

ANTIMICROBIAL ACTIVITY OF NANOEMULSION AGAINST CLINICAL ISOLATES FROM CYSTIC FIBROSIS PATIENTS

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ABSTRACT

Background: Cystic fibrosis (CF) patients are prone to respiratory infections and biofilm formation in the lungs. Systemically-administered antibiotics are inefficient and lead to resistant species. Nanoemulsions (NE) are oil-in-water emulsions stabilized by surfactants with an average droplet size of ~400 nm. They have broad-spectrum antimicrobial activity and kill pathogens by interacting with their membranes. This physical kill-on-contact mechanism significantly reduces the possibility of the emergence of resistant strains. The NE is formulated from pharmaceutically acceptable ingredients. In this study we evaluated the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the nanoemulsion NB-400 against four genera of bacteria commonly infecting CF patients: *Pseudomonas*, *Burkholderia*, *Acinetobacter* and *Stenotrophomonas*. We also tested for potential synergy between NB-400 and colistin or tobramycin.

Methods: Multidrug-resistant Gram-negative bacteria isolated from CF patients were tested in this study. These organisms include *Pseudomonas*, *Burkholderia*, *Acinetobacter* and *Stenotrophomonas*. The *P. aeruginosa* isolates had defined lipid A modifications¹ that have been documented in many CF patients. MICs and MBCs were determined using CLSI guidelines and standard methods M7-A7² and M100-S17³. The addition of alamar blue, a redox indicator that yields a colorimetric change in response to metabolic activity, was used to determine the MICs of NB-400 because of NE opacity at higher concentrations. Synergy between NB-400 and other antimicrobials was assessed using checkerboard synergy microtiter-based assays. The microbicidal activity of NB-400 has been visualized using transmission electron microscopy.

Results: The MIC₉₀/MBC₉₀ values for NB-400 were 8/64 µg/ml for *P. aeruginosa*, 64/514 µg/ml for *B. cenocepacia*, 8/64 µg/ml for *A. baumannii* and 8/32 µg/ml for *S. maltophilia*. Colistin had MIC₉₀/MBC₉₀ values of 2/8, >32/>32, 1/>16 and >32/>32 for *P. aeruginosa*, *B. cenocepacia*, *A. baumannii* and *S. maltophilia*, respectively. Cefepime, imipenem, levofloxacin and tobramycin had MIC₉₀/MBC₉₀ values of ≥32/>32, ≥32/>32, 16/16 and >32/>32 µg/ml, respectively, against all strains. NB-400 was synergistic *in vitro* with colistin and not antagonistic to tobramycin. Transmission electron microscopy verified that NB-400 rapidly and effectively disrupts these CF pathogens.

Conclusions: NB-400 was effective against strains that were multidrug-resistant, including colistin-resistant isolates of *Burkholderia* and *Stenotrophomonas*. None of the described lipid A modifications in *Pseudomonas* species impacted the MIC/MBCs with NB-400. Further studies are ongoing to investigate the nebulization of NB-400 for the treatment of pulmonary infection in cystic fibrosis patients.

BACKGROUND

Antimicrobial nanoemulsions are novel oil-in-water emulsions with nanometer-sized droplets that physically interact with and disrupt bacteria (Fig. 1). A specific nanoemulsion (NB-400) has been developed for CF using ingredients known to be safe for use in humans.

Despite significant advances in the treatment of CF, 85% percent of CF patients die of respiratory failure due to bacterial infection. Many of these infections are due to organisms that are resistant to some or all of the currently available antibiotics.

We hypothesized that the novel physical mechanism of action of nanoemulsions would result in killing of bacteria that were resistant to other antimicrobial agents. To test this hypothesis, we tested NB-400 against clinical isolates from CF patients, including multidrug and pan resistant organisms.

