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In Vitro and Murine Efficacy and Toxicity Studies of Nebulized SCC1, a Methylated Caffeine-Silver(I) Complex, for Treatment of Pulmonary Infections[∇]

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The expanding clinical challenge of respiratory tract infections due to resistant bacteria necessitates the development of new forms of therapy. The development of a compound composed of silver coupled to a methylated caffeine carrier (silver carbene complex 1 [SCC1]) that demonstrated in vitro efficacy against bacteria, including drug-resistant organisms, isolated from patients with respiratory tract infections was described previously. The findings of current in vitro studies now suggest that bactericidal concentrations of SCC1 are not toxic to airway epithelial cells in primary culture. Thus, it was hypothesized that SCC1 could be administered by the aerosolized route to concentrate delivery to the lung while minimizing systemic toxicity. In vivo, aerosolized SCC1 delivered to mice resulted in mild aversion behavior, but it was otherwise well tolerated and did not cause lung inflammation following administration over a 5-day period. The therapeutic efficacy of SCC1 compared to that of water was shown in a 3-day prophylaxis protocol, in which mice infected with a clinical strain of *Pseudomonas aeruginosa* had increased survival, decreased amounts of bacteria in the lung, and a lower prevalence of bacteremia. Similarly, by using an airway infection model in which bacteria were impacted in the airways by agarose beads, the administration of SCC1 was significantly superior to water in decreasing the lung bacterial burden and the levels of bacteremia and markers of airway inflammation. These observations indicate that aerosolized SCC1, a novel antimicrobial agent, warrants further study as a potential therapy for bacterial respiratory tract infections.

Respiratory tract infections are a major cause of morbidity and mortality worldwide (8). This trend has been exacerbated by drug-resistant bacterial strains that are rapidly increasing in community-acquired as well as hospital-acquired infections. Moreover, hospital-acquired pneumonia and ventilator-associated pneumonia are the leading causes of death from nosocomial infections and affect up to 25% of all intensive care unit patients, with mortality rates being as high as 70% (4). Accordingly, the therapy currently recommended for hospitalized patients with pneumonia includes broad-spectrum antibiotics active against organisms with multidrug resistance (2). Furthermore, these serious pulmonary infections require the systemic delivery of high doses of antibiotics to overcome not only the potential resistance of the infecting organisms but also the low level of lung tissue penetration of many agents. A more attractive alternative for the treatment of infections caused by these resistant organisms, as well as organisms sensitive to antimicrobials delivered through the typical oral and intravenous routes, is the aerosol route of drug delivery, which is uniquely possible for respiratory tract infections. The deposition of drug in the lungs via the inhaled route

can result in drug concentrations much higher than those that can be achieved through intravenous administration and avoids systemic exposure and toxicity (32). Nonetheless, inhaled antimicrobials are not routinely used for the treatment of acute pneumonia, in part due to the availability of effective systemic therapy for the treatment of infections caused by sensitive organisms.

The emergence of multidrug-resistant organisms has rekindled an interest in inhaled antimicrobials as adjunct therapy, especially in select populations with chronic lung disease and in those with nosocomial hospital-acquired pneumonia and ventilator-associated pneumonia (25). The delivery of tobramycin by inhalation is well established for the treatment of *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF) lung disease (32) and is also used to treat non-CF patients (11). The organisms responsible for nosocomial pneumonias vary, but they commonly include resistant strains of *Pseudomonas* and methicillin (meticillin)-resistant strains of *Staphylococcus aureus* (22). These pathogens are also found to be the causes of other diseases that are marked by persistent airways infections, such as non-CF bronchiectasis and chronic obstructive pulmonary disease (21, 30). Thus, novel antimicrobials that are active against these strains of resistant organisms and that can be delivered by the inhalation route are likely to have utility for the treatment of a broad range of patient populations.

Silver is an antimicrobial agent that is attractive as a potential therapy for respiratory tract infections. The broad-spectrum antimicrobial effects of silver have been appreciated for centuries (35). Although the medicinal use of silver waned

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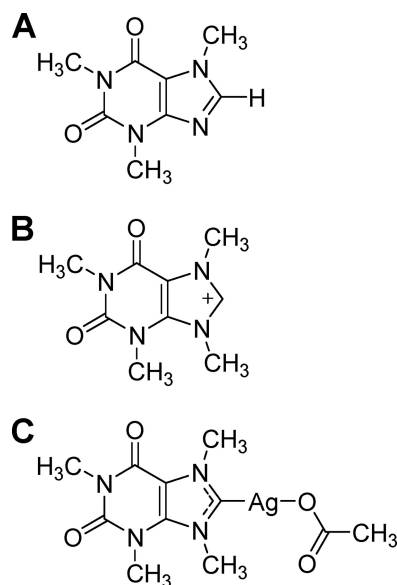


FIG. 1. Structures of caffeine (A), methylated caffeine used as a carrier in SCC1 (B), and the methylated caffeine silver acetate designated SCC1 (C).

after the advent of antibiotics, silver-containing products and compounds, such as silver nitrate, silver sulfadiazine, and silver-impregnated bandages, continue to be used for the treatment of burns and wounds (24) and are particularly effective against difficult-to-treat bacteria, such as *P. aeruginosa*. More recently, silver-impregnated catheters and endotracheal tubes have been developed to decrease the likelihood of catheter-associated, hospital-acquired infections and ventilator-associated pneumonia, respectively, and are currently under investigation (7, 34). Despite the encouraging results obtained in studies of silver-impregnated devices, no silver-based antimicrobials have been developed for direct delivery to the lung.

To achieve clinical utility, a nebulized silver compound must have demonstrated efficacy against respiratory pathogens without toxicity to the host. The toxicity of silver used in clinical materials is reported to be minimal, with the notable rare cosmetic side effect of gray skin known as argyria (23). The toxicity of silver compounds, however, can often be linked to the carrier molecules (13). Consequently, the rational design of a new silver-based antimicrobial must ideally include coordination of the use of the bioactive metal with a nontoxic carrier. We have previously reported the synthesis and *in vitro* antimicrobial properties of the *N*-heterocyclic carbene silver complex designated SCC1, for silver carbene complex 1, formed from the coupling of silver to the xanthine derivative, methylated caffeine (20). Xanthine derivatives have been used for multiple purposes, including bronchodilation. Caffeine (Fig. 1A) is readily available and has low toxicity, properties which are attractive as a carrier for the delivery of silver to the lung (3). Thus, SCC1 appears to have properties ideal for use as a nebulized antimicrobial. To begin to assess the suitability of SCC1 as a nebulized antimicrobial, the *in vitro* cytotoxicity of SCC1 in airway epithelial cells in primary culture and *in vivo* efficacy of SCC1 in mouse models have been examined.

