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ORIGINAL ARTICLE

Comparing the action of HT61 and chlorhexidine on natural and model *Staphylococcus aureus* membranes

Alasdair TM Hubbard[1,3], Anthony RM Coates[1] and Richard D Harvey[2,4]

HT61 and chlorhexidine (CHX) are both putative membrane-active antimicrobials, which non-specifically target the anionic lipids abundant in bacterial membranes. In model systems, the ability of these antimicrobials to partition into lipid monolayers and increase the permeability of lipid bilayers is dependent upon the presence and proportion of anionic lipids such as phosphatidylglycerol. Despite their apparent similarity in membrane affinity, we have found that HT61 and CHX differ in the extent to which they affect membrane integrity. HT61 was found to be capable of severely disrupting the lipid bilayer, resulting in lysis of *Staphylococcus aureus* membranes and the release of ATP from protoplasts. CHX, by contrast, does not disrupt the lipid bilayer to a sufficiently large degree to result in lysis of the membrane or release of ATP from *S. aureus* protoplasts. This suggests that although antimicrobials that interact with the membrane often have a common target, the action they have on the membrane may differ widely and may not be the primary mode of action of the antimicrobial.
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INTRODUCTION

Bacterial membranes constitute a good target for antimicrobial action as their integrity is essential for the survival of both multiplying and non-multiplying bacteria. The presence of anionic lipids such as phosphatidylglycerol (PG) and the zwitterionic phosphatidylethanolamine,[1] which are not usually found on the outer surface of mammalian cells, represents the basis for the selectivity of antimicrobials for these bacterial membrane targets.[2] Indeed, several cationic membrane-active antimicrobials have been shown to exhibit non-specific affinity for anionic lipids.[3–6] The disruption to the cytoplasmic membrane leads to increased permeability, depolarization, leakage of intracellular components and cell death.[1,3,7–10] However, the ability for membrane-active antimicrobials to interact with the membrane also depends on the physiochemical properties of a lipid bilayer, which can alter due to the properties of the different constituent lipids, including lipid charge, head group size, tail chain length and degree of saturation[11,12] and directly affect the lipid packing and overall charge of the membrane.[11,12] For example, the cytoplasmic membrane of *Staphylococcus aureus* is fairly unique in that it contains negligible amounts of zwitterionic phospholipids, but instead is comprised of a mixture of PG, cardiolipin (CL) and the cationic lipid lysyl-phosphatidylglycerol (lysyl-PG).[13,14] Changes in the ratios of these lipids within the cytoplasmic membrane can result in a degree of resistance to membrane-active antimicrobials.[15–19] The abundance of PG within the *S. aureus* membrane means that cationic membrane-acting antimicrobials are particularly active towards this pathogen.[3,7–9,20,21]

There are a number of marketed and developmental membrane-active antimicrobials including daptomycin,[10] telavancin[8] and chlorhexidine (CHX).[3,7] These membrane-active antimicrobials have rapid bactericidal activity and have been shown to facilitate perturbation of cytoplasmic membranes, as daptomycin does.[22] Many of these antimicrobials that are also active against the cytoplasmic membrane are known to be active against non-multiplying bacteria,[3,21,23] referred to as persister cells, which have been implicated as the cause of recurrent infections which most conventional antimicrobials that inhibit specific metabolic processes are not active against.[24,25] It has been observed for a number of membrane-active antimicrobials that it is difficult to produce bacteria resistant to these antimicrobials *in vitro*, a phenomenon which is attributed to their rapid bactericidal activity and their non-specificity due to the complexity of the lipid bilayer.[26,27] This apparent lack of resistance has led to membrane-active antimicrobials being an attractive prospect for future drug development. However, resistant strains of *S. aureus* and *Enterococci* have been described for the membrane-active antibiotic, daptomycin,[15,17,26,28] and there is also intrinsic resistance to cationic membrane-active antimicrobials seen in Gram-negative bacteria due to the outer membrane and Gram-positive bacteria that lack a high abundance of anionic lipids in the cytoplasmic membrane.[29]

