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Short Note

Induced Pseudomonas Aeruginosa Biofilm-Matrix Collapse Fluidizes Cystic Fibrosis Sputum

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SHORT NOTE

Bacterial biofilms interfere with otherwise effective antimicrobials, exemplified by persistent colonization by *Pseudomonas aeruginosa* of cystic fibrosis (CF) lungs despite aggressive antibiotic treatment [1-3]. Slime matrix protection of oral bacterial pathogens within dental plaques, the archetypical biofilms, imparts resistance to chlorhexidine disinfection in mechanically ventilated patients with respiratory infections [4]. Here we show that that a rinse that reduces gingivitis by physically collapsing the plaque biofilms' supporting matrices also strips the viscous slime layers from *P. aeruginosa's* self-produced polymeric matrix, and fluidizes thick sputum of active CF patients. We have analyzed 30 de-identified specimens of sputum donated by CF patients at Women and Children's Hospital of Buffalo before and after in vitro treatment. The results document a hitherto unknown effectiveness of the non-antimicrobial reagent delmopinol [(+-) 3-(4-propylheptyl) -4-(2-hydroxyethyl) morpholine hydrochloride], an FDA-approved C10 analog [5] of similarly clinically effective plaque-reducing compound octapinol [6], at concentrations from 0.2% to 100%. Delmopinol spontaneously reduces immobile CF sputum masses to flow able suspensions by physical coagulation of CF sputum biofilm-stabilizing polymers and liquefaction of the remaining sputum mass. We speculate that this biofilm-matrix stripping agent may be useful as an adjunct to antibiotic treatment of CF patients and others with biofilm-related infections [7-10] while minimizing risks of developing further antibiotic resistance.

Pseudomonas aeruginosa [1] and a variety of multi-drug resistant pathogens grow as a biofilms in the airways of patients with cystic fibrosis (CF). Treatment of CF-associated airways infection is not always successful. Targeting biofilms, rather than the organisms that produce them, has been proposed as a possible new strategy to control lung disease in CF [11]. *Streptococcus mutans* is the principal plaque-forming pathogen of the oral cavity [12]. We studied the cultured prototype strain of *P. aeruginosa* PA01, and CF-related mucoid strains M-4 and M-5, as well as *S. mutans* strain 10449 and *Streptococcus* strain G9B, exposing their mature biofilms to 0.2% delmopinol solutions and examining them by standard microbiological techniques, as well by spectroscopic and microscopic techniques before and after such rinses. No overt bacterial killing was observed, although bacterial re-growth in culture was delayed by about 24-hours after the delmopinol rinse. In the mucoid *P. aeruginosa* strains M-4 and M-5, the protective matrix seen earlier [1] around microbial clusters, and also shown here in Figure (1A), was

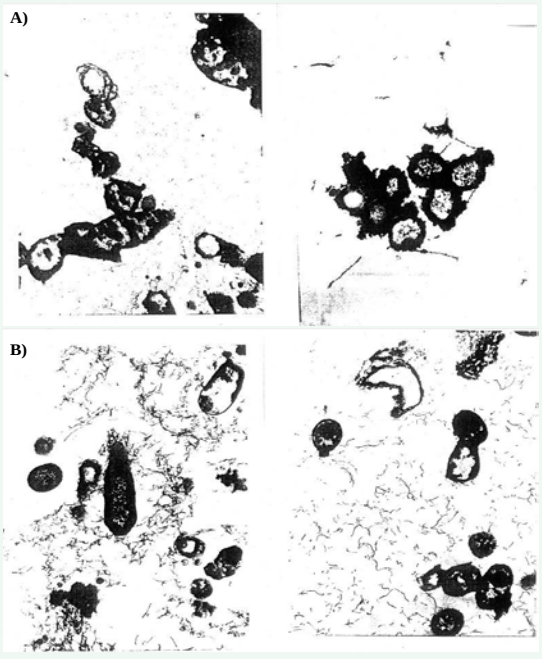

Figure 1 Transmission electron microscopic sections through *Pseudomonas aeruginosa* Strain M5 biofilm segments before (top) and after (bottom) exposure to 0.2% solution of delmopinol. Note breakdown and dispersal of initial thick polymeric matrix into collapsed, coagulated strands, fluidizing the preparation and exposing the still culturable bacteria. Compare with Figure (1) in Reference 1. Diameters of *Pseudomonas aeruginosa* are 0.5 - 1.0 micrometer.

disaggregated and dispersed during delmopinol exposure, as seen in Figure (1B). *S. mutans* biofilms were physically dispersed by delmopinol droplet application treatments and sodium alginate gels simulating the major *S. mutans* polymeric exudates were liquefied by delmopinol droplet addition. The effect of delmopinol on the protective matrices of biofilms in general is quite broad. For example, it has been found to strip the polymeric matrix from biofilms formed in tanks of seawater harboring multiple marine organisms, simulating natural bioinvasive films [13].

