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Evaluation of synergistic activity of bovine lactoferricin with antibiotics in corneal infection

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Objectives: The objectives of this study were to determine whether a synergistic effect could be obtained *in vitro* between bovine lactoferricin (B-LFcin) and antibiotics against *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolates from ocular infections, and to evaluate the use of B-LFcin as an adjunct to the antibiotic treatment of corneal infection *in vivo*.

Methods: Chequerboard and time–kill assays were performed to investigate the combined effects of B-LFcin and conventional antibiotics, including ciprofloxacin, ceftazidime and gentamicin, against 17 strains of *P. aeruginosa* (8) and *S. aureus* (9) isolated from ocular infection and inflammation, and 1 reference strain of *S. aureus*. Corneas of C57BL/6 mice were topically challenged with a multidrug-resistant strain of *P. aeruginosa*. Nine hours post-challenge, mice were treated topically and hourly with either vehicle, B-LFcin, ciprofloxacin or ciprofloxacin containing B-LFcin for 8 h. Corneas were then clinically examined, and bacterial numbers and levels of myeloperoxidase (MPO) evaluated.

Results: Synergy between B-LFcin and ciprofloxacin or ceftazidime was identified in most *P. aeruginosa* isolates, including multidrug-resistant strains, whereas no synergistic effect was seen between B-LFcin and gentamicin. Synergy was only observed with B-LFcin and ciprofloxacin against 2/10 *S. aureus* strains, and there was no synergy between B-LFcin and any of the other antibiotics tested. Combined B-LFcin and ciprofloxacin treatment significantly improved the clinical outcome, and reduced bacterial numbers and MPO in infected mouse corneas. B-LFcin alone was also able to reduce levels of MPO in infected corneas.

Conclusions: These findings indicate that B-LFcin may have advantages as an adjunct therapy with both antimicrobial and anti-inflammatory properties in the treatment of corneal infection.

Keywords: keratitis, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, ciprofloxacin

Introduction

Since their introduction, antibiotics have been the mainstay of treatment for bacterial infections.[1] However, increasing bacterial resistance to antimicrobials is rapidly becoming a major public health concern.[2,3] In response to increasing bacterial resistance, the fluoridated 4-quinolones, such as ciprofloxacin, were introduced in the 1980s. Now, there are increasing reports of resistance to this class of drugs in clinical isolates, particularly *Pseudomonas aeruginosa* and *Staphylococcus aureus*, with high levels of resistance being reported in cases of microbial keratitis.[4]

Microbial keratitis is a relatively rare but severe disease of the cornea, which can lead to blindness and vision loss as a result of scarring or perforation of the cornea if appropriate antibiotic therapy is not rapidly instituted. *S. aureus* is the most common pathogen associated with microbial keratitis of all causes,[5] while *P. aeruginosa* is regarded as a major causative pathogen for contact lens-associated microbial keratitis and represents 40%–70% of clinical isolates from microbial keratitis associated with the use of soft contact lenses.[6] These organisms are particularly difficult to treat, as they have the ability to easily acquire resistance to many antibiotics. Further, there are increasing reports of microbial resistance to conventional antibiotics during ocular infection,[7–10] including resistance to fluoroquinolones, which are currently the monotherapy of choice for the treatment of keratitis.[4,9,11] It is of further concern that keratitis caused by methicillin-resistant *S. aureus* has recently been reported.[12]

Combinations of antibiotics have been widely used to overcome resistance to a particular antimicrobial agent.[9,13,14] Moreover, Mouton[15] reported that inhibition of microbial growth is achieved at concentrations below that for each agent alone, which may reduce the possibility of antibiotic-resistant strains developing or allow the use of toxic agents where dose reductions are possible. In addition, a clinical study reported that an improvement in the mortality rate occurred when combination antibiotic therapies were used.[16]

It has been proposed that cationic peptides are potential candidates for use as adjunct agents with conventional antibiotics.[17,18] Cationic peptides kill bacteria rapidly in comparison to conventional antibiotics and usually act primarily by the disruption of cell membranes.[19] They are also thought to have a limited potential to induce resistant mutants *in vitro*.[20] The cationic protein lactoferrin and its related peptides are known to have a broad spectrum of antimicrobial activities.[21] Bovine lactoferricin (B-LFcin) is released from the N-terminal domain of bovine lactoferrin by acid pepsin digestion, and this peptide is responsible for the majority of the antimicrobial activity of lactoferrin towards Gram-positive and Gram-negative bacteria.[22,23] There are some species differences in lactoferricin, with B-LFcin being reportedly more active than those of human, murine and caprine origin.[24]

In this study, the ability of B-LFcin to act as an adjunct agent to potentiate the activity of conventional antibiotics that act by different mechanisms against clinical ocular isolates of *P. aeruginosa* and *S. aureus in vitro* was investigated. *In vitro* findings have been further investigated to determine the usefulness of B-LFcin as an adjunct agent to antibiotic treatment for antibiotic-resistant *P. aeruginosa* corneal infection *in vivo*.