References:

- Ernst, R.K., Adams, K.N., Moskowitz, S.M., Kraig, G.M., Kawasaki, K., Stead, C.M., Trent, M.S., Miller, S.I. 2005. "The *Pseudomonas aeruginosa* Lipid A Deacylase: Selection for Expression and Loss within the Cystic Fibrosis Airway." *Journal of Bacteriology*, 188 (1): 191-201.
- Clinical and Laboratory Standard Institute. 2006. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-seventh edition. CLSI document M7-A7 (ISBN 1-56238-587-9).
- Clinical and Laboratory Standard Institute. 2007. Performance Standards for Antimicrobial Susceptibility and Testing; Seventeenth Information Supplement. CLSI document M100-S17 (ISBN 1-56238-625-5).

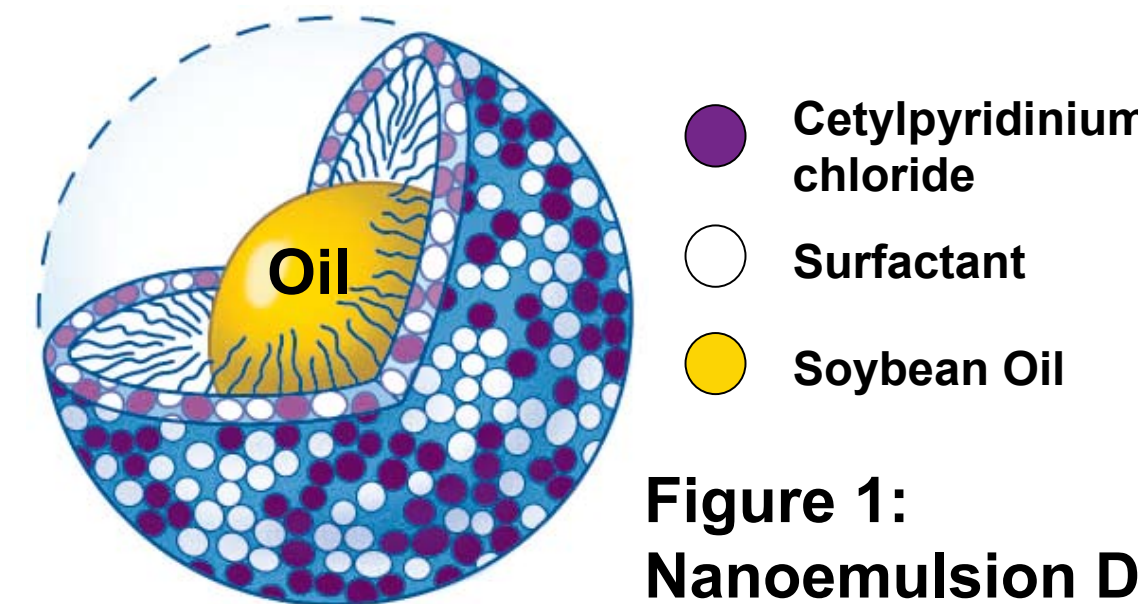


Figure 1:
Nanoemulsion Droplet

METHODS

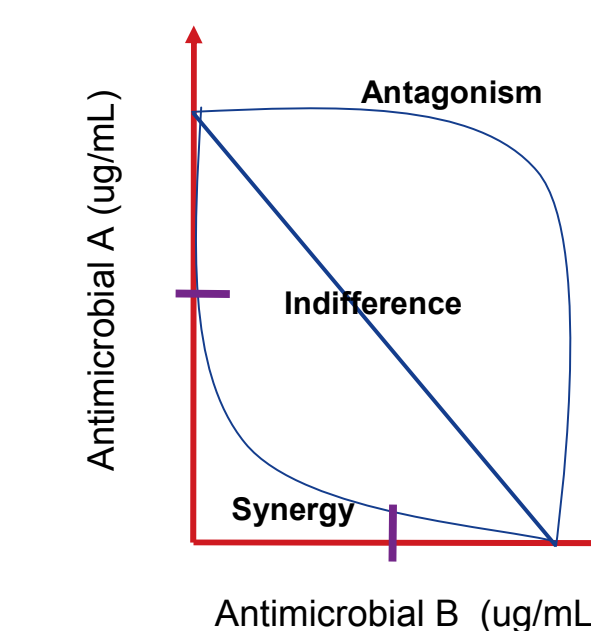
MIC and MBC were assayed for multidrug-resistant Gram-negative bacteria (*Acinetobacter*, *Stenotrophomonas*, *Pseudomonas* and *Burkholderia*) isolated from CF patients using NB-400 and a battery of antibiotics.