Sputum samples (2-10ml) were obtained from CF patients in the Adult CF Clinic of Women and Children's Hospital of Buffalo, by voluntary expectoration, with informed consent under a protocol approved by the SUNY Buffalo Health Sciences Institutional Review Board. Employing the analytical technique of Multiple Attenuated Internal Reflection Infrared (MAIR-IR) spectroscopy [14], sputum specimens were found to comprise glycoproteinaceous and proteoglycan-rich mucopolymers similar to the anionic mucopolysaccharide "slimes" present in cultured bacterial biofilms and in clinical dental plaques. The ratios of the protein to carbohydrate components present in sputum specimens varied from patient to patient, but all but one were susceptible to breakdown by delmopinol as described here. These analyses supported our hypotheses that the reagents successful in dis-aggregating dental plaque should also be successful in fluidizing CF sputum for easier clearance and expectoration.

Employing the analytical technique of digital viscometry, sputum specimens were found to vary from "too viscous to measure" [instrumentation limit of 1000 centipoise (cps)] to lower than 60 cps, but most tested specimens were thixotropic showing "shear thinning" behavior as constant shear stress was applied. Dilution of CF-patient sputum samples with distilled water provided a short-term (minutes) reduction in viscosity, but the sputum persisted in thixotropic behavior as the water was incorporated into the motion-resisting polymeric gel. Thus, we concluded that simple dilution of CF-patient sputum is not sufficient to reduce its viscosity or to eliminate the extended gel-forming polymer network responsible for thixotropic behavior.

Employing the analytical technique of contact angle goniometry, sputum specimens were found to vary in operational surface tensions (from about 55 mN/m to about 25 mN/m), the lowest values associated with the most strongly gelled sputum masses that could not spontaneously recede over a laboratory standard polytetrafluoroethylene substratum [14].

These tests showed that low operational surface tensions of CF-patient sputum do not directly correlate with easy flow of sputum masses.

CF patient sputum aliquots taken up, with difficulty, into Pasteur pipettes (tips subsequently sealed), were observed to remain as stiff gels when the pipettes were held horizontally, as seen in Figure (2A). Paired specimens were compared, one as a control treated only with water or an inert (fumed silica) powder and the other treated with the test reagents, and observed side-by-side for changes in response to gravitational forces as well as ability to spontaneously penetrate back down the pipette tips from which they were driven by the tip-sealing step. Figure (2B) illustrates the prompt effectiveness (usually within minutes) of 0.2% delmopinol treatment (bottom pipette in Figure (2B)) compared with the lesser effect of a distilled water aliquot of the same volume (one 50 µl droplet).

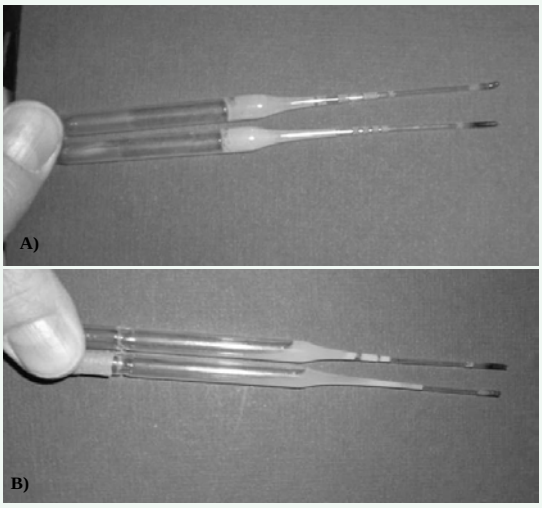

Figure 2 Photographs of the same CF patient sputum aliquots taken up into Pasteur pipettes before and after in-place treatment from the open end of each pipette. (a) non-flow able when tubes are horizontal; (b) same samples within minutes after being treated with either distilled water (upper tube) or 0.2% delmopinol (lower tube). Note additional gravitational flattening of the sputum profile in the delmopinol-treated specimen and its spontaneous penetration into the narrow pipette region, facilitated by the specimen's fluidization and decreased surface tension.

The primary treatment reagent was a fine dry powder of [(+-) 3-(4-propylheptyl)-4-(2-hydroxyethyl) morpholine hydrochloride] originally supplied by Ferrosan AB, a Swedish corporation, used (a) neat (100%) as a sprinkled aerosol over sputum specimens; (b) as a 10% solution in distilled water added drop wise to the sputum specimens; (c) as a sprayed 10% concentration in water aerosol mist over a sputum sample; and (d) as drops of the 0.2% Decapinol mouth rinse commercial product donated by Sinclair Pharmaceuticals of England. The efficacy of the primary reagent to liquefy the sputum samples was convincingly demonstrated over the range of 0.2% to 100% concentrations, in dry neat powder aerosol, liquid 10% (solution droplets and aerosol), and liquid 0.2% (droplets).

Comparison reagents, all having the capability to reduce the surface tensions of aqueous fluids to the same low values of approximately 40-50 mN/m produced by added delmopinol, were these: sodium lauryl sulfate (100% dry powder), Pluronic F68 (100% dry powder and 10% commercially prepared solution), and Triton X100 (100% liquid). These powerful surfactants often have been used in contact with biological systems, and were selected as comparison reagents for delmopinol's fluidization effect.

