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Lactoferricin-inspired peptide AMC-109 augments the effect of ciprofloxacin against *Pseudomonas aeruginosa* biofilm in chronic murine wounds

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ABSTRACT

Objectives: Chronic wounds are characterised by prolonged inflammation, low mitogenic activity, high protease/low inhibitor activity, microbiota changes and biofilm formation, combined with the aetiology of the original insult. One strategy to promote healing is to terminate the parasitism-like relationship between the biofilm-growing pathogen and host response. Antimicrobial peptide AMC-109 is a potential treatment with low resistance potential and broad-spectrum coverage with rapid bactericidal effect. We aimed to investigate whether adjunctive AMC-109 could augment the ciprofloxacin effect in a chronic *Pseudomonas aeruginosa* wound model.

Methods: Third-degree burns were inflicted on 33 BALB/c mice. *Pseudomonas aeruginosa* embedded in seaweed alginate was injected sub-eschar to mimic biofilm. Mice were randomised to receive AMC-109, combined AMC-109 and ciprofloxacin, ciprofloxacin, or placebo for 5 days followed by sample collection.

Results: A lower bacterial load was seen in the double-treated group compared with either monotherapy group (AMC-109, $p = 0.0076$; ciprofloxacin, $p = 0.0266$). To evaluate the innate host response, cytokines and growth factors were quantified. The pro-inflammatory response was dampened in the double-treated mice compared with the mono-ciprofloxacin-treated group ($p = 0.0009$). Lower mobilisation of neutrophils from the bone marrow was indicated by reduced G-CSF in all treatment groups compared with placebo. Improved tissue remodelling was indicated by the highest level of tissue inhibitor of metalloproteases and low metalloprotease level in the double-treated group.

Conclusion: AMC-109 showed adjunctive antipseudomonal abilities augmenting the antimicrobial effect of ciprofloxacin in this wound model. The study indicates a potential role for AMC-109 in treating chronic wounds with complicating biofilm infections.

1. Introduction

Chronic wounds, defined as wounds not healed within 3 months, are a substantial burden for individual patients and are negatively associated with physical, social and psychiatric functions [1]. In recent years, biofilm formation deep in non-healing wounds has been shown to contribute to wound pathogenesis and has been reported to be present in 78% of non-healing wounds [2].

Antibiotics are often used in an attempt to eradicate chronic biofilm infections, albeit with limited success. The reason for the lack of antibiotic efficacy can be attributed to several mechanisms. Biofilm-growing bacteria develop tolerance to antibiotics and may have a 10–1000-fold increase in the minimum bactericidal concentration (MBC) compared with planktonic bacteria. This can be fur-

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ther complicated by the acquisition of resistance based on mutations [3]. Unfavourable distribution of the antibiotic in the wound bed is often further complicated by the underlying condition of the patient, e.g. diabetes, arteriosclerosis or oedema, and is an additional challenge limiting antimicrobial efficacy [4]. Furthermore, there is a substantial lack of knowledge about the host response to the recalcitrant biofilm in the non-healing wound. All of these complicating factors, combined with the compromised health of the typical chronic wound patient, are the main reasons delaying the establishment of a general and efficient treatment regimen for chronic wounds. Therefore, it is crucial to identify novel treatment modalities that can improve the healing of chronic wounds by enhancing antimicrobial therapies in order to eradicate biofilm infections.

Peptidic components of the innate immune system such as antimicrobial peptides (AMPs) are potent agents with a wide range of activities. AMPs usually have high selectivity towards bacterial cells versus human cells, fast bacterial killing kinetics comparable with or often superior to conventional antibiotics, and exhibit efficiency in low doses [5]. AMPs may have different modes of action depending on the pathogen. They may upregulate transmembrane pores, interfere with bacterial cell wall, DNA, RNA or protein synthesis [6,7], produce proteolytic enzymes, and have antimetabolic functions. In addition, some AMPs function as immune regulators by stimulating neutrophil chemotaxis and modifying gene expression in the host [8].

One synthetic cationic amphiphilic peptide inspired by lactoferricin is AMC-109, previously known as LTX-109 (Amicoat AS, Tromsø, Norway). AMC-109 is currently being evaluated as an AMP for use as an adjuvant in medical devices. AMC-109 binds to the negatively charged bacterial membrane, with subsequent membrane disruption and cell lysis. No cross-resistance with other drugs has been detected and the development of resistance is rare [9,10]. Unlike most naturally occurring AMPs, the synthetic peptide is stable against protease degradation. AMC-109 has been tested in different in vivo and in vitro experiments against a wide range of pathogens, including Gram-negative *Pseudomonas aeruginosa*, which is a key pathogen in chronic wounds [11]. The antimicrobial activity of AMC-109 against *P. aeruginosa* has been investigated in vitro by Amicoat, where the minimum inhibitory concentration (MIC) ranged from 8–16 μg/mL [12]. AMC-109 also has efficient biofilm-eradicating properties against staphylococci [13,14].

So far, no safety issues regarding AMC-109 have been reported in clinical trials. In a trial of methicillin-resistant *Staphylococcus aureus* (MRSA) decolonisation, systemic exposure was low with a maximum drug concentration in plasma (C_{max}) of 3.72–11.7 ng/mL at 1–2 h post-dosing. The peptide displayed low bioavailability when applied in the nostrils when formulated in a hydrogel [14]. One reported side effect, especially important for a potential wound treatment, was minimal reversible epithelial lesions in the nasal cavity during an MRSA decolonisation trial [14].

The aim of the present study was investigate the adjunctive effect of AMC-109 in combination with ciprofloxacin on recalcitrant *P. aeruginosa* biofilm infected wounds using an established murine chronic wound model.

