Topical nanoemulsion therapy reduces bacterial wound infection and inflammation after burn injury

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Background. Nanoemulsions are broadly antimicrobial oil-in-water emulsions containing nanometer-sized droplets stabilized with surfactants. We hypothesize that topical application of a nanoemulsion compound (NB-201) can attenuate burn wound infection. In addition to reducing infection, nanoemulsion therapy may modulate dermal inflammatory signaling and thereby lessen inflammation following thermal injury.

Methods. Male Sprague-Dawley rats underwent a 20% total body surface area scald burn to create a partial-thickness burn injury. Animals were resuscitated with Ringer’s lactate solution and the wound covered with an occlusive dressing. At 8 hours after injury, the burn wound was inoculated with $1 \times 10^6$ colony-forming units (CFUs) of Pseudomonas aeruginosa. NB-201, NB-201 placebo, 5% mafenide acetate solution, or 0.9% saline (control) was applied onto the wound at 16 and 24 hours after burn injury. Skin was harvested 32 hours postburn for quantitative wound culture and determination of inflammatory mediators in tissue homogenates.

Results. NB-201 decreased mean bacterial growth in the burn wound by 1,000-fold, with only 13% (3/23) of animals having $P. \ aeruginosa$ counts greater than $10^5$ CFU/g tissue versus 91% (29/32) in the control group ($P < .0001$). Treatment with NB-201 attenuated neutrophil sequestration in the treatment group as measured by myeloperoxidase assay and by histology. It also significantly decreased levels of proinflammatory cytokines (interleukin [IL]-1β and IL-6) and the degree of hair follicle cell apoptosis in skin compared to saline-treated controls.

Conclusion. Topical NB-201 substantially decreased bacterial growth in a partial-thickness burn model. This decrease in the level of wound infection was associated with an attenuation of the local dermal inflammatory response and diminished neutrophil sequestration. NB-201 represents a novel potent antimicrobial and anti-inflammatory treatment for use in burn wounds. (Surgery 2010;148:499-509.)

Contemporary burn wound management involves early debridement and reconstruction of clearly nonviable skin coupled with provision of supportive care

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Topical antimicrobial dressing changes to partial-thickness burn wounds. The goal of modern burn wound care is to provide an optimal environment for epidermal renewal. During the period of epidermal renewal, it is important to avoid further injury to the skin, abrogate burn wound progression, and minimize secondary complications such as wound infection.

Poplar topical antimicrobial agents include silver sulfadiazine (Silvadene), mafenide acetate (Sulfamylon), and colloidal silver-impregnated dressings (Acticoat, Silverlon). Each of these agents has potential limitations, such as variable ability to penetrate eschar, uneven efficacy against both Gram-negative and Gram-positive bacteria, and potential toxicity to host immune cells. A need exists to develop a new generation of topical, broad-spectrum antimicrobial agents that can penetrate deeper
into the burn wound. These agents could potentially be combined with anti-inflammatory drugs to minimize early burn wound inflammation and tissue edema. In addition to local effects, severe dermal burns are known to induce systemic inflammatory response syndrome, which results in a high risk of end-organ dysfunction.5

Antimicrobial nanoemulsions are mixtures of oil-in-water droplets in which the droplets range from 200 to 600 nm in size. These emulsions are stabilized by surfactants and alcohol. The active ingredients are approved for over-the-counter human application and are on the U.S. Food and Drug Administration (FDA) Generally Recognized as Safe (GRAS) list. A high-energy state is formed in the particle during manufacture using a high-speed mixer.

In vitro testing of these agents has confirmed that they have broad antimicrobial activity against Gram-negative and Gram-positive bacteria, enveloped viruses, fungi, spores, and protozoa. The liquid nanoemulsion particles are thermodynamically driven to fuse with lipid-containing organisms. Fusion with cell membranes is enhanced by the electrostatic attraction between the cationic charge of the emulsion and the anionic charge of the pathogen. When critical concentrations of nanoparticles fuse with the cell membrane, they release energy trapped within the emulsion, which destabilizes the pathogen lipid membrane and results in cell lysis and death.4–8

NB-201 is an antimicrobial nanoemulsion formulation consisting of emulsification of vegetable oil and water with surfactants and alcohol. We hypothesized that treatment of burn wounds with this innovative nanoemulsion compound would attenuate the development of wound infection based on quantitative wound culture, with a positive result defined as growth of bacteria at greater than $1 \times 10^5$ colony-forming units (CFUs) per gram of tissue.9–11 Treatment with topical nanoemulsion will also result in less dermal inflammation and decrease hair follicle apoptosis in an animal model of partial-thickness scald injury.

