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

Catlyn Blanchard[*], Lauren Brooks[*], Andrew Beckley, Jennifer Colquhoun, Stephen Dewhurst, and Paul M. Dunman[**]

Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642

*Authors contributed equally to this work

[**]Corresponding author

Paul M. Dunman

Department of Microbiology and Immunology

601 Elmwood Ave; Box 672

Rochester, NY 14642

P- 585.276.5700

F- 585.473.9573

E- paul_dunman@urmc.rochester.edu

Keywords: *Staphylococcus aureus*, mupirocin, neomycin sulfate, decolonization, wound

Running title: Prevention and treatment of *S. aureus* infections

ABSTRACT (word count 234)

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 an F.D.A. 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 the inhibition 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.

INTRODUCTION

Staphylococcus aureus has been designated as one of the six ESKAPE bacterial pathogens of greatest U.S. healthcare concern (1). The organism is a predominant cause of nosocomial- and community- associated bacterial infections and has developed resistance to all currently available antibiotics (2). *S. aureus* annual U.S. mortality rates have already surpassed that of HIV/AIDS and are 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 principle ecological niche for *S. aureus* and nasal carriage is a recognized risk factor for staphylococcal disease, particularly among patient populations undergoing surgical procedures, hemodialysis, or requiring long term intensive care unit stays [reviewed in (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). The agent displays excellent antibacterial activity toward most Gram-positive species, lacks cross resistance to current antibiotics, but 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 pre-surgical nasal decolonization [Reviewed in (14)]. Indeed, mupirocin mediated nasal decolonization has been shown to be effective in reducing infections in burn wound, dialysis, and surgical patient

79 populations, as well as *S. aureus* transmission among healthcare workers and intensive care
80 unit patients (15-21). In addition to nasal decolonization, topical mupirocin has been used to
81 successfully treat hemodialysis central venous catheter exit sites, impetigo, eczema, surgical
82 wound sites, skin and soft tissue wounds, the breasts of breast-feeding mothers, and tympanic
83 membrane lesions (22-27). However, the emergence of *S. aureus* mupirocin resistance has
84 reduced the agent's efficacy both as a nasal decolonization agent and as a treatment option for
85 skin and wound infections.
86
87 Low level mupirocin resistant (LL-MR) *S. aureus* strains are defined as exhibiting an MIC of 8 to
88 256 μg ml^{-1} due to point mutations in the organism's native isoleucyl tRNA synthetase gene
89 (*ileRS*) and develop rapidly in both the laboratory and clinical settings (28). High level mupirocin
90 resistance (HL-MR; MIC of > 256 μg ml^{-1}) occurs less frequently and is attributable to the
91 acquisition of a mobile genetic elements harboring either *mupA*, which codes for an alternate
92 isoleucyl tRNA synthetase, or the less-characterized *mupB* gene (29, 30). Both LL-MR and HL-
93 MR lead to mupirocin treatment failure (31). Indeed, while low level resistant strains initially
94 respond to therapy they frequently re-emerge quickly; relapse is hypothesized to be due to
95 latent LL-MR subpopulations that are not eradicated by mupirocin dosing (31, 32). Conversely,
96 HL-MR are recalcitrant to mupirocin ointments (31). Thus, the emergence of mupriocin
97 resistance has prompted renewed interest in developing alternative decolonization and wound
98 infection treatment strategies.
99
100 *S. aureus* RNase P is an essential riboprotein complex consisting of RnpA and ribozyme *rnpB*
101 that acts upstream of tRNA synthetases in the transfer RNA maturation pathway (33, 34). More
102 specifically RNase P is hypothesized to catalyze removal of the 5' leader sequences from
103 precursor tRNA species thereby creating mature tRNA substrates for tRNA synthetases,
104 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 (F.D.A.) 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, confers an additive antimicrobial advantage to mupirocin and 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, in comparison to either agent when tested 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 otherwise indicated, strains were grown overnight in tryptic soy broth (TSB) then used to inoculate a fresh (1:100 dilution) media, grown to early exponential phase ($1x10^8$ 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. Polyethylene glycol (PEG) ointment-base was prepared by mixing PEG 400 (70% w/v) with PEG 3350 (30% w/v) as described by the United States 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. Mixtures were then added directly to 5 g of PEG ointment pre-liquefied by heating at 60°C for 30 min to create 2% mupirocin, 1% neomycin suspensions 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 DMSO.

Screen of Selleck Library. Members of the Selleck Library of Food and Drug Association approved drugs (Selleck Chemicals, Houston TX, L1300) were screened for agents that potentiate the antimicrobial activity of mupirocin toward *S. aureus* strain UAMS-1. To do so, $1x10^5$ colony forming units of UAMS-1 were added to individual wells of a 96-well microtiter plate, mixed with 0.03 μg ml^{-1} mupirocin (0.5x minimum inhibitory concentration) and 50 μM of test agent in Mueller Hinton broth (MHB; 100 μL total well volume). Microtiter plates were incubated at 37˚C for 16 hr, 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 then added (2.5 pmol) to 5 pmol of $ptRNA^{Tyr}$, 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 2x 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 7M 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 $tRNA^{Tyr}$ signal/mock $tRNA^{Tyr}$ signal.

Antimicrobial Susceptibility Testing. Minimum inhibitory concentration (MIC) was tested in accordance with the Clinical and Laboratory Standards Institute guidelines. Briefly, $1x10^5$ CFU of the indicated *S. aureus* strain was added to individual wells of a microtiter plate containing 88 µL of MHB media and two-fold increasing concentrations of mupirocin or test agent (0 – 128 µg ml^{-1}). Plates were incubated for 16 hr at 37˚C and wells were visually inspected for growth. The lowest concentration of mupirocin or test agent that inhibited *S. aureus* growth was considered to be the minimum inhibitory concentration. 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 x 10^5 CFU of *S. aureus* strain UAMS-1 was added and the plate was incubated at 37˚C for 16 hr. 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 ≤ 0.5, additive as FIC value 0.5 – 1.0, no interaction as an FIC of 1-4, or an antagonistic interaction FIC > 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 $1x10^8$ CFU ml^{-1} of *S. aureus* was spread on TSA plates. Plates were dried for 10 min and 40 µL of ointment was pipetted onto the center of the plate. Plates were incubated at 37˚C for 16 hr 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 a *S. aureus* nasal colonization model as previously described (45), but with modifications. The nostrils of awake mice were inoculated with 1 x 10^7 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. Mice nostrils 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 post inoculation and treatments were repeated every 8 hr for three 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 PBS. Samples were homogenized for five minutes, serially diluted, and plated on Mannitol Salt agar (MSA, ThermoScientific, Waltham MA). Plates were incubated for 16 hr and the number of *S. aureus* were enumerated.