2. Materials and methods

2.1. In vivo study design

Twelve-week-old, pathogen-free, female BALB/c mice ($n = 33$) (Janvier Labs, Le Genest-Saint-Isle, France) were acclimatised in the animal facility at the Biotech Research & Innovation Centre of the University of Copenhagen (Copenhagen, Denmark) 1 week prior to the start of the experiment. Mice were clinically scored daily by accredited personnel and humane endpoints were set prior to the start of the experiment [15].

On experiment Day 0, the sacral area was shaved with an electric razor and a full-thickness sacral burn wound (1.7 × 1.7 cm) was inflicted on all mice by hot air (EasyHeat 500; Robert Bosch GmbH, Gerlingen, Germany) for 5seconds, after subcutaneously (s.c.) administering 0.1 mL/10 g of Hypnorm/midazolam (mixture of 1 mL fentanyl 0.315 mg/mL + fluanisone 10 mg/mL, 2 mL sterile water and 1 mL midazolam 5mg/mL) per mouse [16]. To prevent dehydration and hypothermia of the mice, liquid therapy was applied (s.c.) in the neck region (NaCl 0.9%, 200 μL) and animals were placed on an 35°C heating mat during the 24-h post-surgery recovery period. During this period, standard post-procedure analgesia to prevent nociceptor activity in the area bordering up to the third-degree burn was initiated with s.c. injections of buprenorphine (100 μL 30 μg/mL) every sixth hour. This borderline zone has previously histopathologically been determined to be <100 μm wide [17], making the analgesia a precautionary measure. Mice were kept in individually ventilated cages to avoid potential gnawing of wounds. They had free access to chow, water and cage enrichment materials such as toys and nest-building materials.

On Day 4 post thermal infliction, 100 μL of a 10^7 CFU/mL solution of *P. aeruginosa* (strain PAO1, Iglewski) was injected under the eschar to produce a monobacterial biofilm infection. Prior to the injection, PAO1 was propagated overnight in Luria–Bertani medium at 37°C. The overnight culture was centrifuged, re-suspended and mixed in a 1% alginate solution. The solution was then transferred to a syringe attached to a pump (model 3100; Graseby, London, UK) and was pumped out through an encapsulation nozzle (Var J30; Nisco Engineering, Zurich, Switzerland) into a $CaCl_2$ and Tris–HCl buffer (pH 7.0). Beads were filtered and a washing procedure was performed twice in 0.9% NaCl with added $CaCl_2$ to avoid bursting of the beads. To determine CFU, beads were dissolved in 0.1 M citric acid buffer (pH 5.0) and were plated to reach a bacterial concentration 1.0 × 10^7 for the sub-eschar injections the following day. Mice were observed and clinically scored on a regular basis. No casualties due to the burn or analgesia procedure were observed. The 33 mice were allocated randomly into the following groups after the a priori sample size was calculated using the Bland–Altman nomogram: (I) 100 μL of AMC-109 (1% hydrogel formulation) injected sub-eschar and 500 μL of saline s.c. ($n = 9$); (II) 100 μL of AMC-109 (1% hydrogel formulation) sub-eschar and 500 μL of ciprofloxacin (2 mg/mL s.c.) (Fresenius Kabi, Copenhagen, Denmark; MIC = 0.75–1.00 μg/mL) ($n = 10$); (III) 500 μL of ciprofloxacin (2 mg/mL s.c.) and 100 μL of saline sub-eschar ($n = 8$); and (IV) placebo, comprising 100 μL of saline sub-eschar and 500 μL of saline s.c. ($n = 6$).

All injections of ciprofloxacin (or respective placebo) were administered s.c. in the abdomen once daily, with the first dosing 24 h after establishment of the *P. aeruginosa* infection.

We aimed to investigate whether two different compounds improve the effect when administered simultaneously. The specific dosages both of ciprofloxacin and AMC-109 were based on initial studies testing different dosages of the compounds in our specific model to ensure a certain, although not complete, bacterial killing. Working with antibiotic dosages below the MIC when correlating the efficiency of antibiotics in experimental research and the clinic is an acknowledged approach. At the end of the dosing interval, plasma concentrations drop significantly below the MIC in the patient, which is mimicked by sub-MIC dosing in the experimental setup. In addition, some patients have higher clearance rates due to altered clearance function, and different penetration rates of affected tissue are also seen. Suboptimal penetration is also seen in biofilm, unrelated to tissue type [18].

AMC-109 was administered in a hydrogel formulation composed of AMC-109 (1%), glycerol (10%), propylene glycol (20%), ben-

