

In Vitro Antibacterial Activity of NB-003 against *Propionibacterium acnes*[▽]

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NB-003 and NB-003 gel formulations are oil-in-water nanoemulsions designed for use in bacterial infections. *In vitro* susceptibility of *Propionibacterium acnes* to NB-003 formulations and comparator drugs was evaluated. Both NB-003 formulations were bactericidal against all *P. acnes* isolates, including those that were erythromycin, clindamycin, and/or tetracycline resistant. In the absence of sebum, the MIC_{90s}/minimum bactericidal concentrations (MBC_{90s}) for NB-003, NB-003 gel, salicylic acid (SA), and benzoyl peroxide (BPO) were 0.5/2.0, 1.0/2.0, 1,000/2,000, and 50/200 µg/ml, respectively. In the presence of 50% sebum, the MIC_{90s}/MBC_{90s} of NB003 and BPOs increased to 128/1,024 and 400/1,600 µg/ml, respectively. The MIC_{90s}/MBC_{90s} of SA were not significantly impacted by the presence of sebum. A reduction in the MBC_{90s} for NB-003 and BPO was observed when 2% SA or 0.5% BPO was integrated into the formulation, resulting in MIC_{90s}/MBC_{90s} of 128/256 µg/ml for NB003 and 214/428 µg/ml for BPO. The addition of EDTA enhanced the *in vitro* efficacy of 0.5% NB-003 in the presence or absence of 25% sebum. The addition of 5 mM EDTA to each well of the microtiter plate resulted in a >16- and >256-fold decrease in MIC₉₀ and MBC₉₀, yielding a more potent MIC₉₀/MBC₉₀ of ≤1/<1 µg/ml. The kinetics of bactericidal activity of NB-003 against *P. acnes* were compared to those of a commercially available product of BPO. Electron micrographs of *P. acnes* treated with NB-003 showed complete disruption of bacteria. Assessment of spontaneous resistance of *P. acnes* revealed no stably resistant mutant strains.

Propionibacterium acnes is a Gram-positive, non-spore-forming, anaerobic bacillus and resides in normal facial and nasal microflora. *P. acnes* is one of the primary factors involved in the pathogenesis of acne vulgaris. It is a predominant microorganism of the pilosebaceous glands of human skin, with up to 10⁷ viable organisms isolated from a single sebaceous unit (37, 38). Although aerotolerant, *P. acnes* typically grows in the anaerobic environment of the infrainfundibulum of the pilosebaceous unit, where it releases lipases and digests local accumulations of the skin and sebum (oily substance produced by sebaceous glands). Sebum is primarily composed of glycerides, triglycerides, wax esters, squalene, and free fatty acids (40, 61, 62).

Most cases of mild acne are treated with topical antibacterials containing benzoyl peroxide, erythromycin, clindamycin, or tetracycline with salicylic acid as a keratolytic agent (4).

If inflammatory acne lesions are present, the Global Alliance to Improve Outcomes in Acne recommends a combination therapy of an antibacterial with a topical retinoid that exhibits both direct anti-inflammatory and comedolytic activities as first-line therapy in patients with mild, moderate, and, in many cases, severe acne (12). Each year, 5 million prescriptions for oral antibiotics and 1.4 million prescriptions of oral isotretinoin are dispensed for treatment of moderate to severe acne (56). A combination of topical benzoyl peroxide (BPO) and a retinoid can cause erythema, scaling, burning, and stinging in some patients, particularly during the first 2 to 3 weeks of treatment. Therefore, there have been numerous attempts to minimize the irritation associated with BPO, and various

drug delivery technologies have been developed (13, 14, 25, 64). Studies have confirmed that the combination of benzoyl peroxide and a topical antibiotic (e.g., clindamycin, erythromycin) is just slightly more effective than either agent alone, with lesser skin irritation observed than with benzoyl peroxide monotherapy (57). In more severe acne (grades III and IV) characterized by numerous papules and/or cysts, oral therapy consisting of an oral retinoid and/or oral antibiotic (erythromycin, tetracycline, minocycline, doxycycline) is prescribed. Oral retinoids (isotretinoin) shrink the sebaceous glands but have severe side effects (major birth defects, depression, hair loss, etc.) and are only used in very severe or cystic acne (52, 59).