Materials and methods

Bacterial strains and culture conditions

A total of 17 clinical isolates (Table 1) consisting of 8 strains of *P. aeruginosa* and 9 strains of *S. aureus*, and a methicillin-resistant strain of *S. aureus* ATCC 6538 were used. These strains were selected for their various susceptibilities to test antibiotics. The clinical isolates were all from cases of ocular infection and inflammation. Bacterial stocks were kept frozen at −80°C. Strains were inoculated on chocolate agar plates and incubated at 37°C overnight. Colonies from agar plates were resuspended into Mueller–Hinton broth (MHB; Oxoid Ltd, Basingstoke, UK) to OD_{660} 0.1 (~1×10^8 cfu/mL, matching a 0.5 McFarland turbidity standard), and serially diluted in MHB to 10^6 cfu/mL for MIC, chequerboard and time–kill assays.

Antimicrobial agents

Three antibiotics representing agents from fluoroquinolone, β-lactam and aminoglycoside classes used to treat ocular infections were used. Ciprofloxacin was purchased from ICN Biochemicals (Germany), and ceftazidime and gentamicin from Sigma (St Louis, MO, USA). B-LFcin with 11 amino acids (Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Glu) was obtained commercially from American Peptide Company (Sunnyvale, CA, USA).

Determination of the minimal inhibitory concentrations

A broth microdilution method was used to determine the MIC of test antimicrobial agents following the guidelines described by the CLSI (formerly NCCLS).[25] Briefly, serial 2-fold dilutions of antimicrobial agents were prepared in MHB to obtain the required concentrations. A positive control containing broth without antibiotics was included. Fifty microlitres of prepared dilutions were dispensed in triplicate into individual wells of a 96-well plate. Fifty microlitres of bacterial inocula, prepared as above, were added to each well and then the plate was incubated at 37°C for 24 h. Bacterial growth was examined by measurement of the optical density at 660 nm using a microtitre plate reader. All the tests were repeated at least twice. The MIC was defined as the lowest concentration of the drug that inhibited the growth of the test microorganism by >90%. Organisms were defined as resistant, intermediate or susceptible to the individual antibiotics based on the breakpoints defined by the CLSI.[26]

Microdilution chequerboard assay

Interactions between B-LFcin and the test antibiotics were assessed using a microbroth chequerboard technique. Chequerboards were prepared in sterile 96-well microtitre plates (Greiner, Austria). Serial 2-fold dilutions of each antibiotic and B-LFcin were prepared in MHB (Oxoid, Basingstoke, UK) encompassing 1/32×MIC to 4×MIC against the organism to be tested.[27,28] The chequerboard plates were inoculated with 0.5×10^6 cfu/mL of the appropriate bacterial strain and incubated for 18–20 h at 37°C. For each combination of drugs, the fractional inhibitory concentration (FIC) was calculated as the ratio of the MIC of agents A and B in combination to the MIC of agent A (or B) alone. The FIC index (FICI) was then calculated by summation of the FICs of agents A and B. Synergy was defined when the FICI was ≤0.5, no interaction when the FICI was >0.5–4 and antagonism when the FICI was >4.[29]

Time–kill assay

Synergy between B-LFcin and ciprofloxacin or ceftazidime against three strains of *P. aeruginosa* (strains Paer 032, 037 and 6294) was further investigated by the time–kill test. Time–kill curves were performed in MHB inoculated with the test strains to a final concentration of 0.5×10^6 cfu/mL. Ciprofloxacin or ceftazidime were tested alone or in combination with B-LFcin at concentrations of 0.25, 0.5 and 1×MIC for time periods up to 24 h at 37°C. Samples were taken at 0, 6, 12 and 24 h post-inoculation. Viable bacterial counts were enumerated by a standard dilution and plating method. Time–kill curves were constructed by plotting $\log_{10}$ cfu/mL against the time of incubation over 24 h. Synergy was defined as a ≥2 $\log_{10}$ cfu/mL decrease in viable counts with the combination as compared with its most active single agent after 24 h of incubation, and the number of surviving organisms in the presence of the combination was ≥2 $\log_{10}$ cfu/mL below the starting inoculum.[30] Antagonism was defined as an increase in viable counts of ≥2 $\log_{10}$ cfu/mL by the combination compared with the most active single agent alone at 24 h.[30,31]

Effect of B-LFcin on treatment of P. aeruginosa keratitis in a mouse model

The institutional Animal Care and Ethics Committees approved all protocols for animal use and animals were monitored during each experiment.

Infection of mice

Stock cultures of *P. aeruginosa* 037, stored in 30% glycerol at −80°C, were inoculated into 10 mL of tryptone soya broth (Oxoid, Basingstoke, UK). Cultures grown overnight were washed twice in phosphate-buffered saline (PBS, pH 7.4) and the concentration adjusted turbidometrically to ~1×10^9 cfu/mL. Bacterial numbers were confirmed retrospectively by viable counts.

