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Antimicrobial Activity of Nanoemulsions Tested Against Seven Gram-Negative Species

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ABSTRACT

Background: Nanoemulsions (NEs) are composed of pharmaceutically approved substances emulsified to nanodroplets with an average diameter of 180 or 350 nm. Previous studies have shown that NEs significantly penetrate the skin or mucosa into underlying tissues without any signs of inflammation. Three NEs were evaluated against seven gram-negative species. Many of these species, especially *Pseudomonas aeruginosa* and *Burkholderia cepacia,* are problematic in cystic fibrosis patients.

Methods: MICs and MBCs were determined using CLSI standard methods. MICs to NEs were determined in the presence of 5 mM EDTA, a known enhancer of NE activity. 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 NEs that are opaque at higher concentrations.

Results: All 3 NEs had activity against gram-negative isolates (EDTA alone inhibited 3 and 5 strains of *A. baumannii* and *S. maltophilia,* respectively). This included isolates that were resistant to comparator agents. MBCs were performed for 23 isolates; W_{20}5EC, P_{407}5EC and W_{20}5GBA$_2$ED were bactericidal against 85%, 95% and 90% of the isolates, respectively.

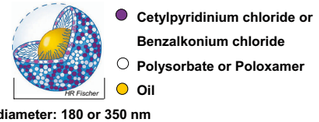
Conclusions: NEs were broadly active against gram-negative species, including multidrug-resistant isolates. The documented MICs were within the range of concentrations achievable with topical application to skin or mucosal tissues. One or more of the nanoemulsions may be useful for prophylaxis of chronic pulmonary infections in cystic fibrosis patients.

BACKGROUND

Cystic Fibrosis (CF) is one of the most common fatal genetic disorders in the United States. A mutation in a gene that encodes a chloride channel, the CF transmembrane conductance regulator, produces partially functional or completely dysfunctional channels. CF is characterized by chronic respiratory infections that begin early in life with *Staphylococcus aureus and Haemophilus influenzae*; later, colonization with mucoid strains of *Pseudomonas aeruginosa* occurs. In CF lung disease, early colonization with *Burkholderia cepacia* correlates with a poor prognosis for the CF patient. Patients are often prescribed inhaled tobramycin to prevent exacerbations of bacterial infections. With time, patients can become unresponsive to tobramycin therapy/prophylaxis. New inhaled topical agents that are not cross-resistant to known antibiotics would be valued as an alternative to inhaled tobramycin.

Several topical nanoemulsions were evaluated for microbiological activity against gram-negative isolates, including *P. aeruginosa* and *B. cepacia*. Nanoemulsions are oil-in-water emulsions composed of nanometer-sized droplets (Figure 1). All three nanoemulsions kill rapidly in the presence of an outer membrane permeabilizer such as EDTA (Figure 2). The nanoemulsions were evaluated against 23 of the 35 gram-negative isolates for cidal activity and all were bactericidal (85-95%). Thus, there is a potential for one or more of these novel nanoemulsions to be used to prevent exacerbation of chronic pulmonary infections.

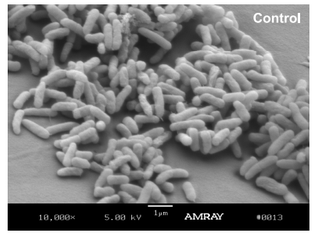
Figure 1. Nanoemulsion droplet.



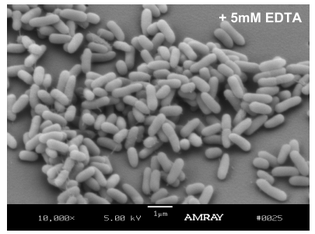
Average diameter: 180 or 350 nm

RESULTS

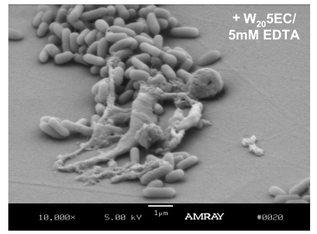
Figure 2. Scanning electron micrographs of *Pseudomonas aeruginosa* treated with W_{20}5EC ± EDTA.

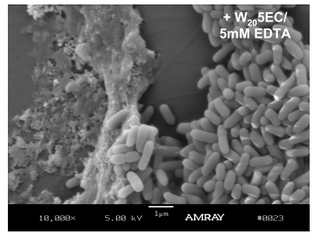


A. Control (no drug treatment)



B. Treatment with 5 mM EDTA for 1 minute





C, D. Treatment with 4 µg/ml W_{20}5EC + 5 mM EDTA for 1 minute (about 2 log reduction in cfu/ml)

Table 1. Susceptibility of seven gram-negative isolates to three nanoemulsions and comparators.

