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J. Pannu, A. McCarthy, A. Martin and J. Sutcliffe  
NanoBio Corp., Ann Arbor, MI, USA

For additional information contact:  
John Coffey, Jr.  
Phone: (734) 302-9107  
E-mail: john.coffey@nanobio.com

## ABSTRACT

**Background.** Antibiotic resistance has become an enormous clinical problem in the treatment of acne. NB-003 is an antimicrobial oil-in-water emulsion with an average droplet diameter of ~200 nm and a composition that allows for selective penetration into the pilosebaceous unit, the site of acne pathogenesis. The mechanism of action of NB-003 is physical via membrane destabilization, making the emergence of resistance improbable. This study sought to determine the susceptibility of *P. acnes* to NB-003 formulations in the presence of sebum, a major component of the pilosebaceous unit and a nutrient source for *P. acnes*.

**Methods.** Sixteen clinical isolates of *P. acnes* with defined resistance mechanisms to erythromycin, clindamycin and/or tetracycline were used. Antimicrobial susceptibility testing was done in the presence and absence of 50% artificial sebum under anaerobic conditions. NB-003 was made by high speed emulsification of Tween 20, ethanol, soybean oil, cetylpyridinium chloride and water. Other formulations assessed were NB-003 containing either 0.5% benzoyl peroxide (NB/BPO) or 2% salicylic acid (NB/SA).

**Results.** NB-003 was bactericidal for all strains of *P. acnes* with MIC<sub>90</sub>/MBC<sub>90</sub> values of 0.5/2 µg/ml in the absence of sebum. The MIC<sub>90</sub>/MBC<sub>90</sub> values in the presence of 50% sebum increased to 128/1024 µg/ml. A reduction in the MBC<sub>90</sub> for NB-00X occurred when BPO or SA was integrated into the formulation, resulting in a MIC<sub>90</sub>/MBC<sub>90</sub> of 128/256 µg/ml. The MIC<sub>90</sub>/MBC<sub>90</sub> values of SA (1000/2000 µg/ml) were not significantly impacted by the presence of sebum, but the MIC<sub>90</sub>/MBC<sub>90</sub> values of BPO increased eight-fold in the presence of sebum (400/1600 µg/ml).

**Conclusions.** NB-00X had relevant microbiological and bactericidal activity against a collection of recent clinical isolates of *P. acnes*, including multidrug-resistant strains. The combinations of NB/BPO or NB/SA were synergistic in the presence of sebum suggesting novel combinations for the treatment of acne.

## BACKGROUND

### *Propionibacterium acnes*

- Is a gram-positive, non-spore forming, anaerobic bacillus and one of the primary factors involved in the pathogenesis of acne vulgaris. It is the predominant microorganism of the pilosebaceous glands of human skin, with up to 10<sup>7</sup> viable organisms isolated from a single sebaceous unit.

### Drug-resistant *Propionibacterium acnes* on the rise

- Recent studies in six European countries identified that acne patients carried *P. acnes* isolates with at least one resistance determinant, 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.

### NB-003

- Is a clinical stage nanoemulsion, currently under development for the treatment of microbial skin infections, including acne vulgaris. Previous studies have shown that NB-003 nanodroplets are concentrated in the pilosebaceous unit where *P. acnes* migrates to enjoy a rich source of food (sebum) and a preferred anaerobic environment.

### Our objectives

- Were to determine broth-based MICs and MBCs for NB-003 alone and in combination with other anti-acne agents against susceptible and drug-resistant strains of *P. acnes*.

