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Efficacy of a synthetic antimicrobial peptidomimetic versus vancomycin in a *Staphylococcus epidermidis* device-related murine peritonitis model

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Objectives: Biofilm-forming *Staphylococcus epidermidis* is a prevalent cause of peritonitis during peritoneal dialysis. We compared the efficacy of a synthetic antimicrobial peptidomimetic (Ltx21) versus vancomycin in a murine model mimicking a device-related peritonitis.

Methods: Silicone implants, pre-colonized with an *S. epidermidis* biofilm, were inserted into the peritoneal cavity of BALB/c mice. Three groups (36 mice in each) with pre-colonized implants received intraperitoneal treatment with Ltx21, vancomycin or placebo. Mice were euthanized on day 3 ($n=12$), day 6 ($n=12$) or day 8 ($n=12$) post-implantation. Controls were mice with sterile implants ($n=18$) and mice without surgery ($n=6$). Bacterial reductions in cfu were analysed from implants and peritoneal fluid (PF). Inflammatory responses in serum and PF were measured.

Results: Vancomycin resulted in a stronger reduction in cfu counts, both on pre-colonized implants and in PF, compared with Ltx21 and placebo. Complete bacterial clearance of the implants was not achieved in any of the groups. The implants pre-colonized with *S. epidermidis* 1457 resulted in a low-grade peritonitis. We observed, only on day 6, a significant increase in the PF leucocyte count in the group with pre-colonized implants compared with the group with sterile implants ($P=0.0364$).

Conclusions: Treatment with vancomycin or Ltx21 was not sufficient to achieve complete bacterial clearance of implants, underlining the difficulties of treating such infections. The low-grade infection may attenuate the inflammatory response and contribute to impaired bacterial clearance.

Keywords: biofilms, device-related peritonitis, mouse model

Introduction

Gram-positive bacteria, in particular coagulase-negative staphylococci, are a prevalent cause of peritoneal dialysis catheter-related peritonitis.[1] This is a serious complication that may lead to catheter removal, peritoneal membrane dysfunction and transfer to haemodialysis.[2] Peritonitis is believed to occur by bacterial entrance to the peritoneal cavity through catheter colonization.[2] Staphylococcal biofilm formation on catheters results in a reduced ability to combat such infections, both by the host immune system and by conventional treatment with antibiotics.[3,4] Thus these infections are difficult to treat and are often caused by bacteria resistant to conventional antimicrobial agents. The search for new therapeutic agents is therefore crucial.

Synthetic antimicrobial peptidomimetics (SAMPs) are novel antimicrobial agents derived from cationic antimicrobial peptides that are widespread in nature.[5] Their modes of action are not completely resolved. However, a central mechanism is bacterial membrane disruption, affecting both dormant and dividing bacteria.[6] A previous *in vitro* study with SAMPs showed a superior killing of biofilm-embedded bacteria compared with vancomycin.[7]

This study aimed to investigate the efficacy of a SAMP (Ltx21) versus vancomycin in a murine model mimicking device-related *Staphylococcus epidermidis* biofilm-associated peritonitis. We assessed bacterial clearance and the host innate immune response to understand the pathophysiological mechanisms involved.

Methods

Bacterial isolates and MICs

S. epidermidis 1457, used in this experiment, was originally isolated from a central venous catheter infection. *S. epidermidis* 1457 forms a thick biofilm under the *in vitro* growth conditions used in this study.[8] The MIC of vancomycin was 2 mg/L, determined by Etest (AB Biodisk, Sweden). Ltx21 is structurally similar to the previously tested SAMPs Ltx5 and Ltx9.[7] All three SAMPs have the same tripeptide sequence, with two arginine moieties providing their cationic properties and a modified tryptophan providing the lipophilic bulk. The SAMPs differ by C-terminal modifications, of which Ltx21 has an additional phenylalanine attached compared with Ltx9 and Ltx5. For Ltx21 the MIC was 6 mg/L (determined by the microbroth dilution method) and the minimal biofilm inhibitory concentration was 60 mg/L (determined by the Alamar blue method), comparable to those values previously reported for Ltx5 and Ltx9.[7] Species confirmation of small colony variants (SCVs) was performed with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer.

