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Synthesis and in vitro Efficacy Studies of Silver Carbene Complexes on Biosafety Level 3 Bacteria

Matthew J. Panzner,[a] Arpaporn Deeraksa,[b] Alyssa Smith,[c] Brian D. Wright,[a] Khadijah M. Hindi,[a] Aysegul Kascatan-Nebioglu,[a] Alfredo G. Torres,[d] Barbara M. Judy,[b] Christine E. Hovis,[e] Julia K. Hilliard,[e] Rebekah J. Mallett,[e] Emily Cope,[c] D. Mark Estes,*[b] Carolyn L. Cannon,*[e] Jeff G. Leid,*[c] and Wiley J. Youngs*[a]

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A series of N-heterocyclic carbene silver complexes have been synthesized and tested against the select group of biosafety level 3 bacteria *Burkholderia pseudomallei*, *Burkholderia mallei*, *Bacillus anthracis*, methicillin-resistant *Staphylococcus aureus* and *Yersinia pestis*. Minimal inhibitory concentrations, minimal bactericidal and killing assays demonstrated the exceptional efficacy of the complexes against these potentially weaponizable pathogens.

Introduction

Silver carbene complexes (SCCs), in particular those of N-heterocyclic carbenes, have gained a significant amount of interest in the past few years.[1,2] Much of this interest stems from recent studies demonstrating the exceptional antimicrobial efficacy of these complexes against a broad spectrum of both gram-positive and gram-negative bacteria as well as fungi.[3–9] The successful in vitro and in vivo studies of SCCs against virulent and antibiotic resistant bacteria associated with cystic fibrosis lung infections has led us to investigate their efficacy against the biosafety level 3 (BSL3) bacteria *Burkholderia pseudomallei*, *Burkholderia mallei*, *Bacillus anthracis*, and *Yersinia pestis*, as well as methicillin-resistant *Staphylococcus aureus* (MRSA). Due to their high virulence by the respiratory route, these pathogens are considered potential bioterrorism agents, excluding MRSA, and are classified as such in list B by the Centers for Disease Control and Prevention.[10,11]

[a] Department of Chemistry, University of Akron,
Akron, OH 44325-3601, USA
Fax: +1-330-972-6085
E-mail: youngs@uakron.edu
[b] Department of Pediatrics, University of Texas Medical Branch,
Galveston, Texas 77555, USA
[c] Department of Biological Sciences, Northern Arizona University,
Flagstaff, Arizona 86011, USA
[d] Department of Microbiology and Immunology, University of Texas Medical Branch,
Galveston, Texas 77555, USA
[e] Department of Pediatrics and Molecular Microbiology and Microbial Pathogenesis, Washington University School of Medicine,
St. Louis, MO 63110, USA
E-mail: cannon_c@kids.wustl.edu

B. pseudomalleus is the gram-negative motile bacterium responsible for melioidosis. Melioidosis is a life-threatening disease that is mainly acquired through skin inoculation or pulmonary contamination, although other routes have been documented. This saprophyte inhabitant of telluric environments is mainly encountered in Southeast Asia and northern Australia, but is sporadically isolated in subtropical and temperate countries. *B. mallei*, a gram-negative nonmotile bacterium, is the causative agent of glanders. This disease mainly affects horses; humans can be infected after prolonged and close contact with these animals. Both *Burkholderia* species are highly pathogenic and are listed in biological risk class 3. *Burkholderia* infections are difficult to treat with antibiotics and no vaccine exists for either animal or human.

Bacillus anthracis is part of the natural ecology of ruminants and the microorganism that causes the disease anthrax. *B. anthracis* has been used as a biological weapon throughout history. Reemergence of its use as a bioterrorism agent as in the Anthrax Letter Attacks in the United States has lifted the importance of this pathogen to new levels. Recently, we demonstrated that *B. anthracis* readily formed biofilms under static and shear conditions and that these biofilms were quickly resistant to the common clinical antibiotics ciprofloxacin and doxycycline.[12]