MATERIALS AND METHODS

Drugs. The silver carbene complex designated SCC1 (Fig. 1C) is composed of silver complexed to a methylated caffeine carrier (Fig. 1B) (20). SCC1 and the methylated caffeine SCC1 precursor (Fig. 1B) were both reconstituted in water (Milli-Q synthesis system; Millipore Corp., Billerica, MA) to a concentration of 10 mg/ml (K_{sp} of SCC1, 11 mg/ml) and stored at -80°C until use.

Mice. Male C57BL/6J mice (age, 8 to 10 weeks; Jackson Laboratories, Bar Harbor, ME) were used for these studies, which were approved by the Washington University School of Medicine (St. Louis, MO) Animal Studies Committee. The animals were housed in a barrier facility under pathogen-free conditions until they were inoculated with bacteria.

Bacteria. A mucoid clinical isolate of *P. aeruginosa* designated PA M57-15 was provided by Thomas Ferkol (Washington University). For the infection model experiments, bacteria were streaked from frozen stocks onto tryptic soy agar (TSA) and incubated overnight at 37°C . Cells from the fresh plates were suspended in Luria broth (LB) 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 $\sim 2 \times 10^8$ CFU/ml for *P. aeruginosa*, as confirmed by the plating of serial dilutions.

mTEC cultures. Highly differentiated mouse tracheal epithelial cell (mTEC) cultures were established by using air-liquid interface conditions, as described previously (37). Briefly, cells were harvested from the tracheas by pronase digestion, and epithelial cells were selected from fibroblasts by differential adherence. To initiate the cultures, cells were seeded on supported membranes (Transwell; Costar-Corning, Corning, NY) and proliferated in growth factor-enriched medium maintained in the upper and lower chambers until the cell layer was confluent and sufficient to exclude medium from leaking into the apical compartment. The air-liquid interface condition was then created for the induction of cell differentiation by aspirating the medium from the apical chamber and providing fresh medium to the lower chamber every 3 days. The cells used in these studies were cultured under these conditions for at least 7 days, at which time ciliated (approximately 30% to 50%) secretory and basal cells were present.

***In vitro* cytotoxicity studies.** Concentrations of SCC1 ranging from 0 to 500 $\mu\text{g/ml}$ (0, 5, 10, 50, 100, 200, 300, 400, and 500 $\mu\text{g/ml}$) were applied to the apical surface of mTEC cultures, and the cultures were allowed to incubate for 24 h. Subsequently, the cells on the Transwell membranes were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 min at 25°C . The membranes containing the cells were mounted on glass slides by use of a medium that contains 4',6-diamidino-2-phenylindole (DAPI) to stain intracellular DNA (Vectashield; Vector Laboratories, Burlingame, CA). Microscopy was performed with a epifluorescent microscope (DM5000; Leica, Wetzlar, Germany) with a Retiga 200R charge-coupled-device camera (Q-Imaging, Surrey, British Columbia, Canada) interfaced with Q-Capture Pro software (Q-Imaging). Cell enumeration was performed by counting the number of DAPI-stained nuclei per high-power field in at least three fields per well of cells. Each SCC1 concentration was applied to at least two wells of the mTEC cultures. The average number of nuclei per high-power field in the wells that were not treated with SCC1 served as the control against which the number of cells in the treated wells were normalized, as follows: percent surviving cells = (average number of nuclei in treated wells/average number of nuclei in untreated wells) \times 100%. Data are presented as the averages and standard deviations (SDs) of the normalized cell numbers.

Mouse toxicity studies. Two groups of five mice each were placed in a multidosing animal chamber (Buxco Research Systems, Wilmington, NC). After accommodation to the chamber for 5 min, the animals were exposed for 5 min to 0.5 ml of nebulized water or SCC1 dissolved in water at a concentration of 10 mg/ml. Both SCC1 and water were delivered via an Aeroneb Lab apparatus (Aerogen Inc., Galway, Ireland) connected directly through the lid of the multidosing chamber. The Aeroneb Lab nebulizer is based on micropump technology that produces fine particles (1 to 5 μm) in a low-velocity aerosol (26). The treatment with either nebulized SCC1 or water was repeated twice daily for a total of 5 days. The mice were weighed daily and were monitored during the exposure periods for activity, including their location in the multidosing chamber relative to the location of the nebulizer and grooming or jumping behaviors. On the last day of treatment, the mice were placed in the chamber and photographed from an aerial view prior to treatment and every 15 s beginning 5 s after the onset of aerosol production. Their position within the chamber was then plotted relative to the position of nebulizer. At the end of the 5-day exposure period, the mice were euthanized and the lungs were collected for histological examination. The experiment was repeated with similar groups of mice.

Prophylaxis protocol. Evaluation of the efficacy of SCC1 by using both the acute and agarose bead infection models employed identical prophylaxis proto-

cols. The mice were pretreated with either 0.5 ml of nebulized water as a control or 0.5 ml of SCC1 at a concentration of 10 mg/ml. The mice were then immediately anesthetized intraperitoneally with 2.5% tribromoethanol (Avertin) at a dose of 0.015 ml/g of mouse body weight. The tracheae were exposed by dissection and then cannulated with a 22-gauge angiocatheter through which 50 μ l of *P. aeruginosa* strain PA M57-15 grown as described above was injected into the airways. The first dose and inoculation were completed within 30 min and together were considered to be time zero in the experiment. Subsequent doses were administered according to the following schedule: doses 2 and 3 at 4 and 8 h, respectively; doses 4, 5, and 6 at 24, 28, and 32 h, respectively; and doses 7, 8, and 9 at 48, 52, and 56 h, respectively. The mice were evaluated at 72 h after dose 1 and inoculation (see Fig. 4).

