebrates by defending the host against invading microorganisms.^{4,5,6} Although these peptides differ in sequence and structure, they are predominantly short (10–50 amino acids) amphipathic molecules.⁶ The most extensively studied host-defence/antimicrobial peptide in humans is the cathelicidin-derived peptide LL-37.⁶ LL-37 exhibits strong bactericidal properties, but at the same time neutralizes pathogenic factors released during injury/infection including lipopolysaccharide (LPS) or lipoprotein (LP).⁷ In addition to the interaction with PAMPs (pathogen associated molecular patterns), LL-37 modulates the inflammatory response induced by DAMPs and, hence, modulates many physiological host functions including inflammation, angiogenesis and wound healing.^{6,8} Thus, host-defence/antimicrobial peptides are attractive candidates for the development of novel therapeutic interventions in infectious and inflammatory diseases.⁶ The systemic application of

LL-37 as a potential drug in man, however, is limited by its toxicity.^{9,10} The challenge is to develop synthetic host-defence/antimicrobial peptides (mimetics) that have little or no adverse effects. Peptide 19-4LF (Pep19-4LF) is one of several new synthetic host-defence/antimicrobial peptides, which belongs to the class of synthetic anti-lipopolysaccharide peptides (SALP=synthetic anti-LPS peptides).^{11,12} However, in addition to binding LPS, these peptides exhibit potent anti-inflammatory effects in experimental sepsis by interacting with a variety of PAMPs and DAMPs.^{13,12,14,15}

The role of host-defence/antimicrobial peptides in HS is unknown. Therefore, the aims of the present study were to (i) investigate the plasma levels of LL-37 in patients with trauma with or without HS and (ii) to explore the effects of Pep19-4LF on the organ injury/dysfunction associated with HS. We report here for the first time, that (i) the plasma levels of LL-37 are elevated in patients with trauma/HS (when compared with trauma without HS) and that (ii) Pep19-4LF attenuates the HS-associated organ injury/dysfunction. Mechanistically, Pep19-4LF has pro-survival and anti-inflammatory properties, as it activates the Akt/eNOS cell survival pathways and attenuates the activation of the nuclear factor kappa B (NF- κ B) pathway in the rat *in vivo*. Moreover, Pep19-4LF exhibits its anti-inflammatory activity, at least in part, by directly interacting/binding to the DAMP heparan sulfate in human mononuclear cells (MNCs) *in vitro*. These data suggest that Pep19-4LF may prevent the MOF in patients caused by trauma-associated HS, which, in turn, may improve outcome in these patients.

METHODS

Additional details relating to materials and methodology are provided in the supplemental.

Use of human subjects-ethic statement

All patients or their legal representative gave written informed consent. Before inclusion of the first individual, the local National Health Service Research Ethics Committee (REC: 07/Q0603/29) approved this study, which was performed in accordance with the Declaration of Helsinki in its latest form. The use of plasma from healthy volunteers was approved by the ethics committee of the University Hospital Aachen (EC Nr.206_09, 5 January 2010).

Use of experimental animals-ethic statement

The experimental protocols used in this study have been approved by the Animal Welfare Ethics Review Board (AWERB) of Queen Mary University of London and the study was performed under license issued by home office (Procedure Project License; PPL:70/7348). Animal care was in accordance with the Home Office guidance on Operation of Animals (Scientific Procedures Act 1986) published by Her Majesty's Stationery Office and the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Hemorrhagic shock and quantification of organ injury and dysfunction

This study was carried out on 46 male Wistar rats (Charles River Ltd, Margate, UK) weighing 230-350 g receiving a standard diet and water *ad libitum*. Hemorrhagic shock and quantification of organ injury and dysfunction were performed as described previously in this journal (*supplemental Fig. 1*).¹⁶

Experimental design

The following groups were studied (total n=45): Sham + vehicle (n=11), sham + Pep19-4LF (n=6), HS + vehicle (n=12), HS + Pep19-4LF-LD (n=8), HS + Pep19-4LF-HD (n=8). Rats were administered vehicle (saline 1.5 ml/kg/h) or Pep19-4LF (low dose (LD) = 66 µg/kg x h; high dose (HD) = 333 µg/kg x h) continuously for 4h after resuscitation using infusion pump for rodents