Animals and animal ethics

One hundred and thirty-two female BALB/c mice (Taconic M&B A/S Ry, Denmark), aged 7–8 weeks, were used. Information on the experimental treatment of the animals and the duration of experiments (days) is provided in Table 1. The animal studies were carried out in accordance with the European Convention and Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the Danish law on animal experimentation. All animal experiments were approved by the National Animal Ethics Committee, Denmark.

Device-related *S. epidermidis* biofilm-associated peritonitis model

Silicone implants (5 mm×5 mm×2 mm; Ole Dich, Denmark) were inserted into the murine intraperitoneal cavity in order to mimic a device-related peritonitis. Briefly, the implants were prepared by incubation for 120 h in an *S. epidermidis* 1457 culture. The inoculum was adjusted to an optical density at 600 nm that was equivalent to that of a 2 McFarland standard in 0.9% NaCl and further suspended in tryptic soy broth (TSB) with 1% glucose to induce biofilm formation. Every 24 h the implants were rinsed in PBS and transferred to a sterile flask containing fresh medium (TSB with 1% glucose). Mice were anaethetized by subcutaneous injections (0.15 mL) in the groin area with a mixture of 0.0375 mL of 0.315 mg/mL fentanyl/10 mg/mL fluanisone (VetaPharma Ltd, UK) and 0.0375 mL of 5 mg/L midazolam (Hameln Pharmaceuticals, Germany) in 0.075 mL of sterile water. Insertion of implants and intraperitoneal treatment were performed as previously described.[9,10] Vancomycin (Sandoz, Australia) and Ltx21 (Lytix Biopharma AS, Tromsø, Norway) were both dissolved in 0.9% NaCl to a final concentration of 1 mg/mL and 0.5 mg/mL, respectively. All animals received intraperitoneal injections (400 μL) every 24 h for up to 7 days. Treatment was initiated 2 h post-implantation. The vancomycin dose was 20 mg/kg, based on previous studies.[11,12] The Ltx21 dose was 10 mg/kg, based on previous toxicology studies, a pilot treatment study and *in vitro* MIC studies. On the days of implant removal, mice were anaethetized by subcutaneous injection of 0.1 mL of pentobarbital (200 mg/mL) (KVL, Denmark). After general anaesthesia, blood was drawn by cardiac puncture and transferred to tubes containing heparin for fluorescence-activated cell sorting (FACS) ($n=6$ animals) or 50 mg/L lepirudin (Refludan, Hoechst, Germany) for complement analysis ($n=6$ animals). Peritoneal lavage was performed by injecting 5 mL of PBS into the peritoneal cavity, followed by gently massaging the abdomen before withdrawing the peritoneal fluid (PF). Implants were removed from the peritoneal cavity and transferred to tubes containing 1 mL of NaCl and 20 glass beads (Lenz, Laborglasinstrumente, Germany). Mice were euthanized by removal of the heart under general anaesthesia.

Table 1. Treatments

Surgery	Treatment	Number of animals		
		day 3	day 6	day 8
Pre-colonized implant	vancomycin	12[a]	12	12
Pre-colonized implant	Ltx21	12	12	12
Pre-colonized implant	NaCl (placebo)	12	12	12
Sterile implant	Ltx21	3	3	3
Sterile implant	NaCl (placebo)	3	3	3
No surgery	none	2	2	2

Mice with pre-colonized implants ($n=108$) were divided into three treatment groups (Ltx21, vancomycin and placebo). Mice with sterile implants ($n=18$) received Ltx21 or placebo. One group of mice without surgery ($n=6$) was included for baseline data. Samples were analysed on days 3, 6 and 8.

[a]On each experimental day all 12 animals were analysed for cfu count on implants and in PF. Six animals each were analysed for either (i) complement activation or (ii) haematological parameters and chemokines/cytokines.

Bacteriology

Implants removed from the animals were vortexed for 30 s followed by 5 min of sonication at 40 kHz in an ultrasound bath (Bransonic 3510, Branso Ultrasonic Corporation, USA). One hundred microlitres of both the implant-derived suspension and PF was serial diluted and plated on blood agar plates (SSI, Denmark) for bacterial enumeration. The cfu counts were determined after incubation at 37°C overnight. Prolonged incubation was necessary to detect SCVs.

