Selective toxicity of fluorinated dipeptides against *Pseudomonas aeruginosa* biofilms *in vitro*

A Senior Thesis Presented to

The Faculty of the Department of Molecular Biology

Colorado College

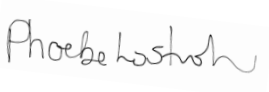
By Myriam Ortiz Isuiza

Bachelor of Arts Degree in Molecular Biology

Submitted on May 17th, 2023

Dr. Olivia Hatton

Primary Thesis Advisor



Dr. Phoebe Lostroh

Secondary Thesis Advisor

ABSTRACT

According to the Health World Organization, *Pseudomonas aeruginosa (Pae*) is a multi-drug resistant bacteria urgently needing new antibiotics. *Pae* infections are particularly common in cystic fibrosis patients, due to their immunocompromised status. The ability of this bacteria to infect this vulnerable population lies in its capacity to form biofilms with a self-produced matrix of extracellular polymeric substances (EPS). Previous research on biofilm formation of several gram-negative bacteria, including *Pae*, concluded that D-leucine, D-methionine, D-tyrosine, and D-tryptophan were produced to inhibit biofilm formation. Based on these early findings, the Drug Distributed Discovery (D3) project, which purpose is to inexpensively synthesize compounds to treat neglected diseases from the developing world, repeated these experiments to finally conclude that only L-amino acids caused a reduction in *Pae* biofilm formation. More than 150 pro-drug formulations were synthesized for further testing, due to concerns regarding selective toxicity.

In this research paper, I investigated the selective toxicity of two fluorinated dipeptides from the D3 project, against *Pae* strain 14 (PA14). After conducting biofilm and cell viability assays of increasing concentrations of selected compounds, it was determined that these compounds do exhibit selective toxicity against PA14 *in vitro*.

INTRODUCTION

The current multi-drug resistant nature of bacteria belonging to the *Pseudomonas* genus has caused the Health World Organization to categorize it as a critical Priority 1 bacteria with an urgent need for new antibiotics[1]. *Pseudomonas* are now resistant to carbapenems and third generations cephalosporins, the strongest antibiotics known in the market[1]. *Pseudomonas aeruginosa (Pae*) infections are the most common cause of respiratory infections in genetic cystic fibrosis (CF) patients[2]. Its robust biofilm-growing mucoid, mainly composed of the polysaccharide alginate, is the main cause for lung infections in CF patients[3]. An advantageous characteristic of bacteria that tend to form biofilm relies on its ability to resist antibiotics[4]. Biofilms also reduce innate and adaptive immune responses such as phagocytosis[4].

Following the International Union of Pure and Applied Chemistry (IUPAC) recommendations, biofilms are defined as 'aggregates of microorganisms in which cells are frequently embedded in a self--produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface'[5]. *Pae* biofilms, specifically, behave as a type of intercellular bacterial communication on solid surfaces. In this way, *Pae* biofilms prevent damage from external environmental factors, via the aid of flagella, type IV pili or soluble products that form an extracellular biofilm matrix[6]. In particular, the self-produced EPS matrix, mainly composed of polysaccharides, proteins, lipids, and extracellular DNA (eDNA), allows for the biofilm's 3D structure, cell-to-cell communication, versatile external digestive system, recycling of DNA, desiccation prevention and a protection barrier for various antimicrobial agents[7].