Although antibiotic therapy has been used for more than 40 years to treat acne, resistance to antibiotics became noticeably prevalent in 1970 (36). Recent studies in six European countries identified that acne patients carried *P. acnes* isolates with at least one resistance determinate, with frequencies ranging from 51% in Hungary to 94% in Spain. Combined resistance to erythromycin and clindamycin was very common, with 91% of the isolates in Spain having this resistance phenotype (53, 54). Another study in the United Kingdom showed that the proportion of patients carrying strains resistant to one or more commonly used antiacne antibiotics rose steadily from 34.5% in 1991 to a peak of 64% in 1997. The prevalence dropped to 50.5% during 1999 and then rose again to 55.5% in 2000 (9). Resistance to erythromycin was the most common, and the majority of erythromycin-resistant strains were cross-resistant to clindamycin. Thus, any new agent seeking to treat acne by reducing the *P. acnes* burden should not be cross-resistant to erythromycin, clindamycin, or tetracycline, the most commonly used antibiotics for acne treatment. Quaternary ammonium compounds showed the *in vitro* MICs comparable to relevant antibiotics used in acne treatment, suggesting the possibility of

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replacing antibiotics in the antimicrobial therapy of acne by quaternary ammonium compounds (18).

Nanomaterials have unique structural properties, such as a large surface-area-to-mass ratio and high affinity of binding to surfaces. Nanoparticle-based therapeutics have been approved for clinical use, such as liposome and polymer-based technology (65). Antimicrobial nanoemulsions are highly stable oil-in-water emulsions composed of nanometer-sized, positively charged droplets that have broad-spectrum activity against enveloped viruses, fungi, and bacteria (2, 17, 19–21, 41, 47–50, 58). NB-003 (NanoStat, NanoBio Corp., Ann Arbor, MI) contains the cationic quaternary ammonium compound cetylpyridinium chloride (CPC) oriented at the oil-water interface, which also stabilizes the nanoemulsion droplets, contributes to the anti-infective activity, and serves as a marker for the pharmacokinetic, pharmacodynamic, and toxicological studies.

These studies assessed the *in vitro* antibacterial activity of NB-003 with and without other conventional antimicrobial agents against *P. acnes*, including isolates resistant to one or more common antibiotics used in the treatment of acne. Antibacterial synergy of NB-003 with other antiacne compounds was also evaluated, as was the effect of EDTA. Spontaneous resistance to NB-003 was also assessed.

MATERIALS AND METHODS

Nanoemulsion manufacturing and potency. NB-003 is an oil-in-water nanoemulsion manufactured from ingredients that are on the FDA list of inactive ingredients of approved drug products. The emulsion is made by high-speed emulsification of highly purified oil, ethanol, polysorbate 20, cetylpyridinium chloride (CPC), and water. NB-003 gel formulation is modified to include a thickening agent. Placebo (vehicle) nanoemulsions were made from the same ingredients without CPC. The mean nanoemulsion droplet size is 180 nm as measured by dynamic light scattering using a Malvern Zetasizer Nano ZS3600 (Malvern Instruments Ltd., Worcestershire, United Kingdom). The relative activity of NB-003 is expressed in terms of the concentration of cationic surfactant ($\mu\text{g CPC/ml}$).

Source of comparator compounds. Erythromycin and tetracycline were purchased from Sigma Chemicals (St. Louis, MO), clindamycin was obtained from USP (Rockville, MD), and salicylic acid was purchased from J.T. Baker (Phillipsburg, NJ). Benzoyl peroxide (BPO) was obtained from commercial products: Persa-Gel 10 (Johnson & Johnson, Skillman, NJ), Clearasil (Reckitt Benckiser, Parsippany, NJ), and Proactiv (Proactiv Solution, Des Moines, IA).

Source of *P. acnes* isolates. Three ATCC isolates were obtained from American Type Culture Collection (Manassas, VA). Thirteen clinical isolates of *P. acnes* were obtained from Basilea Pharmaceutica (Basel, Switzerland) and have been previously described by Heller et al. (23). Twelve of these isolates had defined resistance mechanisms to erythromycin, clindamycin, and/or tetracycline. The resistance mechanisms as shown in Table 2 were mutations in either the 16S or 23S rRNA conferring tetracycline resistance or erythromycin resistance with or without clindamycin resistance, respectively, or resistance was conferred by an *erm(X)* methylase that dimethylates residue A2058 in 23S rRNA (*Escherichia coli* numbering of 23S rRNA residue), conferring high-level erythromycin and clindamycin resistance (23). Resistance is defined as erythromycin, $\geq 0.5 \mu\text{g/ml}$; clindamycin, $\geq 0.25 \mu\text{g/ml}$; or tetracycline, $\geq 2 \mu\text{g/ml}$ (44, 45).

