Enhancement by novel anti-methicillin-resistant *Staphylococcus aureus* compound HT61 of the activity of neomycin, gentamicin, mupirocin and chlorhexidine: *in vitro* and *in vivo* studies

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Objectives: Previously, we described a small quinoline-derived compound that exhibited selective bactericidal activity against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). It depolarizes the bacterial cell membrane. In this study, we investigated if HT61 was able to enhance the potency of other antibiotics, namely neomycin, gentamicin and mupirocin, and an antiseptic, namely chlorhexidine, against clinical isolates of MSSA and MRSA *in vitro* and *in vivo*.

Methods: The MICs were determined by the broth microdilution method. The effect of combinations was examined using the chequerboard method and time–kill curves. A murine skin infection model was used to evaluate the enhancement by HT61 of other antimicrobials.

Results: Using the fractional inhibitory concentration index, no interaction was seen in both MSSA and MRSA for the pair HT61 and gentamicin or the pair HT61 and neomycin. Synergism was seen for 65% of both MSSA and MRSA when HT61 was combined with chlorhexidine. There was also no interaction between HT61 and mupirocin. Time–kill analysis demonstrated significant synergistic activities when a low level of HT61 was combined with neomycin, gentamicin or chlorhexidine. The effect was more dramatic against non-multiplying bacteria against which the antimicrobials used were inactive on their own. Significant synergistic effects were also seen on mouse infected skin.

Conclusions: We demonstrate that HT61, developed as a topical agent, acts as an enhancer that accelerates the activities of other antimicrobial agents against both MSSA and MRSA.

Keywords: MRSA, *S. aureus*, enhancers, antibiotic combinations, mouse skin infection models, topical agents

Introduction

Staphylococcus aureus is one of the commonest pathogens to be implicated in both hospital- and community-acquired infections. Methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) cause surgical site infections, bacteraemia, pneumonia and catheter-associated infections[1,2] and are responsible for significant morbidity and mortality. Currently, Bactroban (2% mupirocin) is used for the decolonization of nasal MRSA and for the treatment of skin infections with MRSA.[3] Chlorhexidine digluconate is used to decolonize *S. aureus* from the skin.[4] The combined effects of mupirocin and chlorhexidine on different carriage sites have successfully controlled endemic and epidemic MRSA infections in intensive care units and significantly reduce surgical site infections.[5–7] However, this strategy cannot produce a long-term clearance of MRSA carriage in most of the carriers,[8–11] which may be due to latent bacteria that are insensitive to mupirocin or are persistent in its presence.[12] With respect to MRSA, mupirocin resistance was discovered shortly after the drug was commercialized and its subsequent spread severely restricted the use of mupirocin across the world.[13–15] Chlorhexidine, which is not suitable for all patients, is also affected by resistance, particularly in intensive care units.[7,14] Naseptin is an alternative topical agent for the elimination of *S. aureus* in the nasal vestibule, and contains 0.1% chlorhexidine hydrochloride and 0.5% neomycin sulphate, but is not as effective as Bactroban for MRSA decolonization.[16] Gentamicin is used topically to treat eye and ear infections.[17] Resistance to neomycin and gentamicin is escalating.[18,19] Therefore, new topical prophylactic agents that exhibit rapid, potent

and direct bactericidal activity against MSSA and MRSA are urgently needed.

We have developed a novel antibiotic, HT61, a small quinoline-derived compound[20] that is active against both multiplying and non-multiplying bacteria. Its spectrum of action encompasses both MSSA and MRSA as well as Panton–Valentine leucocidin-carrying strains. Importantly, it also kills mupirocin-resistant MRSA. In experimental models, no HT61-resistant strains were found after 50 passages of exposure to suboptimal concentrations of HT61. The drug kills bacteria quickly, reducing a culture of >6 log cfu to zero in 6 h at a concentration of 10 mg/L.[20] The drug targets the bacterial cell membrane, leading to membrane depolarization and cell wall destruction.[20] Both of these make bacterial resistance development difficult.[21] The drug exhibited a strong therapeutic efficacy against MSSA and MRSA in mouse skin colonization and infection models.[20] Currently, HT61, developed as a topical agent, is in clinical trials with the aim of decolonizing S. aureus, including MRSA, from the nasal cavity.

As a therapeutic strategy, molecules that target the cell membrane or cell walls are most likely to synergize with conventional antibiotics or antiseptics by weakening the cell envelope and increasing cellular permeability.[22,23] We tested this hypothesis in vitro by combining HT61 with mupirocin, neomycin, gentamicin and chlorhexidine against MSSA and MRSA clinical isolates. Here, we describe, for the first time, studies of the enhancement of the activity of old antimicrobial agents by HT61 in vitro and in vivo. We found that there were significant synergistic activities when HT61 was combined with most of these tested drugs in vitro, especially against non-multiplying bacteria. Most importantly, we found significant therapeutic activities of these combinations in infection models.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are as follows: Oxford, S. aureus NCTC 6571 (methicillin susceptible), MRSA NCTC 12493, MRSA strains (117 clinical isolates from St George's Hospital, London) and MSSA strains (102 clinical isolates from St George's Hospital, London). The strains were grown in nutrient broth (Oxoid) or on blood agar and trypticase soy agar (Oxoid) plates.

Susceptibility tests of antibiotics against exponentially growing bacteria

The MICs were determined by the broth dilution method in Iso-Sensitest broth (Oxoid) following the CLSI guidelines for broth microdilution MICs.[24] The MICs were determined using 96-well microtitre plates. The antibiotics were diluted with 2-fold serial dilutions in triplicate followed by the addition of a standard bacterial suspension of $1-5\times10^5$ cfu/mL to make the final antibiotic concentrations starting from 256 or 16 to 0 mg/L. After 24 h of incubation at 37°C, the optical density (OD) readings were determined using an ELx800 Absorbance Microplate Reader (BioTek). The lowest concentration of an antibiotic with a similar OD reading as the control (medium only) was determined as the MIC. The antibiotics neomycin, gentamicin and mupirocin and the antiseptic chlorhexidine were obtained from Sigma (UK).