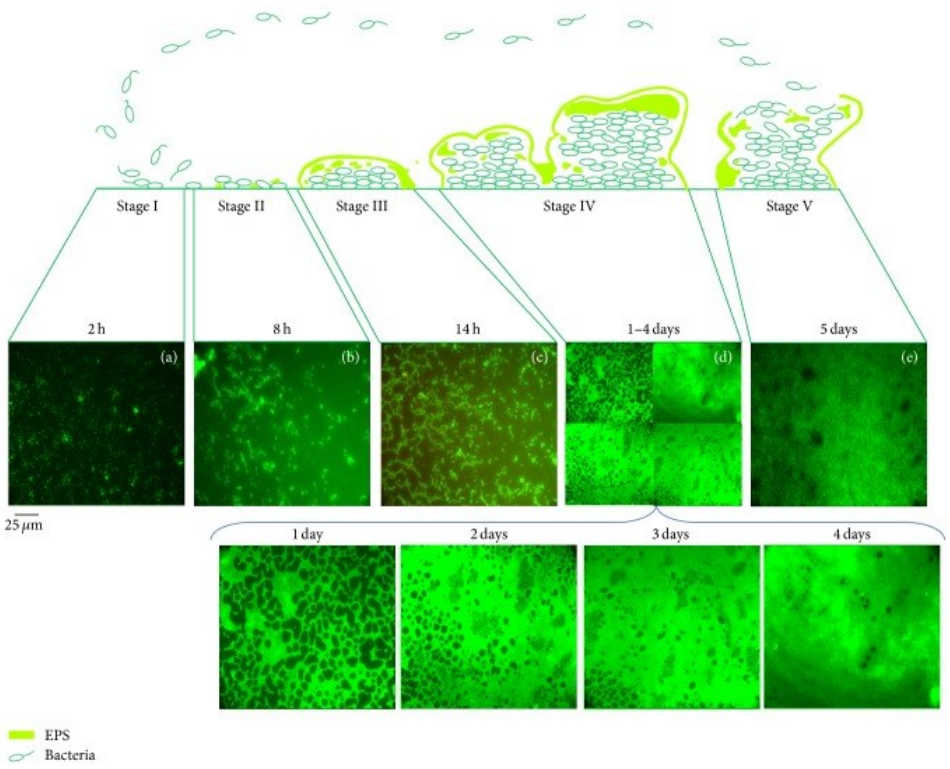


Fig 1. Biofilm lifestyle cycle of P. aeruginosa PAO1 grown in glucose minimal media. In stage I, planktonic bacteria initiate attachment to an abiotic surface, which becomes irreversible in stage II. Stage III corresponds to microcolony formation. Stage IV corresponds to biofilm maturation and growth of the three-dimensional community. Dispersion occurs in stage V and planktonic bacteria that are released from the biofilm to colonize other sites. The biofilm formation by P. aeruginosa PAO1 was revealed with Syto9 and visualized in Leica DM IRE2 inverted fluorescence microscope with 400x magnification at 2 h (Stage I), 8 h (Stage II), 14 h (Stage III), 1 to 4 days (Stage IV), and 5 days (Stage V). Images represent a 250 × 250-μm field. Quoted from Fig 1 of Rasamiravaka[8].

Developmental stages of *Pae* biofilm formation

Based on the study of *Pae* strain PA01 grown in glucose minimal media *in vitro*, the biofilm life cycle of *Pae* can be divided into 5 distinct stages (Fig 1). Stage I would correspond to the planktonic stage, in which free-living bacterial cells are not attached to the surface. Stages II and III are considered to be the attachment stages, characterized by a microcolony formation and biofilm initiation via structures like adhesins, type IV pili and lipopolysaccharide (LPS)[9]. The EPS matrix characteristic to biofilms starts to form at the end of this stage. Stage IV is considered the maturation

stage where three-dimensional communities are finally observed with the usual EPS matrix fully formed around them after 1 to 4 days of incubation. The signaling system quorum sensing (QS) is also developed to reinforce the biofilm resistance to outer environmental stresses[9]. After 5 days, the biofilm enters the detachment stage, in which some bacterial cells start to disperse and reenter the planktonic stage to colonize other surfaces. Developmental stages of *Pae* strain 01 biofilm are used as the standard for other strains of *Pae*.

Extracellular matrix of *Pae* biofilms

One of the ways in which *Pae* achieves antibiotic resistance is through adaptive resistance, specifically the formation of a biofilm matrix. Biofilm-mediated resistance is achieved by the components of the biofilm matrix: extrapolysaccharides (EPS), DNA and proteins. Polysaccharide alginate, a copolymer of partly O-acetylated D-mannuronic and L-glucuronic acids, serves as a factor used to distinguish between mucoid or non-mucoid *Pae* biofilms. Alginate is known to retain water and nutrients and supply antibiotic resistance and immune evasion to the biofilm[9]. A high percentage of alginate-containing strains present a mutation of the gene *mucA*, responsible to produce MucA protein, responsible for blocking one of the alternative sigma factors on which alginate operon expression depends. Mannose rich polysaccharide Psl accumulates on the outside of the biofilms during maturation phase, and it is essential for initiation and maintenance of biofilm. Psl also interacts with eDNA to form a web that contributes to structural support of the biofilm and increases survival[9]. Glucose rich polysaccharide, Pel, needed for the formation of pellicles at the air-liquid interface and solid surface associated biofilms in P.a. In this way, Pel serves as a platform for

biofilm structure to deliver further protection against aminoglucoside antibiotics[9]. Psl and Pel rely on 2 regulatory systems; the quorum sensing (QS), LasI/LasR and RhlI/RhlR; which establishes the basis for bacterial social communication and controls several genes of the bacterial genome.

Similarly, extracellular DNA (eDNA) also performs crucial functions for structural reinforcement of biofilm and maintenance of bacterial resistance to antibiotics and other antimicrobial substances. DNA molecules form a barrier against beta-lactam antibiotics, aminoglycosides, and cationic proteins by binding them via anionic termini. It is also known that eDNA plays roles in the formation of cations gradients, antibiotic resistance, nutrient source, and early biofilm development[9].