As noted earlier, MAIR-IR spectroscopy revealed that CF patient-donated sputum is dominated by polymeric substances similar to those associated with antiseptic-resistant and antibiotic-resistant biofilm exopolymers. In these sputa, delmopinol

bound spontaneously to the polymers in a water-rinse-resistant manner to coagulate them into isolated, insoluble masses. MAIR-IR also showed that such binding did not occur with any other surfactants tested. Thus, delmopinol reacts uniquely among surfactants, being the only tested reagent to spontaneously bind with and coagulate CF patient sputum polymers.

Digital viscometry showed that all the tested surfactant reagents could reduce the viscosity of CF patient-donated sputum, but only delmopinol also eliminated the thixotropic behavior of CF sputum masses. Delmopinol treatment of thin viscous layers of CF sputum produced isolated coagulated fragments that were, themselves, of substantially higher viscosity than the original sputum, within a water-like free-flowing phase. Only delmopinol collapsed the gel-forming polymers from CF patient-donated sputum volumes to produce a free flowing fluid phase and an irreversibly coagulated polymer mass.

Contact angle measurements showed that all the tested surfactants reduced the operational surface tensions of CF patient-donated sputum to values between 40 and 50mN/m. Therefore, the differential results of exopolymer coagulation and irreversible fluidization of CF patient-donated sputum cannot be attributed to surfactancy as the main principle of action. Rather, the unique mechanism proposed for the breakdown of bacterial biofilms is the preferential binding of the cationic delmopinol to anionic sites of the protective slime polymers, adding a hydrophobic hydrocarbon "umbrella" that helps shed water from the collapsing matrix while exposing the microbes to a now-fluidized environment (Figure 3).

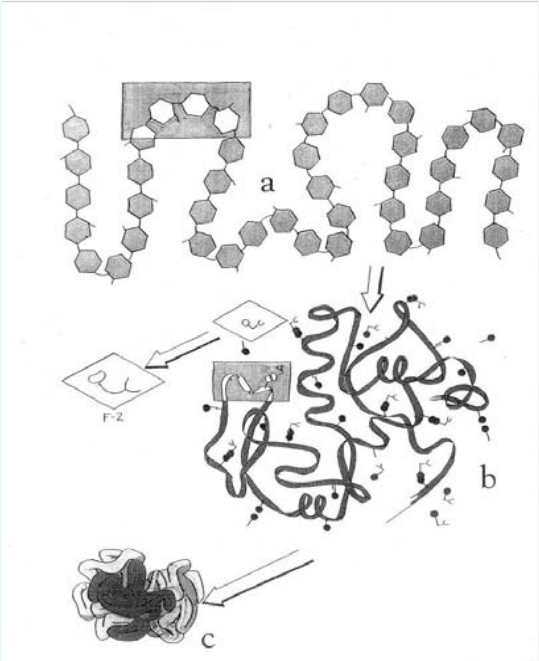

Figure 3 Schematic of the mechanism identified for the biofilm matrix breakdown phenomena illustrated in Figure (1,2). (a) a stylized molecular-level segment of the anionic, hydrophilic polysaccharide slime layer comprising most biofilm matrices; (b) a ribbon version of the same structure, showing its interaction with compound F-2 (experimental designation for delmopinol in 1990) by cation-to-anion association and conversion of the originally hydrophilic, anionic sites to neutral hydrophobic sites, followed by (c) spontaneous physical collapse of the dehydrated polymer/reagent adduct into a condensed structure no longer capable of protecting microbes or supporting biofilm architectures.

In the spirit of more rapidly identifying and translating cross-disciplinary research findings to broader patient benefit, we speculate that similar biofilm-matrix stripping will occur with delmopinol pretreatments in cases of otitis media with effusion [8], wound healing [9], and catheter-based infections, overcoming their notable resistance to conventional antibiotics [10].

In summary, we have shown that delmopinol appears to have unique properties that cause increased fluidity of sputum from patients with CF. We speculate that this biofilm-matrix stripping agent may be useful as an adjunct to antibiotic treatment of CF patients and others with biofilm-related infections [7-10] while minimizing risks of developing further antibiotic resistance.

AUTHOR INFORMATION

Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to baier@buffalo.edu

AUTHOR CONTRIBUTIONS

REB, AEM, MJA, and JMM performed the biofilm studies with support from the Cystic Fibrosis Foundation and New York State Center of Advanced Technology, Healthcare Instruments and Devices Institute (CAT-HIDI), in 1987-1988. REB developed the mechanistic interpretation illustrated in Figure (3), under a research contract with Biosurfaces AB, Sweden in 1990. DSB and TFM facilitated the CF sputum portion of the study as an activity of the Buffalo Clinical and Translational Research Center (CTRC) in 2011, and all authors participated in the sputum treatment studies, data review, and manuscript preparation.

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