Organic &
Biomolecular
Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 6367

www.rsc.org/obc

PAPER

Examination of a synthetic benzophenone membrane-targeted antibiotic

Sunil K. Vooturi, Mahender B. Dewal and Steven M. Firestine*

Received 25th April 2011, Accepted 15th June 2011
DOI: 10.1039/c1ob05643c

The enormous success of antibiotics is seriously threatened by the development of resistance to most of the drugs available on the market. Thus, novel antibiotics are needed that are less prone to bacterial resistance and are directed toward novel biological targets. Antimicrobial peptides (AMPs) have attracted considerable attention due to their unique mode of action and broad spectrum activity. However, these agents suffer from liability to proteases and the high cost of manufacturing has impeded their development. Previously, we have reported on a novel class of benzophenone-based antibiotics and early studies suggested that these agents might target the bacterial membrane. In this study, we present our work on the mechanism of action of these novel membrane targeted antibiotics. These compounds have good affinities to polyanionic components of the cell wall such as lipoteichoic acid (LTA) and lipopolysaccharide (LPS). We found that these agents release potassium ions from treated bacteria; thus, resulting in disruption of the bacterial membrane potential. Benzophenone-based membrane targeted antibiotics (BPMTAs) cause membrane disruption in synthetic lipid vesicles that mimic Gram-positive or Gram-negative bacteria. The compounds display no hemolytic activity up to a concentration that is 100 times the MIC values and they are capable of curing mice of a lethal MRSA infection. Repeated attempts to develop a mutant resistant to these agents has failed. Taken together, BPMTAs represent a promising new class of membrane-targeted antibacterial agents.

Introduction

The rapid emergence of bacterial infections that are resistant to many drugs presents a difficult challenge for healthcare workers. To aid in this challenge, antibiotics are needed which are less prone to the development of bacterial resistance and are directed towards novel biological targets.[1,2] One method towards developing novel antibiotics is by emulating the host defence antimicrobial peptides (AMPs). AMPs from innate immunity have broad spectrum activity and are selective against pathogens.[3,4] AMPs can be quite varied but generally contain cationic amino acids and display amphiphilicity. The overall positive charge of many AMPs ensures accumulation at polyanionic microbial cell surfaces, and the amphiphilic nature of the peptide helps to facilitate pore formation in bacterial membranes leading to cell death.[5] While resistance to some antimicrobial peptides has been detected, it is generally believed that resistance is less common with these classes of antibiotics because it requires substantial changes to the membrane and its components.[4] Although three major models ('barrel-stave', 'toroidal pore', and 'carpet') have been proposed for the membrane–peptide interaction, the exact molecular mechanism by which the AMPs form pores in the membrane is still not fully understood.[5] Despite the promise of these peptides, several barriers remain for the development of AMPs into medicinal agents. First, AMPs are liable to proteases, creating potentially unfavorable pharmacokinetics. Second, AMPs are costly to synthesize and finally, AMPs have only been used topically rather than systemically (parenteral or oral), indicating a possible toxicity issue with systemic use.[6] To circumvent these problems, researchers have designed and investigated synthetic molecules that are mimics of AMPs.[6,7]

Recently, we discovered a class of benzophenone-based membrane targeted antibiotics (BPMTAs) with excellent activity against antibiotic-resistant strains like MRSA and VRSA (Fig. 1). These agents possess a MIC of 0.68 μM (0.5 mg L^{-1}) to 1.36 μM (1.0 mg L^{-1}) against Gram-positive bacteria and initial studies revealed that these antibiotics targeted the bacterial membrane

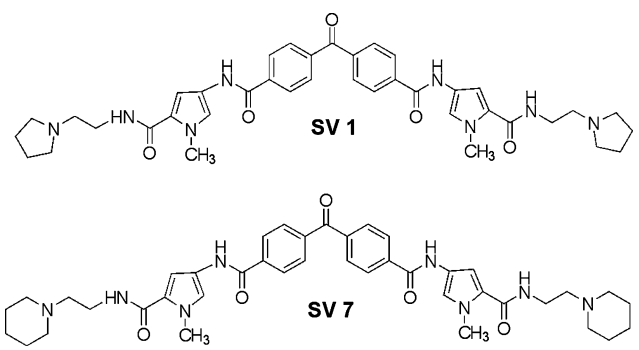

Fig. 1 Structures of novel membrane-targeted antibiotics.

Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, 259 Mack Avenue, Detroit, MI, 48201, USA. E-mail: sfirestine@wayne.edu; Fax: +1 313-577-2033; Tel: +1 313-577-0455

and disrupted the membrane potential.[7] Beyond these simple observations, little was known on the mechanism of action of BPMTA and how this agent affects the membrane. In this paper, we describe our studies aimed at addressing these issues. We find that BPMTAs are selective for bacterial cells and fail to lyse red blood cells even when the concentration of the drug is 100 times the MIC value. Our studies also demonstrate that BPMTAs bind to anionic cell wall components like lipoteichoic acids and lipopolysaccharides and result in a time- and dose-dependent release of intracellular potassium from the bacterial cells. BPMTA promotes dye release from synthetic membrane vesicles, indicating that these agents either disrupt membrane packing or form a transmembrane pore.

Results

LTA and LPS binding studies

Previous work on **SV 7** and **SV 1** revealed that these compounds induced membrane disruption without affecting macromolecular biosynthesis.[7] This suggests that **SV 7** and **SV 1** disrupt the bacterial membrane. Bacterial membranes from both Gram-positive and Gram-negative microbes are negatively charged and also contain additional, negatively charged polymeric molecules on their exterior. Thus, before **SV1** and **SV7** can access the bacterial membrane, they must first interact with these polymers. The most common polymers are lipoteichoic acids (LTA), which are present in Gram-positive membranes and lipopolysaccharides (LPS) which are part of Gram-negative membranes. Both polymers are negatively charged at physiological pH and it has been demonstrated that the charge of these molecules plays an important role in the recognition of bacteria to other cells and surfaces. Furthermore, it is possible that these polymers could serve to enhance the local concentration of the cationic **SV 7** and **SV 1** and thus play a role in the mechanism of action of these agents. To determine whether **SV1** and **SV7** are capable of binding to these polymers, we conducted binding assays to both LTA and LPS. Binding of **SV 1** and **SV 7** to LTA was measured by competition with the dansyl conjugate of Polymixin B (DPX), which decreases fluorescence when displaced from LTA.[8] As shown in Fig. 2a, both **SV 1** and **SV 7** displace DPX and thus bind to LTA, although the maximal displacement was approximately 80% of DPX. No displacement was observed for the negative controls (buffer or methanol (used to dissolve **SV1** and **SV7**)). This suggests that **SV 1** and **SV 7** are not capable of binding to all of the potential binding sites on LTA. Analysis of the data revealed that both compounds have approximately equal affinity for LTA with **SV 7** displaying an IC_{50} of 12.7 μM (9.33 mg L^{-1}) while **SV 1** has an IC_{50} of 16.7 μM (11.81 mg L^{-1}).

Next we investigated binding to LPS. Here, we expected to observe weaker binding since both **SV 7** and **SV 1** are less potent against Gram-negative microbes. However, since LPS is a potent endotoxin, we thought that the ability of the compounds to bind to LPS would be important to determine since such an effect could provide additional medicinal benefits by neutralizing LPS and thereby preventing the inflammatory response.[9] Binding of antibacterial compounds **SV 7** and **SV 1** to LPS was examined using the displacement assay based on binding of BODIPY–cadaverine.[9] Fluorescence of BODIPY decreases when it is bound to the LPS and thus an addition of the antibacterial compounds should lead to displacement of the bound BODIPY resulting in an increase in fluorescence. As expected, these experiments (Fig. 2) reveal that both **SV 1** and **SV 7** bind to LPS with **SV 7** displaying a higher affinity (IC_{50} = 43.6 μM (32 mg L^{-1})) compared to **SV 1** (IC_{50} = 55 μM (39 mg L^{-1})). Again, no displacement was seen for the negative controls.

Membrane depolarization studies

Previous studies using diSC3(5) dye revealed that **SV 7** depolarizes the membrane potential of the cell in a time-dependent manner.[7] **SV 7** gradually depolarized the membrane potential. To further explore the depolarizing affect of **SV 7**, we determined whether **SV 7** displayed a dose-dependent phenomenon. Several concentrations ranging from 0.68 μM (0.5 mg L^{-1}) to 74.9 μM (55 mg L^{-1}) were done in duplicate and the membrane potential was measured after 60 min of incubation with **SV 7**. We found that **SV 7** depolarized the membrane potential in a dose-dependent fashion (Fig. 3). It is interesting to note that a higher concentration of **SV 7** was required to achieve the maximum depolarization compared to its bactericidal activity.