Acute infection model. The acute infection model was used to mimic the pathology seen in acute bacterial pneumonia. Following administration of the pretreatment dose of either SCC1 or water, groups of mice were anesthetized intraperitoneally with 2.5% tribromoethanol at a dose of 0.015 ml/g of mouse. *P. aeruginosa* strain PA M57-15 was delivered to each mouse intratracheally, as described above. The dose ranged from 2.5×10^6 to 3.25×10^7 CFU per mouse in each of five separate experiments with about five mice per treatment group. The data from these experiments were pooled so that data for a total of 25 mice were included in the SCC1 treatment group and data for 26 mice were included in the water control group. Mice were weighed daily and were treated three times daily by using the prophylaxis protocol. The surviving mice were then euthanized on day 3 by carbon dioxide inhalation. Bronchoalveolar lavage (BAL) fluid samples were obtained as described previously and separated (4,000 \times g, 10 min, 4°C) into cell-free supernatants and cells (36). The cell pellets from the BAL fluid were used for white blood cell total and differential cell counts, and the supernatants were frozen for cytokine analysis. Total white blood cell counts were determined on a Hemavet 950 apparatus (Drew Scientific, Oxford, CT). Differentials were performed by staining the cells with Diff-Quick (Hema 3 manual staining system; Fisher Scientific), followed by microscopy. The BAL fluid was analyzed for the cytokines MIP-2 and KC by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). The lungs were harvested for quantitative bacteriology as described previously (36). The spleens were homogenized in 1 ml of PBS solution (PowerGen 125 homogenizer; Fischer Scientific), and 10 μ l of the homogenate was placed on a TSA plate overnight at 37°C. The presence or the absence of bacterial colonies was noted to determine bacteremia. The lower level of detection for both lung and spleen homogenates by this technique is 10^2 CFU/ml. One of the set of lung plates (see Fig. 4C) and three sets of plates of spleen homogenates (see Fig. 4D) were eliminated from the analysis because they appeared to be contaminated.

Time course of treatment effects in acute infection model. To investigate the efficacy of SCC1 on a day-to-day basis, a cohort of 24 mice was subjected to the prophylaxis protocol, with 12 mice receiving nebulized doses of SCC1 (5 mg per dose) and the other 12 mice receiving water. All mice were infected with 3×10^6 CFU per mouse of PA M57-15, according to the acute infection model. However, as treatment proceeded, a group of mice ($n = 4$) was evaluated daily to monitor the mice for changes in lung bacterial burden; the influx of leukocytes, as determined by evaluation of BAL fluid; and bacteremia. On days 1 and 2, the lungs of one animal from each group were harvested for histological examination, and the lungs from the remaining three mice were harvested to determine the lung bacterial burden. Because two animals in the water-treated group died between days 2 and 3, the lungs of all of the remaining mice were harvested for quantitative bacteriology but lungs of none of the mice were evaluated for histology.

Agarose bead model. The agarose bead model of chronic *P. aeruginosa* infection was used to achieve an airway infection that mimics that seen in patients with CF (36). To produce the bacterium-laden beads, a sterile flask with a 2% agarose gel was melted, equilibrated to 55°C, and mixed with *P. aeruginosa* strain PA M57-15 grown in tryptic soy broth. An aliquot of the infected agarose was quickly added to a beaker of rapidly stirring mineral oil and then gradually cooled. The beads were next washed once in a separatory funnel with warm 0.5% sodium deoxycholate, then once in warm 0.25% sodium deoxycholate, and finally, four times in sterile PBS solution. The beads were cultured on a TSA plate to determine the bacterial load, and the concentrated slurry was stored overnight at 4°C. On the following morning, the numbers of CFU were counted and the bead bacterial load was calculated. The bead slurry was diluted with PBS (typically 2:5 or 3:5) to obtain the desired dose of bacteria in a 50- μ l aliquot. A cohort of eight mice was pretreated with either SCC1 ($n = 4$) or water ($n = 4$). Subsequently, the mice were anesthetized with 2.5% tribromoethanol, and the tracheae were exposed by cutdown and cannulated with a 22-gauge angiocatheter, followed by intratracheal instillation of the aliquot of *P. aeruginosa* PA M57-15-impregnated agarose beads (dose, 3×10^5 CFU per mouse). The mice were weighed daily and

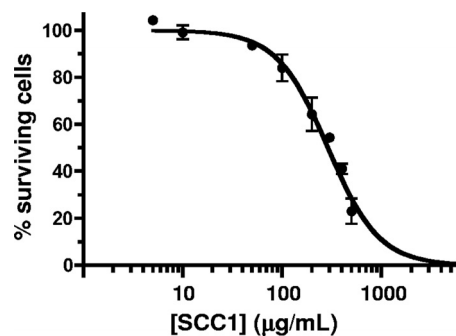


FIG. 2. In vitro cytotoxicity of SCC1. SCC1 was applied to the apical surface of primary cultured, differentiated mTECs for 24 h, followed by quantification of the cell number to determine cytotoxicity. The number of cells surviving at each treatment concentration was compared to the number of cells in control medium-treated samples. The results are the means \pm SDs of the percent surviving cells in replicate images of low-power fields and are representative of the results of at least two independent experiments.

were treated according to the prophylaxis protocol. At 72 h after infection, the mice were euthanized, the BAL fluid specimens were obtained and analyzed as described for the acute infection model, the spleens were harvested for qualitative bacteriology, and the lungs were harvested for histology (two mice per group) and quantitative bacteriology (two mice per group). The results of two separate experiments were pooled, for total results for eight mice per group, except for the one mouse that died during anesthesia.

Histology. The lungs were inflation fixed under 20 cm of water in situ via the intratracheal instillation of 2% paraformaldehyde and were then embedded in paraffin blocks. Sections (5 μ m) were mounted on slides and stained with hematoxylin-eosin. Microscopy images were acquired by the use of Axiovision (version 3.1) software (Carl Zeiss, Thornwood, NY).

Statistical analysis. All analyses were performed with the Prism (version 4) program (GraphPad Software, San Diego, CA). The in vitro cytotoxicities of multiple doses of SCC1, as well as in vivo aversion data, were compared by analysis of variance and post hoc analysis to determine statistical differences. The in vivo survival curves in the acute infection model were compared by a log-rank test. Changes in animal weights in the acute and agarose bead infection models, the lung bacterial burden in the acute infection model, and cytokine concentrations were compared by independent groups *t* tests. In the agarose bead model, bacterial clearance was measured as a categorical variable and the groups were compared by Fisher's exact test of a contingency table (the number of animals with bacteria in the lungs versus the number of animals that cleared the bacteria). Similarly, the levels of bacteremia in SCC1- and water-treated animals were compared by Fisher's exact test of the corresponding contingency table.