The gram-negative bacterium responsible for plague, *Y. pestis* caused millions of deaths during the three major pandemics. Although natural contraction of this pathogen has resulted in only a mean of 7 cases per year in the United States since 1950 and fewer deaths, *Y. pestis* is considered to be a potentially weaponizable pathogen which could be utilized in a bioterrorism attack.[13] *Y. pestis* causes three

distinct patterns of illness in humans: bubonic plague, pneumonic plague and septicemic plague. Without treatment, the mortality from bubonic plague is about 50%, and that of pneumonic and septecemic forms of plague are almost 100%. With treatment, however, the mortality drops to 5%.[14,15]

On the other hand, *S. aureus* kills tens of thousands of people in the US every year. One of the major underlying themes of *S. aureus* mortality is the increasing ability of the bacteria to resist common antibiotics such as oxacillin and methicillin, and its emerging resistance to the antibiotic of last resort, vancomycin. Indeed, a recent report documented that methicillin-resistant *S. aureus* (MRSA) now kills more people in the United States than HIV.[16] Similar to *B. anthracis*, *S. aureus* readily grows as a biofilm community, which renders even susceptible organisms resistant to common antibiotics, and likely contributes to the overall morbidity and mortality in patients.

It is clear that new classes of antimicrobial agents are needed to combat either the weaponized use of these pathogens or for improving the basic health of the world's population. Here we report the activity of SCCs against the bacteria *B. pseudomallei*, *B. mallei*, *B. anthracis*, MRSA, and attenuated strains of *Y. pestis*.

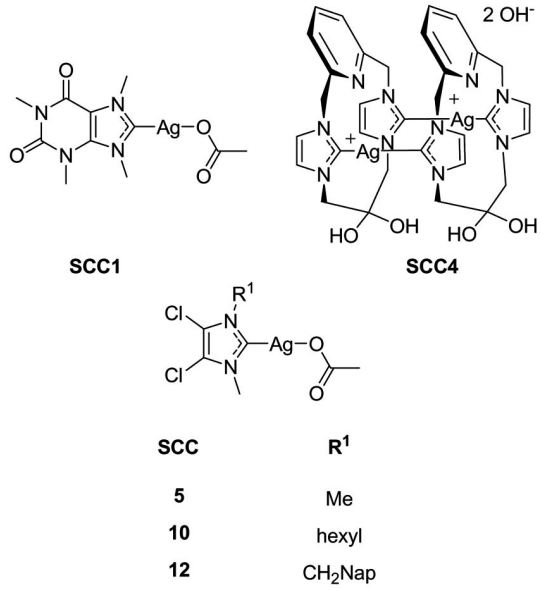
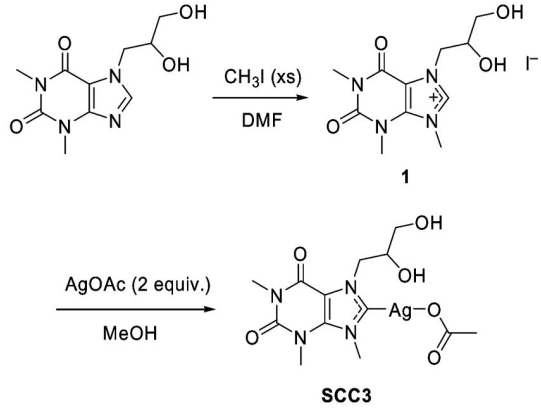


Figure 1. Previously reported SCCs.