The mode of action of CHX has long been suggested to be the disruption of the membrane,[3,7] however this is only at low concentrations and following relatively long exposure[30] and at higher concentrations CHX is bactericidal and causes the precipitation of the cytoplasm[31] meaning the primary mode of action may actually be difficult to pinpoint. Both HT61 and CHX are active against methicillin sensitive and resistant *S. aureus* membranes[32] and unlike

[1]Infection and Immunity Research Centre, Division of Clinical Sciences, St George's, University of London, London, UK and [2]Institute of Pharmaceutical Science, King's College London, London, UK
[3]Current address: Nuffield Department of Clinical Medicine, University of Oxford, Oxford OX3 9DU, UK
[4]Current address: Institute of Pharmacy, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany.
Correspondence: Dr RD Harvey, Institute of Pharmacy, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany.
E-mail: richard.harvey@pharmazie.uni-halle.de
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membrane-active antimicrobial peptides, are small molecules. A recent study into the mode of action of HT61, which is currently in efficacy clinical trials, highlighted that these two compounds resulted in a similar degree of membrane depolarization and release of ATP from *S. aureus*,[3] despite a difference in the structures of the compounds. However some differences between the two compounds were evident, although CHX has a lower minimum inhibitory concentration toward *S. aureus* (minimum inhibitory concentration 4 μg ml^{-1}) than HT61 (8 μg ml^{-1}) it was clear that HT61 was more active toward the bacterial membranes than CHX.[3] Therefore, the effect that HT61 and CHX have on the membrane, and the overall mode of action, may in fact be quite different. In this study, using a mixture of microbiological and biophysical methods, we compared the action of HT61 and CHX and the extent of which they interact with and affect natural *S. aureus* plasma membranes and simple *S. aureus*-mimetic model membranes, in order to gain a greater insight into how these two antimicrobials actually interact with lipid bilayers. Our model *S. aureus* membrane systems required a substitute for the alkali-labile lysyl-PG[13] and therefore contained the more stable cationic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-ethylphosphocholine (POePC) in various mixtures with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG), to simulate the natural lipid mixtures in the model monolayers and bilayers.

MATERIALS AND METHODS

Materials

CHX diacetate (Sigma, Poole, Dorset, UK) and HT61 mesylate (Helperby Therapeutics, London, UK) were both used as supplied and diluted in ultrapure water obtained from a Milli-Q 16 ultrapure water system (Merck Millipore, Billerica, MA, USA) with a specific resistivity of 18.2 MΩ cm^{-1} to a stock concertation of 10 g l^{-1}. Penicillin G (Sigma) was diluted to a stock concentration of 2 g l^{-1} in ultrapure water.

Brain heart infusion and nutrient broth were obtained from Oxoid (Basingstoke, Hampshire, UK). Chloroform, 5(6)-carboxyfluorescein, HEPES, glucose, PBS, Tris base, acetic acid, sucrose, sodium chloride, sodium deoxycholate, lysozyme, lysostaphin, dimethyl sulfoxide, bovine pancreatic DNase and Triton X-100 were all obtained from Sigma Aldrich (Poole, Dorset, UK). Propidium iodide (PI) and SYTO 9 were obtained from Invitrogen (Paisley, UK).

The lipids POPG and POePC were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

Bactericidal assay

A culture of Oxford *S. aureus* (NCTC 6571) was grown at 37 °C in nutrient broth at 100 r.p.m. for 18 h and then centrifuged at 10 000 × *g* for 10 min, the pellet was resuspended in 1 ml of HEPES buffer (5 mM HEPES, 5 mM glucose, pH 7.2) and diluted to 0.2 OD_{600}. Separately, CHX, HT61, penicillin G or ultrapure water (negative control) were added to 1 ml of the culture to give final concentrations of 16 μg ml^{-1}, and incubated at 25 °C for 20 min. PI and SYTO 9 from the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Paisley, UK) were mixed together in equal amounts, 3 μl of this dye mixture was added to the treated culture and incubated in the dark for 15 min. Using a microscope slide, 5 μl of this sample was added under a cover slip and fixed using lacquer. The cells were visualized using a Zeiss Axiovert 200M inverted microscope (Zeiss, Cambridge, UK) using the specific emission/excitation wavelengths required for each dye; 488/506 nm (SYTO 9) and 538/619 nm (PI).

