Antimicrobial peptide novicidin synergises with rifampicin, ceftriaxone and ceftazidime against antibiotic-resistant *Enterobacteriaceae in vitro*

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ABSTRACT

The spread of antibiotic resistance amongst Gram-negative bacteria is a serious clinical threat and infections with these organisms are a leading cause of mortality worldwide. Traditional novel drug development inevitably leads to the emergence of new resistant strains, rendering the new drugs ineffective. Therefore, reviving the therapeutic potentials of existing antibiotics represents an attractive novel strategy. Novicidin, a novel cationic antimicrobial peptide, is effective against Gram-negative bacteria. Here, we investigated novicidin as a possible antibiotic enhancer. The actions of novicidin in combination with rifampicin, ceftriaxone and ceftazidime were investigated against 94

antibiotic resistant clinical Gram-negative isolates and 7 strains expressing New Delhi metallo-β-lactamase-1 (NDM-1). Using the chequerboard method, novicidin combined with rifampicin showed synergy with over 70% of the strains, reducing the minimum inhibitory concentrations (MIC) significantly. The combination of novicidin with ceftriaxone and ceftazidime was synergistic against 89.7% of ceftriaxone-resistant strains and 94.1% of ceftazidime-resistant strains. Synergistic interactions were confirmed using time kill studies with multiple strains. Furthermore, novicidin increased the post-antibiotic effect (PAE) when combined with rifampicin or ceftriaxone. Membrane depolarisation assays revealed that novicidin alters the cytoplasmic membrane potential of Gram-negative bacteria. *In vitro* toxicology tests showed novicidin to have low haemolytic activity and no detrimental effect on cell cultures. We demonstrated that novicidin strongly rejuvenates the therapeutic potencies of ceftriaxone or ceftazidime against resistant Gram-negative bacteria *in vitro*. In addition, novicidin boosted the activity of rifampicin. This strategy can have major clinical implications in our fight against antibiotic resistant bacterial infections.

INTRODUCTION

Bacterial infections remain one of the leading causes of death worldwide. The ever escalating problem of antibiotic resistance leads to the redundancy of many antibiotics, resulting in increased morbidity and mortality in both developed and developing countries. In particular, the effectiveness of antimicrobial agents against Gram-negative pathogens, for example *Enterobacteriaceae*, are being compromised at an alarming rate (1).

Bacteria in the *Enterobacteriaceae* family cause an arsenal of serious infections including pneumonia, wound infections, meningitis, urinary tract infections, intra-abdominal infections (1) and nosocomial bacteriema (2). Extended spectrum β-lactamase (ESBL) producing strains now predominate in many areas, conferring resistance to cephalosporins and remaining sensitive only to carbapenems and the older, more toxic polymyxin antibiotics such as colistin (3). Furthermore, since 2007, infections with

New Dehli metallo-β-lactamase 1 (NDM-1) producing 'superbugs' have emerged. For these infections virtually all antibiotics, including carbapenems, are ineffective. Most NDM-1 strains are usually susceptible only to 'last line' drugs like colistin, which exhibits nepro- and neuro-toxicity (4), and the bacteriostatic glycylcycline tigecycline (5). The most optimal strategy to overcome resistant infections is to use novel antimicrobial agents. However, the traditional strategy of antibiotic discovery cannot maintain pace with the rapid rate of resistance emergence and resistance occurs just a few years after market release (6). In addition, the discovery of novel antibiotics is costly and arduous which means producing large numbers of antibiotic classes within a short period of time is extremely challenging (7-9).

Reviving the potency of existing antibiotics by combining them with novel agents is an extremely desirable strategy to tackle resistance (10). Antimicrobial peptides, in particular those targeting the bacterial cell envelope, have been shown to synergise with conventional antibiotics (11). The dual action of weakening of the cell envelope and increasing permeability may allow the intracellular antibiotic concentration to reach a lethal dose, which is unachievable by the antibiotic alone. Furthermore, the use of multiple agents in combination may reduce or retard the emergence of resistance to the individual antimicrobial components (10, 12).

It has been suggested that novicidin, a novel 18-residue cationic antimicrobial peptide, acts by inserting itself into the head group region of the selectively targeted bacterial membrane bilayer. This subsequently causes membrane perturbation, transient pore formation, and is bactericidal via the resulting leakage of bacterial cell contents (13-15). Significant antimicrobial effects have been noted with several Gram-negative organisms, such as *Escherichia coli* and *Salmonella enterica* (15). Novicidin was developed from ovispirin, which in turn originated from an ovine cathelicidin known as sheep myeloid antimicrobial peptide (SMAP)-29 (13). This derivement allowed for construction of a peptide more suitable for use as a therapeutic agent (14).

In this study, we aimed to investigate the effects of novicidin in combination with conventional antibiotics, namely rifampicin and third generation cephalosporins, ceftriaxone and ceftazidime, against 101 Gram-negative strains including resistant *E. coli* and bacteria in the *Klebsiella-Enterobacter-Serratia* (KES) group. Additionally, investigations were carried out to determine the mechanism of action, haemolytic activity and cytotoxicity of novicidin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used were 94 antibiotic resistant Gram-negative clinical isolates including 61 *E. coli* and 33 isolates in the KES group from St Georges Hospital, London. In addition, 7 strains harbouring the bla_{NDM} plasmid were used: ATCC BAA-2468, BAA-2469, BAA-2470, BAA-2471, BAA-2472 and BAA-2473 and NCTC 13443. Strain ATCC BAA-2468 is identified as *Enterobacter cloacae*; ATCC BAA-2469 and BAA-2471 as *E. coli*; and ATCC BAA-2470, BAA-2472, BAA-2473 and NCTC 13443 as *Klebsiella pneumoniae*. Bacterial strains were grown in nutrient broth no. 2 (Oxoid, UK) and on tryptone soya agar plates (Oxoid, UK). Antibiotics used were as follows: rifampicin (Sanofi), ceftriaxone (Stravencon), ceftazidime (Wockhardt), cefixime (Suprax) and cefotaxime (Reig Jofre). Antibiotics were prepared in water or the provided solvent to an appropriate concentration. Novicidin was kindly provided by Novozymes A/S, Denmark.

