Hybrid Derivative of Cathelicidin and Human Beta Defensin-2 Against Gram-Positive Bacteria: A Novel Approach for the Treatment of Bacterial Keratitis

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ABSTRACT

Purpose: Bacterial keratitis (BK) represents the leading cause of corneal blindness worldwide. This study aimed to generate potent hybridized human-derived host defense peptides (HDPs) as novel topical antimicrobial therapy for bacterial keratitis.

Methods: Hybrid peptides were rationally designed through combination of functional amino acids in parent HDPs, including LL-37 and human beta-defensin (HBD)-1 to -3. Minimal inhibitory concentrations (MICs) and time-kill kinetics assay were performed to determine the concentration- and time-dependent antimicrobial activity and cytotoxicity was evaluated against human corneal epithelial cells (HCE-2) and erythrocytes. In vivo safety and efficacy of the most promising peptide was examined in the corneal wound healing and *Staphylococcus aureus* (ATCC SA29213) keratitis murine models, respectively.

Results: A second-generation hybrid peptide (CaD23), based on rational hybridization of the middle residues of LL-37 and C-terminal of HBD-2, demonstrated good efficacy against methicillin-sensitive and methicillin-resistant *S. aureus* [MIC=12.5-25.0μg/ml (5.2-10.4μM)] and *S. epidermidis* [MIC=3.1-12.5μg/ml (1.3-5.2μM)], and moderate efficacy against *P. aeruginosa* [MIC=50μg/ml (20.9μM)]. CaD23 (at 25μg/ml or 2x MIC) killed all the bacteria within 30 mins, which was 8 times faster than amikacin (25μg/ml or 20x MIC). At 200μg/ml (16x MIC), CaD23 was shown to be relatively safe against HCE-2 (<30% toxicity) and erythrocytes (<10% toxicity). Pre-clinical murine studies showed that CaD23 0.05% (500μg/ml) achieved a median reduction of *S. aureus* bioburden by 94% (or 1.2 $\log_{10}$ CFU/ml) while not impeding corneal healing.

Conclusions: Rational hybridization of human-derived HDPs has led to generation of a potentially efficacious and safe topical antimicrobial agent for treating Gram-positive BK.

Key words: Antimicrobial peptide; bacterial keratitis; cathelicidin; corneal infection; host defense peptide; human beta-defensin; infectious keratitis; *Staphylococcus aureus*

1. INTRODUCTION

Infectious keratitis (IK) represents a major cause of corneal blindness worldwide, with an incidence ranging from 2.5-799 per 100,000 population-year.[1, 2] It is a common, yet potentially sight-threatening, ophthalmic emergency that often warrants hospital admission for intensive antibiotic treatment and monitoring. IK can be caused by a wide array of microorganisms, including bacteria, fungi, protozoa and viruses. Among all, bacterial keratitis (BK) has been shown to be the main cause for IK in many developed countries, including the US and the UK (>90% cases), with *Staphylococci spp.* (30-60%) and *Pseudomonas aeruginosa* (10-25%) being the two most common bacteria reported.[1-6] In view of the diverse causative microorganisms, intensive broad-spectrum topical antibiotics are often administered during the initial treatment of IK. In refractory cases of IK, adjuvant therapy such as photoactivated chromophore-corneal cross-linking (PACK-CXL), amniotic membrane transplant, and therapeutic keratoplasty, are often warranted.[7-9]

The current management of IK is challenged by several factors, including the low culture yield,[1, 2] polymicrobial infection,[10, 11] and emerging trend of antimicrobial resistance (AMR).[1, 12, 13] In the Antibiotic Resistance Monitoring in Ocular Microorganisms (ARMOR) 10-year prospective study, it was shown that 35-50% of the *Staphylococci spp.* were methicillin-resistant, with ~75% of them being multidrug resistant (i.e. resistant to ≥ 3 antibiotic classes).[4] More compellingly, the increased level of AMR of the causative microorganism has been shown to negatively influence the corneal healing time and final visual outcome.[14, 15] Furthermore, complications such as corneal melt, perforation and endophthalmitis may ensue despite timely and intensive antibiotic treatment, necessitating eye-saving surgeries such as tectonic keratoplasty.[8, 16] All these issues highlight an unmet need for new antimicrobial treatment for IK, particularly that no new class of antibiotic has been discovered since 1987.[17]

Host defense peptides (HDPs), or previously known as antimicrobial peptides (AMPs), play

vital roles in the innate immune system.[18, 19] They are a group of evolutionary conserved small molecules that are ubiquitously expressed by the immune cells and the front-line defense structures, including the ocular surface (OS).[20, 21] They have recently shown promise as potential therapeutic agents due to their broad-spectrum antimicrobial properties with minimal risk of developing AMR.[22] These molecules are usually highly cationic and amphiphilic, with 30-50% of hydrophobicity. The cationic amino acid (AA) residues facilitate the binding of HDPs onto the anionic bacterial membrane (*via* electrostatic interaction), while the hydrophobic residues interact with the lipid tail region of the membrane, culminating in membrane disruption, leakage of cytoplasmic contents and subsequent cell death.[23]

Previous studies have demonstrated that numerous HDPs, including LL-37, human beta-defensin (HBD)-2 and -3, are expressed at the OS and upregulated during IK.[20, 24-28] HBD-9, on the other hand, was shown to be downregulated during IK.[29] In addition, it was demonstrated that the modulated levels of HBD-3 and HBD-9 on OS during BK returned to normal baseline following complete healing of ulcers.[30] Furthermore, HDPs exhibit moderate *in vitro* antimicrobial activity [minimum inhibitory concentration (MIC) of around 50-100 μg/mL) against common ocular surface pathogenic isolates such as *S. aureus* and *P. aeruginosa*,[31, 32] highlighting their essential functions in human OS defense.

Despite their promising potential as effective antimicrobial therapies, several issues have impeded the successful translation of HDPs to clinical use. These include their complex structure-activity relationship (SAR), susceptibility to host / bacterial proteases, physiological conditions, and toxicity to host tissues, amongst others.[22, 33, 34] In view of these issues, a number of novel strategies, including residue substitution, chemical modification, and hybridization, have been proposed to enhance the therapeutic potential of HDPs.[22, 33, 34]

A number of hybrid peptides, including cecropin A-melittin,[35] cecropin A-LL37,[36] melittin-protamine,[37] amongst others,[22] have been previously designed and reported in the literature.

When compared to the parent peptide, these hybrid peptides demonstrated improved antimicrobial efficacy and/or reduced toxicity to host tissues. However, strategies in combining two human-derived HDPs from two different classes (e.g. LL-37 and HBD) have not been previously explored. In this study, we aimed to develop novel topical antimicrobial treatment for Gram-positive BK, particularly *Staphylococcus aureus* (SA) and methicillin-resistant SA (MRSA), using hybridized HDPs derived from different combination of LL-37 and HBD-1 to -3, which are all important HDPs expressed at the OS. The efficacy and safety of the most promising molecule, CaD23 (a hybrid derivative of LL-37 and HBD-2), was further examined and validated in murine corneal wound healing and BK models.

2. MATERIALS AND METHODS

The commercially synthesized peptides and commonly used antibiotics for IK were first examined for their in vitro antimicrobial efficacy against a range of bacteria. In vitro cytotoxicity of these antimicrobial agents was then determined against human corneal epithelial cells (HCE-2, CRL-1135, ATCC, UK) using cell viability assay and cytotoxicity assay, and against human erythrocytes using hemolytic assay. The most promising synthetic peptide, CaD23, was further examined for its time- and concentration-dependent in vitro antimicrobial activity. Finally, in vivo efficacy and safety of CaD23 were evaluated in corneal wound healing and *S. aureus* keratitis murine models. All the assays described in this study were conducted in biological duplicate and in at least two independent experiments, with appropriate positive controls (PCs) and negative controls (NCs).

2.1. Design and synthesis of HDPs

A template-based design method was used to design our human-derived HDPs. The native peptide sequences were obtained from an established protein bank database (https://www.uniprot.org/). Several human-derived HDPs, specifically HBD-1, -2, and -3, and cathelicidin (LL-37), were subject to testing and hybridization. The physicochemical properties of the designed peptides, including cationic charges, hydrophobicity and

amphiphilicity, were analyzed using a computational programme called HeliQuest (http://heliquest.ipmc.cnrs.fr). Various strategies, including residue substitution and hybridization, were employed for SAR analysis and for improving the therapeutic index (i.e. increasing the antimicrobial efficacy and reducing the toxicity profile). The native and synthetic peptide sequences are shown in **Table 1**.

All antibiotics were purchased from Sigma-Aldrich, United Kingdom. The peptides were commercially produced by Mimotopes (Mimotopes Pty. Ltd., Mulgrave Victoria, Australia) via traditional solid phase Fmoc synthesis method. All the synthetic peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) to >95% purity and characterized by mass spectrometry. The peptides were then sent to our lab in lyophilized form and stored at -80 °C until further usage. In view of the hydrophobicity, the peptides were first fully dissolved in 50 μl of dimethyl sulfoxide (DMSO) followed by dilution in sterile, de-ionized water to achieve a final concentration of 1 mg/ml peptide in 0.5% DMSO. Further dilution was performed for specific assays as required.