Six-to-eight-week-old C57BL/6 mice (C57BL/6) were challenged with *P. aeruginosa* 037, as previously described.[27] Briefly, mice were

anaesthetized with tribromoethanol (Avertin, 125 mg/kg, intraperitoneally) and the surfaces of their corneas were incised with a sterile 27 gauge needle. Then, 5 μL of the above bacterial suspension was pipetted directly onto the wounded cornea of only the left eye. The right eye of each animal served as a control and was scratched but not infected. After 9 h, a time at which initial clinical symptoms are able to be observed, mice were divided randomly into four groups. Mice were treated topically hourly with either PBS, 2.5 mg/mL B-LFcin, Ciloxan ophthalmic drops (Alcon, USA; containing 0.3% ciprofloxacin HCl as the active ingredient) diluted 1:1 with PBS (0.15% ciprofloxacin HCl), or diluted Ciloxan containing 0.15% ciprofloxacin HCl and 2.5 mg/mL B-LFcin for 8 h. Mice were then clinically examined by a blinded observer, as described below. Mice were sacrificed 1 h after the final topical dose.

Clinical and histological examination

A minimum of 20 mice per group were used. Data presented for clinical scores and bacterial numbers are combined from all experiments. Mice were examined prior to bacterial challenge, immediately subsequent to bacterial challenge, at the commencement of treatment and at the termination of the experiment by an observer blinded to the treatment group. The animals were anaesthetized for examination, as described above, and the corneas were examined at 25× magnification under white light using a biomicroscope (Leica, Australia). A clinical score was generated based on that previously described by Cole *et al.*[27] Briefly, each of four parameters (exudate, epithelial defect, corneal infiltrate and corneal oedema) was graded on a scale of 0 (none) to 4 (severe). The parameter grades were totalled to produce a single slit-lamp examination score, ranging from 0 (normal eye) to a theoretical maximum of 16. The clinical scores were analysed with a one-way analysis of variance (ANOVA) test followed by *post hoc* multiple comparisons with Bonferroni correction.

Quantification of viable bacteria

Corneas were removed at 1 h after the final treatment and homogenized in 1 mL of sterile PBS using a hand-held Ultra-Tarrax T-8 dispersing tool (IKA, Rawang, Malaysia). To quantify viable bacteria, a 100 μL aliquot was serially diluted 1/10 in sterile PBS. Triplicate aliquots (20 μL) of each dilution, including the original homogenate, were plated onto nutrient agar (Oxoid). Plates were incubated for 24 h at 37°C before cfu were counted. Results were expressed as mean cfu per eye ± the standard deviation (SD) and analysed with ANOVA followed by *post hoc* multiple comparisons with Bonferroni correction.

Myeloperoxidase (MPO) assays

MPO activity, which is proportional to the number of polymorphonuclear neutrophils (PMNs) present, was determined, as previously described.[27] Briefly, hexadecyltrimethylammonium bromide (10 μL) was added to a final concentration of 0.5% w/v to a 90 μL aliquot of cornea homogenized as described above. Samples were sonicated (3×10 s) on ice and then subjected to three freeze–thaw cycles before centrifugation at 8000 **g** for 20 min in a refrigerated microcentrifuge. Ten microlitre aliquots of the resulting supernatant were pipetted in triplicate into a flat-bottomed microtitre plate, and the reaction was started by the addition of 90 μL of PBS containing 0.0167 g/100 mL *o*-dianisidine dihydrochloride and 0.002% H_2O_2. The change in absorbance at 3 min was determined at 460 nm using a plate reader and was compared with a standard curve on the same plate. The standard curve was prepared from known numbers of mouse PMNs and data are presented as the average number of PMNs per cornea. Results were compared statistically with ANOVA followed by *post hoc* multiple comparisons with Bonferroni correction.

Results

In vitro antibiotic susceptibility and synergism with B-LFcin against challenge strains

The susceptibility of isolates to the antibiotics tested, either alone or in the presence of B-LFcin, is summarized in Table 1. B-LFcin showed inhibition of bacterial growth for 7/8 strains of *P. aeruginosa* and 9/10 strains of *S. aureus* tested at high concentrations. The MIC level of B-LFcin for most of the *P. aeruginosa* and *S. aureus* isolates was 1280 or 2560 mg/L. Based on CLSI breakpoints for susceptibility, five *P. aeruginosa* isolates (Paer 031, 032, 033, 035 and 037) were resistant to ciprofloxacin (MIC ≥ 4 mg/L) and gentamicin (MIC ≥ 16 mg/L), and exhibited intermediate resistance to ceftazidime (8 < MIC < 64 mg/L). Among the nine clinical isolates of *S. aureus* tested, one (Saur 139) was resistant to ciprofloxacin (MIC ≥ 4 mg/L) and gentamicin (MIC ≥ 16 mg/L), two strains (Saur 138 and 139) were resistant (MIC ≥ 32 mg/L) and three strains (Saur 132, 133 and 137) were intermediate resistant to ceftazidime (8 < MIC < 32 mg/L).