(PHD2000, 70-2000; Harvard apparatus Massachusetts, U.S). We excluded 11 rats from data analysis due to surgical/technical issues (n=5) prior to onset of HS and death during resuscitation (n=6). The doses of Pep19-4LF used in this study were based on efficacy seen in previous *in vitro* and *in vivo* studies.^{13,12,14,15}

Statistics

Unless otherwise stated, the data is expressed as median and standard error or described in box and whisker format showing medians, interquartile ranges and full ranges of *n* observations, where *n* represents the number of animals/experiments studied. Statistical analysis was carried out using Prism 6 for Mac OS X (GrapPad, San Diego, CA, USA). The distribution of the data was assessed using D'Agostino's K-squared test or Kolmogorov–Smirnov test. Unless otherwise stated, normal distributed data were assessed by 1 or 2-way analysis of variance followed by Bonferroni post hoc test. Unless otherwise stated, not normally distributed data were analyzed with a non-parametric test (Kruskal-Wallis followed by Dunn test). A $p < 0.05$ was considered to be significant.

RESULTS

Plasma concentrations of the host defence AMP cathelicidin LL-37

Figure 1 shows plasma concentrations of LL-37 in healthy volunteers and trauma patients recruited from an urban major trauma center. The median age of the healthy volunteers were 47 (32-53) years with 80% male. Further demographic and clinical parameters of trauma patients are described in *supplemental Table 1*. Admission blood samples of trauma patients were obtained within 2h of injury (*supplemental Table 1*). When compared to healthy volunteers, trauma patients (n=24) and trauma hemorrhage patients (n=23) (defined as patients who received greater than or equal to two units of packed red blood cells on admission) showed significantly higher plasma levels of LL-37. When compared to trauma patients, the plasma levels of LL-37 were significantly higher in trauma hemorrhage patients (Fig.1). **Increased levels were associated (at time of admission) with (a) low systolic blood pressure, (b) high heart rate, (c) high lactate, and (d) low base deficit (*supplemental Fig.3*). Moreover, we performed a subgroup analysis with focus on patients with an Injury Severity Score (ISS) of > 24. Applying this threshold to the cohort resulted in an equivalent median ISS between the groups (*supplemental Table 2*). Notably, this subgroup analysis shows different LL-37 serum concentrations between trauma patients and trauma hemorrhage patients (*supplemental Fig.2*).**

Pep19-4LF attenuates the decline in blood pressure during resuscitation after HS

When compared to sham-operated rats, HS-rats treated with vehicle showed a significant decline in mean arterial pressure (MAP) after resuscitation (Fig.2). Intravenous administration of high-dose Pep19-4LF significantly attenuated the decline in MAP observed during the resuscitation period in HS-rats, while the low dose of Pep19-4LF had no effect. In contrast, the high dose of Pep19-4LF had no effect of MAP in sham-operated rats (Fig.2).

Pep19-4LF attenuates the organ injury and dysfunction caused by HS

When compared to sham-operated rats, rats subjected to HS treated with vehicle exhibited a renal dysfunction, as indicated by significant increases in serum urea (Fig.3A) and creatinine (Fig.3B), and a significant decline in creatinine clearance (Fig.3C). HS-rats also exhibited significant increases in alanine aminotransferase (ALT), aspartate aminotransferase (AST), amylase and lipase, indicating the development of liver and pancreatic injury, respectively (Fig. 3E-G). In addition, HS-rats exhibited a significant increase in serum lactate, indicating global ischemia (Fig.3D). When compared to vehicle-treated rats, intravenous administration of high-dose Pep19-4LF significantly attenuated the organ injury and dysfunction as well as the rise in lactate caused by HS (Fig.3A-H). Treatment with low dose Pep19-4LF attenuated the increases in serum creatinine, ALT, amylase, and lipase, but did not attenuate the increases in urea, creatinine clearance, lactate or AST caused by HS (Fig.3A-H).

Pep19-4LF attenuates lung inflammation caused by HS

Having shown that Pep19-4LF-treatment attenuates kidney dysfunction and liver injury, we next investigated the effects of Pep19-4LF on lung inflammation measured as recruitment of macrophages (CD68-positive cells) and neutrophil activation (MPO activity) into the lung. When compared to sham-operated rats, we found a significant increase in CD68-positive cells and MPO-activity in lungs of HS-rats treated with vehicle (Fig.4A-C). Treatment of HS-rats with Pep19-4LF significantly attenuated the recruitment of macrophages and neutrophil activation caused by HS (Fig.4A-C).