Protoplast preparation

A 200 ml culture of *S. aureus* was grown for 18 h at 37 °C, shaken continuously at 100 r.p.m. in brain heart infusion broth. The culture was diluted in brain heart infusion broth to an OD_{660} of 0.66, and 200 ml of the culture was centrifuged at 10 000 × *g* for 20 min at 4 °C, washed once in 50 mM Tris-acetic acid (hypotonic buffer) and resuspended in 10 ml of 1 M sucrose, 50 mM Tris-acetic acid (hypertonic buffer) to prevent lysis of the protoplasts due to osmotic pressure. Using 1 ml of the culture, 200 μg ml^{-1} lysozyme, 100 μg ml^{-1} lysostaphin and 25 μg ml^{-1} bovine pancreatic DNase were added and incubated for 1 h at 37 °C, shaken at 100 r.p.m.. The samples were centrifuged at 10 000 *g* for 10 min and washed with hypertonic buffer prior to use in the protoplast lysis and ATP release assays. A Gram stain was performed on the protoplasts to confirm that they were lacking a cell wall.

Protoplast lysis assay

Protoplasts were diluted in hypertonic buffer to an OD_{600} of between 0.22 and 0.23, 270 μl of which was added to a 96-well microplate and the OD at 600 nm was measured on a GloMax^{+} microplate reader (Promega, Southampton, UK) to prevent cell death due to UV exposure. Using a separate 96-well microplate, the OD_{600} of 270 μl of the hypertonic buffer was measured to give the background reading at time zero. To the same plate, 270 μl of hypertonic buffer, 30 μl of CHX, HT61 and penicillin G (final concentration of 16 μg ml^{-1}) and Triton X-100 (5% v/v) were added separately and the OD_{600} was measured to give background readings. To a separate well, 30 μl of ultrapure water was also added to serve as a negative control. To the 270 μl of protoplast culture, 30 μl of each HT61, CHX and Triton X-100 were added in triplicate and incubated at 25 °C for 20 min before the OD_{600} of each well was measured using a GloMax^{+} microplate reader.

ATP release from protoplasts

Release of ATP from protoplasts was used as an indication of change in loss of membrane integrity. CHX, HT61 and penicillin G were added to 0.5 ml 0.2 OD_{600} protoplast samples to give final concentrations of 16 μg ml^{-1} and Triton X-100 added separately to give a final concentration of 5% (v/v) and the amount of ATP released from protoplasts was analyzed as previously described.[3]

Lipid monolayer partitioning

The ability of the drugs to partition into *S. aureus* membranes was assessed using biomimetic synthetic lipid monolayers with molar ratios of 45:55, 40:60 and 35:65 of POePC and POPG (Avanti Lipids, Alabaster, AL, USA). This was performed as previously described.[3] The surface pressure was continual recorded for up to 1600s and the maximum change in surface pressure over this time was recorded to directly compare the degree of partitioning into the monolayer.

Liposome preparation

POePC and POPG were combined at molar ratios of 35:65, 40:60 and 45:55, to create *S. aureus* lipid membrane mimetic liposomes. The lipid mixtures with a total mass of 10 mg ml^{-1} were dissolved in chloroform in order to form a thin film by evaporating the solvent under vacuum using a Rotorvapor RII (Buchi, Flawil, Switzerland) and an N820 FT.18 Laboport diaphragm pump (KNF, Cambridge, UK). To each resultant lipid film, 2.5 ml of a 40 mM 5(6) carboxyfluorescein dye solution (osmolality 140 mOsmol Kg^{-1}) was added and vortex mixed for 30 seconds to produce a turbid liposome dispersion, before being sonicated using a Labsonic L ultrasonicator (Sartorius BBI Systems GmbH, Melsungen, Germany) until the dispersion became clear, after which it was allowed to anneal at 25 °C for at least 20 min. Unentrapped dye was separated from the liposomes using a PD-10 size-exclusion column (GE Healthcare, Little Chalfont, UK) which was equilibrated with three 5 ml washes with 70 mM sodium chloride (osmolality 140 mOsmol Kg^{-1}) and 1 ml of the liposome mixture was eluted through the column and washed through with 5 × 1 ml aliquots of 70 mM sodium chloride and the eluted fractions were collected, fraction 4 was used in the dye release assay.