In vitro susceptibility of novicidin and antibiotics. The minimum inhibitory concentration (MIC) of novicidin, rifampicin, ceftriaxone, ceftazidime, cefixime and cefotaxime for the 101 strains were calculated using the broth micro dilution method. The MIC for each agent was identified as the lowest concentration required to inhibit bacterial growth. The MIC50 and MIC90 were calculated, defined as the lowest concentration required to inhibit growth in 50% and 90% of the strains respectively.

Chequerboard assays to measure combination effects of novicidin and antibiotics. The chequerboard assay method was used for the measurement of combination effects of novicidin with

the antibiotics. Combinations of two drugs were prepared in 96 well plates (Fisher Scientific UK) using drug concentrations starting from two fold higher than their MIC values, then serially diluted in a two-fold manner. After addition of a log-phase bacterial inoculum of 1-5 x 10^5 colony forming units (CFU)/ml, plates were incubated at 37°C for 24 hours. and then read using ELx800 absorbance microplate reader (BioTek). The effects of the combinations were examined by calculating the fractional inhibitory concentration index (FICI) of each combination as follows: (MIC of drug A, tested in combination)/(MIC of drug A, tested alone) + (MIC of drug B, tested in combination)/(MIC of drug B, tested alone). The profile of the combination was defined as synergistic if the FICI was ≤0.5, indifferent if the FICI was >0.5 but ≤4.0 and antagonistic if the FICI was >4 (16).

Time kill curves of antibiotics alone and in combination with novicidin. Two-fold serial drug dilutions were prepared and added to a 96-well plate alone and in combination, and incubated at 37°C with a log-phase bacterial inoculum of 1-5 × 10^7 CFU/ml. At 0, 1, 2, 4, 7 and 24 hours of incubation, viability expressed as CFU/ml was determined by plating 100 µl of serial dilutions onto tryptone soya agar (Oxoid) plates followed by incubation at 37°C for 24 hours. Colonies were counted using aCOLyte colony counter (Synbiosis) and analysed using the accompanying software. Synergistic activity was defined as a ≥2-$\log_{10}$ decrease in CFU counts at 24 hours of the combination compared with the most effective single agent, in addition to ≥2-$\log_{10}$ decrease compared with the 0 hour count. Indifference was defined as a ≤1-$\log_{10}$ fold change in CFU counts, and antagonism as a ≥2-$\log_{10}$ increase in CFU at 24 hours, of the combination compared with the most effective single agent (17).

Measurement of bacterial cytoplasmic membrane potential. The permeability of the bacterial cytoplasmic membrane after drug treatment was assessed using a fluorescence assay as previously described (18, 19). Log-phase cultures were washed twice and resuspended in a rejuvenating buffer (5 mM HEPES, pH 7.2, 20mM glucose) to an optical density of 0.05 at 600 nm. Membrane potential sensitive dye DiSC3(5) (3'3-Dipropylthiadicarbocyanine iodide, Sigma) was added to the resuspended cells to a final concentration of 0.4 µM and incubated until a stable reduction in fluorescence was

achieved as a result of DiSC3(5) uptake and cell quenching due to an intact membrane. 100 mM of KCl was added to equilibrate the K+ ion concentration intra- and extracellularly. The bacterial cell suspension was added to a 96-well microtitre plate, followed by addition of drugs in triplicate. Fluorescence was measured using GloMax-Multi+ microplate reader (Promega) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Any drug induced disruption of the cytoplasmic membrane resulted in an increase in measurable florescence.

Post-Antibiotic Effect (PAE) of antibiotics alone and in combination with novicidin. Bacteria were cultured overnight at 37°C in nutrient broth. 1 ml of the culture was transferred to fresh nutrient broth medium containing single or combinatory drugs. For the single drugs, 2, 5 or 10 fold higher than MIC values of the drug were utilised. For the combinations, 5 fold higher than the minimal enhancement concentrations of both drugs were selected according to the chequerboard results. After 1 hour of drug exposure, the cultures were washed three times to remove the antimicrobial agents. The bacterial cells were resuspended into nutrient broth and grown at 37°C with continuous shaking at 100 rpm. Bacterial viability was determined by CFU counting at 0, 1, 2, 3, 4, 6 and 8 hours. The PAE was calculated as follows: PAE = T – C, whereby T= time taken for drug exposed culture to increase by 1 log CFU counts, and C = time taken for control culture to increase by 1 log CFU counts (20).

***Ex vivo* haemolysis assay.** A venous blood sample from a male human donor was collected shortly before testing. 10 µl aliquots of the heparinized blood were added to 0.5 ml of saline solution (0.9% NaCl) containing different concentrations of novicidin in triplicate. After 1 hour of incubation at 37°C, the mixtures were centrifuged for 5 minutes at 5000 × g to sediment intact cells. The supernatants were isolated and the absorbance values were measured at a wavelength of 545 nm. Haemolysis of novicidin was analysed against negative (0% lysis) and positive controls (100% lysis) to calculate the percentage of haemolysed cells, using the formulae as follows: haemolysis = (OD_{test} - $OD_{negative\ control}$) / ($OD_{postitive\ control}$ – $OD_{negative\ control}$) x 100. An ethic approval (H-D-2007-0055) was obtained from Danish National Committee on Health Research Ethics for using human blood.