Dermal Wound Model of Infection and Treatment of Mice. The effects of ointment compilations were evaluated for *in vivo* antimicrobial activity using a *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 1 g body weight. Pain relief in the form of 20 µl 0.5% Sensorcaine (APP Pharmaceuticals, Schaumburg, IL) was administered prior to dermal wounding. The dorsal mid-section 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 minutes. 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 $1x10^7$ 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 post inoculation; treatments were repeated every 12 hr for three days. Mice were then euthanized via CO_2 asphyxiation and cervical dislocation, as per UCAR approved methodology, the wound and underlying muscle was excised with an 8 mm biopsy punch and placed in microcentrifuge tubes containing 1 mL of freshly made PBS. Samples were homogenized for five minutes, serially diluted, and plated on MSA. Plates were incubated for 16 hr 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 vehicle, 2% mupirocin, 1% neomycin, or 2% mupirocin plus 1% neomycin combination ointments twice daily for 14 days. Mice were weighed, assessed for grooming and alertness, and images of the wound were obtained daily to measure wound contraction using Image J (NIH). Wound contraction was calculated as percentage of wound area reduction using the formula: WC*d*= (1-WA*d*/WA0)x100, where WC is wound contraction, WA is wound area, *d* is day, and 0 indicates initial day, as previously described (47).

Statistical Analyses. 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 ANOVA analysis to determine the statistical power.

RESULTS

Agents that potentiate the antimicrobial activity of mupirocin. Members of the Selleck library of 853 F.D.A. approved drugs were screened for agents that potentiate the activity of mupirocin. To do so, the antibiotic susceptible *S. aureus* strain UAMS-1 was inoculated into individual wells of a microtiter plate containing 0.5X the strain's mupirocin minimum inhibitory concentration (MIC; 0.0625 μg ml^{-1}) and 50 μM of library material. A total of 101 library members (11.8%), including 61 antibiotics, inhibited bacterial growth suggesting that they may represent agents that: 1. potentiate the antimicrobial activity of mupirocin, 2. exhibit mupirocin-independent antimicrobial activity, or 3. both (**Supplemental Table 1**).

To distinguish between these possibilities, the MIC of each compound was determined in medium lacking or containing 0.5X the strain's mupirocin MIC. Ninety-eight of the 101 compounds (97%) evaluated displayed similar antimicrobial activities regardless of whether mupriocin 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 (FIC's = 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 0.5X 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 0.5X 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 rifampicin, vancomycin, sulfamethoxazole, meropenem, minocycline, ciprofloxacin, ceftriaxone, or erythromycin (data not shown), the additive effects between neomycin and mupirocin appeared to be combination specific.

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*, *Porphyromas 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 *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 combination (2% mupirocin + 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 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 that of 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% of kanamycin, vancomycin, erythromycin, or oxacillin. Representative results for vancomycin and erythromycin, which exhibited antagonistic and no improvement in combination, respectively, toward the strain are shown in **Figs. 2D** and **2E**. These results indicate that the additive effects of the mupirocin + 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 14.0 (± 4) cm^2 zone of growth inhibition. Interestingly, 1% neomycin ointment produced a small (4.3 (± 0.01) cm^2) halo-like zone of inhibition 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 (± 3.4) cm^2) in comparison to 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 in comparison to both UAMS-1 and USA300 but did generate a small zone of growth inhibition (3.6 (± 0.86) cm^2). Conversely, 1% neomycin ointment elicited a clear zone of inhibition (4.9 (± 1.1) cm^2), which was significantly increased by combination treatment (7.3 (± 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 + 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.

The 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, neomycin, and the two agents when applied in combination. To do so, the nasal passages of Balb/C mice were inoculated with ~1 x 10^7 colony forming units of *S. aureus* then treated three times a day for a total of three days, at which point the bacterial burden was measured and the antibiotic susceptibility of ten isolates from each animal was measured by MIC testing.

Consistent with previous reports, 2% mupirocin treatment resulted in a 1.1-log 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 4-fold increase in mupirocin resistance (MIC of 0.5 μg ml^{-1}) in comparison 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) dosing selected for low-level resistant derivatives. One percent neomycin treatment displayed a slight, although not statistically significant, 0.5-log reduction in bacterial burden in comparison to vehicle alone, whereas combination treatment with 2% mupirocin + 1% neomycin resulted in the greatest reduction in *S. aureus* colonization (1.7-log) 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 decrease in bacterial burden, whereas treatment with 1% neomycin (alone) resulted in nearly a 1.8-log reduction in USA300 burden. The combination of mupirocin and neomycin appeared to consistently reduce bacterial burden to the greatest extent (1.7-log 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) following mupirocin (alone) treatment, a 0.9 log reduction in 1% neomycin treated animals and a 1.2-log reduction following combination treatment. 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.

The 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 + 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 bacterial burden was measured.

As shown in **Fig. 4A**, three day treatment with 2% mupirocin resulted in an approximately 6-log reduction in UAMS-1 colonization (8.7 x 10^1 cfu per lesion) of the wound site in comparison to animals that were treated with vehicle alone (4.8 x 10^7 cfu per lesion). One percent neomycin treatment exhibited improved clearance in comparison to mupirocin (alone), resulting in a 1.4 x 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 + 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 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 combination treated group, 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 in comparison to vehicle treated cells, whereas neomycin treatment (alone) resulted in a 4.9-log decrease in recoverable bacteria. The combination of mupirocin + neomycin produced the greatest reduction in colonization, resulting in a 6.1-log decrease in wound site bacteria and no recoverable bacteria in 3 of the 10 (30%) animals tested. These results indicate that mupirocin + 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.

The 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, in comparison 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 + 1% neomycin using *A. baumannii* and *P. 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 PA01. Conversely, neomycin, both alone and in combination with mupirocin, restricted growth of both organisms, indicating that the combination of 2% mupirocin + 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 *S. epidermidis*, *Escherichia coli*, and *Streptococcus pyogenes* strains tested (data not 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, in comparison 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 F.D.A. 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), in comparison to vehicle containing ointment (**Figs. 6A** and **6B**). Regardless of ointment used, wound size increased 3 days post-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 U.S. annually and up to 20 percent of those patients acquire a postoperative nosocomial infection, resulting in increased rates of morbidity and mortality, systemic antibiotic use, and healthcare 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 MRSA carriers should 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 healthcare 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* (MRSA) 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 U.S. 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 0-80% have been recorded in the U.S. (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 + salmeterol), Advicor (niacin + lovastatin), Combivir (azidothymidine + lamivudine) and Trizivir (azidothymidine + lamivudine + abacavir) (64-66). In that regard, we set out to improve the performance of mupirocin ointment via the addition of an F.D.A. 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 F.D.A approved drug library identified 101 agents that displayed antimicrobial activity against the antibiotic susceptible test strain, UAMS-1. Yet only three of those agents, nitazoxanide, nitrofurazone, and the antibiotic neomycin sulfate, were found to exhibit increased anti-staphylococcal activity when combined with mupirocin. Of these, neomycin displayed the greatest potency, both alone and in the presence of mupirocin, and is currently available as 0.25%-4% weight per volume 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 then are expected of the enzyme within bacterial cells (buffer conditions and co-factors). 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 ≥ 1% neomycin proved to display improved antimicrobial activity in zone of inhibition assays designed to measure the combination's performance in topical format, in comparison to either agent when tested alone. For that reason, all studies were conducted with 2% mupirocin and/or 1% neomycin. As noted earlier, combinations of 2%

mupirocin and 1% of other antibiotics evaluated did not exhibit improved antimicrobial effects or caused 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 + 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 x 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 less cells and/or were anesthetized at the time of inoculation. Moreover, testing of various dosing regiments showed that optimal mupirocin decolonization was observed following 3 nasal treatments per day (data not shown), and 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 varying 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 over-ride the resistance phenotype of the strain and/or the mupirocin resistant determinant may only be 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.