MATERIAL AND METHODS

Reagents. Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. Male-specific, pathogen-free Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing approximately 250 to 300 g were used in all experiments. All experiments were performed in accordance with the National Institutes of Health (NIH) guidelines for care and use of animals. Approval for the experimental protocol was obtained from the University of Michigan Animal Care and Use Committee.

Burn model. The procedure was performed according to a previously established method to produce partial-thickness burn injury.12,13 In brief, animals were anesthetized with a 40 mg/kg intraperitoneal (ip) injection of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL). Dorsal hair was closely clipped and then removed using depilatory cream (Nair; Church & Dwight Inc., Princeton, NJ). Each rat was placed in an insulated, custom-made mold, which exposed the dorsal region over 20% of the total body surface area. Partial-thickness scald burn injury was achieved by placing the exposed skin of the rat in a 60°C water bath for 25 seconds. Sham burn animals received the same treatment except they were immersed in room temperature water (range, 21–24°C).

The burn wound was scrub debrided with dry sterile gauze and rinsed with 0.9% sterile NaCl. Each animal was resuscitated with 4 mL Ringer’s lactate solution per percent total body surface area burn per kilogram of body weight. One half of this fluid volume was injected ip and half subcutaneously immediately after the burn injury. After drying, an occlusive dressing of sterile Telfa (Kendall Co., Mansfield, MA) and Tegaderm HP (3M HealthCare, St Paul, MN) was applied to prevent wound contamination. During the experiment, each rat was singly housed and received 0.01 mg/kg buprenorphine subcutaneously at the time of burn and at 16 hours for postburn pain control.

Local wound treatment. Stock nanoemulsion compound NB-201 was obtained from NanoBio Corporation (Ann Arbor, MI). This nanoemulsion was manufactured by emulsification of super-refined soybean oil and water with surfactants and alcohol. The resultant droplets had a mean particle diameter of 350 nm. The experimental solution was made by diluting 1 mL of the 60% stock formulation with 4.88 mL sterile saline and adding 120 μL of 1 M ethylenediaminetetraacetic acid (EDTA), giving a final concentration of 10% NB-201 and 20 mM EDTA. A placebo nanoemulsion compound was manufactured in the same manner as the NB-201, but 1 of the active ingredients was deleted from the formulation (benzalkonium chloride).

A 5% mafenide acetate (Sulfamylon; UDL Laboratories Inc., Rockford, IL) solution was formulated by mixing 50 g of mafenide acetate powder in 1 L of 0.9% sterile saline. The control reagent used was 0.9% sterile saline. Experimental groups...
consisted of sham, burn, burn + NB-201, burn + bacteria + saline, burn + bacteria + placebo, burn + bacteria + NB-201, and burn + bacteria + Sulfamylon. At 16 hours after burn injury, animals were anesthetized with inhaled isoflurane.

The occlusive dressing and Telfa was removed. Nanoemulsion (NB-201), placebo, Sulfamylon, or sterile saline was applied in a uniform fashion to the burn wound surface using a spray bottle. Although animals in the sham or burn group received no topical treatment, they underwent dressing change while anesthetized. The burn wound was then redressed with Telfa and a Tegaderm occlusive dressing. This treatment and dressing change was repeated at 24 hours after burn injury.

**Bacterial culture and inoculation.** Pseudomonas aeruginosa isolated from a human burn patient was provided by the Department of Pathology at the University of Michigan. This bacterial isolate is sensitive to the topical agent Silvadene and Sulfamylon. A bacterial inoculum was prepared by thawing an aliquot (0.5 mL, stored in 50% skim milk at −80°C) in 40 mL of Trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ), and grown overnight at 37°C with constant shaking at 275 rpm. A sample of the resulting stationary-phase culture was transferred to 35 mL fresh Trypticase soy broth and incubated for 2.5 hours to reach the log phase. This subculture was transferred to a 50-mL conical polystyrene tube and centrifuged at 3,000 g for 20 minutes at 4°C.