Pep19-4LF attenuates the increase in interleukin (IL)-6 and monocyte chemotactic protein-1 (MCP-1) caused by HS

We next investigated the effects of Pep19-4LF on the formation of pro- and anti-inflammatory cytokines caused by HS. When compared to sham-operated rats, HS-rats treated with vehicle developed a significant increase in serum IL-6 and MCP-1. In HS-rats, we also observed increases in serum IL-10 and C-X-C motif ligand 1 (CXCL1), but these effects were not significant (Fig.5A,B). Treatment of HS-rats with Pep19-4LF abolished the increases in IL-6, MCP-1, IL-10, and CXCL1 caused by HS (Fig.5A-D).

Pep19-4LF attenuates the activation of NF- κ B (liver and kidney) caused by HS

Having shown that Pep19-4LF significantly attenuates kidney dysfunction and liver injury caused by HS, we next explored the potential mechanism(s) underlying the observed beneficial effects of high dose of Pep19-4LF. When compared to sham-operated rats, HS-rats treated with vehicle exhibited a significant increase in phosphorylation of I κ B kinase α and β (IKK α/β), which is essential for I κ B phosphorylation (Fig.6A,F). Following HS-induced phosphorylation of I κ B (Fig.6B,G), the p65 subunit of NF- κ B is freed to translocate to the nucleus, as shown in Fig. 6C, H. The intravenous administration of high-dose Pep19-4LF attenuated the phosphorylation of IKK α/β on Ser^{176/180}, and of I κ B α on Ser^{32/36} and, accordingly, the nuclear translocation of the NF- κ B subunit p65 in both liver and kidney (Fig.6A,B,C,F,G,H).

Pep19-4LF increases activation of Akt and eNOS in kidney and liver after HS

As activation of the Akt-survival pathway is known to reduce HS-induced organ dysfunction^{16,17} we next investigated whether the high dose of Pep19-4LF activates Akt in kidney and liver of HS-rats (Fig.6D,I). As activation of the Akt-survival pathway is known to reduce HS-induced organ dysfunction^{16,17} we next investigated whether the high dose of Pep19-4LF activates Akt in kidney and liver of HS-rats (Fig.6D,I). When compared to sham-operated rats, HS-rats treated with vehicle showed a significant reduction in the phosphorylation of Akt on Ser⁴⁷³ in both kidney

and liver (Fig.6D,I). This effect was associated with a reduction in the Akt-mediated eNOS phosphorylation at Ser¹¹⁷⁷. In contrast, treatment of HS-rats with Pep19-4LF attenuated the decline in Akt phosphorylation on Ser⁴⁷³ and of eNOS phosphorylation on Ser¹¹⁷⁷ in kidney and liver, when compared to HS rats treated with vehicle (Fig.6E,J).

Pep19-4LF inhibits the heparan sulfate-induced TNF α secretion in human peripheral blood mononuclear cells

As discussed above, traumatic injury and trauma-associated HS result in the release of a variety of endogenous TLR ligands, including heparan sulfate¹⁸. We report here that heparan sulfate stimulates the release of TNF α from human MNCs, and that this effect is reduced/abolished in a concentration-dependent manner by Pep19-4LF (Fig. 7A).

Pep19-4LF exhibits binding to heparan sulfate

To gain a better understanding of the mechanism(s) by which Pep19-4LF reduces the formation of TNF α in human MNC challenged with heparan sulfate, we investigated the potential binding of Pep19-4LF to heparan sulfate by isothermal titration calorimetry. There was an exothermic reaction between the two reactants, running into a saturation of binding at higher mass ratios (Fig.7B). The rather steep slope of the sigmoidal curve indicates distinct binding of the reaction partners and may explain that the binding epitopes of heparan sulfate to receptors (i.e. TLR4) are hidden by the peptide.

Pep19-4LF does not show hemolytic activity

Finally, possible cytotoxic effects of Pep19-4LF were studied in the hemolysis assay with RBC as sensitive target cells for cytotoxicity. Pep19-4LF caused no considerable degree of hemolysis in concentrations of up to 100 μ g/ml (Fig.7C).

DISCUSSION

We report here for the first time that trauma leads (within 2h) to a significant increase in the plasma levels of the host-defence/antimicrobial peptide LL-37. Most notably, the highest levels of LL-37 were found in patients with trauma complicated by severe hemorrhage (Fig.1) and were associated with low systolic blood pressure, high heart rate, high lactate and low base deficit.