Chequerboard assays to measure combination effects of drugs against log-phase bacteria

The chequerboard method was used for the measurement of combination effects of HT61 with neomycin, gentamicin, mupirocin or chlorhexidine. The combinations of two drugs were prepared using 96-well plates using drug concentrations starting from 2-fold higher than their MIC values and then serially diluting 2-fold to zero. The effects of combination 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 interaction of the combination was defined as showing synergy if the FICI was ≤0.5, no interaction if the FICI was >0.5 but <4.0 and antagonism if the FICI was >4.0.[25]

Time–kill curve tests of antibiotics against log-phase and stationary-phase non-multiplying bacteria

The MSSA and MRSA strains were cultured in nutrient broth overnight at 37°C. Samples (200 μL) of the overnight cultures were used to inoculate 100 mL of nutrient broth. Then, the cultures were continuously shaken at 110 rpm at 37°C for 5–6 days. The viability was determined by plating 100 μL of serial dilutions onto nutrient agar (Oxoid) plates or blood agar plates and was expressed as cfu/mL. The cfu were counted using an aCOLyte colony counter (Synbiosis) and analysed using the counter's software. To test antibiotic activities against log-phase cultures, nutrient broth was inoculated with an overnight culture to obtain a cell suspension of 10^7 cfu/mL. Different concentrations of antibiotics alone or in combination were added to the cell suspension. To test antibiotic activities against stationary-phase non-multiplying bacteria, the 5–6-day-old cultures were washed with PBS and diluted in the same buffer to 10^6 or 10^7 cfu/mL, which served as cell suspensions for drug susceptibility tests. The cell suspensions were incubated with different concentrations of the drugs individually or in combination. The activities of the drugs or drug combinations were determined by cfu counting.

Measurement of bacterial cytoplasmic membrane potential

The bacterial cytoplasmic membrane permeability after drug treatment was measured using a fluorescent assay with a membrane potential-sensitive cyanine dye, dipropylthiacarbocyanine [DiSC$_3$(5)], as described previously.[20,26] Bacterial cells from log-phase or stationary-phase cultures were harvested and washed with PBS. The bacterial cells were resuspended with PBS to an OD of 0.05 at 600 nm. The cell suspension was incubated with 0.4 μM DiSC$_3$(5) (Sigma) until a stable (∼90%) reduction in fluorescence was reached as a result of DiSC$_3$(5) uptake and quenching in the cell due to an intact membrane potential. Then, 100 mM KCl was added into the cell suspension to equilibrate the intracellular and external K$^+$ concentrations. The treated bacterial cell suspensions were placed into the wells of a 96-well flat-bottomed fluorescence microtitre plate (Fischer Scientific UK) followed by the addition of different concentrations of drugs individually or in combination in triplicate. The fluorescence was monitored using a fluorescence spectrophotometer (Applied Biosystems) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The induction of fluorescence, which resulted from the disruption of the cytoplasmic membrane by different concentrations of drugs, was recorded. The background was subtracted using a control that contained only the cells and the dye.

Superficial skin bacterial colonization and infection models

The skin bacterial colonization and infection models were performed using female ICR mice (6–8 weeks old; Harlan, UK). The animal

husbandry and animal care guidelines were followed according to the UK Animals Scientific Procedures Act, 1986. The study was specifically approved by the Animal Ethics Committee of St George's, University of London. The superficial damaged skin infection model was performed as described previously.[20,27] ICR mice were anaesthetized by intraperitoneal injection of 200 µL of a 1:1:2 mixture of 100 mg/mL ketamine hydrochloride, 20 mg/mL xylazine and sterile water. The fur of the mice on the back was removed by an electric clipper. An area of 2 cm^2 skin was tape stripped using autoclave tape. The skin was stripped 10 times in succession. This procedure damaged the skin by removing the top dermal layers, which became red and shiny, but without observable bleeding. Buprenorphine was given at 0.2 mg/kg body weight during the anaesthetic period and every 12 h for up to 3 days to reduce pain. Bacterial infection was induced by the addition of 10–25 µL of log-phase culture containing 10^7 bacterial cells on the stripped skin. At 24 h after infection, treatment with gels or ointment was initiated. At different timepoints after infection and treatment, three or four mice were sacrificed by cervical dislocation. The skin, ~2 cm^2, was cut and added to 2 mL tubes that contained 1 mL of water and glass beads (1 mm). The skin was homogenized using a reciprocal shaker (Fisher Scientific UK) for 45 s (at speed 6.5). Antibiotic remaining on the skin was removed by washing three times with water. The cfu counts were performed on serial dilutions of the homogenates.

Results

Chequerboard analysis of combination effects

The combination activities between HT61 and neomycin, gentamicin, mupirocin or chlorhexidine were determined using the broth microdilution chequerboard assay against 102 strains of MSSA and 117 strains of MRSA, respectively. These strains included 5 gentamicin-resistant MSSA (MIC 8–256 mg/L) and 10 mupirocin-resistant MRSA (MIC 64–256 mg/L). The values of the FICI for all these combinations are shown in Table 1. The HT61/gentamicin combination demonstrated a FICI of 0.625–1, showing no interaction with all the MSSA and MRSA strains. The HT61/neomycin combination had a FICI range that was very similar to that of the HT61/gentamicin combination (Table 1). The most synergistic combination (FICI ≤0.5) was HT61/chlorhexidine, which was seen in 65% of both MSSA and MSSA. The rest of the strains exhibited no interaction with the combination. There was no interaction between HT61 and mupirocin for all the MSSA and MRSA strains tested. No antagonism was observed against any of the MSSA and MRSA strains.