Full synergy (FICI ≤ 0.5) was observed with the combination of B-LFcin and ciprofloxacin against five resistant strains of *P. aeruginosa* (Paer 031, 032, 033, 035 and 037), with the MIC of ciprofloxacin decreasing by 4-fold from 16 to 4 mg/L in comparison to the antibiotic alone (Table 1). The combination of B-LFcin and ceftazidime resulted in full synergy in three resistant strains of *P. aeruginosa* (Paer 031, 032 and 037), and FICI levels between 0.63 and 0.75 in strains 6294, and Paer 033 and 035 (Table 1). There was no synergistic effect between B-LFcin and gentamicin against any of the test strains of *P. aeruginosa* (Table 1).

The only significant synergy observed for *S. aureus* was with ciprofloxacin and B-LFcin against two clinical isolates, Saur 41 and 137 (Table 1). Two other *S. aureus* strains (Saur 31 and 142) showed an FICI of 0.75. When ceftazidime or gentamicin was used in combination with B-LFcin, there was no synergy observed in any of the *S. aureus* strains tested. The three resistant (Saur 133, 137 and 139) and one susceptible strain (Saur 41) showed 0.5 < FICI < 1 with the combination of ceftazidime and B-LFcin. Five test strains (Saur 31, 41, 133, 139 and 140) displayed an FICI of 0.75, with the MIC levels being reduced mostly by 4-fold against these strains in the presence of B-LFcin (Table 1) compared with the activity of gentamicin alone.

Representative *P. aeruginosa* strains Paer 032 and 037 (showing synergy) and strain 6294 (showing no synergy between B-LFcin and ciprofloxacin or ceftazidime) were chosen and tested in time–kill assays for confirmation. The synergies observed between the antibiotics and B-LFcin in the chequerboard assays against the two resistant strains of *P. aeruginosa* (Paer 032 and 037) were also observed with the time–kill curves, which test bactericidal activity. In these assays, the combination regimens (either ciprofloxacin or ceftazidime in combination with B-LFcin) at 0.5×MIC resulted in synergy after a 24 h incubation, providing a ≥2 $\log_{10}$ higher rate of killing compared with either single agent. At 1×MIC levels, synergy was even observed at 6 h for B-LFcin combined with either ciprofloxacin or ceftazidime against the two test strains (Paer 032 and 037), indicating that the combination kills bacteria more rapidly than each single agent (Figure 1a and b). Synergistic action was also achieved for the combination of B-LFcin and

Table 1. MICs of antibiotics against test strains in the absence or presence of bovine lactoferricin

		MIC alone (mg/L)				MIC with B-LFcin (mg/L)			FICI		
Strain	Source	B-LFcin	CIP	CAZ	GEN	CIP	CAZ	GEN	CIP	CAZ	GEN
P. aeruginosa											
001	CLARE	>2560	0.063	2	0.625	0.063	0.5	0.039	ND	ND	ND
031	MK	2560	16	16	1280	4	4	640	0.5	0.5	1
032	MK	1280	16	16	640	4	4	320	0.5	0.5	1
033	MK	1280	16	16	1280	4	4	640	0.5	0.75	1
035	MK	1280	16	16	1280	4	2	640	0.5	0.63	1
037	MK	640	16	16	1280	4	4	640	0.5	0.5	1
6206	MK	1280	0.125	2	0.625	0.031	1	0.312	1.25	1	1
6294	MK	1280	0.125	2	0.625	0.031	0.25	0.312	0.75	0.63	1
S. aureus											
31	CLPU	1280	0.5	8	0.625	0.125	8	0.156	0.75	2	0.75
41	CLPU	2560	0.5	8	0.312	0.125	4	0.156	0.5	0.75	0.75
132	MK	2560	0.5	16	0.625	0.25	8	0.312	1	1	1
133	MK	1280	0.5	16	0.625	0.25	8	0.156	1	0.75	0.75
137	CONJ	2560	0.5	16	0.312	0.125	4	0.156	0.5	0.75	1
138	CONJ	>2560	0.25	64	0.625	0.125	32	0.156	ND	ND	ND
139	CONJ	2560	32	256	64	16	32	16	1	0.63	0.75
140	CONJ	2560	0.25	8	0.625	0.125	4	0.156	1	1	0.75
142	MK	2560	0.25	8	0.312	0.063	4	0.312	0.75	1	1
ATCC 6538	HL	640	0.25	8	0.125	0.125	1	0.063	0.75	1.13	1.13

CLARE, contact lens-related acute red eye; MK, microbial keratitis; CONJ, conjunctivitis; CLPU, contact lens-related peripheral ulcer; HL, human lesion; MIC, minimum inhibitory concentration; B-LFcin, bovine lactoferricin; CIP, ciprofloxacin; CAZ, ceftazidime; GEN, gentamicin; ND, not determinable.
FICI, factional inhibitory concentration index (synergy: FICI $\leq$0.5; no interaction: 0.5$<$FICI$\leq$4; and antagonism: FICI$>$4).
Respective CLSI (formerly NCCLS) breakpoints for susceptible and resistant to: ciprofloxacin $\leq$1 and $\geq$4 mg/L for *P. aeruginosa*, and $\leq$1 and $\geq$4 mg/L for *S. aureus*; ceftazidime $\leq$8 and $\geq$64 mg/L for *P. aeruginosa*, and $\leq$8 and $\geq$32 mg/L for *S. aureus*; and gentamicin $\leq$4 and $\geq$16 mg/L for both *P. aeruginosa* and *S. aureus*.

ceftazidime at 0.5×MIC against *P. aeruginosa* 6294, but not for B-LFcin and ciprofloxacin.