MIC/minimum bactericidal concentration (MBC) determination. MICs in the presence or absence of 50% artificial sebum (40) were determined using the broth microdilution method in Wilkins-Chalgren broth (Becton, Dickinson & Co., Sparks, MD) as described in the Clinical and Laboratory Standards Institute (CLSI) methodology for anaerobic bacteria (7, 55). Sixteen *P. acnes* isolates were tested once per isolate, and then the MIC₅₀s and MIC₉₀s were calculated accordingly. Premade drug plates were inoculated with a culture suspension of *P. acnes* to give $\sim 10^6$ CFU/ml. Two quality control (QC) isolates, ATCC 11827 and ATCC 11828, were included on each day of testing. Inoculum concentration was verified by plating the serially diluted inoculum on Trypticase soy agar containing 5% sheep blood (Becton, Dickinson & Co., Sparks, MD). Microtiter plates and blood agar plates (BAP) were incubated at 35 to 37°C in a 7-liter anaerobic incubation jar (Mitsubishi Gas Chemical, New York, NY) fitted with an anaerobic

gas-generating system (Mitsubishi Gas Chemical, New York, NY) and a dry anaerobic indicator strip (Becton, Dickinson & Co., Sparks, MD). MICs were read after 48 h using an illuminated microtiter plate reader fitted with a magnifying mirror (Biodesign, New York, NY). Verification of viable counts of inocula was made after 72 h of incubation.

MBC was determined by using the standard CLSI methodology (8). Briefly, 10 μl was removed from the well that defined the MIC and four or more concentrations above the MIC and was plated onto blood agar plates. In our study, the antibacterial effect of CPC was neutralized by the ingredients present in the Wilkins-Chalgren broth used in the 96-well plate and by the dilution effect of spreading the 10 μl on the blood agar plates. Additionally, to avoid antibacterial carryover, the aliquots were allowed to soak into the agar and then were spread for isolation, thus removing the cells from the drug source. MBC was defined as the lowest concentration of drug that reduced the initial inoculum by ≥ 3 logs. A compound is considered bactericidal if the MBC/MIC ratio is ≤ 4 . Bacteriostatic compounds have an MBC/MIC ratio of >4 (8). The MBC₅₀ and the MBC₉₀ are the lowest concentrations of an antimicrobial agent that collectively kill (≥ 3 log reduction) 50% or 90% of the isolates tested, respectively. Tolerance was defined as an MBC/MIC ratio of ≥ 32 (8, 51).

The effect of EDTA on the antibacterial activity of the nanoemulsion formulations was evaluated in MIC/MBC assays by adding 1, 5, 10, or 20 mM EDTA to each well.

Determination of spontaneous resistance frequencies. Agar-based single-step mutation studies were performed as described in the literature (1, 11, 29, 30, 42, 46). Blood agar plates containing 1, 2, 4, or 8 times the respective agar-based MIC of NB-003, clindamycin, tetracycline, or BPO were inoculated with 10^9 to 10^{10} CFU of *P. acnes* and incubated for 3 to 4 days at 35°C. The spontaneous resistance frequency for an isolate-drug combination was calculated from the number of colonies that grew on plates containing drug versus the number of colonies that grew on drug-free agar. To ensure that drug-selective pressure was maintained, randomly selected colonies recovered from the plates were subcultured to blood agar plates containing the compound at the selecting concentration and used as inoculum for broth-based MIC determination.

Time-kill studies. *In vitro* time-kill studies were performed in broth as well as on the surface of pig skin. Assessment of bacterial reduction in broth was performed in a test tube using 10 times the MBC. Nanoemulsion samples were prepared at 2 times the final test concentration in deionized water, and a culture suspension in 2 \times broth was added in a 1:1 ratio to achieve the final drug concentration and inoculum. At predetermined time points, samples were serially diluted and 100 μl of each sample was plated onto a BAP. Colony counts were assessed after 3 to 5 days under anaerobic conditions.

Time-kill studies were also performed after incubation of *P. acnes* on the surface of swine skin. Swine abdominal skin was obtained from Sinclair Research Center (Auxvasse, MO). The subcutaneous fat was removed manually such that the pilosebaceous units were left intact. Glass rings (2-cm diameter by 1-cm height) were glued to the skin surface with super glue and placed in 6-well tissue culture plates and hydrated with 500 μl of sterile water and stored at 2 to 8°C overnight prior to use. The next day, water was removed from wells containing skin samples, and 30 μl of a *P. acnes* culture was applied to the surface of the pig skin within the confines of the glass ring to give a concentration of 6 to 7 logs of CFU/cm². Inoculated skin samples were incubated under anaerobic conditions for 1 h to allow absorption and adherence of bacteria to the skin surface. Following 1 h of equilibration, 50 μl of drug was applied to the skin samples (17.7 $\mu\text{l/cm}^2$ of skin); this dose of drug provided an application similar to the topical application of BPO used in humans. Drug-treated skin samples were incubated under anaerobic conditions and at predetermined times samples were removed from the incubator. Each skin sample was rinsed with 1 ml cold saline twice to collect any viable bacteria still existing on the skin. The combined rinses were pooled and serially diluted, and 100 μl of all dilutions, including the initial pooled rinse, was plated onto BAP and incubated anaerobically at 35 to 37°C. Control experiments determined that samples containing NB-002 and BPO had to be diluted 1:10 to remove residual activity (data not shown).

