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In vitro antimicrobial studies of silver carbene complexes: activity of free and nanoparticle carbene formulations against clinical isolates of pathogenic bacteria

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Objectives: Silver carbenes may represent novel, broad-spectrum antimicrobial agents that have low toxicity while providing varying chemistry for targeted applications. Here, the bactericidal activity of four silver carbene complexes (SCCs) with different formulations, including nanoparticles (NPs) and micelles, was tested against a panel of clinical strains of bacteria and fungi that are the causative agents of many skin and soft tissue, respiratory, wound, blood, and nosocomial infections.

Methods: MIC, MBC and multidose experiments were conducted against a broad range of bacteria and fungi. Time-release and cytotoxicity studies of the compounds were also carried out. Free SCCs and SCC NPs were tested against a panel of medically important pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Acinetobacter baumannii* (MRAB), *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Klebsiella pneumoniae*.

Results: All four SCCs demonstrated strong efficacy in concentration ranges of 0.5–90 mg/L. Clinical bacterial isolates with high inherent resistance to purified compounds were more effectively treated either with an NP formulation of these compounds or by repeated dosing. Overall, the compounds were active against highly resistant bacterial strains, such as MRSA and MRAB, and were active against the biodefence pathogens *Bacillus anthracis* and *Yersinia pestis*. All of the medically important bacterial strains tested play a role in many different infectious diseases.

Conclusions: The four SCCs described here, including their development as NP therapies, show great promise for treating a wide variety of bacterial and fungal pathogens that are not easily killed by routine antimicrobial agents.

Keywords: antibacterial, antifungal, MIC/MBC, clinical isolates

Introduction

Many bacterial and fungal species play an important role in skin and soft tissue, wound, blood, and respiratory infections. The main challenge in treating these common infections is the increasing resistance of pathogenic bacteria to many commonly used antibiotics.[1,2] For example, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of nosocomial infections. MRSA infections cause a number of other serious diseases, including osteomyelitis,[3,4] bacteraemia,[3,5,6] chronic wound infection,[7–11] septic arthritis,[12] skin and soft tissue infections,[8–10,13] chronic rhinosinusitis (CRS),[14–17] and pneumonia.[2,3,5] Such staphylococcal infections are becomingly increasingly difficult to treat, due to the inability of current antibiotics (e.g. vancomycin) to effectively clear MRSA infections.[1,18–20] The MRSA story represents only one example of the important challenges that are associated with increasing antibiotic resistance in bacteria.

In skin and soft tissue infections associated with deployed military troops, MRSA, multidrug-resistant *Acinetobacter baumannii* (MRAB), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter* spp. are the most common organisms leading to increased morbidity.[6,8–10,13,21] In cystic fibrosis (CF) patients, *P. aeruginosa* and *Burkholderia cepacia* complex organisms play a significant role in the pathogenesis of pulmonary infections.[22–24] CRS, a disease that afflicts ~46 million people in the USA, is often associated with repeated antimicrobial therapy that ultimately fails, leading to the need for surgery.[14–17] Indeed, even the most aggressive antimicrobial therapies are insufficient in clearing microbial communities from pulmonary infections in the CF lung, sinus infections in CRS and wound infections caused by burns or trauma.[2,7,9,11,14–17,19,25–27] These are just a few examples of diseases that we feel represent the dramatic challenge of antimicrobial resistance and underscore the need for the development of novel antimicrobials that effectively kill the bacteria that cause a multitude of diseases.[1,20]

Novel N-heterocyclic silver carbene complexes (SCCs) have displayed *in vitro* antimicrobial activity against a range of pathogenic bacteria.[28] They have also demonstrated efficacy *in vivo* in mouse models of pneumonia.[28] While pure SCCs have shown broad-spectrum antibacterial activity,[28] they do not preferentially accumulate in the lung tissue or ensure a sustained therapeutic concentration.[23] The encapsulation of SCCs into biodegradable nanoparticles (NPs) provides an effective means to circumvent the above-mentioned problems, since NPs exhibit a size-dependent accumulation in lung tissue following nebulization.[29,30] Additionally, the accumulation of NPs in tissue results in a controlled release of SCCs at the site of infection, thereby maintaining effective therapeutic levels of the antimicrobial compound while minimizing potential toxicity.[23]

Recently, L-tyrosine polyphosphate (LTP) NPs have been formulated to encapsulate SCCs and provide a sustained delivery of these compounds.[23] LTP, a polymer synthesized from the amino acid L-tyrosine, can hydrolytically degrade within 7 days without lowering the local pH or producing toxic by-products.[29,31] These SCC-loaded LTP NPs are ~1 μm in diameter.[23] LTP NPs loaded with a hexyl functionalized SCC have shown a survival advantage of ~25% in an infected mouse lung model, but a higher survival advantage is desired.[23] The efficacy of poly(ethylene glycol)-poly(lactic acid) (PEG-PLA) micelles as a delivery system for SCCs has also been explored. The advantage of PEG-PLA, an amphiphilic polymer, is that it is commonly used for drug delivery systems, is biocompatible, is FDA approved and degrades into lactic acid and PEG, both of which are biocompatible products. Here, we demonstrate that purified SCCs, or those incorporated into LTP or PEG-PLA, provide broad and effective antimicrobial activity. These compounds are active against MRSA, MRAB, *P. aeruginosa*, *B. cepacia*, *K. pneumoniae*, *Burkholderia* species, *Candida albicans* and other medically important bacteria *in vitro*. The broad antimicrobial activity of these compounds, including their activity across microbial kingdoms, may offer promising, new therapeutics that can be successfully employed against hard-to-treat diseases.

Materials and methods

SCCs

For this study, we utilized four different SCC compounds (SCC1, SCC8, SCC22 and SCC23) in pure and NP formulations (see Figure 1). These compounds have been previously characterized.[23,28,32–35]