563

564 Similarly, the combination exhibited pronounced improvement in a murine wound model of *S.*
565 *aureus* decolonization, in comparison to either mupirocin or neomycin alone. In this model,
566 twice a day mupirocin dosing consistently exhibited efficacy toward the mupirocin susceptible
567 strains evaluated, thus, each topical formulation was tested twice daily (as opposed to 3x daily
568 for nasal decolonization studies). Indeed, while twice a day 2% mupirocin treatment
569 dramatically reduced *S. aureus* strain UAMS-1 and USA300 wound site colonization, the agent
570 lacked efficacy toward the high-level mupirocin resistant strain tested, mimicking what occurs in
571 the clinical setting. One percent neomycin (alone) exhibited excellent decolonization activity
572 toward UAMS-1 and BAA 1708 but no significant activity toward the neomycin resistant strain
573 USA300. The combination nearly eradicated each *S. aureus* strain tested, with either no
574 measurable viable colony forming units or a single colony detected in 100% of UAMS-1, 90% of
575 USA300 and 60% of BAA-1708 inoculated wounds.

576

577 Interestingly, as noted above, we observed differing antimicrobial effects of neomycin (alone)
578 and mupirocin (alone) toward strains USA300 and BAA-1708, respectively, in the two animal
579 model systems. The application of neomycin three times a day exhibited mild antimicrobial
580 activity toward the nenomycin resistant strain, USA300, in the in the nasal decolonization model
581 but no activity toward the strain in the wound model when applied twice daily. Similarly, three
582 times a day mupirocin dosing was associated with reduction in BAA-1708 nasal colonization,
583 but had no effect on wound decolonization. While there are likely to be vast differences
584 between the bacterial physiology and host-pathogen dynamics in these two settings that may
585 account for the observed differences in antibiotic susceptibility these results could also suggest
586 that more frequent antibiotic application may allow drug accumulation to an extent that overrides
587 each strain's resistance phenotype and may have corresponding clinical implications.
588 Likewise, it is possible that extended time-course or more frequent dosing may further improve

Antimicrobial Agents and Chemotherapy

589 the combination's effects. Wound contraction and overt cytotoxic measures indicate that each
590 agent, when used alone or in combination, is well tolerated over the course of 14 days when
591 applied either twice or three times (not shown) a day to wound sites.
592
593 Taken together the results presented indicate that the topical combinations of mupirocin and
594 neomycin are likely to be superior to currently available mupirocin ointments in terms of
595 promoting *S. aureus* nasal and wound site decolonization, and may be particularly valuable in
596 areas where high-level mupirocin resistance has emerged. Such combination therapies may
597 offer a much needed option for improving *S. aureus* infection prevention, limiting disease
598 progression and, consequently, systemic antibiotic usage. Further, by virtue of the increased
599 spectrum of activity toward problematic Gram-negative organisms, such as *A. baumannii* and *P.*
600 *aeruginosa*, neomycin and mupirocin combinations may provide the option to develop similar
601 strategies for reducing the incidence of these organisms as well as additional options for
602 treatment of polymicrobial infections.
603
604 In considering the development of any clinical candidate, including a combination ointment, one
605 must also take into account that resistant isolates can and will emerge (if they don't already
606 exist). In that regard, neither mupirocin or neomycin sulfate are routinely used for systemic
607 treatment purposes, thus corresponding resistance surveillance data is sparse. However, a
608 comprehensive assessment of gentamycin resistant isolates collected between 1997 and 2002
609 in the U.S. revealed that all high-level mupirocin resistant isolates collected were susceptible to
610 neomycin, indicating that they would be responsive to mupirocin and neomycin combination
611 therapeutics (67). The study also indicated that while neomycin resistance was observed
612 frequently (31%) within *S. aureus* isolates collected less than 1% of those strains were capable
613 of tolerating 1:100th the level of neomycin present in topical formulations and would thus
614 ostensibly be treatable by mupirocin + 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 anti-staphylococcal 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 neomycin containing topical preparations use should be avoided or closely monitored for multiallergic individuals but advocated for 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).

ACKNOWLEDGEMENTS

This work was supported, in part, by University of Rochester Technology Development Award and the National Institute of Allergy and Infectious Diseases (NIAID) grant number AI103507 (PMD). SD and LB were supported by the University of Rochester Center for AIDS Research grant P30AI078498 and AI094511 (NIH/NIAID). Additionally, this publication was made possible by grant number GM068411-11 from Institutional Ruth L. Kirschstein National Research Service Award (JC), the Phileoever Foundation (AB) and the Training Program in Oral Sciences T90FR021985 (CB). We would also like to thank Connie Winters and Kun Hyoe Rhoo for their technical support.

REFERENCES

1. **Rice LB.** 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. J Infect Dis **197:**1079-1081.
2. **Pendleton JN, Gorman SP, Gilmore BF.** 2013. Clinical relevance of the ESKAPE pathogens. Expert review of anti-infective therapy **11:**297-308.
3. **Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK.** 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA **298:**1763-1771.
4. **Projan SJ, Shlaes DM.** 2004. Antibacterial drug discovery: is it all downhill from here? Clinical microbiology and infection. The official publication of the European Society of Clinical Microbiology and Infectious Diseases **10 Suppl 4:**18-22.
5. **Kluytmans J, van Belkum A, Verbrugh H.** 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev **10:**505-520.
6. **Hughes J, Mellows G.** 1978. On the mode of action of pseudomonic acid: inhibition of protein synthesis in *Staphylococcus aureus*. The Journal of antibiotics **31:**330-335.
7. **Hughes J, Mellows G.** 1978. Inhibition of isoleucyl-transfer ribonucleic acid synthetase in *Escherichia coli* by pseudomonic acid. The Biochemical journal **176:**305-318.
8. **Hughes J, Mellows G.** 1980. Interaction of pseudomonic acid A with *Escherichia coli* B isoleucyl-tRNA synthetase. The Biochemical journal **191:**209-219.
9. **Sutherland R, Boon RJ, Griffin KE, Masters PJ, Slocombe B, White AR.** 1985. Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. Antimicrob Agents Chemother **27:**495-498.