Electron microscopy. To observe the morphology of *P. acnes* after different times of nanoemulsion exposure, 450- μl samples from different time points of a time-kill study were mixed with 113 μl of fixative (10% aqueous solution of glutaraldehyde). Mixtures were vortexed and placed at 4°C for at least 18 h. Samples were further processed for scanning electron microscopy as described previously (48).

Determination of interactions between drugs. Interactions between NB-003 and BPO in the presence of 50% artificial sebum were determined using checkerboard methodology (3, 63). The fractional inhibitory concentration (FIC) for each drug was calculated for each strain as follows: FIC for NB-003 = MIC of

TABLE 1. MIC/MBCs of NB-003 and comparators against *P. acnes*

Agent(s)	MIC/MBC (µg/ml)					
	Without sebum (n = 16 isolates)			With 50% sebum (n = 16 isolates)		
	90%	50%	Range	90%	50%	Range
NB-003	0.5/2	0.5/2	0.25–1/0.5–2	128/1,024	128/1,024	64–256/256–1,024
NB-003 gel	1/2	0.5/21	0.5–1/1–4	128/1,024	128/1,024	64–256/128–>1,024
Erythromycin	1,000/>1,000	4/>1,000	≤0.25–>1,000/62.5–>1,000	1,000/>1,000	4/1,000	≤0.25–>1,000/62.5–>1,000
Clindamycin	500/>1,000	4/1,000	≤0.125–500/125–>1,000	500/>1,000	1/100	≤0.125–500/62.5–>1,000
Tetracycline	31/1,000	0.5/250	≤0.063–31.3/31.3–1,000	31.3/1,000	0.5/250	≤0.063–31.3/31.3–1,000
Benzoyl peroxide	50/200	50/200	≤50–100/100–400	400/1,600	200/800	≤50–400/800–1,600
Salicylic acid	1,000/2,000	1,000/2,000	50–1,000/2,000–>2,000	1,000/2,000	1,000/2,000	500–1,000/2,000–>2,000
0.3% NB-003 gel + 0.5% BPO	0.5/4	0.5/4	0.5–1/2–4	128/256	128/128	128/128–256
0.3% NB-003 gel + 2% salicylic acid	0.5/2	0.5/2	0.25–1/0.5–2	128/256	128/128	128/128–256

NB-003 in combination/MIC of NB-003 alone; FIC of BPO = MIC of BPO in combination/MIC of BPO alone.

The average FIC index for each isolate was calculated using FIC indices of all tested drug combinations: FIC index (Σ FIC) = FIC of NB-003 + FIC of BPO.

The fractional bactericidal concentrations (FBC) and FBC index were calculated using MBCs instead of MICs.

Interpretation. Synergism is a Σ FIC of ≤0.5; indifference is a Σ FIC of >0.5 and ≤4; and antagonism is a Σ FIC of >4.

RESULTS

NB-003 is bactericidal for *P. acnes*. The antimicrobial activities of NB-003 and other antiacne agents, such as erythromycin, clindamycin, tetracycline, BPO, salicylic acid, and chlorhexidine, were evaluated using clinical isolates of *P. acnes*. In the absence of sebum, the MICs of NB-003 or NB-003 gel formulations ranged from 0.25 to 1.0 µg/ml, and the MBCs ranged from 0.5 to 4 µg/ml (Table 1). The MIC₉₀s and MBC₉₀s were 0.5 µg/ml and 2.0 µg/ml for 0.3% NB-003 and 1 µg/ml and 2 µg/ml for 0.3% NB-003 gel, respectively. More than 50% of the isolates tested in this study were resistant to erythromycin and clindamycin; 44% of the isolates were resistant to tetracycline. The NB-003 formulations were bactericidal against all the isolates, including strains that were erythromycin, clindamycin, and/or tetracycline resistant. The placebo formulation did not exhibit any antibacterial activity.

Synergism between NB-003 and BPO. Since NB-003 is a nanoemulsion and is preferentially taken up by the transfollicular route (5), incorporation of another antiacne drug into the nanodroplets may also be effectively codelivered to the site of infection. Thus, we investigated the antibacterial activity of NB-003 gel formulated with either benzoyl peroxide or salicylic acid and compared the MICs and MBCs of the combination nanoemulsions to those of benzoyl peroxide or salicylic acid alone. In the presence of 50% artificial sebum, a decrease in MBCs of NB-003, BPO, and salicylic acid to *P. acnes* was observed. The two combination nanoemulsions (NB-003–BPO or NB-003–salicylic acid) had 4-times-lower MBCs (256 µg/ml) than NB-003 (1,024 µg/ml).