NP synthesis

SCC22 and SCC23 were encapsulated within NPs formulated from a blend of LTP (MW = 8 kDa), PEG grafted to chitosan (PEG-g-CHN, CarboMer Inc., 80% acetylation, San Diego, CA, USA) and linear polyethylenimine (LPEI, MW = 25 kDa, PolyScience Inc., Warrington, PA, USA) prepared using a double emulsion of water-in-oil-in-water (w/o/w) by impeller and solvent evaporation, as previously described by Hindi *et al.*[23] Briefly, this procedure for the blank LTP NP formulation consisted of a primary emulsion of 294 mg of LTP dissolved in chloroform (100 mg/mL), 3.0 mg of PEG-g-CHN dissolved in 0.9 mL of 0.1 N acetic acid, 3 mg of LPEI in 1 mL of autoclaved and deionized H_2O, and 1 mL of autoclaved and deionized H_2O. The initial emulsion of SCC-loaded NPs contained 30.0 mg of SCC22 dissolved in 1 mL of autoclaved and deionized H_2O (SCC22-LTP NPs), 30.0 mg of SCC23 dissolved in 3 mL of chloroform (SCC23-LTP NPs) or both SCC22 and SCC23 (SCC22/SCC23-LTP NPs). The initial water-in-oil emulsion was vortexed for 1 min at 3000 rpm by an impeller (Yamato Lab-Stirrer LR400D, Santa Clara, CA, USA). The second water-in-oil-in-water emulsion added 100 mL of 10% polyvinylpyrrolidone (Sigma–Aldrich, St Louis, MO, USA) containing 10 mg/mL $NaNO_3$ and was mixed for 3 min at 1600 rpm. Chloroform was evaporated for 5 h while the NPs were stirred and vented. The NPs were collected and washed with deionized and autoclaved H_2O by centrifugation at 12 000 **g** for 20 min. Lastly, NPs were shell frozen and lyophilized (Labconco Freezone 4.5, Kansas City, MO, USA). Control NPs formulated from poly(lactic-glycolic acid) (PLGA, inherent viscosity = 0.59 dL/g in hexafluoroisopropanol at 30°C, MW = 40 kDa, Lactel Absorbable Polymers International, Pelham, AL, USA) were produced by the same protocol except for an absence of LTP and LPEI.

The PEG-PLA polymer was synthesized by ring opening polymerization of L-lactide with stannous octoate utilizing the hydroxyl group of 2000 MW PEG as the initiator. The resulting polymer had a PEG:PLA ratio of 1:1 confirmed by ^{1}H-NMR spectroscopy. The polymer was used to formulate SCC23 encapsulated in PEG-PLA micelles. These are self-assembled particles that take advantage of the amphiphilic nature of the polymer. The PEG-PLA polymer and SCC23 drug are dissolved in tetrahydrofuran, and slowly dripped into water with vigorous stirring. The hydrophobic PLA block of the polymer and SCC23 associate with the organic solvent, and the hydrophilic PEG associates with the water phase. Once the critical micelle concentration is reached, the polymers self-assemble to form a spherical particle with the PLA block polymer and hydrophobic SCC23 in the core, and PEG on the surface interacting with the water. The particles are dialysed against bulk water and lyophilized to yield SCC23-loaded PEG-PLA micelles.

Scanning electron microscopy of NPs

Scanning electron microscopy (Hitachi S2150, Krefeld, Germany) was used to image the size and morphology of the NPs. The scanning electron microscopy samples were prepared by suspending 1 mg of NPs in 1 mL of deionized and autoclaved H_2O. Then, 200 μL of the suspended NPs was pipetted onto a stub, dehydrated, sputter coated with silver/palladium and examined.

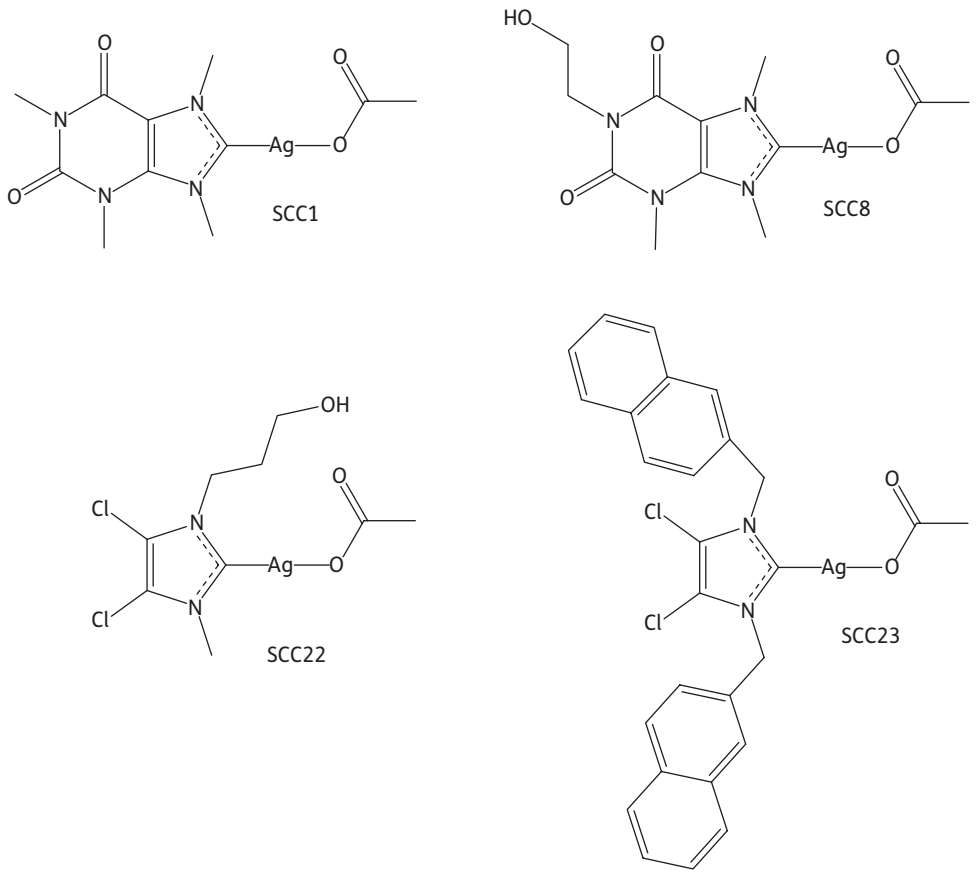

Figure 1. Chemical structures of SCCs 1, 8, 22 and 23.

Dynamic light scattering of NPs

Dynamic light scattering quantified the size of the NPs. These NP samples were prepared by suspending 1 mg of NPs in 10 mL of deionized and autoclaved H_2O. A dynamic laser light-scattering system (Brookhaven Instruments BI-200SM, Holtsville, NY, USA) calculated the NP diameter by the regularized non-negatively constrained least squares (CONTIN) method. The range of NP size was reported as differential distribution values.

Loading into and release from LTP NPs of SCCs

The loading of SCCs in NPs was determined by the absorbance measurements of SCCs extracted from the NPs, as previously described by Hindi *et al*.[23] Briefly, 1 mg of both blank and SCC-loaded NPs was dissolved in 0.4 mL of chloroform, emulsified with 0.4 mL of 0.1 N HCl, and phase separated. Then, the aqueous phase was sampled and its absorbance was measured at a wavelength of 450 nm to detect the Ag(I) ion by spectrophotometry (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). The concentration of the SCCs was calculated according to a standard curve.