Proteins also participate in *Pae* biofilm formation, one of them being CdrA (cyclic diguanylate-regulated partner A). CdrA binds to Psl polysaccharide filaments to increase biofilm stability. Lectins LecA and LecB also participate in the biofilm formation process by binding matrix polysaccharides. Flagella, made up of flagellin proteins, also act as an adhesin, to initiate bacterial attachment to the surface. All these components cooperate to contribute to antibiotic-resistant mechanisms that retard the distribution of antibiotics to infected tissues compared to planktonic cells of the same species[9].

Cystic fibrosis and *Pae* biofilms

To understand how *Pae* can act as an opportunistic pathogen, it is important to understand the defects in host immunity in CF patients and how they relate to high incidence of *Pae* infections. The “low volume hypothesis” suggests that the absence of CFTR in CF patients stops the secretion of chloride and bicarbonate ions into the

airway lumen, affecting normal sodium reabsorption. Disruption of this balance causes increased water uptake by the respiratory epithelium which results in dehydration of the airway surface liquid (ASL)[10]. The ASL is a key component of the innate immune response, so dehydrated mucus affects its own clearance and provides an excellent location for opportunistic pathogens[11].

The reduction of bicarbonate secretion into the airway lumen also results in a decreased pH of ALS in CF patients compared to healthy individuals[12]. Studies performed in CF pig and mice; it was shown that increased acidity causes cationic antimicrobial peptides (AMPs) to be less efficient at killing bacteria[13]. AMPs are small proteins, present within epithelial and leukocytes secretions, with antimicrobial activity towards bacterial and viral pathogens used as part of the innate immune system response[14].

During innate immune response, macrophages and neutrophils are activated after direct contact with bacteria. Phagocytic cells have difficulty penetrating the extracellular matrix due to the usually big size of *Pae* bacterial biofilm, which allows them to be inactivated by bacterial enzymes more easily. Lysis of neutrophils, for instance, leads to an accumulation of harmful compounds which are responsible for tissue damage in infected patients[15]. Also, it is known that autophagy is impaired in epithelial cells and macrophages derived from CF patients[16]; contributing to patients' increased risk of *Pae* infections. All these innate characteristics in the CF lung make it a suitable environment for biofilm-mediated *Pae* infections.

The destabilization of *Pae* biofilm and the D3 program

Earlier studies in Gram-positive bacteria and Gram-negative bacteria, including *Pseudomonas aeruginosa*, proved that endogenously produced D-amino acid factor mixture (D-leucine, D-methionine, D-tyrosine, and D-tryptophan) triggered its own biofilm disassembly in a synergistic manner[17]. The findings from this research inspired the creation of the Distributed Drug Discovery (D3) program. The D3 program focuses on developing compounds that could serve as treatment to eradicate neglected diseases of the developing world[18]. After repeating this previous study, it was concluded that only L-amino acids, natural L-Phenylalanine specifically, showed greater inhibition of *Pae* biofilm formation. Based on recent research that found that halogenation of amino acids could cause an increase of antimicrobial properties[19], two fluorinated L-amino acids (active compounds) derived from L-Phenylalanine were synthesized and confirmed to show antimicrobial properties on *Pae* biofilm formation. However, fluorinated amino acids are considered antimetabolites, which means that they are toxic to bacteria and possibly to humans[20]. After concerns of these active compounds not presenting selective toxicity, a catalog of more than 150 fluorinated dipeptide derivatives (pro-drugs) were synthesized via combinatorial chemistry.

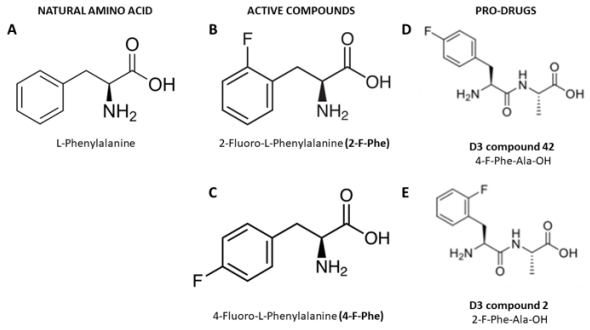


Fig 2. Chemical structures of D3 compounds to be tested in this study. Natural L-amino acid L-Phenylalanine(A), known for its antimicrobial properties against *Pae* biofilms, was used to synthesize active compounds (B, C) and pro-drug formulations (D, E) Structures were retrieved from the D3 catalog provided by Professor Amy Dounay.