2.2. Range of microorganisms being tested

A range of Gram-positive and Gram-negative laboratory- and clinical-strain bacteria were used for the experiments. These included laboratory-strain methicillin-sensitive *S. aureus* (MSSA; including SH1000 and ATCC SA29213), laboratory-strain MRSA (ATCC MRSA43300), clinical-strain MRSA, laboratory-strain methicillin-sensitive *Staphylococcus epidermidis* (MSSE; ATCC SE12228), clinical-strain methicillin-resistant SE (MRSE), and laboratory invasive-strain *P. aeruginosa* (PAO1-L).

2.3. Determination of antimicrobial efficacy using MIC assay

In vitro antimicrobial efficacy of the antibiotics and designed HDPs was determined using an established MIC assay with broth microdilution method approved by the Clinical and Laboratory Standards Institute (CLSI).[38] Briefly, the microorganisms were cultured on

Tryptone Soya Agar (TSA) and incubated overnight for 18-21 hours at 37 °C. Bacterial inoculums were subsequently prepared using the direct colony suspension method.[38] Three to five bacterial colonies were obtained from the agar plate and inoculated into an Eppendorf tube containing 1 ml of cation-adjusted Muller-Hinton broth (caMHB), consisting of 20-25 mg/L calcium ions (Ca^{2+}) and 10-12.5 mg/L magnesium ions (Mg^{2+}). The bacterial suspension was adjusted to achieve a turbidity equivalent to 0.1 OD_{600} or 0.5 MacFarland, containing ~1.5 x 10^8 colony-forming unit (CFU)/ml, which was then further diluted in 1:150 in caMHB to reach a final bacterial concentration of ~1x10^6 colony forming units (CFU)/ml. Subsequently, 50 μl of 1x10^6 CFU/ml bacteria and 50 μl of treatment / controls were added into each well for the MIC assay. As the HDPs are known to be influenced by the salt content,[22] the MIC assay was also performed in the presence of physiological tear salt concentration (150 mM NaCl). The MIC values, defined as the lowest concentration of the antimicrobial agent that prevented any visible growth of bacteria, were determined after 24 hours of incubation with treatment.

2.4. Cell viability and cytotoxicity assays

Cell viability and cytotoxicity of antibiotics and peptides were determined against human corneal epithelial cells (HCE-2, CRL11135, ATCC, Manassas, Virginia, USA) using cell-counting-kit-8 (CCK-8) assay (Sigma Aldrich, Merck Life Science UK Limited, Dorset, UK) and lactate dehydrogenase (LDH) assay (ThermoFisher Scientific, UK), respectively, as per manufacturer's guidelines. HCE-2 cells were cultured and seeded into a 96-well plate at 7.5 x 10^3 cells/well, and grew to 80-90% confluency, in the presence of growth media [consisting of keratinocyte serum free medium (KSFM) supplemented with human recombinant epidermal growth factor, bovine pituitary extract, hydrocortisone, and insulin]. The HCE-2 cells were then starved for 16 hours in KSFM to remove the effect of serum and to synchronize the cell cycle. Subsequently, the cells were incubated with treatment for 3 hours before measurement was taken using BMG Clariostar microplate reader (BMG Labtech Ltd., Aylesbury, UK). Appropriate controls were used, including 0.1% Triton X-100 as positive

control and KSFM as negative control. Cell viability was calculated using the following formula: [(($I_{treatment}$ – I_{NC}) / (I_{NC})) x 100; I=intensity] and cytotoxicity was calculated using the following formula: [(($I_{treatment}$ – I_{NC}) / (I_{PC} – I_{NC})) x 100].

2.5. Hemolytic assay

Ethical approval was obtained from the Local Research Ethics Committee of University of Nottingham prior to the experiment (Ref: 176-1812). Hemolytic assay was performed according to the previously established protocol.[39] Briefly, 5 ml of fresh human blood was collected from healthy participants with informed consent, in an EDTA tube and centrifuged for 10 mins at 1300 g at 10 °C for separation of plasma. The remaining erythrocytes were rinsed and centrifuged further three times in Ca^{2+}/Mg^{2+} free Dulbecco's phosphate-buffered saline (DPBS). Subsequently, erythrocytes were diluted to 8% v/v in DPBS and incubated with 100 μl of treatment, positive control (1% Triton X-100) and negative control (DPBS), all in 1:1 ratio, for 1 hour (final erythrocytes concentration = 4% v/v). After 1 hour of incubation, the plate was centrifuged at 500 x g for 5 mins and 100 μl of supernatant of each well was transferred into a 96-well plate for measurement at OD_{540}. Hemolysis (%) was calculated as [($I_{treatment}$ – I_{NC}) / (I_{PC} – I_{NC})] x 100.

2.6. Time-kill kinetics assay

Time-kill kinetics assay was performed to determine the time-dependent and concentration-dependent in vitro antimicrobial effects of the peptide (0.25x and 2x MIC) and amikacin, a commonly used topical antibiotics for BK (8x and 20x MIC), against 100 μl of ~1 x 10^6 CFU/ml of SH1000 at various time points, including 0 min (pre-treatment), 15 mins, 30 mins, 60 mins, 2 hours, 4 hours, and 24 hours. At each time point, 10 μl of the treated bacteria was transferred to an Eppendorf tube containing 90 μl of PBS, which was then serially diluted in 1:10 concentration for inoculation on agar plates and incubated overnight for 18-21 hours at 37 °C for enumeration of CFU.

2.7. In vivo efficacy and safety studies

The in vivo studies were conducted in two stages, namely the corneal wound healing study (for safety) and BK study (for efficacy), based on previously established protocols.[40, 41] Wild-type C57BL/6J mice (8-9 weeks old, male, average weight of 25g) were used in view of the consistent and reproducible results demonstrated in previous studies. The mice were maintained and treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council) and the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. All animal studies were conducted at the Singapore Eye Research Institute, Singapore, and were approved by the Animal Welfare & Ethical Review Body (AWERB), University of Nottingham, UK (Ref: UoN-Non-UK #16), the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) of SingHealth, Singapore (Ref: 2019/SHS/1491). General anesthesia was administered using intraperitoneal injections of xylazine (10 mg/kg) and ketamine (80 mg/kg)], and a drop of topical proxymetacaine hydrochloride 0.5% was administered immediately before wounding and/or infecting the corneas. Upon completion of the experiments, all mice were sacrificed according to the method of humane killing set out in Animals (Scientific Procedures) Act 1986 Schedule 1 using overdose of general anesthesia via intraperitoneal route.

2.7.1. In vivo corneal wound healing study

Drug drainage, blinking and tear film were considered during dosing translation from in vitro to in vivo use.[42] Based on the MIC of CaD23 against *S. aureus* ATCC 29213 (= 25 μg/ml), a range of concentration of CaD23 was chosen, including 0.03% (12x MIC), 0.05% (20x MIC) and 0.1% (40x MIC).

In vivo safety of CaD23 (in 0.03%, 0.05% or 0.1%), and PBS (negative control) was first determined in a mouse corneal epithelial wound healing model. Four mice were randomly allocated to each of the four treatment groups. Prior to the study, all eyes were examined with slit-lamp biomicroscopy to confirm the health of corneas. Under general and topical

anesthesia, the central 2 mm corneal epithelium was gently debrided with sterile Beaver mini-blades, leaving the basal lamina intact. Each treatment was applied immediately after wounding, then 4 times a day at 3-hour interval for 3 days (total dose of treatment per mouse = 14). Corneal epithelial defect was assessed using a cobalt-blue filter-equipped slit-lamp biomicroscopy and photography with staining with topical sodium fluorescein 1% at baseline (immediately post-debridement) and daily up to 3 days post-treatment. Fluorescein-stained images of the corneal wound defect were analyzed using ImageJ software. The main outcome measure was the wound size at the end of day 1, 2 and 3 [expressed as % of the original wound size in mean and standard deviation (SD)]. Difference in the wound size between groups was analysed using one-way ANOVA with Dunnett's post hoc test (PBS as the control group).

2.7.2. In vivo *S. aureus* keratitis study

Based on the in vivo safety data, the highest tolerable concentration, 0.05%, of CaD23 was used in the subsequent *S. aureus* keratitis murine model. Levofloxacin 0.5% (a commonly used antibiotic for BK in clinical setting) and PBS were used as the positive and negative controls, respectively. Five mice were randomly allocated to each treatment group. Slit-lamp examination was performed before the start of experiment to confirm the health of corneas. Under general and topical anesthesia, the central 2 mm corneal epithelium was gently removed with sterile Beaver mini-blades. 10 μl of ~$1x10^8$ CFU/ml of ATCC SA29213 was applied topically onto the cornea and the lid was held shut for 1 min. At 6 hours post-infection, 10 μl of treatment was applied directly onto the infected corneas with a dose regimen of 4 times a day at 3-hour interval for 3 days (total dose of treatment per mouse = 12). The eyes were monitored daily using slit-lamp biomicroscopy and photography. At the end of day 3, all animals were sacrificed and infected eyes were enucleated. The whole corneas were subsequently dissected, and homogenized in 1 ml of sterile PBS using sterile glass micro-beads. The homogenised infected corneal tissue suspension was serially diluted in 1:10 and plated on TSA plates in triplicates for enumeration of CFU after 24 hours

incubation at 37 °C. The main outcome measure was the residual bacterial load at 3-day post-treatment (expressed as $\log_{10}$ CFU/ml, which was the same as $\log_{10}$ CFU/cornea) and the difference between groups was analyzed using one-way ANOVA with Dunnett's post hoc test (PBS as the control group).