10. **Beale AS, Gisby J, Sutherland R.** 1989. Efficacy of mupirocin calcium ointment in the treatment of experimental wound infections caused by methicillin-resistant strains of Staphylococcus aureus. Journal of chemotherapy **1:**397-398.

11. **Moy JA, Caldwell-Brown D, Lin AN, Pappa KA, Carter DM.** 1990. Mupirocin-resistant *Staphylococcus aureus* after long-term treatment of patients with epidermolysis bullosa. Journal of the American Academy of Dermatology **22:**893-895.

12. **Rode H, de Wet PM, Millar AJ, Cywes S.** 1988. Bactericidal efficacy of mupirocin in multi-antibiotic resistant *Staphylococcus aureus* burn wound infection. The Journal of antimicrobial chemotherapy **21:**589-595.

13. **Rode H, Hanslo D, de Wet PM, Millar AJ, Cywes S.** 1989. Efficacy of mupirocin in methicillin-resistant *Staphylococcus aureus* burn wound infection. Antimicrob Agents Chemother **33:**1358-1361.

14. **Coates T, Bax R, Coates A.** 2009. Nasal decolonization of *Staphylococcus aureus* with mupirocin: strengths, weaknesses and future prospects. The Journal of antimicrobial chemotherapy **64:**9-15.

15. 1996. Nasal mupirocin prevents *Staphylococcus aureus* exit-site infection during peritoneal dialysis. Mupirocin Study Group. Journal of the American Society of Nephrology **7:**2403-2408.

16. **Gaspar MC, Uribe P, Sanchez P, Coello R, Cruzet F.** 1992. [Hospital personnel who are nasal carriers of methicillin-resistant *Staphylococcus aureus*. Usefulness of treatment with mupirocin]. Enfermedades infecciosas y microbiologia clinica **10:**107-110.

17. **Gernaat-van der Sluis AJ, Hoogenboom-Verdegaal AM, Edixhoven PJ, Spies-van Rooijen NH.** 1998. Prophylactic mupirocin could reduce orthopedic wound infections. 1,044 patients treated with mupirocin compared with 1,260 historical controls. Acta orthopaedica Scandinavica **69:**412-414.

18. **Kluytmans JA, Mouton JW, VandenBergh MF, Manders MJ, Maat AP, Wagenvoort JH, Michel MF, Verbrugh HA.** 1996. Reduction of surgical-site infections in cardiothoracic surgery by elimination of nasal carriage of *Staphylococcus aureus*. Infection control and hospital epidemiology: the official journal of the Society of Hospital Epidemiologists of America **17:**780-785.

19. **Mackie DP, van Hertum WA, Schumburg TH, Kuijper EC, Knape P, Massaro F.** 1994. Reduction in *Staphylococcus aureus* wound colonization using nasal mupirocin and selective decontamination of the digestive tract in extensive burns. Burns : journal of the International Society for Burn Injuries **20 Suppl 1:**S14-17; discussion S17-18.

20. **Talon D, Rouget C, Cailleaux V, Bailly P, Thouverez M, Barale F, Michel-Briand Y.** 1995. Nasal carriage of *Staphylococcus aureus* and cross-contamination in a surgical intensive care unit: efficacy of mupirocin ointment. The Journal of hospital infection **30:**39-49.

21. **Wenisch C, Laferl H, Szell M, Smolle KH, Grisold A, Bertha G, Krause R.** 2006. A holistic approach to MRSA eradication in critically ill patients with MRSA pneumonia. Infection **34:**148-154.

22. **McCann M, Moore ZE.** 2010. Interventions for preventing infectious complications in haemodialysis patients with central venous catheters. The Cochrane database of systematic reviews**:**CD006894.

23. **Walsh EE, Greene L, Kirshner R.** 2011. Sustained reduction in methicillin-resistant *Staphylococcus aureus* wound infections after cardiothoracic surgery. Archives of internal medicine **171:**68-73.

24. **Hood R, Shermock KM, Emerman C.** 2004. A prospective, randomized pilot evaluation of topical triple antibiotic versus mupirocin for the prevention of uncomplicated soft tissue wound infection. The American journal of emergency medicine **22:**1-3.

25. **Furukawa M, Minekawa A, Haruyama T, Narui Y, Sugita G, Sugita R, Kusunoki T, Ikeda K.** 2008. Clinical effectiveness of ototopical application of mupirocin ointment in methicillin-resistant Staphylococcus aureus otorrhea. Otology & neurotology : official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology **29:**676-678.

26. **Bass JW, Chan DS, Creamer KM, Thompson MW, Malone FJ, Becker TM, Marks SN.** 1997. Comparison of oral cephalexin, topical mupirocin and topical bacitracin for treatment of impetigo. The Pediatric infectious disease journal **16:**708-710.

27. **Gong JQ, Lin L, Lin T, Hao F, Zeng FQ, Bi ZG, Yi D, Zhao B.** 2006. Skin colonization by *Staphylococcus aureus* in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial. The British journal of dermatology **155:**680-687.

28. **Lee AS, Gizard Y, Empel J, Bonetti EJ, Harbarth S, Francois P.** 2014. Mupirocin-induced mutations in *ileS* in various genetic backgrounds of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol **52:**3749-3754.

29. **Fierobe L, Decre D, Muller C, Lucet JC, Marmuse JP, Mantz J, Desmonts JM.** 1999. Methicillin-resistant *Staphylococcus aureus* as a causative agent of postoperative intra-abdominal infection: relation to nasal colonization. Clin Infect Dis **29:**1231-1238.

30. **Seah C, Alexander DC, Louie L, Simor A, Low DE, Longtin J, Melano RG.** 2012. MupB, a new high-level mupirocin resistance mechanism in *Staphylococcus aureus*. Antimicrob Agents Chemother **56:**1916-1920.

31. **Walker ES, Vasquez JE, Dula R, Bullock H, Sarubbi FA.** 2003. Mupirocin-resistant, methicillin-resistant *Staphylococcus aureus*: does mupirocin remain effective? Infection control and hospital epidemiology. The official journal of the Society of Hospital Epidemiologists of America **24:**342-346.