Absorbance measurements of the supernatant acquired from suspended and incubated NPs generated the release profile of SCCs from NPs, as previously described by Hindi *et al*.[23] Release samples were prepared by suspending 5 mg of NPs in 1 mL of phosphate-buffered saline (pH=7.4) and incubating at 37°C under rotation. At pre-determined intervals of time, the samples were centrifuged at 8000 **g** and 900 μL of buffer was removed and exchanged with fresh buffer. The samples were lyophilized, suspended in chloroform, emulsified with 0.1 N HCl and phase separated. The absorbance of the aqueous phase was measured at a wavelength of 450 nm using spectrophotometry (SpectraMax M2) and the SCC concentration was calculated according to a standard curve.

Bacterial and fungal strains

Clinical bacterial and fungal strains were isolated from various human tissue sources, including blood, sputum, throat and sinuses. Bacterial strains were kept at Northern Arizona University and securely stored cryogenically at −80°C. When testing new antimicrobials, we feel it is vital to test these compounds on recent clinical isolates that have not been serially passaged, as this procedure has been shown to completely change the virulence characteristics of microbes.[36] Thus, the results presented here represent the activity of these silver carbenes against current pathogenic strains of microbes.

The laboratory strain PA01-V was provided by Dr Maynard Olson (University of Washington, Seattle, WA, USA). The mucoid clinical isolate of *P. aeruginosa* PA M57-15 was provided by Dr Thomas Ferkol (Washington University, St Louis, MO, USA). The PA HP3, PA RR05, PA LF05, AX RE05, AX 22, SM AH06, SA EH06 and SA LL06 strains were cultured from the sputum of CF patients at St Louis Children's Hospital. The silver-susceptible and silver-resistant *E. coli* strains J53 and J53+pMG101 were provided by Dr Simon Silver (University of Chicago, Chicago, IL, USA). The J53 strain is known to be susceptible to killing by silver cations and serves as a positive control. In contrast, the J53+pMG101 is a J53 derivative that harbours the pMG101 plasmid

originally conferring silver resistance to a burns ward isolate of *Salmonella* and serves as a negative control. All bacterial strains were maintained as glycerol stocks at −80°C.

MIC determinations of free SCC formulations for planktonic bacteria at $\sim 1 \times 10^6$ cfu

MICs of SCC1, SCC8, SCC22 and SCC23 were determined for 29 strains (16 species) of clinical bacterial and fungal isolates from various human tissue sources (blood, sputum, nasal, throat, eye, CSF and sinus). Bacterial and fungal strains were streaked onto the appropriate media and incubated overnight at 37°C. Single colonies were selected and inoculated into 10 mL of sterile broth. Inoculated broth was incubated at 37°C overnight under constant agitation. Overnight cultures were diluted 1:100 in broth, resulting in $\sim 1 \times 10^6$ cfu, as confirmed by plating serial dilutions. A mass of 10 mg of each pure formulation of SCCs was resuspended in 1 mL of sterile deionized water (SCC23 was resuspended in 1 mL of DMSO) and diluted in Mueller–Hinton (M–H) broth to achieve a concentration of 2048 mg/L SCC. DMSO alone was utilized as a control for these studies and did not exhibit substantial killing of bacteria compared with the SCCs. Next, 200 μL of diluted SCC was added to the first well of a 96-well plate (the experiment was performed in triplicate). One hundred microlitres of M–H broth was added to the 11 subsequent wells. Serial dilutions were performed on SCCs and 100 μL of diluted bacteria at $\sim 1 \times 10^6$ cfu was added to all of the wells. The final concentrations of SCCs were 725, 363, 181.3, 90.7, 45.4, 22.7, 11.4, 5.9, 2.7, 1.5, 0.71 and 0.35 mg/L. Plates were incubated in a shaker at 37°C for 18–24 h. MICs were determined visually as the lowest concentration at which each of the triplicate wells was clear. Wells containing only broth and bacteria were used as a control. The reported MIC is the lowest concentration in which growth was inhibited in three replicate experiments.

MIC and MBC determination for CF clinical isolates

MICs were determined using a broth microdilution method, as previously described in a standard CLSI protocol. Briefly, bacteria were streaked from frozen glycerol stocks onto tryptic soy agar (TSA) or blood agar plates and incubated overnight at 37°C. Colonies from the fresh plates were suspended in the CLSI standard M–H broth to an optical density at 650 nm (OD_{650}) of 0.2 and grown at 37°C in a shaking incubator at 200 rpm to an OD_{650} of 0.4, which corresponds to $\sim 5 \times 10^8$ cfu/mL. The bacteria were diluted in the broth to a concentration of 10^5 in 100 μL, which was added to triplicate wells of a 96-well plate, containing 100 μL of SCC22 or SCC23 diluted in sterile water to various concentrations from the 10 mg/mL stock. The final concentrations tested were 0.125, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 15 and 20 mg/L. The plates were incubated overnight at 37°C. The MIC was the lowest of these concentrations at which each of the triplicate wells in each 96-well plate was clear after 16–24 h of incubation. Each triplicate measurement was performed at least in duplicate for a minimum of six separate measurements. The reported MIC is the lowest concentration at which bacterial growth was inhibited in three replicate experiments. The MBC of SCC22 and SCC23 was determined by plating the wells with growth inhibition (clear) on TSA plates, and noting the lowest concentration that resulted in no growth after an overnight incubation at 37°C.

MIC and MBC determination of pure SCC and NP formulations at $\sim 1 \times 10^5$ cfu

MICs and MBCs of pure SCC1, SCC8, SCC22 and SCC23, SCC22-LTP NPs, SCC23-LTP NPs, SCC22/23-LTP NPs, and SCC23-PEG-PLA micelles were determined for seven strains of clinical bacterial isolates. Bacterial strains were streaked from frozen glycerol stocks onto appropriate media and grown overnight at 37°C. Colonies were scraped from the plate and diluted in M–H broth to obtain an optical density at 600 nm (OD_{600}) of 0.2 and grown in a 37°C shaker to achieve log-phase growth at OD_{600} of 0.4 (resulting in $\sim 2 \times 10^8$ cfu/mL, as confirmed by plating serial dilutions). The bacteria were further diluted in M–H broth to a concentration corresponding to $\sim 1 \times 10^5$ cfu/mL. Pure SCCs, SCC-LTP NPs and SCC23-PEG-PLA micelles were diluted in sterile deionized water (pure SCC23 in DMSO) and treated 1:1 with bacteria, resulting in SCC concentrations of 10, 8, 6, 4, 2 and 1 mg/L. The plates were incubated at 37°C for 18–24 h. MICs were determined visually as the lowest concentration at which each of the triplicate wells was clear. Separate wells containing blank LTP and micelle NPs, broth only, and bacteria only were used as controls.