3. RESULTS

3.1. In vitro antimicrobial efficacy of HDPs

A total of 12 synthetic HDPs were rationally designed and synthesized based on the templates of native LL-37 and HBD-1 to -3 (**Table 1**). The MIC values (in μg/ml and μM) of all the tested antibiotics, single and hybrid peptides, in the absence and presence of 150 mM NaCl are summarized in **Table 2**. All 3 single linear, short-sequenced (6-12 amino acids) peptides (based on HBD-2, HBD-3 and LL-37) and 6 first-generation hybrid HDPs (based on different combinations of LL-37, and HBD-1 to -3) did not demonstrate any significant antimicrobial efficacy (MIC>200 μg/ml) against Gram-positive and Gram-negative bacteria. We further generated 3 second-generation peptides (derived from CaD2 sequence) through rational modification: (1) CaD21: substitution of cysteine with alanine; (2) CaD22: substitution of phenylalanine with tryptophan (to increase hydrophobicity); and (3) CaD23: substitution of phenylalanine and proline with tryptophan (to further enhance hydrophobicity) and substitution of cysteine with leucine. CaD21 and CaD22 demonstrated slight improvement in the antimicrobial efficacy whereas CaD23 exhibited good antimicrobial efficacy against MSSA (MIC=12.5-25.0 μg/ml), MRSA (MIC=25 μg/ml), MSSE (MIC=12.5 μg/ml) and MRSE (MIC=3.1 μg/ml), highlighting the importance of increased hydrophobicity against Gram-positive bacteria (**Table 1**). Moderate efficacy of CaD23 was observed against *P. aeruginosa* (MIC=50 μg/ml). When tested in the presence of physiological tear salt concentration, the MIC of CaD23 against MSSA, MRSA and MRSE increased by 2- to 4-fold, and remained unchanged for MSSE and *P aeruginosa*.

3.2. In vitro cell viability and cytotoxicity

Amikacin and CaD2 did not demonstrate any lethal effect on HCE-2 cell viability at 200 μg/ml (**Figure 1A**). The IC_{50} (concentration that inhibits 50% of the cell viability) of CaD21, CaD22 and CaD23 were >200 μg/ml, >200 μg/ml and 54.6 ± 11.7 μg/ml, respectively. This demonstrated that the increased hydrophobicity with tryptophan residues enhanced the antimicrobial efficacy of CaD23 but with increased negative effect on the cell viability of HCE-2 cells. In terms of cytotoxicity, amikacin and CaD2 did not show any sign of toxicity for HCE-2 cells at 200μg/ml and CaD23 showed 30.4 ± 7.8% cytotoxicity at 200 μg/ml (**Figure 1B**). The LC_{50} (concentration that kills 50% of the cells) of CaD23 was >200μg/ml, which yielded a therapeutic index (defined by LC_{50} divided by the MIC value) of >8 for treating *S. aureus* ATCC29213. Hemolytic assay demonstrated minimal (7.1 ± 3.0%) hemolytic activity of CaD23 at 200 μg/ml (**Figure 2**). A summary of the cell viability, cytotoxicity and haemolytic results of CaD23 is provided in **Table 3**. In addition, a summary of the therapeutic index of CaD23 (defined as IC_{50}, LC_{50} or HC_{50} divided by the MIC value) is provided in **Table 4**.

3.3. Time-kill kinetics

Time- and concentration-dependent antimicrobial activity of CaD23 was determined against MSSA (SH1000) using time-kill kinetics assay. When CaD23 was used at 2x MIC (25 μg/ml) against SH1000, it was able to achieve significant killing (99.9% or 3 $\log_{10}$ CFU/ml reduction) with 15 mins and complete killing (100%) within 30 mins of treatment (**Figure 3**). In contrast, amikacin (used at 20x MIC; 25 μg/ml) could only achieve significant and/or complete killing (99.9-100%) of SH1000 within 4 hours of treatment, which was 8 times slower than CaD23. The antimicrobial efficacy of CaD23 (25 μg/ml) and amikacin (both 10 μg/ml and 25 μg/ml) were maintained at 24 hours' time-point, with no evidence of bacterial re-growth.

3.4. In vivo safety of CaD23

In view of the known discrepancy between in vitro and in vivo results, the safety of CaD23 was further determined in a murine corneal epithelial wound healing model. When compared

to the PBS group, both CaD23 0.03% (or 300 μg/ml) and CaD23 0.05% (or 500 μg/ml) groups did not show any significant difference in the rate of corneal re-epithelialization (all healed within 2-3 days), suggesting that both concentrations were safe for topical application (**Figure 4**). However, significant delay in corneal re-epithelialization was observed in the CaD23 0.1% group, with a mean wound size of 28.5 ± 19.9% at day 3 post-injury (p=0.004).

3.5. In vivo efficacy of CaD23

Based on the in vivo safety results, the highest tolerable concentration of CaD23 0.05% was subjected to subsequent in vivo efficacy testing in a murine *S. aureus* ATCC 29213 BK model. As the data was not normally distributed and was log-transformed, the results were reported in median ± interquartile range (IQR). When compared to the *S. aureus* bacterial viability in the PBS group (4.2 ± 1.3 $\log_{10}$ CFU/ml), there was a considerable reduction of bacterial viability in the CaD23 0.05% and levofloxacin 0.5% groups by 94% (3.0 ± 2.4 $\log_{10}$ CFU/ml; p=0.72) and 98% (2.4 ± 2.2 $\log_{10}$ CFU/ml; p=0.08), respectively, though statistical significance was not achieved (**Figure 5**).

4. DISCUSSION

IK represents a persistent and uncurbed burden on human health at a global level. Topical antibiotics are the current mainstay of treatment for BK but the efficacy is being increasingly challenged by the emergence of antimicrobial resistance.[2, 12] In addition, patients with BK often require long duration of treatment and sight-threatening complications may still ensue despite timely intervention,[8] highlighting an unmet need for newer and better treatment. HDPs serve as an attractive class of antibiotics for treating IK based on the following reasons: (1) the broad-spectrum activity of HDPs can provide comprehensive coverage to a wide range of microorganisms, particularly when mixed infection is relatively common (5-20%) in IK[2]; (2) the rapid antimicrobial action help reduce the microbial load and limit the damage to the cornea more effectively, ultimately have a better chance of preserving the vision, as well as reducing the risk of developing antimicrobial resistance, which is currently

an emerging issue in ocular infection[12]; and (3) HDPs can be used as synergistic or additive agents to the current conventional antibiotics, enhancing the therapeutic index by increasing the antimicrobial efficacy and reducing the dose-related toxicity.[39, 43]

In this study, we highlight a body of work in designing and developing human-derived hybrid HDP as topical treatment for BK. Hybridization strategy has been previously employed by several research groups to improve the therapeutic index of HDPs[36, 37, 44] but the strategy of using human-derived hybrid HDPs (with LL-37 and HBD-2) is first of its kind. The initial concept of developing synthetic HDPs derived from LL-37 and HBD was founded on the observation of the upregulation of these key HDPs at the ocular surface during IK.[20, 24, 30, 45] Furthermore, LL-37 and HBD-2 and -3 were shown to exhibit good antimicrobial efficacy against a range of organisms.[31, 45] We have selected the mid-region of LL-37, consisting of residues 18-29 of LL-37 (i.e. KR-12) as part of the hybrid template as studies have demonstrated that KR-12, though much shorter than parent LL-37, exhibited similar antimicrobial activity against *E. coli* (MIC = ~64 μg/ml) with reduced toxicity to host cells.[46] C-terminal of HBD1-3 was used as the other part of the hybrid template in view of its rich content of cationic residues, which have been shown to play an important role in interacting with anionic bacterial membrane and killing of bacteria.[47, 48]

Our initial attempt of engineering single linear HDPs (including LL-37 and HBD-2 and -3) and hybrid HDPs (based on LL-37 and HBD-1 to -3) did not yield any compound with good antimicrobial efficacy. However, with systematic SAR analysis and modification, particularly through substitution of proline and phenylalanine with tryptophan residues (to increase hydrophobicity and membrane partitioning), ensued the development of peptide with enhanced antimicrobial efficacy against MSSA (MIC=12.5μg/ml) and MRSA (MIC=25μg/ml), serving as a proof-of-concept of this novel design strategy. Interestingly, increased hydrophobicity augmented the antimicrobial activity of HDP against Gram-positive bacteria but not Gram-negative bacteria. This is likely attributed to the different compositions in the

bacterial membrane between Gram-positive bacteria, which consist of a thick peptidoglycan layer, and Gram-negative bacteria, which comprises an additional outer membrane with abundance of negatively charged lipopolysaccharide for which cationicity of the peptide plays a more important role.[49] In addition, the efficacy of CaD23 (18 amino acids in length) is at least equal to or stronger than the full-length parent LL37 (MIC = 25-50 μg/ml) and HBD-1 to -3 peptides (MIC = 50-100 μg/ml).[31, 39] Peptides with shorter sequence not only have the advantage of lower manufacturing cost but may also have a lower risk of inducing immunogenicity.[50] Moreover, the antimicrobial efficacy of CaD23 is comparable to some of the HDPs that are developed for ocular surface infection, including esculentin-1a(1-21)NH2,[51] RP444,[42] melimine and its derivatives,[37, 52] and ε-lysylated Mel-4.[41]

In the time-kill kinetics study, we demonstrated that CaD23 at 2x MIC was able to achieve complete killing of *S. aureus* within 30 mins as compared to 4 hours with amikacin at 20x MIC (i.e. 8 times slower than CaD23). We also observed that increasing the concentration of amikacin from 8x MIC to 20x MIC did not expedite its anti-bacterial action against *S. aureus*, suggesting that the speed of bacterial killing is more related to the underlying mechanism of action than the concentration of antibiotic. The rapid killing action of CaD23 is likely related to a membrane perturbation effect, which is in contrast to amikacin where it exerts its anti-bacterial activity via intracellular inhibition of the 30S subunit of bacterial ribosome.[53] Theoretically, the rapid and membrane disruptive action of CaD23 should result in a lower risk of developing antimicrobial resistance as the bacteria has less time to adapt and require substantial modification of genome to develop effective resistant mechanisms, which are shown in conventional antibiotics.[41] Further studies examining the underlying mechanism of action of CaD23 and its tendency to develop antimicrobial resistance would be valuable.