The bacterial pellet was washed with 0.9% sterile saline, and resuspended in 10 mL of ice-cold saline. The optical density (OD) of the suspension was measured at 620 nm, and bacterial concentration (CFU/mL) was calculated using the formula

\[
\text{CFU/mL} = \frac{\text{OD}_{620} \times 2.5 \times 10^8}{\text{CFU}}
\]

The bacterial suspension was diluted with 0.9% sterile saline to a final concentration of 1 × 10^6 CFU per 100 μL. At 8 hours after burn injury, animals were anesthetized with inhaled isoflurane. The rats then underwent topical application of 1 × 10^6 CFUs of log-phase P. aeruginosa in 100 μL sterile saline pipetted onto a piece of Telfa in a uniform fashion followed by coverage with a Tegaderm occlusive dressing.

**Tissue harvest.** At 32 hours after thermal injury, the animals were killed, and skin tissue samples were harvested employing standard sterile techniques. Skin samples were used immediately or frozen in liquid nitrogen.

**Quantitation of bacterial wound infection.** A 100-mg piece of excised skin tissue was mechanically homogenized in 1 mL of 0.9 NaCl. This homogenate was then further diluted with 9 mL of sterile saline. Serial dilutions were performed, and skin homogenates were plated in triplicate on blood agar plates (Becton Dickinson). Culture plates were incubated for 24 hours at 37°C, and CFUs were counted.

**Dermal cytokine analysis (ELISA).** A 100-mg sample of dorsal skin was homogenized in 1 mL ice-cold lysis buffer consisting of 50 mL phosphate-buffered saline and protease inhibitor (Complete X; Roche, Indianapolis, IN) and 50 μL Triton X (Roche). Homogenates were centrifuged at 3000g for 5 minutes, and the supernatants were collected and stored frozen at −80°C until use. Rat interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, cytokine-induced neutrophil chemoattractant (CINC)-1, CINC-3, IL-10, and tumor growth factor (TGf)-β were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using antibodies and reagents from R&D Systems Inc. (Minneapolis, MN). Results were adjusted for previous dilution and expressed as picograms per milliliter (pg/mL).

**Detection of neutrophil sequestration (Myeloperoxidase assay).** A sample of skin tissue (100 mg) was mechanically homogenized in 1 mL ice-cold potassium phosphate buffer consisting of 115 mM monobasic potassium phosphate (Sigma-Aldrich, Milwaukee, WI). Homogenates were centrifuged at 3,000g for 10 minutes at 4°C, the supernatants were removed, and the pellets were resuspended in 1 mL CTAB buffer consisting of di-basic potassium phosphate, cetyltrimethylammonium bromide, and acetic acid (Sigma-Aldrich).

The suspensions were sonicated (Sonifer 250; Branson Ultrasonics, Danbury, CT) on ice for 40 seconds. Homogenates were centrifuged at 3000g for 10 minutes at 4°C, and the supernatant was collected. Supernatants were incubated for 2 hours in a water bath (Shaker Bath 2568; Forma Scientific, Marietta, OH) at 60°C. Samples were either stored at −80°C until needed or assayed immediately.

Aliquots of 20 μL of standards (Calbiochem, Gibbstown, NJ) or samples were added to a 96-well immunosorbent microplate (NUNC, Rochester, NY), followed by the addition of 155 μL of 20 mM TMB/DMF (3,3′,5,5′-tetramethylbenzidine/N,N-dimethylformamide) substrate in 115 mM potassium phosphate buffer (Fischer Scientific, Pittsburgh, PA) to each well. The samples were mixed well, after which 20 μL of 3 mM H₂O₂ was rapidly added to each well. The reaction was stopped immediately by adding 50 μL/well of 0.061 mg/mL catalase (Roche). The plates were read using a microplate reader (Biotek Instruments, Winooski, VT) at 620 nm. Myeloperoxidase concentrations were calculated using a linear standard

\[
\text{OD}_{620} = \frac{\text{A}_{620} - \text{B}}{C} \times \frac{\text{D}}{\text{E}}
\]

where A is the absorbance of the sample, B is the absorbance of the blank, C is the dilution factor, D is the optical density of the standard, and E is the dilution factor of the standard.
curve and adjusted for previous dilutions. The final concentrations were expressed as micrograms per microliter (µg/mL).

**Determination of dermal capillary leak and tissue edema (Evans blue assay).** Burn wounds are associated with significant levels of capillary leak. This leakage can lead to depletion of the intravascular volume and a need for large amounts of intravenous crystalloid fluid administration. To assess whether our therapy decreased capillary leak in conjunction with decreasing inflammation, we used the Evans blue assay, which is a measure of vascular permeability.13

Animals were anesthetized 90 minutes before tissue harvest. A total of 50 mg/kg body weight of 10% Evans blue (Merck KgaA, Darmstadt, Germany) was injected ip into the burned animal at 30.5 hours after thermal injury. At the tissue harvest time point, animals were exsanguinated by incision of the inferior vena cava. Systemic Evans blue was flushed out with a total of 4 × the blood volume (7.46 mL/100 g body weight) of 0.9 NaCl with 100 U/mL heparin.