The active compounds manufactured were 2-Fluoro-L-Phenylalanine (2-F-Phe) and 4-Fluro-L-Phenyalanine(4-F-Phe), with compelling evidence that confirms they reduce *Pae* biofilm formation (Fig 2). Preliminary studies that tested several of the unnatural dipeptides concluded that compound 42, 4-F-Phe-Ala, was found to be the most potent compound at inhibiting *Pae* biofilm formation[21].

Selective toxicity and the development of *Pae* antimicrobial drugs

Part of the research and development of antimicrobial drugs involves discovering therapies that present selective toxicity. Selective toxicity can be defined as the ability of a compound to inhibit or kill pathogenic microorganisms without adversely affecting the host[22]. Initial *in vitro* studies should prove that possible antimicrobial drugs, at a certain concentration range, reduce bacterial formation without affecting the host. The plate biofilm assay is the most widely studied approach to examine biofilm formation *in vitro*[23]. Other approaches can be utilized to explore the effects of antimicrobial drugs on human cells by performing plate studies where human cell strains are grown in the presence of such drugs for later viability analysis.

It is reasonable to speculate that newly synthesized D3 fluorinated dipeptides could have an effect of *Pae* biofilm formation, however, it is unknown the concentrations at which they present antimicrobial properties or whether they can be toxic towards human cells. Previously mentioned approaches could be used to test the selective toxicity properties D3 fluorinated dipeptides against *Pae*.

METHODOLOGY

Bacterial Strain and Growth Conditions

Pseudomonas aeruginosa PA14 strain was used for all biofilm studies, and this was previously grown in Luria Broth for 24 hours, shaking, at 37 ˚C degrees before incubation in plate. Expected optical density (OD) between 0.060 and 0.080 was expected in order to continue with biofilm assay on plate.

D3 Reagents

Increasing concentrations of 5, 1, 0.5 and 0.2 µg/mL fluorinated dipeptides 42 and 2 (Fig 2) were prepared with 10% DMSO as a vehicle. 5 µg/mL dilutions of active compounds 4-F-Phe and 2-F-Phe were also prepared using 10% DMSO as a vehicle. 10 mg/mL gentamycin and 1.25 mM etoposide were previously prepared. All stock was kept in freezer at degrees -20˚C.

Biofilm Formation Assay

After 24 hours incubation of PA14 in Luria Broth, the effect of D3 compounds on PA14 biofilm formation was assessed. 1:1000 PA14 liquid culture to minimal broth medium(M63) with 1 M $MgSO_4$ and 20% (w/v) Arginine was prepared and incubated 24 hours solution with was grown in sterilized 24-well plate with appropriate treatment (490 µL of PA14 dilution and 10 µL of drug) for 24 hours at 37 ˚C. Biofilm formation was assayed with 0.1% crystal violet staining of each content in plate for 10 minutes at room temperature, washed twice with diH2O and later, wells with stained biofilms were incubated with 30% acetic acid (1000 µL per well) for 15 minutes. The

absorbance of plate was read at 600 nm by using FLUOStar Optima. Qualitative data of plates was gathered through photographs of the plates. Percentage of normalized biofilm formation was calculated using the following formula: Sample OD / (Bacteria OD - Control OD) *100. Sample OD refers to the OD values of all experimental groups, Bacteria OD refers to the cultures with bacteria and vehicle compound (10% DMSO) and Control OD refers to the cultures with just M63 media and 10% DMSO.

Cell line and Treatments

Ramos (RA 1) cells, a B lymphocyte cell line from Burkitt's Lymphoma were grown in RPMI 1640 medium containing heat-inactivated fetal bovine serum (10% final) and added 1000X penicillin/streptomycin (1X final) at 37˚C, 5% CO_2.

$7x10^6$ Ramos cells were added in 48-well plate and treated with increasing concentrations of D3 dipeptides 42 and 2 and control compounds 0.5 µg/mL 4-F-Phe, 0.5 µg/mL 2-F-Phe and 1.25 mM Etoposide; for 48 hours of incubation at 37 ˚C, 5% CO_2. RPMI 1640 was used as medium.

Flow Cytometry and Viability Assay

Cells from each well were individually stained with LIVE/DEAD™ Fixable Read Dead Cell Stain Kit for Excitation at 488 nm and later incubated for 30 minutes at room temperature in the dark. Cells were washed twice with 1X PBS and 100uL of 1X PBS was added to each tube for individual analysis using the guava EasyCyte flow cytometer, with settings for red channel.

Statistical analysis

ANOVA analysis was performed between all experimental groups, where statistical significance was decided by a p-value lower than 0.05 at a 95% confidence interval.