Dye release from liposomes

Drug-induced release of carboxyfluorescein from liposomes indicates an ability to severely disrupt or lyse the lipid bilayer. In each sample, 50 μl of liposomes were added to 2350 μl of 70 mM sodium chloride in a 10 × 10 mm pathlength optical polystyrene macro fluorescence cuvette (Kartell Labware, Milan, Italy), fluorescence was monitored using a Cary Eclipse fluorimeter

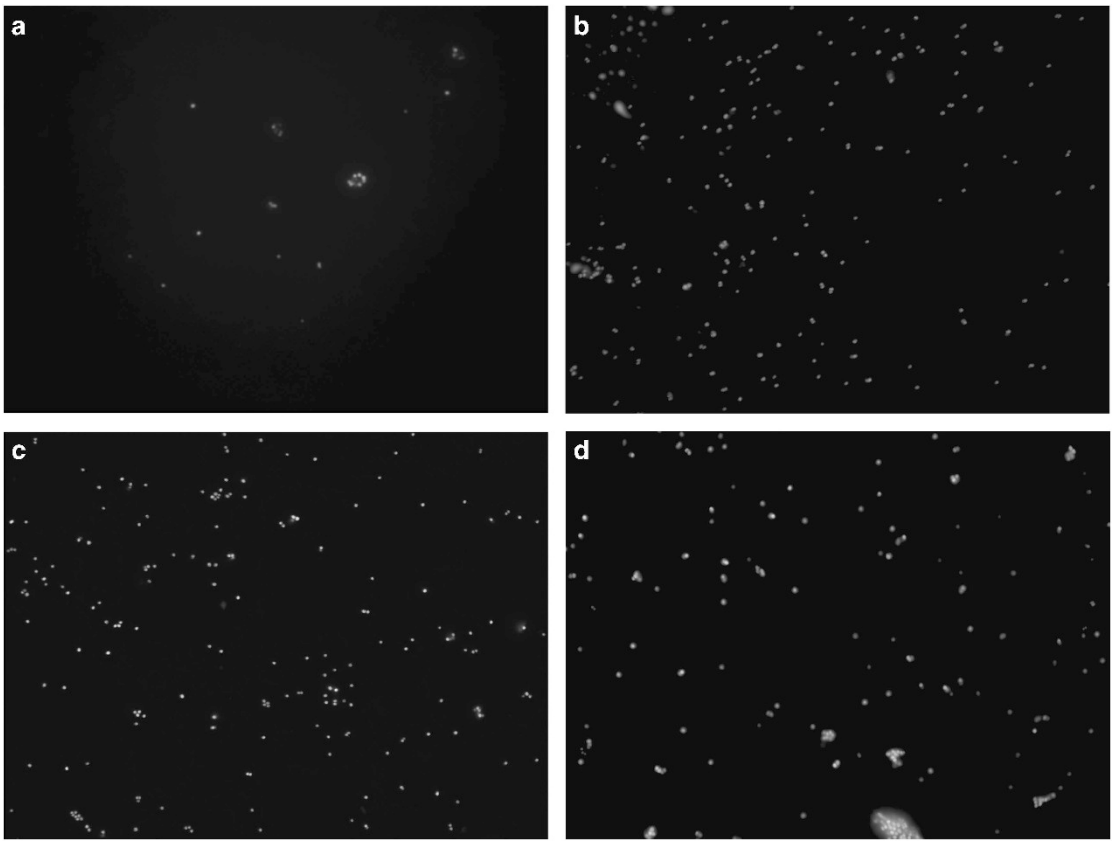

Figure 1 Micrographs of membrane damage of logarithmic phase *S. aureus* using the nucleic acid dyes SYTO 9 (green fluorescence) and propidium iodide (red fluorescence) after 20 min of exposure at a ×40 magnification to (**a**) HT61, (**b**) CHX, (**c**) penicillin G and (**d**) ultrapure water. These pictures are representatives of three repeats of each antimicrobial and negative control. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