Based on the LDH cytotoxicity assay, CaD23 was shown to be relatively non-cytotoxic (20-30% toxicity) at 200 μg/ml, with a therapeutic index of >8-16 against *S. aureus* (defined by LC_{50} concentration divided by MIC value). In addition, the hemolytic activity of CaD23 at 200

μg/ml was only less than 10%. Interestingly, the IC_{50} value (based on cell viability assay) was around 50 μg/ml, considerably lower than the LC_{50} value. This discrepancy may be due to the fact that some of the cells were apoptotic instead of necrotic, which was detected by the cell viability assay but not the cytotoxicity assay. That said, our in vivo corneal epithelial wound healing study showed that CaD23 did not demonstrate any significant toxicity when used at 500 μg/ml (or 0.05%), which was 10 times higher than the IC_{50} value. This suggests that in vitro plate-based static assays such as LDH or cell viability assays could potentially overestimate the in vivo cytotoxicity of drugs that are developed for ocular topical application. The observed discrepancy between in vitro and in vivo findings is likely attributed to the inherent dynamic environment of ocular surface with eye blinking, high tears turnover, and drainage.[54] The shortcoming of in vitro assays may be addressed by the recently developed novel ex vivo biomimetic model where it could simulate the dynamic and complex interface between the ocular surface and the external environment, allowing a better prediction of the in vivo effect.[55]

Our in vivo BK study demonstrated that CaD23 0.05% (20x MIC) was able to reduce the bacterial viability of *S. aureus* by 94% when compared to the untreated control group, though the effect was not statistically significant. The insignificant result could be due to the fact that two mice did not respond well to the CaD23 treatment, though three mice had a 90-99% median reduction of bacterial load. In addition, the result of CaD23 was comparable to the levofloxacin, which is a commonly used antibiotic for BK in clinical setting. Future studies evaluating various drug dosage regimen of CaD23, including treatment frequency and duration, would be beneficial.

One potential approach to improve the therapeutic effect of HDP-derived antimicrobial treatment is to use them in combination with antibiotics as peptide-antibiotic synergism has been demonstrated in several studies.[39, 43, 56] This attractive antimicrobial strategy not only helps extend the lifespan and broaden the antibacterial spectrum of conventional antibiotics,

but also reduce the dose-dependent toxicity associated with HDPs and antibiotics.[57] Recently, our group has also shown that FK16, a cathelicidin-derived molecule, could improve the antimicrobial efficacy of vancomycin, a glycopeptide antibiotic, against PA by 8-fold.[39] This is likely ascribed to the different mechanisms of action between FK16 and vancomycin where the membrane disruptive action of FK16 facilitates the diffusion of vancomycin across the bacterial membrane. Hence, future work investigating the potential synergism between CaD23 and commonly used antibiotics for BK would be useful.

At present, our aim is to develop the human-derived hybrid HDPs as topical treatment for BK. However, it is interesting to note that the hemolytic activity of CaD23 was very low (7.1% at 200 μg/ml), suggesting that it can potentially be developed for treating systemic infection. It also reduces the concern of systemic toxicity when it is applied to the ocular surface because systemic absorption of ocular topical treatment can occur, particularly when the drugs are hydrophobic.[54] The considerable disparity between the hemolytic activity and cytotoxicity against corneal epithelial cells could be attributed to the difference in the membrane structure (particularly the cytoskeleton) and the metabolic structures of the non-nucleated cells (e.g. red blood cells) and nucleated cells (e.g. corneal epithelial cells).[58] Further studies examining the efficacy and stability of CaD23 in serum as well as its interaction with blood proteins will be conducted to determine its therapeutic potential for treating systemic infection.

In conclusion, we demonstrated that rational hybridization of LL-37 and HBD-2 serves as a novel strategy in translating the therapeutic potential of human-derived HDPs. Future work examining the efficacy and safety of combined CaD23-antibiotic therapy would be beneficial.

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FIGURE LEGENDS

Figure 1. Cytotoxicity of synthetic peptides and amikacin (a commonly used antibiotic for bacterial keratitis) in various concentrations against human corneal epithelial cells (HCE-2), presented as dose-response curves (normalized, variable slope). Percentage cell viability is presented as mean ± standard deviation (depicted in error bars) of two independent experiments performed in biological duplicate. Some error bars are missing due to small standard deviation values. **(A)** Cell viability assay (using cell counting kit-8 assay) demonstrating normal metabolic activity of epithelial cells in CaD2 and amikacin but reduced activity in CaD23 (IC_{50} = 54.6 ± 11.7 μg/ml) after 3 h of treatment. IC_{50} (concentration of treatment inhibiting 50% of cell viability) is shown in a black dotted line. **(B)** Cytotoxicity assay (using lactate dehydrogenase assay) demonstrating no sign of cytotoxicity of epithelial cells in amikacin and CaD2, and low level of cytotoxicity in CaD23 (30.4 ± 7.8% at 200 μg/ml; LC_{50} > 200 μg/ml) after 3 h of treatment.

Figure 2. Hemolytic effect of CaD23 and levofloxacin (a commonly used antibiotic for bacterial keratitis) in various concentrations against fresh human erythrocytes, determined after 1 hour of treatment. Percentage hemolysis is presented as mean ± standard deviation (depicted in error bars) of two independent experiments performed in biological duplicate. Some error bars are missing due to small standard deviation values. The graph demonstrating minimal hemolytic effect of CaD23 against fresh human erythrocytes (only 7.1 ± 3.0% at 200 μg/ml).

Figure 3. Time-kill kinetics of CaD23 [at 3.1 μg/ml (or 0.25x MIC) and 25 μg/ml (or 2x MIC)] and amikacin [at 10 μg/ml (or 8x MIC) and 25 μg/ml (or 20x MIC)] against *S. aureus* (SH1000) over 24 hours. Phosphate buffer solution (PBS) group serves as the untreated control. “0 min” represents the starting inoculum, which is around 6 log_{10} CFU/ml. The red dotted horizontal line at 3 log_{10} CFU/ml signifies the threshold of significant bacterial killing (defined as 99.9% or 3 log_{10} CFU/ml reduction of the bacterial viability compared to the

starting inoculum). Data is presented as mean ± standard deviation (depicted in error bars) of two independent experiments performed in biological duplicate. CaD23 [at 25 μg/ml (or 2x MIC)) was able to achieve complete (100%) killing of SH1000 within 30 mins of treatment whereas amikacin [at 10 μg/ml (or 8x MIC) and 25 μg/ml (or 20x MIC)] was only able to achieve complete killing of SH1000 within 4 hours of treatment. The antimicrobial efficacy of CaD23 and amikacin was maintained at 24 hours post-treatment.

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Figure 4. In vivo safety of CaD23 in various concentrations [0.03% (or 300 μg/ml), 0.05% (or 500 μg/ml), and 0.1% (1 mg/ml)] and phosphate buffer solution (PBS) assessed in a murine corneal epithelial wound healing model (n = 4 mice / treatment group). **(A)** Representative slit-lamp images showing the daily progress of corneal wound healing of each treatment group. The green color-stained area depicts the corneal epithelial defect. Complete corneal re-epithelialization was observed in all treatment groups, except CaD23 0.1% group, by day 3. **(B)** Graphical summary of the progress of corneal re-epithelialization of each treatment group over 3 days. The corneal epithelial wound size at various time points is calculated based on the original 100% wound size at baseline. Data is presented as mean ± standard deviation.

Figure 5. In vivo efficacy of CaD23 0.05% (500 μg/ml), levofloxacin 0.5% (positive control) and phosphate buffer solution (PBS; negative control) in a murine *S. aureus* ATCC SA29213 bacterial keratitis model (n = 5 mice / treatment group). **(A)** Representative slit-lamp images showing the corneal appearance over 3 days post-infection in each treatment group. Note the significant infiltrative changes of cornea in the PBS group as compared to the CaD23 0.05% and levofloxacin 0.5% groups. **(B)** Scatter plot showing the bacterial viability of *S. aureus* (in $\log_{10}$ CFU/ml) after 3 days of treatment. In view of the wide range of results, data is presented as median ± interquartile range.

Table 2. A summary of the minimum inhibitory concentrations (MICs) of various antibiotics and synthetic human-derived hybrid host defense peptides. All experiments were performed in full-strength cationic Muller-Hinton broth (i.e. MHB-2) and in the absence or presence of physiological tear salt concentration (150mM NaCl). The MIC values are presented in μg/ml (μM). When the MIC level was >200 μg/ml in the absence of salt, the peptide was not subjected to testing in the presence of salt.