Assessment of cytotoxicity using neutral-red uptake assay. To assess the effects of cytotoxicity of novicidin, the L929 mouse fibroblast cell line was utilised. Cells were grown in Eagle's minimum essential medium (EMEM) with 10% foetal bovine serum (EBS) to 80% confluence. Adherent cells were harvested and seeded at a concentration 5×10^5 cells per well into a 96 well microtitre plate which was incubated for 24 hours at 37°C. Different concentrations of novicidin were added to the cells and incubated at 37°C for 24 and 72 hours. Neutral red (25 mg/L) was added post treatment for 3 hours at 37°C and removed by washing the cells twice with phosphate buffered saline containing $CaCl_2/MgCl_2$. Intracellular neutral red was extracted using neutral red removal solution (50% ethanol, 1% acetic acid and 49% water) for 15 minutes. Neutral red uptake was measured at 540 nm and cell viability was determined as percentage of the untreated control. Sodium dodecyl sulphate (SDS) was used as a positive control.

RESULTS

***In vitro* susceptibility of novicidin and the antibiotics.** The MIC for novicidin, rifampicin, ceftriaxone, ceftazidime, cefixime and cefotaxime were assessed for the 94 Gram-negative clinical isolates and 7 NDM-1 strains. As shown in Table 1, the MIC for novicidin for the 101 strains ranged from 1 to 8 mg/L with an MIC50 and MIC90 at 2 and 4 mg/L, respectively. The MIC for rifampicin varied between 4 to >1024 mg/L. The MIC50 and MIC90 were 16 mg/L and 64 mg/L, respectively. The MIC for ceftriaxone, ceftazidime, cefixime and cefotaxime ranged between 0.03125 and 2048 mg/L. The MIC50 and MIC90 were 1024 and 2048 mg/L for ceftriaxone, 128 and 1024 mg/L for ceftazidime, 256 and 2048 mg/L for cefixime, and 512 and 2048 mg/L for cefotaxime, respectively.

Chequerboard analysis of combination effects. The combination effects of novicidin combined with rifampicin, ceftriaxone and ceftazidime were determined using the broth microdilution chequerboard assay against 94 clinical isolates and 7 NDM-1 strains. The FIC indices for the combinations are shown in Table 2. The combination of novicidin with rifampicin was shown to have

synergistic activity with over 70% of both *E. coli* and isolates in KES group, with FIC indices between 0.018 and 0.5. In addition, the combination was shown to have synergistic effects with all 7 NDM-1 strains. Novicidin reduced the MIC of rifampicin between 2 to 512-fold, with the majority of strains exhibiting 4 or 8-fold reductions in MIC values (Supplementary Table 1, 2 and 3). Novicidin combined with ceftriaxone showed synergy with 57.4% of the *E. coli* strains and 69.7% of isolates in KES group. The combination of novicidin with ceftazidime presented synergy with 63.9% of the *E. coli* strains and 78.8% of isolates in KES group. The FIC indices for the NDM-1 strains were unable to be determined as the MIC for ceftriaxone and ceftazidime was higher than the maximum achievable chequerboard concentration of 2048 mg/L. As revealed in Table 3, synergistic activities were shown in the majority (89.7%) of the ceftriaxone-resistant strains compared to a minority of the ceftriaxone-sensitive strains (16.7%). A similar pattern was observed with the novicidin and ceftazidime combination, whereby synergy was seen in 94.1% of resistant strains compared with 3.8% of sensitive strains. Novicidin reduced the MIC of ceftriaxone or ceftazidime between 2 to more than 2048-fold (Supplementary Table 1 and 2).

Time kill assays confirming synergy of novicidin combined with rifampicin, ceftriaxone or ceftazidime. Time kill assays were performed to examine the activities of novicidin in combination with rifampicin, ceftriaxone and ceftazidime against 5 strains of *E. coli* and KES group clinical strains which represented an FIC index <0.5 for each drug combination. The combination of rifampicin and novicidin was also tested against the 7 NDM-1 strains. A range of different concentrations was tested according to chequerboard analysis and the most effective and synergistic activities are shown. As seen in Figure 1, rifampicin at 2 mg/L (Fig. 1A and Fig. 1B) and at 256 mg/L (Fig. 1C and 1D) failed to reduce the viability of the clinical isolate and the NDM-1 *E. coli*, novicidin at 0.5 or 0.25 mg/L (Fig. 1A and 1B) and at 4 or 2 mg/L (Fig. 1C and 1D) showed initial kill of the bacteria but regrowth was seen. However, when rifampicin at 2 mg/L combined with novicidin at 0.5 (Fig. 1A) or 0.25 (Fig. 1B) mg/L, 100% kill of the *E. coli* cells was achieved at 2 and 4 hours post treatment, respectively.

196 Similarly, when rifampicin at 256 mg/L combined with novicidin at 4 (Fig. 1C) or 2 (Fig. 1D) mg/L,
197 complete kill of the NDM-1 *E. coli* was seen at 4, 7 and 24 hours post treatment, respectively. There
198 were significant differences in the reduction of CFU counts between the combination of novicidin
199 with rifampicin and each of the single drug (rifampicin or novicidin) treatment ($P<0.0001$).