RESULTS

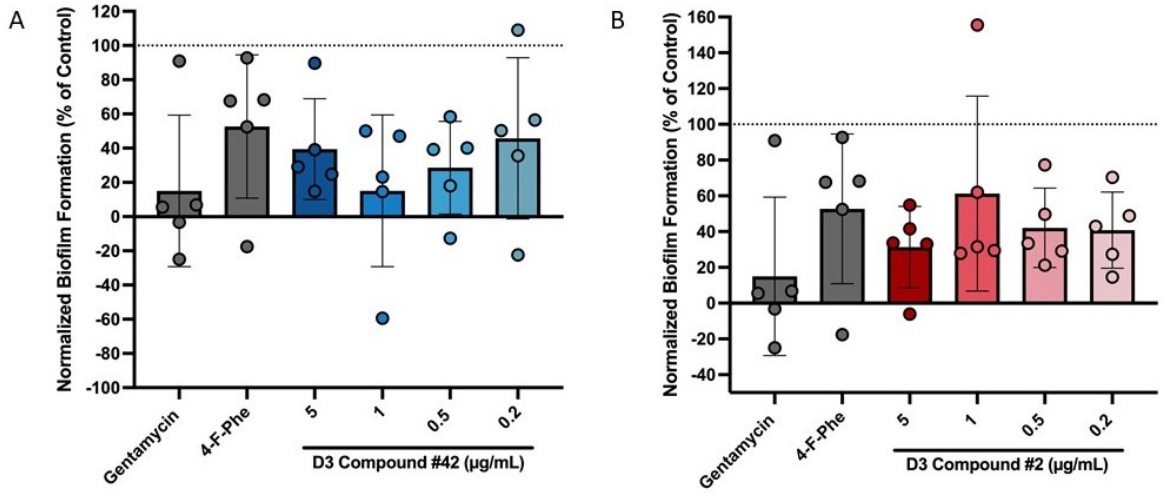


Fig 3. D3 compound #42 and #2 caused a reduction in *P. aeruginosa* biofilm formation. PA14 biofilm formation in the presence of ascending concentrations of D3 compounds 42(A) and 2(B) were measured after violet staining via absorbance detection at 600nm compared to control (Bacteria grown with DMSO only). ANOVA analysis was performed. The dotted line at 100% indicates control group's absorbance.

To determine the antimicrobial effects of D3 compounds 42 and 2, we carried out a 24 hour-biofilm formation assay adding increasing concentrations of drugs before incubation, and later recorded data of biofilm formation via absorbance after crystal violet staining. It is apparent from Figure 3 that, at all increasing concentrations of compounds 42 and 2, there is a reduction of PA14 biofilm formation. Our results also confirm previous studies that state that active compound 4-F-Phe reduces *Pae* biofilm formation, with only a 52.7% of biofilm formation compared to control group without treatment. Also, the percentages of biofilm formation that resulted from the active compound and the descending concentration of both D3 compounds show no significant difference. These results suggest that active compound 4-F-Phe and pro-drugs 42 and 2 at concentrations of 5, 1, 0.5 and 0.2 µg/mL have very similar effects on PA14.

Investigating the effect of increasing concentrations of the D3 compounds provides a broader picture of the concentration range at which these compounds behave in an antimicrobial manner. We found that there were no significant differences between the normalized biofilm formation percentages of the four concentrations tested in this study for the two D3 compounds, so no trend was identified.