## RESULTS

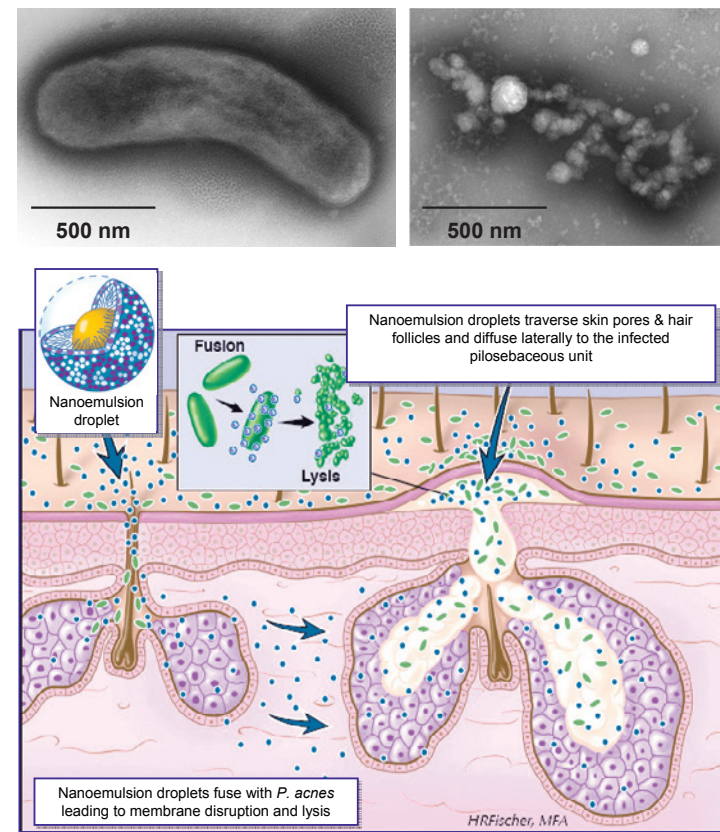


Figure 1. Mechanism of action of NB-003 against *P. acnes*

Table 1. MIC<sub>90</sub>/MBC<sub>90</sub> values (µg/ml) of NB-003 and comparators against *P. acnes* with defined resistance to erythromycin, clindamycin and/or tetracycline (n=16)

Compound	MIC <sub>90</sub>		MBC <sub>90</sub>		MIC <sub>50</sub>		MBC <sub>50</sub>		MIC Range		MBC Range	
	No sebum	+ 50% sebum <sup>a</sup>	No sebum	+ 50% sebum	No sebum	+ 50% sebum	No sebum	+ 50% sebum	No sebum	+ 50% sebum	No sebum	+ 50% sebum
NB-00X	0.5	128	2	1024	0.5	128	2	1024	0.25-1.0	64-256	0.5-2	256-1024
NB-00X gel	1	128	2	1024	0.5	128	2	1024	0.5-1	64-256	1-4	128->1024
Benzoyl peroxide	50	400	200	1600	50	200	200	800	≤50-100	≤50-400	100-400	800-1600
Salicylic Acid	1000	1000	2000	2000	1000	1000	2000	2000	500-1000	500-1000	2000	2000->2000
0.3% NB-00X gel + 0.5% BPO	0.5	128	4	256	0.5	128	4	128	0.5-1	128	2-4	128-256
0.3% NB-00X gel + 2% Salicylic acid	0.5	128	2	256	0.5	128	2	128	0.25-1.0	128	0.5-2	128-256

Table 2. Checkerboard synergy by broth microdilution

GC	A 0.5	A 1	A 2	A 4	A 8	A 16	A 32	A 64	A 128	A 256	A 512
B 6.3	A0.5 + B6.3	A 1 + B6.3	A 2 + B6.3	A 4 + B6.3	A 8 + B6.3	A 16 + B6.3	A 32 + B6.3	A 64 + B6.3	A 128 + B6.3	A 256 + B6.3	A 512 + B6.3
B 12	A 0.5 + B 12	A 1 + B 12	A 2 + B 12	A 4 + B 12	A 8 + B 12	A 16 + B 12	A 32 + B 12	A 64 + B 12	A 128 + B 12	A 256 + B 12	A 512 + B 12
B 25	A 0.5 + B 25	A 1 + B 25	A 2 + B 25	A 4 + B 25	A 8 + B 25	A 16 + B 25	A 32 + B 25	A 64 + B 25	A 128 + B 25	A 256 + B 25	A 512 + B 25
B 50	A 0.5 + B 50	A 1 + B 50	A 2 + B 50	A 4 + B 50	A 8 + B 50	A 16 + B 50	A 32 + B 50	A 64 + B 50	A 128 + B 50	A 256 + B 50	A 512 + B 50
B 100	A 0.5 + B100	A 1 + B100	A 2 + B100	A 4 + B100	A 8 + B100	A 16 + B100	A 32 + B100	A 64 + B100	A 128 + B100	A 256 + B100	A 512 + B100
B 200	A 0.5 + B200	A 1 + B200	A 2 + B200	A 4 + B200	A 8 + B200	A 16 + B200	A 32 + B200	A 64 + B200	A 128 + B200	A 256 + B200	A 512 + B200
B 400	A 0.5 + B400	A 1 + B400	A 2 + B400	A 4 + B400	A 8 + B400	A 16 + B400	A 32 + B400	A 64 + B400	A 128 + B400	A 256 + B400	BC

GC = growth control; BC = broth control; A = NB-003, concentration in µg/ml; B = Benzoyl peroxide; concentration in µg/ml

Table 3. Composition of artificial sebum<sup>a</sup>

Sebum Ingredient	w/w %
Oleic acid	1.4
Palmitoleic acid	5.0
Squalene	15
Olive oil	10
Cottonseed oil	25
Cholesterol	1.2
Cholesterol oleate	2.4
Palmitic acid	5.0
Spermaceti wax	15
Paraffin wax (mp 58-62°C)	10
Coconut oil	10

<sup>a</sup>Li, G., et al. 2009. Comparison of artificial sebum with human and hamster sebum samples. Int. J. Pharm. 367:37-43. Epub date, Sept. 24, 2008