Using a reverse translational approach, we investigated whether pharmacological intervention with synthetic host-defence/antimicrobial peptides attenuates the MOF associated with HS in rats. As the therapeutic use of LL37 in man is limited by its systemic toxicity in therapeutic doses,^{9,10} we synthesized Pep19-4LF, which does not cause any significant adverse effects (hemolysis) in the doses used (Fig.7C). The administration of Pep19-4LF significantly attenuated the fall in blood pressure (Fig.2) as well as the rise in serum lactate caused by HS (Fig.3D). Thus, Pep19-4LF reduces the delayed vascular decompensation and organ/tissue ischemia probably due to increased microvascular perfusion secondary to increased perfusion pressure. Moreover, Pep19-4LF significantly attenuated the liver injury, renal dysfunction, pancreatic injury and lung inflammation caused by HS (Fig.3A,B,C,E,F,G,H). As neutrophils and macrophages play an important role in HS-associated lung inflammation, we evaluated the degree of macrophage infiltration (measured as number of CD68-positive cells) and the degree of neutrophil activation (measured as MPO-activity) in the lung. HS resulted in a significant increase in the number of macrophages in the lung as well as a significant increase in MPO-activity, both of which was attenuated by the treatment of HS-rats with Pep19-4LF (Fig.4). Neutrophils and macrophages release (via degranulation) pro-inflammatory cytokines, such as IL-6 or MCP-1 which importantly contribute to acute lung injury/inflammation.¹⁹ The pro-inflammatory cytokines IL-6 and MCP-1 are important mediators of alterations associated with organ dysfunction and even lethality following HS and resuscitation.^{20,21,22,23} For instance, a

monoclonal antibody against IL-6 reduces the organ dysfunction and inflammation caused by HS.⁴² Indeed, we report here that Pep19-4LF also attenuates the rise in serum IL-6 and MCP-1 caused by HS in the rat (Fig.5).

What, then, are the mechanisms by which Pep19-4LF attenuates HS-associated organ injury/dysfunction? There is good evidence that PAMPs and DAMPs released during trauma-HS interact with Toll-like receptors (i.e. TLR2,4) resulting in activation of NF- κ B.^{24,25,26} Indeed, we observed a significant increase in (a) the degree of phosphorylation of IKK α/β on Ser^{176/180} and (b) of I κ B α on Ser^{32/36}, thus resulting in (c) increased nuclear translocation of NF- κ B subunit p65 (Fig.6) in liver/kidneys of rats with HS. This activation of NF- κ B in key target organs was attenuated in HS-rats treated with Pep19-4LF during resuscitation. I κ B α masks the nuclear localization signals of NF- κ B proteins and sequesters NF- κ B as an inactive complex in the cytoplasm, thereby inhibiting NF- κ B.^{27,28} Signal-induced proteolytic degradation of I κ B α , which has been phosphorylated by I κ B kinases (IKK α/β) liberates NF- κ B to translocate to the nucleus.²⁸ Subsequently, NF- κ B activates the transcription of a number of genes involved in producing pro-inflammatory cytokines and chemokines known to result in the transcription of a multitude of pro-inflammatory cytokines, chemokines and proteins that are widely implicated in the pathophysiology of MOF.^{17,16} Thus, the organ protective effects of Pep19-4LF in HS are associated with a significant reduction in the activation of the NF- κ B pathway, which in turn accounts for the reduced formation of IL-6 and MCP-1.

We also investigated the effects of HS with or without Pep19-4LF on the degree of activation of the Akt-survival pathway (Fig.6). When compared to sham rats, HS rats treated with vehicle showed a significantly decreased phosphorylation of Akt on Ser⁴⁷³ (indicating reduction in activity of this kinase) in kidney and liver, which makes these organs less resistant to organ injury. In

