Small-molecule Inhibitors of *Pseudomonas aeruginosa* Biofilm Development

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Shinerama is one of the hallmark events of McMaster's Welcome Week. Through this student-led initiative, Mac students raised $60,000 last year for cystic fibrosis research. Interestingly, and as some undergraduates would agree, that money is being put to great use right here at McMaster's laboratories. Here we present the exciting new discoveries of one such group: the laboratory of Dr. Lori Burrows.

Matthew A. Chong is a third year student in the Bachelor of Health Sciences (Honours) Program. Funded by a generous studentship from the Canadian Cystic Fibrosis Foundation, Matthew spent his summer researching biofilm development by opportunistic pathogen Pseudomonas aeruginosa. The central focus of his project was to characterize the structure activity relationship of potent biofilm inhibitor palmitoyl-DL-carnitine.

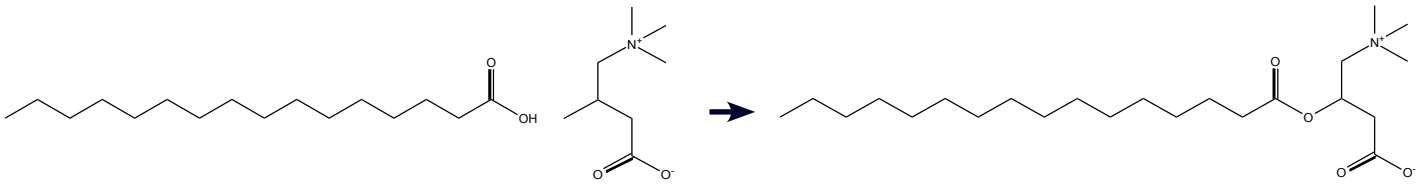
With a prevalence of 1 in 2500 live births, cystic fibrosis (CF) is the most common hereditary disease affecting Caucasians.[1] CF normally manifests itself as a chronic, recurring lung infection that results in irreversible lung damage and ultimately respiratory failure. Pulmonary infections in patients with CF are notoriously difficult to treat because *P. aeruginosa*—the primary pathogen implicated in CF—exists as a surface-associated community known as a **biofilm**.[1] In addition to being involved in the pathophysiology of CF, biofilms are associated with a long-list of diseases that include periodontitis, middle-ear infections, endocarditis, and infections of implanted medical devices such as catheters.[2]

Bacterial biofilms are defined as a surface-associated community of cells enclosed in a self-produced polymeric matrix.[2] Biofilm formation, a ubiquitous characteristic of bacteria, allows them to better survive in the real-world where nutrients and environmental conditions are in a constant state of flux.[3] Schematically, biofilm formation involves arrival at the surface, initial attachment, the formation of microcolonies (small aggregates of cells), biofilm maturation, and dispersal (leaving the biofilm to begin the cycle again).[2,4] After microcolony formation begins, the developing biofilm initiates the synthesis of a slimy and highly-organized matrix composed primarily of DNA, protein and polysaccharides.[2,3]

In addition to providing structural support for the biofilm, the matrix protects biofilm cells from the host immune system by shielding free radicals employed by neutrophils, hindering phagocytosis, and interfering with opsonization (tagging of bacteria by antibodies).[5,6] The biofilm matrix also binds antibiotics and contains antibiotic-degrading enzymes.[3,7] Lastly, the matrix gives rise to metabolic heterogeneity (different rates of metabolism) of biofilm cells—a consequence of gradients in nutrient, waste product, and signaling molecule levels—that renders the cells themselves highly resistant to antibiotics, which generally depend on the disruption of active metabolism.[2] To use an analogy, not only are biofilm cells hiding in a protective castle (the matrix) but they are also wearing armor (intrinsic antibiotic resistance). Indeed, biofilm cells are up to 1000 times more resistant to antibiotics compared to motile cells allowing them to persist—and subsequently recolonize the host—despite cycles of antibiotic therapy.[2,8] Therefore, the development of inhibitors of biofilm formation represents a viable strategy to ameliorate biofilm-associated infections such as those experienced by CF patients.

SMALL-MOLECULES AS INHIBITORS OF *P. aeruginosa* BIOFILM FORMATION

Previous small-molecule screening by Master's candidate Iwona Wenderska of the laboratory of Dr. Lori Burrows identified the eukaryotic kinase inhibitor **palmitoyl-DL-carnitine** (**pDLc**) as a potent disruptor of *P. aeruginosa* biofilm formation. The purpose of this project was to characterize the structure-activity relationship of pDLc by exploring whether related compounds possessed similar inhibitory properties. By understanding how certain chemical moieties (groups) of pDLc contribute to the overall inhibition, we can gain a better understanding



Hexadecanoic Acid Carnitine Palmitoyl-DL-Carnitine

FIGURE 1: Palmitoyl-DL-Carnitine contains two chemical moieties—carnitine and hexadecanoic acid—joined by an ester linkage. Note that hexadecanoic acid is also called the non-IUPAC name palmitic acid, and that is where the palmitoyl portion of palmitoyl-DL-carnitine's name originates.

of the biofilm formation process and possibly identify more potent inhibitors.