(Varian Ltd, Walton-on-Thames, UK) using excitation and emission wavelengths of 490 and 510 nm, respectively, with excitation and emission slit widths of 2.5 nm. After 50 s, 100 µl of either HT61 or CHX, diluted in 70 mM sodium chloride, was injected (to give a final concentration of 9 µg ml^{-1}) and data were collected for another 500 s. After a total time of 550 s, 100 µl of a 5% w/v sodium deoxycholate solution was added to lyse any intact liposomes. Using the maximum fluorescence intensity following addition of sodium deoxycholate, the percentage dye release caused by the antimicrobials was calculated. An increase in fluorescence intensity over time indicated disruption to the bilayer of the liposome caused by the antimicrobial.

RESULTS

Bactericidal assay

The BacLight assay employs two nucleic acid dyes to detect the loss of membrane integrity. SYTO 9 can pass across the membrane to enter living cells and intercalate with DNA, emitting a green fluorescence, whereas PI can only enter cells and intercalate with DNA with a damaged membrane, emitting a red fluorescence.[8,9,33,34] Therefore, penicillin G (Figure 1c), a cell wall synthesis inhibitor added at concentrations eight times the minimum inhibitory concentration[3,35,36] did not damage or increase membrane permeability as penicillin G mediated lysis should not occur during the time scale of this experiment, resulting in the visible green fluorescence of SYTO 9. It is likely that CHX (Figure 1b) and HT61 (Figure 1a) both disrupt the membranes of *S. aureus* to a certain degree at a concentration of 16 µg ml^{-1} to allow the influx of PI, but there were also a number of cells that remained with intact membranes, so clearly this effect is not comprehensive. Interestingly, the orange fluorescence seen following CHX exposure is due to both SYTO 9 and PI binding to the nucleic acid, indicating that although CHX causes some degree of damage to the membranes, it is to a lesser extent than HT61 as PI is unable to fully displace or quench SYTO 9.[33,34] The few visible cells present in the sample treated with HT61, in contrast to the large number of cells present following CHX exposure, could indicate a potential lytic activity, but this could not be determined from this data set alone.

Protoplast lysis and ATP release

Direct membrane damage, rather than a by-product of inhibition of cell wall synthesis, can be measured through the lysis of bacterial protoplasts and is a good indicator of the extent of solubilization of the membrane.[20,37] Penicillin G treatment was able to lyse a small percentage of protoplasts (Figure 2) which is likely to be due to localized osmotic effect following the addition of the antimicrobial to the sample. However, penicillin G could not induce release of ATP (Figure 3) during the time frame of the assay indicating it has no direct lytic activity. As expected, the detergent Triton X-100, which can solubilize membranes, was capable of significant lysis (95%) and of inducing the release of 94% of ATP from the protoplasts. Despite being able to increase the permeability of *S. aureus* membranes, CHX did not lyse the protoplast membranes or induce release of ATP (Figures 2 and 3), suggesting that CHX is not capable of lysing *S. aureus* membranes. In contrast, HT61 did have a solubilising effect on protoplast membranes, but much reduced compared with Triton X-100, only lysing ~25% (Figure 2) and inducing the release of

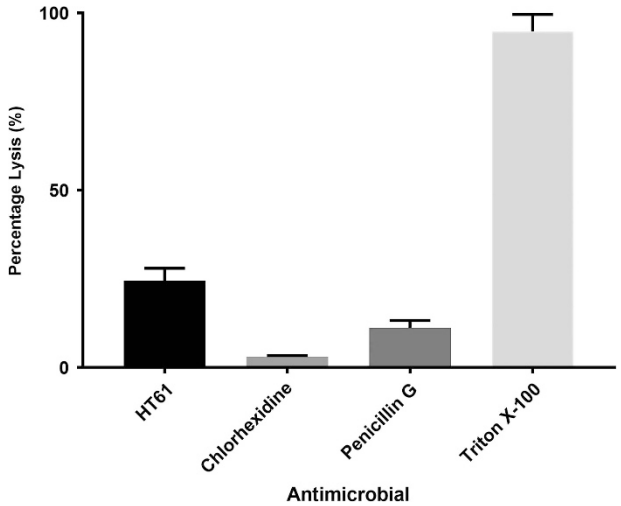

Figure 2 Percentage lysis of protoplasts following exposure to 16 μg ml^{-1} of HT61, CHX, penicillin G and Triton X-100 for 20 min. Error bars represent the s.e. of the mean ($n=3$).

