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Neomycin Sulfate Improves the Antimicrobial Activity of Mupirocin-Based Antibacterial Ointments

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In the midst of the current antimicrobial pipeline void, alternative approaches are needed to reduce the incidence of infection and decrease reliance on last-resort antibiotics for the therapeutic intervention of bacterial pathogens. In that regard, mupirocin ointment-based decolonization and wound maintenance practices have proven effective in reducing *Staphylococcus aureus* transmission and mitigating invasive disease. However, the emergence of mupirocin-resistant strains has compromised the agent's efficacy, necessitating new strategies for the prevention of staphylococcal infections. Herein, we set out to improve the performance of mupirocin-based ointments. A screen of a Food and Drug Administration (FDA)-approved drug library revealed that the antibiotic neomycin sulfate potentiates the antimicrobial activity of mupirocin, whereas other library antibiotics did not. Preliminary mechanism of action studies indicate that neomycin's potentiating activity may be mediated by inhibition of the organism's RNase P function, an enzyme that is believed to participate in the tRNA processing pathway immediately upstream of the primary target of mupirocin. The improved antimicrobial activity of neomycin and mupirocin was maintained in ointment formulations and reduced *S. aureus* bacterial burden in murine models of nasal colonization and wound site infections. Combination therapy improved upon the effects of either agent alone and was effective in the treatment of contemporary methicillin-susceptible, methicillin-resistant, and high-level mupirocin-resistant *S. aureus* strains. From these perspectives, combination mupirocin-and-neomycin ointments appear to be superior to that of mupirocin alone and warrant further development.

*S*taphylococcus aureus has been designated one of the six ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) bacterial pathogens of greatest concern to health care in the United States (1). The organism is a predominant cause of nosocomial and community-associated bacterial infections and has developed resistance to all currently available antibiotics (2). The *S. aureus* annual U.S. mortality rate has already surpassed that of HIV/AIDS and is predicted to worsen given the downsizing of most pharmaceutical antimicrobial programs (3, 4). Consequently, new strategies are needed for the prevention and treatment of staphylococcal infections.

The anterior nares of humans is a principal ecological niche for *S. aureus*, and nasal carriage is a recognized risk factor for staphylococcal disease, particularly among patient populations undergoing surgical procedures or hemodialysis or requiring long-term intensive care unit stays (reviewed in reference 5). *S. aureus* nasal decolonization reduces colonization of other body sites, the risk of transmission, and subsequent infection (5). Consequently, infection control practices routinely include nasal decolonization procedures as a means to prevent staphylococcal disease.

Mupirocin is an antimicrobial agent that inhibits bacterial isoleucyl-tRNA synthetase-mediated Ile-tRNA aminoacylation and protein translation (6–8). This agent displays excellent antibacterial activity toward most Gram-positive species and lacks cross-resistance to current antibiotics, but it is also unstable *in vivo* and thus not well-suited for systemic use in humans (9). However, mupirocin-based ointments have proven effective for the treatment of *S. aureus* skin and wound infections (9–13) and have also recently emerged as the standard of care for presurgical nasal decolonization (reviewed in reference 14). Indeed, mupirocin-mediated nasal decolonization has been shown to be effective in re-

ducing infections in burn wound, dialysis, and surgical patient populations, as well as *S. aureus* transmission among health care workers and intensive care unit patients (15–21). In addition to nasal decolonization, topical mupirocin has been used to successfully treat hemodialysis central venous catheter exit sites, impetigo, eczema, surgical wound sites, skin and soft tissue wounds, the breasts of breast-feeding mothers, and tympanic membrane lesions (22–27). However, the emergence of *S. aureus* mupirocin resistance has reduced the agent's efficacy both as a nasal decolonization agent and as a treatment option for skin and wound infections.

Low-level mupirocin-resistant (LL-MR) *S. aureus* strains are defined as exhibiting an MIC of 8 to 256 μg ml^{-1} due to point mutations in the organism's native isoleucyl-tRNA synthetase gene (*ileRS*) and develop rapidly in both the laboratory and clinical settings (28). High-level mupirocin resistance (HL-MR) (MIC of >256 μg ml^{-1}) occurs less frequently and is attributable to the acquisition of a mobile genetic element harboring either *mupA*, which codes for an alternate isoleucyl-tRNA synthetase, or

the less-characterized *mupB* gene (29, 30). Both LL-MR and HL-MR lead to mupirocin treatment failure (31). Indeed, while low-level resistant strains initially respond to therapy, they frequently reemerge quickly; relapse is hypothesized to be due to latent LL-MR subpopulations that are not eradicated by mupirocin dosing (31, 32). Conversely, HL-MR *S. aureus* bacteria are recalcitrant to mupirocin ointments (31). Thus, the emergence of mupirocin resistance has prompted renewed interest in developing alternative decolonization and wound infection treatment strategies.

S. aureus RNase P is an essential riboprotein complex consisting of RnpA and ribozyme *rnpB* that acts upstream of tRNA synthetases in the tRNA maturation pathway (33, 34). More specifically, RNase P is hypothesized to catalyze removal of the 5′ leader sequences from precursor tRNA species, thereby creating mature tRNA substrates for tRNA synthetases, including isoleucyl-tRNA synthetase (the cellular target for mupirocin) (33–39). Recognizing that two antimicrobials targeting independent steps in the same metabolic pathway can have combined antibacterial effects, it has been hypothesized that combination therapies involving mixtures of RNase P inhibitors together with mupirocin would display increased antimicrobial efficacy and the potential to overcome mupirocin resistance (33, 40).

Herein, we report the results of a screen of a Food and Drug Administration (FDA)-approved drug library for agents that potentiate the antimicrobial properties of mupirocin toward *S. aureus*. The antibiotic neomycin sulfate, which is approved for topical use and previously shown to inhibit *Escherichia coli* RNase P, was among the hits identified (41). Assays revealed that neomycin also inhibits *S. aureus in vitro* RNase P function, that it confers an additive antimicrobial advantage to mupirocin, and that the combination could be effectively formulated in ointment format. Topical application of the combination displayed significantly improved murine nasal decolonization toward a panel of *S. aureus* strains compared to either agent alone. Likewise, the combination led to the near eradication of contemporary methicillin-susceptible, methicillin-resistant, and high-level mupirocin-resistant strains in a murine wound model of colonization.

MATERIALS AND METHODS

Bacterial strains and animals. All bacterial studies were performed with *S. aureus* strain UAMS-1, a well-characterized antibiotic-susceptible clinical isolate commonly used to study the organism's biofilm formation and colonization properties (42), USA300, a neomycin- and methicillin-resistant community-acquired clinical isolate (43), or BAA-1708, a high-level mupirocin-resistant strain containing *mupA* obtained from the American Type Culture Collection (Manassas, VA). Unless indicated otherwise, strains were grown overnight in tryptic soy broth (TSB) and were then used to inoculate fresh (1:100 dilution) medium, grown to early exponential phase (1×10^8 CFU/ml), and processed as described below. Female BALB/c mice 4 to 6 weeks of age were obtained from Charles River (Wilmington MA) and housed according to approved University of Rochester Medical Center Council on Animal Research (UCAR) protocol UCAR-2013-024.