Time–kill analysis of the combinations against log-phase bacteria

Time–kill assays were performed for HT61 in combination with neomycin, gentamicin or chlorhexidine for 10 MSSA and 10 MRSA strains. A range of concentrations was used for each treatment, either with a single antimicrobial agent or combination therapy. The data shown were derived from the combinations that gave maximum synergistic activity. As shown in Figure 1 (representative data derived from one of the MSSA or MRSA strains), neomycin at 2 mg/L (Figure 1a and b) reduced the initial inocula by 99% (2 log) at ~3 h post-treatment. Gentamicin (Figure 1c and d) at 1 mg/L and chlorhexidine (Figure 1e and f) at 2 mg/L showed a similar effect. The kill rate slowed after 4 h, then the cfu counts eventually reached a peak at 24 h. Bacterial regrowth after 8 h of incubation with antibiotics commonly occurs during time–kill curve experiments.[28–31] This could be due to drug degradation or microorganism adaptation.[29,30] However, in combination with 4 mg/L HT61, there was a 100% reduction of cfu counts at 2–4 h for MSSA and at 4–8 h for MRSA for all the combinations, whilst HT61 showed no activity. The cfu counts remained at zero for 24 h. The time–kill assay demonstrated that there was a significant synergistic activity between HT61 and gentamicin, neomycin or chlorhexidine for the 20 strains tested.
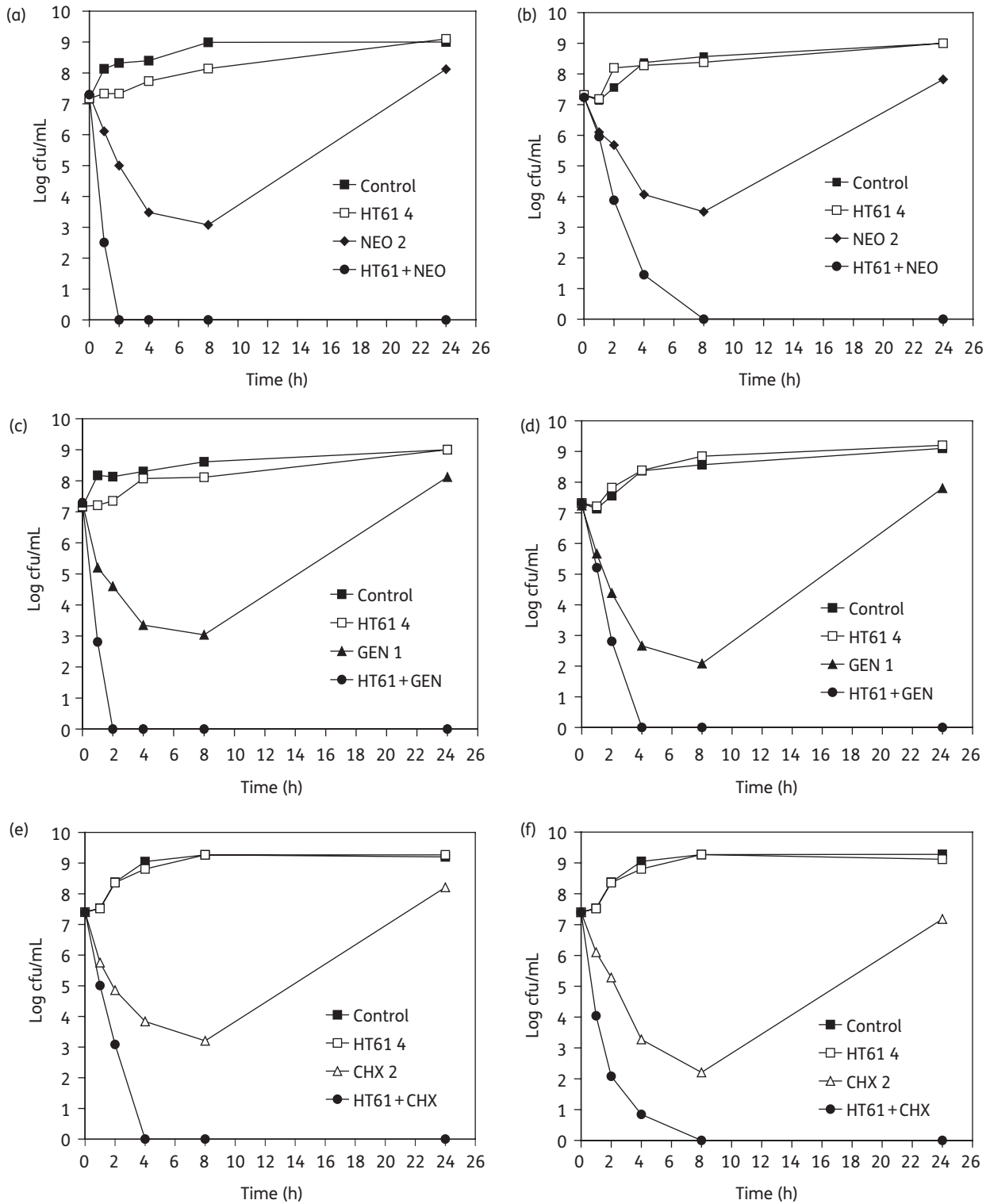
Time–kill analysis of the combinations against non-multiplying (stationary-phase) bacteria

Synergistic activity was also demonstrated against stationary-phase non-multiplying cultures of MSSA and MRSA using the same strains as for the log-phase cultures. Figure 2 shows that neomycin and gentamicin alone at 8 mg/L had no effect against both stationary-phase MSSA (Figure 2a and c) and MRSA (Figure 2b and d). HT61 alone at 4 mg/L killed ~2.5 log of bacteria over 8 h. However, the combination of HT61 at 4 mg/L and neomycin or gentamicin at 8 mg/L reduced the cfu counts to zero at 2 h of incubation for MSSA and at 6–8 h for MRSA. Chlorhexidine at concentrations of 8 mg/L reduced cfu counts ~3.5 log over the period of 8 h (Figure 2e and f). However, in combination with HT61, the cfu counts reduced to zero at 2 h for MSSA and at 4 h for MRSA. The 20 strains tested showed very similar kill profiles with the different pairs of HT61 combinations.