Interactions of NB-003 and BPO in the presence of 50% artificial sebum were evaluated by checkerboard microdilution methodology. Ten isolates of *P. acnes* were tested for susceptibility to NB-003–BPO combinations. The FIC and FBC indices for the NB-003–BPO combination ranged from 0.4 to 0.6

and 0.2 to 0.7, respectively (Table 2). This combination showed synergy against 90% of isolates based on MICs and 80% of isolates based on MBCs, with none of the isolates showing antagonism.

EDTA enhances the activity of NB-003. Broth-based MICs of NB-003 with different concentrations of EDTA were evaluated in the presence of 25% artificial sebum. The addition of EDTA reduced the MIC and MBC of NB-003 in the presence of 25% artificial sebum (Table 3). In the absence of EDTA, the MIC₉₀/MBC₉₀ was 0.5/2 µg/ml for NB-003 without sebum and increased to 32/512 µg/ml in the presence of 25% sebum. With the addition of 1 mM to 20 mM EDTA to each well of a microtiter plate containing 0.5% NB-003, MBC₉₀s and MBC₅₀s ranged from >16 to ≤1 µg/ml. The addition of 5 mM EDTA resulted in an MBC₉₀ of 4 µg/ml, a more than 256-fold decrease in MBC₉₀ compared to the MBC₉₀ of NB-003 alone.

TABLE 2. Resistance phenotype characterization and FIC/FBC index values of NB-003–BPO against *P. acnes* isolates

Strain no.	Resistance phenotype ^a	Average synergy indices ^b	
		FIC	FBC
PAC-001	Ery ^s Cli ^s Tet ^s (ATCC6919)	0.4	0.2
PAC-003	Ery ^s Cli ^s Tet ^s (ATCC11828)	0.4	0.4
PAC-004	Ery ^s Cli ^s Tet ^s	0.6	0.5
PAC-005	Ery ^s Cli ^r Tet ^s (unknown)	0.4	0.7
PAC-006	Ery ^r Cli ^r Tet ^r (G2057A + G1058C)	0.5	0.3
PAC-007	Ery ^r Cli ^r Tet ^r (A2058G + G1058C)	0.4	0.3
PAC-008	Ery ^r Cli ^r Tet ^r (A2058G + G1058C)	0.4	0.4
PAC-009	Ery ^r Cli ^r Tet ^s (A2059G)	0.5	0.6
PAC-010	Ery ^r Cli ^r Tet ^r (A2059G + G1058C)	0.5	0.4
PAC-011	Ery ^r Cli ^r Tet ^s (<i>ermX</i>)	0.4	0.4
PAC-002	Ery ^s Cli ^s Tet ^s (ATCC11827)	NA	NA
PAC-012	Ery ^s Cli ^s Tet ^r (G1058C)	NA	NA
PAC-013	Ery ^r Cli ^r Tet ^r (unknown)	NA	NA
PAC-014	Ery ^r Cli ^r Tet ^r (<i>ermX</i>)	NA	NA
PAC-015	Ery ^r Cli ^r Tet ^s (G2057A)	NA	NA
PAC-016	Ery ^r Cli ^r Tet ^s (A2058G)	NA	NA

^a Ery^{s/r}, erythromycin susceptible/resistant; Cli^{s/r}, clindamycin susceptible/resistant; Tet^{s/r}, tetracycline susceptible/resistant. The resistance breakpoints are erythromycin, ≥0.5 µg/ml; clindamycin, ≥0.25 µg/ml; tetracycline, ≥2 µg/ml. Unknown, the genetic basis of antibiotic resistance is unknown.

^b NA, not tested in synergy study.

TABLE 3. MIC/MBCs of NB-003 tested with different concentrations of EDTA

Agent(s)	MIC/MBC ($\mu\text{g/ml}$) ^a					
	Without sebum ($n = 10$ isolates)			With 25% sebum ($n = 10$ isolates)		
	90%	50%	Range	90%	50%	Range
0.5% NB003	0.5/2	0.5/2	0.25–0.5/1–2	32/512	16/256	16–32/128–>512
0.5% NB-003 + 1 mM EDTA/well	$\leq 0.063/2$	$\leq 0.063/>1$	$\leq 0.063/1->1$	$\leq 1/>16$	$\leq 1/>16$	$\leq 1/16->16$
0.5% NB-003 + 5 mM EDTA/well	$\leq 0.063/0.5$	$\leq 0.063/0.125$	$\leq 0.063/\leq 0.063-0.5$	$\leq 1/4$	$\leq 1/<1$	$\leq 1/<1-4$
0.5% NB-003 + 10 mM EDTA/well	$\leq 0.063/\leq 0.063$	$0.063/\leq 0.063$	$0.063/\leq 0.063$	ND/ ≤ 1	ND/ ≤ 1	ND/ ≤ 1
0.5% NB-003 + 20 mM EDTA/well	$\leq 0.063/\leq 0.063$	$0.063/\leq 0.063$	$0.063/\leq 0.063$	ND/ ≤ 1	ND/ ≤ 1	ND/ ≤ 1

^a ND, not done.