Effect of B-LFcin on treatment of P. aeruginosa keratitis in a mouse model

Clinical and histological examination of corneas

Average macroscopic scores generated from the observations of an observer blinded to the treatment group showed that the macroscopic ocular response was significantly less severe in the mice treated with the combination of ciprofloxacin and B-LFcin (7.33 ± 3.38) compared with those treated either with PBS (10.77 ± 2.41, $P=0.000$) or ciprofloxacin alone (9.22 ± 2.69, $P=0.029$). There was no significant difference in the clinical appearance of corneas in groups treated with the combination compared with B-LFcin alone (7.52 ± 2.08, $P=1.000$) or between the group treated with ciprofloxacin alone and PBS ($P=0.132$).

The response of the corneas of the PBS-treated mice showed generalized cellular infiltration of the cornea, extending to the periphery. Epithelial loss was extensive (Figure 2a) and the central cornea showed thinning. Histologically, mice treated with ciprofloxacin alone showed oedema and generalized cellular infiltration of the cornea with smaller or no epithelial defect evident (Figure 2b). However, mice receiving B-LFcin alone or in combination with ciprofloxacin (Ciloxan) were observed to have reduced density of infiltration of the cornea both clinically ($P=0.016$ and $P=0.004$) and histologically (Figure 2c and d) compared with mice treated with ciprofloxacin alone. In addition, treatment with B-LFcin alone or in combination with ciprofloxacin reduced scores for the extent of corneal epithelial defect ($P=0.012$ and $P=0.018$, respectively) compared with mice treated with ciprofloxacin alone.

Bacterial counts

At 1 h following the final treatment of mice infected with *P. aeruginosa* strain 037, viable bacterial counts were not significantly different between the group treated with PBS and that treated with B-LFcin ($P=1.000$; Figure 3a). Treatment with the combination of ciprofloxacin and B-LFcin resulted in a 30-fold decrease in bacterial numbers compared with treatment with vehicle ($P=0.000$) and a 3-fold reduction compared with treatment with ciprofloxacin alone ($P=0.031$; Figure 3a). There were no bacteria found in the corneas of the scratch controls (data not shown).

MPO levels

Numbers of neutrophils in the cornea were estimated using MPO assay results. There was no significant reduction in the levels of

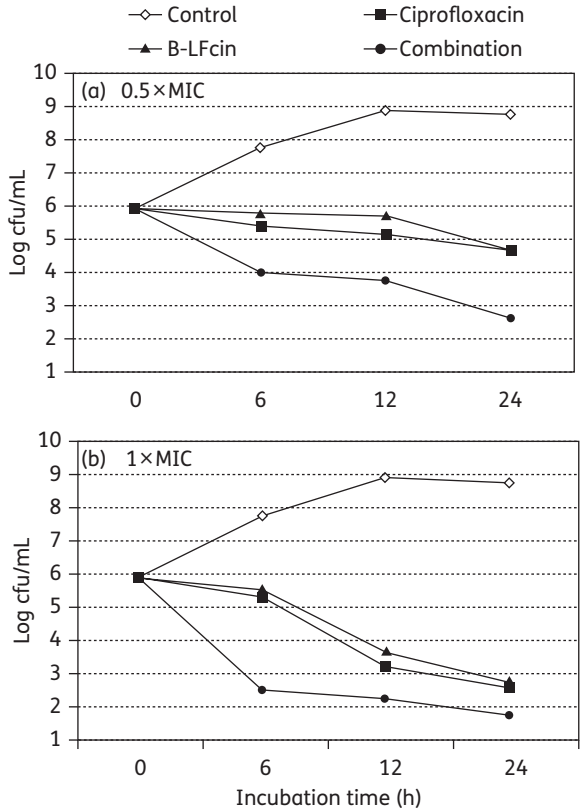

Figure 1. Time–kill curve of representative strain *P. aeruginosa* 037 grown in the presence of ciprofloxacin or bovine lactoferricin (B-LFcin) alone or in combination at (a) 0.5×MIC, or (b) 1×MIC.