200 Novicidin and ceftriaxone combinations were tested against ceftriaxone resistant *E. coli* and KES
201 group clinical isolates. As seen in Fig. 1E, 1F, 1G and 1H, ceftriaxone at 2048 mg/L was unable to
202 reduce the CFU counts of both strains. However, when novicidin was added in the culture at 1 or 0.5
203 mg/L and 2 or 1 mg/L, the bacterial cells were rapidly killed showing 100% reduction in CFU count at
204 2 or 4 hours post treatment for the *E. coli* isolate respectively (Fig. 1E and 1F), and at 1 or 2 hours
205 post treatment for the KES group strain (Fig. 1G and 1H) respectively, demonstrating significant
206 synergy. There were significant differences in the reduction of CFU counts between combination of
207 novicidin with ceftriaxone and each of the single drug (ceftriaxone or novicidin) treatment
208 ($P<0.0001$). The ability of novicidin enhancement to rifampicin or ceftriaxone was also compared
209 with another defensin, plectasin which was neither bactericidal on its own nor boosting the activity of
210 rifampicin (Fig. 1I) or ceftriaxone (Fig. 1J) against Gram-negative bacteria to validate the assay.
211 Similar patterns of combination activities were observed for the strains tested when novicidin was
212 combined with ceftazidime (data not shown).

213 **Membrane permeabilising effects of novicidin against *E. coli* and KES group isolates.** The effects
214 of novicidin at the cytoplasmic bacterial membrane with both *E. coli* and an isolate from the KES
215 group were investigated with fluorescence assays. Immediately after novicidin exposure, a sharp
216 concentration dependent increase in fluorescence occurs with the *E. coli* strain (Fig. 2) indicating
217 disruption of the bacterial membrane which led to the leakage of the fluorescent dye. A similar effect
218 was observed when novicidin was used to treat the strain in the KES group despite only high
219 concentrations of novicidin such as 64 and 32 mg/L resulting in an increase in fluorescence (data not
220 shown).

Post-antibiotic effect of novicidin and novicidin-antibiotic combinations. The PAE of novicidin, rifampicin or ceftriaxone singly and in combination was determined; rifampicin was used at 5-fold higher than the MIC level and ceftriaxone at 10-fold higher than the MIC level. As novicidin was rapidly bactericidal at 5-fold higher than its MIC concentration, 2-fold higher than MIC level was used to induce the PAE. Due to their enhanced synergistic activities, the same concentrations for novicidin and rifampicin or ceftriaxone used singly for PAE induction would completely kill all the bacterial cells within 1 hour if combined. Therefore, to induce PAE with combination treatment, 5-fold higher than the minimal enhancement concentrations for novicidin and rifampicin or ceftriazone were used, chosen from chequerboard results. As shown in Fig. 3A, the PAE of both novicidin and rifampicin was estimated as 52.8 minutes for the *E. coli* strain. The novicidin and rifampicin combination doubled the PAE to 121.8 minutes despite substantially lower concentrations being used ($P<0.0001$). As shown in Fig. 3B, the PAE of novicidin was 84 minutes and ceftriaxone produced no PAE. The novicidin and ceftriaxone combination exhibited a prolonged PAE of 117 minutes ($P<0.0001$).

Haemolytic effects of novicidin. Haemolysis of novicidin was tested using human blood. As shown in Table 4, at the lowest tested novicidin concentration of 125 mg/L, haemolysis occurred at a rate of 4.4% ranging up to 19.9% at the highest tested concentration of 1000 mg/L. The 50% haemolytic concentration could not be accurately predicated due to the non-linear correlation between novicidin concentration and haemolysis, however is shown to be >1000 mg/L from the current data. Extrapolation provides an estimate of between 2500 and 3000 mg/L. 100% haemolysis was seen when the blood was added into distilled water (Table 4). The experiments were repeated twice with reproducible results.

Determination of cytotoxicity by neutral-red uptake. To assess the cytotoxicity of novicidin, neutral-red uptake was measured after treatment of the murine fibroblasts with different concentrations of novicidin. As seen in Table 5, cell viability was well conserved and remained

between 93% to 99% after 24 hours of novicidin exposure, and 98% to 102% after 72 hours exposure for all tested concentrations. This indicates low levels of general cytotoxicity even with prolonged exposure. SDS was used as a positive control: concentrations of 80, 100 and 120 mg/L reduced cell viability to 80%, 9% and 0% at 24 hours and 55%, 0% and 0% at 72 hours, respectively, confirming the validity of the assay. The experiments were repeated twice with reproducible results.

DISCUSSION

Novicidin is a newly derived antimicrobial peptide. In this study, we demonstrated for the first time that novicidin synergised with rifampicin and third generation cephalosporins (ceftriaxone and ceftazidime) against Gram-negative antibiotic-resistant bacterial strains *in vitro*. The 94 clinical isolates from the *Enterobacteriaceae* family covered a broad host distribution in the South London area and the 7 NDM-1 strains represented the most resistant type of Gram-negative bacteria. Most of the ceftriaxone and ceftazidime resistant bacteria were also resistant to cefotaxime and cefixime indicating these were ESBL producing strains.

Rifampicin is an important component of the combination regimen used for the treatment of tuberculosis and many Gram-positive bacterial infections (21). Rifampicin is not considered to be standard treatment for *Enterobacteriaceae* infections, and thus a breakpoint for resistance is not available. Our results showed that the MIC50 and MIC90 for rifampicin were 16 and 32 mg/L, respectively. Recently, rifampicin has been introduced in combination therapy for the treatment of infections caused by multi-drug resistant Gram-negative bacteria (22, 23). Our chequerboard analysis reveals that the combination of novicidin and rifampicin showed synergistic effects with over 70% of the tested strains with marginally higher effectiveness with the bacterial strains in KES group compared with *E. coli*. Novicidin was able to revive the activity of rifampicin by reduction of rifampicin MIC between 2 to 512 fold. The combination was also synergistic with all of the strains harbouring NDM-1 plasmids. Synergistic activity of novicidin with rifampicin was confirmed using

time kill assays, a method allowing for a more dynamic analysis of bactericidal and combinatorial effects. Time kill assays were performed with multiple strains, repeatedly demonstrating that at concentrations at which both novicidin and rifampicin were ineffective alone, when combined, rapid bactericidal activities were seen with 100% elimination of the bacterial cells within a few hours of drug exposure, which substantially speeded up the treatment duration. Rifampicin alone required higher concentrations such as 128 mg/L to completely eradicate *E. coli* cells in culture (data not shown) and this concentration was only able to reduce the CFU counts of a KES group strain by 2 logs (data not shown). However when combined with novicidin at 0.5 or 1 mg/L, rifampicin at concentrations of just 2 mg/L killed 100% of the bacterial cells at 4 or 2 hours post treatment (Fig. 1A and 1B). The combination was also able to enhance the activities of rifampicin against the NDM-1 strains (Fig. 1C and 1D), however required high rifampicin concentrations.