Class of agent	**Agents**	**SH1000**		**SA29213**		**MRSA**		**MRSA43300**		**SE12228**		**MRSE**		**PAO1-L**	
	μg/ml (μM)	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*
Antibiotics	Amikacin	1.25 (2.13)	10 (17.1)	-	-	10 (17.1)	10 (17.1)	-	-	-	-	1.25 (2.13)	5 (8.53)	0.63 (1.08)	1.25 (2.13)
	Levofloxacin	0.31 (0.86)	0.31 (0.86)	-	-	0.31 (0.86)	0.31 (0.86)	-	-	-	-	0.31 (0.86)	0.31 (0.86)	0.31 (0.86)	0.31 (0.86)
First-generation hybrid HDPs	DD12	>200 (>115)	-	-	-	>200 (>115)	-	-	-	-	-	>200 (>115)	-	>200 (>115)	-
	DD13	>200 (>92.3)	-	-	-	>200 (>92.3)	-	-	-	-	-	>200 (>92.3)	-	>200 (>92.3)	-
	D32	>200 (>101)	-	-	-	>200 (>101)	-	-	-	-	-	>200 (>101)	-	>200 (>101)	-
	CaD1	>200 (>94.7)	-	-	-	>200 (>94.7)	-	-	-	-	-	>200 (>94.7)	-	>200 (>94.7)	-
	CaD2	>200 (>88.5)	-	-	-	>200 (>88.5)	-	-	-	-	-	>200 (>88.5)	-	>200 (>88.5)	-
	CaD3	>200 (>91.8)	-	-	-	>200 (>91.8)	-	-	-	-	-	>200 (>91.8)	-	>200 (>91.8)	-
Second-generation hybrid HDPs	CaD21	200 (89.8)	>200 (>89.8)	-	-	>200 (>89.8)	-	-	-	-	-	50 (22.4)	100 (44.9)	100 (44.9)	50 (22.4)
	CaD22	100 (43.5)	>200 (87.0)	-	-	>200 (>87.0)	-	-	-	-	-	6.3 (2.7)	25 (10.9)	200 (87.0)	200 (87.0)
	CaD23*	12.5 (5.2)	50 (20.8)	25 (10.4)	50 (20.8)	25 (10.4)	100 (41.7)	25 (10.4)	100 (41.7)	12.5 (5.2)	12.5 (5.2)	3.1 (1.3)	6.3 (2.6)	50 (20.8)	50 (20.8)

MIC refers to the lowest concentration of antibiotic / peptide that prevents any visible bacterial growth after 24 hours of incubation with treatment.

Data represent the mean of two biological duplicate from two to three independent experiments.

*The hybrid HDP that demonstrated good antimicrobial efficacy against Gram-positive bacteria and moderate efficacy against Gram-negative bacteria.

Table 3. Summary of the cytotoxicity, cell viability and hemolytic results of antibiotics and synthetic peptides (in µg/ml concentration). The cytotoxicity and cell viability results were obtained after 3 hours of treatment whereas hemolytic effect of treatment was examined after 1 hour of treatment.

Types	Agents	LC_{50}	L_{max} (%)	IC_{50}	I_{max} (%)	HC_{50}	H_{max} (%)
Antibiotics	Amikacin	>200	0.0 (0.1)	>200	100.1 (7.3)	>200	0.0
	Levofloxacin	>200	1.2 (0.7)	>200	108.0 (2.8)	>200	0 (0.0)
First-generation peptides	DD12	-	-	-	-	-	-
	DD13	-	-	-	-	-	-
	DD32	-	-	-	-	-	-
	CaD1	-	-	-	-	-	-
	CaD2	>200	2.9 (2.2)	>200	98.1 (7.3)	-	-
	CaD3	-	-	-	-	-	-
Second-generation peptides	CaD21	-	-	>200	56.1 (1.0)	-	-
	CaD22	-	-	>200	65.7 (8.2)	-	-
	CaD23	>200	26.6 (6.5)	54.6 (11.7)	30.4 (7.8)	>200	7.1 (3.0)

LC_{50} = Concentration of treatment causing 50% cytotoxicity; L_{max} (%) = Percentage of cytotoxicity at 200 µg/ml treatment concentration; IC_{50} = Concentration of treatment causing 50% inhibition of cell viability; I_{max} (%) = Percentage of inhibition of cell viability at 200 µg/ml treatment concentration; HC_{50} = Concentration of treatment causing 50% haemolysis; H_{max} (%) = Percentage of haemolysis at 200 µg/ml treatment concentration

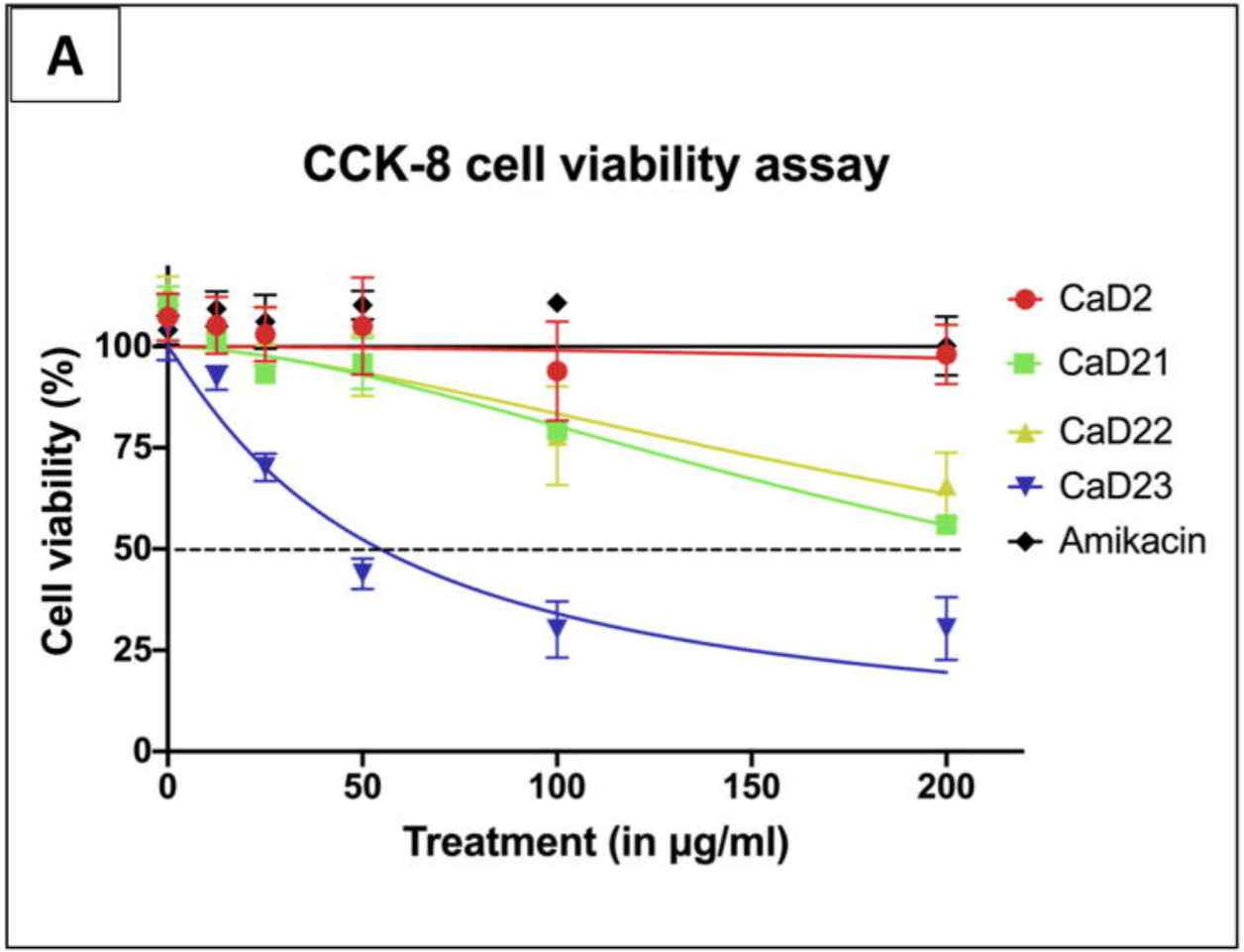
Results are presented in mean (SD) of two independent experiments performed in biological duplicate. Toxicity results of some peptides were missing because their antimicrobial efficacy was poor and hence toxicity was not determined.

Table 4. Summary of therapeutic index of CaD23 determined based on cytotoxicity, cell viability, and hemolytic results. The minimum inhibitory concentration (MIC) values and therapeutic index (TI) values are presented in mean values.