contrast, Pep19-4LF attenuated the decline in Akt-activation caused by HS (Fig.6D,H). Akt is a member of the phosphoinositide-3-kinase (PI3K) signal transduction enzyme family. When activated (phosphorylated on Ser⁴⁷³) by its upstream regulator PI3K, Akt controls inflammatory response, chemotaxis and apoptosis.²⁹ Most notably, activation of the Akt-survival pathway reduces organ injury in many conditions associated with ischemia/inflammation including ventilation-induced lung injury, sepsis-induced organ dysfunction, myocardial infarction, and HS-induced organ dysfunction.^{30,31,32,33,16} Moreover, activation of Akt results in phosphorylation and activation of eNOS at Ser¹¹⁷⁷ that enhances the formation of small amounts of NO, which is pivotal for the preservation of microvascular perfusion and, hence, reducing organ injury.^{34,31,35} We report here that the degree of eNOS phosphorylation on Ser¹¹⁷⁷ is significantly higher in HS-rats treated with Pep19-4LF indicating activation of eNOS and enhanced formation of NO in the microcirculation at least of kidney and liver. We propose that the enhanced formation of NO by eNOS in HS-rats treated with Pep19-4LF contributes to improved microcirculatory perfusion resulting in better tissue oxygenation and lower lactate levels (Fig.3D). Thus, the organ protective effects of Pep19-4LF in HS are associated with a significant activation of the Akt/eNOS survival pathway.

Traumatic injury and trauma-associated HS result in the release of a variety of endogenous TLR ligands, including heparan sulfates.^{24,25,26,36} Moreover, the degradation of the glycocalyx (and subsequent liberation of heparan sulfates) induces remote organ injury after trauma/hemorrhagic shock,^{37, 38,39,40} suggesting heparan sulfate as a potential therapeutic target for Pep19-4LF. Indeed, using isothermal titration calorimetry, we found a strong Coulomb interaction between Pep19-4LF and heparan sulfate, as indicated by strong exothermic reaction running into a saturation characteristic (Fig.7B). To investigate whether Pep19-4LF inhibits the release of TNF α caused by heparan sulfate in human cells, we exposed human MNCs to heparan sulfate

in the presence or absence of Pep19-4LF. Most notably, Pep19-4LF attenuated the release of $\text{TNF}\alpha$ caused by heparan sulfate in these cells in a dose-related fashion (Fig.7A) indicating that Pep19-4LF is able to prevent the activation of human cells by the DAMP heparan sulfate. Thus, these findings indicate, that the organ protective and anti-inflammatory effects of Pep19-4LF in HS are, at least partly, associated with its interaction with relevant DAMPs, such as heparan sulfate.

Limitations of our study: We used an acute model of HS, which leads to vascular decompensation (e.g. a fall in MAP despite fluid resuscitation), MOF and systemic inflammation within 4h of the onset of resuscitation. Even at this relatively early time-point, HS and resuscitation led to organ dysfunction and activation (kidney/liver) of NF- κ B and expression of NF- κ B-dependent proteins. Although Pep19-4LF showed some very striking, beneficial effects in this acute setting, further experiments with trauma/hemorrhage and recovery (for 24-48h) are necessary to confirm that the observed early reduction in MOF does indeed translate to improved outcome and ultimately reduced mortality. In addition, future studies in large animals (pigs) may be useful to confirm efficacy and to explore other aspects of the potential mechanism(s) of action (i.e. effects on microcirculation and blood gas analyses) of Pep19-4LF in HS. It would have been very useful to investigate whether Pep19-4LF does inhibit iNOS expression, improve microcirculatory blood flow or improves cardiac contractility (in order to understand the improvements in MAP seen during resuscitation). Lack of the above data does, however, not hinder the clinical translation of our findings, as approval by the regulatory authorities (MHRA in the UK) will primarily depend on the availability of the relevant preclinical and phase I safety data rather than further efficacy and/or mechanistic studies in a higher species. Given the pilot character of our clinical study investigating LL-37 levels in patients with trauma and trauma hemorrhage in men, our findings should be confirmed and extended by a measurements of LL37 at several time points over an extended period in order to provide a

dynamic picture of the changes in plasma LL-37 in patients with trauma and trauma hemorrhage. It would also be useful to investigate the influence of gender on the level of LL-37 in trauma/haemorrhage, although this study would need to be done in a much larger patient cohort.

In conclusion, we report here for the first time that trauma and trauma-haemorrhage result in a significant release of the host-defence/antimicrobial peptide LL-37. As the systemic administration of higher doses of LL-37 leads to adverse effects, we have synthesized a small host-defence/antimicrobial peptide, Pep19-4L. Like LL-37, Pep19-4LF neutralizes the effects of LPS and lipoproteins.¹² LL-37 also interacts with and attenuates the effects of several DAMPs.^{6,8} We report here that Pep19-4LF abolishes the release of $\text{TNF}\alpha$ caused by heparan sulfate in human mononuclear cells. In addition, Pep19-4LF attenuates the organ injury/dysfunction caused by severe hemorrhage and resuscitation in the anesthetized rat. This protective effect of Pep19-4LF was associated with activation of the Akt/eNOS-survival pathway (kidney and liver), which increases the resistance of these organs to injury. In addition, Pep19-4LF also attenuates the activation of NF- κ B in these organs, resulting in the reduced formation of the pro-inflammatory cytokines IL-6 and MCP-1. Thus, we propose that Pep19-4LF may be useful to reduce the organ injury/dysfunction and inflammation caused by severe hemorrhage and resuscitations in patients with trauma.