RESULTS

In vitro cytotoxicity of SCC1. Prior to in vivo testing, the in vitro margin of safety of SCC1 was assessed. Following exposure to concentrations of SCC1 that included the MIC and minimum bactericidal concentration of *P. aeruginosa* PA M57-15 (2 and 4 μ g/ml, respectively), cell numbers were determined in a primary cell culture model of the respiratory epithelium. The cell numbers remained unchanged at concentrations corresponding to the MIC₉₀ of SCC1, but cytotoxicity was significant by the time that a concentration of 200 μ g/ml was tested (Fig. 2). As determined by curve fitting of the semilogarithmically transformed data, the 50% lethal dose of SCC1 in this model is 289 μ g/ml, which is 48-fold greater than the MIC₉₀ for all of the *P. aeruginosa* strains previously tested and 145-fold above the MIC for PA M57-15 (20).

Effect of nebulized SCC1 solution on mouse activity and lung inflammation. As an initial in vivo evaluation of the suit-

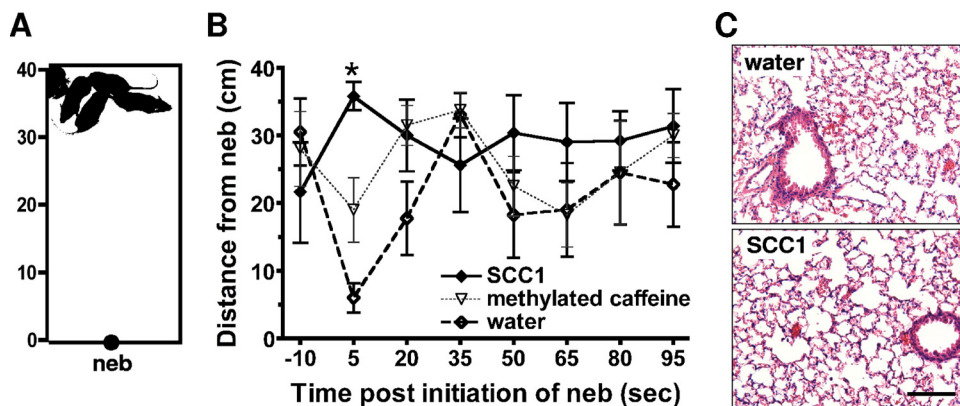


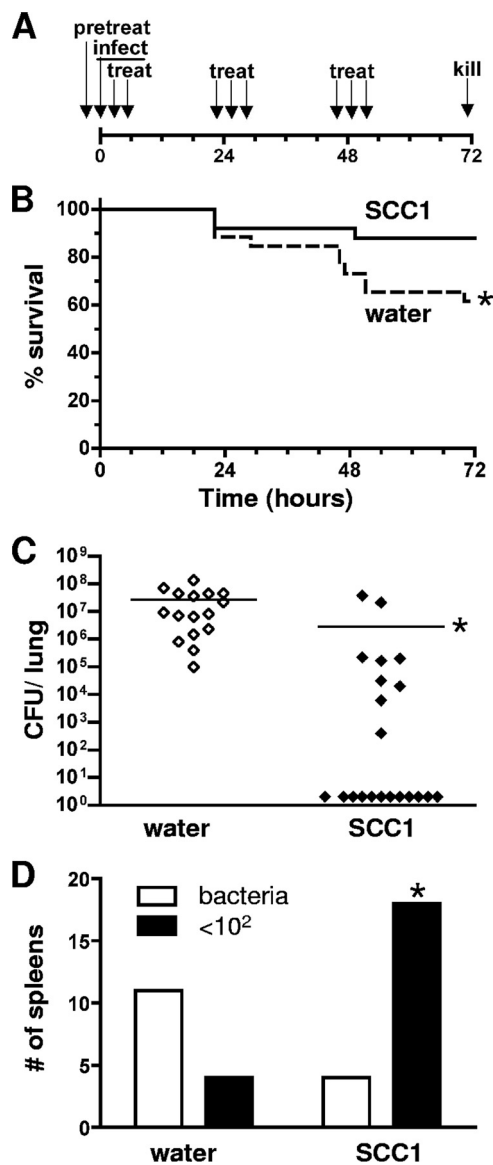
FIG. 3. Effect of nebulized SCC1 solution on mouse position relative to aerosol source and on lung inflammation. Mice were exposed to aerosolized water or SCC1 twice daily for 5 days in a multidosing chamber designed with a nebulizer (neb) at one end. (A) Representation of the location of mice obtained 5 s after the initiation of an SCC1 aerosol treatment. (B) Means \pm standard errors of mouse position relative to that of the nebulizer device at the indicated times after the initiation of nebulization. *, $P < 0.01$, SCC1 versus water. (C) Representative lung sections from water- and SCC1-treated mice after day 5 of treatment. The sections were stained with hematoxylin-eosin. Bar, 100 μm .

ability of SCC1 as a nebulized therapy, mice were placed in a multidosing chamber and observed during their exposure to aerosolized water or SCC1 twice daily over a 5-day period. The animals that were exposed to water approached the nebulizer (Fig. 3B; time, 5 s) and then moved to the opposite side of the chamber in the direction of the leading edge of the mist cloud (Fig. 3B; times, 20 s and 35 s). Once the concentration throughout the dosing chamber equilibrated, as indicated by diffusion of the mist cloud, the mice dispersed throughout the chamber. In contrast, animals exposed to SCC1 immediately moved away from the mist and congregated at the opposite end of the dosing chamber (Fig. 3A and B; time, 5 s), although they subsequently dispersed after the chamber filled with mist (Fig. 3B; time, >35 s). To determine the portion of the SCC1 molecule that was aversive, animals previously exposed to nebulized water were exposed to the nebulized methylated caffeine carrier (Fig. 1B). Even though these animals had previously approached the water mist, they moved to the opposite side of the chamber following initiation of the carrier compound aerosol, although not as quickly as the naïve animals when they were exposed to SCC1 itself (Fig. 3B). Notably, the animals exhibited no weight loss or changes in grooming or jumping behaviors after repeated dosing of nebulized water or SCC1 (5 mg/dose SCC1, 5 min exposure, twice daily) over a period of 5 days (data not shown). After the 5-day treatment period, the lungs were harvested for histologic evaluation. The lungs from both water- and SCC1-treated mice appeared to be histologically normal. Specifically, the lungs from the SCC1-treated animals did not show evidence of increased immune cell infiltration (Fig. 3C). Accordingly, we next tested the efficacy of SCC1 for the treatment of lung infections in two different mouse models, an acute pneumonia model and a chronic airway infection model.