Dorsal skin samples were harvested, and a 100-mg sample was placed in 4 mL 99.5% formamide in polyethylene tubes. The tubes were placed on a shaker at room temperature for 48 hours for Evans blue extraction. Supernatants were collected and the absorbance was read on a microplate reader at 620 nm. Concentrations were calculated from a standard curve of Evans blue in formamide. Results were expressed as micrograms of Evans blue per milligram of skin tissue.

**Histology.** Skin samples were fixed in 10% buffered formalin and embedded in paraffin. A total of 8-µm thick sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to assess morphologic changes.

**Detection of hair follicle cell apoptosis (TUNEL assay).** Animals were anesthetized and underwent the creation of a 20% partial-thickness scald burn wound or sham injury. Treatment groups consisted of sham, burn + saline, burn + placebo, and burn + NB-201. Treatment and dressing changes were performed at 0 and 8 hours postburn. No bacterial infection was created in this experiment. Full-thickness skin samples were taken from 3 locations across the entire burn wound at 12 and 24 hours post-thermal injury for determination of hair follicle cell apoptosis. There were 4 animals per treatment group per time sample.

As described previously, apoptosis was detected in situ with fluorescein-based labeling of DNA strand breaks using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (ApopTag; Chemicon International Inc., Temecula, CA).13 The 3 fresh skin samples for each animal were placed in disposable vinyl cryomolds filled with optimal cutting temperature compound (Sakura Finetek USA Inc., Torrance, CA) and frozen at −80°C until ready for use. Frozen, embedded skin specimens were cut into 4-µm-thick serial sections in a cryostat and collected on Superfrost Plus glass slides (Fisher Scientific). Sections were fixed and stained according to the manufacturer’s instructions.

The TUNEL assay slides were blinded to experimental treatment groups. Under the microscope, appropriate hair follicle cells in a randomly chosen high-power field were identified. Appropriate hair follicles for analysis were those sectioned in the mid-sagittal or mid-coronal plane. A total of 3–6 hair follicles were selected from among the 3 skin samples present on a slide. Fluorescent-labeled TUNEL slides were captured digitally at

![Fig 1. Topical application of NB-201 decreases *Pseudomonas aeruginosa* growth in burn wounds. Male Sprague-Dawley rats received partial-thickness burn wounds. At 8 hours postinjury, animals were inoculated with 10⁶ colony-forming units (CFUs) *P. aeruginosa*. At 16 and 24 hours postburn, the animals were treated with topical saline (control), placebo (NB-201 without benzalkonium chloride), NB-201 (nanoemulsion), or Sulfamylon (mafenide acetate). At 32 hours, animals were killed, skin samples obtained and homogenized, plated, and CFUs counted. The scatterplot represents cultured CFUs for each individual animal. The median value for each group is plotted as a horizontal line. There was minimal pathogen growth in 12 of 23 of the NB-201–treated animals. A majority of the control (29/32) and placebo (9/12) animals with burn injury had evidence of wound infection based on a positive quantitative wound culture with significantly more bacteria present in the wound than those animals treated with NB-201. *P < .0001, Kruskal-Wallis test; P < .05 for saline vs NB-201, placebo vs NB-201, and saline vs Sulfamylon, Dunn multiple comparison test.*
identical times postlabeling to control fading of fluorescence using an Olympus BX51 fluorescence microscope (Olympus, Melville, NY) at fixed-image capture settings and 40× magnification.

Each hair follicle was selected and first digitally captured by visualizing counterstained nuclei present using the DAPI (4',6-diamidino-2-phenylindole) excitation/emission channel. Then, for each hair follicle analyzed, the excitation/filter channel was changed to visualize the fluorescein-labeled, TUNEL-positive cells, and images were again digitally captured. Within the captured images a region of interest (ROI) was digitally defined, and set to include only hair follicle cells and exclude bright fluorescing hair shafts and surrounding cells (ImageJ; NIH, Bethesda, MD). Fluorescence of TUNEL-positive cells was quantified, normalized to ROI size, and expressed as pixels per area fraction, controlling for differences in ROI size.