PMN infiltration of corneas between those treated with PBS and those treated with ciprofloxacin alone ($P=0.1$). However, treatment with B-LFcin alone resulted in an ~2.5-fold reduction in the numbers of infiltrating PMN ($P=0.009$; Figure 3b). Treatment with the combination of B-LFcin and ciprofloxacin resulted in a 4-fold reduction in the numbers of infiltrating PMNs in corneas ($P=0.0004$; Figure 3b) compared with PBS and a 2.5-fold reduction compared with treatment with ciprofloxacin alone ($P=0.015$). However, there was no significant difference between treatment with the combination of ciprofloxacin and B-LFcin compared with treatment with B-LFcin alone ($P=1.000$).

Discussion

The research presented here shows both *in vitro* and *in vivo* that B-LFcin has excellent potential for pharmacological use as an adjunct agent to conventional antibiotic treatment for ocular infection. It acts synergistically with the antimicrobial agent to increase antimicrobial efficacy and may also act to reduce excessive inflammation, to which the cornea is particularly vulnerable.

Previous research has provided substantial evidence for the *in vitro* antimicrobial activity of B-LFcin and its related cationic peptides.[22,23,32,33] The primary structure of B-LFcin is well established as a 25 residue peptide that forms into a looped structure through an intramolecular disulphide bond.[22] The B-LFcin used in the current study comprises 11 residues of basic amino acid-rich region. This short peptide has been reported to have similar antimicrobial activities while losing most of the haemolytic activities as compared with the 25 residue peptide.[34] Low toxicity of this peptide was observed and confirmed in a standard cell growth inhibition test (data not shown). B-LFcin only showed a marginal antibacterial efficacy against *P. aeruginosa* and *S. aureus* with much higher MIC levels. The discrepancy in the various MIC levels may be attributed to the different culture media used in the studies. Most of the previous studies for determination of the MIC of B-LFcin for bacterial strains were performed in 1% Bacto Peptone water.[34,35] However, MHB was used as a standard culture medium for the determination of MICs of antibiotics in the present study. Although the MHB used was non-cation-adjusted, it still contains low concentrations of Mg^{2+} (~6 mg/L) and Ca^{2+} (~5 mg/L). Similar to polymyxins, a group of polypeptide antibiotics, B-LFcin is a cationic detergent-like molecule that disrupts the outer membrane of bacteria, resulting in altered membrane permeability. It is believed that the antimicrobial activity of B-LFcin is initiated from binding to the negatively charged bacterial surfaces [lipopolysaccharide (LPS) of Gram-negative bacteria or teichoic acid of Gram-positive bacteria].[33] It is possible that cations such as Ca^{2+} or Mg^{2+} may act to neutralize negatively charged target sites on the surface of the bacterial membrane and reduce the affinity of B-LFcin to the target sites.[36] The B-LFcin with a dense positive charge used in the current study may be more sensitive to the effect of Ca^{2+} or Mg^{2+} ions on its binding. Nevertheless, it is difficult to compare the antimicrobial activity of peptides from different studies, since different peptide structures, bacterial strains and experimental procedures are used.

Despite a number of *in vitro* reports on synergistic activities between B-LFcin and conventional antibiotics against *Escherichia coli*, studies are limited with respect to the synergistic combination of B-LFcin with antibiotics against other Gram-negative bacteria, especially *P. aeruginosa* strains. However, in this study we have shown that for the treatment of ocular isolates, particularly *P. aeruginosa* where 5/9 resistant strains showed synergy and a reduced MIC of ciprofloxacin, there may be a therapeutic advantage in using B-LFcin as an adjunct treatment. To our knowledge this is the first report of synergy between B-LFcin and ciprofloxacin or ceftazidime against multidrug-resistant *P. aeruginosa* isolated from a corneal infection. It is interesting to notice that synergy can be achieved with B-LFcin and ciprofloxacin/ceftazidime against *P. aeruginosa* early (6 h) in a time–kill assay. These findings indicate that the use of B-LFcin as an adjunct to antibiotics in the treatment of infection may result in a rapid reduction of pathogens.

The mechanisms that contribute to synergistic combinatorial therapy with B-LFcin are thought to occur via cell membrane interactions with the bacteria, increasing permeability.[23,35,37,38] The enhanced effect of ciprofloxacin and ceftazidime in *P. aeruginosa* can be explained by the B-LFcin-mediated compromise in membrane permeability that allows antibiotics easier access through the bacterial membrane. Amphipathic cations are regarded as preferred substrates of multidrug resistance pumps in bacteria.[39] Similar to other cationic peptides,[39,40] B-LFcin may act as an efflux pump inhibitor in *P. aeruginosa* and, consequently, enhance the accumulation of ciprofloxacin and ceftazidime in the cytoplasm. Our results showing a particular synergic effect between B-LFcin and ciprofloxacin may also be consistent with the proposal