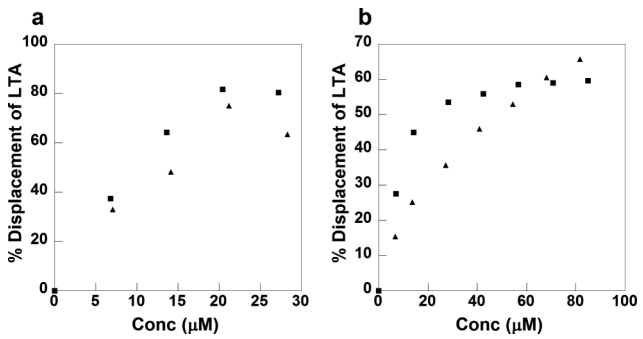

Fig. 2 Binding affinities of antibacterial compounds **SV 7** (■) and **SV 1** (▲) for LTA and LPS as measured by the DPX displacement assay and BODIPY–cadaverine displacement assay respectively. a) LTA (5 mg L^{-1}) was incubated with 2.5 μM DPX, and the fluorescence was measured at 485 nm. b) LPS (5 mg L^{-1}) was incubated with 1.0 μM BODIPY–cadaverine, and the fluorescence was measured at 580 nm.

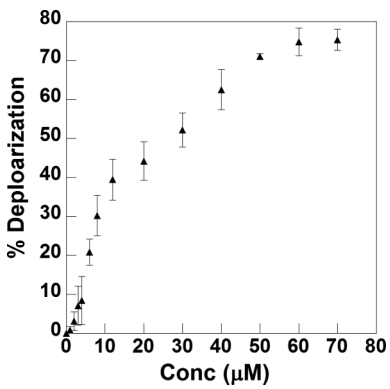

Fig. 3 Dose-dependent depolarization studies of **SV 7** using *S. aureus* as measured by the membrane potential-sensitive dye diSC3(5). Various concentrations of **SV 7** ranging from 0.68 μM (0.5 mg L^{-1}) to 74.9 μM (55 mg L^{-1}) were used. Changes in membrane potential were calculated as a percentage of depolarization due to nisin which was set to 100%.

Potassium release studies

Disruption of bacterial membranes is often accompanied by the release of ions from inside the cell, most commonly potassium. To determine if potassium is released, we incubated early exponential phase *Staphylococcus aureus* cells with **SV 7** and monitored the release of potassium using a potassium selective electrode. In these experiments, nisin was used as a positive control as it is known to permeabilize the bacterial membrane resulting in the release of cellular contents.[10] As shown in Fig. 4a, **SV 7** (13.6 μM (10 mg L^{-1})) results in a time-dependent release of potassium which plateaus after 30 min. No potassium release was seen for either buffer alone or 5% DMSO. This pattern is similar to the time-dependent depolarization studies of **SV 7**.[7] Indeed, the extent of depolarization and potassium release is the same. Based on the results from these two studies, we suggest that there is a temporal correlation between depolarization studies and the potassium release. Fig. 4b, shows the dose-dependent release of potassium using **SV 7**. Six concentrations of **SV 7** were used ranging from 0.68 μM (0.5 mg L^{-1}) to 27 μM (20.0 mg L^{-1}), and the potassium levels were measured after 30 min of incubation. We found that **SV 7** exhibited dose-dependent potassium release substantiating the evidence that our antibacterials act by releasing potassium ions from the bacteria.

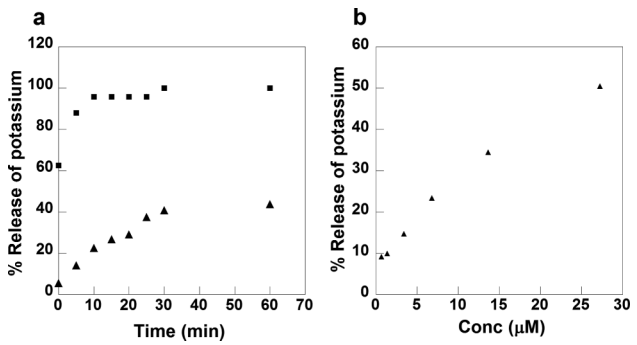

Fig. 4 Potassium release studies of **SV 7**. *S. aureus* cells were suspended in HEPES buffer (5 mM HEPES, 5 mM glucose, pH 7.2) and potassium release was monitored using a potassium selective electrode at 37 °C. a) Time-dependent potassium release was performed using 13.6 μM (10.0 mg L^{-1}) of **SV 7** (▲). Potassium release is monitored every 5 min up to 60 min of incubation, while nisin (■) was used as a positive control. b) Dose-dependent potassium release was measured using six different concentrations of **SV 7** (0.68 μM (0.5 mg L^{-1}) to 27 μM (20.0 mg L^{-1})). Potassium release was monitored after 30 min of incubation.