Preparation of test articles. The polyethylene glycol (PEG) ointment base was prepared by mixing PEG 400 (70%, wt/vol) with PEG 3350 (30%, wt/vol) as described by the U.S. Pharmacopeia and The National Formulary (USP 24-NF 19). Mupirocin (AppliChem, Chicago, IL) and neomycin (Sigma, St. Louis, MO) were suspended in 250 μl of dimethyl sulfoxide (DMSO) to create working concentrations of 100 mg and 50 mg, respectively. The mixtures were then added directly to 5 g of PEG ointment preliquefied by heating at 60°C for 30 min to create suspensions contain-

ing both 2% mupirocin and 1% neomycin and then cooled to room temperature to solidify the suspension. The same procedure was used to create DMSO vehicle control and 2% mupirocin–1% neomycin PEG mixtures by adding a combination of 100 mg mupirocin and 50 mg neomycin in a total 250-μl volume of DMSO.

Screen of Selleck library. Members of the Selleck library of Food and Drug Administration-approved drugs (catalog no. L1300; Selleck Chemicals, Houston, TX) were screened for agents that potentiate the antimicrobial activity of mupirocin toward *S. aureus* strain UAMS-1. To do so, 1×10^5 CFU of *S. aureus* UAMS-1 was added to individual wells of a 96-well microtiter plate, mixed with 0.03 μg ml^{-1} mupirocin ($0.5\times$ MIC) and 50 μM test agent in Mueller-Hinton broth (MHB) (100-μl total well volume). Microtiter plates were incubated at 37°C for 16 h, and individual wells were inspected for growth. Wells lacking growth were considered to represent agents that either potentiated the antimicrobial properties of mupirocin or mupirocin-independent antimicrobial microbial properties. All drugs that resulted in no growth were confirmed in duplicate and were plated without mupirocin to measure their inherent antimicrobial activity.

RNase P ptRNA processing assay. *S. aureus* RNase P activity assays were performed as previously described (33). Briefly, RNase P was first reconstituted by mixing an equimolar ratio of denatured *rnpB* and RnpA for 15 min at 37°C, and 2.5 pmol of this mixture was then added to 5 pmol of precursor tRNA$^{\text{Tyr}}$ (ptRNA$^{\text{Tyr}}$) and increasing concentrations of the indicated concentration of neomycin or the known RNase P inhibitor RNPA2000 (33) in a total volume of 20 μl. Mixtures were incubated for 5 min at 37°C, stopped by adding 20 μl of $2\times$ RNA loading dye (95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.5 mM EDTA), and 30 μl of each sample was electrophoresed in a 7 M urea–8% polyacrylamide gel and stained with ethidium bromide (0.5 μg ml^{-1}). A FluorChem 5500 imaging system was used to visualize RNA products and quantified using ImageJ software (National Institutes of Health, Bethesda, MD). The percent RNase P activity was then calculated using the following equation: test compound tRNA$^{\text{Tyr}}$ signal/mock tRNA$^{\text{Tyr}}$ signal.

Antimicrobial susceptibility testing. MIC was tested in accordance with the Clinical and Laboratory Standards Institute guidelines. Briefly, 1×10^5 CFU of the indicated *S. aureus* strain was added to individual wells of a microtiter plate containing 88 μl of MHB medium and 2-fold increasing concentrations of mupirocin or test agent (0 to 128 μg ml^{-1}). The plates were incubated for 16 h at 37°C, and the wells were visually inspected for growth. The lowest concentration of mupirocin or test agent that inhibited *S. aureus* growth was considered to be the MIC. Fractional inhibitory concentration index (FIC) testing was performed to measure interactions between mupirocin and neomycin, as previously described (44). Briefly, in checkerboard format, each row of the plate contained increasing concentrations of mupirocin (2-fold increments; 0 to 32 μg ml^{-1}), whereas each column contained increasing concentrations of neomycin (2-fold increments; 0 to 32 μg ml^{-1}). To every well (100-μl total volume), MHB containing 3×10^5 CFU of *S. aureus* strain UAMS-1 was added, and the plate was incubated at 37°C for 16 h. The FIC was determined using the following formula: (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone) = FIC. A synergistic interaction was defined as an FIC value of ≤ 0.5, an additive interaction was defined as an FIC value of 0.5 to 1.0, no interaction was defined as an FIC of 1 to 4, and an antagonistic interaction was defined as an FIC of >4.

***In vitro* ointment antimicrobial testing.** Antimicrobial zones of inhibition were measured for PEG ointment compilations using the indicated *S. aureus* strains. To do so, 100 μl of 1×10^8 CFU ml^{-1} of *S. aureus* was spread on tryptic soy agar (TSA) plates. The plates were dried for 10 min, and 40 μl of ointment was pipetted onto the center of the plate. The plates were incubated at 37°C for 16 h, and zones of bacterial clearance were measured using ImageJ software (NIH).

Nasal colonization and treatment of mice. Ointments were evaluated for *in vivo* antimicrobial activity using an *S. aureus* nasal colonization model as previously described (45) but with modifications. The nostrils of awake mice were inoculated with 1×10^7 CFU of the indicated *S. aureus* strain by pipetting 10 μl of culture directly into the nostrils and confirmed by the visualization of air bubbles appearing as the mouse breathed in and out. The nostrils of the mice were then treated with 10 μl PEG ointment (brought to 55°C in a heat block to liquefy) containing either vehicle alone or the indicated antibiotic 45 min postinoculation, and treatments were repeated every 8 h for 3 days. Mice were then euthanized via CO_2 asphyxiation and cervical dislocation. The full nares from the back of the soft palate to the tip of the nostrils was collected by gross dissection and placed in microcentrifuge tubes containing 1 ml of freshly made phosphate-buffered saline (PBS). Samples were homogenized for 5 min, serially diluted, and plated on mannitol salt agar (MSA) (Thermo Scientific, Waltham MA). The plates were incubated for 16 h, and the number of *S. aureus* was determined.

Dermal wound model of infection and treatment of mice. The effects of ointment compilations were evaluated for *in vivo* antimicrobial activity using an *S. aureus* dermal wound model (46) but with modifications. Mice were anesthetized by intraperitoneal injection with a mixture of 100 mg ml^{-1} ketamine (Hospira Inc., Lake Forest, IL) and 20 mg ml^{-1} Xylazine (Lloyd Laboratories, Shenandoah, IA) in 0.9% NaCl at 5 μl per g of body weight. Pain relief in the form of 20 μl of 0.5% Sensorcaine (APP Pharmaceuticals, Schaumburg, IL) was administered prior to dermal wounding. The dorsal midsection of the mouse was shaved and cleaned with a series of betadine scrub (Fisher Scientific), povidone-iodine pads (Professional Disposables International Inc.; Orangeburg, NY), and isopropyl alcohol pads (Fisher Scientific) for a total contact time of 2 min. A single wound was created in this sterile field on the mouse with a 6-mm biopsy punch (Fisher Scientific) to remove only the dermal layer and not disrupt the underlying musculature. The wounds of the mice were inoculated with 1×10^7 CFU of the indicated *S. aureus* strain by pipetting 10 μl of culture directly onto the wound. Mice were then treated with ointment formulations (50 μl) containing either vehicle alone, or indicated antibiotics 45 min postinoculation; treatments were repeated every 12 h for 3 days. Mice were then euthanized via CO_2 asphyxiation and cervical dislocation, per UCAR-approved methodology, the wound and underlying muscle were excised with an 8-mm biopsy punch and placed in microcentrifuge tubes containing 1 ml of freshly made PBS. Samples were homogenized for 5 min, serially diluted, and plated on MSA. The plates were incubated for 16 h, and the number of *S. aureus* was enumerated.