Time-kill and mechanism-of-action studies. The kinetics of bactericidal activity of NB-003 and BPO were evaluated against two isolates of *P. acnes*, ATCC 6919 and multidrug-resistant clinical isolate PAC-008. Figure 1 shows that NB-003 at a concentration of 20 $\mu\text{g/ml}$ is rapidly bactericidal *in vitro* (test tube), causing a more than 2-log reduction on contact (time zero) and a more than 6-log reduction after 10 min of exposure time. In a pig skin model, there was dose-dependent killing of *P. acnes* in the presence of 0.001 to 0.25% NB-003, and 0.1% and 0.25% NB-003 were shown to be more potent in killing *P. acnes* on pig skin than 2.50% BPO (Fig. 2). Both 0.1% and 0.25% NB-003 (3.8- and 4.2-log reduction, respectively) reduced the bacterial counts significantly more effectively than Clearasil (5% BPO) (2.6-log reduction) or Proactiv (2.5% BPO) (2.5-log reduction). Figure 3 shows the morphology of *P. acnes* bacterium by using a scanning electron microscope before and after a 1-h treatment with 20 $\mu\text{g/ml}$ (or 0.002%) NB-003. The integrity of the multidrug-resistant rod-shaped bacterium is rapidly destroyed, leaving no doubt that NB-003 is rapidly bactericidal.

Spontaneous resistance frequency. Sixteen isolates tested for agar-based MIC showed MIC ranges of 4 to 16, 781, 0.5 to >64, and 0.5 to >64 $\mu\text{g/ml}$ for NB003, BPO, clindamycin, and tetracycline, respectively. Twelve to 16 isolates of *P. acnes* were evaluated for spontaneous resistance frequency on agar plates

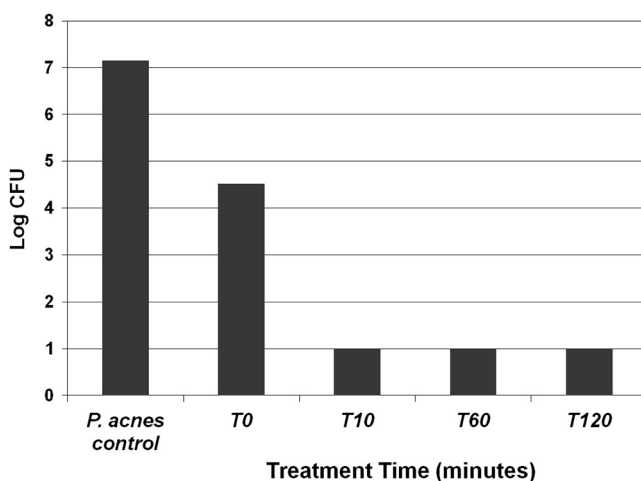


FIG. 1. The impact of NB-003 on the viability of *P. acnes* PAC-001. Time course of *P. acnes* in broth treated with 20 $\mu\text{g/ml}$ NB-003. The lower limit of detection was 10 CFU, because a 1:10 dilution was necessary to neutralize the antibacterial carryover.

containing 1, 2, 4, or 8 times the respective MIC of NB-003, tetracycline, clindamycin, or BPO. At 2 \times the MIC of tested drugs, phenotypic mutants appeared at a frequency of 2.5×10^{-11} , $>3.8 \times 10^{-7}$, and $>2.2 \times 10^{-7}$ for NB-003, tetracycline, and clindamycin, respectively. No mutants appeared on any plates containing BPO. The frequencies of mutants phenotypically resistant to NB-003 at 1 \times , 2 \times , 4 \times , and 8 \times MIC are given in Table 4. The ranges of spontaneous resistance frequency for NB-003, tetracycline, and clindamycin were 2.5×10^{-11} to $<2.7 \times 10^{-10}$, $<2.5 \times 10^{-11}$ to $>3.8 \times 10^{-7}$, and $<3.4 \times 10^{-11}$ to $>2.2 \times 10^{-7}$, respectively.

Despite the growth on agar plates containing higher concentrations of selecting compound, most of the phenotypically resistant isolates had MICs that were ± 2 -fold the initial MIC for all compounds when retested in broth. One mutant selected with tetracycline had a >16-fold increase in MIC for tetracycline compared to that of the parent isolate. MICs of recovered isolates tested by microtiter broth ranged from 0.25 to 0.5 $\mu\text{g/ml}$, ≤ 0.125 to 32 $\mu\text{g/ml}$, ≤ 0.125 to >64 $\mu\text{g/ml}$, and 24 to 98 $\mu\text{g/ml}$ for NB-003, tetracycline, clindamycin, and BPO, respectively.