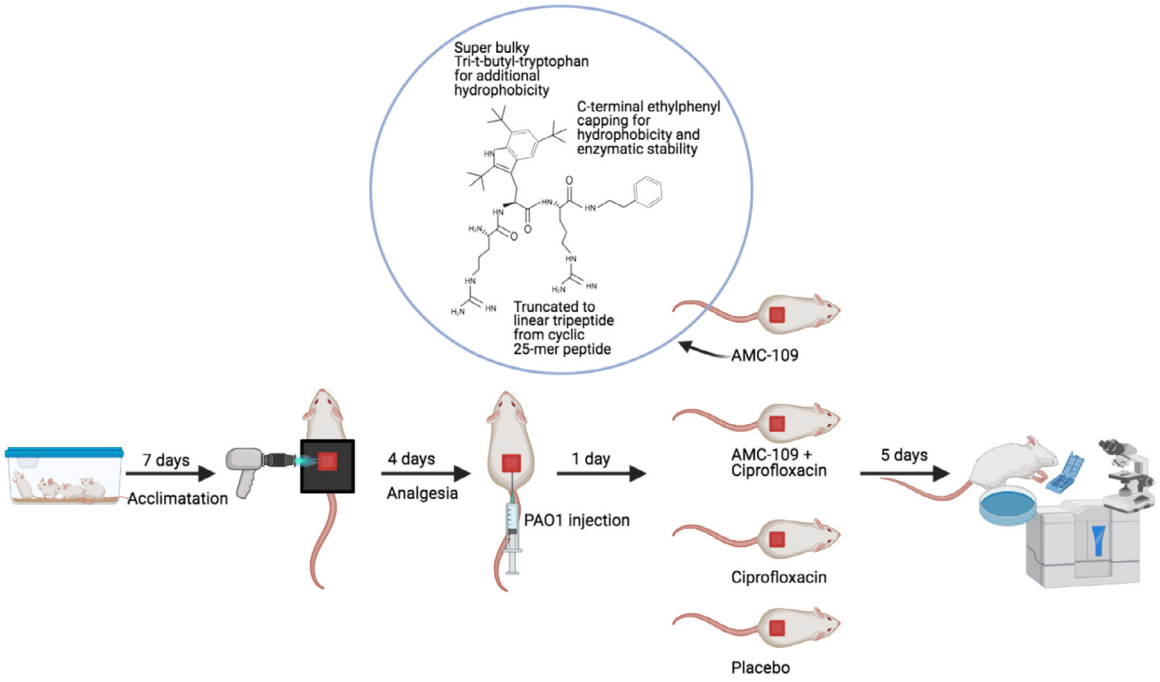

Fig. 1. Experimental setup. Twelve-week-old BALB/c mice (n = 33) were inflicted with a full-thickness sacral burn wound under analgesia/anaesthesia and were infected sub-eschar with *Pseudomonas aeruginosa* embedded in alginate to mimic a biofilm. Mice were randomised into four groups as follows: (I) 100 μL of AMC-109 (1% hydrogel formulation) injected sub-eschar and 500 μL of saline s.c. (n = 9); (II) 100 μL of AMC-109 (1% hydrogel formulation) sub-eschar and 500 μL of ciprofloxacin (2 mg/mL s.c.) (n = 10); (III) 500 μL of ciprofloxacin (2 mg/mL s.c.) and 100 μL of saline sub-eschar (n = 8); and (IV) placebo, comprising 100 μL of saline sub-eschar and 500 μL of saline s.c. (n = 6). Mice were treated until sacrifice on Day 10 after wound establishment. Wounds were photographed for size evaluation. Wounds were collected and homogenised for quantitative bacteriology and host factor measurements by means of Luminex®. (s.c., subcutaneous.)

zyl alcohol (2%) and hydroxyethyl cellulose (2%). The pH of the hydrogel was adjusted to pH=5 by addition of sodium hydroxide. Two AMC-109 treatments were administered daily (starting at the same time as ciprofloxacin) for 5 days.

On the last day, all mice were sacrificed and wounds were collected (Day 10 after burn procedure) (Fig. 1). No adverse events occurred during the course of the experiment.

2.2. In vitro study design

To evaluate a possible in vitro synergism between the AMP and ciprofloxacin, two checkerboard assays were constructed, one investigating the effect of AMC-109 and ciprofloxacin on planktonic *P. aeruginosa* and one on biofilm-mimicking bacteria embedded in alginate.

A 96-well microtitre plate was inoculated with 100 μL of planktonic PAO1 (10^7 CFU/mL). Luria–Bertani medium with increasing concentrations of AMC-109 (0 to 40 μg/mL) and ciprofloxacin (0 to 4 μg/mL) were added to the wells. Each well held a unique concentration combination of the two compounds.

The same setup was designed for the PAO1 embedded in alginate. Plates were inoculated with 100 μL of PAO1 (10^7 CFU/mL) in beads and the concentrations of AMC-109 were increased to 160 μg/mL. The optical density was measured once every hour for 8 h (with incubation in between) and plates were then incubated overnight. The following day, the optical density was read again and the wells were plated to determine CFU. Fractional inhibitory concentration (FIC) values were determined to evaluate a possible additive or synergistic effect [19]. Both experimental setups were performed in triplicate.

2.3. Digital photoplanimetry

Wounds were photographed post-mortem for evaluation of macroscopic wound healing. Digital image analysis was performed by two independent researchers using the program ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.4. Wound collection

Wounds were collected post-mortem in individual tubes containing 1.0 mL of saline using sterile surgical instruments. Wounds were homogenised for 20 seconds at 14 000 rpm (Heidolph Silent Crusher M; Heidolph Instruments, Schwabach, Germany). After microbiological quantification, samples were centrifuged for 10 min at 5000 rpm and the supernatant was kept at –80°C for cytokine analysis.

2.5. Quantitative bacteriology

Ten-fold serial dilutions of the homogenate were prepared and plated on modified Conradi–Drigalski substrate ('blue plates') (Statens Serum Institut, Copenhagen, Denmark) and incubated overnight. CFU were counted and multiplied by the dilution factor.

2.6. Cytokines and growth factors

Relevant cytokines and growth factors were quantified to determine the wound response using a Luminex® 200™ Platform (Luminex Corp., Austin, TX, USA) with customised multiplex assays (Bio-Techne R&D Systems, Minneapolis, MN, USA).