766 32. **Coates A, Hu Y, Bax R, Page C.** 2002. The future challenges facing the development of
767 new antimicrobial drugs. Nat Rev Drug Discov **1:**895-910.
768 33. **Eidem TM, Lounsbury N, Emery JF, Bulger J, Smith A, Abou-Gharbia M, Childers**
769 **W, Dunman PM.** 2015. Small-molecule inhibitors of *Staphylococcus aureus* RnpA-
770 mediated RNA turnover and tRNA processing. Antimicrob Agents Chemother **59:**2016-
771 2028.
772 34. **Spitzfaden C, Nicholson N, Jones JJ, Guth S, Lehr R, Prescott CD, Hegg LA,**
773 **Eggleston DS.** 2000. The structure of ribonuclease P protein from *Staphylococcus*
774 *aureus* reveals a unique binding site for single-stranded RNA. Journal of molecular
775 biology **295:**105-115.
776 35. **Buck AH, Dalby AB, Poole AW, Kazantsev AV, Pace NR.** 2005. Protein activation of a
777 ribozyme: the role of bacterial RNase P protein. The EMBO journal **24:**3360-3368.
778 36. **Guerrier-Takada C, Altman S.** 1984. Catalytic activity of an RNA molecule prepared by
779 transcription *in vitro*. Science **223:**285-286.
780 37. **Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S.** 1983. The RNA moiety
781 of ribonuclease P is the catalytic subunit of the enzyme. Cell **35:**849-857.
782 38. **Kazantsev AV, Pace NR.** 2006. Bacterial RNase P: a new view of an ancient enzyme.
783 Nat Rev Microbiol **4:**729-740.
784 39. **Niranjanakumari S, Kurz JC, Fierke CA.** 1998. Expression, purification and
785 characterization of the recombinant ribonuclease P protein component from *Bacillus*
786 *subtilis*. Nucleic acids research **26:**3090-3096.
787 40. **Potter VR, Simonson H.** 1951. Sequential blocking of metabolic pathways in vivo.
788 Proceedings of the Society for Experimental Biology and Medicine. Society for
789 Experimental Biology and Medicine (New York, N.Y.) **76:**41-46.
790 41. **Mikkelsen NE, Brannvall M, Virtanen A, Kirsebom LA.** 1999. Inhibition of RNase P
791 RNA cleavage by aminoglycosides. Proc Natl Acad Sci U S A **96:**6155-6160.

792 42. **Gillaspy AF, Hickmon SG, Skinner RA, Thomas JR, Nelson CL, Smeltzer MS.** 1995.
793 Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal
794 osteomyelitis. Infect Immun **63:**3373-3380.
795 43. **McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC.**
796 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus*
797 *aureus* isolates from the United States: establishing a national database. J Clin Microbiol
798 **41:**5113-5120.
799 44. **Odds FC.** 2003. Synergy, antagonism, and what the chequerboard puts between them.
800 The Journal of antimicrobial chemotherapy **52:**1.
801 45. **Kiser KB, Cantey-Kiser JM, Lee JC.** 1999. Development and characterization of a
802 *Staphylococcus aureus* nasal colonization model in mice. Infect Immun **67:**5001-5006.
803 46. **Guthrie KM, Agarwal A, Tackes DS, Johnson KW, Abbott NL, Murphy CJ,**
804 **Czuprynski CJ, Kierski PR, Schurr MJ, McAnulty JF.** 2012. Antibacterial efficacy of
805 silver-impregnated polyelectrolyte multilayers immobilized on a biological dressing in a
806 murine wound infection model. Annals of surgery **256:**371-377.
807 47. **Amegbor K, Metowogo K, Eklu-Gadegbeku K, Agbonon A, Aklikokou KA, Napo-**
808 **Koura G, Gbeassor M.** 2012. Preliminary evaluation of the wound healing effect of Vitex
809 doniana sweet (Verbenaceae) in mice. African journal of traditional, complementary, and
810 alternative medicines **9:**584-590.
811 48. **Mikkelsen NE, Johansson K, Virtanen A, Kirsebom LA.** 2001. Aminoglycoside
812 binding displaces a divalent metal ion in a tRNA-neomycin B complex. Nature structural
813 biology **8:**510-514.
814 49. **Tok JB, Cho J, Rando RR.** 1999. Aminoglycoside antibiotics are able to specifically
815 bind the 5'-untranslated region of thymidylate synthase messenger RNA. Biochemistry
816 **38:**199-206.

50. **von Ahsen U, Davies J, Schroeder R.** 1992. Non-competitive inhibition of group I intron RNA self-splicing by aminoglycoside antibiotics. Journal of molecular biology **226:**935-941.

51. **Eubank TD, Biswas R, Jovanovic M, Litovchick A, Lapidot A, Gopalan V.** 2002. Inhibition of bacterial RNase P by aminoglycoside-arginine conjugates. FEBS letters **511:**107-112.

52. **Liu X, Chen Y, Fierke CA.** 2014. A real-time fluorescence polarization activity assay to screen for inhibitors of bacterial ribonuclease P. Nucleic acids research **42:**e159.

53. **Rouse MS, Rotger M, Piper KE, Steckelberg JM, Scholz M, Andrews J, Patel R.** 2005. *In vitro* and *in vivo* evaluations of the activities of lauric acid monoester formulations against *Staphylococcus aureus*. Antimicrob Agents Chemother **49:**3187-3191.

54. **Kokai-Kun JF, Walsh SM, Chanturiya T, Mond JJ.** 2003. Lysostaphin cream eradicates *Staphylococcus aureus* nasal colonization in a cotton rat model. Antimicrob Agents Chemother **47:**1589-1597.

55. **Noskin GA, Rubin RJ, Schentag JJ, Kluytmans J, Hedblom EC, Smulders M, Lapetina E, Gemmen E.** 2005. The burden of *Staphylococcus aureus* infections on hospitals in the United States: an analysis of the 2000 and 2001 Nationwide Inpatient Sample Database. Archives of internal medicine **165:**1756-1761.

56. **van Rijen MM, Bonten M, Wenzel RP, Kluytmans JA.** 2008. Intranasal mupirocin for reduction of *Staphylococcus aureus* infections in surgical patients with nasal carriage: a systematic review. The Journal of antimicrobial chemotherapy **61:**254-261.

57. **Coia JE, Duckworth GJ, Edwards DI, Farrington M, Fry C, Humphreys H, Mallaghan C, Tucker DR.** 2006. Guidelines for the control and prevention of meticillin-resistant *Staphylococcus aureus* (MRSA) in healthcare facilities. The Journal of hospital infection **63 Suppl 1:**S1-44.

58. **Hetem DJ, Bonten MJ.** 2013. Clinical relevance of mupirocin resistance in *Staphylococcus aureus*. The Journal of hospital infection **85:**249-256.

59. **Antonov NK, Garzon MC, Morel KD, Whittier S, Planet PJ, Lauren CT.** 2015. High prevalence of mupirocin resistance in *Staphylococcus aureus* isolates from a pediatric population. Antimicrob Agents Chemother **59:**3350-3356.

60. **Park SH, Kim SY, Lee JH, Park C, Lee DG.** 2013. Community-genotype strains of methicillin-resistant *Staphylococcus aureus* with high-level mupirocin resistance in a neonatal intensive care unit. Early human development **89:**661-665.

61. **Keith CT, Borisy AA, Stockwell BR.** 2005. Multicomponent therapeutics for networked systems. Nat Rev Drug Discov **4:**71-78.