Effect of nebulized SCC1 on survival, bacterial lung burden, and dissemination following acute in vivo infection. Mice were given a single prophylactic dose of nebulized SCC1 and were then intratracheally infected with *P. aeruginosa*; this procedure was followed by the administration of eight additional doses of SCC1 over the next 72 h (Fig. 4A). Mice that received SCC1

according to this protocol had improved survival compared to that of mice that received treatment with the vehicle control, water (Fig. 4B). Of the SCC1-treated animals, 22 of 25 survived (88%), whereas 16 of 26 water-treated mice survived (62%), for a 26% survival advantage in this model ($P = 0.04$). Death in the water-treated mice occurred especially at 48 h postinfection. At 72 h, no statistically significant difference in weight change was noted between the groups (data not shown). Analysis of the lung bacterial burden in the surviving mice after 72 h showed a distinct difference between treatment groups characterized by a total absence of bacteria in the lungs of more than half of the SCC1-treated survivors (12 of 21 [57%]; one set of plates appeared to be contaminated with a species other than *Pseudomonas*, and those plates were eliminated from the analysis). In contrast, the lungs of all surviving mice treated with water had greater than 10^5 CFU/lung (Fig. 4C; water-treated mice [$n = 16$], $2.7 \times 10^7 \pm 0.9 \times 10^7$ CFU/lung; SCC1-treated mice [$n = 21$], $2.9 \times 10^6 \pm 2.0 \times 10^6$ CFU/lung; $P = 0.004$). Furthermore, there was a significant increase in the number of water-treated mice from which bacteria were recovered from the spleen compared to the number in the SCC1-treated group (Fig. 4D; water-treated group, 9 of 13 mice [three sets of spleens appeared to grow a species other than *Pseudomonas* and were eliminated from the analysis]; SCC1-treated group, 4 of 22 mice; $P = 0.002$). For the majority of the SCC1-treated mice (82%), bacteria could not be cultured from the spleen.

Effect of nebulized SCC1 on serial changes in inflammation, bacterial lung burden, and dissemination following acute in vivo infection. To investigate the efficacy of SCC1 on a day-to-day basis in the acute infection model, 24 mice were infected with 3×10^6 CFU per mouse of strain PA M57-15 and subjected to the prophylaxis protocol (Fig. 4A), with half of the mice receiving nebulized doses of SCC1 and the other half receiving water. As treatment proceeded, a group of mice ($n = 3$ to 4) from each treatment group was evaluated daily to monitor changes in the lung bacterial burden, the influx of leukocytes as determined by BAL, and bacteremia. Animals in both treatment groups lost weight at the same rate until day 3,



when the SCC1-treated animals began to gain weight (data not shown). After 24 h, the bacterial burden in the lungs were similar in the water- and SCC1-treated groups (water-treated group [*n* = 3], $0.41 \times 10^6 \pm 0.1 \times 10^6$ CFU/lung; SCC1-treated group [*n* = 3], $1.9 \times 10^6 \pm 1.7 \times 10^6$ CFU/lung; *P* = 0.42). By the second day of treatment with SCC1, the lungs of the mice had 100-fold less bacteria than the lungs of the water-treated

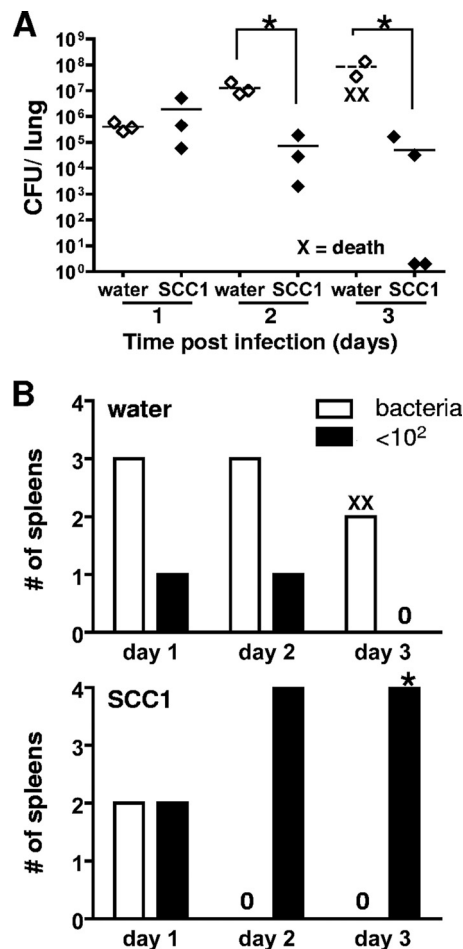


FIG. 5. Effects of nebulized SCC1 on serial changes in bacterial lung burden and dissemination following acute in vivo infection. A cohort of 24 mice was subjected to the prophylaxis protocol described in the legend to Fig. 4A; however, a group of four mice in each treatment group was evaluated daily (three mice for quantitative bacteriology, one mouse for histology). (A) Concentration of bacteria recovered from the lungs of mice evaluated at 24, 48, and 72 h after inoculation. Bars, mean concentrations; *, *P* = 0.005. Mice that died between 48 and 72 h are represented by an X and were not analyzed. (B) Bacteremia as determined by the recovery of bacteria from the spleens of mice evaluated at 24, 48, and 72 h. *, *P* = 0.01.

mice (Fig. 5A; water-treated group [*n* = 3], $1.2 \times 10^7 \pm 0.4 \times 10^7$ CFU/lung; SCC1-treated group [*n* = 3], $7.3 \times 10^4 \pm 5.8 \times 10^4$ CFU/lung; *P* = 0.04). By day 3, the difference was 1,000-fold (water-treated group [*n* = 2], $8.5 \times 10^7 \pm 4.9 \times 10^7$ CFU/lung; SCC1-treated group [*n* = 4], $5.0 \times 10^4 \pm 4.0 \times 10^4$ CFU/lung; *P* = 0.005). Two of the animals in the water-treated group died overnight on day 2 before their lungs could be assayed. Thus, the difference in the lung bacterial burdens is likely underestimated. By day 3, all of the surviving animals in the water-treated group but none of the SCC1-treated animals had bacteria in their spleens (Fig. 5B; day 3, *n* = 4 in each group; *P* = 0.01, contingency table). Thus, consistent with the changes in survival noted after day 2, SCC1 treatment resulted in the clearance of bacteria from the lungs and the prevention of bacteremia between days 2 and 3.