***In vivo* toxicity testing.** Ointment toxicity was tested in a modified dermal wound model. Mice in groups of three per indicated treatment group were wounded as described above but were not inoculated with *S. aureus*. The wound was treated with ointments containing vehicle, 2% mupirocin, 1% neomycin, or 2% mupirocin plus 1% neomycin combination twice daily for 14 days. Mice were weighed and assessed for grooming and alertness, and images of the wound were obtained daily to measure wound contraction using ImageJ (NIH). Wound contraction was calculated as a percentage of wound area reduction using the following formula: $WCd = (1 - WAd/WA0) \times 100$, where WCd is the wound contraction on day d, WAd is the wound area on day d, and $WA0$ is the wound area on the initial day, as previously described (47).

Statistical analyses. Statistical analyses were performed using Graphpad Prism software version 6.0. For zone of inhibition assays, a Student t test was used to determine the statistical power between each treatment group. For murine studies, measures were log transformed and subjected to an one-way analysis of variance (ANOVA) analysis to determine the statistical power.

RESULTS

Agents that potentiate the antimicrobial activity of mupirocin. Members of the Selleck library of 853 FDA-approved drugs were screened for agents that potentiate the activity of mupirocin. To

TABLE 1 Selleck library members with mupirocin-associated improved activity[a]

Drug	MIC (μg ml^{-1}) of drug[b]	
	(−) Mup	(+) Mup
Nitazoxanide	16	8
Nitrofurazone	16	8
Neomycin sulfate	0.5	0.25

[a] The fractional inhibitory concentration index (FIC) was 0.75 for all three drugs.
[b] The MIC of the drug was determined in the presence (+) or absence (−) of 0.5× mupirocin (Mup) MIC (0.0625 μg ml^{-1}).

do so, the antibiotic-susceptible *S. aureus* strain UAMS-1 was inoculated into individual wells of a microtiter plate containing 0.5× the strain's mupirocin MIC (0.0625 μg ml^{-1}) and 50 μM library material. A total of 101 library members (11.8%), including 61 antibiotics, inhibited bacterial growth, suggesting that they may represent agents that (i) potentiate the antimicrobial activity of mupirocin, (ii) exhibit mupirocin-independent antimicrobial activity, or (iii) both (see Table S1 in the supplemental material).

To distinguish between these possibilities, the MIC of each compound was determined in medium lacking or containing 0.5× the strain's mupirocin MIC. Of the 101 compounds, 98 (97%) evaluated displayed similar antimicrobial activities regardless of whether mupirocin was present, indicating that they do not potentiate the antibacterial effects of mupirocin. Conversely, the antimicrobial activity of nitazoxanide, nitrofurazone, and neomycin sulfate increased in the presence of mupirocin. Fractional inhibitory concentration index (FIC) measures confirmed that each agent displayed an additive effect (FICs = 0.75) when combined with mupirocin indicating that they have the capacity to potentiate the activity of mupirocin (Table 1). More specifically, nitazoxanide and nitrofurazone reproducibly displayed modest antimicrobial activities of 16 μg ml^{-1} and 8 μg ml^{-1} in the absence and presence of 0.5× MIC mupirocin, respectively. The aminoglycoside antibiotic neomycin sulfate exhibited the most potent activity against the test strain in the absence (0.5 μg ml^{-1}) and presence (0.125 to 0.25 μg ml^{-1}) of 0.5× MIC mupirocin (0.0625 μg ml^{-1}). Given that no other antibiotics within the Selleck library, including other aminoglycosides, displayed improved antimicrobial properties in the presence of mupirocin, and expanded FIC testing revealed that neomycin did not improve the antimicrobial activity of rifampin, vancomycin, sulfamethoxazole, meropenem, minocycline, ciprofloxacin, ceftriaxone, or erythromycin (data not shown), the additive effects between neomycin and mupirocin appeared to be specific to this combination of antibiotics.

Neomycin inhibits *S. aureus* RNase P *in vitro* activity. As noted above, it has been hypothesized that inhibitors of RNase P function would display improved antimicrobial effects when combined with mupirocin. In that regard, aminoglycoside antibiotics bind the major groove of the 16S rRNA to disrupt the fidelity of tRNA selection and block protein translation, but recent studies have revealed that they can also bind and affect the function of mRNAs, tRNAs, and catalytic RNAs (41, 48–50). Indeed, neomycin B and/or derivatives have been shown to bind to the *rnpB* component of RNase P and/or precursor tRNA molecules in a manner that inhibits *Escherichia coli*, *Neisseria gonorrhoeae*, *Porphyromonas gingivalis*, *Streptococcus pneumoniae*, and *Bacillus subtilis* RNase P function (41, 51, 52). Accordingly, we evaluated whether neomycin also inhibits *S. aureus* RNase P activity using an
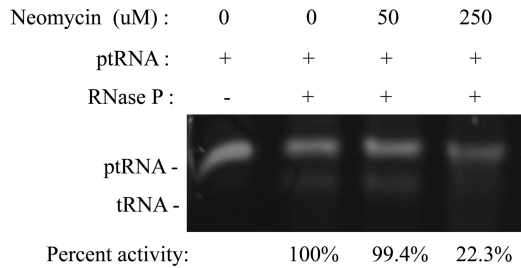
Neomycin (uM): 0 0 50 250
ptRNA: + + + +
RNase P: − + + +

ptRNA −
tRNA −

Percent activity: 100% 99.4% 22.3%

FIG 1 Effects of neomycin on *S. aureus* RNase P-mediated ptRNA^{Tyr} processing. The mobility of precursor tRNA^{Tyr} in the presence (+) and absence (−) of *S. aureus* RNase P enzyme and the indicated concentration of neomycin (in micromolar) is shown. Densitometry measured percent activity shown (tRNA product formed) normalized to the value for DMSO-treated enzyme alone.

in vitro precursor tRNA processing assay (33). As shown in Fig. 1, results revealed that high concentrations (250 μM) of neomycin inhibit *S. aureus* RNase P's ability to catalyze the maturation of precursor tRNA^{Tyr}, suggesting that the agent's ability to potentiate mupirocin may, in part, be mediated by its ability to inhibit the organism's RNase P activity.

Antimicrobial effects of mupirocin and neomycin combination in ointment formation. Because neomycin improves the antimicrobial potency of mupirocin and the two antibiotics have differing mechanisms of action, we reasoned that combination ointments containing both agents would overcome mupirocin resistance. As a first test of this hypothesis, antimicrobial plate assays

were used to monitor the antimicrobial effects of PEG-based ointments containing either DMSO (vehicle), 2% mupirocin, 1% neomycin, or the combination of 2% mupirocin plus 1% neomycin toward a neomycin- and mupirocin-susceptible clinical isolate (UAMS-1), a neomycin-resistant clinical isolate (USA300; MIC > 128 μg ml^{-1}; data not shown), and a strain containing the *mupA* gene that confers high-level mupirocin resistance (BAA-1708; MIC > 256 μg ml^{-1}; data not shown).