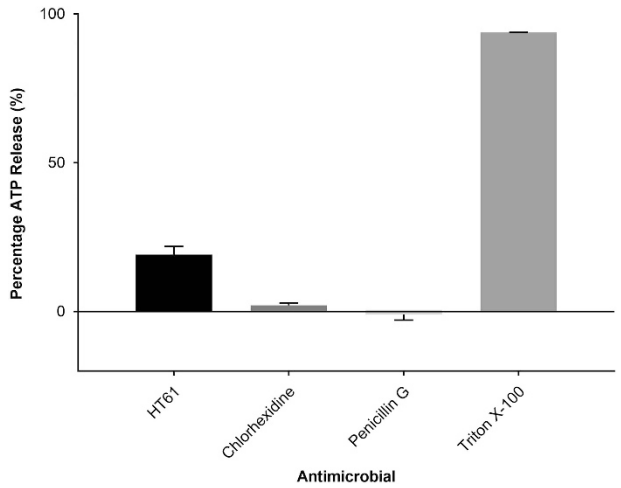

Figure 3 ATP release from *S. aureus* protoplasts 20 min after exposure to 16 μg ml^{-1} of HT61, CHX, penicillin G and Triton X-100. Error bars represent the s.e. of the mean ($n=3$).

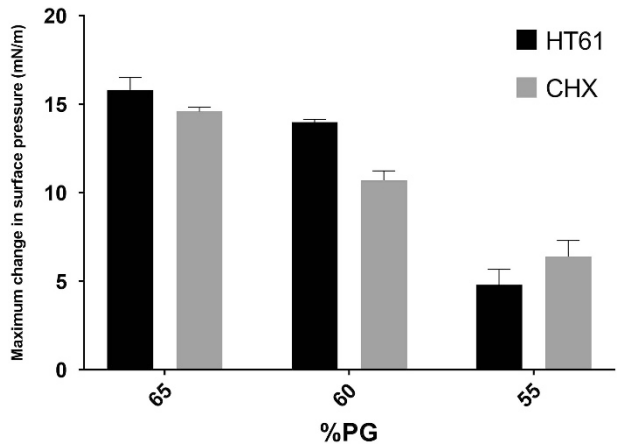

Figure 4 Maximum change in surface pressure of monolayers with a molar ratio of 35:65, 40:60 and 45:55 molar ratio POePC/POPG following injection of 9.1 μg ml^{-1} HT61 and CHX under the water-lipid interface.

approximately 20% of ATP (Figure 3). With respect to statistical analysis, performed using the paired two-sample *t*-test function in Microsoft Excel (2016),[38] which is relevant for small data sets if normality of the data is assumed,[39] a significant difference was found between the degree of protoplast lysis ($P=0.03$) and ATP release data ($P=0.03$) elicited by HT61 and CHX. Thus, HT61 demonstrated a significantly greater ability to lyse the protoplasts than CHX, suggesting an obvious difference in the effect of the two antimicrobials on the *S. aureus* membrane and the amount of resulting damage. It is clear from these results that the modes of action of CHX and HT61 deviate in terms of the degree of damage they can cause to the *S. aureus* plasma membrane.