MBCs were obtained by plating wells with no bacterial growth (clear) on appropriate media. The plates were incubated at 37°C overnight. MBCs were determined by observing the lowest concentration of SCC resulting in no growth.

MIC determinations for planktonic bacteria: 72 h after a single dose and 72 h after a triple dose

MICs of SCC22-LTP NPs and SCC23-LTP NPs were determined for 14 strains (13 species) of clinical bacterial isolates. Overnight cultures were made by selecting an isolated colony and placing it in 10 mL of broth. The overnight culture was diluted 1:100 in broth, resulting in $\sim 1 \times 10^6$ cfu, as confirmed by plating serial dilutions. SCC-LTP NPs were resuspended in 1 mL of water and diluted in M–H broth to achieve a concentration of 2048 mg/L NP (174 mg/L SCC). Serial dilutions were performed and 100 μL of diluted bacteria was added to all of the wells. The final concentrations of SCC were 87, 43.5, 21.7, 10.9, 5.4, 2.7, 1.4, 0.68, 0.34, 0.17, 0.085 and 0.0425 mg/L. One 96-well plate was treated once and incubated in a shaker at 37°C for 72 h. A second 96-well plate was treated every 24 h for 72 h and incubated in a shaker at 37°C for 72 h. MICs were determined visually on the first plate at 72 h. For the second plate, MICs were determined prior to each dose, and 24 h after the third and final dose of SCC-LTP NPs. Wells containing blank LTP NPs, broth only and bacteria only were used as controls.

Cytotoxicity of SCC-loaded NPs determined using an MTT assay

The *in vitro* cytotoxicity of SCC-loaded NPs was determined with an MTT assay (Trevigen, Gaithersburg, MA, USA). Primary human dermal fibroblasts (a gift from Judy Fulton, Akron General Medical Center) were seeded onto 96-well tissue culture plates (Falcon BD Bioscience, San Jose, CA, USA) at a density of 5000 cells/well and maintained overnight at 37°C with 0.1 mL of feeding medium (90% Dulbecco's and 10% fetal calf serum containing 1% antimycotic). Each well was incubated with serial dilutions of NPs based on loading data, such that concentrations of SCCs were 0, 12.5, 25.0, 50.0, 100.0, 200.0 and 400.0 μM. After 3 and 7 days, an MTT assay was performed according to the manufacturer's instructions and the LD_{50} of each NP formulation was determined. The controls were 100 μL of medium, blank PLGA and LTP NPs, and 2 mM H_2O_2.

Statistical analysis

Where appropriate, analysis of variance and Tukey's Honest Significant Difference (HSD) tests were performed to determine whether SCC activity was significantly different among the tested groups of bacteria and fungi. Student's *t*-test was used to determine significance between SCC-LTP NPs and blank NPs, single and triple doses, and SCC-PEG-PLA micelles and blank micelles. JMP8 statistical software was used for these analyses and significance was noted when $P < 0.05$.

Results

Scanning electron microscopy of SCC NPs

Scanning electron microscopy was used to examine the size, shape and morphology of the SCC22- and SCC23-LTP NPs. The NPs appeared spherical in shape and smooth in morphology (Figure 2). The approximate diameters ranged between 500 nm and 3 μm (Figure 2), which represents an appropriate size range for different treatment regimes, including nebulization.

Dynamic light scattering of NPs

Dynamic light scattering was used to quantify the size range of the NP formulations. This technique demonstrated that all NP formulations had diameters ranging between 500 nm and 3 μm, with mean diameters of ∼1 μm (Figure 3). The encapsulation of both the hydrophobic SCC23 and the hydrophilic SCC22 did not alter the mean diameter of either LTP NP formulation (Figure 3). The resulting mean diameters of LTP NPs are 1450 ± 50 nm for blank LTP NPs, 1998 ± 697 nm for SCC22-LTP

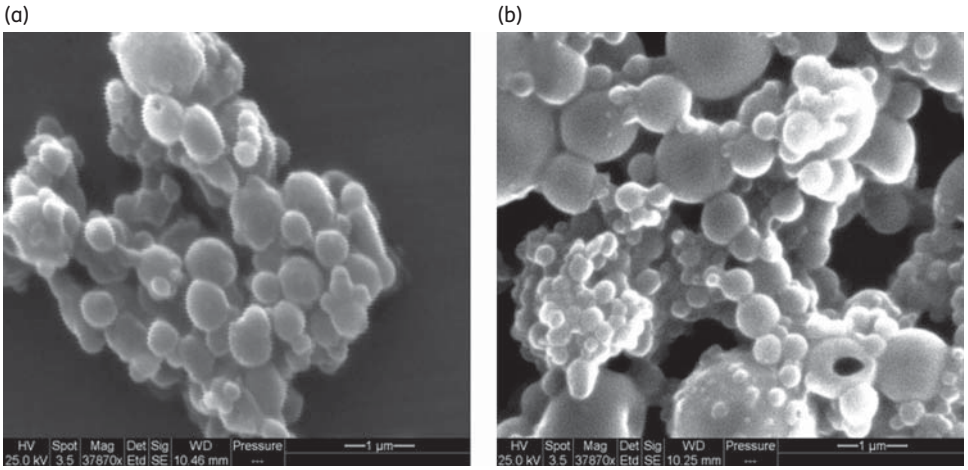

Figure 2. Scanning electron microscopy of (a) SCC22-LTP NPs (magnification: 40000×) and (b) SCC23-LTP NPs (magnification: 20000×).