Table 1. Combination activities of HT61 with gentamicin, neomycin, chlorhexidine and mupirocin against 219 staphylococcal strains

| Strain | Combination activity | FICI | Total no. (%) of strains detected after | | | |
			HT61/GEN	HT61/NEO	HT61/CHX	HT61/MUP
MSSA	synergy	≤0.5	0	0	67 (65.69%)	0
	no interaction	0.625–1	102 (100%)	102 (100%)	35 (34.31%)	0
		2	0	0	0	102 (100%)
MRSA	synergy	≤0.5	0	0	77 (65.81%)	0
	no interaction	0.625–1	117 (100%)	117 (100%)	40 (24.18%)	0
		2	0	0	0	117 (100%)

GEN, gentamicin; NEO, neomycin; CHX, chlorhexidine; MUP, mupirocin.

Figure 1. Time–kill analysis showing the effects of HT61 in combination with gentamicin, neomycin and chlorhexidine against log-phase MSSA and MRSA. The tested agents alone or each combined with HT61 were added to the log-phase cultures and cfu counts were carried out at different timepoints. Combination of HT61 and neomycin (NEO) against MSSA (a) and MRSA (b). Combination of HT61 and gentamicin (GEN) against MRSA (c) and MRSA (d). Combination of HT61 and chlorhexidine (CHX) against MSSA (e) and MRSA (f). The concentration used for each agent: HT61, 4 mg/L; neomycin, 2 mg/L; gentamicin, 1 mg/L; and chlorhexidine, 2 mg/L. These results were confirmed in two independent experiments.
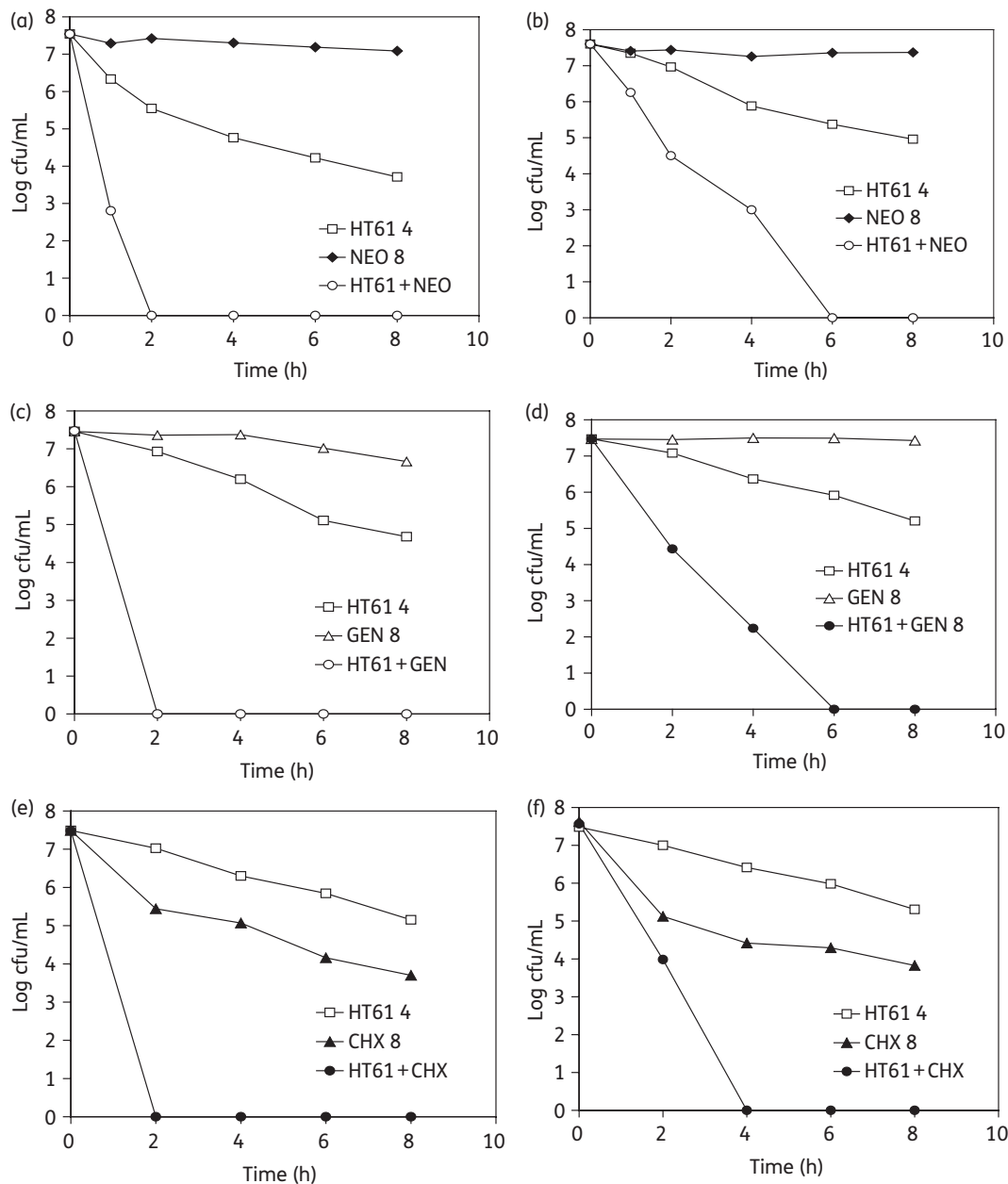
Figure 2. Time–kill analysis showing the effects of HT61 in combination with gentamicin, neomycin and chlorhexidine against non-multiplying stationary-phase MSSA and MRSA. The tested agents alone or each combined with HT61 were added to the cultures and cfu counts were carried out at different timepoints. Combination of HT61 and neomycin (NEO) against MSSA (a) and MRSA (b). Combination of HT61 and gentamicin (GEN) against MRSA (c) and MRSA (d). Combination of HT61 and chlorhexidine (CHX) against MSSA (e) and MRSA (f). The concentration used for each agent: HT61, 4 mg/L; neomycin, 8 mg/L; gentamicin, 8 mg/L; and chlorhexidine, 8 mg/L. These results were confirmed in two independent experiments.