Novicidin also enhanced the activities of ceftriaxone and ceftazidime. Interestingly, the majority of synergy was observed with those strains showing resistance to ceftriaxone or ceftazidime. This was also confirmed with time kill assays tested against multiple strains. Ceftriaxone has a long half-life and is used to treat septicaemia, pneumonia, meningitis and urinary tract infections. Clinical pharmacokinetic data revealed that after a single intravenous injection of a standard 2000 mg dose, the plasma Cmax was approximately 257 mg/L and at 24 hours post administration, the plasma concentration was approximately 15 mg/L. However, in the urine, the Cmax of ceftriaxone was approximately 2692 mg/L within 2 hours following 2000 mg intravenously administrated (24). Ceftazidime, like ceftriaxone, has broad spectrum activity and is one of the few agents in this class to be used clinically against *Pseudomonas* spp. Ceftazidime pharmacokinetic data shows comparative serum Cmax, as a 1000 mg intravenous dose produced a peak concentration of approximately 140 mg/L. Similarly, much higher concentrations are present in the urine. Up to 6 hours post infusion of a 50 mg/kg dose of ceftazidime, the concentration in collected urine samples ranged from 2370 to 11, 340 mg/L, with approximately 75% of the drug being recovered unchanged (25). Based on this data, it

295 may be argued that novicidin-cephalosporin combinations may not be clinically appropriate for the
296 treatment of septicaemia as 2048 mg/L appears to be an unattainable serum concentration. However,
297 pharmacokinetic analysis of novicidin in combination with the antibiotics may give more realistic
298 estimations of the concentrations required to achieve synergistic and bactericidal effect. Nevertheless,
299 the extremely high concentrations of both ceftriaxone and ceftazidime in the urine indicate that either
300 of these in combination with novicidin may be clinically applicable in treating urinary tract infections.

301 The combination of novicidin with rifampicin or ceftriaxone was able to suppress bacterial growth
302 against our tested bacterial strains after the drugs had been removed. Interestingly, although
303 ceftriaxone alone was unable to produce a PAE (26), a prolonged PAE was generated in the
304 combination with novicidin. Therefore, novicidin and the antibiotic combinations, possibly by
305 prolonging the PAE, are able to reduce the likelihood of resistance development. A longer PAE also
306 contributes a therapeutic advantage in devising dosing intervals for drug regimens. Generally a longer
307 PAE enables less frequent drug doses whilst maintaining therapeutic efficacy; this can reduce adverse
308 effects and increase patient compliance (20).

309 The precise mechanism underlying the antibiotic enhancing activities of novicidin is unclear. Due to
310 decreased cell envelope permeability and altered efflux-pump systems, Gram-negative bacteria are
311 intrinsically resistant to many antibiotics, such as rifampicin. Rifampicin inhibits bacterial DNA-
312 dependant RNA polymerase (23) and its action on bacterial cells is concentration dependent. It has
313 been shown that compounds which target the cell wall or cell membrane were found to potentiate the
314 activities of other antibiotics (11, 18, 27, 28). Previous work on artificial membranes showed that low
315 concentrations of novicidin resulted in transient pore formation and increased concentrations cause
316 cell membrane disruption (13, 29). It is also suggested that novicidin accumulates on the membrane
317 surface until a detergent-like disintegration occurred (known as the carpet mechanism) (13).
318 Consistent with this finding, we showed that novicidin disturbed the cytoplasmic membrane potential
319 by depolarising the membrane, and even at very low concentrations, significant fluorescence release

was observed. It is likely that the enhanced activities of rifampicin by novicidin was due to increased cell membrane permeability against the Gram-negative bacteria leading to higher intracellular accumulation of rifampicin (30, 31).

Cephalosporins are β-lactam antibiotics and interact with transpeptidases also known as penicillin binding proteins (PBP) (32), blocking the terminal step in bacterial cell wall biosynthesis (33). Accordingly, the synergy between novicidin and ceftriaxone or ceftazidime may be attributed to a 'double hit' mechanism: (1) the disruption of the membrane by novicidin, and (2) the inhibition of cell well biosynthesis by ceftriaxone or ceftazidime, which may be sufficient in reducing the integrity of the cell envelope, resulting in cell death. Our chequerboard analysis showed that synergy was more likely with ceftriaxone or ceftazidime resistant strains, and resistance to such agents is usually due to the acquisition of plasmids carrying ESBL genes, producing enzymes which hydrolyse the β-lactam ring of antibiotics. It is unclear how novicidin enhances the activities of these cephalosporins against resistant strains. We hypothesized that the enhanced antibiotic activities was likely due to the action of pore formation by novicidin, leading to the elimination of enzymes or plasmids, the resistance determinants. However, this notion needs to be further tested.