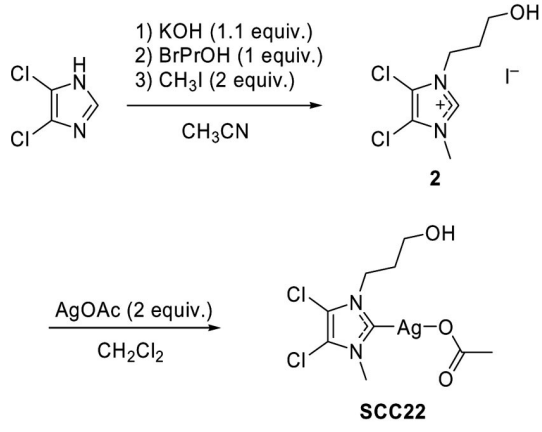
Results and Discussion

Synthesis of SCCs

SCC1, **SCC4**, **SCC5**, **SCC10**, and **SCC12** were prepared by methods previously published (Figure 1).[3,5,8,17] **SCC3** was prepared in a similar manner to **SCC1** by the methylation of 7-(2,3-dihydroxypropyl)theophylline to give 7-(2,3-dihydroxypropyl)-1,3,9-trimethylxanthinium iodide (**1**) followed by metallation with two equivalents of silver acetate to give the final product in 44% yield (Scheme 1).[5] **SCC22** was prepared in a manner similar to that of **SCC5**, **SCC10**, and **SCC12**.[8,17] In this procedure 4,5-dichloroimidazole was deprotonated with KOH and reacted with 3-bromopropanol. Subsequent methylation afforded the imidazolium salt **2** which was treated with two equivalents of silver acetate to give **SCC22** in 75% yield (Scheme 2). In general the typical yields for the investigated SCCs ranged between 44–88%.



Scheme 1. Synthesis of silver complex **SCC3**.



Scheme 2. Synthesis of silver complex **SCC22**.

For both compounds, ^{1}H and ^{13}C NMR were used to confirm the formation of the silver carbenes during synthesis. A loss of the peak corresponding to the xanthinium or imidazolium proton (9.34 and 9.46 ppm, respectively) in the ^{1}H NMR and a shift of the xanthinium C8 carbon atom (139.4 to 186.3 ppm) or the imidazolium C2 carbon atom (136.5 to 179.7 ppm) in the ^{13}C NMR were used to confirm the formation of the complexes. The chemical structures of **SCC3** and **SCC22** were also verified by the isolation and analysis of single crystals of each of the compounds.

Single crystals of **SCC3** suitable for single-crystal X-ray analysis were obtained by slow evaporation from a concentrated sample in methanol/water (Figure 2). Bond lengths and angles pertaining to the metal center to the carbene carbon atom and one oxygen atom of the acetate were consistent with those of previously reported **SCC1**.[5] The structure contains one solvent water molecule per asymmetric

unit forming a hydrogen bonding network with one water molecule bonding to three **SCC3** molecules.

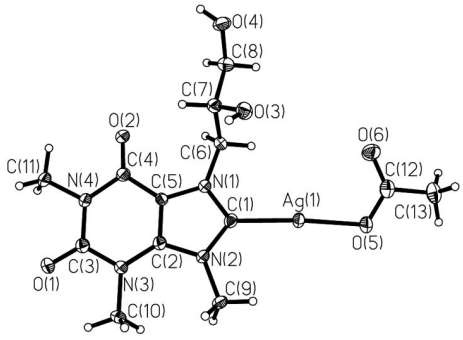


Figure 2. Thermal ellipsoid plot of **SCC3** with thermal ellipsoids shown at 50% probability.

Single crystals of **SCC22** suitable for single-crystal X-ray analysis were grown by precipitation of a concentrated sample from hot acetone (Figure 3). Bond lengths and angles from the metal center to the carbene carbon atom and one oxygen atom of the acetate were consistent with those of the previously reported analogous structures **SCC5**, **SCC10**, and **SCC12**.[8,17] The asymmetric unit of the crystal structure consists of two **SCC22** molecules with a short Ag–Ag contact of ca. 3.2 Å.

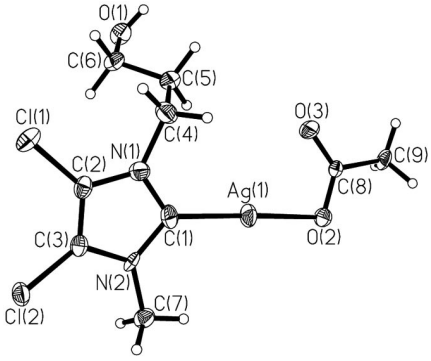


Figure 3. Thermal ellipsoid plot of **SCC22** with thermal ellipsoids shown at 50% probability.

Rationale for Carbene Carrier Structures

The silver carbene complexes used in this investigation can be subdivided into three basic categories based on the fundamental building block that constitutes the carbene center of each. Basic chemical structures and substituents were selected to produce complexes with stability in water (rate of silver dissociation), varying degrees of hydrophilicity or hydrophobicity, and low projected toxicities.