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AUTHOR CONTRIBUTIONS

Conception and design: NY, LM, and CT; Animal experiments: NY; Cell culture experiments: LH, TG, WC, and KB; Human sample analysis: NY, LM, EZ, GM, TS; Animal sample analyses: NY, LM, GP, FC, BV, MC, and TS; Clinical study and patient data analyses: LM, JS, KB, GM, and TS; Peptide development and synthesis: KB, LH, TG, WC. Statistical analyses: NY, LM, and CT; Drafting the manuscript for important intellectual content: NY, LM, EZ, GP, FC, BV, MC, JS, LH, TG, WC, KB, GM, TS, KB, and CT; All authors reviewed and approved the manuscript.

ADDITIONAL INFORMATION

Supplementary information accompanies this paper.

Competing financial interests: KB has a patent for the structure of the synthetic antimicrobial peptide 19-4LF (Brandenburg Antiinfektiva, Borstel, Germany): Patent-No: PCT/EP2009/002565. KB and LH are chief scientific officer and TS is chief medical officer of Brandenburg Antiinfektiva GmbH. All the other authors declare no conflicts of interest.

LEGENDS TO FIGURES

FIGURE 1. LL-37 plasma levels in human healthy volunteers and trauma patients

Plasma concentrations of the cathelicidin LL-37 were assessed in control healthy volunteers ($n = 10$) and in trauma patients ($n = 24$) as well as in trauma hemorrhagic patients ($n = 23$). Data are expressed as box and whisker min to max for n number of observations. + = mean value. * $P < 0.05$ vs. healthy; $^{\S}P < 0.05$ vs. trauma (Kruskall-Wallis test with Dunn's multiple comparisons test).

FIGURE 2. Pep19-4LF attenuates the decline in MAP during resuscitation after HS.

HS-rats received continuous administration of low-dose (LD; $66 \mu\text{g/kg} \times \text{h}$) or high-dose (HD; $333 \mu\text{g/kg} \times \text{h}$) of Pep19-4LF or saline (vehicle) throughout 4h after resuscitation. Sham animal were used as control and received saline or high-dose of Pep19-4LF. The MAP was recorded during the whole experiment. The following groups were studied: sham + vehicle ($n = 11$); sham + Pep19-4LF-HD ($n = 6$); HS + vehicle ($n = 12$), HS + Pep19-4LF-LD ($n = 4$); HS + Pep19-4LF-HD ($n = 8$). Data are expressed as mean \pm SEM for n number of observations. Statistical analysis was performed using 2-way ANOVA followed by Bonferroni post hoc test. * $P < 0.05$ vs HS + vehicle. ANOVA indicated analysis of variance; SEM, standard error of the mean.

FIGURE 3. Pep19-4LF attenuates the organ injury and dysfunction caused by HS.

(A) Serum urea, (B) serum creatinine, (C) creatinine clearance (CCr), (D) serum lactate, (E) serum alanine transaminase (ALT), (F) serum aspartate transaminase (AST), (G) serum amylase, and (H) serum lipase of HS or sham-operated rats. All parameters were assessed 4h subsequent to HS. HS-rats received continuous administration of low-dose (LD; $66 \mu\text{g/kg} \times \text{h}$) or high-dose (HD; $333 \mu\text{g/kg} \times \text{h}$) of Pep19-4LF or saline (vehicle) throughout 4h after resuscitation.

Sham animal were used as control and received saline or high-dose of Pep19-4LF. Data are shown as box and whiskers, showing medians, interquartile range, and full range. The following groups were studied: sham + vehicle (n = 11); sham + Pep19-4LF-HD (n = 6); HS + vehicle (n = 12), HS + Pep19-4LF-LD (n = 8); HS + Pep19-4LF-HD (n = 8). Statistical analysis was performed using 1-way ANOVA followed by Bonferroni post hoc test. § $P < 0.05$ vs sham + vehicle and * $P < 0.05$ vs HS + vehicle. ANOVA indicated analysis of variance.