2.7. Ethics

The in vivo experiment was approved by the Danish Animal Experiments Inspectorate. The experiment complied with the ARRIVE guidelines and was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

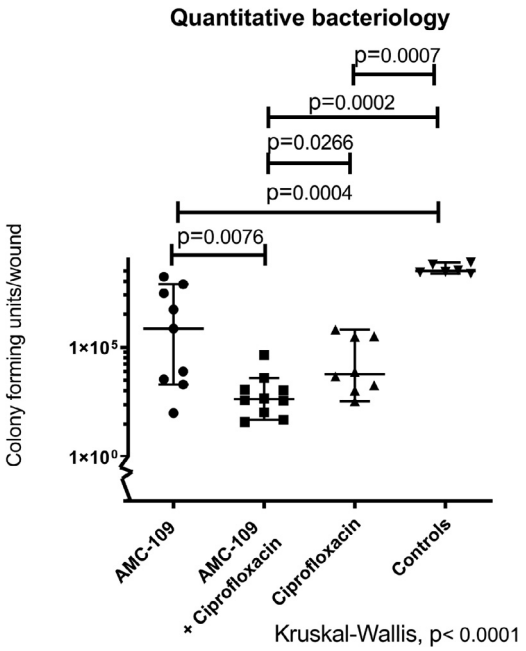

Fig. 2. Quantification of colony-forming units (CFU). Female BALB/c mice were inflicted with a burn wound and 4 days post-infliction were infected sub-eschar with 10^6 CFU of *Pseudomonas aeruginosa*. Treatment with AMC-109 (sub-eschar), ciprofloxacin (subcutaneous) or both was applied twice daily for 5 days. Animals were sacrificed (at Day 10 post burn), wounds were removed and homogenised, and quantitative bacteriology was performed. The lowest bacterial count was in the double-treated group [4.30×10^2 ($50.00–4.00 \times 10^3$)], followed by the ciprofloxacin monotherapy group [6.07×10^3 ($3.50 \times 10^2–6.35 \times 10^5$)] and the AMC-109 monotherapy group [7.10×10^5 ($2.00 \times 10^3–7.55 \times 10^7$)]. The highest CFU was detected in the placebo group [3.05×10^8 ($2.3 \times 10^8–7.40 \times 10^8$)]. In the dichotomous analyses, statistically significant differences were identified between the double-treated group and the two monotherapy groups. All CFU levels from the three treatment groups were, when individually analysed, different from the level of the placebo group. Displayed are the CFU of individual mice and the median and 95% confidence interval of the group.

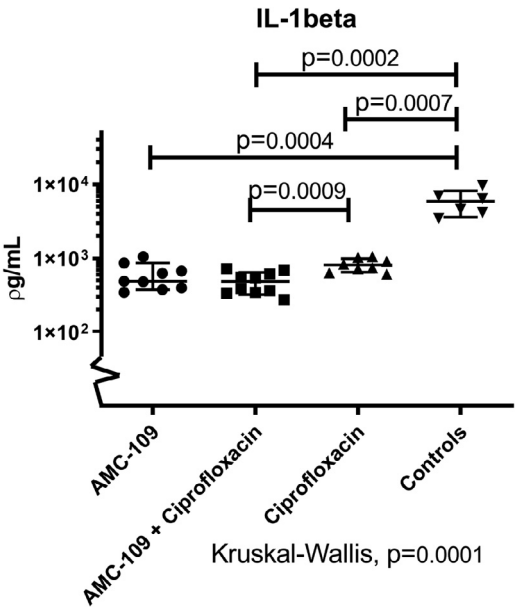

Fig. 3. Levels of the pro-inflammatory cytokine interleukin-1 beta (IL-1β) in wound supernatants from the four groups analysed using a Luminex®. Levels of IL-1β were significantly lower in mice receiving different combinations of treatment [from left on *x*-axis, 4.87×10^2 ($3.75 \times 10^2–8.59 \times 10^2$), 4.65×10^2 ($3.34 \times 10^2–6.86 \times 10^2$) and 7.85×10^2 ($6.09 \times 10^2–1.05 \times 10^3$)] compared with the level in the placebo group [5.50×10^3 ($3.66 \times 10^3–9.65 \times 10^3$)]. In a dichotomous analysis, the IL-1β proinflammatory response was further dampened in the double-treated group compared with the ciprofloxacin monotherapy group. Displayed are the individual measured cytokine concentrations in pg/mL for every mouse as well as median and 95% confidence interval for the group.

2.8. Statistical analysis

Non-parametric statistical methods were employed, including the Kruskal–Wallis by ranks for multiple comparisons. If a significant difference was detected, further Mann–Whitney tests where used to compare groups dichotomously. A *P*-value of ≤ 0.05 was considered statistically significant.

Statistical analyses of cytokine and growth factor levels were performed on the observed concentration directly from the Luminex® platform. When measurements were just outside the standard curve, the automated extrapolated values from the Luminex® software were employed. Wound tissue densities were calculated as CFU/wound. All values are reported as the median with 95% confidence interval (CI).

Statistical analyses were performed using Microsoft Excel v.15.41 (Microsoft Corp., Redmond, WA, USA) and GraphPad Prism v.7.02 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Quantitative bacteriology of Pseudomonas aeruginosa-infected wounds

A significantly lower bacterial count was seen in the group receiving the combination of the antimicrobial peptide AMC-109 and ciprofloxacin compared with the other groups (Fig. 2), with a log reduction in CFU of 1.15 from the ciprofloxacin mono-treated group, 3.22 from the AMC-109 group and 5.58 from the placebo group to the dual-treated group.

Statistically significant differences were also found between the two mono-treated groups (AMC-109 or ciprofloxacin) and the double-treated group. A pronounced dichotomous distribution of CFU was seen in the group receiving AMC-109 monotherapy that was not detected in any of the other groups. Lastly, all three treatment groups had significantly lower bacterial levels compared with the biofilm control group.

3.2. Evaluation of the innate host response

The interleukin-1 beta (IL-1β) level was significantly lower in all of the treatment groups compared with the placebo (control) group (Fig. 3). The lowest level of IL-1β was detected in the combined treatment group, with a significant difference from the level in the ciprofloxacin monotherapy group but not from the level in the AMC-109 monotherapy group.