SCC4 is a silver(I)-imidazole cyclophane gem-diol complex, which when encapsulated in electrospun tecophilic nanofibers had previously shown antimicrobial efficacy against both bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) and fungi (*Candida albicans, Aspergillus niger, and Saccharomyces cerevisiae*).[3] This complex was designed to be water and alcohol soluble, properties which aided in its incorporation into electrospun fibers. However, the stability of free **SCC4** in water is relatively low with a deposition of a silver precipitate observed within minutes. Preliminary acute toxicity studies showed the carrier itself to have an LD_{50} of 100 mg/kg in rats making the complexes potential in vivo use uncertain.[3]

Complexes **SCC1** and **SCC3** are derived from a basic xanthine scaffold chosen because of their occurrence in biological systems and use in medicine as diuretics, central nervous system stimulants and as bronchodilating agents.[18,19] **SCC1** is derived from the naturally occurring molecule caffeine and **SCC3** from an N(7)-functionalized theophylline. **SCC1** is sparingly soluble in water (11.6 mg/mL) while **SCC3** has moderate solubility (82 mg/mL) which can be attributed to the 2, 3-dihydroxypropyl substituent on the N(7) nitrogen atom of the xanthine ring. Both complexes have similar stabilities in water with ca. 60% decomposition observed over a 24 h period. These complexes were selected based on their low anticipated in vivo toxicities. In fact, preliminary toxicity studies showed the intravenous LD_{50} of the xanthinium iodide salt of methylated caffeine to be 1.068 g/kg in rats. Additionally, no adverse effects were observed after the injection of **SCC1** in rats, although this may be partially attributed to the solubility limited dose that was administered.[5] Preliminary in vitro cytotoxicity studies have shown **SCC3** to be more toxic to immortalized airway cells than **SCC1**.

SCC5, **SCC10**, **SCC12**, and **SCC22** are derivatives of 4,5-dichloroimidazole and were selected for their exceptional water stability degrading less than 20% in water over a 24 h period. The increased stability of these complexes is likely attributed to σ-withdrawing and π-donating properties of the chlorine substituents lessening the σ-donating ability of the carbene carbon atom making it less susceptible to protonation in aqueous solution.[8] The complexes have varying degrees of water solubility ranging from <10 mg/mL for **SCC12** to ca. 110 mg/mL for **SCC22**. Anticancer studies using an ovarian cancer xenograft model in athymic nude mice has demonstrated that **SCC5** can be administered in doses of 333 mg/kg subcutaneously with no acute toxicity observed.[17] The in vivo toxicities of the remaining complexes of this type are currently being investigated.

MIC and MBC of SCC1, 5, 10, 12 and 22 against *Burkholderia pseudomallei* and *Burkholderia mallei*

Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of the methylated caffeine silver acetate complex **SCC1**, as well as **SCC5**, **SCC10**, **SCC12** and **SCC22** against two potential respiratory pathogens, *B. pseudomallei* K96243 and *B. mallei* ATCC23344, are shown in Table 1. The MICs and MBCs of all compounds against *B. pseudomallei* were 4–6 μg/mL and 6–10 μg/mL or higher, respectively. Whereas in the case of *B. mallei*, the MICs and MBCs of all compounds were 1–4 μg/mL and 6 μg/mL, respectively. These data suggested that *B. mallei* is more susceptible to silver carbene com-

plexes, especially **SCC1** and **SCC22**, than *B. pseudomallei*. The MIC_{90}s of **SCC1** and **SCC22** against *B. pseudomallei* were 8 and 6 μg/mL, respectively. **SCC5** appeared to be the best candidate to treat *B. pseudomallei* (Table 1). Whereas **SCC5** and **SCC22** were great candidates for *B. mallei* as MIC_{50}s were only 2 μg/mL for both complexes.

Table 1. MIC and MBC of **SCC1**, **SCC5**, **SCC10**, **SCC12** and **SCC22**.

Species	Strain	Compound	MIC[a]	MBC[a]
Burkholderia pseudomallei	K96243	**SCC1**	6	>10
		SCC5	4	6
		SCC10	6	10
		SCC12	6	10
		SCC22	4	>10
Burkholderia mallei	ATCC23344	**SCC1**	4	6
		SCC5	1	6
		SCC10	2	6
		SCC12	2	6
		SCC22	1	6

[a] μg/mL.