Lipid monolayer partitioning

To examine the membrane activities of both HT61 and CHX in more detail, we investigated the interaction and partitioning into air/liquid interface monolayers containing three different molar ratios of POePC and POPG, 35:65, 40:60 and 45:55 to mimic the *S. aureus* membrane composition under different conditions. The cationic antimicrobials HT61 and CHX both noticeably interact electrostatically with the anionic lipids within the monolayer, as is evident by the direct correlation between the increase in maximum change in surface pressure and increase in %PG in the monolayer (Figure 4). Interestingly, the affinity to the monolayer and the subsequent partitioning by HT61 and CHX are also very similar, resulting in maximum changes in surface pressure of 15.8 and 14.6 mN m^{-1}, respectively, in monolayers with 65% PG content (Figure 4). The interaction and partitioning of HT61 and CHX was reduced as the % PG content decreased, resulting in the lowest maximum changes in surface pressure of 4.8 and 6.4 mN m^{-1}, respectively, at 55% PG. The degree of interactions of HT61 and CHX with the monolayer are of a comparable magnitude and, using the paired two-sample *t*-test function in Microsoft Excel (2016),[38] overall there is no significant difference between the action of the two antimicrobials on the 65% PG monolayer ($P=0.21$). In the case of the 60% PG monolayer, there is a small increase in HT61 partitioning, over that of CHX, which is statistically significant ($P=0.01$). The reverse of this results was observed for the 55% PG monolayer, whereby CHX demonstrated a small but significant ($P=0.005$) increase in partitioning over that of HT61. These fluctuations suggest that the two antimicrobials may undergo a similar initial interaction with the monolayer, resulting in a broadly comparable degree of partitioning into the monolayer.

Dye release from synthetic liposomes

HT61 and CHX's ability to disrupt the lipid bilayer sufficiently to cause leakage, was investigated using liposomes composed of the same molar ratios of POePC and POPG (35:65, 40:60 and 45:55) as were used to assess lipid monolayer partitioning. Despite the fact that both HT61 and CHX were capable of interacting and partitioning into a lipid monolayer to the same degree, as well as increasing the permeability of the cytoplasmic membrane in protoplasts, only HT61 demonstrated lytic activity towards the liposomes (Figure 5). In line with the protoplast lysis assay, HT61 was capable of disrupting the lipid bilayer to a large enough degree to result in the release of 19% of the entrapped carboxyfluorescein from liposomes containing 65% PG (Figure 5). As previously shown in the lipid partitioning assay, HT61's activity against the bilayer was reduced as the content of %PG within the bilayer also deceased, only inducing the release of 13% and 16% of the entrapped dye at 55% and 60% PG, respectively (Figure 5). CHX's activity on the lipid bilayer was much reduced

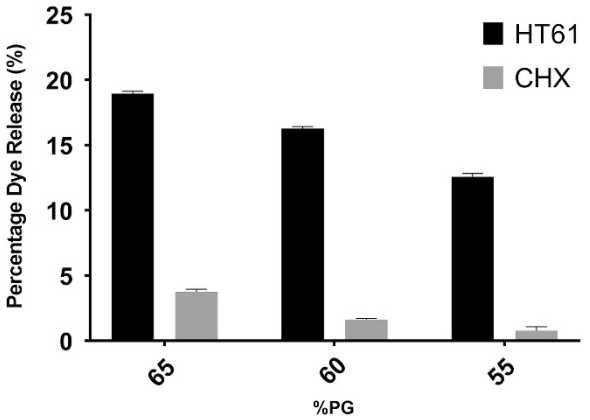

Figure 5 Maximum percentage dye release from 35:65, 40:60 and 45:55 molar ratio POePC/POPG liposomes over 500 s following challenge with 9 μg ml^{-1} HT61 and CHX.

compared with that of HT61. At the highest %PG, CHX could only induce 4% carboxyfluorescein leakage from the liposomes, a quarter of what was released following challenge with HT61 (Figure 5). This slight affect CHX had on the 65% PG lipid bilayers was further attenuated when the %PG within the bilayer was also reduced, resulting in the release of 1 and 2% entrapped dye from liposomes containing 55% and 60% PG, respectively (Figure 5).

DISCUSSION

In recent years, a number of antimicrobials that target and disrupt the bacterial cytoplasmic membrane have been investigated[1,7–10,23] with several, such as daptomycin,[10] coming to market and being routinely used to treat invasive bacterial infections. HT61 and CHX have both previously been shown to be active against the bacterial cytoplasmic membrane,[3] non-specifically targeting anionic lipids, disrupting the membrane to a degree that results in the release of the constituents of the cytoplasm, such as ATP and K^+.[3,7] However, it is clear from a previous study into the mode of action of HT61[3] there is a noticeable difference in membrane activity of the two antimicrobials, as their minimum inhibitory concentrations and relative effects on *S. aureus* membranes did not correlate as expected.