Table 4. Synergy Indices for *P. acnes*

<i>P. acnes</i> Isolate #	Average FIC index	Average FBC index
PAC-001	0.4	0.2
PAC-003	0.4	0.4
PAC-004	0.6	0.5
PAC-005	0.4	0.7
PAC-006	0.5	0.3
PAC-007	0.4	0.3
PAC-008	0.4	0.4
PAC-009	0.5	0.6
PAC-010	0.5	0.4
PAC-011	0.4	0.4
<b>Synergy</b>	<b>90%</b>	<b>80%</b>

Calculations of Fractional Inhibitory (FIC) and Fractional Bactericidal Concentration (FBC). FIC for each drug was calculated as: FIC for NB-00X = MIC of NB-00X in combination/MIC of NB-00X alone. FIC of BPO = MIC of BPO in combination/MIC of BPO alone; FIC index (Σ FIC) = FIC of NB-00X + FIC of BPO. The average FIC index for each isolate was calculated using FIC indices of all tested combinations. The Fractional Bactericidal Concentration and FBC index were calculated using MBC values instead of MICs. Interpretation: Synergism = Σ FIC is ≤0.5. Indifference = Σ FIC is >0.5 and ≤4. Antagonism = Σ FIC is >4.

## METHODS

**Emulsion manufacturing and source of BPO.** NB-00X is an oil-in-water emulsion manufactured from ingredients that are on the FDA list of recognized inactive ingredients in drug substances. The emulsion is formed from highly purified oil, ethanol, polysorbate, CPC and water. The average droplet size was ~200 nm as measured by dynamic light scattering. The antibacterial activity of NB-00X is expressed in microgram CPC/ml. BPO in the form of Clearasil® cream was purchased over-the-counter. Salicylic acid was formulated with NB-00X at a final concentration of 0.3% NB-00X/2% salicylic acid.

**Source of *P. acnes* isolates.** Clinical isolates of *P. acnes* were obtained from Stuart Shapiro, Basilea Pharmaceutica, AG, Basel, Switzerland. The resistance mechanisms were mutations in either the 16S or 23S rRNA of the small or large ribosomal subunit conferring tetracycline or erythromycin ± clindamycin resistance, respectively, or resistance was conferred by an *erm(X)* methylase that dimethylates residue A2058 in 23S rRNA, conferring high level erythromycin and clindamycin resistance. Three isolates were obtained from ATCC, Manassas, Virginia, USA.

**MIC/MBC determinations.** Minimum inhibitory concentrations (MIC) and bactericidal concentrations (MBC) to clinical isolates of *P. acnes* were determined anaerobically using standard methodology (Clinical and Laboratory Standards Institute). Because of the opacity of BPO, 20 µl of CellTiter-Blue (Alamar blue from Promega G8080) was added after 48 hrs; the plates were incubated for an additional hour prior to reading. Colony-forming units were counted after 72 h of incubation to ensure that the initial inocula were between 2-5 x 10<sup>6</sup> cfu/ml. The minimal bactericidal concentrations (MBC) for *P. acnes* were determined by plating 10 µl from the well determined to be the MIC plus 4 wells above the MIC on blood-supplemented Mueller-Hinton agar plate. Inoculated petri plates were incubated for 72 h at 35°C under anaerobic conditions. The MBC was calculated as the concentration of drug that gave ≥3-log reduction from the initial inoculum concentration.

**Checkerboard synergy experiments.** Synergy between NB-00X and BPO was determined using standard methodology (Verma, P. 2007). Methods for determining bactericidal activity and antimicrobial interactions: synergy testing, time-kill curves, and population analysis, p. 275-298. In R. Schwalbe, L. Steele-Moore and A.C. Goodwin (ed.), Antimicrobial Susceptibility Testing Protocols, CRC Press, Boca Raton, Florida). Briefly the BPO stock was serially diluted, 1:1 in 4X Wilkenson-Chalgren broth. To prepare a microtiter plate, 25 µL of the NB-00X stock solution (4x the highest concentration) was added to column 12, 25 µL of the first dilution in column 11, and so on until column 2. Sterile water with no NB-002 was placed in column 1. On the same plate 25 µL of the BPO stock solution (8x the highest tested concentration) was added in row H, and the second dilution in row G, and so on until row B. Only the four times concentrated broth was added to row A. Finally 45 µL of artificial sebum (was added to every well to give about ~50% of sebum. Well A1 was used as growth control and H12 as sterility control.

## CONCLUSIONS

- NB-003 was bactericidal for all strains of *P. acnes* tested, including multidrug-resistant isolates.

- NB-003 is synergistic with benzoyl peroxide or salicylic acid in the presence of artificial sebum

- A phase I clinical study testing the *in vivo* activity of NB-003 against *P. acnes* is currently underway in the U.S.