The Burrows laboratory uses a highly optimized assay to quantify the biofilm formation by *P. aeruginosa*. The biofilms are grown overnight (19hs) on plastic pegs immersed in culture containing *P. aeruginosa* strain PAO1. After this incubation period, the pegs are washed to remove non-adherent bacteria and then stained using crystal violet dye (a dye also used in gram-staining) to quantify the amount of biofilm.

The starting point of the project was to determine the biofilm inhibitory properties of palmitoyl-DL-carnitine's (pDLc) chemical moieties: the polar carnitine head-group and hexadecanoic acid. Interestingly, only hexadecanoic acid (and not carnitine) was able to inhibit biofilm formation. Since these results suggested that the lipid moiety of pDLc was of particular importance, other saturated fatty acids of varying chain length were tested for their biofilm-inhibitory properties.

Only a subset of fatty acids is able to inhibit *P. aeruginosa* biofilm formation at micromolar concentrations (Table 2). Interestingly, the naturally occurring 14- and 16-carbon fatty acids, but not the synthetic 15-carbon acid, exhibited the most potent biofilm inhibition properties; this suggests that even modest differences in fatty acid chain length may affect their signaling properties. Hexadecanoic acid is found in nature as an antimicrobial present in plant extracts.[9]

Fatty acids are similar to detergents in so far as both compounds are known to make surfaces slippery (act as surfactants), which may physically interfere with bacterial attachment and subsequent biofilm formation. The surfactant properties of fatty acids made it unclear as to whether their inhibitory action was due to this physical effect or specific signaling mechanisms. Therefore, to elucidate the nature of the observed inhibition, the ability of common laboratory

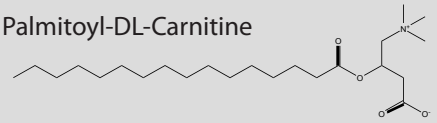
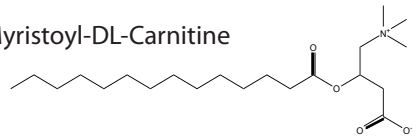
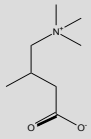
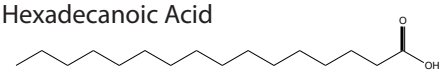
Compound	EC_{50}
Palmitoyl-DL-Carnitine 	4μM
Myristoyl-DL-Carnitine 	15μM
Carnitine 	Not efffective
Hexadecanoic Acid 	30μM

TABLE 1: Summary of the effects of pDLc and its derivatives on biofilm formation. With the exception of the carnitine, all compounds shown inhibited biofilm formation by PAO1 in a concentration-dependent manner. The EC_{50} is defined as the concentration of the test compound at which biofilm formation is reduced to 50% of the vehicle-treated control. EC_{50} values below 25μM are generally considered to be excellent.

Systemic Name	Chain Length	EC_{50}
Nonanoic Acid	9	Not effective
Decanoic Acid	10	Not effective
Dodecanoic Acid	12	Not effective
Tridecanoic Acid	13	Not effective
Tetradecanoic Acid	14	25 μM
Pentadecanoic Acid	15	Not effective
Hexadecanoic Acid	16	30 μM
Heptadecanoic Acid	17	Not effective
Octadecanoic Acid	18	Not effective

TABLE 2: Fatty acids differ in their ability to inhibit PAO1 biofilm formation. The EC_{50} is defined as the concentration of the test compound at which biofilm formation is reduced to 50% of the vehicle-treated control.

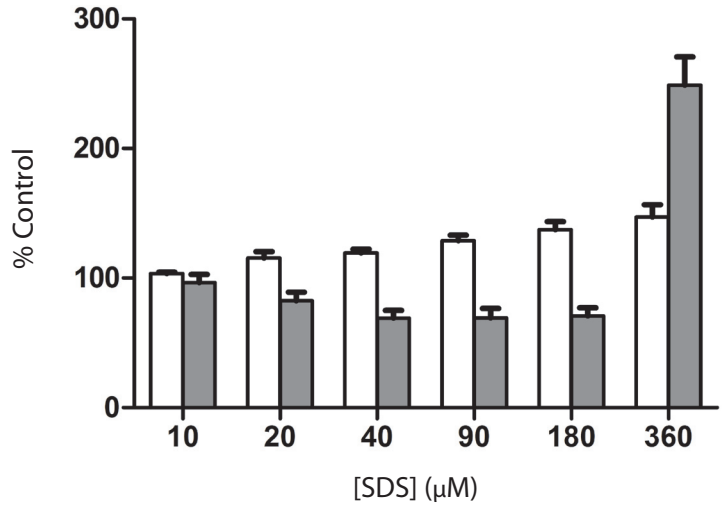


FIGURE 2: Detergent-induced biofilm formation.
The response to SDS is similar to the other detergents screened, and none were as effective as the fatty acids or pDLc at inhibiting biofilm formation. Planktonic growth is defined as growth in the culture medium (motile, non-biofilm cells) and is measured to ensure that test compounds are not inhibiting biofilm formation by simply killing the bacteria.

detergents to inhibit biofilm formation was investigated (as an example, Figure 1 shows data for the common detergent sodium-dodecyl sulfate). The detergents had only modest effects on biofilm formation (30% inhibition), and at higher concentrations they counterintuitively induced biofilm formation (>200% increase).