As shown in Fig. 2A, measures of each treatment's zone of inhibition revealed that while vehicle alone did not affect *S. aureus* UAMS-1 growth, both antibiotics, alone and in combination, produced zones of growth inhibition, suggesting that the ointment formulation did not antagonize the antimicrobial properties of either agent. More specifically, 2% mupirocin generated a zone of inhibition of 20 (±2) cm^2, whereas 1% neomycin exhibited an average zone of clearance of 9.4 (±1.1) cm^2. The combination of 2% mupirocin and 1% neomycin displayed the greatest zone of inhibition (24.3 ± 1 cm^2), which was statistically improved over the zone of inhibition caused by mupirocin or neomycin alone. We considered that the improved activity of the combination could be attributed to either the additive effects of the specific antibiotic combination or merely reflect an overall increase in active antimicrobial ingredients. However, similar improvements in antimicrobial clearance were not observed in tests of 2% mupirocin in combination with 1% concentration of kanamycin, vancomycin, erythromycin, or oxacillin. Representative results for vancomycin and erythromycin, which exhibited an antagonistic
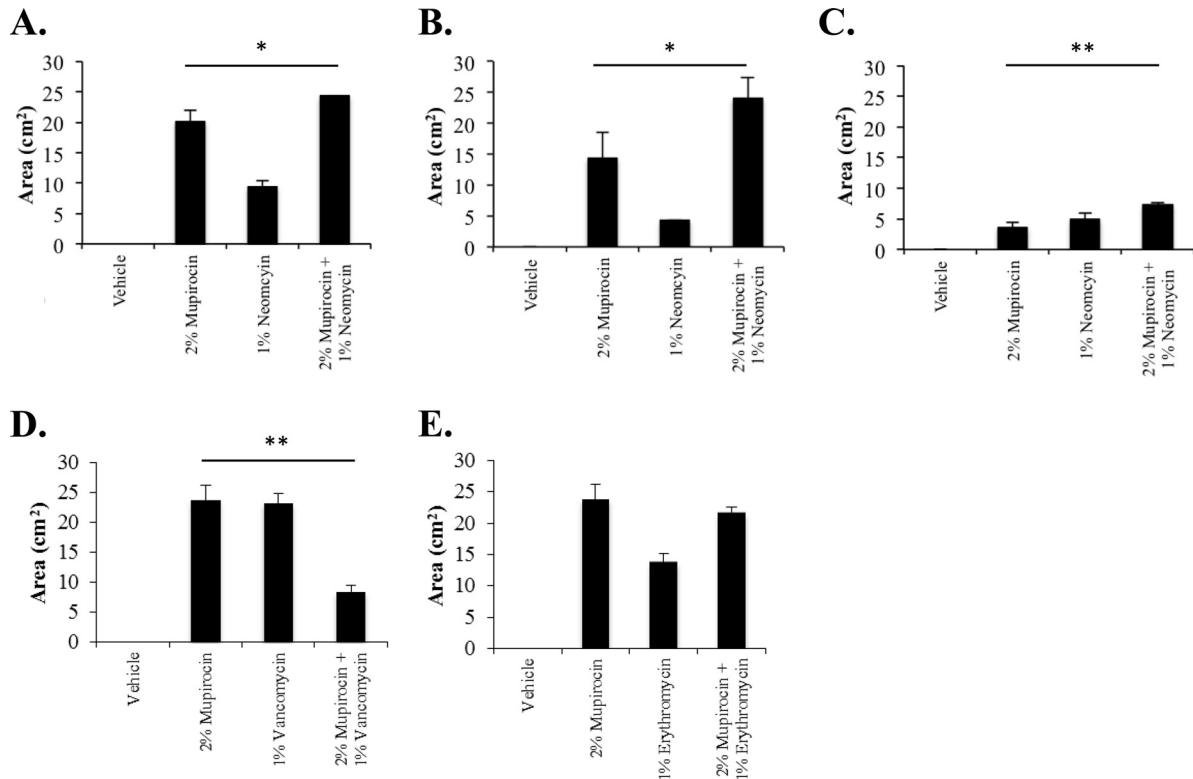

FIG 2 Antimicrobial zone of growth inhibition measures. (A to E) The average zones of inhibition (*y* axis; in square centimeters) of PEG-based ointments containing the indicated antibiotic or antibiotic mixture (*x* axis) toward *S. aureus* strain UAMS-1 (A, D, and E), USA300 (B) or BAA-1708 (C) are plotted. Significant increases in growth inhibition zones by Student's *t* test (*n* = 4), compared to the growth inhibition zones observed with 2% mupirocin, are indicated by bars and asterisks as follows: *, *P* ≤ 0.1; **, *P* ≤ 0.05.
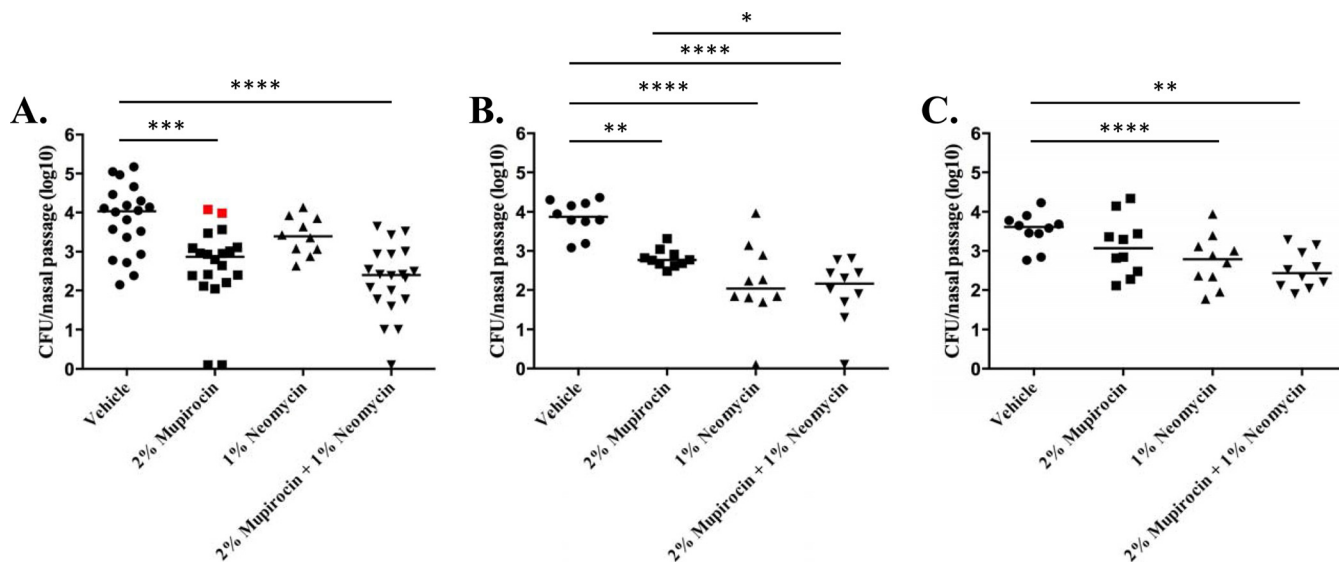
FIG 3 Murine nasal decolonization measures. (A to C) The numbers of CFU per mouse nasal passage (*y* axis) after 3 days of dosing with PEG-based ointment containing the indicated antibiotic or antibiotic mixture (*x* axis) are plotted. Results for *S. aureus* strain UAMS-1 (A), USA300 (B), and BAA-1708 (C) are shown; red data points indicate low-level mupirocin-resistant isolates. Each symbol represents the value for an individual mouse. Each horizontal bar is the mean for the group of mice. Significant reductions in bacterial burden compared to the bacterial burden for mice treated with vehicle by one-way ANOVA are indicated by bars and asterisks as follows: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

interaction and no improvement in combination, respectively, toward the strain are shown in Fig. 2D and E. These results indicate that the additive effects of the mupirocin-plus-neomycin combination observed in liquid culture conditions also occur in ointment format.