The fact that both HT61 and CHX interact with the cytoplasmic membrane is not in dispute, this study and studies before it quite clearly show that HT61 and CHX electrostatically interact with anionic lipids that are present in the membrane, resulting in bilayer partitioning and increased permeability. This interaction increases as the content of the anionic lipid increases, confirming the specificity of the antimicrobials toward bacterial membranes. This type of activity is characteristic of many different membrane-active antimicrobials, such as daptomycin.[8–10] However, what is less apparent is whether this measurable membrane interaction is the primary mode of action of both HT61 and CHX, as has previously been proposed.[3] In the experiments we have conducted, the liposomes and protoplasts used were dispersed in isosmotic buffers to prevent osmotic lysis prior to challenge with the antimicrobials. This meant that any ATP release or lysis of *S. aureus* protoplasts, or release of carboxyfluorescein from liposomes could only be due to a disruptive lytic effect rather than purely as by-product of the partitioning of antimicrobial into the bilayer. HT61 clearly has some lytic effect on the bacterial membrane, specifically in this case that of *S. aureus*, as it induced lysis of protoplast membranes and a significant release of ATP. HT61 was also able to induce the release of carboxyfluorescein from liposomes made to represent a simple model of *S. aureus* lipid membranes. This suggests that HT61's action on the membrane may be comparable to a weak detergent and confirms HT61 as a membrane-acting antimicrobial and provide an explanation for HT61's high activity against non-multiplying *S. aureus*,[27] although these data do not prove that HT61's primary mode of action is on the membrane. A previously study found that there is no difference in PG, cardiolipin or lysyl-PG content in methicillin-resistant and sensitive strains of *S. aureus* membranes[40] and, as HT61 is actually more active against methicillin-resistant *S. aureus* than the methicillin sensitive Oxford strain of *S. aureus*,[27] it seems reasonable to assume that HT61 would have the same action on methicillin-resistant *S. aureus* membranes as were observed in this study. CHX did not lyse the *S. aureus* protoplast membranes or result in the release of ATP and did not induce significant release of carboxyfluorescein from liposomes. This also raises questions about whether the primary mode of action of CHX is indeed activity against the bacterial membrane or whether there is also an internal target. It has previously been suggested that CHX causes cytoplasmic precipitation and is bactericidal at high concentrations and affects the membrane and is bacteriostatic at low concentrations,[31] but this has recently been superseded by the proposal that the primary mode of action is at the membrane due to the overwhelming evidence of membrane activity.[3,7] However, it seems that osmotic pressure in assays and systems that measure membrane activity may enhance the activity of membrane-active antimicrobials and could therefore mask the true mode of action of these antimicrobials and would need to be taken into account when drawing conclusions from these assays. CHX tolerance has been thought to be mediated through a proton motive force-dependent efflux pump, which would suggest CHX has an internal target.[41] A membrane-active antimicrobial that directly and catastrophically affects the bacterial membrane would be able to circumvent a resistance mechanism such as an efflux pump. Indeed, it has been recently shown that HT61 can act in synergy with CHX against *S. aureus*, increasing the potency of the two antimicrobials.[32] However, this study has shown that resistance, or at least tolerance, to membrane-acting antimicrobials is a real possibility if there are changes in the composition of the lipid bilayer by reduction of the amount of anionic lipid present.[15–19] This suggests that the primary target for CHX may indeed be within the cell interior as has been previously proposed (where it causes precipitation of cytoplasmic material), and that the membrane disruption is actually an off-target side-effect of it gaining entry to the cell.[42]

CONCLUSIONS

HT61 and CHX are both membrane-active antimicrobials that non-specifically target anionic lipids and partition into the lipid bilayer, conferring specificity to the cytoplasmic membrane of bacteria, resulting in increased permeability. However, HT61 also has a demonstrable lytic effect on both bacterial and model membranes, which results in the leakage of the intracellular components and lysis. This action is absent from bacterial and model membranes following challenge with CHX, raising questions over the suggestion that CHX and HT61 primary mode of action is via damage to the cytoplasmic membrane.

CONFLICT OF INTEREST

ARMC is a director and shareholder of Helperby Therapeutics Group plc. The remaining authors declare no conflict of interest.

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