Dye release from LUVs

To further study the effects of **SV 7** on the membrane, we utilized fluorescent dye release studies using synthetic lipid vesicles that mimic both bacterial and mammalian cell membranes. In this study, we examined the release of calcein dye from large unilamellar vesicles (LUVs) composed of either 80 : 20 DOPE : DOPG as a surrogate for Gram-negative bacteria membranes, 58 : 42 DOPG : CL as a Gram-positive bacteria membrane mimetic or 100% DOPC as a mammalian membrane analog.

The dye release data are shown in Fig. 5. As seen in Fig. 5, **SV 7** is capable of releasing dye from bacterial membranes, whereas, little dye is released when **SV 7** is added to mammalian LUVs.

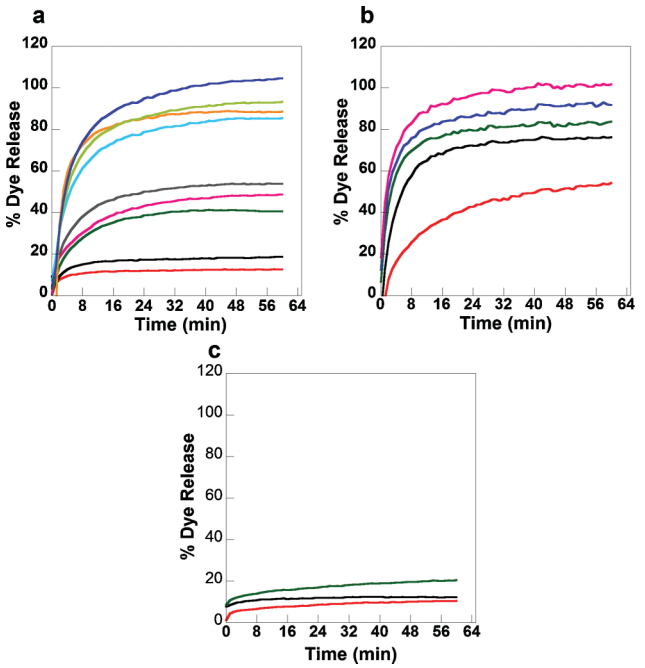

Fig. 5 Calcein dye release by **SV 7** from the LUVs that mimic **a)** Gram-positive bacteria membrane LUVs. **SV 7** concentration ranges from 1.4 μM (1 mg L^{-1}, red) to 6.8 μM (5 mg L^{-1}, blue) in 0.68 μM (0.5 mg L^{-1}) intervals. **b)** Gram-negative bacteria membrane LUVs. **SV 7** concentration ranges from 6.8 μM (5 mg L^{-1}, red) to 34 μM (25 mg L^{-1}, pink) in 6.8 μM (5 mg L^{-1}) intervals. **c)** Mammalian cell membrane LUVs. **SV 7** concentration ranges from 13.6 μM (10 mg L^{-1}, red) to 41 μM (30 mg L^{-1}, green) in 13.6 μM (10 mg L^{-1}) intervals.

This is in agreement with our previous results which indicate that **SV 7** is a non-cytotoxic, antibacterial agent. The data also show that **SV 7** is more efficacious against Gram-positive LUVs *versus* Gram-negative membrane analogs. **SV 7** causes 100% dye release at 5 mg L^{-1} for the Gram-positive LUVs, whereas 25 mg L^{-1} is needed for complete dye release with Gram-negative LUVs. These data are in agreement with antibacterial selectivity of **SV 7**.

Hemolysis, animal and resistance studies

Hemolysis is one of the main side effects caused by membrane-bound antibiotics. To examine whether our compounds have any hemolytic activity, we tested both **SV 7** and **SV 1** for their ability to lyse red blood cells. As seen in Fig. 6, we found that neither compound resulted in the lysis of red blood cells even up to a concentration of 100 times the MIC value. Thus, these compounds act selectively on bacterial membranes.