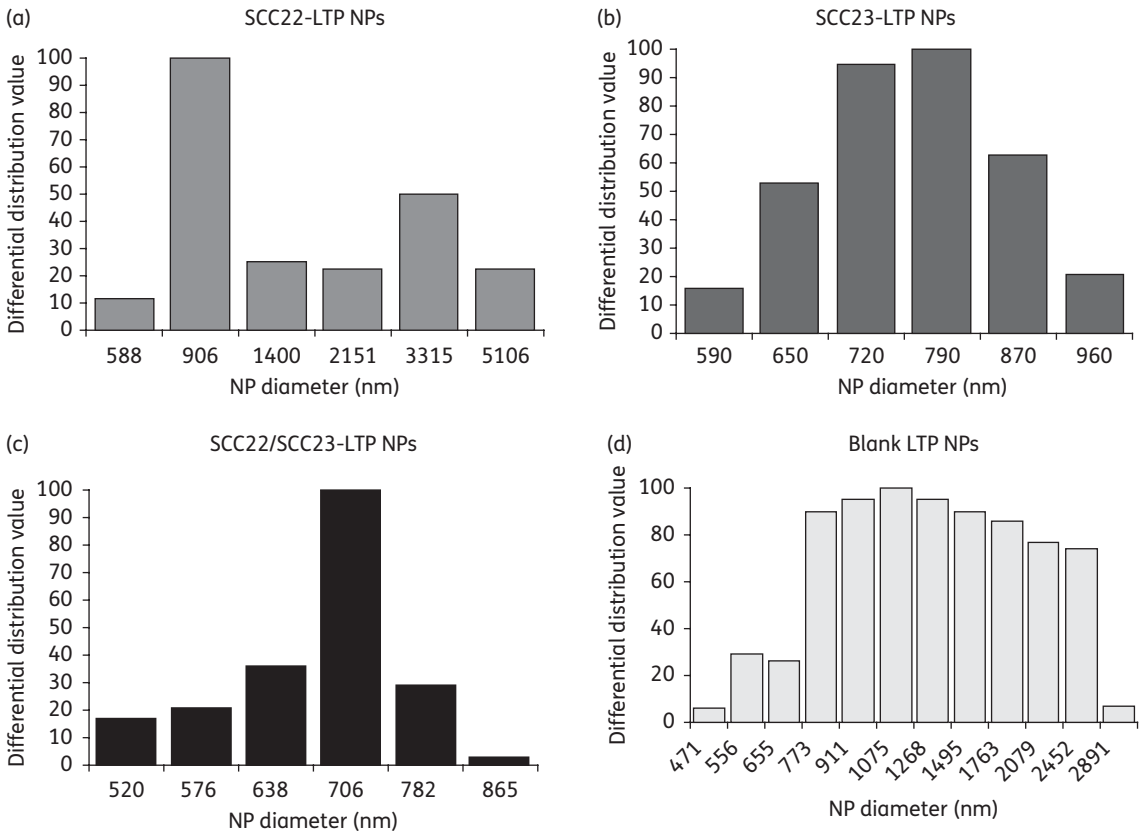

Figure 3. Diameter distribution of (a) SCC22-LTP NPs, (b) SCC23-LTP NPs, (c) SCC22/SCC23-LTP NPs and (d) blank LTP NPs determined by dynamic light scattering.

NPs, 765 ± 56 nm for SCC23-LTP NPs and 679 ± 58 nm for SCC22/SCC23-LTP NPs (Figure 3).

Loading into and release from NPs of SCCs

The loading of SCCs in the NPs was determined by measuring the absorbance of SCCs extracted from the NPs. The loading percentages of SCC22-LTP NPs, SCC23-LTP NPs and SCC22/SCC23-LTP NPs were 8.5% ± 0.2%, 8.3% ± 0.2% and 17% ± 0.2%, respectively (Table 1 and Figure 4). Since these NPs were formulated with either 10% or 20% (w/w) of SCCs, the encapsulation efficiencies of SCC22-LTP NPs, SCC23-LTP NPs and SCC22/SCC23-LTP NPs were 85% ± 2%, 83% ± 2% and 85% ± 2%, respectively (Table 1). Furthermore, these loading efficiencies were used to titrate the amount of SCC NPs necessary for *in vitro* MIC and MBC studies.

Measuring the absorbance of supernatant fluid acquired from suspended NPs was used to generate the release profile of SCCs from the NPs. These studies demonstrated a week-long controlled near-linear release of either SCC22 or SCC23 from the LTP NPs (Table 2 and Figure 4). Meanwhile, the release profile of SCCs from SCC22/SCC23-LTP NPs demonstrated a burst release pattern over the first 24 h that levelled off after 1 week (Table 2 and Figure 4). The cumulative release of SCC from all the NP formulations corresponded to the loading study of SCC in the NP (Table 2 and Figure 4), in which no significant differences exist ($P > 0.947$).

MICs of pure SCCs for planktonic bacteria ($\sim 1 \times 10^6$ cfu/mL)

A wide range of clinical respiratory, enteric, opportunistic and antibiotic-resistant pathogens were tested. All four SCCs in pure and NP form displayed antimicrobial activity against fungi, Gram-positive bacteria and Gram-negative bacteria isolated from different human tissue sources, including sputum, blood, throat, nasal, eye and CSF. The resulting averages of MICs across all clinical pathogens were 17.63 mg/L for SCC23, 25.51 mg/L for SCC22, 30.58 mg/L for SCC1 and 49.68 mg/L for SCC8 (Table 3). An analysis of variance demonstrated that the means across clinical organisms were significantly different ($P = 0.001$). Tukey's HSD test determined that SCC8 was significantly different from SCC22 and SCC23 ($P < 0.05$), and all other comparisons were not significant.

Table 1. Loading and encapsulation efficiencies of various SCC NP formulations

SCC	Loading	Encapsulation efficiency
SCC22	8.5% ± 0.2%	85% ± 2%
SCC23	8.3% ± 0.2%	83% ± 2%
SCC22/SCC23	17.0% ± 0.2%	85% ± 2%

MICs of pure SCCs for planktonic bacteria ($\sim 1 \times 10^5$ cfu/mL)

The MICs were determined visually after $\sim 1 \times 10^5$ cells were dosed once with pure SCC1, SCC8, SCC22 or SCC23, with SCC concentrations ranging from 1 to 10 mg/L. The resulting averages of MICs across all clinical microorganisms were 1.86 mg/L for SCC23 ($P = 0.02$), 2.43 mg/L for SCC22 ($P = 0.03$), 3.14 mg/L for SCC1 and 3.71 mg/L for SCC8 (Table 4).

MICs of pure SCCs for CF clinical isolates ($\sim 1 \times 10^5$ cfu/mL)

The MIC_{90} of both SCC22 and SCC23 was 2 mg/L for the non-silver-resistant organisms tested. The MIC for the J53 strain of *E. coli*, which lacks a silver-resistant plasmid, was 1 mg/L for both SCC22 and SCC23. In contrast, the MIC of SCC22 for J53 containing pMG101 was >20 mg/L, demonstrating that the antimicrobial activity of the SCC is primarily due to the silver functionality. Surprisingly, the MIC of pure SCC23 for the J53 strain containing the pMG101 was 6 mg/L, suggesting that the parent carbene compound may play an important role in the observed antimicrobial effect. The MBCs of SCC22 and SCC23 for the tested strains were determined. With the exception of the silver-resistant *E. coli* strain, SCC22 was bactericidal for all of the strains tested. The same pattern was seen with SCC23, including bactericidal activity against the pMG101 strain. These results indicate that both SCCs are capable of killing numerous bacterial strains at clinically achievable concentrations (Table 5).