MICs and MBCs were determined using CLSI guidelines and standard methods M7-A7² and M100-S17³. NB-400 was supplemented with additional EDTA added to each well. Due to NB-400 opacity at higher concentrations, CellTiter Blue (Promega), was used as a colorimetric metabolic indicator to determine MICs of NB-400.

Time kill studies were performed to determine NB-400 antimicrobial activity against *Burkholderia*.

Samples from time killed studies were stained with 1% uranyl acetate and examined using a Philips CM-100 Transmission Electron Microscope (TEM).

Synergy between NB-400 and other commonly used antibiotics (colistin and tobramycin) was tested in microtiter plates. Shift in MIC or MBC (FIC and FBC) was measured and categorized as synergistic, indifferent or antagonistic.



The fractional inhibitory concentration index (FIC) examines the ratio of the MIC of a single drug when in combination with another to the MIC of that drug alone.

$$FIC = \frac{MIC \text{ of Drug in combination}}{MIC \text{ of Drug alone}}$$

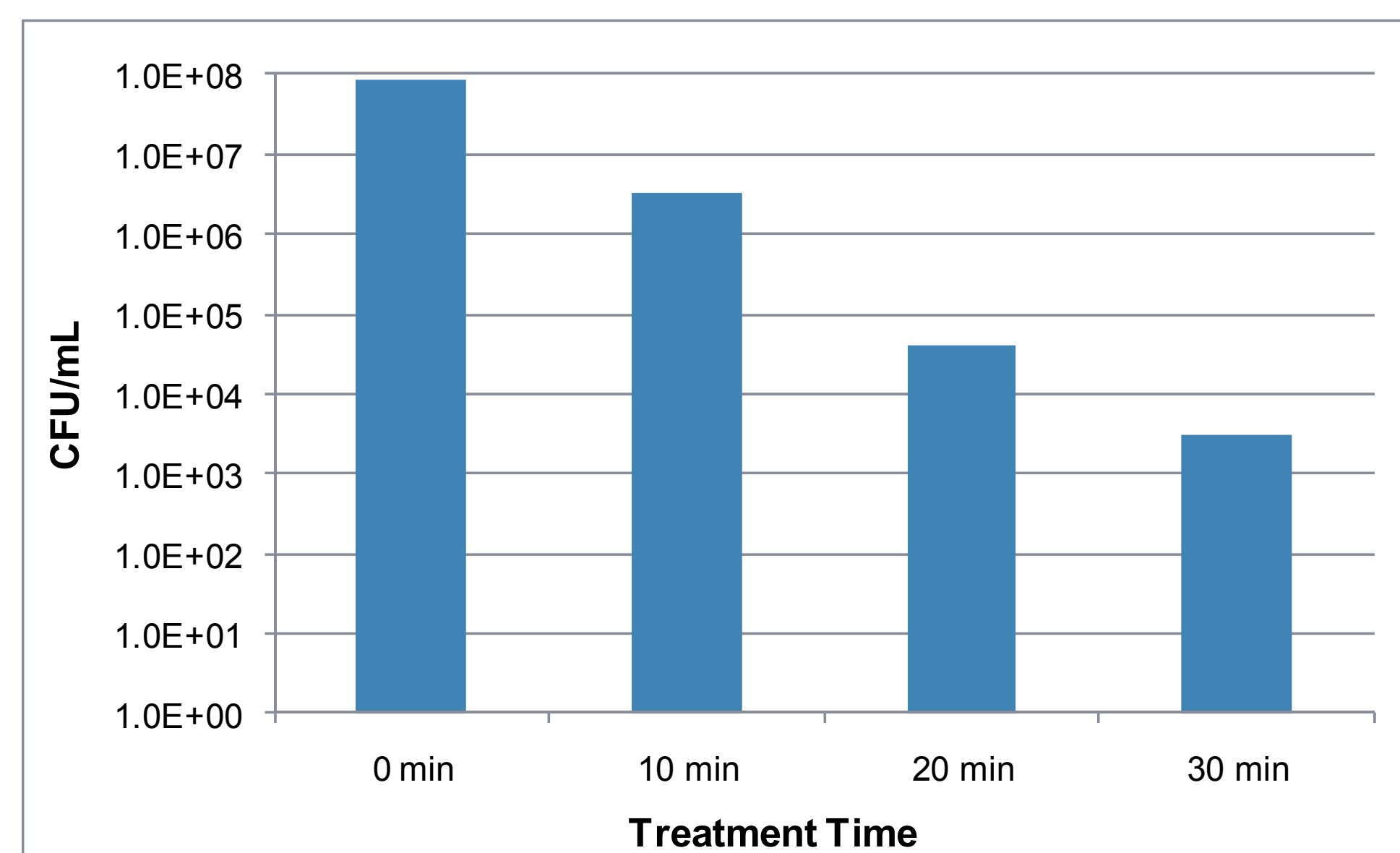
This ratio is calculated for both drug A and drug B. The fractions are added together. The summation is compared to the following ranges:

Synergism: Sum of FIC for the two drugs ≤ 0.5
Indifference: Sum of FIC for the two drugs > 0.5 to ≤ 4
Antagonism: Sum of FIC for the two drugs > 4

FBC is calculated using MBC instead of MIC

RESULTS

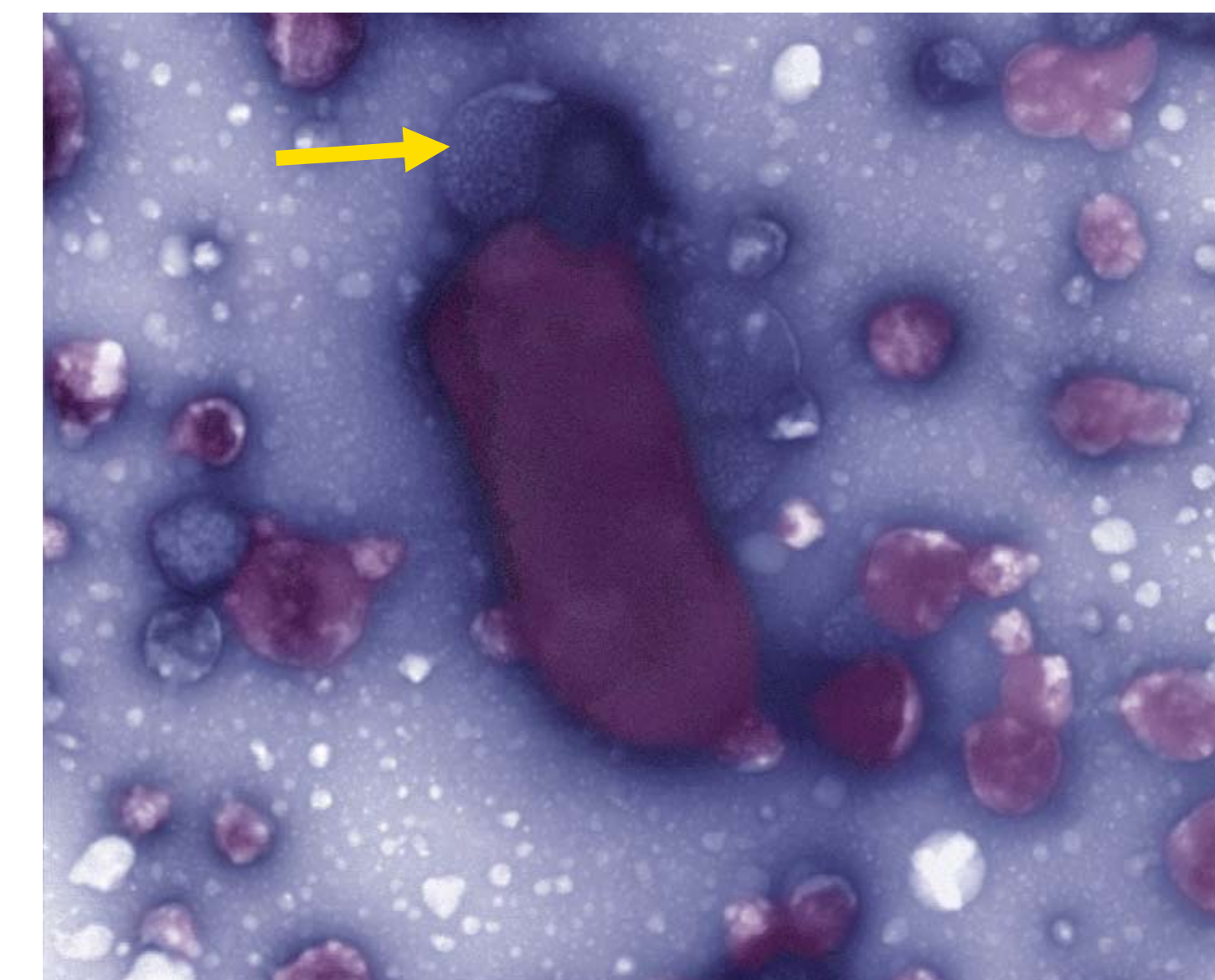
Figure 2: Time kill studies and corresponding transmission electron micrographs for *Burkholderia* treated with the NB-400