Next, we conducted animal studies to examine whether **SV 7** was efficacious in an animal model of infection. In this model, groups of 6 mice were injected with a solution of MRSA (ATCC 33591, 7×10^7 CFU mL^{-1}) in PBS supplemented with 5% hog gastric mucin. Subcutaneous injections of various concentrations of **SV 7** were administered and the ED_{50} under these conditions is 24 mg/kg. **SV 7** was able to cure mice of MRSA infection when given at a dose of 50 mg kg^{-1} and thus was as effective as the positive control (30 mg kg^{-1} vancomycin). These data indicate that **SV 7** has the potential to be a useful agent in the treatment of Gram-positive antibiotic resistant infections.

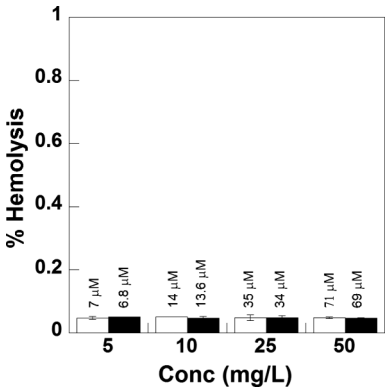

Fig. 6 Hemolysis data of **SV 7** (unshaded) and **SV 1** (shaded) using sheep erythrocytes. Four different concentrations ranging from 5 mg L^{-1} to 50 mg L^{-1} were used. Hemolysis was calculated as a percentage of the release due to 1% Triton-100 which was set to 100%. The molar concentration of each agent is listed above the bar.

Finally, we examined the ability of *S. aureus* to develop resistance to **SV 7**. Cultures of *S. aureus* were serially inoculated for 21 consecutive days in the presence of sub-lethal doses (0.5-0.75 X MIC) of **SV 7**. After each overnight incubation, a portion of the culture was added to a fresh media containing **SV 7** at its MIC value to determine if any bacteria were capable of growing in the presence of a lethal amount of **SV 7**. We observed no bacteria which became resistant to **SV 7**. We repeated the same experiment using gradient agar plates in which the level of **SV 7** across the plate varied from 0–1.5 X MIC. Again, no **SV 7**-resistant bacteria were observed. We thus conclude that **SV 7** has a low potential for the development of resistance.

Discussion

Structural and mechanistic similarities between BPMTAs and AMPs

AMPs are quite diverse, with peptides differing in chain length, charge, composition, and secondary structure. Yet, most AMPs have two common structural motifs, namely a cationic nature and amphiphilicity. The overall positive charge ensures accumulation of peptide at polyanionic microbial surfaces, whereas the amphiphilic nature facilitates pore formation or membrane disruption in the bacteria. Like AMPs, BPMTAs contain two cationic residues and thus bind well to anionic component of the cell wall (LTA and LPS). Indeed they have better affinities for these components compared to chlorohexidine (~ 84.6 μM (43.0 mg L^{-1}) against LPS and ~ 93 μM (47.0 mg L^{-1}) against LTA), which is a popular biocide used in antiseptic products.[9] BPMTAs also display amphiphilicity where the two cationic moieties are separated by a hydrophobic patch. Moreover, BPMTAs also exhibit facial-edge amphiphilicity such that polar moieties are aligned on the edges and non polar moieties are aligned on the faces of the molecule.

The antimicrobial mechanism of AMPs is a subject of much speculation; however, it is generally believed that AMPs kill bacteria by inducing membrane damage. We have shown here that BPMTAs cause depolarization and release of potassium ions from bacterial cells and result in dye release from synthetic vesicles. All of these effects have been observed for naturally occurring AMPs. We have also shown that BPMTAs disrupt lipid vesicles mimicking the lipid compositions of Gram-negative and Gram-positive cell membranes. In contrast, BPMTAs do not result in substantial dye release from the lipid vesicles that mimic mammalian cell membrane, a result which is corroborated by the lack of hemolytic activity. There have been published reports on other small molecule, membrane targeted antibiotics.[11,12] While these compounds pioneered the field and also displayed impressive *in vivo* activity, it is worth noting that the compounds discussed here are more potent *in vitro*, display less hemolysis, and have a lower molecular weight.