Cytokines, chemokines and complement

Quantification of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 2 (MIP-2) and monocyte chemotactic protein-1 (MCP-1/CCL2) was performed on plasma and/or PF using the Fluorokine MAP system (R&D Systems, UK) in combination with a dual-laser, flow-based sorting and detection analyser (Luminex Corporation, USA), according to the manufacturer's description. Complement factors C3a and C5a from plasma and PF were quantified using ELISA kits (USCN Life Science Inc., Wuhan, China) according to the manufacturer's instructions.

Haematological parameters and flow cytometry

The total leucocyte concentration and the fractions of granulocytes and macrophages were estimated in PF and blood as previously described.[13] Briefly, the fixed samples were analysed using FACS Canto (Becton Dickinson, USA). Light scatter and logarithmically amplified fluorescence parameters from at least 10000 events were recorded in list mode after gating on forward light scatter to avoid debris, cell aggregates and bacteria.

Statistics

The data were analysed using GraphPad Prism version 5 (GraphPad Software, Inc., San Diego, CA, USA) or IMB SPSS Statistics 19. We used the two-way analysis of variance (ANOVA) test with Bonferroni corrections for multiple comparisons. The non-parametric Mann–Whitney *U*-test was

applied when comparing two groups. For all analyses a P value <0.05 was considered statistically significant.

Results

Clinical observation

Independent of treatment groups the mice exhibited signs of illness such as ruffled fur and reduced activity levels during the first 2 days. The mortality rate for mice with infected implants was 6/108 (vancomycin=2, Ltx21=2 and placebo=2). The death of these mice was not anticipated, and compared with surviving mice they did not exhibit signs of illness or distress prior to death.

Bacteriology

Untreated pre-colonized implants had cfu counts of 3×10^8 cfu/implant. Treatment *in vivo* with vancomycin and Ltx21 resulted in moderate reductions in the bacterial counts on the implants (Figure 1), but neither treatment was able to achieve complete bacterial clearance. However, mice treated with vancomycin had significantly lower cfu counts on their implants compared with both Ltx21 and placebo on day 6 ($P=0.045$). In the Ltx21-treated group we observed a significant reduction in the cfu counts on the implants from day 3 to day 8 ($P=0.0134$). In PF a significant reduction in cfu counts was observed in all three groups (Ltx21, vancomycin and placebo) from day 3 to day 8. On day 8 the median cfu count was zero in both the active treatment groups (Table 2). From day 6 we observed SCVs with reduced size, growth rate and loss of pigmentation in all treatment groups. The cfu counts include both wild-type colonies and SCVs.

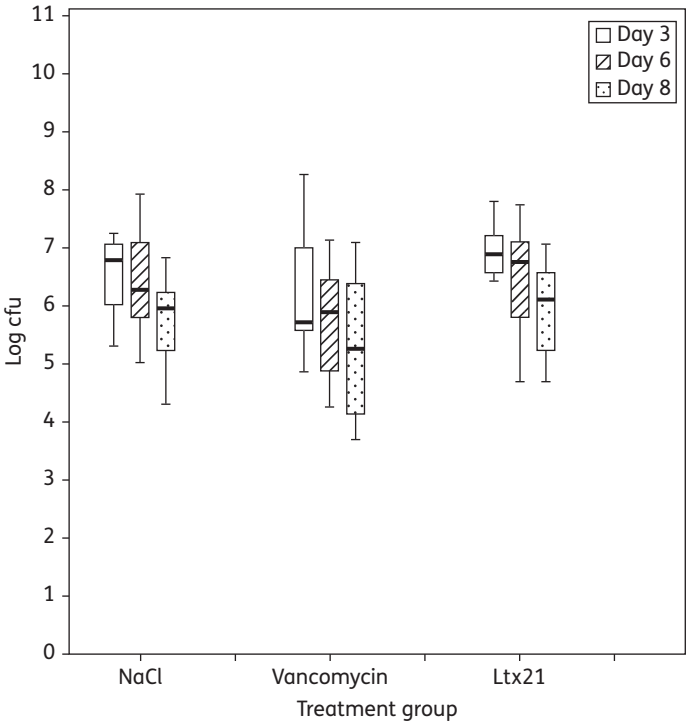

Figure 1. Clearance of implants pre-colonized with *S. epidermidis* 1457 treated with Ltx21, vancomycin or placebo on days 3, 6 and 8 post-implantation. Compared with Ltx21 and placebo, there was a greater reduction in cfu counts in the vancomycin group on day 6 ($P=0.045$).