HT61 in combination with mupirocin, neomycin and gentamicin kills MSSA and MRSA on mouse skin

We have developed HT61 as a topical agent named HY50A to clear MSSA and MRSA.[20] HY50A is a gel formulation containing 1% HT61. We investigated if HT61 synergized with marketed topical formulations such as Bactroban® (2% mupirocin, GlaxoSmithKline), Naseptin® (0.5% neomycin sulphate and 0.1% chlorhexidine hydrochloride, Alliance) and Genticin® (0.3% gentamicin, Amdipharm) against MSSA and MRSA on mouse skin. As shown previously, a bacterial infection was established after 24 h when the bacterial numbers on the infected skin remained constant.[20] Treatment with 45 μL of each of HY50A, Bactroban, Naseptin, Genticin, placebo and HY50A plus Bactroban, Naseptin or Genticin was initiated and cfu counts were estimated after 24 h of treatment. As seen in Figure 3, the effects of HY50A,
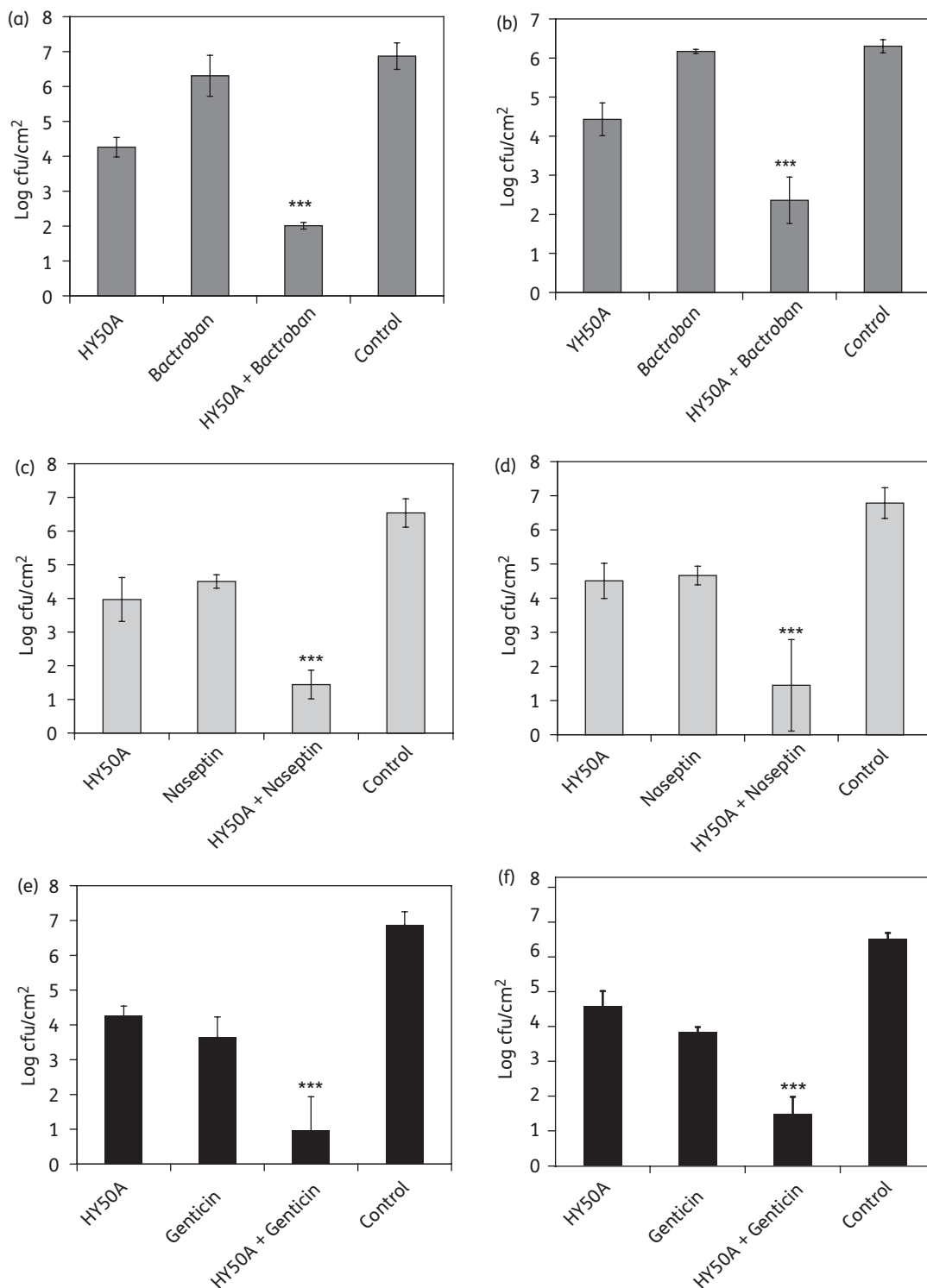
Figure 3. Effect of HT61 in combination with mupirocin, neomycin and gentamicin against MSSA and MRSA in a murine skin bacterial infection model. Viability of the bacteria was determined after 24 h of treatment. Treatment with HY50A, Bactroban, placebo or HY50A with Bactroban against MSSA (a) and MRSA (b). Treatment with HY50A, Naseptin, placebo or HY50A with Naseptin against MSSA (c) and MRSA (d). Treatment with HY50A, Genticin, placebo or HY50A with Genticin against MSSA (e) and MRSA (f). ***$P<0.001$. These results were confirmed in two independent experiments.