MIC and MBC of SCC1 and SCC5 against *Yersinia pestis*

MICs and MBCs of **SCC1** and **SCC5** against two attenuated strains of *Y. pestis*, YP1–1 and YP8–1, which are Lcr- and Pgm-derivatives, respectively, of the fully virulent CO92 strain, are shown in Table 2. Because the activity of antimicrobials including the SCCs may be affected by the milieu, the MICs and MBCs of both strains were tested in the standard Mueller–Hinton broth (MH), as well as the more highly enriched broth typically used to grow *Y. pestis* in the laboratory, Brain Heart Infusion (BHI).[5] The MICs of both **SCC1** and **SCC5** against both strains of *Y. pestis* tested in either media were uniformly 1 μg/mL. Similarly, the MBCs of both **SCC1** and **SCC5** did not differ appreciably when tested in either MH or BHI, and at 1–6 μg/mL, were significantly lower than that for either *B. pseudomallei* or *B. mallei*. Thus, *Y. pestis* appears more susceptible to killing by silver carbenes than the *Burkholderia* species tested. The Pgm-strain appears more susceptible than the Lcr-strain to killing by SCCs, which suggests that the *pgm* locus may encode proteins involved in Ag^+ detoxification. The 102-kb locus, however, is composed of several parts including the high-pathogenicity island involved in iron acquisition, a fimbriae gene cluster, the genes for a two-component regulatory system and the hemin storage locus, none of which have been linked to heavy metal detoxification.[20]

Table 2. MIC and MBC of **SCC1** and **SCC5** against *Yersinia pestis*.

Strain	SCC	MIC[a] MH[b]	MBC MH	MIC BHI[c]	MBC BHI
YP1-1 (*lcr*-)	**SCC1**	1	6	1	nd[d]
	SCC5	1	4	1	4
YP8-1 (*pgm*-)	**SCC1**	1	4	1	nd
	SCC5	1	2	1	1

[a] All concentrations are μg/mL. [b] Mueller–Hinton broth. [c] Brain Heart Infusion broth. [d] Not done.

Effect of SCC1, SCC3, and SCC4 on *Bacillus anthracis* and Methicillin-Resistant *Staphylococcus aureus* Planktonic and Biofilm Communities

To determine the effect of compounds **SCC1**, **SCC3** and **SCC4** on *B. anthracis* and MRSA, we challenged planktonic and biofilm communities with various concentrations. Because our previous data suggested that the maturation stage of the biofilm and the number of stationary phase cells (planktonic) affected antibiotic treatment, microbial populations were grown for 1, 3, 5 and 7 d (biofilm and planktonic) and these populations were challenged with serial dilutions of the SCC compounds. In all cases, the reductions in CFU were based on the number of CFU present in the growth media. For planktonic assays gentamicin, a clinical antibiotic, was used as a control. It is important to note that like all aminoglycoside antibiotics, gentamicin can cause nephro and ototoxicity, some effects of which are irreversible.[21] These adverse side effects are dramatically reduced with silver-related compounds.

Overall, the SCC compounds were most active against 3 d incubated planktonic *B. anthracis*, 7 d incubated planktonic MRSA, and 1 d old *B. anthracis* and MRSA biofilms (see Figures 4, 5, 6, and 7). The SCCs were more active against the planktonic microbes than the biofilm organisms,

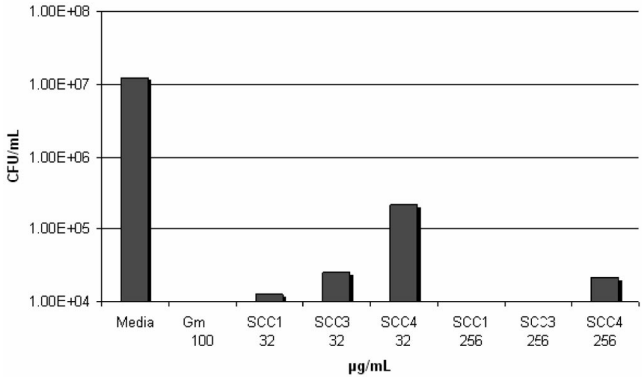


Figure 4. Killing assay of SCCs on a 3-d incubated planktonic *B. anthracis* to SCCs. Cell growth media was used as a control as well as the clinical antibiotic gentamicin.