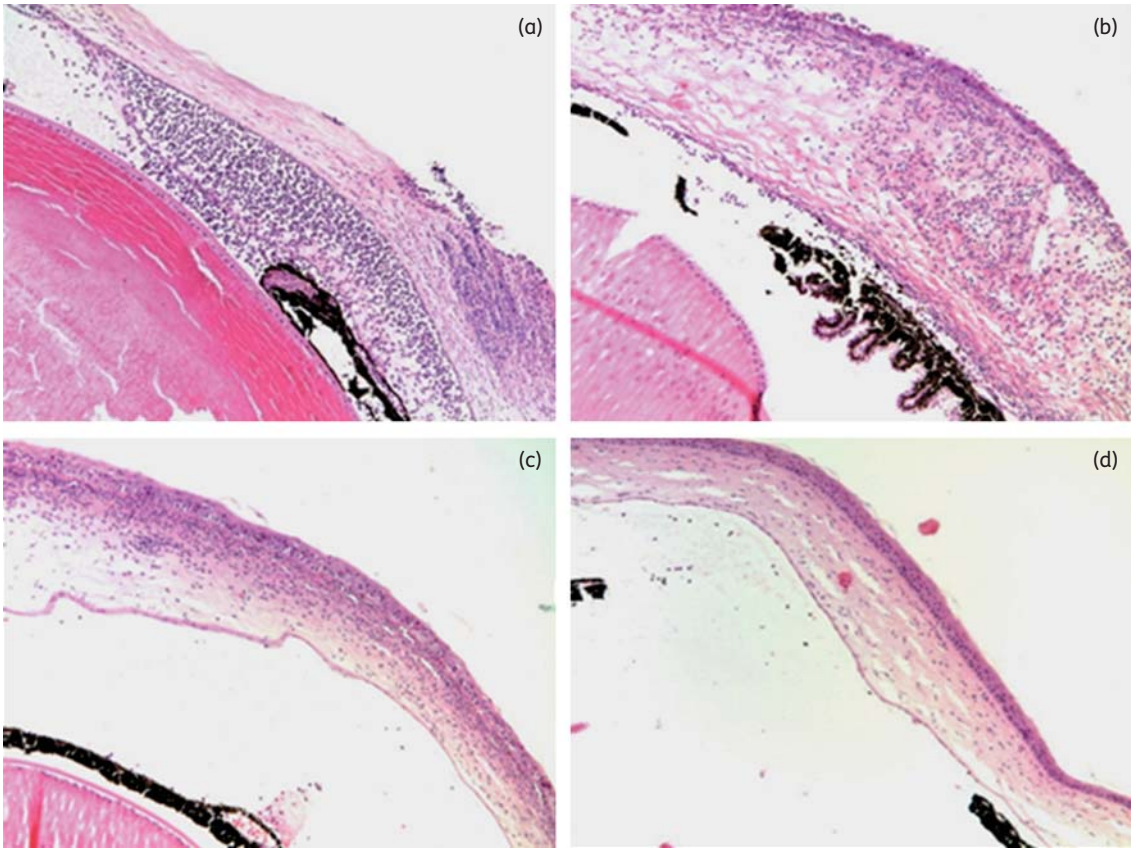

Figure 2. Corneal histology of mouse eyes infected with antibiotic-resistant *P. aeruginosa*. Representative corneas from the group of mice treated with PBS (a), ciprofloxacin (b), bovine lactoferricin (B-LFcin) (c) or the combination of B-LFcin and ciprofloxacin (d). This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*.

that ciprofloxacin increases the binding of antimicrobial peptides to the outer membrane of Gram-negative bacteria.[41] An additional mode of action may be postulated in that B-LFcin could penetrate the bacterial nuclear envelope and bind to DNA,[33,42] in turn, increasing the effect of ciprofloxacin on DNA synthesis. Conversely, gentamicin, which possesses a net positive charge and binds avidly to membranes containing acidic phospholipids,[43] may compete for binding sites with B-LFcin on the membrane lipids or lipid-associating proteins on *P. aeruginosa* strains. These may be the explanation for no synergy with the combination of B-LFcin and gentamicin.

The mechanism(s) of action of B-LFcin against *S. aureus* are not well known. Most likely, the B-LFcin binds and crosses the negatively charged teichoic acid layer and exerts its effects intracellularly.[42,44] Recent studies indicate that B-LFcin is capable of inhibiting the synthesis of macromolecules, such as DNA, RNA and proteins, in both Gram-positive and Gram-negative bacteria.[42] Our findings of no synergy with combinations of B-LFcin and most of the test antibiotics against *S. aureus*, and synergy between B-LFcin and ciprofloxacin against limited strains of *S. aureus* are consistent with those previously reported,[35,37,45,46] and further highlight the complex interplay of bacterial strain characteristics and choice of antimicrobial therapy.