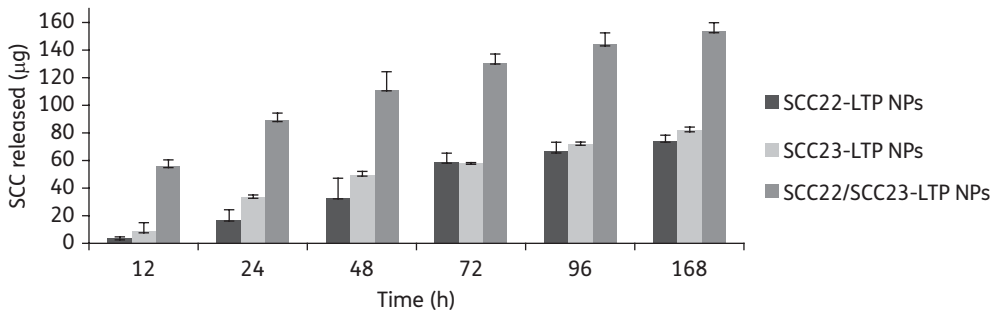

Figure 4. Cumulative SCC release per 1 mg of NPs over 168 h. Note that the dual-loaded NPs released approximately twice the amount of SCC per 1 mg over the time period measured.

Table 2. Release of SCCs from NPs at 12, 24, 72, 96 and 168 h

	Cumulative SCC release per 1 mg of NPs (μg)					
	12 h	24 h	48 h	72 h	96 h	168 h
SCC22-LTP NPs	4±1	17±7	33±14	59±6	66±7	74±4
SCC23-LTP NPs	9±6	34±1	50±2	58±1	72±1	82±2
SCC22/SCC23-LTP NPs	56±4	89±5	111±13	131±6	144±8	153±6

Table 3. *In vitro* antibacterial activity of pure SCCs against various medically important pathogens (~1×10^6 cfu/mL)

		MIC (mg/L)			
Source	Species	SCC1	SCC8	SCC22	SCC23
Sputum	*Acinetobacter baumannii*	5.7	11.4	5.7	11.4
Blood	*Acinetobacter baumannii*	22.7	45.4	22.7	11.4
Unknown	*Bordetella bronchiseptica*	45.4	11.4	45.4	2.9
Sinus	*Burkholderia cepacia*	2.9	22.7	1.5	5.7
Unknown	*Candida albicans*	22.7	45.4	11.4	11.4
Blood	*Enterobacter cloacae*	11.4	22.7	5.7	11.4
Blood	*Enterobacter sakazakii*	5.7	45.4	5.7	5.7
Sputum	*Escherichia coli*	11.4	45.4	11.4	22.7
Throat	group A *Streptococcus*	34.1	22.7	17.1	5.7
CSF	group B *Streptococcus*	56.7	45.4	51.1	4.3
Sputum	*Haemophilus influenzae*	25.6	45.4	14.2	6.45
Eye	*Haemophilus parainfluenzae*	45.4	22.7	45.4	22.7
Throat	*Klebsiella oxytoca*	45.4	90.7	34.1	136
Blood	*Klebsiella pneumoniae*	11.4	45.4	11.4	22.7
Sputum	MRSA	45.4	90.7	45.4	5.7
Blood	MRSA	45.4	90.7	45.4	5.7
Nasal	MRSA	45.4	90.7	11.4	5.7
Blood	methicillin-susceptible *Staphylococcus aureus*	22.7	45.4	22.7	22.7
Blood	*Micrococcus* sp.	5.7	45.4	2.9	1.5
Nasal	*Moraxella catarrhalis*	11.4	11.4	11.4	5.7
Sputum	*Pseudomonas aeruginosa*	2.9	22.7	2.9	5.7
Sputum	*Pseudomonas aeruginosa*-mucoid	2.9	11.4	1.5	2.9
Sputum	*Serratia marcescens*	34.1	45.4	28.4	68.1
Blood	*Staphylococcus epidermidis*	34.1	45.4	28.4	34.1
Blood	*Staphylococcus haemolyticus*	38.1	90.7	38.1	9.5
Unknown	*Staphylococcus saprophyticus*	45.4	45.4	45.4	9.5
Blood	*Stenotrophomonas maltophilia*	2.7	11.4	2.9	5.7
Unknown	*Streptococcus pseudopneumoniae*	68.1	90.7	34.1	6.7
Blood	VRE	136	181.3	136	41.6

MICs and MBCs of SCC-LTP NPs and SCC-PEG-PLA micelles for planktonic bacteria (~1×10^5 cfu/mL)

The resulting average of the MICs for all tested organisms (excluding MICs of >10 mg/L) was 6.33 mg/L for SCC22-LTP NPs (the SCC22 concentration is 0.54 mg/L), 6.0 mg/L for SCC23-LTP NPs (the SCC23 concentration is 0.50 mg/L) and 4.71 mg/L for SCC22-23-LTP NPs (the concentration of SCCs is 0.80 mg/L). The MICs of blank LTP NPs were all >10 mg/L ($P<0.05$; Table 6). The average MIC of SCC23-PEG-PLA micelles was 2.57 mg/L. The MICs of blank PEG-PLA micelles were all >10 mg/L ($P<0.001$; Table 7). The MBCs of all NPs for every bacterium, except *P. aeruginosa*-mucoid, were >10 mg/L. The MBC for *P. aeruginosa*-mucoid was 6 mg/L for SCC22-LTP NPs, 8 mg/L for SCC23-LTP NPs and 8 mg/L for SCC22/23-LTP NPs (Table 6). These results correspond to SCC concentrations of

Table 4. MICs and MBCs of pure SCCs for medically important pathogens (~1×10^5 cfu/mL); all concentrations are in mg/L

	SCC1		SCC8		SCC22		SCC23 (in DMSO)	
Species	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Acinetobacter baumannii	1	1	1	1	1	1	1	1
Bacillus anthracis	1	2	1	2	1	1	1	1
Burkholderia cepacia	1	2	2	8	2	4	2	6
Klebsiella pneumoniae	6	8	6	10	4	>10	4	10
MRSA	6	8	10	NA	6	10	1	6
Pseudomonas aeruginosa-mucoid	1	4	2	6	1	8	2	8
VRE	6	>10	4	10	2	4	2	6

NA, not applicable.