Naseptin or Genticin alone were very similar for both MSSA and MRSA, with ~2 log kill. Bactroban showed very little activity. However, when Bactroban, Naseptin or Genticin were combined with HY50A, significant synergistic activities were seen: an ~5 log reduction of cfu counts for both MSSA and MRSA recovered from the infected skin compared with the placebo control.

Mechanism of action of the combinations

The effect of HT61 in combination with neomycin, gentamicin and chlorhexidine on the cytoplasmic membrane of bacterial cells was investigated. As shown in Figure 4, gentamicin (Figure 4a and b) and neomycin (Figure 4c and d) at 16 mg/L had no effect on the cell membrane potential of both log-phase and stationary-phase *S. aureus*. One minute after the addition of 4 mg/L HT61, the fluorescence values increased to peak levels, which confirmed the previous study.[20] The addition of either neomycin or gentamicin to HT61 showed no influence on the fluorescence value. Chlorhexidine at 16 mg/L led to a release of fluorescence to a similar extent as did HT61 at 4 mg/L for both log-phase and stationary-phase cultures (Figure 4e and f). Interestingly, the combination of HT61 and chlorhexidine resulted in an increased cytoplasmic membrane depolarization (Figure 4e and f), indicating an enhanced cell membrane damage.

Discussion

Previously, we have demonstrated that HT61 is active against Gram-positive bacteria, especially MSSA and MRSA.[20] It selectively targets non-multiplying bacteria and shows reduced activity against multiplying organisms. Why does a membrane-active compound work more effectively against non-multiplying bacteria than actively growing ones? A number of drugs, such as pyrazinamide, specifically target non-multiplying bacteria.[32] It is thought that pyrazinamide enters *Mycobacterium tuberculosis* by passive diffusion. It is converted into pyrazinoic acid, which is excreted by a weak efflux pump and protonated pyrazinoic acid is reabsorbed into the bacteria under acidic conditions. In non-multiplying cells that are oxygen limited, the efflux pump activity is down-regulated and so the acid accumulates inside the cell, leading to a fall in the intracellular pH, which is an important part of the proton-motive force. This leads to a disruption of membrane transport and energetics, followed by death of the non-multiplying cells.[33] Pyrazinamide has poor activity against multiplying cells. Whilst there is no evidence that HT61 reduces the intracellular pH, it is unlikely that its depolarizing action alone is responsible for its bactericidal activity. For example, although daptomycin also depolarizes the cell membrane, it is not thought that this kills Gram-positive bacteria;[34] rather, death is caused by multiple effects, which may include disturbance of the membrane and the rapid inhibition of protein, lipoteichoic acid, RNA and DNA synthesis.

More likely, in our view, is that HT61, by disrupting the membrane, also disturbs membrane-embedded enzymes that are involved in anaerobic metabolism and redox reactions. Non-multiplying bacterial cells have to switch on their anaerobic metabolism pathways when they are oxygen deprived and so become susceptible to killing by drugs that poison the respiratory chain. For example, derivatives of prodrug nitroheterocyclic antibiotics, the nitroimidazoles PA-824 and OPC-67683, are effective against anaerobic bacteria and are active against non-multiplying *M. tuberculosis*.[35] The reduction of the precursor in the bacterium leads to the formation of reactive oxygen or nitrogen intermediates, which damage multiple targets, such as the cell envelope lipids, proteins and DNA. Cytochrome oxidases are inhibited, the redox status of the cells is changed and

intracellular ATP is significantly reduced. Death of the non-multiplying cells follows. Because multiplying cells are in an oxygen-rich environment, they do not switch on anaerobic metabolism and so are not as susceptible to agents such as PA-824.

In this study, we tested the combination effect of HT61 with several antibiotics and one antiseptic against clinical isolates of MSSA and MRSA. There were significant synergistic activities when HT61 was combined with gentamicin, neomycin or chlorhexidine.

Upon examination of 219 clinical isolates of MSSA and MRSA using chequerboard array analysis, synergistic activity was only seen with HT61 and chlorhexidine for the majority of both the MSSA and MRSA. HT61 and neomycin or gentamicin exhibited no interaction. However, significant synergistic activities were seen with each of the HT61 combinations using time–kill analysis. Many studies have highlighted the differences between chequerboard and time–kill methods to examine combination activity.[28,30,36–38] The time–kill curve is superior to the chequerboard assay, with dynamic and detailed viability measurement over time. Synergistic combinations were more frequently observed with the time–kill curve methodology compared with chequerboard studies.[28,30] In the latter, assays were read at 24 h of incubation, while in the former, cfu counts were performed at 1, 2, 4, 8 and 24 h. Consequently, any rapid bactericidal activities detected by time–kill curve would be missed by the chequerboard assay. In addition, the incubation conditions between these two methods were different. In the chequerboard studies, the plates were incubated without disturbance, so the bacteria would adhere to the surface of the wells to form biofilms and multiply despite the presence of lower concentrations of antibiotics.[30] However, in the time–kill assay, constant shaking of the cultures was achieved, which prevented bacterial adhesion and allowed the bacteria to remain in a multiplying stage in which they were more susceptible to the antibiotic treatment.