Statistical methods. All statistical analysis and graphs were performed using GraphPad Prism software (version 5.0; GraphPad Software, La Jolla, CA). Results are presented as mean values ± the SEM unless otherwise noted. Continuous variables were analyzed using an unpaired 2-tailed Student t test and/or 1-way analysis of variance (ANOVA) followed by Tukey post-test comparisons. The Kruskal-Wallis test with the Dunn multiple comparison test was used to evaluate differences in medians for data with a nonparametric distribution. Discrete variables were compared using the Fisher exact test. Statistical significance was defined as a P value < .05.

![Fig 2. NB-201 treatment after burn injury attenuates dermal proinflammatory cytokine expression.](image)

Groups were sham, burn, and burn with NB-201 treatment (n = 8–10 per group). (A) Interleukin (IL)-1β (P = .02, 1-way analysis of variance [ANOVA]); (B) IL-6 (P = .8); (C) tumor necrosis factor (TNF)-α (P = .5); (D) cytokine-induced neutrophil chemoattractant (CINC)-1 (P = .04); (E) CINC-3 (P = .005); and (F) myeloperoxidase (P = .07). Burn-injured animals treated with nanoemulsion compared to untreated partial-thickness burned animals had decreased levels of IL-1β (1,773 ± 516 vs 5,625 ± 1,743 pg/mL, respectively) and CINC-3 (225 ± 66 vs 1,589 ± 527 pg/mL, respectively). *P < .05, the Student t test or Tukey multiple comparison test.
RESULTS

Topical application of NB-201 nanoemulsion decreases *P. aeruginosa* growth in burn wounds. Animals treated with nanoemulsion had a decreased mean (6.5 × 10^4 vs 7.9 × 10^7; P = .07) and median (0 vs 4.4 × 10^6; P < .05) number of CFUs of bacteria per gram of skin tissue compared to the saline-treated controls (Fig 1). A similar reduction in skin bacterial counts was found for NB-201-treated animals versus those treated with placebo (mean, 6.5 × 10^4 vs 5.5 × 10^6; P = .02). When performing quantitative wound culture on clinical tissue samples, a positive result is generally considered to be growth of organisms at greater than 1 × 10^5 CFUs per gram of tissue.9-11

Using these criteria, of the 32 animals in the control group, 29 exhibited evidence of a positive quantitative wound culture; of the 23 animals in the nanoemulsion group, only 3 demonstrated proof of this level of wound infection (91% vs 13%; P < .0001). Positive quantitative wound culture results for the placebo group were 9 of 12 animals (75%) and for the Sulfamylon group were 2 of 10 animals (20%). The Sulfamylon-treated animals also demonstrated a significant decrease in both the median wound bacterial level and the positive quantitative wound culture rate compared to the saline controls (3 × 10^4 vs 4.4 × 10^6 [P < .05] and 20% vs 91% [P < .0001], respectively).

Treatment with nanoemulsion or Sulfamylon produced a similar decrease in the level of *P. aeruginosa* cultured from the burn wound compared to the saline-treated animals. However, there was no statistically significant difference between the placebo and Sulfamylon groups, whereas there was a difference between the NB-201 and placebo groups.

Fig 3. NB-201 treatment after burn wound infection with *Pseudomonas aeruginosa* attenuates dermal proinflammatory cytokine expression. All animals received burn injury, and groups were saline, placebo, NB-201, and Sulfamylon (n = 10–30 per group). (A) Interleukin (IL)-1β (P = .001, 1-way analysis of variance [ANOVA]); (B) IL-6 (P = .07); (C) tumor necrosis factor (TNF)-α (P = .3); (D) cytokine-induced neutrophil chemoattractant (CINC)-1 (P = .8); (E) CINC-3 (P = .4); and (F) myeloperoxidase (P = .0001). Burn wound–infected animals treated with nanoemulsion had decreased levels of IL-1β (1,007 ± 157 vs 3,054 ± 499 pg/mL) and IL-6 (244 ± 51 vs 485 ± 73 pg/mL) compared to saline treated controls. The NB-201 and Sulfamylon treatment groups demonstrated decreased dermal neutrophil sequestration compared to saline and placebo as evidenced by myeloperoxidase assay (NB-201, 0.09 ± 0.02; Sulfamylon, 0.08 ± 0.02; saline, 0.40 ± 0.06; placebo, 0.45 ± 0.11 μg/mL). *P < .05, Tukey multiple comparison test.
Skin homogenates from the nanoemulsion-treated group had levels of IL-1β and IL-6 that were considerably diminished compared to the levels measured in the saline-treated animals (Fig 3, A and B). There was no statistically significant difference seen in the level of TNF-α between the 2 experimental groups of animals (Fig 2, C, and Fig 3, C). Treatment of the infected burn wound with Sulfamylon did not result in any significant alteration of dermal levels of the measured proinflammatory cytokines (IL-1β, IL-6, TNF-α, CINC-1, or CINC-3) compared to controls. Treatment with either NB-201 or Sulfamylon decreased the level of myeloperoxidase found in the infected burn wound at 32 hours postinjury. This observation suggests that treatment with an antimicrobial decreases the level of neutrophil sequestration into the partial-thickness burn wound.