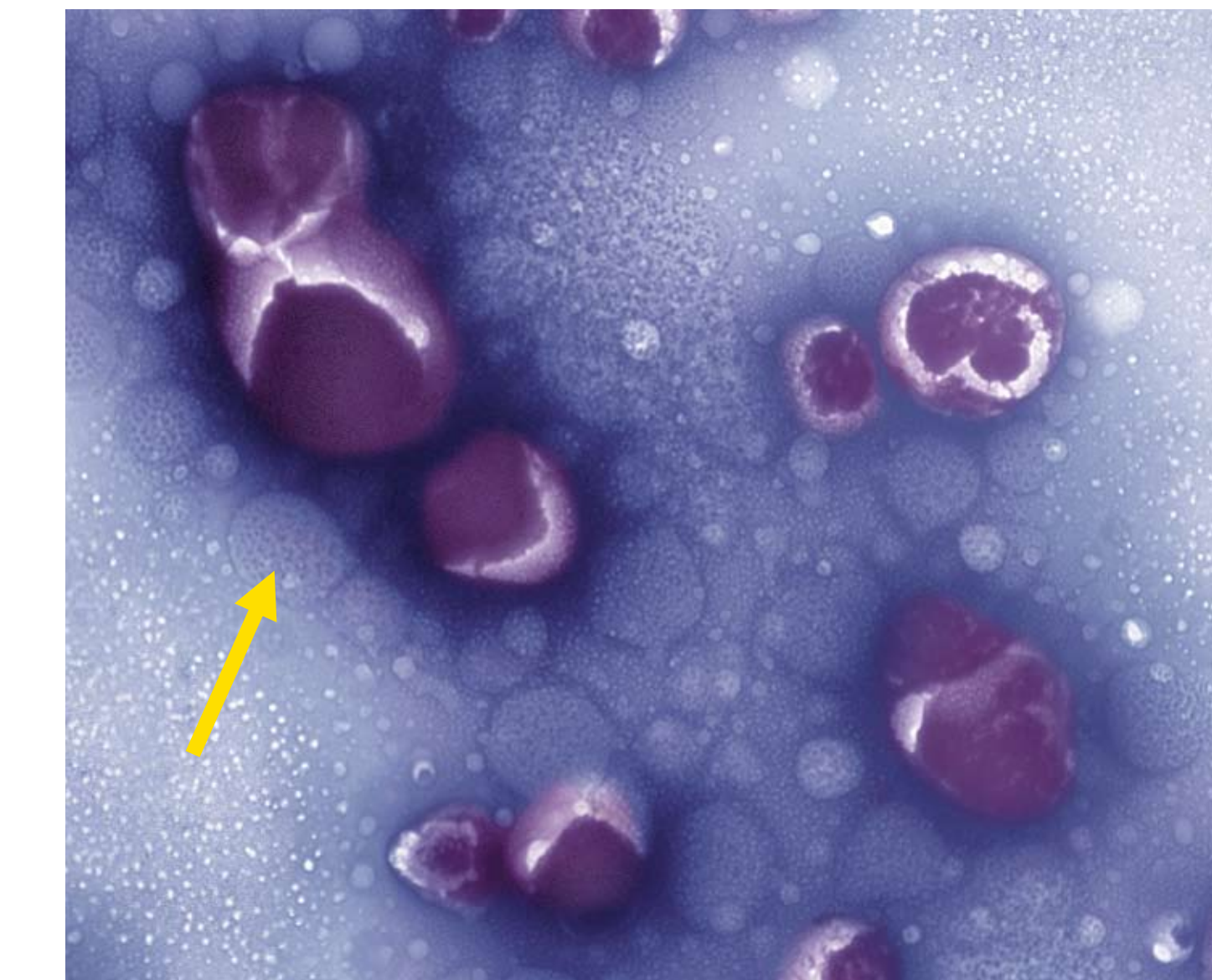
Microbial Reduction of *Burkholderia* Following NB-400 Treatment *in vitro*