As shown in Fig. 2B, tests of the neomycin-resistant strain USA300 revealed that mupirocin elicited a zone of growth inhibition of 14.0 ($\pm$ 4) cm^2. Interestingly, 1% neomycin ointment produced a small halo-like zone of inhibition (4.3 [$\pm$ 0.01] cm^2) despite the strain's resistance to the agent, indicating that the concentration tested is able to overcome the organism's resistance phenotype to a certain extent. Moreover, the combination treatment showed a significantly increased inhibition zone (24.0 [$\pm$ 3.4] cm^2) compared to the inhibition zone for either agent alone. Testing of the high-level mupirocin-resistant strain BAA-1708 (Fig. 2C) demonstrated that the strain was resistant to 2% mupirocin ointment compared to both UAMS-1 and USA300 but did generate a small zone of growth inhibition (3.6 [$\pm$ 0.86] cm^2). Conversely, 1% neomycin ointment elicited a clear zone of inhibition (4.9 [$\pm$ 1.1] cm^2), which was significantly increased by combination treatment (7.3 [$\pm$ 0.4] cm^2).

Taken together, these results indicate that mupirocin and neomycin are compatible in the ointment format tested here. Further, the combination of 2% mupirocin plus 1% neomycin exhibited increased antimicrobial activity in comparison to either agent alone and displayed activity against all strains irrespective of their resistance profile. From these perspectives, we hypothesized that the combination would be similarly therapeutically beneficial in host environments that mupirocin alone is typically used for the prevention and/or therapeutic intervention of staphylococcal infections.

Effects of mupirocin and neomycin on *S. aureus* nasal decolonization. A murine model of *S. aureus* nasal colonization was used to compare the antimicrobial efficacy of mupirocin, neomy-cin, and the two agents when applied in combination. To do so, the nasal passages of BALB/c mice were inoculated with ~1×10^7 CFU of *S. aureus* and then treated three times a day for a total of 3 days, at which point the bacterial burden was measured and the antibiotic susceptibility of 10 isolates from each animal was measured by MIC testing.

Consistent with previous reports, 2% mupirocin treatment resulted in a 1.1-log-unit reduction in *S. aureus* strain UAMS-1 nasal colonization (Fig. 3A) (53). However, two mice displayed uncharacteristically high burdens; upon testing, these isolates were found to exhibit an 4-fold increase in mupirocin resistance (MIC of 0.5 μg ml^{-1}) compared to the inoculating strain as well as isolates from the other animals within the treatment group (MIC of 0.125 μg ml^{-1}), suggesting that mupirocin alone selected for low-level resistant derivatives. One percent neomycin treatment displayed a slight, although not statistically significant, 0.5-log-unit reduction in bacterial burden compared to the bacterial burden in animals treated with vehicle alone, whereas combination treatment with 2% mupirocin plus 1% neomycin resulted in the greatest reduction in *S. aureus* colonization (1.7 log units) and did not appear to select for low-level mupirocin resistance. Similar results were observed for USA300 nasal decolonization (Fig. 3B). More specifically, 2% mupirocin treatment resulted in a 1-log-unit decrease in bacterial burden, whereas treatment with 1% neomycin alone resulted in nearly a 1.8-log-unit reduction in USA300 burden. The combination of mupirocin and neomycin appeared to consistently reduce bacterial burden to the greatest extent (1.7-log-unit reduction). Likewise, combination treatment exhibited increased efficacy toward *S. aureus* strain BAA-1708, in comparison to each agent alone (Fig. 3C). Despite displaying a high-level mupirocin-resistant phenotype, the strain exhibited a moderate reduction in burden (0.54-log-unit reduction) following mupirocin (alone) treatment, a 0.9-log-unit reduction in 1% neomycin-treated animals and a 1.2-log-unit reduction following combination treat-
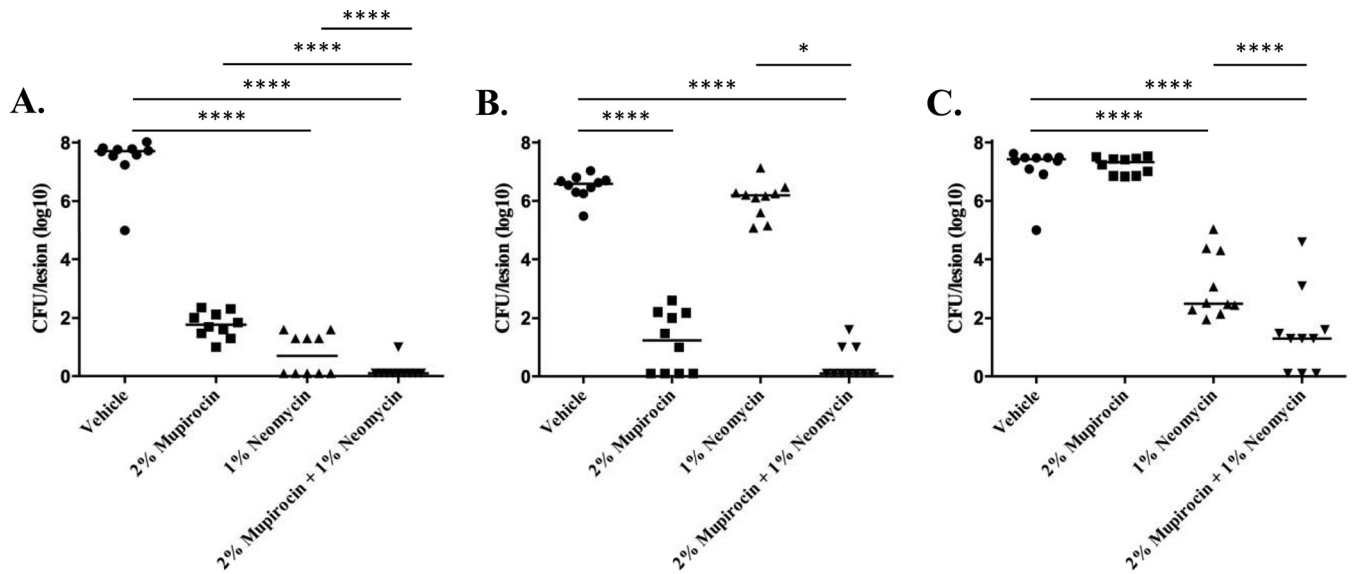
FIG 4 Murine wound decolonization measures. Shown are the numbers of CFU per lesion (*y* axis) following 3 days of dosing with PEG-based ointment containing the indicated antibiotic or antibiotic mixture (*x* axis). Results for *S. aureus* strains UAMS-1 (A), USA300 (B), and BAA-1708 (C) are shown. Significant reductions in bacterial burden between treatment groups by one-way ANOVA are indicated by bars and asterisks as follows: *, $P \leq 0.05$; ****, $P \leq 0.0001$.

ment. The observed improved activity of the combination toward each strain, combined with the notoriously low resolution of the nasal models available (45, 53, 54), prompted us to evaluate the combination's ability to reduce *S. aureus* wound site colonization.