Cellular response

We found no indication of an elevated systemic cellular response, either in mice with pre-colonized implants or in mice with sterile implants (data not shown). A local cellular response was observed in PF. Higher levels of leucocytes in PF from mice with pre-colonized implants receiving Ltx21, vancomycin or placebo were observed on day 6 ($P=0.0364$) when compared with animals with sterile implants (Table 3). There were no significant differences in local cellular response between groups treated with vancomycin, Ltx21 or placebo. In mice with sterile implants, a significant increase in granulocytes was observed on day 3 ($P=0.020$), day 6 ($P=0.020$) and day 8 ($P=0.040$) compared with mice without surgery. A significant increase in macrophages was observed on day 6 ($P=0.020$) in mice with sterile implants compared with mice with pre-colonized implants.

Cytokine response

Levels of TNF-α, IL-1β, GM-CSF, MIP-2 and MCP-1/CCL2 were measured in plasma and in PF on days 3, 6 and 8 post-implantation (data not shown). No significant differences were observed between the groups on any days regarding levels of IL-1β, MIP-2 and TNF-α. Mice with sterile implants showed significantly higher levels of MCP-1 in PF ($P=0.005$) on day 6 compared with mice with pre-colonized implants treated with placebo. GM-CSF was measured in blood only. Significantly increased values of GM-CSF were detected on days 3, 6 and 8 in mice with pre-colonized implants ($P=0.0028$) receiving Ltx21, vancomycin or placebo compared with mice with sterile implants. Significantly increased values of GM-CSF were also found on day 3 ($P=0.02$) and day 6 ($P=0.04$) in animals with sterile implants compared with animals without surgery.

Complement activation

The activation products of the complement system, C3a and C5a, were measured in plasma and PF. No significant activation of complement was observed within the groups with pre-colonized implants compared with the control groups without surgery.

Discussion

This murine model mimicking a device-related *S. epidermidis* biofilm-associated peritonitis enabled us to study the effects of two different treatment regimens and the host innate immune response. Our aim was to investigate whether Ltx21 could eradicate pre-formed *S. epidermidis* biofilms on peritoneal implants.

Table 2. Bacterial clearance in PF

	cfu, median (IQR)			
Treatment	day 3	day 6	day 8	P
Vancomycin ($n=11$)	200 (0–1100)	90 (20–9000)	0 (0–0)	0.01
Ltx21 ($n=12$)	990 (30–7700)	70 (0–920)	0 (0–1)	0.01
NaCl (placebo) ($n=11$)	960 (5–13600)	175 (18–708)	1 (0–25)	0.02

Table 3. Haematological parameters measured on days 3, 6 and 8 in PF

Haematological parameter	Day 3		Day 6		Day 8	
	sterile	pre-colonized	sterile	pre-colonized	sterile	pre-colonized
Total leucocytes	1.2×10^6 (8.6×10^5–1.2×10^6)	1.5×10^6 (1.0×10^6–1.8×10^6)	1.2×10^6[a] (1.1×10^6–1.8×10^6)	1.9×10^6 (1.5×10^6–2.3×10^6)	1.4×10^6 (9.9×10^5–1.4×10^6)	1.5×10^6 (8.8×10^5–1.7×10^6)
Granulocytes	8.0×10^4 (1.2×10^4–8.0×10^4)	3.0×10^3 (6.6×10^2–1.5×10^5)	6.9×10^4 (2.3×10^4–6.9×10^4)	3.4×10^5 (2.7×10^5–1.2×10^6)	1.8×10^4 (1.5×10^4–1.8×10^4)	1.1×10^5 (4.3×10^4–3.8×10^5)
Macrophages	5.6×10^4 (5.2×10^4–5.6×10^4)	9.6×10^4 (4.0×10^4–2.2×10^5)	3.6×10^5 (1.5×10^5–3.6×10^5)	4.6×10^5 (3.3×10^5–4.8×10^5)	6.6×10^4 (5.1×10^4–6.6×10^4)	4.5×10^4 (3.2×10^4–6.0×10^4)

Data are presented as medians and IQRs.
[a]Sterile significantly lower than pre-colonized, $P=0.036$.