62. **Stein GE, Gurwith MJ.** 1984. Amoxicillin-potassium clavulanate, a beta-lactamase-resistant antibiotic combination. Clinical pharmacy **3:**591-599.

63. **Csonka GW, Knight GJ.** 1967. Therapeutic trial of trimethoprim as a potentiator of sulphonamides in gonorrhoea. The British journal of venereal diseases **43:**161-165.

64. **Kavuru M, Melamed J, Gross G, Laforce C, House K, Prillaman B, Baitinger L, Woodring A, Shah T.** 2000. Salmeterol and fluticasone propionate combined in a new powder inhalation device for the treatment of asthma: a randomized, double-blind, placebo-controlled trial. The Journal of allergy and clinical immunology **105:**1108-1116.

65. **Bays HE, Dujovne CA, McGovern ME, White TE, Kashyap ML, Hutcheson AG, Crouse JR.** 2003. Comparison of once-daily, niacin extended-release/lovastatin with standard doses of atorvastatin and simvastatin (the ADvicor Versus Other Cholesterol-Modulating Agents Trial Evaluation [ADVOCATE]). The American journal of cardiology **91:**667-672.

66. **Larder BA, Kemp SD, Harrigan PR.** 1995. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science **269:**696-699.

67. **Jones RN, Li Q, Kohut B, Biedenbach DJ, Bell J, Turnidge JD.** 2006. Contemporary antimicrobial activity of triple antibiotic ointment: a multiphased study of recent clinical isolates in the United States and Australia. Diagnostic microbiology and infectious disease **54:**63-71.

68. **Fraki JE, Peltonen L, Hopsu-Havu VK.** 1979. Allergy to various components of topical preparations in stasis dermatitis and leg ulcer. Contact dermatitis **5:**97-100.

69. **Bonomo RA, Van Zile PS, Li Q, Shermock KM, McCormick WG, Kohut B.** 2007. Topical triple-antibiotic ointment as a novel therapeutic choice in wound management and infection prevention: a practical perspective. Expert review of anti-infective therapy **5:**773-782.

70. **Epstein E.** 1966. Allergy to dermatologic agents. JAMA **198:**517-520.

Table 1. Selleck Library Members with Mupirocin-Associated Improved Activity

	MIC (μg ml^{-1})		
Drug	**(-) Mup**	**(+) Mup[1]**	**Fractional Inhibitory Concentration Index**
Nitazoxanide	16	8	0.75
Nitrofurazone	16	8	0.75
Neomycin sulfate	0.5	0.25	0.75

[1] Performed in the presence of 0.5x Mupirocin MIC (0.0625 μg ml^{-1})

914 **FIGURE LEGENDS**

915

916 **Fig. 1. Effects of neomycin on *S. aureus* RNase P mediated ptRNA^{Tyr} processing.** Shown
917 are the mobility of precursor tRNA^{Tyr} in the absence and presence of *S. aureus* RNase P
918 enzyme and the indicated concentration of neomycin. Densitometry measured percent activity
919 shown (tRNA product formed) normalized to DMSO treated enzyme alone.

920

921 **Fig. 2. Antimicrobial zone of inhibition measures.** Plotted are the average zones of
922 inhibition (*y*-axis; cm^2) of PEG-based ointments containing the indicated antibiotic or antibiotic
923 mixture (*x*-axis) toward *S. aureus* strain UAMS-1 (Panel A, D, and E), USA300 (Panel B) or
924 BAA-1708 (Panel C). Significant increases in growth inhibition zones, in comparison to 2%
925 mupirocin, are indicated (Student's t-test (N=4); * $P \leq 0.1$; ** $P \leq 0.05$).

926

927 **Fig. 3. Murine nasal decolonization measures.** Plotted are the numbers of colony forming
928 units (cfu) per mouse nasal passage (*y*-axis) following 3 days dosing with PEG-based ointment
929 containing the indicated antibiotic or antibiotic mixture (*x*-axis). Results for *S. aureus* strain
930 UAMS-1 (Panel A), USA300 (Panel B), and BAA-1708 (Panel C) are shown; red data points
931 indicate low-level mupirocin resistant isolates. Significant reductions in bacterial burden, in
932 comparison vehicle are indicated (one-way ANOVA; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq$
933 0.0001).

934

935 **Fig. 4. Murine wound decolonization measures.** Shown are the numbers of colony forming
936 units (cfu) per lesion (*y*-axis) following 3 days dosing with PEG-based ointment containing the
937 indicated antibiotic or antibiotic mixture (*x*-axis). Results for S. aureus strain UAMS-1 (Panel A),
938 USA300 (Panel B), and BAA-1708 (Panel C) are shown. Significant reductions in bacterial

939 burden between treatment groups are indicated (one-way ANOVA; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq$
940 0.001; ****$P \leq 0.0001$).
941
942 **Fig. 5. Antimicrobial effects of PEG-based ointments toward *A. baumannii* and *P.**
943 ***aeruginosa*.** Shown are the antimicrobial effects of PEG ointments containing vehicle, 2%
944 mupirocin, 1% neomycin and the combination of 2% mupirocin + 1% neomycin toward *A.*
945 *baumannii* strain 98-37-09 (Top Panels) or *P. aeruginosa* strain PA01 (Bottom Panels).
946
947 **Fig. 6. Effects of PEG-based ointments on wound healing and animal health.**
948 Representative wound healing images following 0, 3, 7, and 14 days of treatment with PEG-
949 base ointments containing vehicle, 2% mupirocin, 1% neomycin or combination (Panel A).
950 Panel B shows average measures (N=3) of corresponding wound contraction corresponding to
951 Panel A conditions. Panel C average body weight of animals (*y*-axis) at the indicated day (*x*-
952 axis) post lesion formation and treatment with PEG-based ointment supplemented with the
953 indicated agent.

Antimicrobial Agents and Chemotherapy

Figure 1.

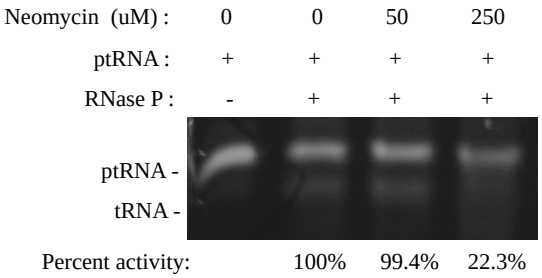

Fig. 1. Effects of neomycin on *S. aureus* RNase P mediated ptRNA^{Tyr} processing. Shown are the mobility of precursor tRNA^{Tyr} in the absence and presence of *S. aureus* RNase P enzyme and the indicated concentration of neomycin.

Figure 2.