Effects of mupirocin and neomycin on *S. aureus* wound clearance. A murine dermal wound model was used to evaluate the decolonization properties of 2% mupirocin, 1% neomycin, and 2% mupirocin plus 1% neomycin. To do so, dermal wounds were created on the backs of BALB/c mice, inoculated with either *S. aureus* strain UAMS-1, USA300, or BAA-1708, and then treated with test agent suspended in PEG-based ointment twice a day for a total of 3 days, at which point the bacterial burden was determined.

As shown in Fig. 4A, 3-day treatment with 2% mupirocin resulted in an approximately 6-log-unit reduction in *S. aureus* UAMS-1 colonization (8.7×10^1 CFU per lesion) of the wound site compared to animals that were treated with vehicle alone (4.8×10^7 CFU per lesion). One percent neomycin treatment exhibited improved clearance compared to mupirocin (alone), resulting in 1.4×10^1 CFU per lesion with no bacteria recovered from 5 of the 10 (50%) of the animals within the treatment group. Combination treatment displayed the greatest efficacy. No bacteria were recovered from 9 of the 10 animals (90%) treated with 2% mupirocin plus 1% neomycin, whereas a single UAMS-1 colony was recovered from the remaining animal.

Testing of the neomycin-resistant strain, USA300, showed that 2% mupirocin was effective, resulting in a 5-log-unit reduction in bacterial wound site burden, with no bacteria recovered from 4 of the 10 (40%) animals in the treatment group (Fig. 4B). As expected, neomycin treatment alone had minimal effects on decolonization, presumably due to the strain's neomycin resistance phenotype, while the greatest efficacy was observed for the group treated with the combination, in which no USA300 cells were recovered from 7 of 10 (70%) of the animals tested. Similarly, the combination of mupirocin and neomycin displayed the greatest

efficacy in tests of the mupirocin-resistant strain BAA-1708 (Fig. 4C). More specifically, as expected, 2% mupirocin treatment alone did not reduce wound site colonization compared to vehicle-treated cells, whereas neomycin treatment alone resulted in a 4.9-log-unit decrease in recoverable bacteria. The combination of mupirocin plus neomycin produced the greatest reduction in colonization, resulting in a 6.1-log-unit decrease in wound site bacteria and no recoverable bacteria in 3 of the 10 (30%) animals tested. These results indicate that mupirocin-plus-neomycin ointments are more effective in reducing wound site *S. aureus* burden than either agent alone and that the combination is capable of overcoming resistance to either agent.

Antimicrobial potential of mupirocin-and-neomycin combination ointment toward other bacterial species. Mupirocin and neomycin are predominantly active toward Gram-positive and Gram-negative species, respectively. Consequently, we predicted that the combination would display increased spectrum of activity, compared to either agent alone, and could improve treatment options for polyclonal wound site infections composed of mixtures of both Gram-positive and -negative organisms.

As a preliminary test of that hypothesis, zone of inhibition assays were performed for 2% mupirocin, 1% neomycin, and 2% mupirocin plus 1% neomycin using *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, two Gram-negative organisms that are frequent causes of wound site infections. As shown in Fig. 5, 2% mupirocin ointment did not appear to restrict growth of *A. baumannii* strain 98-37-09 or *P. aeruginosa* strain PAO1. Conversely, neomycin, both alone and in combination with mupirocin, restricted growth of both organisms, indicating that the combination of 2% mupirocin plus 1% neomycin may be useful in the prevention and/or treatment of complicated wound infections. Both agents, independently and in combination, also limited growth of *Staphylococcus epidermidis*, *Escherichia coli*, and *Streptococcus pyogenes* strains tested (data not shown).
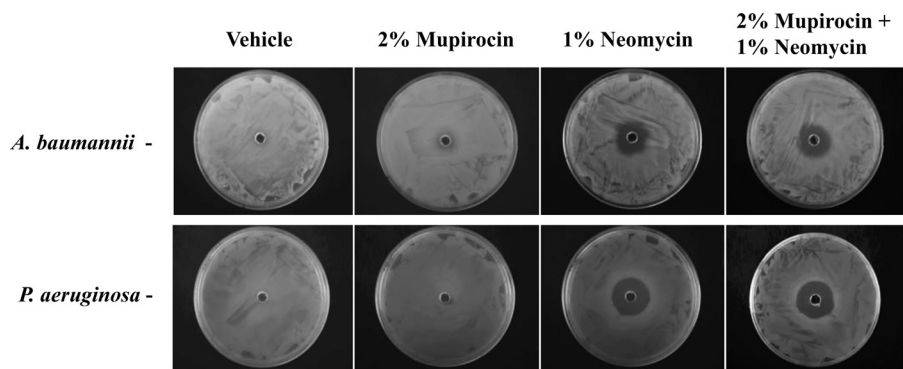
FIG 5 Antimicrobial effects of PEG-based ointments toward *A. baumannii* and *P. aeruginosa*. The antimicrobial effects of PEG ointments containing vehicle, 2% mupirocin, 1% neomycin, and the combination of 2% mupirocin plus 1% neomycin toward *A. baumannii* strain 98-37-09 or *P. aeruginosa* strain PAO1 are shown.

Effects of mupirocin and neomycin on wound healing. The above results indicate that combination ointments comprised of mupirocin and neomycin display improved antimicrobial efficacy, overcome mupirocin resistance, and are likely to exhibit increased spectrum of activity toward other bacterial species compared to mupirocin (alone). Such a combination therapeutic would most likely be of value in the context of the wound setting. In that regard, although both mupirocin and neomycin are FDA-approved antibiotics for topical use, we evaluated whether the mixture of both agents exhibited overt detrimental side effects at the wound site. To do so, dermal wounds were created, and animals were treated with either vehicle, 2% mupirocin, 1% neomycin, or the combination twice daily for a total of 14 days. Each day, animals were assessed for alertness and grooming, weight, and wound size.

No significant differences in wound contraction were observed for any of the treatment groups ($n = 3$ for each treatment) compared to vehicle-containing ointment (Fig. 6A and B). Regardless of the ointment used, the size of the wound increased 3 days after lesion formation and was followed by a linear increase in wound contraction, such that the wound healing was completed and hair growth had been restored at 14 days of treatment. Likewise, no significant differences in weight were recorded for any animals in any of the treatment groups (Fig. 6C).

DISCUSSION

More than 30 million patients undergo surgery in the United States annually, and up to 20% of those patients acquire a postoperative nosocomial infection, resulting in increased rates of morbidity and mortality, systemic antibiotic use, and health care costs of $5 to $10 billion (55, 56). Mupirocin-based ointments (2% mupirocin) have proven successful in the prevention and/or treatment of staphylococcal disease. Indeed, in the United Kingdom, it is recommended that methicillin-resistant *S. aureus* (MRSA) carriers undergo nasal decolonization with mupirocin as a prophylactic measure prior to surgical intervention (57). However, mupirocin use has predictably selected for resistance that has, in turn, mitigated the agent's efficacy.