Although the synergistic combination of antimicrobial agents is regarded as a promising strategy for the treatment of antibiotic-resistant infection,[47] using combination regimens when synergy is demonstrable *in vitro* does not assure an improved clinical outcome due to the complexity of the *in vivo* environment during infection. The clinical relevance of *in vitro* synergy between B-LFcin and ciprofloxacin has been further investigated in the current study. Evaluation of the ability of B-LFcin in combination with ciprofloxacin to improve therapeutic outcome in corneal infection showed improvement of clinical progress in comparison with the eyes treated with the PBS control or ciprofloxacin alone. Interestingly, the infected eyes treated with B-LFcin alone also showed reduced gross pathology and a reduction in the number of infiltrating leucocytes. The improvement in clinical parameters with the administration of B-LFcin in combination with ciprofloxacin might occur by a number of mechanisms in addition to increased killing of the infecting bacteria. It is well known that there are many tear components with antibacterial properties, including lysozyme, lactoferrin, lipocalin and secretory phospholipase A_2.[48,49] The antibacterial activity of B-LFcin may be enhanced in the presence of antimicrobial factors in tears, such as lysozyme.[50,51] It is possible that the interaction of the B-LFcin with the bacterial membrane might modulate the production of toxins or proteases by the bacteria. B-LFcin might interact with the host immune system;[23] or as with other cationic peptides, it might act by interaction with LPS-binding protein.[17] Cationic peptides have been reported to reduce the inflammatory response to LPS *in vitro* and *in vivo*,[52–54] including changes in peripheral leucocyte

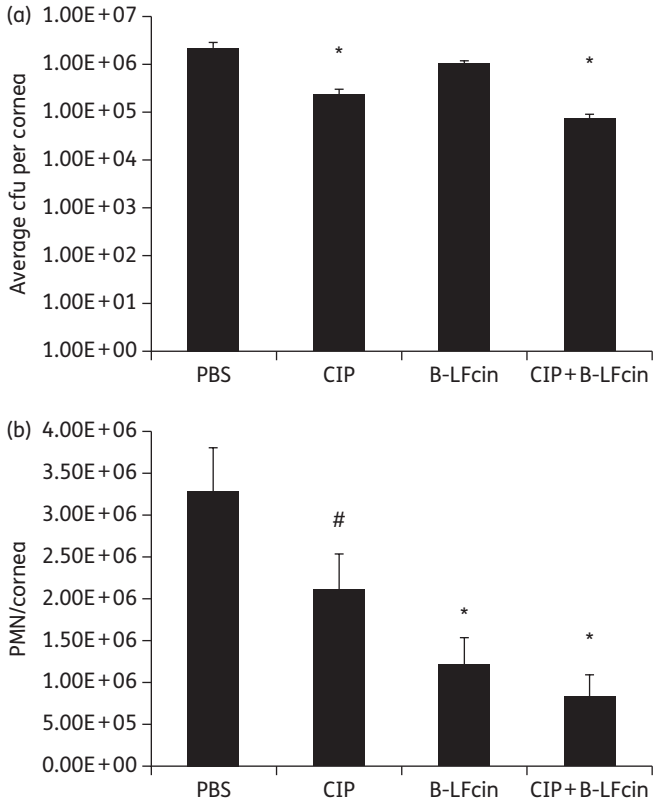

Figure 3. (a) Bacterial numbers recovered from corneas of mice with an antibiotic-resistant *P. aeruginosa* corneal infection treated with PBS, ciprofloxacin (CIP), bovine lactoferricin (B-LFcin) or the combination of CIP and B-LFcin. *$P < 0.03$ versus PBS. (b) Relative myeloperoxidase (MPO) activity per cornea ± SEM at 18 h post-infection with antibiotic-resistant *P. aeruginosa* corneal infection treated with PBS, ciprofloxacin (CIP), bovine lactoferricin (B-LFcin) or the combination of CIP and B-LFcin. #$P = 0.015$, CIP versus CIP + B-LFcin; *$P < 0.009$ versus PBS.

numbers, and activation and reduction in levels of proinflammatory mediators. On the other hand, a bovine lactoferrin peptide has been reported to up-regulate the killing activity of PMN by increasing their superoxide generation and protein kinase C activity.[55] The recruitment of excessive numbers of neutrophils into the cornea is an indicator of a poor corneal outcome.[56] Guarna *et al.*[57] also concluded that cationic peptides can play a major role in the reduction of inflammatory cytokines and other inflammatory mediators of inflammation. Our findings that MPO activity was decreased in the corneas treated with B-LFcin alone and in combination with ciprofloxacin may result from the reduction of inflammatory mediators.[57] In addition, B-LFcin B has exhibited inhibition of the classical complement pathway.[58] However, the exact mechanisms by which the improved clinical outcomes occur remain to be investigated. The findings may be further improved by appropriate formulation of the combinatorial therapy by employing a sustained release strategy using muco-adhesive liquids or an *in situ* gelling system to increase corneal residence time and consequent bioavailability.

This study has demonstrated synergy of B-LFcin, enhancing the activity of ciprofloxacin and ceftazidime against ocular isolates of *P. aeruginosa*. Importantly, our results indicate that B-LFcin increases the antibacterial activity of ciprofloxacin *in vitro* and *in vivo* against multidrug-resistant *P. aeruginosa* and, further, that B-LFcin may also act as an anti-inflammatory agent during an adjunct treatment of corneal infection. Therefore, B-LFcin as an adjunct to antibiotics might offer advantages over current adjunctive therapeutics to treat ocular infection and significantly reduce important clinical indicators of poor visual outcome.

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Transparency declarations

None to declare.

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