The findings from our study demonstrate proof of concept, displaying the potential of peptide-antibiotic combinations which undoubtedly contribute to important clinical applications. Firstly, our demonstration of novicidin as a powerful antibiotic enhancer strongly illustrates that other similar peptides or compounds may potentially be beneficial above and beyond their direct anti-microbial properties. Secondly, addition of novicidin reduced MICs and improved the rate of bactericidal activities of antibiotics, therefore highly resistant Gram-negative bacteria which are extremely difficult to kill can be eliminated from the bacterial culture. Finally, novicidin exhibited a very low haemolytic activity which was in agreement with those found by Dorosz et al (14). In addition, novicidin was non-toxic and cell viability was well conserved after treatment with different concentrations of novicidin. Combination therapy with novicidin shows promise for becoming a novel

and much clinically desired therapeutic option to treat "superbug" infections. *In vivo* work is under way aiming to expose the therapeutic potential of novicidin in the combination regimen to treat infections caused by antibiotic resistant Gram-negative bacteria.

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Table 1. MIC values for novicidin and antibiotics used in this study.

	MIC range (mg/L)	MIC50 (mg/L)	MIC90 (mg/L)
Novicidin	1 - 8	2	4
Rifampicin	4 - >1024	16	32
Ceftriaxone	0.03125 - >2048	1024	2048
Cefixime	0.03125 - >2048	256	2048
Ceftazidime	0.03125 - >2048	128	1024
Cefotaxime	0.03125 - >2048	512	2048

Table 2. Combination activity of novicidin with rifampicin, ceftriaxone and ceftazidime against the 101 Gram-negative *Enterobacteriaceae* strains

Strains	Combination Activity	FICI	Total no. (%) of strains with activity when novicidin combined with		
			Rifampicin	Ceftriaxone	Ceftazidime
E. coli	Synergy	≤0.5	43 (70.5%)	35 (57.4%)	39 (63.9%)
	Indifferent	>0.5 <4	18 (29.5%)	26 (42.6%)	22 (36.1%)
	Antagonism	≥4	0	0	0
Isolates in KES group	Synergy	≤0.5	28 (84.8%)	23 (69.7%)	26 (78.8%)
	Indifferent	>0.5 <4	5 (15.2%)	10 (30.3%)	7 (21.2%)
	Antagonism	≥4	0	0	0
NDM-1 strains	Synergy	≤0.5	7 (100%)	-	-
	Indifferent	>0.5 <4	0	-	-
	Antagonism	≥4	0	-	-

Table 3. Combination activity of novicidin with ceftriaxone and ceftazidime against the 94 Gram-negative clinical isolates

Strains (total no.)	Total no. (%) of strains with activity of novicidin combined with ceftriaxone and ceftazidime		
	Synergy	Indifferent	Antagonism
	FICI ≤0.5	FICI >0.5 <4	FICI ≥4
Ceftriaxone resistant strains [58]	52 (89.7%)	6 (10.3%)	0
Ceftriaxone sensitive strains [36]	6 (16.7%)	30 (83.3%)	0
Ceftazidime resistant strains [68]	64 (94.1%)	4 (5.9%)	0
Ceftazidime sensitive strains [26]	1 (3.8%)	25 (96.2%)	0