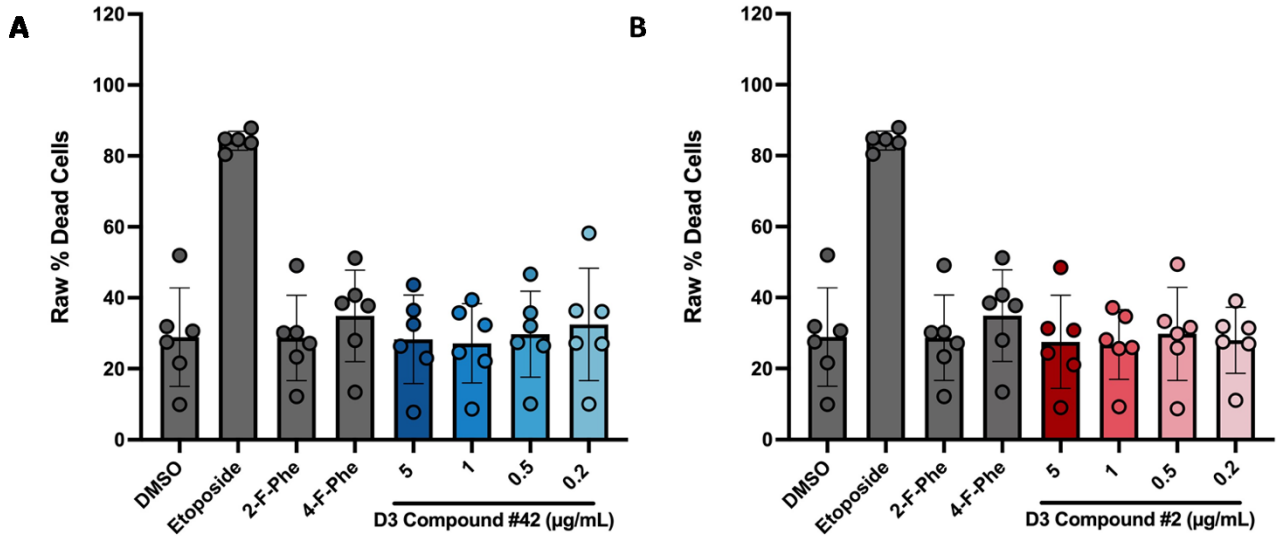


Fig 2. D3 compounds present selective toxicity towards bacteria and not towards mammalian cells. The percentage of dead cells after 48-hour incubation of Ramos cells at ascending concentrations of D3 compound 42(A) and 2(B) via flow cytometry. Error bars are shown as standard deviations. Cells were stained with LIVE/DEAD™ Fixable Read Dead Cell Stain Kit.

After confirming the antimicrobial properties of both D3 compounds, we investigated the effect of these fluorinated dipeptides on human cells, specifically the B lymphocyte cell line Ramos. We conducted a viability assay to figure out the cytotoxic effects of the same increasing concentrations of D3 compounds tested during the biofilm assay, by measuring the percentage of dead cells after treatment via flow cytometry, after stain with LIVE/DEAD™ Fixable Read Dead Cell Stain Kit. Due to previous research concluding that active compounds 2-F-Phe and 4-F-Phe

have an antimicrobial effect on biofilm formation, we decided to also analyze the effect of these two compounds on human cells.

Etoposide, a known chemotherapeutic drug, was used as a positive control for this experiment. As expected, this drug caused high cell death with an average of 84.28% compared to the experimental group with DMSO with 28.91%, values that are significantly different. Similarly, results of death percentage of cells grown with both active compounds and descending concentrations of D3 compounds are all significantly different than the experimental group with etoposide. When looking at the descending concentrations of D3 compounds 42 and 2, there was not a significant difference between dead cell percentages compared to DMSO group, Finally, when comparing cell death of both active compounds with D3 compounds, there was no significant difference among percentages. All of these previous provides evidence that both active compounds 4-F-Phe and 2-F-Phe, as well as both pro-drug formulations, compounds 42 and 2, cause low death percentages in Ramos cells suggesting they present selective toxicity against *Pae*.

DISCUSSION

Pae biofilm formation plays an important role in its resistance to currently available antibiotics in the market. To identify compounds with possible antimicrobial properties against *Pae* that do not affect the host, we carried out an experiment that answered the question of selective toxicity. These results suggest that compounds 42 and 2 present selective toxicity against PA14 strain *in vitro*. It was initially determined that both compounds reduced biofilm formation, at all increasing concentrations tested. Additionally, our findings discovered that both pro-drug compounds at increasing concentrations behave similarly to the active compounds 4-F-Phe, possibly suggesting that lower concentrations of the reagents could be tested and present similar results. This finding is important as the less the concentration of a drug, the lower chances for possible sides effects when testing *in vivo*. Finally, compounds 42 and 2 caused a low dead percentage on B lymphocyte cell strain; which confirms that these fluorinated dipeptides confer selective toxicity against *Pae*.

Antimicrobial properties of phenylalanine-containing compounds have been found before on *Pae* biofilm formation. The catechol-containing amino acid 3,4-dihydroxy-L-phenylalanine (DOPA) has several adhesive properties that inspired its anchoring with antimicrobial peptides (AMPs) to render antimicrobial properties before biofilm formation could even happen. It was found that these coated compounds killed *Pae* planktonic cells upon contact; but it was unknown whether phenylalanine specifically played a role[24].

The initial development of hundreds of pro-drugs derived from the active compounds 4-F-Phe and 2-F-Phe happened due to concerns regarding selective toxicity. Based on our results, 0.5 µg/mL 4-F-Phe does present selective toxicity

against *Pae*, but further experimentation should be carried out in *in vivo* models. Results concluding that toxicity of active compounds and pro-drugs are low and similar to each other suggest that testing with lower concentrations could render the same results. Perhaps there is no need to develop pro-drugs as active form is equally effective. It was interesting to see no visible trends among biofilm formation after addition of increasing concentrations fluorinated compounds. While this phenomenon can be explained by the high variance between duplicates, our results could suggest that these compounds are extremely effective at inhibiting biofilm formation even at lower concentrations. Further studies should seek to investigate selective toxicity of concentrations lower than 0.2 μg/mL. Furthermore, the experiments performed did not explore the actual molecular mechanisms by which these compounds exert their antimicrobial properties on *Pae* biofilm formation.