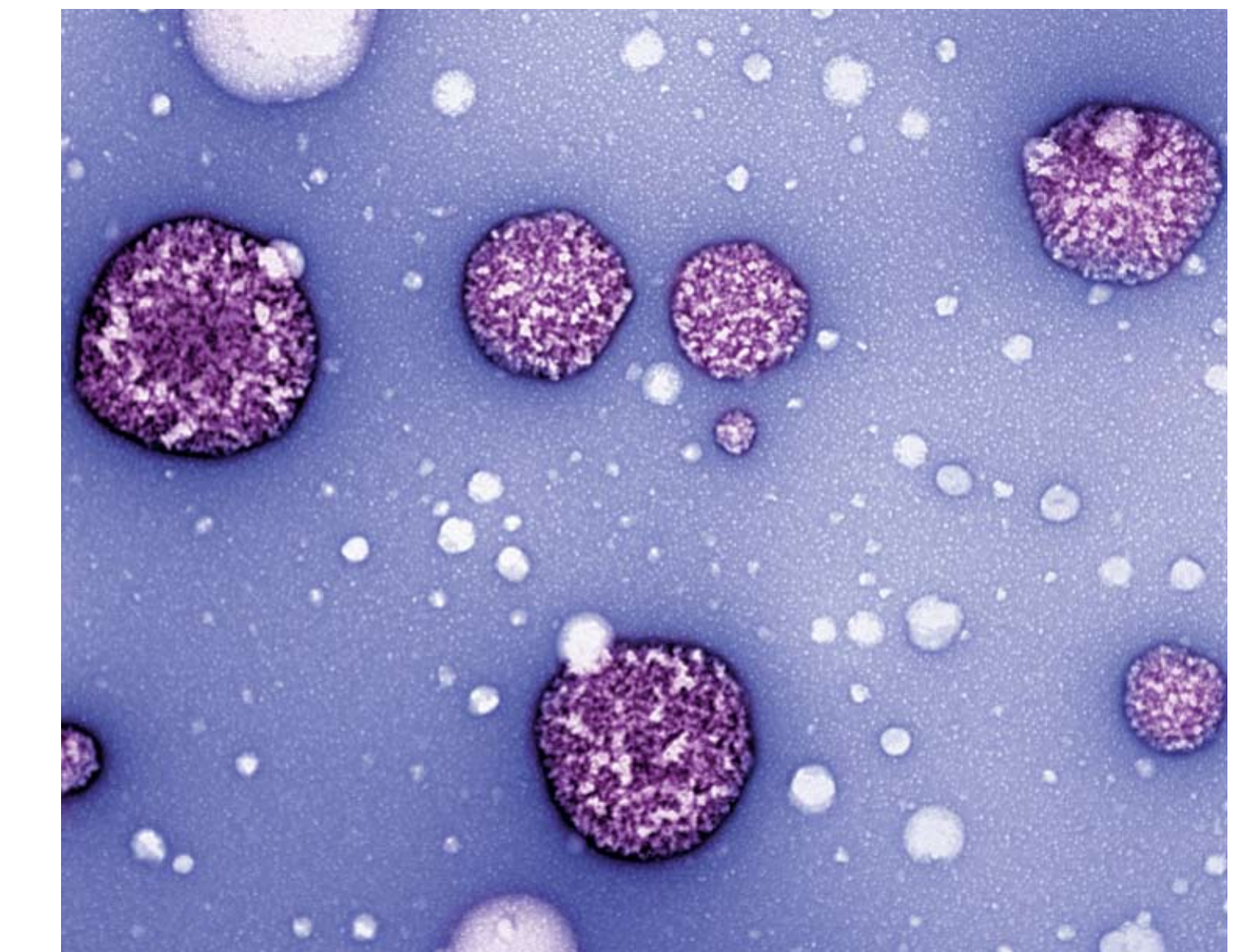
A. Time = 0 Minutes; Untreated



B. 10 Minutes
Burkholderia surrounded by bacterial fragments (purple) and nanoemulsion droplets (arrow)



C. 20 Minutes
Bacterial lysis (purple)
Intact nanoemulsion droplets (arrow)



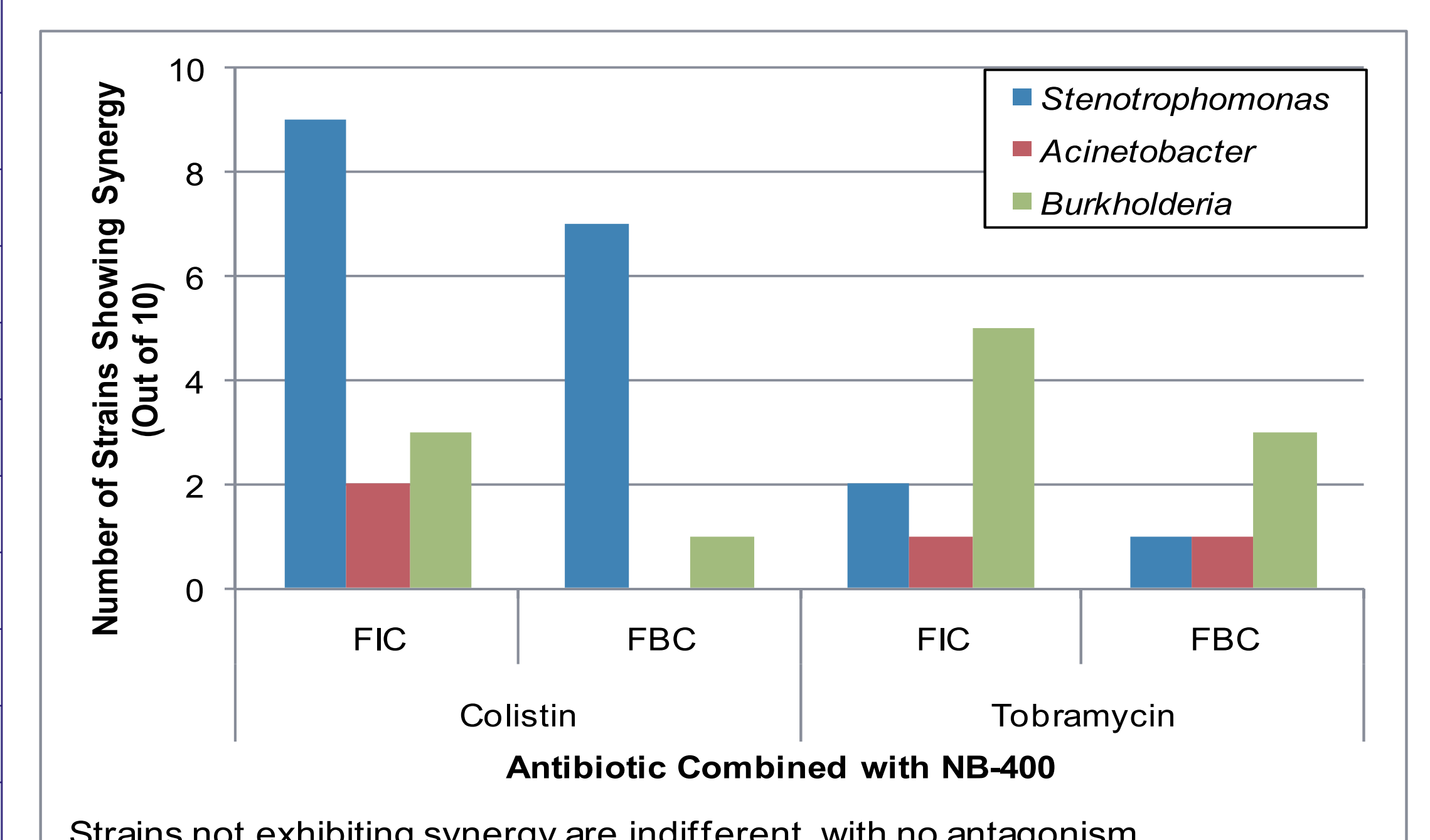
D. 30 Minutes
Bacterial fragments

Number of Isolates Tested	
<i>Pseudomonas aeruginosa</i>	20
<i>Burkholderia cenocepacia</i>	10
<i>Acinetobacter baumannii</i>	10
<i>Stenotrophomonas maltophilia</i>	11
Total Number of Isolates	51
Multi-Drug Resistant Strains	37%
(Resistant to ≥ two classes of antibiotics)	
Pan-Drug Resistant Strains	6%
(Resistant to ≥ four classes of antibiotics)	