FIGURE 4. Pep19-4LF attenuates lung inflammation caused by HS.

Representative images for CD68 as a macrophage marker (200 fold), (B) quantitative analysis of the number of CD68-positive cells/mm², (C) MPO activity in lungs of HS or sham-operated rats. The scale bar represents 100µm. All parameters were assessed 4h subsequent to HS. HS-rats received continuous administration of vehicle (saline) or Pep19-4LF of (333 µg/kg x h) throughout 4h after resuscitation. Sham animal were used as control and received saline. Data are shown as box and whiskers, showing medians, interquartile range, and full range. The following groups were studied: sham + vehicle (n =4); HS + vehicle (n = 6), HS + Pep19-4LF HD (n = 6). Statistical analysis was performed using 1-way ANOVA followed by Bonferroni post hoc test. § $P < 0.05$ vs sham + vehicle and * $P < 0.05$ vs HS + vehicle. ANOVA indicated analysis of variance.

FIGURE 5. Pep19-4LF attenuates the increase in IL-6 and MCP-1 caused by HS.

The serum concentrations of (A) interleukin (IL)-6, (B) monocyte chemotactic protein-1 (MCP-1), (C) IL-10, and (D) C-X-C motif ligand 1 (CXCL1) were determined using a cytometric bead array in sham and HS rats treated with vehicle or Pep19-4LF (333 µg/kg x h) throughout 4h after resuscitation. All parameters were assessed 4h subsequent to HS. Data are presented as box and whiskers format, showing medians, interquartile range, and full range. (E) Heatmap of

measured cytokines. The following groups were studied: sham + vehicle (n = 11); HS + vehicle (n = 12), HS + Pep19-4LF-HD (n = 8). Statistical analysis was performed using 1-way ANOVA followed by Bonferroni post hoc test. §*P* < 0.05 vs sham + vehicle and **P* < 0.05 vs HS + vehicle. ANOVA indicated analysis of variance.

FIGURE 6. Pep19-4LF attenuates the activation of NF-κB and increases the activation of Akt and eNOS in kidney and liver after HS.

The phosphorylation of Ser^{176/180} on IKKα/β (kidney (A), liver (F)), of Ser^{32/36} on IκBα (kidney (B), liver (G)), the nuclear translocation of p65 subunit of NF-κB (kidney (C), liver (H)), and Ser⁴⁷³ on Akt (kidney (D), liver (I)), and Ser¹¹⁷⁷ on eNOS (kidney (E), liver (J)) of sham and HS rats treated with vehicle or high-dose of Pep19-4LF (333 μg/kg x h) upon resuscitation were determined by Western blotting. Protein expression was measured as relative OD. Data are shown as box and whiskers, showing medians, interquartile range, and full range. The following groups were studied: sham + vehicle (n = 4); HS + vehicle (n = 4), HS + Pep19-4LF-HD (n = 4). Statistical analysis was performed using 1-way ANOVA followed by Bonferroni post hoc test. §*P* < 0.05 vs sham + vehicle and **P* < 0.05 vs HS + vehicle. ANOVA indicated analysis of variance; OD, optical density.

FIGURE 7. Pep19-4LF interacts with heparan sulfate

Inhibitory effect of Pep19-4LF on heparan sulfate-induced TNFα release in human peripheral blood mononuclear cells from healthy donors. Pep19-4LF was added at the indicated weight ratios of the concentrations of heparan sulfate to Pep19-4LF. (B) Enthalpy of the Pep19-4LF-heparan sulfate binding. Isothermal calorimetric titration of a 1 – 4 mM Pep19-4LF solution into a 200 μg/ml heparan sulfate dispersion. The enthalpy changes at each injection were measured and the area under the peak was integrated and plotted against the weight ratio of the

concentrations of Pep19-4LF to heparan sulfate. A downward peak corresponds to an exothermic reaction, and an upward peak corresponds to an endothermic reaction. (C) Red blood cells, obtained from citrated human blood were suspended at a concentration equivalent to 5% of the normal hematocrit. Pep19-4LF and melittin (as control) were added at different concentrations and the supernatants were analyzed for haemoglobin. Results are expressed as the percentage released with respect to sonicated controls (100% release) or controls processed without peptide (0% release).