Table 5. MICs and MBCs of SCC22 and SCC23 for CF clinical isolates (~1×10^5 cfu/mL); all concentrations are in mg/L

	SCC22		SCC23	
Species, isolate	MIC	MBC	MIC	MBC
Pseudomonas aeruginosa				
PA 01-V	1	1	1	4
PA M57-15	0.5	4	0.5	1
PA RR05	0.5	1	0.5	1
PA HP3	0.25	2	0.5	1
PA LF05	2	4	0.5	1
Alcaligenes xylosoxidans				
AX 22	0.5	1	1	1
AX RE05	0.5	1	0.5	1
Stenotrophomonas maltophilia				
SM AH08	0.5	1	0.5	1
MRSA				
SA LL06	2	4	2	4
SA EH06	1	4	2	6
Escherichia coli				
J53	1	1	1	1
J53+pMG101	>20	>20	6	8

Table 6. MICs and MBCs of SCC22-LTP, SCC23-LTP and SCC22/23-LTP NPs for medically important pathogens ($\sim 1 \times 10^5$ cfu/mL); MICs are based on the SCC content of SCC-LTP NPs and all concentrations are in mg/L

	SCC22-LTP		SCC23-LTP		SCC22/23-LTP		LTP NPs	
Species	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Acinetobacter baumannii	6	>10	2	>10	1	>10	>10	NA
Bacillus anthracis	2	>10	6	>10	2	>10	>10	NA
Burkholderia cepacia	10	NA	10	NA	4	>10	>10	NA
Klebsiella pneumoniae	>10	NA	>10	NA	10	NA	>10	NA
MRSA	6	>10	4	>10	4	>10	>10	NA
Pseudomonas aeruginosa-mucoid	4	6	4	8	2	8	>10	NA
VRE	10	NA	10	NA	10	NA	>10	NA

NA, not applicable.

Table 7. MICs and MBCs of SCC23-PEG-PLA micelles for medically important pathogens ($\sim 1 \times 10^5$ cfu/mL); MICs are based on the SCC content of SCC-PEG-PLA micelles and all concentrations are in mg/L

	SCC23-PEG-PLA micelles		PEG-PLA micelles blank	
Species	MIC	MBC	MIC	MBC
Acinetobacter baumannii	1	1	>10	NA
Bacillus anthracis	1	>10	>10	NA
Burkholderia cepacia	2	>10	>10	NA
Klebsiella pneumoniae	8	>10	>10	NA
MRSA	1	4	>10	NA
Pseudomonas aeruginosa-mucoid	1	4	>10	NA
VRE	4	10	>10	NA

NA, not applicable.

0.66, 0.51 and 1.36 mg/L, respectively. The MBCs of SCC23-PEG-PLA micelles were >10 mg/L for *Bacillus anthracis*, *B. cepacia* and *K. pneumoniae*, 4 mg/L for MRSA and *P. aeruginosa*-mucoid, 1 mg/L for *A. baumannii*, and 10 mg/L for vancomycin-resistant *Enterococcus* (VRE) (Table 7).

MICs of SCC NPs for planktonic bacteria at $\sim 1 \times 10^6$ cfu after single and triple doses incubated for 72 h

The resulting average MIC was 55.94 mg/L for single-dose SCC22-LTP NPs, 15.75 mg/L for triple-dose SCC22-LTP NPs ($P=0.001$), 43.61 mg/L for single-dose SCC23-LTP NPs and 24.93 mg/L for triple-dose SCC23-LTP NPs after 72 h of incubation ($P=0.08$; Table 8). The actual SCC concentrations to achieve these MICs were 4.76, 1.34, 3.62 and 2.07 mg/L, respectively.

Cytotoxicity of SCC NPs determined using an MTT assay

An MTT assay was performed as an initial examination of the cytotoxicity of the SCC-loaded NPs against primary human dermal fibroblasts at various concentrations. The LD_{50}s of the SCC22 and SCC23 drugs alone were 200 μM for each (Table 9). Meanwhile, the LD_{50}s were 400 μM for the SCC22-LTP NPs, SCC23-LTP NPs and SCC22/SCC23-LTP NPs (Table 9). When calculating the NP concentrations needed to achieve these dosages, the concentrations were 1628, 2842 and 1101 mg/L, respectively. Lastly, the blank LTP NPs did not show any significant toxicity ($P>0.790$).

Discussion

Drug resistance in bacteria, including the rise of multidrug resistance, is an increasing problem in human medicine that may have catastrophic effects if new treatment options are not explored. The need for new drugs that are broadly effective against a number of clinically important bacteria should therefore be a priority. This is especially true for nosocomial infections, chronic infections, such as those associated with CF, CRS as well as infections of the skin and soft tissues that are often correlated with military and diabetic wounds. Here, we tested pure and NP formulations of SCCs against multiple species and strains of clinical bacteria and fungi that are the causative agents of many clinically important diseases. The data indicate these compounds are broadly active against important medical pathogens, including MRSA, MRAB, *P. aeruginosa*, *K. pneumoniae*, *Haemophilus*

Table 8. MICs (mg/L) of SCC22-LTP and SCC23-LTP NPs for medically important pathogens ($\sim 1 \times 10^6$ cfu/mL) 72 h after a single dose and 72 h after three identical doses administered at 24 h intervals; MICs are based on the SCC content of SCC-LTP NPs

		SCC22-LTP NPs		SCC23-LTP NPs	
Source	Species	single dose	triple dose	single dose	triple dose
Unknown	*Candida albicans*	21.8	5.4	21.8	0.68
Blood	*Enterobacter cloacae*	87	21.8	87	43.5
Sputum	*Escherichia coli*	87	43.5	87	43.5
Throat	*Klebsiella oxytoca*	43.5	21.8	1.4	21.8
Blood	*Klebsiella pneumoniae*	43.5	10.9	21.8	43.5
Sputum	MRSA	43.5	10.9	43.5	21.8
Blood	MRSA	43.5	5.4	5.4	10.9
Sputum	methicillin-susceptible *Staphylococcus aureus*	43.5	10.9	43.5	21.8
Sputum	*Pseudomonas aeruginosa*	87	2.7	87	10.9
Sputum	*Pseudomonas aeruginosa*-mucoid	43.5	10.9	5.4	10.9
Blood	*Staphylococcus epidermidis*	21.8	10.9	21.8	10.9
Blood	*Staphylococcus haemolyticus*	43.5	21.8	10.9	21.8
Unknown	*Streptococcus pseudopneumoniae*	87	21.8	87	43.5
Blood	VRE	87	21.8	>87	43.5

Table 9. Toxicity of SCCs and LTP NPs against primary human dermal fibroblasts

	LD_{50} (μM) based on free SCCs	LD_{50} concentration of NP (mg/L)
SCC22	196±12	—
SCC23	197±41	—
SCC22-LTP NPs	442±48	1628±212
SCC23-LTP NPs	413±8	2842±55
SCC22/SCC23-LTP NPs	390±8	1101±22
Blank LTP NPs	>400	>2753

The second column gives the concentrations of free SCCs and the encapsulated SCCs. The third column gives the concentrations of LTP NPs to achieve the LD_{50} values.

influenzae, *Burkholderia* spp. and fungal species such as *C. albicans*. Importantly, pure and NP formulations of SCCs were active across multiple species of pathogens. SCCs encapsulated into NPs provide flexibility in the shipment, storage and delivery of therapeutics across diverse medical settings. Lastly, the active concentrations reported here are clinically achievable, not toxic to fibroblasts and the multiple formulations may provide decreased toxicity for any given dosage route.