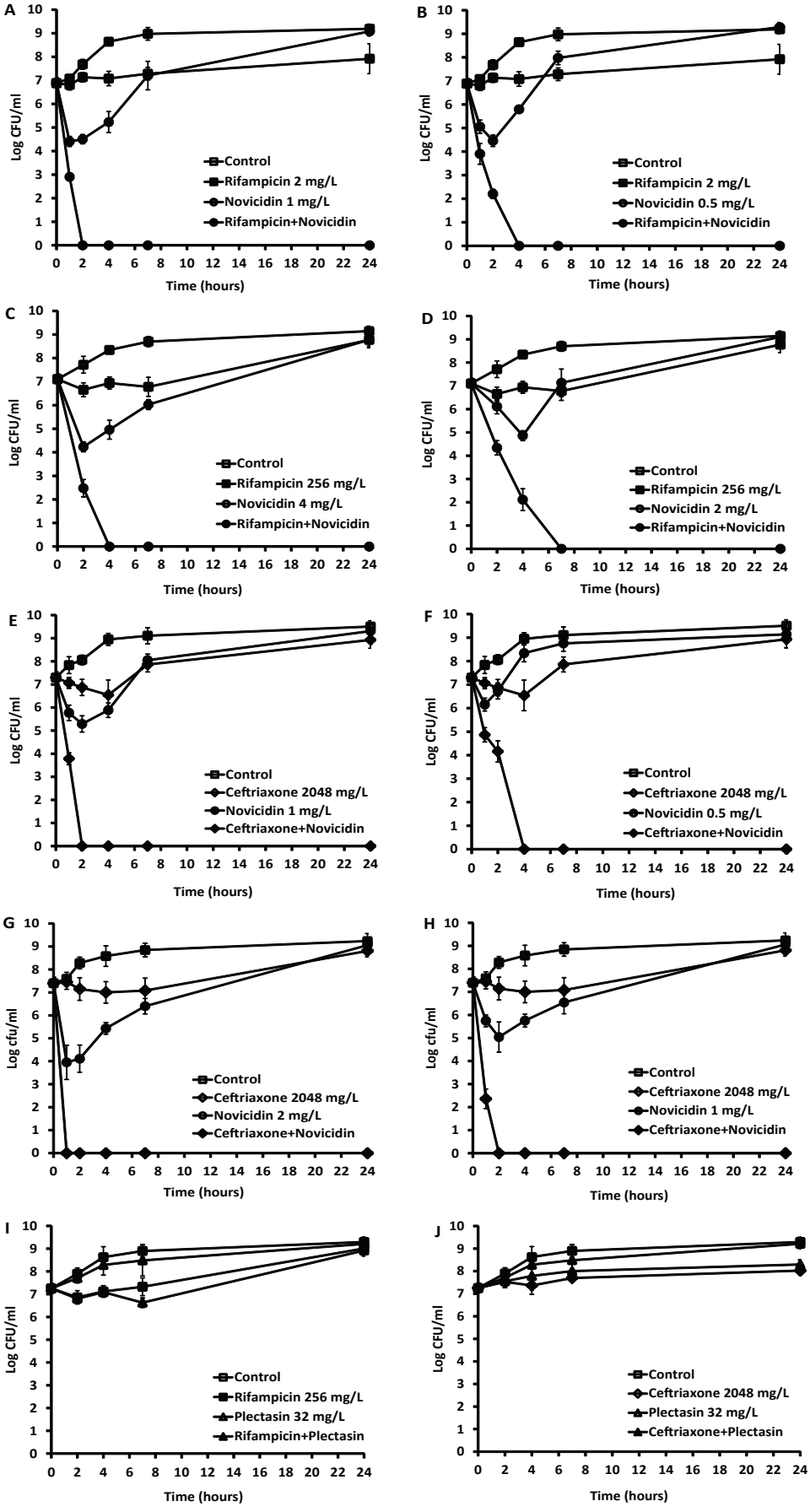

FIG 1 Time-kill analysis showing the effects of novicidin in combination with rifampicin, ceftriaxone and ceftazidime against antibiotic resistant *E. coli* and strains. The peptide and antibiotics alone or each combined with novicidin were added to the bacterial cultures and CFU counts were carried out at different time points. Combination of rifampicin at 2 mg/L and novicidin at 1 mg/L (A) or 0.5 mg/L (B) against a clinical isolate of *E. coli*. Combination of rifampicin at 256 mg/L and novicidin at 4 mg/L (C) or 2 mg/L (D) against a NDM-1 *E. coli*. Combination of ceftriaxone at 2048 mg/L and novicidin at 1 mg/L (E) or 0.5 mg/L (F) against a clinical isolate of *E. coli*. Combination of ceftriaxone at 2048 mg/L and novicidin at 2 mg/L (G) or 1 mg/L (H) against a clinical isolate of the KES group. Negative controls were included as (I) combination of plectasin at 32 mg/L with rifampicin at 256 mg/L against a DNM-1 *E. coli* and (J) combination of plectasin at 32 mg/L with ceftriaxone at 2048 mg/L against a clinical isolate of the KES group. These results shown are mean with standard deviation (SD) of two independent experiments.