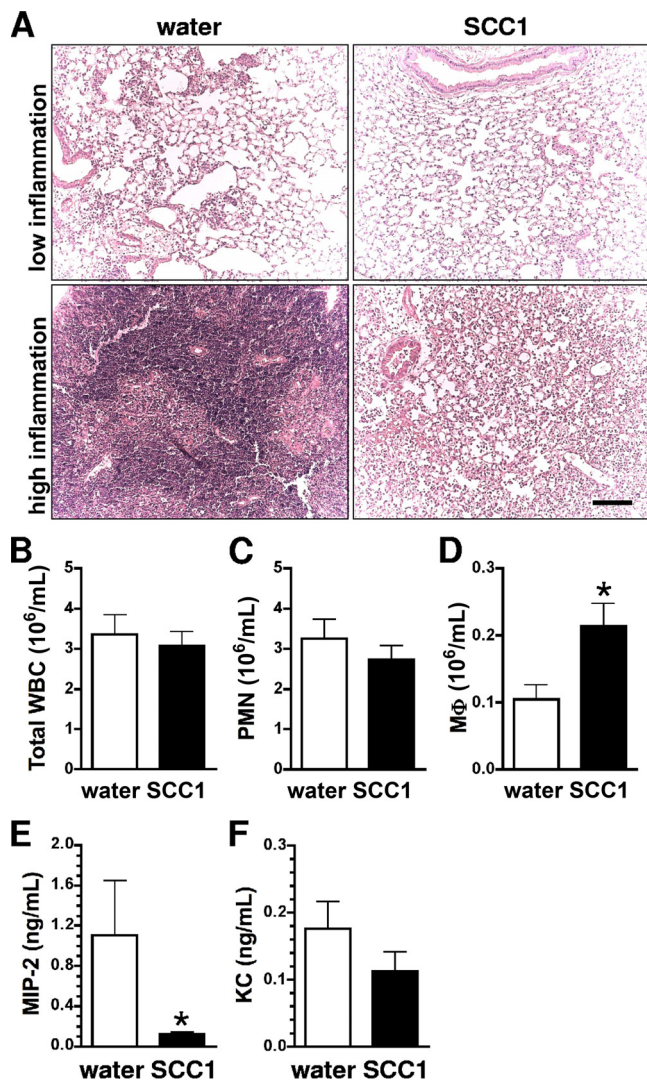


FIG. 6. Effect of nebulized SCC1 on lung inflammatory cells and cytokine expression following acute in vivo infection. The lungs from mice treated as described in the legend to Fig. 4A were analyzed. (A) Representative images of lung sections depicting the range in intensity of inflammation (low to high) observed in the lungs of mice treated with water or SCC1 after infection with *P. aeruginosa*. Sections were stained with hematoxylin-eosin. Bar, 400 μ m. Mice underwent BAL at 72 h after infection with *P. aeruginosa*. (B) Total inflammatory cell numbers are depicted: leukocytes (WBC) (B), neutrophils (polymorphonuclear leukocytes [PMN]) (C), and macrophages (MΦ; *, $P = 0.02$) (D). Also shown are the results for the chemokines MIP-2 (*, $P = 0.02$) (E) and KC (F). Shown are the means \pm SDs for four samples.

Effect of nebulized SCC1 on *P. aeruginosa*-induced inflammation in the acute lung infection model. The inflammatory response in the lungs of mice treated with water or SCC1 72 h after the initial infection was next examined. Lung tissue sections showed regional variation in pneumonitis, with the affected areas demonstrating perivascular, peribronchiolar, and alveolar accumulation of immune cells. By taking these features into account, the range of inflammation between the water- and SCC1-treated groups was increased in the lungs of the water-treated animals compared to that in the lungs of the

SCC1-treated animals (Fig. 6A). Neither the total leukocyte counts nor the neutrophil counts recovered from BAL fluid, however, were different (Fig. 6B and C; for total leukocytes, $3.4 \times 10^6 \pm 0.5 \times 10^6$ in the water-treated group [$n = 16$], $3.1 \times 10^6 \pm 0.3 \times 10^6$ in the SCC1-treated group [$n = 22$] [$P = 0.64$]; for neutrophils, $3.3 \times 10^6 \pm 0.5 \times 10^6$ in the water-treated group [$n = 16$] and $2.7 \times 10^6 \pm 0.3 \times 10^6$ in the SCC1-treated group [$n = 22$] [$P = 0.37$]). The numbers of macrophages recovered in BAL fluid were statistically significantly higher in the SCC1-treated mice than the water-treated mice (Fig. 6D; water-treated group [$n = 16$], $1.0 \times 10^5 \pm 0.2 \times 10^5$; SCC1-treated group [$n = 22$], $2.2 \times 10^5 \pm 0.4 \times 10^5$; $P = 0.02$). In the mouse, MIP-2 and KC are neutrophil chemoattractants homologous to human interleukin-8. While the concentration of MIP-2 was lower in the SCC1-treated mice (Fig. 6E; water-treated group [$n = 16$], 1.1 ± 0.5 ng/ml; SCC1-treated group [$n = 22$], 0.12 ± 0.02 ng/ml; $P = 0.02$), both treatment groups had similar levels of KC in their BAL fluid (Fig. 6F; water-treated group [$n = 16$], $1.8 \times 10^{-1} \pm 0.4 \times 10^{-1}$ ng/ml; SCC1-treated group [$n = 22$], $1.1 \times 10^{-1} \pm 0.3 \times 10^{-1}$ ng/ml; $P = 0.10$). Importantly, these samples were obtained from the mice that survived for 72 h and did not include samples from the animals that died at 48 h.

Effect of nebulized SCC1 on *P. aeruginosa* in the agarose bead infection model. The effect of SCC1 was evaluated in a model of airway infection, the agarose bead infection model, to compare the effects of SCC1 in this model to those seen in the acute infection model. Unlike the acute infection model that allows for alveolar deposition of bacteria more typical of pneumonia, the agarose bead infection model restricts bacteria primarily to the medium and small airways sparing the alveoli (Fig. 4). In contrast to the acute lung infection model, no animals died during the 72-h observation period, and the SCC1-treated mice in this model lost significantly less weight than the water-treated mice (Fig. 7A; water-treated animals [$n = 7$], -16 ± 3 ; SCC1-treated animals [$n = 8$], -4 ± 2 ; $P = 0.008$). Importantly, the burden of bacteria in the lung was also significantly reduced by SCC1 treatment, such that all of the SCC1-treated animals cleared their infections (Fig. 7B; water-treated group [$n = 3$], $3.1 \times 10^6 \pm 5.0 \times 10^6$ CFU/lung; SCC1-treated group [$n = 4$], 0 CFU/lung; $P = 0.03$, contingency table; the lungs of the remaining animals were examined histologically). Similar to the findings from the acute infection model, the clearance of pulmonary bacteria was associated with the clearance of bacteremia. All of the water-treated animals exhibited bacteremia, whereas bacteria were recovered from the spleens of none of the SCC1-treated animals (Fig. 7C; water-treated group, $n = 7$; SCC1-treated group, $n = 8$; $P = 0.0002$, contingency table).