Burn injury caused an increase in the level of the anti-inflammatory cytokine TGF-β, but not IL-10, compared to the sham-injured animals (Fig 4). NB-201 treatment decreased the amount of TGF-β in the infected burn wound compared to the level found in the burn wound alone. This finding suggests that NB-201 not only alters acute burn wound dermal inflammation, but that it could potentially decrease the eventual immunosuppression created by thermal injury.

Histologic examination of skin from the saline-treated control animals showed a loss of most of the epidermis. There was also a diffuse cellular infiltrate in the subepidermal region that extended into the lower dermal connective tissue in which collagen fibrils are separated by the infiltrating leukocytes and edema fluid (Fig 5, A). At an increased magnification (not shown), the cellular infiltrate between the collagen bundles consisted almost entirely of neutrophils. Edema fluid caused separation of the collagen fibrils.

Figure 5, B, shows skin subjected to thermal injury after inoculation with P. aeruginosa, and then application of topical nanoemulsion to the burned area. The keratin layers of the epidermis were separating, and some of the keratin had been lost. There was a barely detectable intradermal presence of neutrophils together with neutrophils that were adhering to the wall of a venule, which had been longitudinally sectioned (as shown in the center of the microphotograph). The changes in this microphotograph were substantially less extensive than those shown in Fig 5, A.

Quantification of capillary leak and tissue edema. Evans blue is a dye that binds to serum albumin and can be quantitated to determine vascular permeability. Topical NB-201 treatment resulted in less Evans blue dye leaking out of the bloodstream and into the skin tissue following thermal injury and bacterial inoculation of the wound when compared to

**Fig 4.** Burn injury upregulates dermal tumor growth factor (TGF)-β expression and treatment with NB-201 in the setting of burn wound infection decreases the level of TGF-β in the wound. Dermal levels of the antiinflammatory cytokines interleukin (IL)-10 and TGF-β were measured in the burn wound 32 hours after thermal injury in animals treated with nanoemulsion and in animals exposed to bacteria and treated with NB-201 or Sulfamylon. (A) IL-10 (P = .4, 1-way analysis of variance [ANOVA]), and (B) TGF-β (P = .0001). Partial-thickness burn increased the presence of dermal TGF-β compared to sham (624 ± 55 vs 232 ± 17 pg/mL). Treatment of an infected burn wound with NB-201 significantly decreased the dermal level of TGF-β compared to the untreated burn group (404 ± 43 vs 624 ± 55 pg/mL). Sulfamylon treatment did not suppress the level of IL-10 or TGF-β in the infected burn wound. *P < .05, Tukey multiple comparison test.

**NB-201 treatment after burn injury attenuates dermal proinflammatory cytokine levels.** Scald injury resulting in a partial-thickness burn produced differences in dermal levels of IL-1β and CINC-3 within skin homogenates obtained 32 hours postinjury compared to sham-injured animals (Fig 2, A and E). Treatment with NB-201 at 16 and 24 hours postburn reduced the dermal level of these 2 inflammatory mediators to the baseline (sham) in the absence of bacterial infection.

In experiments where a bacterial wound infection was not created, a difference in neutrophil sequestration as measured by myeloperoxidase assay was not observed despite the rise in the rat CXC chemokine CINC-3 within burned skin. A difference was found between all 3 groups (sham, burn, and burn + NB-201) for CINC-1 (P = .04, ANOVA); however, the values for intergroup comparison did not reach statistical significance.