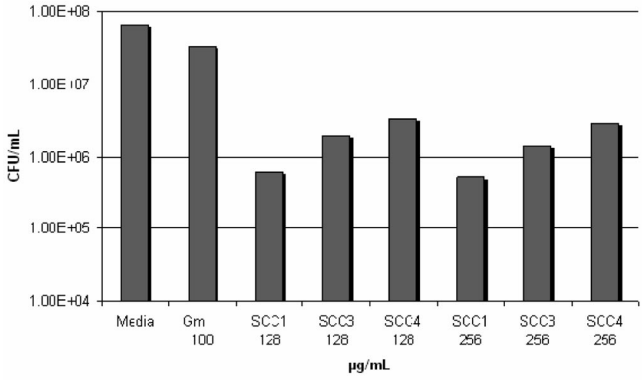


Figure 5. Killing assay of SCCs on a 7-d incubated planktonic MRSA to SCCs. Cell growth media was used as a control as well as the clinical antibiotic gentamicin.

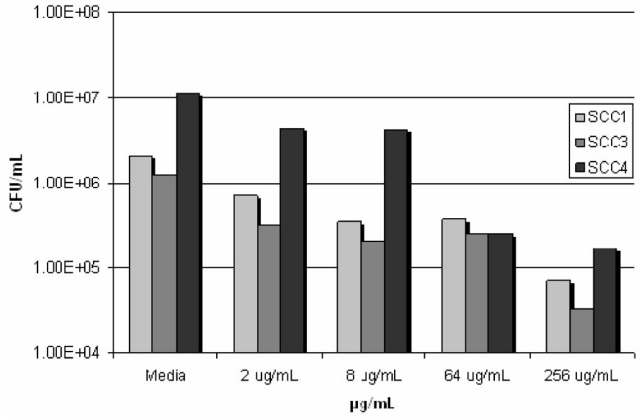


Figure 6. Killing assay of SCCs on a 1-d old biofilm of *B. anthracis* to SCCs. Cell growth media was used as a control.

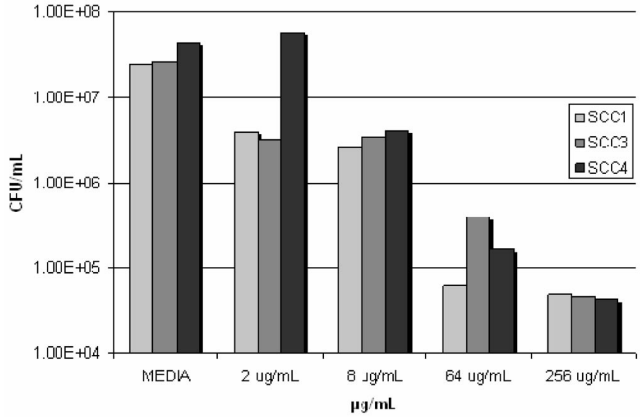


Figure 7. Killing assay of SCCs on a 1 d old biofilm of MRSA. Cell growth media was used as a control.

as is common with many antimicrobials. Interestingly, the biofilm organisms of *B. anthracis* were more susceptible to these silver compounds than the common antibiotics ciprofloxacin and doxycycline.[12] These data suggest that the tested silver antimicrobial compounds are more effective at microbial killing than antibiotics currently being utilized in the clinic. For MRSA biofilms there was marked bacterial killing of 1, 3 and 5 day-old communities. These silver compounds all exhibited greater than one log, and sometimes greater than two logs of killing. The planktonic killing data were interesting in that the 24-h cultures were fairly resistant to silver challenge. Yet, the 7-day planktonic cultures were susceptible, especially to **SCC1**, suggesting that these compounds may also be effective at treating chronic bacterial infections in humans.

Conclusions

We have demonstrated through MIC and MBC determinations as well as killing assays that SCCs have remarkably broad-spectrum activity against MRSA and the potentially weaponizable BSL3 bacteria *B. pseudomallei*, *B. mallei*, *B. anthracis*, and *Y. pestis*. Single-digit µg/mL concentrations were obtained for *B. pseudomallei*, *B. mallei*, and *Y. pestis*. In the case of biofilm organisms of *B. anthracis*, SCCs proved more effective than currently used clinical antibiotics ciprofloxacin and doxycycline. SCCs, depending on conditions, were shown to be equivalent to or more effective than the aminoglycoside antibiotic gentamicin against mature cultures of planktonic *B. anthracis* and MRSA. The in vitro studies did not reveal any remarkable relationship between SCC structure and antimicrobial effectiveness; however, it is our contention that these differences will be born out in future in vivo studies where carrier functionality will play a greater role.