Similarities to synthetic ion channels

The ability of BPMTAs to release ions from inside of the cell is reminiscent of synthetic ion channels. Typically, synthetic ion channels are classified into two main classes.[13] The first class, tubular channels, are commonly composed of a tris-macrocycle in which all three aza-crown ethers stack co-facially creating a transmembrane channel for cation conduction.[14] The second class, aggregate forming channels, are typified by bolaamphiphiles which are two-headed amphiphiles that are long enough to span the lipid bilayer.[15] For bolaamphiphiles, the monomers associate edge-wise to form a water filled pore. The BPMTAs reported here have some similarities to the aggregate forming synthetic ion channels. BPMTAs are bolaamphiphiles containing two polar head groups, molecular modeling studies indicates that BPMTAs are long enough to span the membrane and preliminary NMR studies indicated that these compounds self-associate in a hydrophobic environment.[18] Taken together, we suggest that BPMTAs function by either disrupting membrane packing or by forming a synthetic pore.

Conclusion

We have described mechanistic studies on a novel benzophenone-based membrane-targeting antibiotic that has similarities to the structure and function of AMPs and synthetic ion channels. We have shown that these BPMTAs bind to the anionic membrane components and result in both a dose- and time-dependent release of potassium ions. These agents result in dye release from lipid vesicles that contain a membrane that mimics Gram-positive bacteria. In contrast, higher drug concentrations are needed for dye release from Gram-negative bacteria mimics or mammalian membrane analogs. This suggests that BPMTAs are selective for bacterial membranes and likely form either a small diameter or ion-selective pore in the membrane.

Methods and materials

LTA binding assay

Dansyl conjugates of Polymyxin B (DPX) and Lipoteichoic acid (LTA) were purchased from Invitrogen and Sigma respectively. The binding affinity of compounds for LTA was determined by using the assay described by Scott *et al.*[8] To a 1.5 mL cuvette, 2.5 μM of DPX and 5.0 μg of LTA (1 mg ml^{-1} stock in water) were mixed with 1.0 mL of 5 mM HEPES buffer (pH 7.2). The fluorescence of DPX bound to LTA was determined using a FluoroMax 3 fluorescence spectrophotometer (Horiba Jobin Yvon, Edison, NJ)

at an excitation wavelength of 340 nm and emission wavelength of 485 nm. **SV 7** or **SV1** (1.36 mM (1.0 mg mL^{-1}) stock in methanol) was added to the cuvette and the decrease in DPX fluorescence due to its displacement was determined. Negative controls included addition of either buffer alone or methanol. The IC_{50} value was determined from a plot of percent displacement *versus* drug concentration.

LPS binding assay

BODIPY® cadaverine and lipopolysaccharides (LPS) were purchased from Invitrogen and Sigma respectively. The relative binding affinity of test compounds for LPS was determined by using the assay described by Zorko *et al.*[9] In a 1 cm quartz cuvette, 1 μM of BODIPY® cadaverine in Tris buffer (50 mM, pH 7.4) was mixed with 5.0 mg L^{-1} of LPS. Sequential additions of **SV 7** or **SV1** in 5.0 μL (1.36 mM (1.0 mg mL^{-1}) stock in methanol) volumes were made to cuvette and the resulting decrease in fluorescence were determined at an excitation wavelength of 580 nm and emission wavelength of 620 nm. Negative controls included addition of either buffer alone or methanol. The IC_{50} value was determined from a plot of percent displacement *versus* drug concentration.

Membrane depolarization assay

DiSC3(5) dye and nisin were purchased from Sigma. The assay was done using the method described by Wu *et al.*[10] *Staphylococcus aureus* were grown at 37 °C with shaking to mid-logarithmic phase (OD_{600} = 0.4–0.5). Cells were collected by centrifugation, washed with a buffer (5 mM HEPES, 5 mM glucose, pH 7.2) and resuspended in the same buffer to an OD_{600} of 0.05. The cell suspension was incubated with 0.4 μM diSC3(5) for an hour at 37 °C, and then 100 mM KCl was added to equilibrate, over a 5 min period, the cytoplasmic and external K^+ concentrations. A 1.0 mL cell suspension was placed in a plastic cuvette with a stir bar and placed in a heated (37 °C) cuvette holder. The desired concentration of **SV 7** (in duplicate, dissolved in DMSO) was added and the fluorescence readings at an excitation wavelength of 622 nm and emission wavelength of 670 nm were taken after 60 min. A blank with only cells and dye was used to subtract the background fluorescence.