Bacteria develop resistance to antibiotics and this is associated with an increase in the MIC of one or more antibiotics. This means that when a patient has a clinical disease that is caused by a resistant bacterium, treatment with the antibiotic to which the bacterium is resistant is less effective. Sometimes, it is possible to simply increase the dose of the antibiotic to overcome the bacterial resistance. However, for many antibiotics, such as aminoglycosides, it is not feasible to significantly increase the dose of the antibiotic because of toxic side effects. In these circumstances, benefit for the patient could be achieved by enhancing the effect of the antibiotic against resistant bacteria. Here, we clearly showed that HT61 enhances the activity of neomycin, gentamicin and chlorhexidine. Combination with HT61 reduced the MIC of gentamicin and neomycin 2–4-fold and the MIC of chlorhexidine 4–16-fold. Most interestingly, the bactericidal activities of the tested drugs were significantly enhanced. HT61 at 4 mg/L showed no activity against log-phase bacteria, but acted as an enhancer to increase the potency of other drugs. The concentrations of neomycin or gentamicin that were needed to kill 100% of log-phase *S. aureus* were 4 or 8 mg/L, respectively (data not shown), at 2–4 h. However, in most cases, the bacteria regrew (data not shown) when the drug level was reduced. At a lower dose of 2 mg/L, neomycin killed most but not all of the bacteria at 4 h, after which the curve flattened out. However, when HT61 was combined with
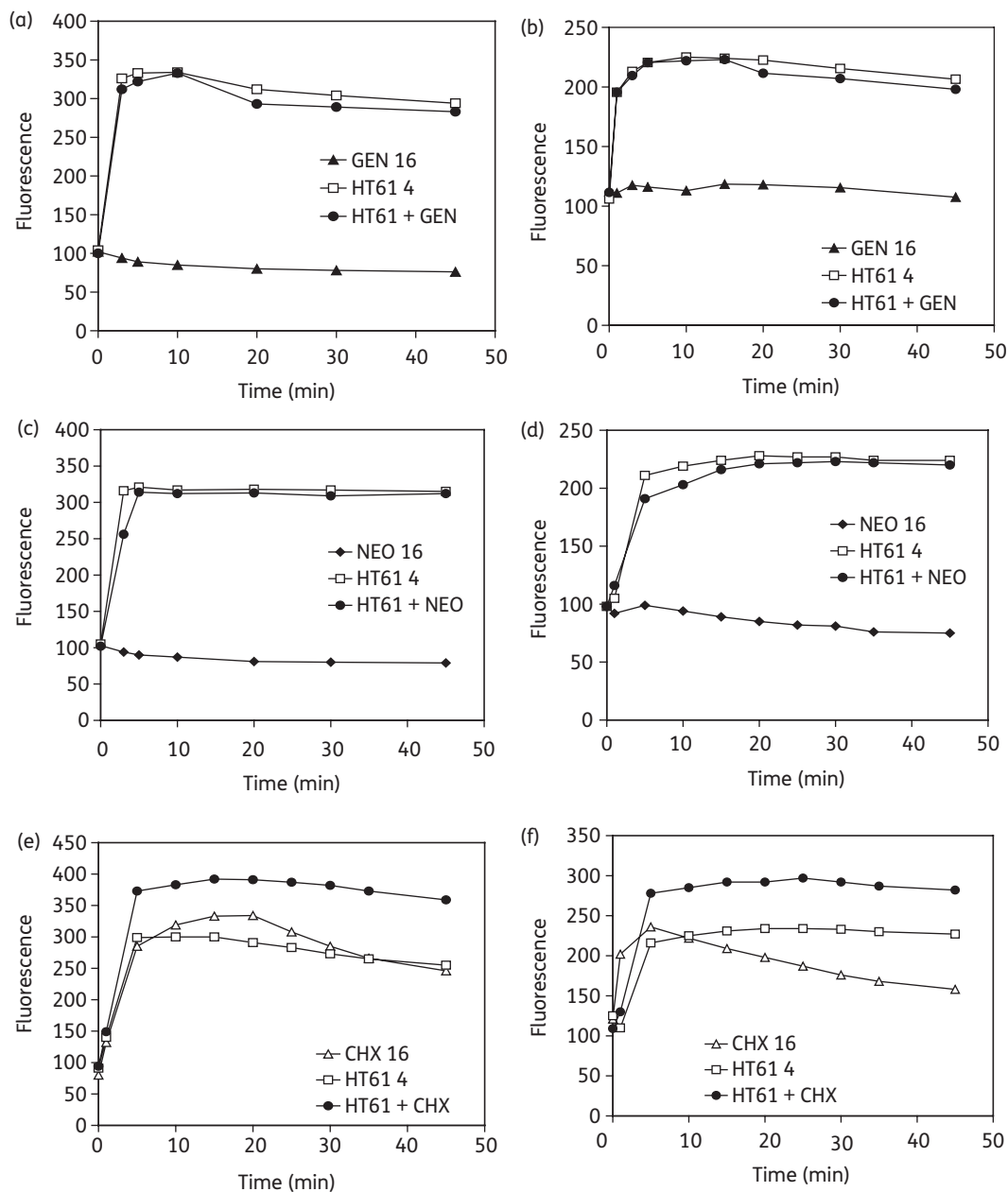
Figure 4. Determination of cytoplasmic membrane potential in the presence of HT61, neomycin, gentamicin and chlorhexidine (individually or in combination). Non-multiplying and log-phase MSSA were incubated with DiSC$_3$(5) to a final concentration of 0.4 μM until no more quenching was detected, which was followed by addition of 0.1 M KCl. HT61, neomycin, gentamicin and chlorhexidine were incubated with the cultures individually or in combination. The changes in fluorescence were monitored at various timepoints. HT61 in combination with gentamicin (GEN) in log-phase culture (a) and stationary-phase culture (b). HT61 in combination with neomycin (NEO) in log-phase culture (c) and stationary-phase culture (d). HT61 in combination with chlorhexidine (CHX) in log-phase culture (e) and stationary-phase culture (f). The concentration used for each agent: HT61, 4 mg/L; gentamicin, 16 mg/L; neomycin, 16 mg/L; and chlorhexidine, 16 mg/L. The results were confirmed in two independent experiments.