The determination of LTP NP size, loading and release is essential for designing and optimizing their *in vitro* and *in vivo* antimicrobial efficacy and cytotoxicity. The optimal size of NPs for drug delivery depends upon the specific application, with sizes ranging from 150 nm to several micrometres.[29,37] Previous studies have shown that LTP NPs and PLGA NPs of this size can be easily nebulized within aerosol droplets for drug delivery into the lungs.[23,29] The scanning electron microscopy and dynamical light scattering data show that these desired diameters can be obtained (Figures 2 and 3). Furthermore, these LTP NPs must possess release rates that maintain the appropriate drug concentration in order to potentially prevent microbial drug resistance. Previous research with PLGA NP and microparticle systems has demonstrated release rates ranging from the order of months to hours.[38] The release studies of the various LTP NP formulations revealed a release rate from several days to 1 week (Table 2). Depending upon the application, a fast or slow release rate could enhance the antimicrobial activity. For example, a release rate of 1–3 days may be effective for the treatment of lung infections, while sustained release may improve skin and soft tissue healing. These latter formulations may potentially be applied within a cream or sterile gauze wrap that can be easily deployed away from the hospital setting.

All four pure SCC compounds exhibited strong activity against a broad range of bacteria and fungi (Table 3) at clinically achievable levels. The effective concentration of these compounds decreased to single digit MICs when one order of magnitude fewer bacteria (10^5 versus 10^6) were challenged for 24 h (Table 4). The CLSI guidelines utilize 10^5 bacteria for determining MICs and we increased this number 10-fold to demonstrate that a single dose of these SCCs often resulted in similar killing. In some skin and soft tissue infections, including chronic wounds, there are likely to be $>10^5$ bacteria present.

The source of the strains, whether from blood, sputum, eye, throat, CSF or even the CF lung (Table 5), did not significantly affect the activity of these pure compounds. This fact is important, as well-defined ATCC laboratory strains often do not represent the current clinical strains that are causing disease in humans.[36] Thus, by testing recent (<2 years) strains that have been minimally passaged in the laboratory, from a variety of sources, we feel that the studies presented here represent a more accurate description of the activity of these compounds against the bacteria that are currently causing disease. Overall, the general trend suggested that SCC1 was the most active

compound in pure form, although all four compounds demonstrated broad clinical efficacy (Tables 3–5). Importantly, even multidrug-resistant strains, such as MRSA, MRAB, *B. cepacia* and VRE, were susceptible to at least one of these SCCs. The compounds were also effective against the biodefence-relevant pathogens *B. anthracis* and *Yersinia pestis* (Table 4).

The increasing use of NP drug therapy led our team to investigate whether these SCCs were effective antimicrobials when formulated as LTPs or PEG-PLA micelles. For these studies, we encapsulated SCC22 or SCC23, alone and in combination, into LTPs or micelles and tested their efficacy against a subset of bacteria from the initial screen. As Table 6 shows, the SCC22- and SCC23-LTP NPs were active against clinically relevant microbes, including multidrug-resistant microbes (MRSA and MRAB) and biodefence/military pathogens. Dual-loaded LTP NPs also demonstrated antimicrobial efficacy at low MICs (Table 6), including some increased efficacy against pathogens such as MRSA, MRAB and *P. aeruginosa*. For several of the microbes, the MICs and MBCs of the NPs were lower than those of the purified compounds. These results are important, because they lower the likelihood of any potential adverse effects that can be associated with NP therapy. The micelle SCC23 formulations were more active against this same panel of microbes than the LTP NPs (Table 7). These results were not due to the micelles, as blank particles did not exhibit any antimicrobial effects. Collectively, these data provide an independent way to administer these compounds. For example, NPs may be most effective as an aerosolized agent whereas purified compounds may be best for direct (or topical) administration. Nonetheless, these compounds and their multiple formulations provide the framework for many different clinical applications.

Because antimicrobial therapy is more often administered as multiple doses, versus the single-dose challenges discussed above, we tested whether three consecutive doses of LTP NPs increased the efficacy of the compounds over time. One of the most striking examples of this was observed with a clinical strain of *Enterococcus* (VRE). This strain was inherently resistant to single doses of these SCCs (Tables 3–7). However, after 24 h consecutive doses, the effective antimicrobial doses decreased by more than half (Table 8). The largest reduction was observed for the fungus *C. albicans*, where multiple doses reduced the MIC to <1 mg/L (Table 8). These experiments demonstrate that the broad antimicrobial activity of SCCs may be increased with consecutive doses, in association with expected clinical applications.

Lastly, the significant difference in SCC-LTP NP toxicity between the bacteria and human fibroblasts demonstrates the feasibility of using LTP NPs in a clinical setting as an antimicrobial delivery device ($P<0.001$; Table 9). Notably, the lethal dosages for antibacterial activities were 10–100 times less than the concentrations needed to obtain the LD_{50} values against human dermal fibroblasts. This differential toxicity is an attractive property of SCCs and the LTP NPs, since both are being translated for clinical applications.

These *in vitro* studies demonstrated that multiple formulations of SCCs are effective in treating a broad range of clinical bacteria and fungi. The concentrations at which these compounds are effective are clinically achievable and may decrease with the administration of consecutive doses. Varying formulations and delivery systems of SCCs broaden the clinical applications for these antimicrobials. The antimicrobial activity against a multitude of virulent bacterial and fungal isolates from various human sources demonstrates their potential to treat a wide range of diseases. The low cytotoxicity and varied formulations for delivery and release, combined with the potent antimicrobial properties of these SCCs indicate that they are a promising alternative to antibiotics that have become increasingly ineffective for treating chronic infections. Current studies are underway to determine their efficacy against the biofilm forms of organisms involved in chronic infections and to test their ability to clear bacteria from different infections in animal models.

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None to declare.

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