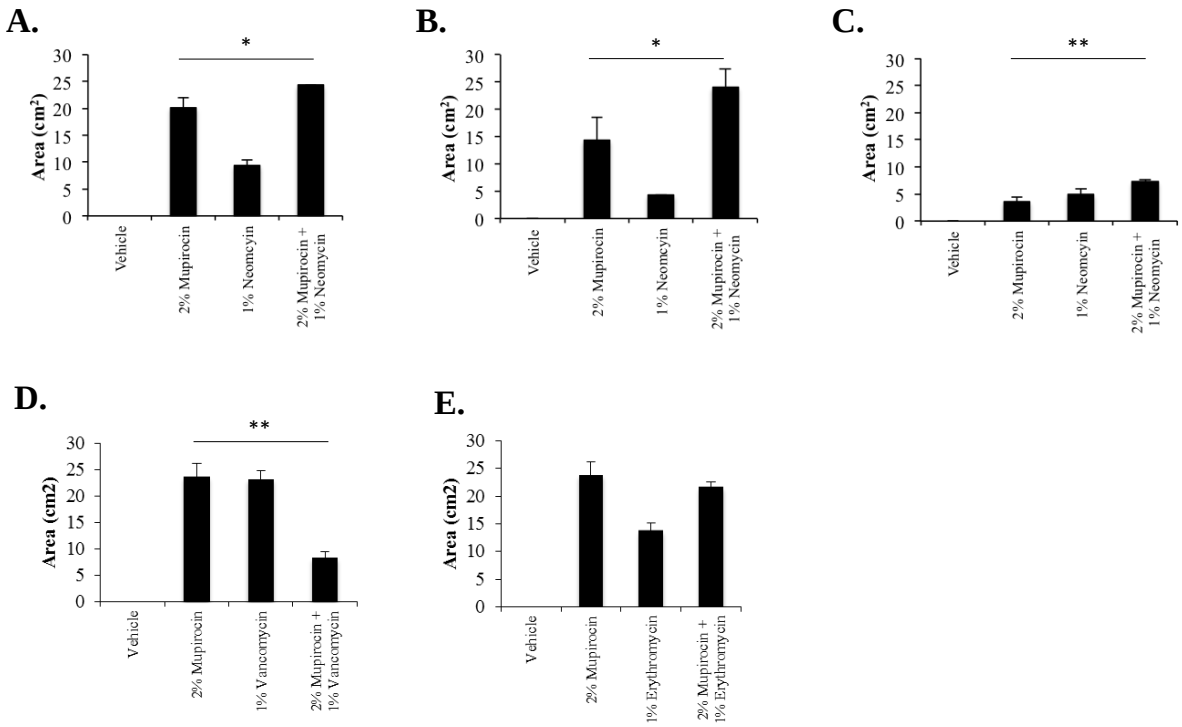

Fig. 2. Antimicrobial zone of inhibition measures. Plotted are the average zones of inhibition (*y*-axis; cm^2) of PEG-based ointments containing the indicated antibiotic or antibiotic mixture (*x*-axis) toward *S. aureus* strain UAMS-1 (Panel A, D, and E), USA300 (Panel B) or BAA-1708 (Panel C). Significant increases in growth inhibition zones, in comparison to 2% mupirocin, are indicated (Student's t-test (N=4); * $P \leq 0.1$; ** $P \leq 0.05$).

Figure 3.

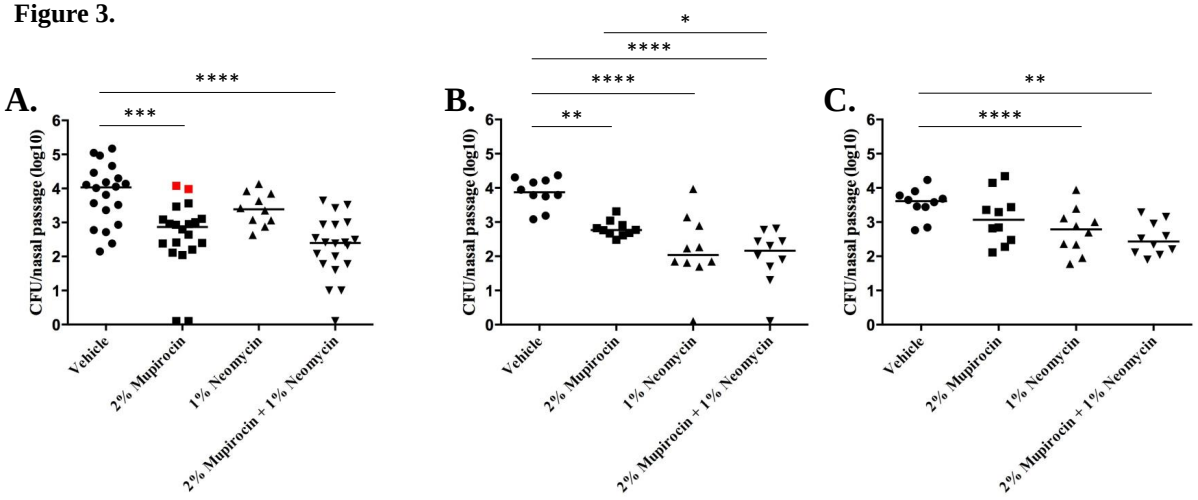

Fig. 3. Murine nasal decolonization measures. Plotted are the numbers of colony forming units (cfu) per mouse nasal passage (*y*-axis) following 3 days dosing with PEG-based ointment containing the indicated antibiotic or antibiotic mixture (*x*-axis). Results for *S. aureus* strain UAMS-1 (Panel A), USA300 (Panel B), and BAA-1708 (Panel C) are shown; red data points indicate low-level mupirocin resistant isolates. Significant reductions in bacterial burden, in comparison vehicle are indicated (one-way ANOVA; **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001; *****P* ≤ 0.0001).

Figure 4.