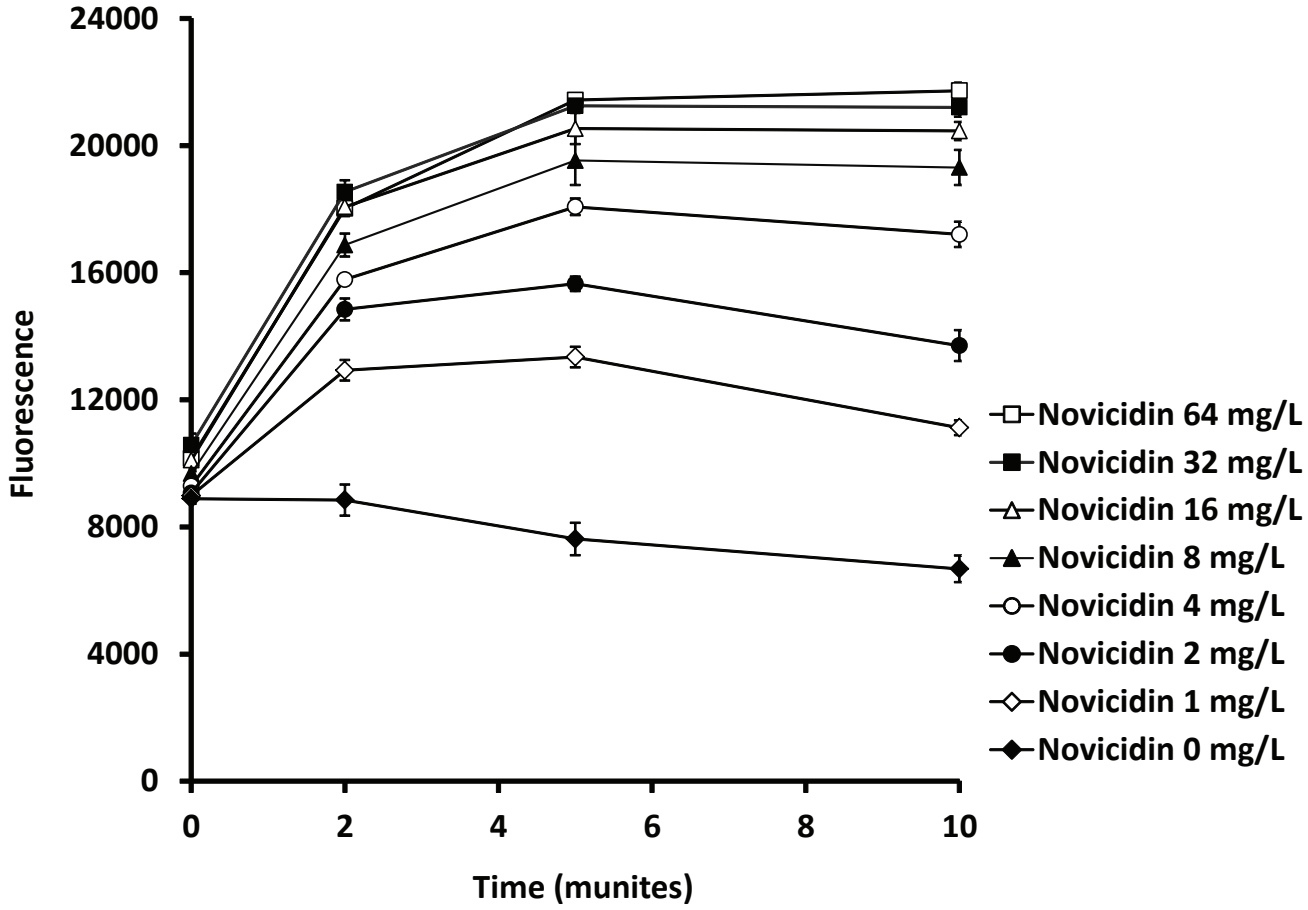

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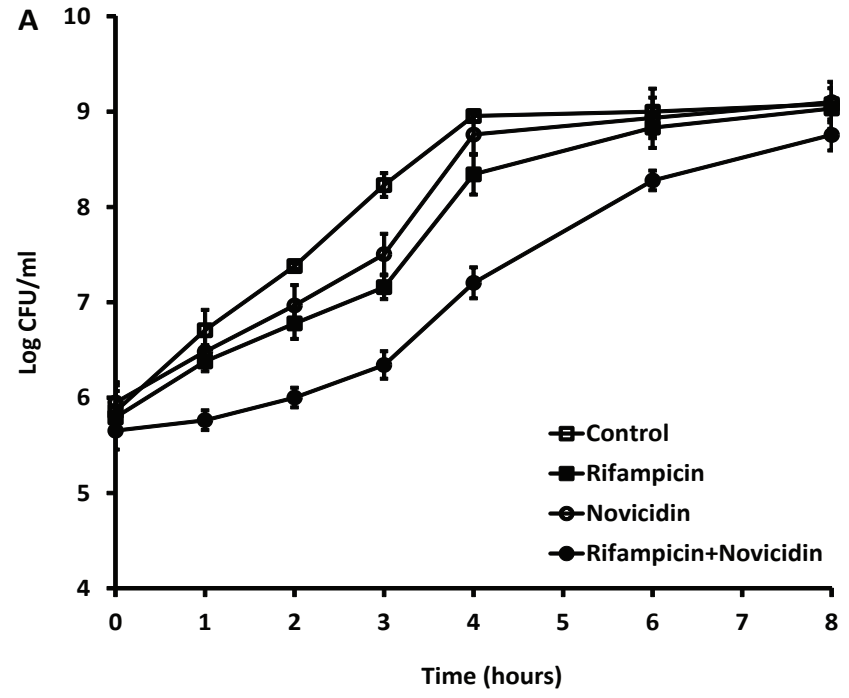
FIG 2 Determination of cytoplasmic membrane potential by novicidin against a clinical isolate of *E. coli*. Log phase *E. coli* culture was incubated with DiSC3(5) to a final concentration of 0.4 μM until no more quenching was detected, which was followed by addition of 0.1 M KCl. Novicidin were incubated with the cultures at different concentrations. The changes in fluorescence were monitored at various time points. The data was mean with SD of two independent experiments.



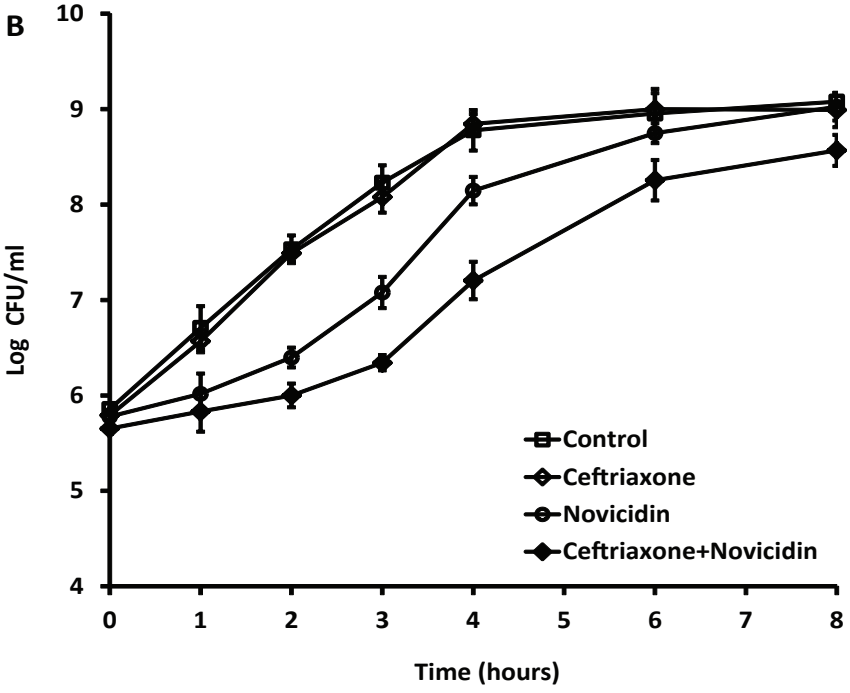


FIG 3 Induction of PAE of rifampicin (A) and ceftriaxone (B) by novicidin against a clinical isolate of *E. coli.* Concentrations used for single drug PAE induction are rifampicin 80 mg/L, novicidin 2 mg/L and ceftriaxone 1024 mg/L. For combination PAE induction, rifampicin was 20 mg/L and novicidin was 0.625 mg/L; ceftriaxone was 640 mg/L and novicidin was 0.625 mg/L. The data was mean with SD of two independent experiments.

Table 4. The haemolytic effects of novicidin at different concentrations

Novicidin concentration (mg/L)	Haemolysis (%)
125	4.4
250	7.7
500	13.2
750	13.3
1000	19.9
Negative control*	0
Positive control**	100

*blood was mixed with saline solution. **blood was mixed with distilled water

Table 5. Cell viability following treatment with novicidin assessed via neutral red uptake

	Concentrations (mg/L)	Viability (%)	
		24 hours	72 hours
Novicidin	0	100	100
	25	95	102
	50	93	102
	100	99	98
	200	99	101
SDS	80	80	55
	100	9	0
	120	0	0