Based on the results, certain fatty acids and pDLc can act as potent inhibitors of *P. aeruginosa* biofilm formation. Furthermore, the observation that common lab detergents are unable to inhibit biofilm formation in the same range of concentrations as the fatty acids and pDLc raises the possibility that the observed effect is not physical but may instead act through specific signaling mechanisms. *P. aeruginosa* has several fatty acid responsive transcription factors that modulate its gene expression.[10] The ability of fatty acids to interact with these transcription factors was shown to be highly dependent on the chain-length of the fatty acid, consistent with our observation that only the 14- and 16- carbon fatty acids appreciably inhibit biofilm formation.[11] Odd-numbered chain length fatty acids, which do not occur naturally, were interestingly less effective at inhibiting biofilm formation than their even-numbered counterparts. The fact that the two chemical moieties of pDLc—hexadecanoic acid and carnitine—were individually less potent than pDLc at inhibiting *P. aeruginosa* biofilm formation illustrates beautifully that the whole is greater than the sum of its parts.

Implicated in the etiology of fatal diseases like CF and many nosocomial infections, biofilms are clearly a widespread healthcare problem.[2] Biofilms also make headlines in industry; for example, the recent contamination of a Maple Leaf Foods plant with *Listeria monocytogenes* biofilms resulted in the deaths of 22 Canadians.[12] On the bright side, biofilms can be harnessed to bioremediate polluted environments and purify sewage.[13] Nonetheless, in a medical context at least, the development of biofilm inhibitors may improve the efficacy of current antibiotics and increase the life expectancy and quality of life of patients with biofilm-associated chronic infections. Further research is currently underway to determine whether pDLc and related compounds increase the susceptibility of *P. aeruginosa* biofilms to antibiotics. ▪

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Canadian Cystic Fibrosis Foundation

Reviewed by Dr. Lori Burrows, Ph.D.

Dr. Lori Burrows is a Professor at McMaster University in the Department of Biochemistry and Biomedical Sciences. Her research interests include type IV pili, peptidoglycan dynamics, and bacterial biofilms. Her lab employs a wide-range of genetic and biochemical techniques with the goal of identifying vulnerabilities in these systems that could be exploited for drug development.

REFERENCES

[1]Hassett DJ, Korfhagen TR, Irvin RT, Schurr MJ, Sauer K, Lau GW, et al. (2010). Pseudomonas aeruginosa biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies. Expert Opin.Ther. Targets, 14(2), 117-130.

[2]Costerton JW, Stewart PS, & Greenberg EP. (1999). Bacterial biofilms: a common cause of persistent infections. Science, 284(5418), 1318-1322.

[3]Karatan E, & Watnick P. (2009). Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiol.Mol.Biol.Rev., 73(2), 310-347.

[4]Glick R, Gilmour C, Tremblay J, Satanower S, Avidan O, Deziel E, et al. (2010). Increase in rhamnolipid synthesis under iron-limiting conditions influences surface motility and biofilm formation in Pseudomonas aeruginosa. J.Bacteriol., 192(12), 2973-2980.

[5]Mah TF, & O'Toole GA. (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol., 9(1), 34-39.

[6]Ryder C, Byrd M, & Wozniak DJ. (2007). Role of polysaccharides in Pseudomonas aeruginosa biofilm development. Curr.Opin.Microbiol., 10(6), 644-648.

[7]Schooling SR, & Beveridge TJ. (2006). Membrane vesicles: an overlooked component of the matrices of biofilms. J.Bacteriol., 188(16), 5945-5957.

[8]Hall-Stoodley L, Costerton JW, & Stoodley P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. Nat.Rev.Microbiol., 2(2), 95-108.

[9]Yff BT, Lindsey KL, Taylor MB, Erasmus DG, & Jager AK. (2002). The pharmacological screening of Pentanisia prunelloides and the isolation of the antibacterial compound palmitic acid. J.Ethnopharmacol., 79(1), 101-107.

[10]Kang Y, Nguyen DT, Son MS, & Hoang TT. (2008). The Pseudomonas aeruginosa PsrA responds to long-chain fatty acid signals to regulate the fadBA5 beta-oxidation operon. Microbiology, 154(Pt 6), 1584-1598.

[11]Kang Y, Lunin VV, Skarina T, Savchenko A, Schurr MJ, & Hoang TT. (2009). The long-chain fatty acid sensor, PsrA, modulates the expression of rpoS and the type III secretion exsCEBA operon in Pseudomonas aeruginosa. Mol.Microbiol., 73(1), 120-136.

[12]Charlebois S, & Horan H. (2010). Institutional and relational determinants in high- and medium-extent food product crises: the inner perspective of a public health crisis. Int.J.Environ.Health Res., 20(4), 299-312.

[13] Cao B, Nagarajan K, & Loh KC. (2009). Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. Appl.Microbiol.Biotechnol., 85(2), 207-228.