**NB-201 treatment after burn wound infection with P. aeruginosa attenuates dermal cytokine levels and results in decreased neutrophil sequestration.** Skin homogenates from the nanoemulsion-treated group had levels of IL-1β and IL-6 that were considerably diminished compared to the levels measured in the saline-treated animals (Fig 3, A and B). There was no statistically significant difference seen in the level of TNF-α between the 2 experimental groups of animals (Fig 2, C, and Fig 3, C). Treatment of the infected burn wound with Sulfamylon did not result in any significant alteration of dermal levels of the measured proinflammatory cytokines (IL-1β, IL-6, TNF-α, CINC-1, or CINC-3) compared to controls. Treatment with either NB-201 or Sulfamylon decreased the level of myeloperoxidase found in the infected burn wound at 32 hours postinjury. This observation suggests that treatment with an antimicrobial decreases the level of neutrophil sequestration into the partial-thickness burn wound.

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Figure 5, B, shows skin subjected to thermal injury after inoculation with P. aeruginosa, and then application of topical nanoemulsion to the burned area. The keratin layers of the epidermis were separating, and some of the keratin had been lost. There was a barely detectable intradermal presence of neutrophils together with neutrophils that were adhering to the wall of a venule, which had been longitudinally sectioned (as shown in the center of the microphotograph). The changes in this microphotograph were substantially less extensive than those shown in Fig 5, A.

Quantification of capillary leak and tissue edema. Evans blue is a dye that binds to serum albumin and can be quantitated to determine vascular permeability. Topical NB-201 treatment resulted in less Evans blue dye leaking out of the bloodstream and into the skin tissue following thermal injury and bacterial inoculation of the wound when compared to
saline treated control animals (1.26 ± 0.05 vs 1.93 ± 0.24 μg Evans blue/mg tissue, n = 8 per group; P = .02, t test). Therefore, nanoemulsion-treated animals had less evidence of postburn capillary leak and tissue edema than the saline-treated controls.

**Treatment with NB-201 decreases burn-induced hair follicle apoptosis.** Dermal apoptosis occurred in the hair follicle cells after thermal injury. Using a fluorescence-labeled TUNEL assay, the burn wounds treated with saline showed evidence of intense fluorescein isothiocyanate (FITC)-labeled/TUNEL-positive cells that appeared green as shown in Fig 6. The DAPI nuclear stain allowed identification of coronal or sagittally sectioned hair follicles with the cells staining blue. FITC-TUNEL-positive cells appear green and are representative of apoptotic cells. In the merged images, the apoptotic hair follicle cells are evident in slides from the burn + saline animals, and these changes are diminished in the burn + NB-201–treated animals.

Counting the pixels of TUNEL-positive cells within a hair follicle ROI allowed quantification of the reduction in hair follicle cell apoptosis by treatment with topical NB-201 (Fig 7). The saline-treated control animals had an increased amount of TUNEL-positive cells compared to the sham burn animals. Both the NB-201 and placebo treatment resulted in a decrease in hair follicle cell apoptosis after partial-thickness burn injury in tissue harvested 12 hours after thermal injury. This difference was not evident in the dermal skin sampled at 24 hours postburn. To summarize, treatment with NB-201 decreased apoptotic cell death in hair follicles in the early postburn period.

**DISCUSSION**

NB-201, the nanoemulsion formulation used in these experiments, is capable of decreasing and, in some cases, eradicating a *P. aeruginosa* wound infection within a partial-thickness burn wound. We found that this decrease in microbial infection was coupled with the generation of lower levels of local dermal proinflammatory cytokines and evidence of decreased neutrophil sequestration into the burn wound. This decrease in burn wound bacterial growth and inflammation also produced less capillary leak in the early post-thermal injury time period. Having the ability to clinically decrease capillary leak and tissue edema in the immediate postburn time period could result in a decreased need for large-volume crystalloid fluid resuscitation and a decrease in the associated sequelae of physiologic volume overload, pulmonary dysfunction, and abdominal compartment syndrome.

Skin that is damaged by thermal injury loses its ability to protect the host against infection from both the loss of physical barrier function and the secondary immunosuppression caused by the thermal injury. Increased production of TGF-β and...
IL-10 during the postburn period can result in immunosuppression. It has been established that treatment of burn-injured animals with anti-TGF-β can improve local and systemic clearance of P. aeruginosa. Inhibition of TGF-β also results in increased survival after bacterial challenge. In our experiments, we found a significant elevation of TGF-β, but not IL-10, in the skin after partial-thickness burn injury. Topical application of NB-201 to the burn wound inoculated with bacteria resulted in a decrease of the level of TGF-β compared to the untreated burn wound.