Effects of nebulized SCC1 on *P. aeruginosa*-induced lung inflammation in the agarose bead infection model. Similar to the findings from the acute lung infection model, lung tissue sections in the agarose bead model of chronic airway infection at 72 h showed regional variations in inflammatory infiltrates. The delivery of sterile beads to the lungs of untreated animals resulted in little or no inflammation (data not shown). However, the level of inflammation was marked in the water-treated animals infected with *P. aeruginosa* and especially occurred in regions marked by the anatomic location of the bacteria-laden beads (Fig. 8A, top panel). The lungs of the

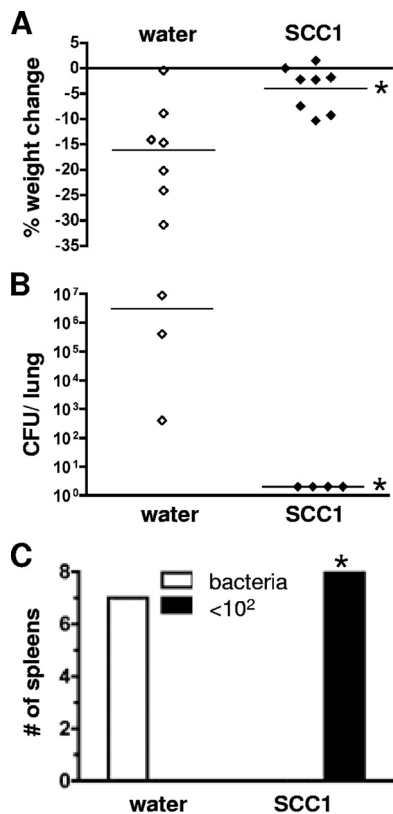


FIG. 7. Effect of nebulized SCC1 in the agarose bead infection model. Mice were treated with water or SCC1 as described in the legend to Fig. 4A before and after infection with *P. aeruginosa* PA M57-15-laden agarose beads. (A) Weight change. Bars, mean percent change from the initial body weight; *, $P = 0.008$. (B) Concentration of bacteria recovered from the lungs of mice surviving at 72 h after inoculation. Bars, mean concentrations; *, $P = 0.03$. (C) Bacteremia determined by recovery of bacteria from the spleen after the indicated treatment in surviving mice at 72 h. *, $P = 0.0002$.

SCC1-treated animals had minimal inflammation that was primarily localized in a peribronchiolar distribution surrounding airways that contained beads (Fig. 8A, lower panel). The majority of the lungs from the SCC1-treated animals exhibited little evidence of infection. This difference in airway inflammation was reflected in the BAL fluid from water- and SCC1-treated animals; the total leukocyte and neutrophil counts recovered from the SCC1-treated animals were significantly reduced (Fig. 8B and C; for total leukocytes, $2.1 \times 10^5 \pm 0.7 \times 10^5$ in the water-treated group [$n = 7$] and $3.2 \times 10^4 \pm 0.8 \times 10^4$ in the SCC1-treated group [$n = 8$] [$P = 0.02$]; for neutrophils, $1.7 \times 10^5 \pm 0.6 \times 10^5$ for the water-treated group [$n = 7$] and $6.0 \times 10^3 \pm 3.0 \times 10^3$ for the SCC1-treated group [$n = 8$] [$P = 0.01$]). The number of macrophages recovered was less for the SCC1-treated animals, but the number was not statistically different from that recovered from the water-treated mice (Fig. 8D; water-treated group [$n = 7$], $3.9 \times 10^4 \pm 1.1 \times 10^4$; SCC1-treated group [$n = 8$], $2.6 \times 10^4 \pm 0.8 \times 10^4$; $P = 0.3$). The concentrations of the mouse neutrophil chemoattractants MIP-2 and KC were both dramatically reduced in the SCC1-treated animals; the concentration of KC was below the level of detection in all SCC1-treated animals (Fig. 8E and F; for MIP-2, 0.78 ± 0.6 ng/ml in the water-treated group [$n = 7$] and 0.011 ± 0.001 ng/ml in the SCC1-treated group [$n = 8$] [$P = 0.04$, contingency table]; for KC, 0.077 ± 0.07 ng/ml in the water-treated group [$n = 7$] and no KC detected in the SCC1-treated group [$n = 8$] [$P = 0.007$, contingency table]).

DISCUSSION

As reported previously, the silver-caffeine compound designated SCC1 was effective for the inhibition of the in vitro growth of numerous respiratory pathogens, including strains of *Pseudomonas aeruginosa* and multidrug-resistant organisms from the *Burkholderia cepacia* complex for which the MIC₉₀

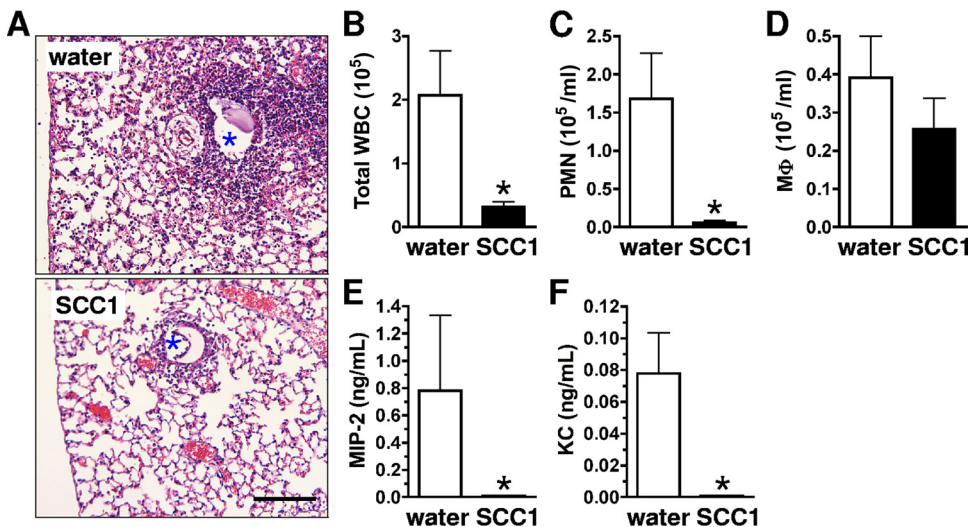


FIG. 8. Effects of nebulized SCC1 on lung inflammation in the agarose bead infection model. The lungs from mice infected with agarose beads impregnated with PA M57-15 and treated as described in the legend to Fig. 7 were analyzed. (A) Representative images of the lungs of mice treated with nebulized water or SCC1 after infection with PA M57-15-laden agarose beads. Blue asterisks, peripheral airways containing beads. Bar, 400 μm. Mice underwent BAL at 72 h after infection with PA M57-15-laden agarose beads. Total inflammatory cell numbers are depicted: leukocytes (WBC; *, $P = 0.02$) (B); neutrophils (polymorphonuclear leukocytes [PMN]; *, $P = 0.01$) (C); and macrophages (MΦ) (D). Also shown are the results for the chemokines MIP-2 (*, $P = 0.04$) (E) and KC (*, $P = 0.007$) (F). Shown are the means ± SDs for four samples.