Potassium release experiment

The potassium combination electrode was purchased from Denver instruments. HPLC water was purchased from EMD chemicals. Overnight *Staphylococcus aureus* cells, grown from Mueller Hinton media, were washed 3 times with a buffer (5 mM HEPES, 5 mM glucose, pH 7.2) and resuspended in the same buffer to an OD_{600} of 0.2 and stored on ice prior to their use. To a 10.0 mL suspension of cells incubated at 37 °C, varying concentrations of **SV 7** (in DMSO) were added, followed immediately by 6 drops of ISA (Ionic Strength Adjuster – 1 M NaCl). The potassium concentrations were monitored by a change in voltage from the electrode after a specific time (either 30 or 60 min). Nisin was used as a positive control, while the negative control were cells incubated with the same concentration of DMSO as the drug treated sample. The total amount of K^+ inside of the bacteria was determined by boiling bacteria for 5 min. To convert voltage into potassium ion concentration, a standard curve using known concentrations of KCl solutions was constructed.[16] For time-dependent studies, readings were taken every 5 min for up to 30 min.

General procedure for the preparation of calcein dye encapsulated large unilamellar vesicles (LUVs)

All the lipids were purchased as $CHCl_3$ solutions from Avanti Polar Lipids. The required amount of the desired lipids were placed in a 10 mL round bottom flask and the solvent was evaporated *in vacuo* without heat. The resulting film was further dried under high vacuum and then hydrated with 1 mL of 40mM Calcein (Fluorexon), 10 mM NaH_2PO_4, pH 7.0 for 1h. The resulting suspension was subjected to five freeze–thaw cycles using a dry ice/acetone bath and warm water bath respectively. The homogeneous solutions of LUVs were prepared by the extrusion method using a polycarbonate membrane (Whatman, pore size 400 nm). External calcein was removed by gel filtration with Sephadex-G50 using 10 mM NaH_2PO_4, 90 mM NaCl, pH 7.0 buffer.

LUVs that mimic the Gram-negative bacteria (*E. coli*) membrane

137 μL of DOPE (1,2-dioleoyl-sn-*glycero*-3-phosphoethanolamine, 1.370 mg, 1.84 μmol) in $CHCl_3$ solution and 32 μL of DOPG (1,2-dioleoyl-sn-*glycero*-3-phospho-(1′-*rac*-glycerol) (sodium salt), 797.02, 0.40 μmol) in $CHCl_3$ solution were used to prepare LUVs that mimic Gram-negative bacteria membrane.

LUVs that mimic the Gram-positive bacteria (*S. aureus*) membrane

92 μL of DOPG (1,2-dioleoyl-sn-*glycero*-3-phospho-(1′-*rac*-glycerol) (sodium salt), 1.15 μmol) in $CHCl_3$ solution and 126 μL of CL (1,1′2,2′-tetra-(9*Z*-octadecenoyl) cardiolipin (sodium salt) or 1′,3′-bis[1,2-dioleoyl-sn-*glycero*-3-phospho]-sn-glycerol (sodium salt), 0.84 μ mol) were used to prepare LUVs that mimic Gram-positive bacteria membrane.

LUVs that mimic the mammalian membrane

160.4 μL of DOPC (1,2-di-(9*Z*-octadecenoyl)-sn-*glycero*-3-phosphocholine) in $CHCl_3$ solution was used to prepare LUVs that mimic the mammalian membrane.

Dye release assay

To a 2.5 mL cuvette, 20 μL of the calcein-loaded LUVs was added along with various concentrations of **SV 7** followed by a buffer (10 mM Na_2HPO_4, 90mM NaCl, pH 7.0) until the total volume was 2.0 mL. The change in fluorescence over time was determined using an excitation wavelength of 450 nm and an emission wavelength of 510 nm. Control experiments were performed using DMSO as a negative control and 20% Triton-X solution as a positive control. All curves were normalized to % dye release using the equation: % dye release = $(I_t - I_0 / I_\alpha - I_0) \times 100$ where I_t = emission intensity with **SV 7**, I_0 = emission intensity with DMSO control (0% release), I_α = emission intensity with Triton-X control (100% release).