Antimicrobial agent	*Escherichia coli* (n = 5)[a]			*Klebsiella pneumoniae* (n = 5)			*Proteus mirabilis* (n = 5)			*Pseudomonas aeruginosa* (n = 5)			*Acinetobacter baumannii* (n = 5)[b]			*Stenotrophomonas maltophilia* (n = 5)[c]			*Burkholderia cepacia* (n = 5)		
	MIC_{50}	Range	% susceptible/ resistant[b]	MIC_{50}	Range	% susceptible/ resistant	MIC_{50}	Range	% susceptible/ resistant	MIC_{50}	Range	% susceptible/ resistant	MIC_{50}	Range	% susceptible/ resistant	MIC_{50}	Range	% susceptible/ resistant	MIC_{50}	Range	% susceptible/ resistant
W_{20}5EC	2	1 – 2	- / -	8	4 – 16	- / -	8	4 – 8	- / -	16	16 – 32	- / -	≤0.12	≤0.12 – 2	- / -	≤0.12	≤0.12	- / -	256	64 – >256	- / -
P_{407}5EC	4	4	- / -	8	8 – 16	- / -	16	8 – 16	- / -	64	32 – 64	- / -	≤0.12	≤0.12 – 4	- / -	≤0.12	≤0.12	- / -	256	128 – >256	- / -
W_{20}5GBA$_2$ED	2	2 – 4	- / -	4	4 – 8	- / -	8	4 – 16	- / -	16	8 – 16	- / -	≤0.12	≤0.12 – 4	- / -	≤0.12	≤0.12	- / -	128	32 – >256	- / -
Ceftazidime	≤1	≤1 – >16	80.0 / 20.0	≤1	≤1 – >16	80.0 / 20.0	≤1	≤1	100.0 / 0.0	2	2 – 4	100.0 / 0.0	4	≤1 – >16	80.0 / 20.0	16	2 – >16	40.0 / 40.0	2	2 – 4	100.0 / 0.0
Cefepime	≤0.12	≤0.12 – >16	80.0 / 20.0	≤0.12	≤0.12 –>16	80.0 / 20.0	≤0.12	≤0.12	100.0 / 0.0	4	1 – 8	100.0 / 0.0	2	≤0.12 –>16	80.0 / 20.0	>16	4 – >16	- / -	8	4 – 16	- / -
Piperacillin/ tazobactam	2	1 – 8	100.0 / 0.0	2	1 – 64	80.0 / 0.0	≤0.5	≤0.5 – 1	100.0 / 0.0	2	2 – 16	100.0 / 0.0	2	≤0.5 – >64	80.0 / 20.0	>64	8 – >64	- / -	4	2 – 32	- / -
Imipenem	0.25	≤0.12 – 0.5	100.0 / 0.0	0.25	≤0.12 –0.25	100.0 / 0.0	1	≤0.12 – 2	100.0 / 0.0	1	1 – >8	80.0 / 20.0	≤0.12	≤0.12 – >8	80.0 / 20.0	>8	4 – >8	- / -	8	4 – 8	- / -
Gentamicin	≤2	≤2 – >8	80.0 / 20.0	≤2	≤2	100.0 / 0.0	≤2	≤2	100.0 / 0.0	≤2	≤2 – 4	100.0 / 0.0	≤2	≤2 – >8	80.0 / 20.0	>8	≤2 – >8	- / -	>8	>8	- / -
Tobramycin	0.5	0.25 – 16	80.0 / 20.0	0.25	0.25 – 16	80.0 / 20.0	1	0.25 – 1	100.0 / 0.0	0.5	0.25 – 1	100.0 / 0.0	0.5	0.25 – >16	80.0 / 20.0	>16	0.5 – >16	- / -	>16	>16	- / -
Levofloxacin	0.03	0.03 – >8	60.0 / 40.0	0.06	0.06 – 8	80.0 / 20.0	0.12	0.06 – 2	100.0 / 0.0	1	0.25 – 4	80.0 / 0.0	0.25	0.06 – >8	80.0 / 20.0	0.5	0.5 – 8	60.0 / 20.0	2	1 – 2	100.0 / 0.0
Tetracycline	>8	4 – >8	20.0 / 80.0	≤2	≤2 – 4	100.0 / 0.0	>8	>8	0.0 / 100.0	8	8 – >8	0.0 / 40.0	≤2	≤2 – >8	80.0 / 20.0	>8	≤2 – >8	- / -	>8	>8	- / -
Colistin	≤0.5	≤0.5	- / -	≤0.5	≤0.5	- / -	>4	>4	- / -	1	≤0.5 – 2	100.0 / 0.0	≤0.5	≤0.5 – 2	100.0 / 0.0	≤0.5	≤0.5 – 1	- / -	-	>4	- / -