Onset of a bacterial infection within a burn wound can delay or even reverse the tissue-healing process. Topical antimicrobial therapy is used to decrease the microbial load in the burn wound and decrease this risk of infection. Current topical agents include silver nitrate (AgNO₃), silver sulfadiazine, mafenide acetate, and nanocrystalline-impregnated silver dressings. Thermal injury initiates dermal inflammatory and proapoptotic cell signaling. None of the above listed agents acts principally to decrease burn wound inflammation.

In this study, topical application of NB-201 resulted in decreased hair follicle cell apoptosis within the dermis of burned skin. This observation suggests that NB-201 may be helpful in decreasing conversion of the partial-thickness burn wound within the "zone of stasis" to regions of full-thickness burn. A limitation of our experimental model is that we created a partial rather than a full-thickness burn wound. We chose this type of burn specifically, so that we could investigate the hair follicle response to the topical treatment agents.

In patients without evidence of inhalational injury, the burn wound itself is the primary source triggering the systemic inflammatory response via the generation of proinflammatory cytokines and the sequestration of neutrophils into the burn wound. Topical application of SB202190, an inhibitor of activated p38 mitogen-activated protein kinase (MAPK), can control the source of inflammation at the level of the dermis, resulting in lower levels of proinflammatory mediators, decreased neutrophil sequestration and microvascular damage, and less epithelial apoptosis in burn wound hair follicle cells. Dermal source control of inflammation also decreases bacterial growth and attenuates the systemic inflammatory response, resulting in less acute lung injury and cardiac dysfunction after partial-thickness burn injury in a rodent model.

Our search for a potential vehicle in which to deliver this compound led us to consider nanoemulsion technology. The ultimate future therapeutic goal...
is to couple an antimicrobial vehicle (NB-201) with a synergistic anti-inflammatory agent (SB202190 or a similar inhibitor of activated p38 MAPK) to decrease local dermal inflammation and the risk of infection within early burn wounds.

During the 1990s, the Michigan Nanotechnology Institute developed a composite material that resulted in a new class of antimicrobial agents with broad activity against gram-positive and gram-negative bacteria, spores, fungi, and viruses.4-7 These nanoemulsions are oil-in-water mixtures containing high-energy, nanometer-sized droplets stabilized by surfactants. The spectrum of antimicrobial activity can be altered depending on which detergents and solvents are added to stabilize the emulsion.6

The addition of ethylene diamine tetraacetic acid (EDTA) permeabilizes the outer membrane of gram-negative bacteria, and enhances the intrinsic activity of the nanoemulsion against P. aeruginosa. The dilute emulsions are milky white in consistency and appearance. Thickeners can be added to increase viscosity and decrease running. This was not done in the current experiment because we wished to avoid mechanical manipulation of the skin surface after inoculation with bacteria.

Pathogen killing is a function of the nanoemulsion particles being thermodynamically driven to fuse with lipid-containing cell walls of bacteria or other organisms. This fusion is enhanced by electrostatic attraction between the cationic-charged emulsion particles and the anionic charge of the pathogen cell wall. When a critical amount of nanoparticles fuse with the pathogen, they release part of the energy trapped within the emulsion. Both the active ingredient and the released energy act to destabilize the pathogen lipid membrane, which results in cell lysis and death.5-7

The nanoemulsion material is selectively toxic to microbes at concentrations that are nonirritating to human skin or mucous membranes. An FDA phase 2b clinical trial for topical treatment of Herpes labialis using a similar formulation has been completed.25,26 No safety issues were identified, and the treatment was both efficacious and well tolerated.27 Because of their small particle size and surface-active properties, nanoemulsions are believed to traverse skin pores and hair follicles, while being excluded from entering the tight junctions of the epithelium.

Fluorescent labeling of a similar nanoemulsion confirmed that it is distributed preferentially in the hair follicles and sebaceous glands after application to human cadaver skin.28 Thus, the nanoemulsion compound can be highly bioavailable in the dermal tissues without disrupting the normal epithelial matrix. This property, coupled with its antimicrobial activity and low toxicity, makes NB-201 a highly desirable topical agent to be used alone or as a vehicle to deliver additional agents to enhance antiinflammatory treatment of burn wounds.

In conclusion, topical nanoemulsion therapy with NB-201 significantly decreased bacterial growth in a partial-thickness burn model. Killing of the inoculated pathogen P. aeruginosa resulted in attenuation of the local dermal inflammatory response and decreased neutrophil infiltration into the burn wound. NB-201 is a novel potent antimicrobial treatment for potential use in clinical burn wounds.

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REFERENCES