µg/ml	***S. aureus* SH1000**		***S. aureus* ATCC29213**		**Clinical MRSA**		**MRSA ATCC43300**		***P. aeruginosa* PA01-L**	
	MIC	**TI**	**MIC**	**TI**	**MIC**	**TI**	**MIC**	**TI**	**MIC**	**TI**
Based on LC50 (= >200)	12.5	>16	25	>8	25	>8	50	>4	50	>4
Based on IC50 (= 55)	12.5	4.4	25	2.2	25	2.2	50	1.1	50	>1.1
Based on HC50 (= >200)	12.5	>16	25	>8	25	>8	50	>4	50	>4

LC_{50} = Concentration of treatment causing 50% cytotoxicity; IC_{50} = Concentration of treatment causing 50% inhibition of cell viability; HC_{50} = Concentration of treatment causing 50% hemolysis

MRSA = methicillin-resistant *Staphylococcus aureus*


A
CCK-8 cell viability assay
Cell viability (%)
100
75
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0
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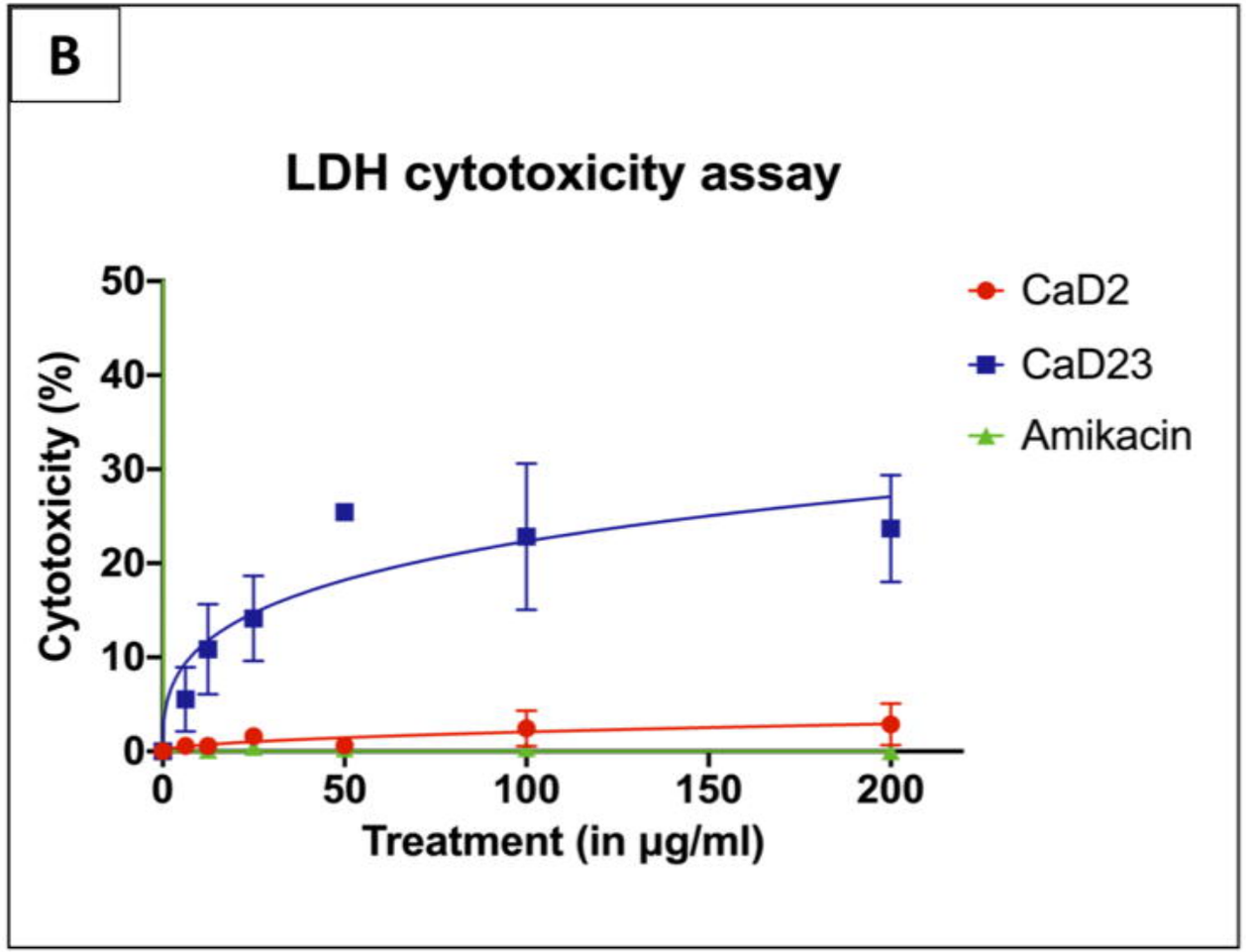
100
150
200
Treatment (in µg/ml)
CaD2
CaD21
CaD22
CaD23
Amikacin


B
LDH cytotoxicity assay
Cytotoxicity (%)
50
40
30
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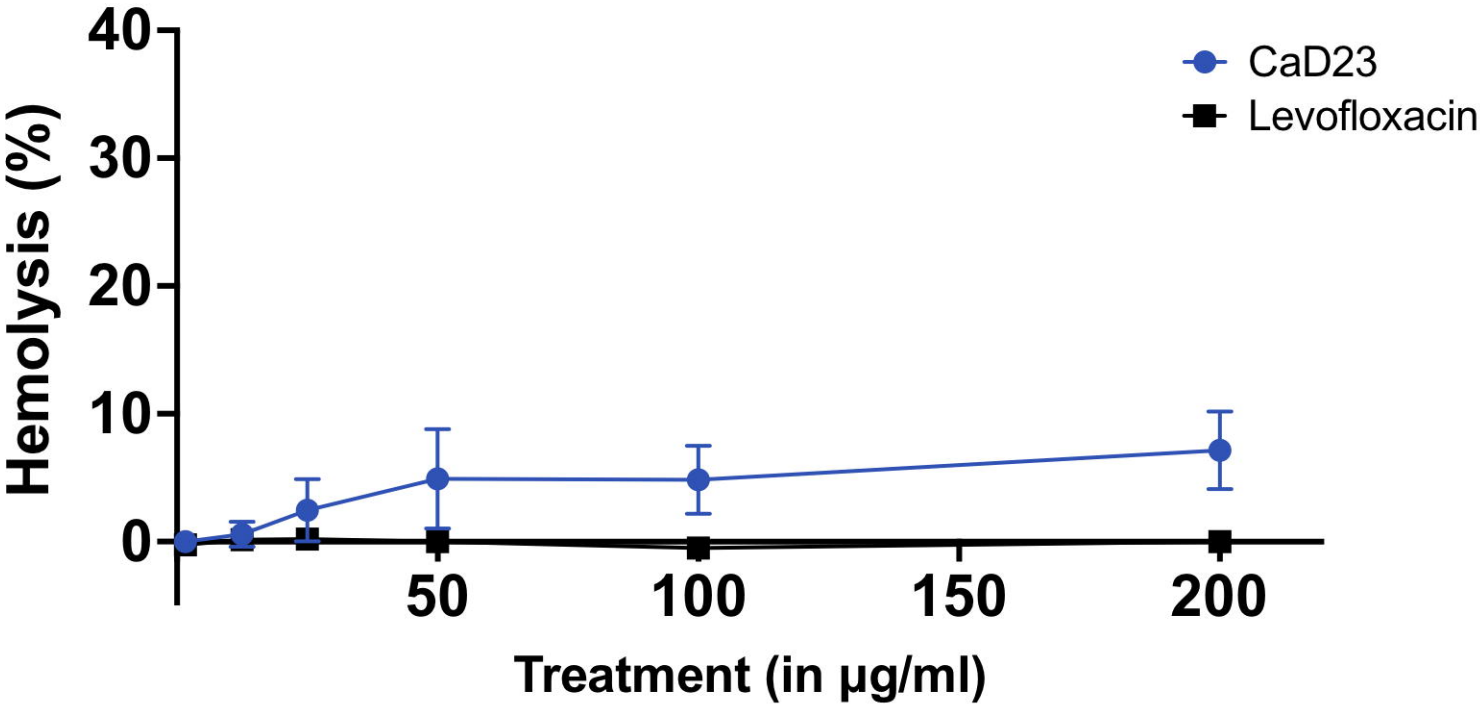
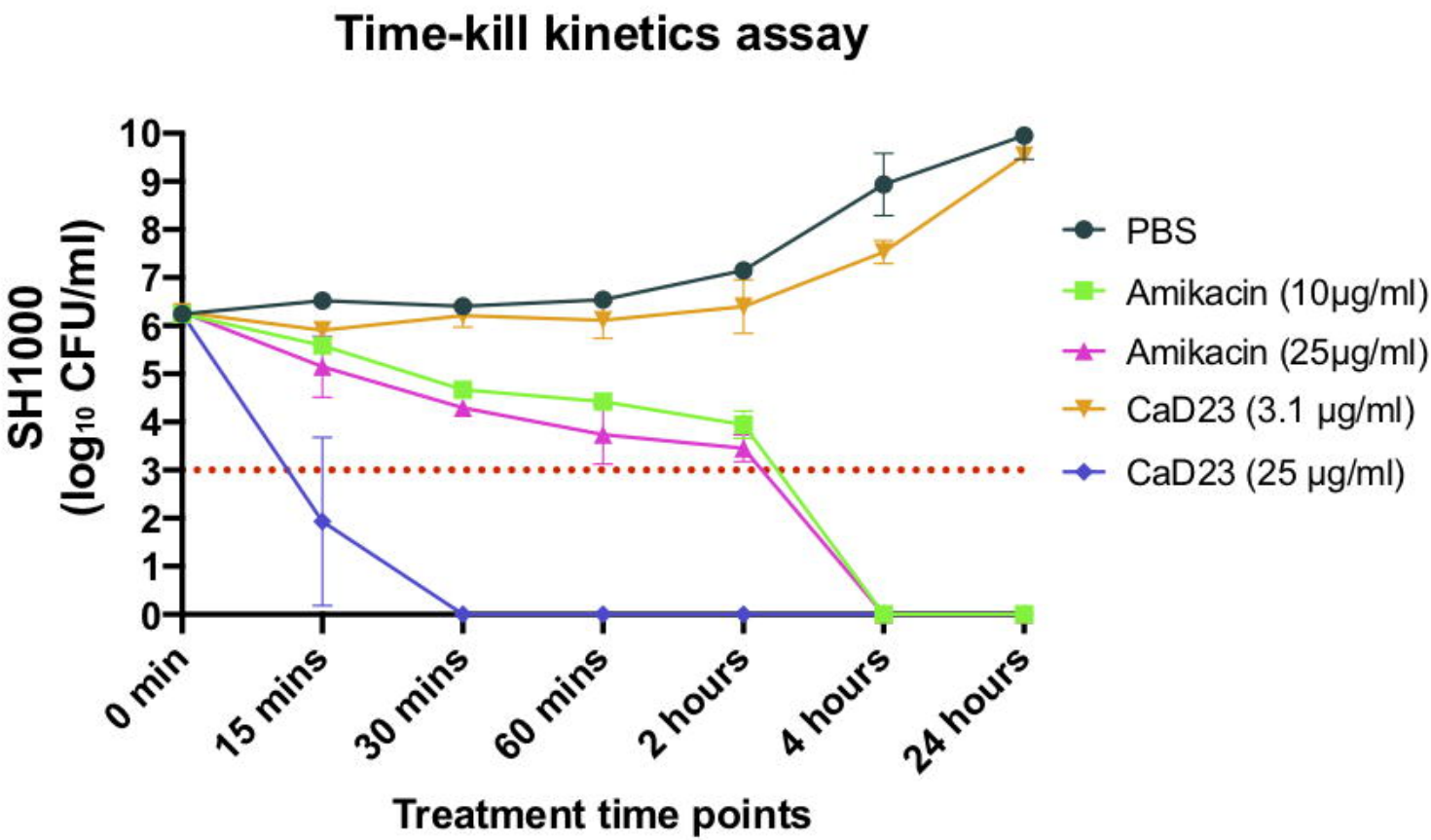
100
150
200
Treatment (in µg/ml)
CaD2
CaD23
Amikacin