The incidence of *S. aureus* low- and high-level mupirocin resistance within individual health care institutions is highly variable and is presumably influenced by differences in corresponding infection control practices and between the strains circulating at local and regional levels. One retrospective survey of methicillin-resistant *S. aureus* nasal and blood isolates collected from 23 U.S. hospitals revealed that 3% and 5% of the strains tested displayed high-level mupirocin resistance, respectively (58). However, single-center studies have recorded higher prevalences both in the United States and abroad. For instance, one New York hospital recently reported that 31% of pediatric isolates tested exhibited high-level resistance (59), and in one extreme case, 47% and 79% of community- and hospital-associated MRSA isolates collected from a Korean neonatal intensive care unit exhibited high-level mupirocin resistance (60). Single-center low-level mupirocin resistance rates of 0 to 80% have been recorded in the United States (58). From these perspectives, it is not surprising that recent studies have called into question the advantageous effects of mupirocin ointments, highlighting the need for new approaches for *S. aureus* decolonization and wound care management.

Drug combinations are a mainstay therapeutic strategy in the treatment of cancer, HIV, asthma, hypercholesterolemia, malaria, and tuberculosis (61). Several current antibiotics represent combination therapeutics, such as sulfonamides and trimethoprim and β-lactam antibiotics in conjunction with β-lactamase inhibitors (62, 63). A central tenet of the combination approach is that the sum of the ingredients is greater than the individual components themselves, and a highly successful strategy for development of multicomponent drugs has been to combine single-compound drugs that already exist; early examples include Advair (fluticasone plus salmeterol), Advicor (niacin plus lovastatin), Combivir (azidothymidine plus lamivudine), and Trizivir (azidothymidine plus lamivudine plus abacavir) (64–66). In that regard, we set out to improve the performance of mupirocin ointment via the addition of an FDA-approved agent with the goal of creating an improved antimicrobial ointment with increased antimicrobial efficacy and capable of overcoming high-level mupirocin resistance.

Numerous studies have made it apparent that the simple addition of two agents does not reliably correlate with improved combined activity. Indeed, that has also been our experience. Screening of an 853-member FDA-approved drug library identified 101 agents that displayed antimicrobial activity against the antibiotic-susceptible test strain UAMS-1. However, only three of those agents, nitazoxanide, nitrofurazone, and the antibiotic neomycin sulfate, were found to exhibit increased antistaphylococcal activity when combined with mupirocin. Of these, neomycin displayed
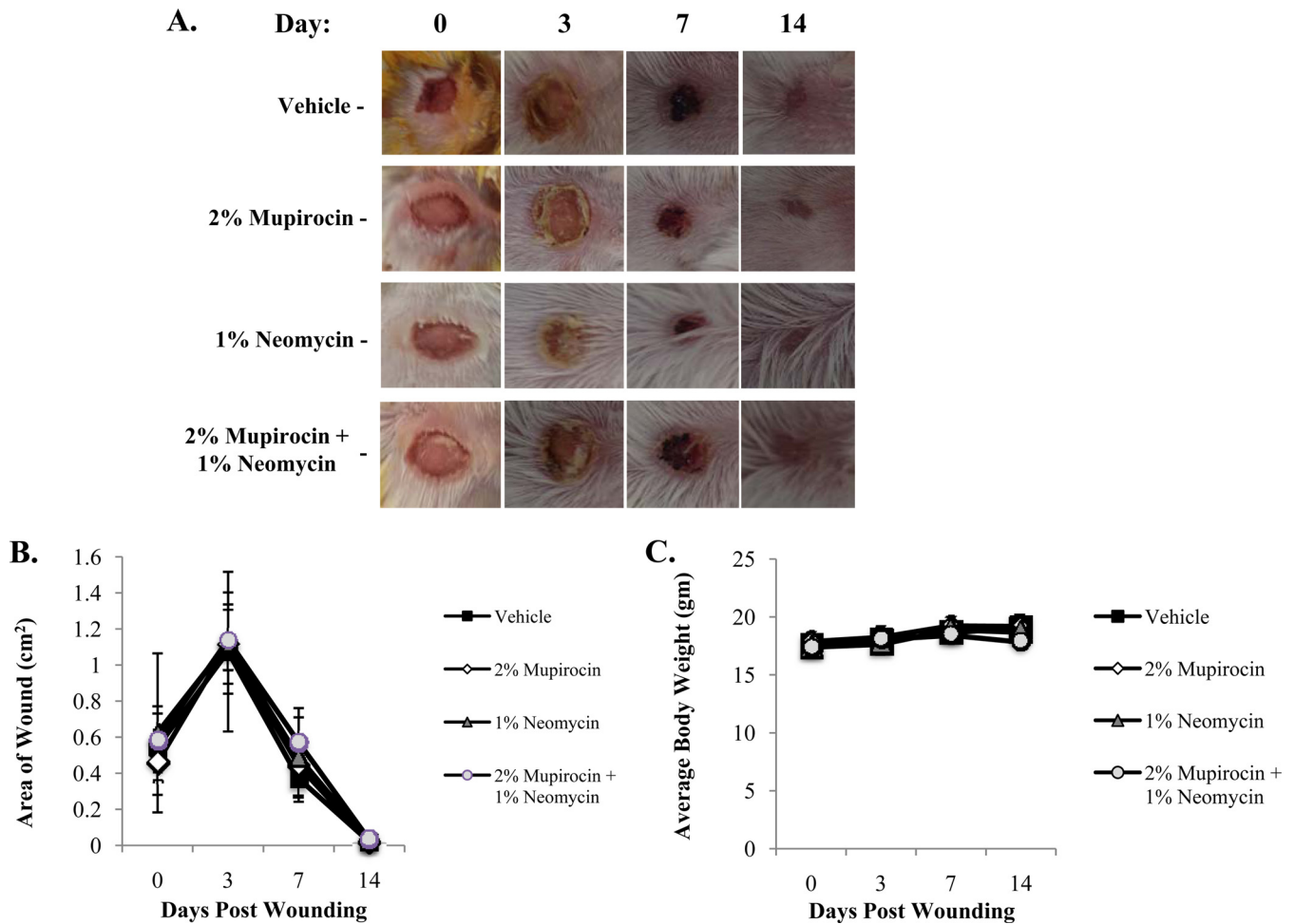
FIG 6 Effects of PEG-based ointments on wound healing and animal health. (A) Representative wound healing images following 0, 3, 7, and 14 days of treatment with PEG-based ointments containing vehicle, 2% mupirocin, 1% neomycin, or the combination of 2% mupirocin plus 1% neomycin. (B) Average measures (*n* = 3) of wound contraction under the same conditions as in panel A. (C) Average body weight of animals (*y* axis) at the indicated day (*x* axis) after lesion formation and treatment with PEG-based ointment supplemented with the indicated agent.

the greatest potency, both alone and in the presence of mupirocin, and is currently available as 0.25% to 4% (wt/vol) ointment for topical antimicrobial use. Thus, we chose to focus effort on characterizing the effects of combinations of mupirocin and neomycin, with the anticipation that they may have the greatest likelihood of having a clinical impact and ease of advancement.