Both the monotherapy and dual therapy groups had lower levels of matrix metalloproteinase 8 (MMP8) and granulocyte-colony stimulating factor (G-CSF) compared with the control group (Figs 4 and 5). MMP8 is an indicator of wound proteolysis, and G-CSF an indicator of mobilisation of polymorphonuclear neutrophils (PMNs) from the bone marrow. No differences between the treatment groups were detected.

Tissue inhibitor matrix metalloproteinase 1 (TIMP-1) levels, an indicator of anti-proteolytic wound activity, was estimated in the wound samples (Fig. 6). TIMP-1 levels in the AMC-109 monotherapy and the double-treated group were similar and were higher than the levels in the ciprofloxacin and placebo groups. When groups were compared dichotomously, a significantly higher level of TIMP-1 was seen in the double-treated group compared with the mono-ciprofloxacin group. No difference was seen between the ciprofloxacin monotherapy and control groups.

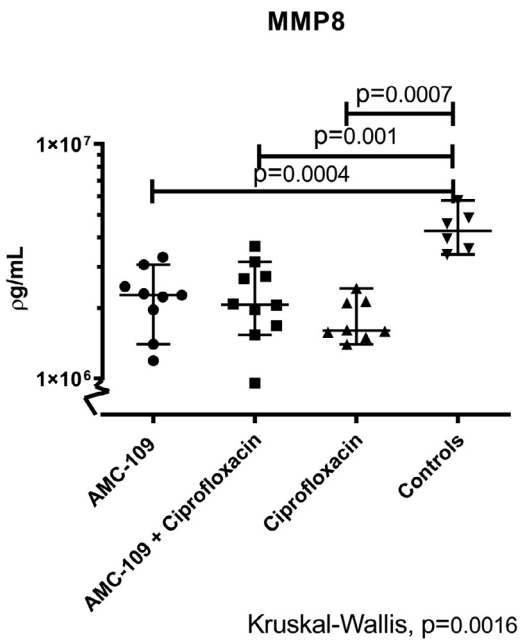

Fig. 4. Levels of metalloproteinase 8 (MMP8), an endopeptidase responsible for degradation of the extracellular matrix in wound supernatants from the four groups analysed using a Luminex®. Levels of MMP8 were lower in the treatment groups [from left on *x*-axis, 2.27×10^6 (1.40×10^6–3.06×10^6), 2.07×10^6 (1.54×10^6–3.15×10^6) and 1.60×10^6 (1.40×10^6–2.43×10^6)] compared with the level in the placebo group [4.26×10^6 (3.38×10^6–5.76×10^6)]. No further differences were seen in the non-parametric unpaired Mann–Whitney analyses. Displayed are the individual measured cytokine concentrations in pg/mL for every mouse as well as the median and 95% confidence interval.

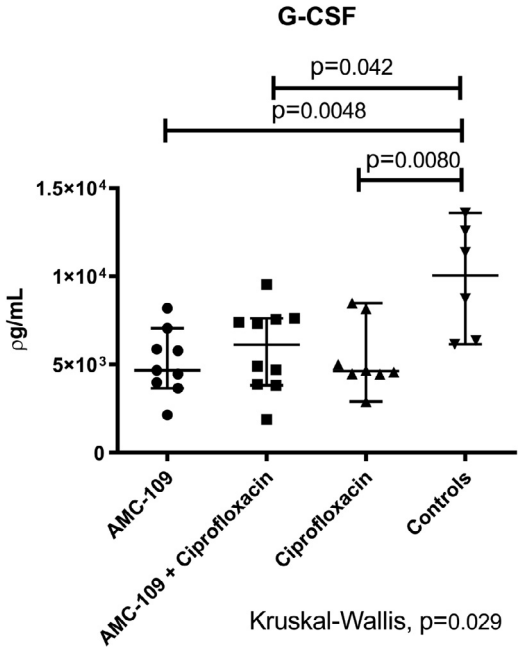

Fig. 5. Levels of granulocyte-colony stimulating factor (G-CSF), a multifaceted glycoprotein involved in differentiation of haematopoietic progenitor cells, collagen deposition, neovascularisation and re-epithelisation in wound supernatants from the four groups analysed using a Luminex®. All groups had different levels of G-CSF in the Kruskal–Wallis multivariate analysis. Levels were lower in the three groups of mice receiving different treatment combinations [from left on *x*-axis, 4.67×10^3 (3.66×10^3–7.06×10^3), 6.12×10^3 (3.81×10^3–7.65×10^3) and 4.62×10^3 (2.91×10^3–8.48×10^3)] compared with mice receiving placebo [1.00×10^4 (6.15×10^3–1.36×10^4)]. No further significant differences were revealed in the dichotomous analyses. Displayed are the individual measured cytokine concentrations in pg/mL for every mouse as well as the median and 95% confidence interval for the group.