Experimental Section

Synthesis of SCC3: Xanthinium salt **1** (0.73 mmol, 0.29 g) was dissolved in methanol (5 mL) and silver acetate (1.22 mmol, 0.20 g) was added. The mixture was stirred at 60 °C for 2 h under refluxing conditions. The reaction was subsequently filtered to remove silver iodide affording a colorless solution. The volatiles were removed by rotary evaporation. **SCC3** (0.32 mmol, 0.14 g, 44%) was obtained as a white solid after washing the crude product with hot ethanol (70 °C–75 °C). m.p. 167–170 °C. ^{1}H NMR (400 MHz, [D$_6$]-DMSO): δ = 5.06 (broad s, 1 H, OH), 4.88 (broad s, 1 H, OH), 4.51 (d, $^2J_{HH}$ = 13.2 Hz, 1 H, NCH_{2A}), 4.31 (d, $^2J_{HH}$ = 13.2 Hz, 1 H, NCH_{2B}), 4.28 (s, 3 H, NCH_3), 3.83 (m, 1 H, CHOH), 3.74 (s, 3 H, NCH_3), 3.41 (m, 2 H, CH_2OH), 3.24 (s, 3 H, NCH_3), 1.80 (s, 3 H, $COOCH_3$) ppm. ^{13}C{^{1}H} NMR (75 MHz, [D$_6$]DMSO): δ = 187.4, 174.1, 153.3, 150.6, 140.7, 108.7, 70.74, 63.5, 53.8, 39.5, 31.7, 28.4, 23.0 ppm. ESI-MS (*m*/*z*): calcd. for $[C_{11}H_{16}AgN_4O_4]^+$ 375.02; found 645.0, $[C_{22}H_{32}AgN_8O_8]^+$. $C_{13}H_{19}AgN_4O_6$: calcd. C 35.88, H 4.40, N 12.87; found C 35.84, H 4.36, N 12.52.

Synthesis of SCC22: Imidazolium salt **2** (10 mmol, 3.37 g) was dissolved in dichloromethane (220 mL) and silver acetate (20 mmol, 3.34 g) was added with stirring. The reaction was stirred for 1 h at room temperature during which time a yellow precipitate of AgI was formed. The reaction mixture was filtered and the volatiles removed by rotary evaporation. Diethyl ether (100 mL) was added to the crude and **SCC22** was collected by vacuum filtration as a white powder (2.83 g, 75%). M.p. 170–171 °C. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 4.82 (broad s, 1 H, OH), 4.22 (t, 2 H, NCH_{2A}), 3.78 (s, 3 H, NCH_3), 3.46 (t, 2 H, NCH_{2C}), 1.87 (m, 2 H, NCH_{2B}), 1.80 (s, 3 H, $COOCH_3$) ppm. ^{13}C{^{1}H} NMR (125 MHz, [D$_6$]DMSO): δ = 179.7, 175.6, 117.0, 116.2, 57.0, 47.6, 37.6, 33.0, 23.2 ppm. ESI-MS (*m*/*z*): Calcd. for $[C_7H_{10}AgCl_2N_2O_1]^+$ 316.9; found 316.9, $[C_7H_{10}AgCl_2N_2O_1]^+$. $C_{13}H_{19}AgN_4O_6$: calcd. C 28.75, H 3.49, N 7.45; found C 28.07, H 3.31, N 7.10.

Bacteria: All bacterial strains were maintained as glycerol stocks at –80 °C. For experiments, the bacteria were streaked onto either Luria Bertani containing 4% glycerol agar (LBG) or blood agar plates and incubated overnight at 37 °C. Dr. Virginia Miller of Chapel Hill, NC kindly provided the *Y. pestis* strains.

Stock Solutions: SCC1, **SCC5** and **SCC22** were diluted in sterile water at a concentration of 10 mg/mL and stored in small aliquots at –80 °C. **SCC10** and **SCC12** were dissolved in dimethyl sulfoxide (DMSO) to give a final concentration of 10 mg/mL. Stock solutions of **SCC10** and **SCC12** were not stored for longer than the day of the experiment.