Hemolysis studies

Sheep erythrocytes were purchased from Lampire Biological Laboratories (catalogue number- 7209011, Pipersville, PA). The

whole blood cells were centrifuged at 3000 rpm for 10 min to separate the plasma and white blood cells, followed by washing twice with TBS buffer (10 mM Tris, 150 mM NaCl, pH 7.0). RBCs (0.3 mL) were diluted to 30 ml with TBS buffer to generate a 1% RBC stock. Hemolysis assays were done in 96-well plates and in duplicate. In each well, 120 µl of RBC stock was taken, the drug stocks (**SV 7** and **SV 1**) were added, and the final volume in each well was made to 150 µl using TBS buffer. For controls, DMSO was used as the negative and 1% Triton was used as the positive control. The 96-well plate was incubated for 1 h at 37 °C and centrifuged at 3800 rpm for 5 min. Supernatant (20 µl) was removed from the plate and diluted to 100 µl with TBS buffer. The absorbance at 540 nm was measured to determine the release of hemoglobin using a plate reader (Biotek Synergy 2 multimode plate reader, Winooski, VT). The absorbance of the negative control solution (DMSO) was set to 0% hemolysis while the absorbance of the Triton solution was set to 100% hemolysis.[17]

Animal efficacy studies

These studies were conducted by Ricerca (Concord, OH). The animal studies were performed in compliance with institutional guidelines (Ricerca internal animal welfare committee) regarding animal welfare. Groups of 6 mice were injected with a solution of MRSA (ATCC 33591, 7×10^7 CFU mL^{-1}) in PBS supplemented with 5% hog gastric mucin. Fifteen minutes later, subcutaneous injections of various concentrations of **SV 7** were administered and the mice were observed over five days. For controls, mice treated with saline represented the negative control while vancomycin (30 mg kg^{-1}) was used as the positive control.

Attempted generation of a resistant mutant

MSSA was grown overnight in MHB and a 1% inoculum was added to a fresh tube of MHB substituted with 0.5–0.75 X MIC of **SV 7**. The culture was grown overnight with shaking at 37 °C and the process was repeated over the course of 21 days. A portion of each overnight culture was also added to a fresh tube of MHB containing 1 X MIC of **SV 7** to determine if any bacteria grew at this concentration of **SV 7**. In no case did bacterial grow at the MIC value of **SV 7** even after repeated passaging of **SV 7**.

References

1 C. Walsh, *Nature*, 2000, **406**, 775–781.
2 R. E. Hancock and H. G. Sahl, *Nat. Biotechnol.*, 2006, **24**, 1551–1557.
3 Y. Chen, C. T. Mant, S. W. Farmer, R. E. Hancock, M. L. Vasil and R. S. Hodges, *J. Biol. Chem.*, 2005, **280**, 12316–12329.
4 M. Zasloff, *N. Engl. J. Med.*, 2002, **347**, 1199–1200.
5 K. A. Brogden, *Nat. Rev. Microbiol.*, 2005, **3**, 238–250.
6 R. W. Scott, W. F. DeGrado and G. N. Tew, *Curr. Opin. Biotechnol.*, 2008, **19**, 620–627.
7 S. K. Vooturi, C. M. Cheung, M. J. Rybak and S. M. Firestine, *J. Med. Chem.*, 2009, **52**, 5020–5031.
8 M. G. Scott, M. R. Gold and R. E. Hancock, *Infect Immun*, 1999, **67**, 6445–6453.
9 M. Zorko and R. Jerala, *J. Antimicrob. Chemother.*, 2008, **62**, 730–737.
10 M. Wu, E. Maier, R. Benz and R. E. Hancock, *Biochemistry*, 1999, **38**, 7235–7242.
11 S. Fernandez-Lopez, H. S. Kim, E. C. Choi, M. Delgado, J. R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D. A. Weinberger, K. M. Wilcoxen and M. R. Ghadiri, *Nature*, 2001, **412**, 452–455.
12 L. Liu, K. Xu, H. Wang, P. K. Tan, W. Fan, S. S. Venkatraman, L. Li and Y. Y. Yang, *Nat. Nanotechnol.*, 2009, **4**, 457–463.
13 T. M. Fyles, *Chem. Soc. Rev.*, 2007, **36**, 335–347.
14 G. W. Gokel and I. A. Carasel, *Chem. Soc. Rev.*, 2007, **36**, 378–389.
15 W. Wang, R. Li and G. W. Gokel, *Chem.–Eur. J.*, 2009, **15**, 10543–10553.
16 E. J. van Kan, R. A. Demel, E. Breukink, A. van der Bent and B. de Kruijff, *Biochemistry*, 2002, **41**, 7529–7539.
17 A. Som and G. N. Tew, *J. Phys. Chem. B*, 2008, **112**, 3495–3502.
18 S. Firestine, unpublished data.