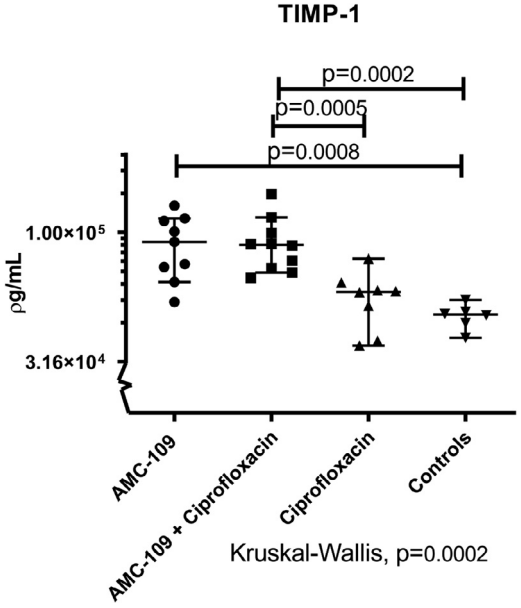

Fig. 6. Levels of tissue inhibitor matrix metalloproteinase 1 (TIMP-1), a glycoprotein representing anti-proteolytic wound activity in the wound supernatants from the four treatment groups analysed using a Luminex®. Levels of TIMP-1 were different in the groups when analysed in the multivariate analysis [from left on *x*-axis, 9.18×10^4 (6.43×10^4–1.13×10^5), 8.94×10^4 (6.99×10^4–1.14×10^5), 5.89×10^4 (3.65×10^4–7.91×10^4 and 4.81×10^4 (3.91–10^4–5.48×10^4)]. A significant difference between the TIMP-1 level of mice receiving double therapy and mice receiving only ciprofloxacin was also seen when analysed dichotomously. Displayed are the individual measured cytokine concentrations in pg/mL for every mouse as well as the median and 95% confidence interval for the groups.

Osteopontin (OPN) was included as a marker of neovascularisation. Quantification of OPN levels showed the lowest level in the control group and the highest level in the double-treated group (Fig. 7). No significant differences between the treatment groups were detected.

The levels of CXCL1/KC (C-X-C motif chemokine ligand 1/keratinocytes-derived chemokine), a potent neutrophil chemoattractant, and RANTES/CCL5 (regulated on activation, normal T-cell expressed and secreted/chemokine ligand 5), representing an adaptive immune chemokine, did not differ in the four groups (data not shown).

3.3. Digital photoplanimetry

A burn wound is shown in Fig. 8. No differences in wound sizes between the four groups were observed at the time of sacrifice (statistical analyses are not shown).

3.4. In vitro quantitative bacteriology

The checkerboard analysis did not reveal any synergism or additive effect when AMC-109 was combined with ciprofloxacin in vitro. Plates inoculated with planktonic bacteria revealed MICs for both compounds (0.125 μg/mL for ciprofloxacin and 40 μg/mL for AMC-109), but the FIC values on all three plates showed indifference meaning that no synergism or antagonism was detected between the two compounds.

Plates inoculated with *P. aeruginosa* embedded in beads showed similar MICs to the planktonic analysis for ciprofloxacin, but AMC-109 did not show inhibition of bacterial growth when the bacteria were embedded in alginate beads.

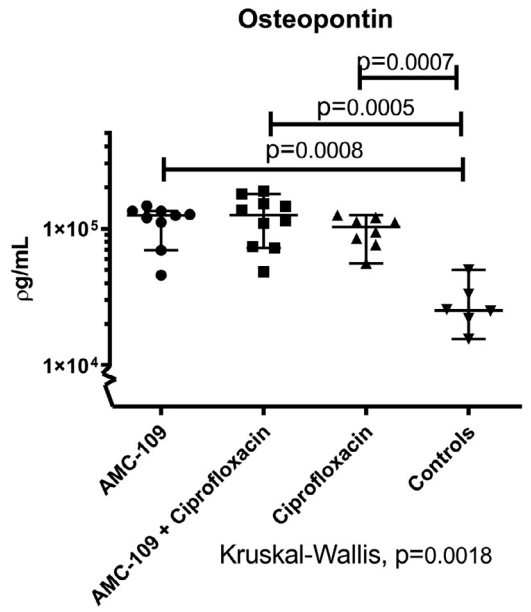

Fig. 7. Levels of osteopontin, a glycoprotein with multiple suggested physiological functions, in the wound supernatants from the four treatment groups analysed using a Luminex®. All groups had different levels when compared in a multivariate analysis [from left on *x*-axis, 1.25×10^5 (6.95×10^4–1.34×10^5), 1.25×10^5 (7.23×10^4–1.00×10^5), 1.203×10^5 (5.58×10^4–1.25×10^5) and 2.52×10^4 (1.56×10^4–4.99×10^4)]. No significant differences between the treatment groups were detected. Displayed are the individual measured cytokine concentrations in pg/mL for every mouse as well as the median and 95% confidence interval for the groups

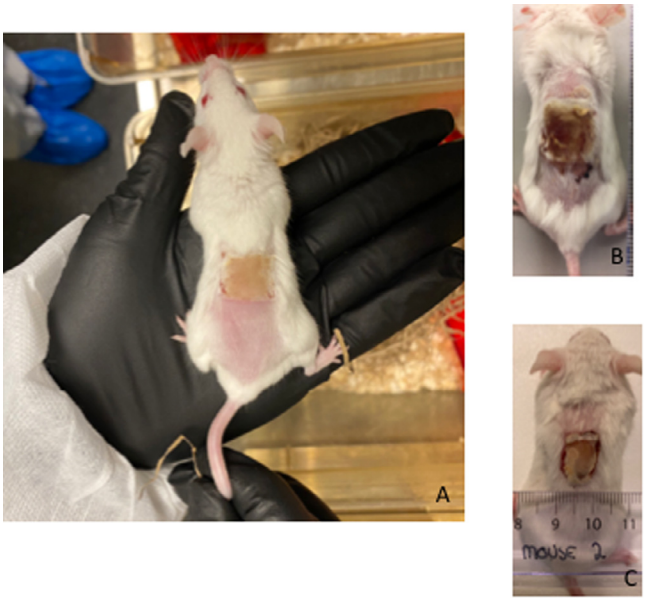

Fig. 8. (A) Visual appearance of the burn wounds 1 day post thermal infliction. All wounds are similar at this point. After biofilm settlement and initiation of the different treatment combinations the wounds develop differently. Mice in (B) and (C) are both photographed on sacrifice day, where wound B appears to have expanded with a likely loss of infection control and wound C has contracted visually. No statistical differences based on wound size were detected between the groups.