MIC and MBC Determinations for *Burkholderia pseudomallei* and *Burkholderia mallei*: Because *B. pseudomallei* and *B. mallei* have

high virulence by the respiratory route and are listed in biological risk class III, all work with these organisms was done in a biosafety level 3 laboratory. MICs were determined by the macro-dilution method.[5] Bacteria are streaked from glycerol-frozen stocks onto Luria Bertani containing 4% glycerol (LBG) agar plates and incubated for 2 d at 37 °C. Cells from the plate were inoculated into LBG broth and incubated for 12 h at 37 °C under shaking condition at 200 rpm. The bacteria were diluted in broth to a concentration corresponding to 10^5 CFU in 50 μL which was added to 3 mL of LBG broth containing various concentrations of **SCC1**, **SCC5**, **SCC10**, **SCC12** or **SCC22**. The final concentrations tested were 1, 2, 4, 6, 8, and 10 μg/mL. The tubes were incubated for 24 h at 37 °C under shaking condition at 200 rpm. Cell densities were measured as an optical density at 600 nm (OD_{600}), and the cultures were then diluted and plated onto LBG agar. The MIC was the lowest of these concentrations, at which cell density at OD_{600} did not increase after incubation. The MIC_{50} is, by definition, the concentration at which growth of 50% of the tested strains is inhibited, and similarly the MIC_{90} is the concentration at which 90% of the tested strains fail to grow. Minimal bactericidal concentrations (MBCs) were the lowest concentration, at which there were no colonies on the plates after incubation for 2 d at 37 °C.

MIC and MBC Determinations for *Yersinia pestis*: While the CO92 *Y. pestis* strain is listed in biological risk class III, the Lcr- and Pgm-derivatives are listed as biosafety level II and were studied using standard BSL2 precautions. MICs and MBCs were determined by a standard Clinical and Laboratory Standards Institute (CLSI) micro-dilution method, as described previously.[5,8,22] Bacteria are streaked from glycerol-frozen stocks onto blood agar plates and incubated overnight at 37 °C. Cells from the fresh plates are suspended in either Brain Heart Infusion broth (BHI) or the CLSI standard Mueller–Hinton broth (MH) to an OD_{650} of 0.25 and grown in a shaking incubator until the OD_{650} is 0.4, which corresponds to ca. 2×10^8 colony forming units (CFU)/mL, confirmed by plating serial dilutions. The bacteria are diluted in broth to a concentration corresponding to 10^5 CFU in 100 μL, which is added to triplicate wells of a 96 well plate containing 100 μL of twice the SCC concentration to be tested. The SCC concentrations tested were again, 1, 2, 4, 6, 8, and 10 μg/mL. The plate was incubated for 18 to 20 h at 37 °C and the MIC determined as the lowest concentration with clear wells. The clear wells were plated on blood agar and the MBC was determined to be the lowest concentration at which there were no colonies after incubation for 2 d at 37 °C.

Planktonic and Biofilm Killing Assays for *Bacillus anthracis* and Methicillin-Resistant *Staphylococcus aureus* (MRSA): *B. anthracis* (Sterne strain, pX02-) and *S. aureus* strains were streaked on BHIA and TSA with 1 μg/mL oxacillin and incubated for 24 h at 37 °C. *B. anthracis* was grown in the presence of 5% CO_2. A single colony of each organism was isolated and inoculated into 10 mL of BHIB and TSB and incubated for 12 to 15 h overnight at 37 °C under constant agitation. Following overnight incubation, each culture was diluted 1:100 in fresh media. *B. anthracis* was incubated at 37 °C under constant agitation for 4 h, MRSA was incubated for 3 h under the same conditions. Following incubation, each culture was diluted 1:50 into fresh media and 100 μL of culture was used to inoculate respective wells in a microtiter plate and incubated at 37 °C under static conditions.

Each silver compound was diluted from a 10 mg/mL stock into Mueller–Hinton broth. The highest concentration was 1.028 mg/mL and these were serially diluted until the concentration reached 1 μg/ml. Each organism was challenged with all available dilutions for 24 h at 37 °C. Control treatments were MH broth and gentamicin (500 μg/mL).

After 24 h incubation with the silver compounds, microtiter plates were inverted and supernatant removed. Biofilms were gently sonicated and remaining bacteria were enumerated by serially diluting and plating on appropriate agar media. Colonies were counted and recorded as CFU.

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