the same concentration of neomycin (2 mg/L), the cfu counts reached zero at 2 and 4 h for MSSA and MRSA, respectively, giving an equivalent potency of 8 mg/L neomycin. In addition, the culture remained sterile after 24 h of incubation, which indicated that all the bacteria were killed. Combination of HT61 with gentamicin and chlorhexidine also showed a similar level of

synergy. It is well established that repeated exposure to amino-glycosides, particularly gentamicin, increases the risk of nephro-toxicity, if used systemically, or ototoxicity, particularly insidious vestibular ototoxicity, which limits the therapeutic usage of this class of antibiotics.[39,40] Are these observations in this study potentially useful in the clinic? Simply, such combinations could

render aminoglycosides more effective in terms of faster action and lower relapse rates. But, perhaps more importantly, these combinations could be used to treat infections that are caused by bacteria that are resistant to aminoglycosides. In other words, it may be possible to rejuvenate some antibiotics by combining with compounds such as HT61. Another possible use of these combinations would be to reduce the dose of an aminoglycoside.

A further important finding was that combinations of antibiotics with HT61 effectively killed non-multiplying bacteria. In bacterial infections, slow or non-multiplying bacteria coexist with fast-growing organisms.[41] Antibiotics are capable of killing actively multiplying bacteria, but are almost always partially active against slowly multiplying or inactive against non-multiplying persistent bacteria.[20,42,43] More than 60% of all microbial infections are associated with non-multiplying bacteria such as those present in biofilms.[44] The persistent bacteria are responsible for recurrent infections, as seen in tuberculosis. With the currently available antibiotics, many persistent infections cannot be eradicated and are therefore associated with poor clinical outcomes.[45,46] Antibiotic tolerance is problematic, because lengthy treatment with multiple doses of antimicrobial agents is required for such bacterial infections, which in turn can lead to an increased frequency of genetic resistance associated with poor patient compliance,[42,47] high cost of treatment and further side effects. Here, we show that neomycin and gentamicin at 8 mg/L have little activity against non-multiplying MSSA and MRSA. However, in combination with 4 mg/L HT61, cfu counts reduced to zero within a short period of time (Figure 2) and remained at baseline for 24 h (data not shown). Although chlorhexidine alone showed a degree of killing, in combination with HT61, cfu counts reduced to zero at 2 and 4 h for MSSA and MRSA, respectively. So, this combination may target drug-tolerant persisters with the potential to reduce the length of antibiotic therapy.

HT61 targets the bacterial cell membrane.[20] The compound acts on the bacterial cell cytoplasmic membrane by disrupting the *S. aureus* cell membrane potential, which leads to the release of the cellular contents.[20] We have shown that HT61 disrupted the cell membrane potential even at 2 mg/L without causing cell death.[20] Treatment with HT61 weakens the cell membrane permeability, which may allow the accumulation of other antibiotics into the cytoplasm. Gentamicin and neomycin are bactericidal antibiotics that inhibit bacterial protein synthesis. Killing by these antibiotics is concentration dependent.[48] We postulate that increased levels of the antibiotics inside the cell as a result of the permeabilizing effect of HT61 in this combination may accelerate bacterial kill. It is known that in *Enterococcus* sp.,[49] streptomycin uptake is enhanced when combined with penicillin or other antibiotics that inhibit cell wall synthesis leading to changes in cell wall permeability. β-Lactam antibiotics can also alter cell surface tension and it is thought that this may enhance the activity of daptomycin.[50] Furthermore, rapid permeabilization of the *Acinetobacter baumannii* outer membrane allowed increased penetration of imipenem and rifampicin.[22] Although it has been suggested that the mode of action of gentamicin affects the outer cell membrane of Gram-negative bacteria, especially *Pseudomonas aeruginosa*,[51] we showed that aminoglycosides, such as gentamicin and neomycin, have no impact on the cell membrane potential. It is likely that the

increased accumulation of gentamicin and neomycin may be lethal to the bacterial cells. In the case of chlorhexidine, it is reported that the mechanism of action of chlorhexidine is membrane disruption.[52] We showed here that chlorhexidine depolarized the cell membrane potential. In combination with HT61, an enhanced bactericidal activity was shown, indicating a possible double hit on the cell membrane (Figure 4e and f). Investigation of the mechanisms of HT61 enhancement is currently underway in our laboratory.

The therapeutic usefulness of HT61 combinations with current topical agents such as Bactroban, Naseptin or Genticin was also demonstrated using a mouse skin model. Developed as a topical agent, HT61 has the potential to clear MRSA and MSSA on mouse intact skin, but with reduced potency against the same bacteria on broken mouse skin.[20] Here, we demonstrate that the combined usage of an HT61 formulation (HY50A) with Bactroban, Naseptin or Genticin augmented the potency of these combinations with significant killing of MSSA and MRSA on mouse skin. There are many advantages of this augmented effect. The fast action of the combined drugs could reduce the duration of Bactroban treatment, which currently is three times a day for 5 days. In addition, fast initial killing of MRSA in the nose could be useful for urgent hospital admissions when invasive treatment, such as surgery, must be given within hours. Such a combination could also reduce the emergence of mupirocin resistance.

In conclusion, HT61 in combination with neomycin, gentamicin, mupirocin and chlorhexidine may be useful in the treatment of infections caused by MSSA and MRSA. The usefulness of such combinations in humans is currently being tested in clinical trials.

Transparency declarations

Y. H. and A. C. are shareholders in Helperby Therapeutics Group plc, and A. C. is a director.

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