was 6 $\mu\text{g/ml}$ (MIC range, 1 to 10 $\mu\text{g/ml}$; the MIC_{90} is the concentration at which 90% of the strains tested fail to grow) (20). Other silver carbene complexes have demonstrated exceptional and comparable broad-spectrum activity against both gram-positive and gram-negative bacteria; fungi; methicillin-resistant *S. aureus*; and the biosafety level 3 bacteria *Burkholderia pseudomallei*, *Burkholderia mallei*, *Bacillus anthracis*, and *Yersinia pestis* (16, 17, 28, 29, 31, 33). In particular, the MIC of SCC1 for the mucoid clinical strain of *P. aeruginosa* studied here, strain PA M57-15, is 2 $\mu\text{g/ml}$. This strain had previously been well studied in a mouse model of airway infection (36).

Prior to the in vivo studies, the in vitro cytotoxicity of SCC1 was investigated. Concentrations of SCC1 encompassing the MIC_{90} noted in previous studies were applied to primary cultures of mTECs without apparent toxicity. While these cell cytotoxicity studies could be supplemented with the investigation of biochemical markers of cell injury, the absence of cell death with the use of concentrations below 200 $\mu\text{g SCC1/ml}$ was notable, particularly because the 24-h direct incubation period was likely greater than that which the respiratory epithelium would encounter in vivo. Moreover, the previously reported microarray analysis of SCC1- and water-treated mTECs showed minimal differences in gene expression after 24 h (20). These findings together suggested that the concentrations of SCC1 that would be required for bacterial growth inhibition were not toxic to airway epithelial cells, and in vivo studies seemed warranted.

Initial in vivo studies were aimed at investigating the potential toxicity of SCC1 delivered to the airways. To deliver SCC1, a micropump nebulizer, which could provide particles capable of delivery to the alveoli ($<5 \mu\text{m}$), was used to provide a static exposure over 5 min two times daily for 5 days (26). The precise amount of silver that was delivered to the lung in these treatment models has yet to be determined, in part because the amount appears to be very low. The immediate harvesting of the lungs after a single dose or after the last of multiple doses followed by homogenization and attempted quantification of Ag(I) by mass spectroscopy failed to demonstrate a signal greater than that found in the lungs of the water-treated control animals (data not shown). It is possible, however, that SCC1 was also delivered orally, because the animals ingested compound that had been deposited by aerosol on their fur through grooming. Although an initial aversion response to the aerosolized SCC1 was noted, our investigations revealed that this was most likely due to the caffeine derivative carrier rather than the silver moiety.

The toxicology of silver has been remarkably understudied, likely because silver causes so few clinically significant problems. At the cellular level, silver salts inhibit the proliferation of some cell types, including lymphocytes and keratinocytes (12, 18). Silver has been described to stimulate the respiratory burst in neutrophils (19), which may enhance bacterial clearance but which could also increase inflammation (9). Suggested mechanisms of silver cytotoxicity include the inhibition of cellular respiration with a loss of ATP (15). Other studies showed that silver had no cytotoxic effects (1, 6). Consequently, the safety of silver-based antimicrobials is still an open question. The studies of the cytotoxicity of SCC1 reported here begin to address this question. Given the broad therapeutic window between the MIC_{90} found for respiratory pathogens, 6

$\mu\text{g/ml}$ (20), and the 50% lethal dose for respiratory epithelial cells treated with SCC1, 289 $\mu\text{g/ml}$, this compound appears to have a very low level of toxicity at the doses delivered. This conclusion is supported by the findings of the in vivo studies, which showed that the administration of SCC1 by nebulization to mice results in no toxic effects, other than a mild aversion to the methylated caffeine carrier.

The efficacy of SCC1 was tested in two specific lung infection protocols that were developed to model two types of disease that might be treated with aerosolized SCC1. The infection achieved by the direct intratracheal instillation of motile bacteria models acute pneumonia in humans, while the instillation of bacteria-laden beads models the chronic infection of the airways seen in bronchiectasis CF patients and non-CF patients. This agarose bead infection model has features similar to the features of clinical infections in patients with CF, namely, obstruction of small airways with bacteria-laden material and the restriction of inflammation primarily to the peribronchial areas (27). More representative of the human disease would be a chronic lung infection model that developed over several months; however, such a model is not readily available (14). Nonetheless, in each model, the SCC1 aerosol decreased the lung bacterial burden, indicating that it had a beneficial antibacterial effect. Furthermore, survival in the SCC1-treated groups in both models appeared to be linked to averting bacteremia, as indicated by the striking difference in the number of animals in each treatment group in which bacteria disseminated to the spleen. The results obtained with the two infection models differed. The biochemical parameters of inflammation did not correlate with the attenuated inflammation seen in the SCC1-treated animals in the acute infection model, whereas they correlated directly with the decreased inflammation of the lungs of the SCC1-treated animals in the agarose bead model. One possible explanation is that the initial inoculum is necessarily lower in the agarose bead model than the acute infection model and may consequently evoke a different time course of inflammation.

Our report on the use of aerosolized silver as an antimicrobial for pulmonary infections joins a recent resurgence in interest in silver compounds that includes the development of several different preparations for topical use, some of which incorporate nanocrystalline silver (5, 10). This report is the first to investigate the direct nebulization of a silver carbene complex in solution as a potential therapy for pulmonary infections. Given that SCC1 appears to be safe and effective in these animal models of lung infection, further studies of SCC1 and other silver carbene compounds, either directly aerosolized in solution or in a nanoparticle suspension (17), appear to be warranted. Nebulized silver-based antimicrobials may serve as useful adjuncts to the current therapies for difficult-to-treat pulmonary infections.

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