µg/mL Active Ingredient	NB-400 A	Cefepime	Colistin	Imipenem	Levo-floxacin	Ceftazidime	Tobramycin	Cefoxitin
Pseudomonas								
MIC 50	4	2	2	4	0.5	Not Tested	1	>256
MIC 90	8	32	2	>32	8	Not Tested	16	>256
MBC 50	16	8	4	16	1	Not Tested	8	>256
MBC 90	64	>32	8	>32	16	Not Tested	>16	>256
Burkholderia								
MIC 50	16	32	>32	>32	2	8	>32	128
MIC 90	64	>32	>32	>32	8	16	>32	256
MBC 50	128	>32	>32	32	4	32	>32	256
MBC 90	>514	>32	>32	>32	>16	>32	>32	>256

µg/mL Active Ingredient	NB-400 B	Cefepime	Colistin	Imipenem	Levo-floxacin	Ceftazidime	Tobramycin	Cefoxitin
Acinetobacter								
MIC 50	4	32	1	4	16	>32	1	>256
MIC 90	8	>32	1	32	>16	>32	16	>256
MBC 50	16	>32	4	8	16	>32	4	>256
MBC 90	64	>32	>16	>32	>16	>32	>32	>256
Stenotrophomonas								
MIC 50	8	>32	16	>32	2	>32	>32	>256
MIC 90	8	>32	>32	>32	8	>32	>32	>256
MBC 50	16	>32	>32	>32	4	>32	>32	>256
MBC 90	32	>32	>32	>32	16	>32	>32	>256

Figure 3: Synergy with NB-400



Strains not exhibiting synergy are indifferent, with no antagonism.

SUMMARY & CONCLUSIONS

- NB-400 was effective against multi-drug resistant strains, including colistin-resistant isolates of *Burkholderia* and *Stenotrophomonas*.
- Synergy was demonstrated between NB-400 and colistin against *Stenotrophomonas*.
- No antagonism was demonstrated between NB-400 and colistin or tobramycin against *Acinetobacter* and *Burkholderia*.
- The TEM images demonstrated the kill on contact effect of NB-400.
- Exploratory studies of inhaled NB-400 in dogs are in progress.