While our results strongly suggest that these two D3 compounds exhibit selective toxicity against *Pae*, one concern about the biofilm assay results involves high variance between the duplicates of several experimental groups. An apparent limitation of this method is the possible disruption of biofilm during the crystal violet staining step. Another possible limitation from this methodology involves the inadequate pipetting technique which could have caused biofilm disruption before proper staining. Even if biofilms were not disturbed after staining, several washes could have reduced the amount of stained biofilm to be tested via absorbance. While microtiter plate assay and dye crystal violet are considered standard methodologies for quantifying biofilm formation, tetrazolium salt assay using the microtiter plate method is more accurate at detecting *Pae* biofilm formation[25]. 2,3,5-triphenyl-tetrazolium chloride (TTC) assay at 0.5% (6 h of incubation and absorbance measurement at 405 nm) and 1% (5 h incubation and absorbance measurement at

490 nm) were proven to be accurate and repeatable methods to detect *Pae* PAO1 biofilm formation when compared to crystal violet (CV) and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)car-bonyl]-2H-tetrazolium hydroxide (XTT) assays[25]. However, some steps of the methodology can be changed in order to reduce error, such as turning the plate over to discard media and planktonic cells and continue staining of biofilm with 0.1% solutions of crystal violet[26]. Further testing could have been carried out to test other mechanical properties of biofilm, such as cohesiveness to surfaces. Further studies might also seek to investigate these fluorinated dipeptides with different testing methods for biofilm formation.

Moreover, the experiment carried out is based on the addition of D3 compounds to planktonic cells before a biofilm is formed. Based on our results, D3 compounds tested caused an effect on biofilm formation only; so, the question of whether these compounds can contribute to the disassembly of *Pae* biofilms remains unanswered. A possible experiment to answer these questions could involve a biofilm assay in which different concentrations of D3 compounds are added after 24 hours, and measure absorbance of violet stained biofilms compared the untreated experimental group. Finally, biofilm analysis was only carried out with PA14 strain so it would be beneficial to repeat these experiments with other strains such as PA01, which is known for its robust biofilms.

For the viability assay, B lymphocyte from Burkitt's Lymphoma cancer cell line, Ramos, was used to test selective toxicity of the tested compounds *in vitro*, due to its availability in the lab. It was initially planned to use the human T lymphocyte cells line, Jurkat, in order to provide a more diverse array of human cells to test and hopefully gather similar results. Due to low expansion levels from these cells, it was decided only to test with Ramos cells. It was realized that Ramos cells still presented a quite

high percentage of death in the control group with vehicle DMSO, and even with this consistency, there was an obvious trend of selective toxicity against bacteria only for tested compounds. It would be appropriate to repeat these same experiments using several cell lines available to determine a continuous trend of selective toxicity in all, if not most, human cells lines; and be able to generalize the results.

As mentioned earlier in this paper, a main influence for researching these compounds against *Pae* is its deadly lung infections in cystic fibrosis patients. Further studies with compounds from the D3 project could involve the use of human cell lines from the lung such as BEAS-2B cell line.

ACKNOWLEDGEMENTS

I would like to thank Dr. Olivia Hatton for her constant support, mentorship and patience throughout this research and my college career. I would also like to thank Dr. Phoebe Lostroh for providing helpful and insightful feedback editing this thesis. Lastly, I would like to thank my friends and family, for their love, inspiration and constant motivation.

REFERENCES

1. WHO publishes list of bacteria for which new antibiotics are urgently needed. https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed. Retrieved 25 February 2023.
2. Waters CM, Goldberg JB. 2019. Pseudomonas aeruginosa in cystic fibrosis: A chronic cheater. Proceedings of the National Academy of Sciences 116:6525–6527.
3. Høiby N, Ciofu O, Bjarnsholt T. 2010. Pseudomonas aeruginosa biofilms in cystic fibrosis. Future Microbiol 5:1663–1674.
4. Høiby N, Krogh Johansen H, Moser C, Song Z, Ciofu O, Kharazmi A. 2001. Pseudomonas aeruginosa and the in vitroand in vivo biofilm mode of growth. Microbes and Infection 3:23–35.
5. Vert M, Doi Y, Hellwich K-H, Hess M, Hodge P, Kubisa P, Rinaudo M, Schué F. 2012. Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). Pure and Applied Chemistry 84:377–410.
6. Mayansky AN, Chebotar IV, Rudneva EI, Chistyakova VP. 2012. Pseudomonas aeruginosa: Characteristics of the biofilm process. Mol Genet Microbiol Virol 27:1–6.
7. Flemming H-C, Wingender J. 2010. The biofilm matrix. 9. Nat Rev Microbiol 8:623–633.
8. Rasamiravaka T, Labtani Q, Duez P, El Jaziri M. 2015. The Formation of Biofilms by Pseudomonas aeruginosa: A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms. BioMed Research International 2015:1–17.