DISCUSSION

NB-003, an oil-in-water nanoemulsion with broad-spectrum antimicrobial activity, was optimized for topical treatment of

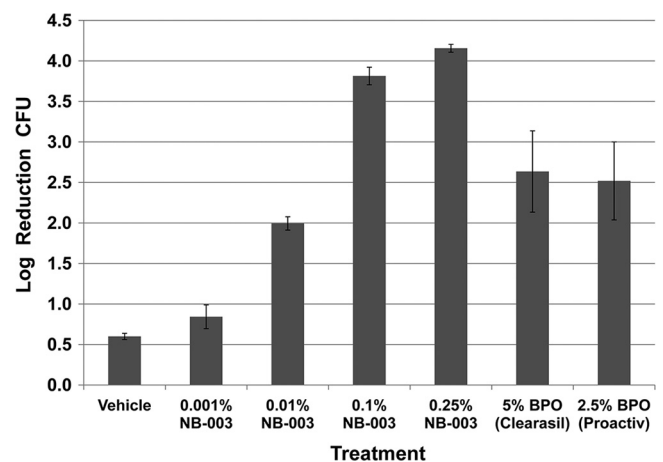


FIG. 2. The impact of NB-003 and BPO on the viability of *P. acnes* PAC-008, a clinical isolate resistant to tetracycline, erythromycin, and clindamycin. Log reduction in *P. acnes* on pig skin surface treated with 0.001% to 0.25% NB-003 and 5% to 2.5% BPO.

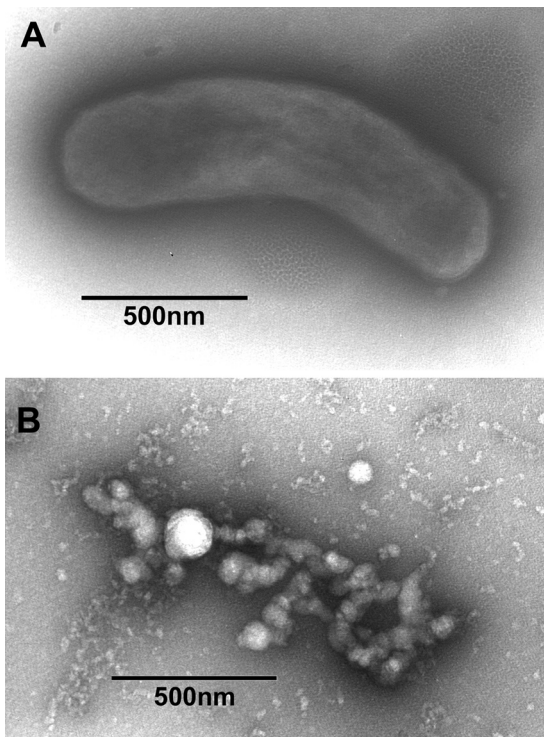


FIG. 3. Scanning electron micrographs of *P. acnes* PAC-001 before (A) and after (B) 1 h of treatment with 20 µg/ml NB-003.

acne vulgaris. NB-003 has a significant bactericidal activity against a collection of recent clinical isolates of *P. acnes*, including multidrug-resistant strains. Comparator drugs that have been used to treat acne, including erythromycin, clindamycin, tetracycline, benzoyl peroxide, and salicylic acid, were less effective against this strain panel. Resistance and cross-resistance of *P. acnes* to commonly used acne drugs are common and prevalent problems (15, 34, 53, 54). Consistent with its physical mechanism of action of interacting with the *P. acne* surface, NB-003 had no cross-resistance with known antiacne agents. Further, stably resistant isolates from cultures of *P. acnes* cultured on plates containing 2, 4, or 8 times the MIC of NB-003 were difficult to maintain.

Use of combination therapy to treat acne has been previously studied (10, 15, 32–35, 39). Benzoyl peroxide-clindamycin demonstrated improved efficacy and similar tolerability as benzoyl peroxide used alone. The efficacy of this combination therapy was similar to that of a benzoyl peroxide-erythromycin combination (10, 32, 35, 39). Erythromycin or clindamycin plus BPO have been reported to reduce drug resistance development compared to use of single antimicrobials.

Other combination strategies of BPO combined with a retinoid cause irritation and are often not well tolerated (35), resulting in discontinuation by patients. Studies on less irritating formulations have been reported; glycerin and dimethicone have been reported to reduce the formation of erythema and dryness caused by BPO and retinoids (14, 64) as well as micronized particles of BPO. In this study, NB-003 showed synergy with BPO for 90% of the isolates tested. Since individual MICs and MBCs of NB-003 and BPO decreased in the pres-

ence of sebum compared to those of NB-003 alone (Table 1), the synergistic interaction of the two drugs presents a potential opportunity for the nanoemulsion combination to be used for acne treatment. Under the best scenario that would likely cause less skin irritation and drying, 0.0025% BPO can be formulated with 0.3% NB-003. Combinations of the nanoemulsion NB-003 with BPO or salicylic acid were as effective as NB-003 alone, but synergy at the bactericidal level (MBCs) were seen when the combinations were used in the presence of sebum. Thus, the power of codelivering synergistic combinations by the transfollicular route conferred with nanoemulsion technology (5) could yield more effective treatment. Due to its kill-on-contact mechanism, NB-003 poses a very low risk for the development of resistance and cross-resistance with other drugs, and if resistance does emerge, it is not stable.