[a]Criteria as published by CLSI, 2008; [b]EDTA alone inhibited three strains of A. baumannii; [c]EDTA alone inhibited five strains of *S. maltophilia*.

Table 2. Pilot study: MIC values of W_{20}5EC ± EDTA.

Bacterial Isolate	MIC (µg/ml) for W_{20}5EC + EDTA				
	0 mM	5 mM	10 mM	15 mM	20 mM
K. pneumoniae 24-5A	64	8-16	8	8	8
E. coli ATCC 25922	16	1	≤0.12	≤0.12	≤0.12
P. mirabilis 119-163A	128	≤0.12[a]	≤0.12	≤0.12	≤0.12
A. baumannii 67-299A	32	≤0.12[a]	≤0.12	≤0.12	≤0.12
P. aeruginosa ATCC 27853	>256	32-64	32	32	32
B. cepacia 30-492A	>256	>256	>256	>256	>256

[a]EDTA alone inhibited these strains.

Table 3. Cidality of nanoemulsions against a subset (23) gram-negative isolates.

		Compound (number of strains)			
	MBC/MIC ratio	W_{20}5EC	P_{407}5EC	W_{20}5G BA_2 ED	Levofloxacin
Cidal	1	7	10	9	9
	2	6	7	7	8
	4	4	2	2	1
Static	8	1	1	1	0
	>8x	2	0	1	5
	No growth[a]	3	3	3	0

[a]EDTA alone inhibited 3 strains of *A. baumannii*

METHODS

Emulsion manufacturing. Nanoemulsions W_{20}5EC, P_{407}5EC, and W_{20}5GBA$_2$ED are oil-in-water emulsions manufactured from ingredients that are Generally Recognized As Safe (GRAS) with a cationic detergent (cetylpyridinium chloride, CPC, or benzalkonium chloride, BA) as active ingredients that have proven safe for human use. The emulsion is formed from highly purified oil, ethanol, a nonionic surfactant and water. The average nanoemulsion droplet size is 180 nm for W_{20}5EC and 350 nm for P_{407}5EC and W_{20}5GBA$_2$ED as measured by dynamic light scattering using a Malvern Zetasizer Nano 3600 (Malvern Instruments Ltd., Worcestershire, UK).
Source of isolates. The source of the clinical isolates was bloodstream or skin and soft tissue isolates collected by JMI Laboratories over the past two years.
MIC and MBC determinations. Initially, MICs of W_{20}5EC ± 0, 5, 10, 15 and 20 mM EDTA were evaluated in cation-adjusted Mueller-Hinton broth by microdilution per M7-A7 (2006). MICs for a larger panel of gram-negative isolates were determined for the nanoemulsions in the presence of 5 mM EDTA. EDTA was used to permeabilize the gram-negative envelope, aiding in fusion of the nanodroplets to the cell membrane, resulting in lysis. Alamar blue was added to the assay panels two hours post-inoculation for enhanced MIC endpoint detection. MBC values were assessed for nanoemulsions and a comparator compound (levofloxacin) by plating the entire broth content from the MIC well and from those four doubling dilutions above the MIC onto blood agar growth media. Quantitative colony counts were performed on the initial inoculum. The lowest concentration of antimicrobial agent that killed ≥ 99.9% of the starting test inoculum was defined as the MBC endpoint. A ratio MBC/MIC of ≤4 is defined as bactericidal.

CONCLUSIONS

- Nanoemulsions W_{20}5EC, P_{407}5EC and W_{20}5GBA$_2$ED administered by inhalation may have potential for prophylactic use in patients with cystic fibrosis or other chronic pulmonary diseases.
- W_{20}5EC, P_{407}5EC and W_{20}5GBA$_2$ED were bactericidal against 85%, 95% and 90% of the isolates, respectively.
- No cross-resistance to any known antibiotic was observed for any of the nanoemulsion antimicrobial agents.

NanoBio Corporation