Hemolytic activity




Time-kill kinetics assay
SH1000
(log10 CFU/ml)
10
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8
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6
5
4
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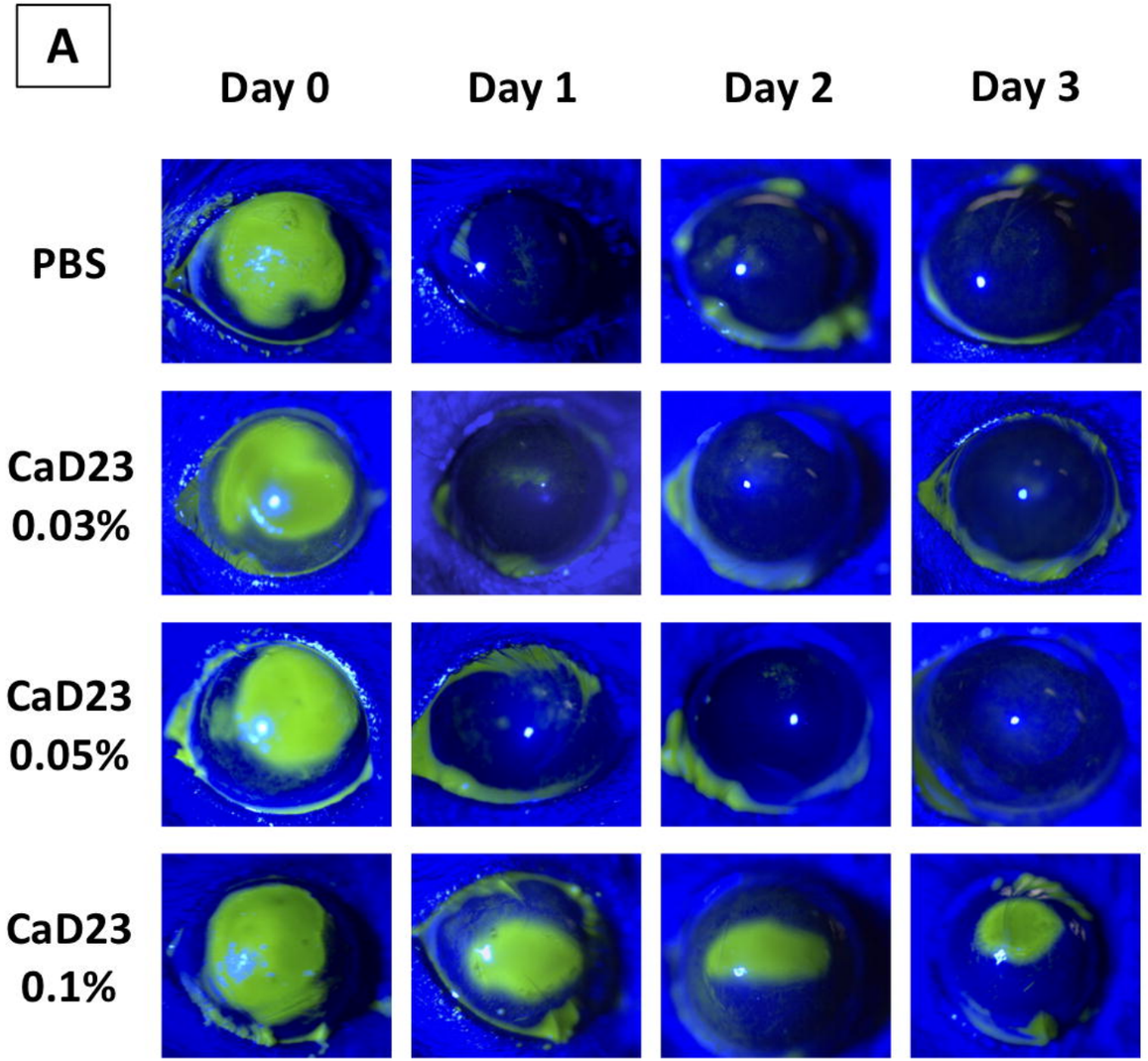
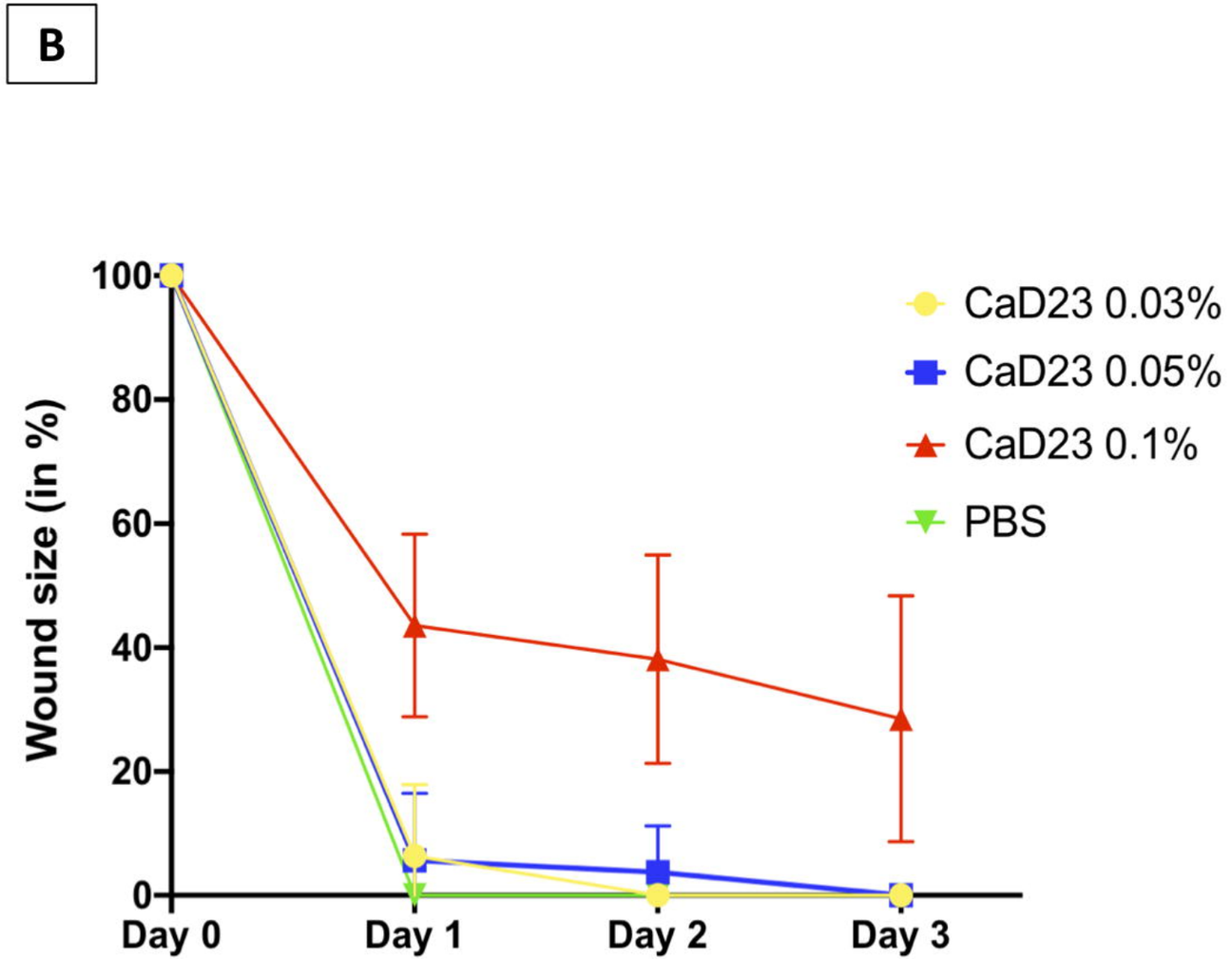
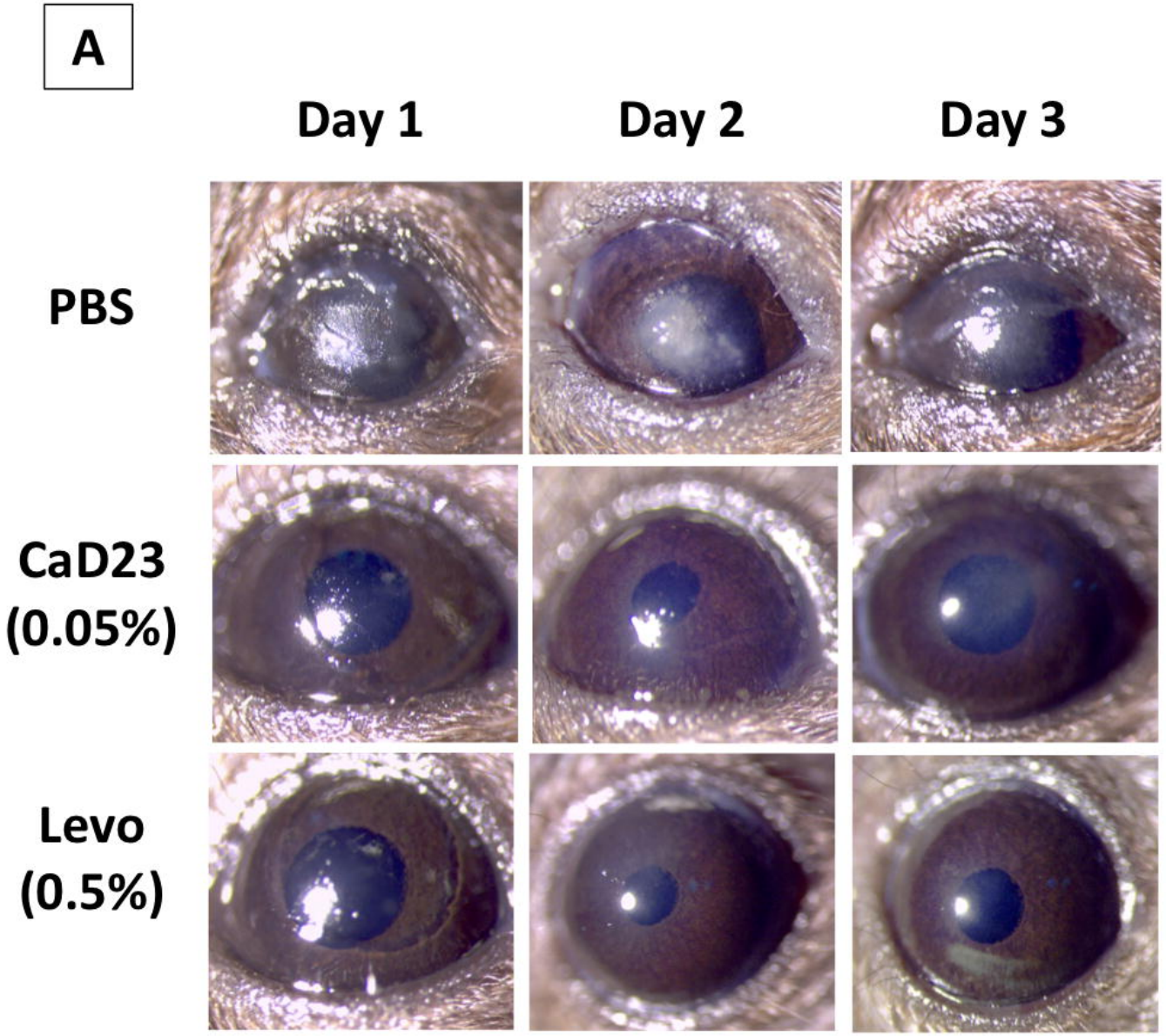
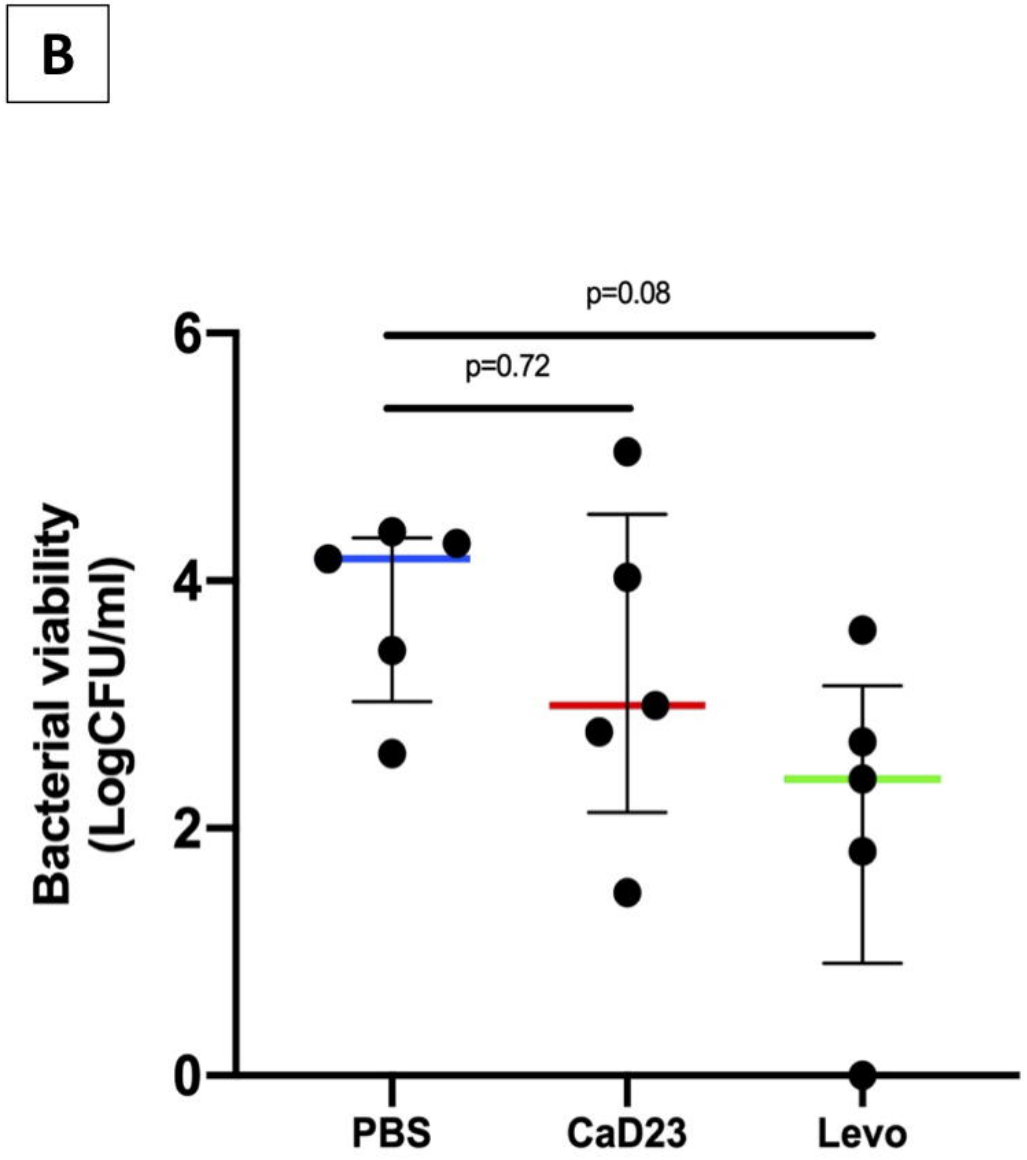
0 min
15 mins
30 mins
60 mins
2 hours
4 hours
24 hours
Treatment time points
PBS
Amikacin (10µg/ml)
Amikacin (25µg/ml)
CaD23 (3.1 µg/ml)
CaD23 (25 µg/ml)


A
Day 0
Day 1
Day 2
Day 3
PBS
CaD23 0.03%
CaD23 0.05%
CaD23 0.1%


B
Wound size (in %)
100
80
60
40
20
0
Day 0
Day 1
Day 2
Day 3
CaD23 0.03%
CaD23 0.05%
CaD23 0.1%
PBS


A
Day 1
Day 2
Day 3
PBS
CaD23
(0.05%)
Levo
(0.5%)


B
p=0.08
p=0.72
Bacterial viability
(LogCFU/ml)
6
4
2
0
PBS
CaD23
Levo