4. Discussion

Complications of chronic wounds owing to biofilm infections have attracted substantial and increasing interest during the latest decades. Although research in this area has resulted in an improved understanding of the mechanisms of pathophysiology and hence could provide novel treatment options, the challenges of non-healing wounds remain sizeable. Due to the significant heterogeneity of patients with chronic wounds, animal experiments in representative models are a substantial source for improving knowledge and identification of treatment candidates for this important clinical problem.

Bacteria assembled in biofilm formation are characterised by increased tolerance to antimicrobial agents and the immune defences of the host. This tolerance is based on decreased metabolic activity thought to be caused by decreased access to nutrients and oxygen as well as increased mutation frequencies within the biofilms. Most biofilm experiments investigate these two issues. Our setup focuses on the efficiency and possible synergy between two compounds, one with known beneficial effects and one new immune player, AMC-109.

The present experimental setup was designed after a previous study performed with 30 mice. In this first study, mice were inflicted with similar *P. aeruginosa* biofilm wounds and were divided in two groups, one group treated with saline as placebo and the second group treated with 200 μL of AMC-109 (1% hydrogel formulation). One-half of each group were terminated at day 1 after infection (day 5 after burn) and the remaining on day 3 (day 7 after burn). Bacterial loads in the wounds were significantly reduced in the group receiving AMC-109 compared with the saline placebo group on day 3 ($p < 0.05$). The present study was designed to further analyse the antimicrobial effect of AMC-109 and a possible adjunctive effect to ciprofloxacin.

We found that topical AMC-109 injected under the eschar as an adjuvant to ciprofloxacin lowered the bacterial load. Dual antibiotic therapy for multidrug-resistant infections has long been considered superior to monotherapy if combined to eradicate the mutant selection window of both compounds [20]. Choosing an advantageous synergistic combination has increased the interest of various AMPs. The advantage that AMPs have over antibiotics is that they have multiple targets and rarely a specific receptor [21]. The most prominent feature of AMPs is their net positive charge that acts on the negatively charged bacterial cell wall and plasma membrane components and, by disrupting this, increases the antibiotic bioavailability of the co-administered drug [22]. Several studies have already been published on this beneficial synergistic combination to eliminate various multidrug-resistant gram-negative bacteria, MRSA [23,24] and even different bacteria congregated in biofilms [25].

Adequate penetration of ciprofloxacin and AMC-109 into the wound base and the maintenance of sufficient serum levels is essential for wound healing in the clinical setting. Biofilms are impermeable by nature and thus result in limited drug availability at the site of infection. Despite this, topically administrated ciprofloxacin has been proven efficient for bacterial elimination [26]. Topical ciprofloxacin is therefore preferable to avoid adverse systemic side effects in the clinical setting. In our experimental setting, we chose to work with subcutaneously administered ciprofloxacin to ensure absorption of the exact dosage in all animals. AMC-109 administered intraperitoneally in mice has a decreased effect in the target tissue because of absorption by the liver. A first pass effect was also seen when the compound was administered intravenously in a rat whole-body autoradiography study [27].

Pseudomonas aeruginosa biofilm in wounds stimulates the host to increase the defence against bacterial virulence factors. Blocking of IL-1β or local treatment with the receptor antagonist have previously been associated with improved wound healing [28]. We found the level of IL-1β to be the lowest in the group treated with a combination of ciprofloxacin and AMC-109, which was significantly lower than in the ciprofloxacin monotherapy and placebo groups. This could indicate that the combination of AMC-109 and

ciprofloxacin promotes a condition in the wounds with reduced inflammatory cellular infiltration and reduced fibrosis [29]. IL-1β also stimulates the transcription and release of the phosphorylated glycoprotein OPN [30]. The matricellular protein OPN has a multifaceted role, which includes control of the immune response at different levels. The protein is classified as pro-inflammatory but also has significant anti-inflammatory action. It has abilities for inducing neutrophilic endothelial adhesion and chemotaxis as well as stimulation of stem cells and participation in apoptosis regulation [31]. OPN modulates the dendritic cell response and increases MMP expression. OPN is also known to have neovascularisation and tissue remodelling/matrix reorganisation potential [32]. Our results, with higher levels of OPN in the treated groups with presumed improved healing versus the non-treated controls with more inhibited healing, is in contrast to the observation that OPN knockout mice show accelerated healing [33]. However, previous studies on OPN and wound healing are based on uninfected mice sacrificed on different days up until 14 days post wound infliction [31,33]. Our BALB/c mice were infected after 4 days and were sacrificed on Day 10 after the burn procedure, resulting in two separate insults activating the immune apparatus, which is also the scenario in the majority of clinical chronic wound cases. These different experimental settings could explain the possible discrepancies in OPN levels, with the lowest OPN levels in the non-treated group with the highest bacteriology. We believe our results illustrate a second OPN boost, underlining the multifaceted role of OPN with a switch in the angiogenic balance towards neovascularisation and thus a move towards the proliferating healing phase. This supports the anti-inflammatory abilities of the protein, but needs to be investigated further.

Overexpression and activation of MMP8 and reduced levels of the inhibitor TIMP-1 in the chronic wound edge (zone with migrating keratinocytes) and in the wound fluid are key participants in the pathogenesis of non-healing chronic wounds. Reactive oxygen species (ROS) and MMPs are released when polymorphonuclear leukocytes (PMNs) respond to the pathogen to disrupt and degrade the biofilm. However, as the biofilm perseveres and PMN chemotaxis is pathologically prolonged, collateral damage to the surrounding healthy tissue follows. The PMNs continually release MMPs, prolonging the inflammatory phase of the wound as well as providing the biofilm with nutrients from the lysed immune cells. The MMP/TIMP interaction and the metalloproteinase activation cascade ratios are highly regulated systems, but when the biofilm persists a normal function of MMP with an initial proteolysis essential for keratinocyte migration and re-epithelialisation turns into tissue destruction and biofilm persistence [34].