While neomycin is known to bind 16S rRNA and inhibit bacterial protein translation, more-recent studies indicate that it also has off-target effects that may contribute to its antimicrobial activity. In that regard, while other translational inhibitors, including several aminoglycosides, exhibited antimicrobial activity toward the test strain, they did not potentiate the activity of mupirocin. Thus, it seemed reasonable to predict that neomycin's off-target effects contribute to its potentiation of mupirocin. Neomycin binding to the *rnpB* component of the RNase P holoenzyme interferes with the enzyme's ability to catabolize precursor tRNA processing and consequently generation of mature tRNA substrates for tRNA synthetases, including the primary cellular target of mupirocin, isoleucyl-tRNA synthetase. Consequently, neomycin may limit *S. aureus* cellular RNase P activity resulting in a limited supply of mature tRNA^{Ile} species, thereby requiring less

mupirocin to generate an antimicrobial phenotype. As a first test of that prediction, it was found that *S. aureus* RNase P activity is inhibited by neomycin (250 μM) during *in vitro* conditions that admittedly may be vastly different than are expected of the enzyme within bacterial cells (buffer conditions and cofactors). Even so, neomycin's RNase P inhibitory activity approximates the concentration required to potentiate mupirocin in liquid format (50 μM) and is well below its potentiating activity in topical format (16 mM), suggesting that the agent's ability to improve mupirocin's antimicrobial effects may be, in part, mediated by the cellular inhibition of RNase P. Further, neomycin did not increase the antimicrobial properties of other antibiotics tested in combination, supporting the notion that the agent's off-target effects may account for its ability to potentiate the antimicrobial activity of mupirocin and that these results are specific to mupirocin.

Combinations of 2% mupirocin and $\geq$1% neomycin proved to display improved antimicrobial activity in zone of inhibition assays designed to measure the combination's performance in topical format, compared to either agent when tested alone. For that reason, all studies were conducted with 2% mupirocin and/or 1% neomycin. As noted earlier, combi-

nations of 2% mupirocin and 1% of other antibiotics evaluated did not exhibit improved antimicrobial effects or cause an antagonistic effect, suggesting that the improved performance of the combination was specific to neomycin and mimicked their performance in liquid format.

Using a murine nasal colonization model that has admittedly proven highly variable in terms of establishing *S. aureus* colonization and measuring the performance of antimicrobial agents, such as mupirocin, in the past, we found that the combination of mupirocin plus neomycin displayed greater efficacy than either agent alone. In initial studies designed to measure the model's performance using *S. aureus* strain UAMS-1, it was found that optimal colonization was achieved using 1×10^7 CFU and when animals were allowed to breathe in and out the inoculum, whereas colonization occurred in ∼70% of the animals challenged with fewer cells and/or animals that were anesthetized at the time of inoculation. Moreover, testing of various dosing regimens showed that optimal mupirocin decolonization was observed following three nasal treatments per day (data not shown), which consequently served as the standard dosing for nasal dosing studies. Complete antimicrobial-associated decolonization was rarely observed and may reflect poor distribution of the test agents throughout the nasal passage. In the model, mupirocin treatment displayed efficacy to various degrees for the three strains evaluated, with greatest decolonization observed for strains UAMS-1 and USA300 and less activity measured for the high-level mupirocin-resistant strain BAA-1708. Presumably, the dosing regimen used may partially override the resistance phenotype of the strain and/or the mupirocin resistance determinant may be only partially expressed during nasal colonization. Similar effects were observed for neomycin (alone) treatment for all strains, including neomycin-resistant USA300. In all cases, the combination of mupirocin and neomycin resulted in the greatest extent of nasal decolonization, and this occurred regardless of the strain used, suggesting that the combination may have greater promise in decolonizing at-risk patient populations than mupirocin (alone) ointments.

Similarly, the combination exhibited pronounced improvement in a murine wound model of *S. aureus* decolonization compared to either mupirocin or neomycin alone. In this model, twice-a-day mupirocin dosing consistently exhibited efficacy toward the mupirocin-susceptible strains evaluated, thus, each topical formulation was tested twice daily (as opposed to three times daily for nasal decolonization studies). Indeed, while twice-a-day 2% mupirocin treatment dramatically reduced *S. aureus* strain UAMS-1 and USA300 wound site colonization, the agent lacked efficacy toward the high-level mupirocin-resistant strain tested, mimicking what occurs in the clinical setting. One percent neomycin alone exhibited excellent decolonization activity toward UAMS-1 and BAA 1708 but no significant activity toward the neomycin-resistant strain USA300. The combination nearly eradicated each *S. aureus* strain tested, with either no measurable viable CFU or a single colony detected in 100% of UAMS-1-inoculated wounds, 90% of USA300-inoculated wounds, and 60% of BAA-1708-inoculated wounds.

Interestingly, as noted above, we observed differing antimicrobial effects of neomycin alone and mupirocin alone toward strains USA300 and BAA-1708, respectively, in the two animal model systems. The application of neomycin three times a day exhibited mild antimicrobial activity toward the neomycin-resistant strain USA300 in the nasal decolonization model but no activity toward the strain in the wound model when applied twice daily. Similarly, mupirocin dosing three times a day was associated with reduction in BAA-1708 nasal colonization but had no effect on wound decolonization. While there are likely to be vast differences between the bacterial physiology and host-pathogen dynamics in these two settings that may account for the observed differences in antibiotic susceptibility, these results could also suggest that more-frequent antibiotic application may allow drug accumulation to an extent that overrides each strain's resistance phenotype and may have corresponding clinical implications. Likewise, it is possible that extended time course or more-frequent dosing may further improve the combination's effects. Wound contraction and overt cytotoxic measures indicate that each agent, when used alone or in combination, is well tolerated over the course of 14 days when applied either twice or three times (not shown) a day to wound sites.

Taken together, the results presented indicate that the topical combinations of mupirocin and neomycin are likely to be superior to currently available mupirocin ointments in terms of promoting *S. aureus* nasal and wound site decolonization and may be particularly valuable in areas where high-level mupirocin resistance has emerged. Such combination therapies may offer a much needed option for improving *S. aureus* infection prevention, limiting disease progression and, consequently, systemic antibiotic usage. Further, by virtue of the increased spectrum of activity toward problematic Gram-negative organisms, such as *A. baumannii* and *P. aeruginosa*, neomycin-and-mupirocin combinations may provide the option to develop similar strategies for reducing the incidence of these organisms as well as additional options for treatment of polymicrobial infections.

In considering the development of any clinical candidate, including a combination ointment, one must also take into account that resistant isolates can and will emerge (if they do not already exist). In that regard, neither mupirocin or neomycin sulfate is routinely used for systemic treatment purposes, thus corresponding resistance surveillance data are sparse. However, a comprehensive assessment of gentamicin-resistant isolates collected between 1997 and 2002 in the United States revealed that all high-level mupirocin-resistant isolates collected were susceptible to neomycin, indicating that they would be responsive to mupirocin-and-neomycin combination therapeutics (67). The study also indicated that while neomycin resistance was observed frequently (31%) within *S. aureus* isolates collected, less than 1% of those strains were capable of tolerating 1:100th the level of neomycin present in topical formulations and would thus ostensibly be treatable by mupirocin-plus-neomycin ointments. Moreover, as noted above, neither agent is routinely used for systemic purposes or is associated with cross-resistance to currently used systemic antibiotics. From these perspectives, it is anticipated that combination neomycin-and-mupirocin ointments may hold great promise in the prevention and treatment of currently circulating *S. aureus* strains and that resistance to the multicomponent mixture will be slow to develop and unlikely to compromise the current antistaphylococcal armament. We also recognize that there will be limitations in the use of such a combination ointment. Indeed, one widely referenced study reported that neomycin-related contact allergy developed in 34% of patients with chronic dermatoses, who were patch tested with 20% neomycin (68). Thus, others have noted that the use of neomycin-

containing topical preparations should be avoided or closely monitored for multiallergic individuals but advocated the use of neomycin in the majority of the general population, in whom the incidence of neomycin sensitivity is estimated to be 0.9% (69, 70).

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