However, the efficacy of Ltx21 administered intraperitoneally *in vivo* did not correspond to the efficacy previously observed *in vitro*.[7] SAMPs have been found to have high levels of serum albumin binding.[14] Although *in vitro* time–kill kinetic studies have demonstrated rapid killing of bacteria,[6] protein binding might occur instantaneously upon administration of such peptides.[14] This reduces the amount of available peptide and might explain the reduced efficacy of Ltx21 *in vivo* compared with the good efficacy observed *in vitro*.[7,14] Vancomycin resulted in better bacterial clearance than Ltx21. However, complete biofilm clearance was not achieved by any of the two study drugs, despite high intraperitoneal dosing.

On days 6 and 8 post-implantation we observed SCVs associated with implants in both treatment groups and in the placebo group. SCVs have previously been associated with persistent, subclinical and resistant infections associated with implanted medical devices.[15] The activity of vancomycin against SCVs has previously been found to be significantly reduced compared with colonies with normal morphology.[16] SCVs of *S. aureus* may persist intracellularly. This may protect the bacteria against the activity of cell-wall-targeting drugs[17] and may potentially explain the relatively poor effect of the cell-wall-active agents vancomycin and Ltx21.

The overall low levels of granulocytes and macrophages in both blood and PF on days 3, 6 and 8 indicated a low-grade infection. Induction of macrophage apoptosis and mechanisms interfering with phagocytosis and macrophage activation has been observed for both *S. epidermidis* and *Staphylococcus haemolyticus*.[18,19] Schommer *et al.*[19] demonstrated that biofilm production by *S. epidermidis* 1457 resulted in reduced phagocytosis and macrophage activation, yielding low activation of the transcription factor NF-κB, leading to a significantly reduced IL-1β synthesis in mouse macrophage-like cells. Furthermore, in a mouse model, an *S. aureus* biofilm induced macrophage death and a significant reduction in IL-1β, TNF-α and MCP-1 production.[20] These observations are in line with findings from our study. In general, we found no consistent increase in cytokine production in the infected groups compared with the sterile groups. In *S. epidermidis* biofilm infections, a recent study reported that granulocytes are recruited and activated, but are not capable of engulfing bacteria embedded in the biofilm.[21] We also found a local immune response with increased levels of granulocytes in the infected groups, but no increase in cytokine production.

No complement activation was observed in this *S. epidermidis* biofilm peritonitis model. In contrast, a previous study by our group demonstrated that *S. epidermidis* 1457 biofilm induced a strong complement activation in an *ex vivo* full blood model.[22] However, in our animal study we found little evidence of systemic inflammation. Besides the obvious difference between an *in vivo* model and an *ex vivo* model, the *ex vivo* model allowed short-term observations only, with complement activation being observed as early as 30 min post-infection. The incongruence regarding complement activation therefore probably reflects the different models employed.

There are limitations with this study. The model used is a suitable peritonitis model allowing simultaneous sampling of several parameters in response to treatment of an implant-associated biofilm infection. However, the current study could have benefitted from inclusion of additional animals, allowing for prolonged observation of persistence. One could argue that the use of pre-colonized implants is clinically irrelevant. However, in order to establish a biofilm infection to study the efficacy of the two different treatment regimens, we found that pre-colonization was necessary in order to obtain an infection in immunocompetent mice.

Conclusions

Our observations demonstrate failure of the novel SAMP Ltx21 and vancomycin in efficiently eradicating the *S. epidermidis* implant-associated biofilm infection. The reduced efficacy of the SAMP *in vivo* compared with previous *in vitro* results reflects the importance of performing animal studies. The presence of a persistent implant infection, which is not cleared by the innate immune system, is demonstrated. We demonstrated that this model allows for study of the complex interplay between the host immune system and the effects of antimicrobial treatment.

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Transparency declarations

None to declare.

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