9. Lee K, Yoon SS. 2017. Pseudomonas aeruginosa Biofilm, a Programmed Bacterial Life for Fitness 27:1053–1064.
10. Garland AL, Walton WG, Coakley RD, Tan CD, Gilmore RC, Hobbs CA, Tripathy A, Clunes LA, Bencharit S, Stutts MJ, Betts L, Redinbo MR, Tarran R. 2013. Molecular basis for pH-dependent mucosal dehydration in cystic fibrosis airways. Proceedings of the National Academy of Sciences 110:15973–15978.
11. Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW, Boucher RC. 1998. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. Cell 95:1005–1015.
12. Song Y, Salinas D, Nielson DW, Verkman AS. 2006. Hyperacidity of secreted fluid from submucosal glands in early cystic fibrosis. American Journal of Physiology-Cell Physiology 290:C741–C749.
13. Pezzulo AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, Karp PH, Wohlford-Lenane CL, Haagsman HP, van Eijk M, Bánfi B, Horswill AR, Stoltz DA, McCray PB, Welsh MJ, Zabner J. 2012. Reduced Airway Surface pH Impairs Bacterial Killing in the Porcine Cystic Fibrosis Lung. Nature 487:109–113.
14. Auvynet C, Rosenstein Y. 2009. Multifunctional host defense peptides: Antimicrobial peptides, the small yet big players in innate and adaptive immunity. The FEBS Journal 276:6497–6508.
15. Watters C, Fleming D, Bishop D, Rumbaugh KP. 2016. Chapter Seven - Host Responses to Biofilm, p. 193–239. *In* San Francisco, M, San Francisco, B (eds.), Progress in Molecular Biology and Translational Science. Academic Press.

16. Cormet-Boyaka E, Caution K, Amer AO, Cormet-Boyaka E, Caution K, Amer AO. 2016. Autophagy in Cystic Fibrosis Pathogenesis and TreatmentAutophagy in Current Trends in Cellular Physiology and Pathology. IntechOpen. https://www.intechopen.com/chapters/51976. Retrieved 2 April 2023.
17. Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R. 2010. D-Amino Acids Trigger Biofilm Disassembly. Science 328:627–629.
18. Scott WL, O'Donnell MJ. 2009. Distributed Drug Discovery, Part 1: Linking Academia and Combinatorial Chemistry to Find Drug Leads for Developing World Diseases. J Comb Chem 11:3–13.
19. Molchanova N, Nielsen JE, Sørensen KB, Prabhala BK, Hansen PR, Lund R, Barron AE, Jenssen H. 2020. Halogenation as a tool to tune antimicrobial activity of peptoids. Sci Rep 10:14805.
20. Park BK, Kitteringham NR, O'Neill PM. 2001. Metabolism of Fluorine-Containing Drugs. Annual Review of Pharmacology and Toxicology 41:443–470.
21. Scott WL, O'Donnell MJ, Samaritoni JG, Marrs K, Anderson GG, Dounay AB, Fuller AA, Miranda L, Montes I, Morales G, Schirch D, Sullivan K. Distributed Drug Discovery (D3) Synthesis and Testing of Multiple Unnatural Dipeptides Identifies a Subset with Potent Antimicrobial Activity against Pseudomonas aeruginosa (Pa).
22. Madigan MT, Brock TD, Martinko JM, Madigan MT, Martinko JM. 2015. Antimicrobial drugs, p. 811–817. In Brock Biology of Microorganisms, 14th ed. Pearson, Boston, Massachusetts.

23. Merritt JH, Kadouri DE, O'Toole GA. 2005. Growing and Analyzing Static Biofilms. Curr Protoc Microbiol 0 1:Unit-1B.1.
24. Hwang YE, Im S, Kim H, Sohn J-H, Cho B-K, Cho JH, Sung BH, Kim SC. 2021. Adhesive Antimicrobial Peptides Containing 3,4-Dihydroxy-L-Phenylalanine Residues for Direct One-Step Surface Coating. Int J Mol Sci 22.
25. Sabaeifard P, Abdi-Ali A, Soudi MR, Dinarvand R. 2014. Optimization of tetrazolium salt assay for Pseudomonas aeruginosa biofilm using microtiter plate method. Journal of Microbiological Methods 105:134–140.
26. O'Toole GA. 2011. Microtiter Dish Biofilm Formation Assay. J Vis Exp 2437.