TIMP-1 and other MMP modulators have previously been suggested as promising topical agents [35,36]. From our results on this dynamic pivotal relationship, it can be interpreted that the excess protease activity from MMP8 can be kept at bay by combining the antimicrobial peptide AMC-109 with ciprofloxacin therapy. This is underlined by the observed increase in the level of the glycoprotein TIMP-1 in the double-treated group.

G-CSF levels were reduced in all treatment groups, whereas it was still high in the control group. These findings support the theory that AMC-109 and ciprofloxacin treatments have resulted in progression to the proliferative phase in the wound healing cascade including a decline in leukocyte migration.

In the present study, the action of AMC-109 alone was able to significantly reduce bacterial CFU compared with the placebo-treated group; furthermore, our results suggest an immunomodulatory role of the peptide. The compound alone was able to reduce the level of IL-1β to similar levels of the double-treated group and also showed an increased presence of TIMP-1 comparable with the double-treated group. The group receiving both AMC-109 and ciprofloxacin differed in TIMP-1 and IL-1β levels accordingly compared with the ciprofloxacin monotherapy group, but not compared with the AMC-109 monotherapy group. This observation strongly indicates an additional immunomodulatory role of AMC-109 and will have to be explored further in the future, including for wounds of a non-infectious aetiology or in chronic wounds where the biofilm infection is under control. These findings correlate with our in vitro findings where a synergistic or additive effect between ciprofloxacin and AMC-109 was not identified when the host response was not present in either planktonic or biofilm bacteria. The fact that the MIC was not determinable for the peptide in the in vitro alginate setup further suggests a boosted host response to eradicate these settled infections in addition to the current antibiotic regimen. However, the absence of synergism in vitro may not be that surprising since the lytic effect of AMC-109 at concentrations above the MIC is rapid and may outcompete the ciprofloxacin effect, whereas lower concentrations closer to the MIC have a non-lytic effect similar to the related peptide Bac8c [37]. It should be noted that the effect profile of AMPs close to the MIC is quite different (non-lytic) from the effect at 4–8 $\times$ MIC, where a direct membranolytic effect is observed. The alginate beads used to mimic the *P. aeruginosa* biofilms in vitro cause binding of free AMC-109 to the polyanionic polymer, resulting in a lower antimicrobial concentration, which possibly explains the lack of in vitro effect [13].

There is a general concern about the development of AMP resistance if used as a clinical drug. Several defence mechanisms by different pathogens have been described, including a change in net surface charge [38], capsule polysaccharides [39] or changed fluidity of the outer membrane [40], introduction of efflux pumps, or influx with AMP degradation in the cytoplasm [41]. Multiple resistance mechanisms have been described specifically in *Pseudomonas* spp. [42]. Combining stable AMPs with conventional antibiotic drugs may prevent the development of resistance to both drugs owing to improved bacterial killing and the fact that it is challenging to develop two different resistance mechanisms simultaneously. Our results support this and, for the biofilm relevance of our chronic wound model, we have shown aggregated bacteria inside the wounds completely resembling similar observations in humans. We also observed an accumulation of inflammation in relation to wound biofilms and that the inflammatory response was arrested in a polymorphonuclear neutrophils (PMN)-dominated state compared with matrix (alginate) without bacteria [43]. We previously looked into the synergy between another compound derived from nature's own defence, the PMN heterodimer S100A8/A9, and ciprofloxacin in a similar setup [44]. As with AMC-109 combined with ciprofloxacin, this experiment showed reduced pathogenic bacterial burden but also a reduced resistance development in infected wounds when both compounds were administered [45].

Another shortcoming of natural AMPs is their large size (usually 10–50 amino acids), resulting in pharmacokinetic concerns such as poor bioavailability, short half-lives and low metabolic stability. This shortcoming has to a large degree been circumvented by the development of short, synthetic variants of AMPs that are sufficiently stable to be used in a clinical situation and additionally retain their antimicrobial activity, acceptable bioavailability and low toxicity [9].

The beneficial effect of AMC-109 on quantitative bacteriology and changes in host factors indicate improved healing. However, such improved healing of the wounds was not observed. This can be explained by the different predominant contraction healing mechanism of rodents and the relatively short observation period. Finally, we used a suboptimal dose of ciprofloxacin as well as AMC-109. This was done intentionally to reveal any potential adjunctive effect of AMC-109 to ciprofloxacin. The dichotomous distribution of CFU in the AMC-109 monotherapy group is a valid visual indicator that this suboptimal dosage was achieved since some animals

showed good effect of the compound, whereas some appeared to not clear the infection.

5. Conclusion

Dual treatment with the synthetic AMP AMC-109 and the antibiotic ciprofloxacin showed a promising antipseudomonal biofilm-eradicating effect in chronic wounds. In addition, a direct anti-inflammatory effect independent of the antibacterial effect was observed. Based on our observations, dual treatment induced increased bacterial killing and reduced inflammation with beneficial immunological alterations in wounds especially compared with the ciprofloxacin monotherapy and placebo groups. Further focus is needed on these synthetic AMPs and their potential in wound care.

Data availability

Raw data are available by contacting the corresponding author.

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Competing interests

JSMS and JPC are employed by Amicoat AS. All other authors declare no competing interests.

Ethical approval

The in vivo experiment was approved by the Danish Animal Experiments Inspectorate [approval no. 2015-15-0201-00618]. The experiment complied with the ARRIVE guidelines and was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

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