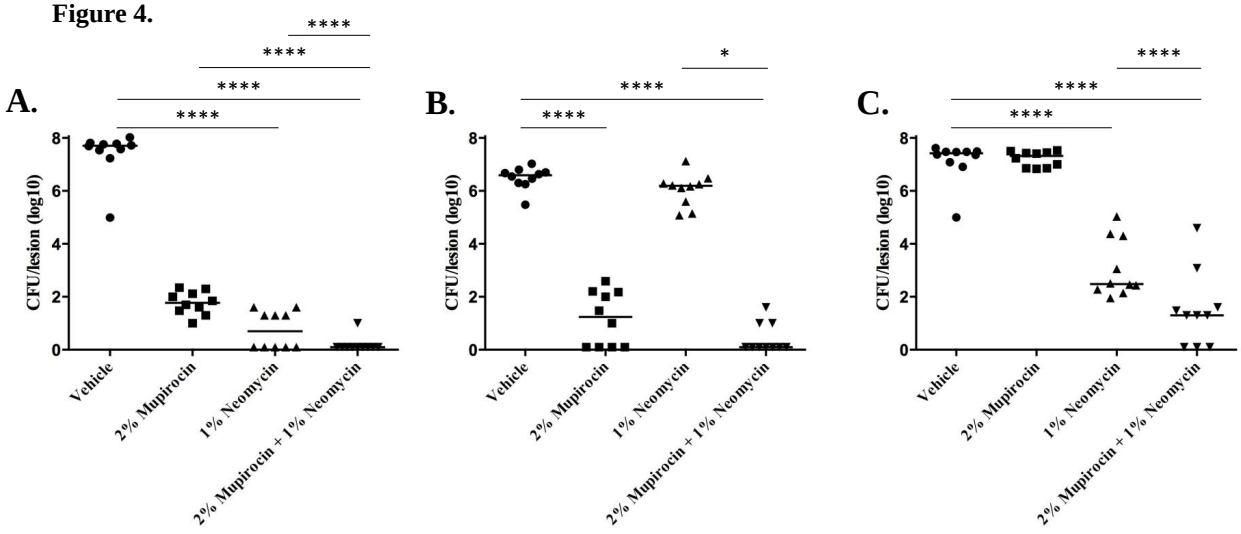

Fig. 4. Murine wound decolonization measures. Shown are the numbers of colony forming units (cfu) per lesion (*y*-axis) following 3 days dosing with PEG-based ointment containing the indicated antibiotic or antibiotic mixture (*x*-axis). Results for S. aureus strain UAMS-1 (Panel A), USA300 (Panel B), and BAA-1708 (Panel C) are shown. Significant reductions in bacterial burden between treatment groups are indicated (one-way ANOVA; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$).

Figure 5.

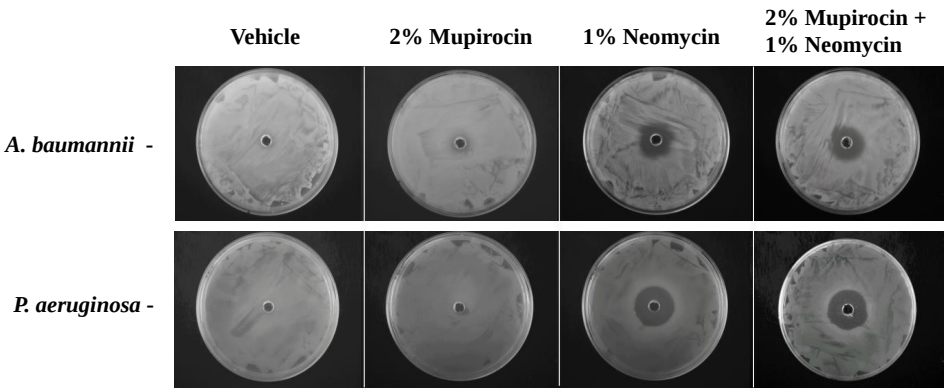

Fig. 5. Antimicrobial effects of PEG-based ointments toward *A. baumannii* and *P. aeruginosa*. Shown are the antimicrobial effects of PEG ointments containing vehicle, 2% mupirocin, 1% neomycin and the combination of 2% mupirocin + 1% neomycin toward *A. baumannii* strain 98-37-09 (Top Panels) or *P. aeruginosa* strain PA01 (Bottom Panels).

Figure 6.

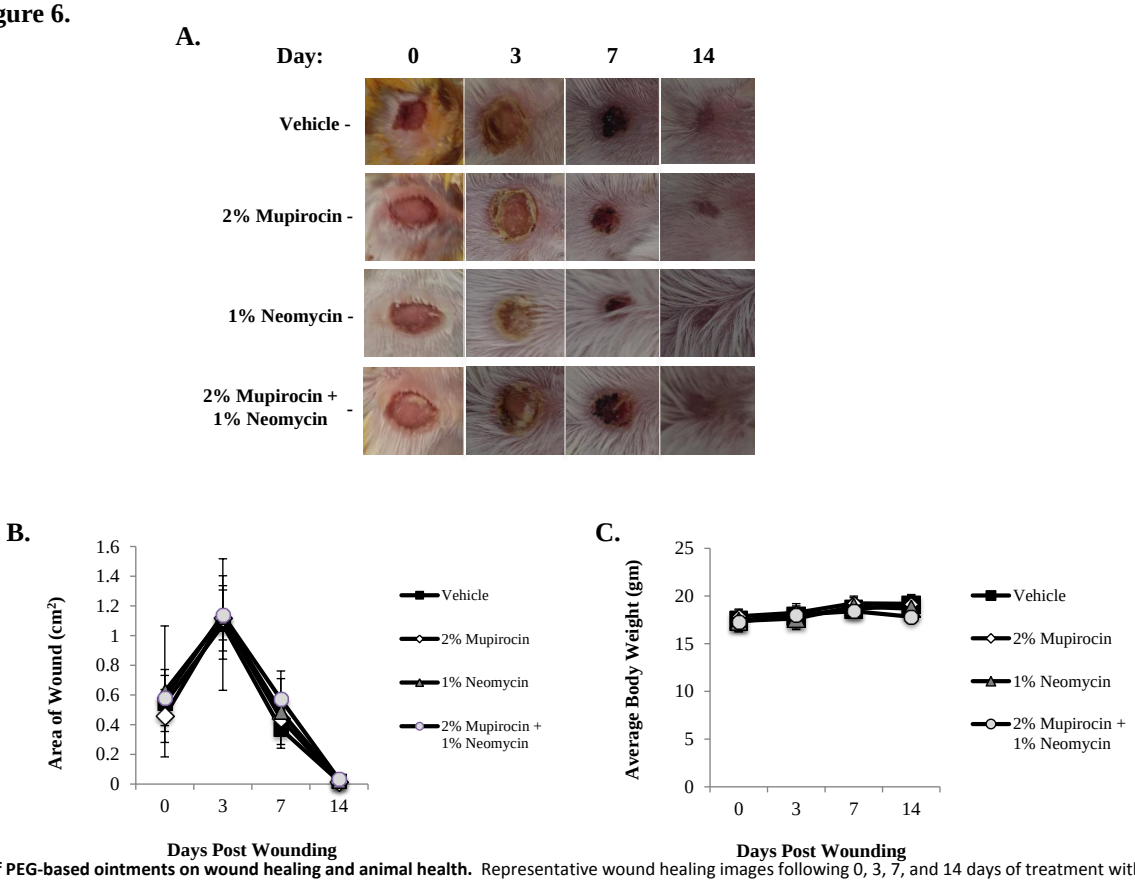

Fig. 6. Effects of PEG-based ointments on wound healing and animal health. Representative wound healing images following 0, 3, 7, and 14 days of treatment with PEG-base ointments containing vehicle, 2% mupirocin, 1% neomycin or combination (Panel A). Panel B shows average measures (N=3) of corresponding wound contraction corresponding to Panel A conditions. Panel C average body weight of animals (*y*-axis) at the indicated day (*x*-axis) post lesion formation and treatment with PEG-based ointment supplemented with the indicated agent.