EDTA, a preservative in the food, drug, and cosmetic industries (16, 22, 28, 31, 43, 60), has been shown to potentiate the antibiotic activity of nanoemulsions containing CPC against Gram-positive and Gram-negative bacteria. Farca et al. have shown the synergistic effect of EDTA with five antimicrobial agents, ampicillin, cephalexin, oxytetracycline, streptomycin, and sulfadimethoxine, on three clinically isolated Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus hominis*, and *Enterococcus faecium*) even in the absence of nanoemulsion delivery (16). We have shown that 20 mM EDTA enhances the activity of NB-003 up to 256-fold against *P. acnes* in the presence of 25% sebum. EDTA may enhance the activity of NB-003 by acting as a permeabilizer and chelating agent and enhancing the transmembrane diffusion of nanoemulsion.

NB-003 is a broad-spectrum topical that is being developed for treatment of diseases in the skin, hair, and nails. Previous work in human cadaver skin samples demonstrated that the antiviral NB-001 nanoemulsion uses a transfollicular route to enter the epidermal and dermal layers and that lateral diffusion occurred along tissue planes to sites distal from the application site (5, 6). Clinical studies for the topical treatment of

TABLE 4. Frequency of spontaneous mutations for 16 strains of *P. acnes*

Strain no.	Spontaneous mutation frequency ^a			
	MIC	2× MIC	4× MIC	8× MIC
PAC-001	7.7×10^{-11}	$<7.7 \times 10^{-11}$	$<7.7 \times 10^{-11}$	$<7.7 \times 10^{-11}$
PAC-002	3.4×10^{-10}	$<6.9 \times 10^{-11}$	$<6.9 \times 10^{-11}$	$<6.9 \times 10^{-11}$
PAC-003	$<5.6 \times 10^{-11}$	$<5.6 \times 10^{-11}$	$<5.6 \times 10^{-11}$	$<5.6 \times 10^{-11}$
PAC-004	2.0×10^{-10}	$<4.0 \times 10^{-11}$	$<4.0 \times 10^{-11}$	$<4.0 \times 10^{-11}$
PAC-005	9.6×10^{-10}	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$
PAC-006	$<9.1 \times 10^{-11}$	$<9.1 \times 10^{-11}$	$<9.1 \times 10^{-11}$	$<9.1 \times 10^{-11}$
PAC-007	$<3.8 \times 10^{-11}$	$<3.8 \times 10^{-11}$	$<3.8 \times 10^{-11}$	$<3.8 \times 10^{-11}$
PAC-008	$<2.7 \times 10^{-11}$	$<2.7 \times 10^{-11}$	5.5×10^{-11}	$<2.7 \times 10^{-11}$
PAC-009	4.4×10^{-08}	$<2.2 \times 10^{-10}$	$<2.2 \times 10^{-10}$	$<2.2 \times 10^{-10}$
PAC-010	$<1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$
PAC-011	$<2.5 \times 10^{-11}$	2.5×10^{-11}	$<2.5 \times 10^{-11}$	$<2.5 \times 10^{-11}$
PAC-012	$<2.7 \times 10^{-10}$	$<2.7 \times 10^{-10}$	$<2.7 \times 10^{-10}$	$<2.7 \times 10^{-10}$
PAC-013	$<3.4 \times 10^{-11}$	$<3.4 \times 10^{-11}$	$<3.4 \times 10^{-11}$	$<3.4 \times 10^{-11}$
PAC-014	$<1.0 \times 10^{-07}$	$<1.0 \times 10^{-07}$	$<1.0 \times 10^{-07}$	$<1.0 \times 10^{-07}$
PAC-015	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$
PAC-016	$<1.0 \times 10^{-07}$	$<1.0 \times 10^{-07}$	$<1.0 \times 10^{-07}$	$<1.0 \times 10^{-07}$

^a Concentration shown as multiples of the MIC of NB-003 against the parent strain.

herpes labialis and onychomycosis confirm that there is no systemic exposure in patients after multiple nanoemulsion applications per day, eliminating worry about drug-drug interactions and undesired systemic side effects (24, 26, 27). Thus, NB-003 offers a topical, empirical alternative to the oral or topical antibiotics used to treat acne, and clinical trials are ongoing to prove its value for the treatment of acne.

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