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TARGETING PRE-FORMED BIOFILMS OF THE NOTORIOUS *PSEUDOMONAS AERUGINOSA* USING ANIDULAFUNGIN AND ANTIBACTERIAL AGENTS IN *IN-VITRO* AND *EX-VIVO* URINARY TRACT INFECTION MODELS

by
NISREEN AMIN GHANEM

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AN ABSTRACT OF THE THESIS

Nisreen Amine Ghanem for Master of Science
Major: Microbiology and Immunology

Title: Targeting Pre-formed Biofilms of the Notorious *Pseudomonas aeruginosa* using Anidulafungin and Antibacterial Agents in *in-vitro* and *ex-vivo* Urinary Tract Infection Models

Background: A crucial problem in patient care is the ability of certain bacteria, including *Pseudomonas aeruginosa*, to form biofilms on implanted and indwelling medical devices. *P. aeruginosa* biofilms play a role in protecting the bacterial community, increasing antimicrobial resistance, and making the bacterium among the most threatening nosocomial pathogens. Hence, it is important to develop therapeutic interventions that would not only inhibit biofilm formation, but also be able to eradicate the pre-formed ones. Several studies investigated the presence of 1,3β-D-glucan, a major component of fungal cell wall, in *P. aeruginosa* biofilms. Moreover, previous studies at the department of Experimental Pathology, Immunology and Microbiology at the American University of Beirut shed light on the importance of an Echinocandin, known as Micafungin, in inhibiting the formation of biofilms in case of *P. aeruginosa*. Here we aimed to investigate the effectiveness of the combination therapy that involves Anidulafungin, another Echinocandin, with either Colistin, Gentamicin, or Ciprofloxacin in eradicating pre-formed *P. aeruginosa* biofilms in *in-vitro* and *ex-vivo* urinary tract infection models.

Methods: A *P. aeruginosa* PAN14 clinical strain was screened for its susceptibility against a panel of six antimicrobial agents (Colistin, Gentamicin, Ciprofloxacin, Tazocin, Ceftazidime and Cefepime). The Minimal Biofilm Inhibitory Concentration (MBIC) and the Minimal Biofilm Eradication Concentration (MBEC) were evaluated for the antimicrobials alone and in combination with Anidulafungin. PAN14 biofilms were grown on catheter sections under static and flow conditions, and on ECV304 epithelial cells to further assess the efficacy of the combination therapy. Crystal Violet stain was performed to ensure the presence of biofilms, and Scanning Electron Microscopy (SEM) in order to visualize any morphological changes that took place following treatment.

Results: Serial broth microdilution assay revealed that PAN14 planktonic cells display susceptibility to all tested antimicrobials. Moreover, combining variable concentrations of Anidulafungin to Colistin, Gentamicin, and Ciprofloxacin lead to a decrease in the MBIC and MBEC values. Crystal Violet stain ensured the presence of biofilms on catheter sections, and SEM images revealed complete eradication of PAN14 preformed biofilms in case of combining Anidulafungin with Colistin or Gentamicin, but not with Ciprofloxacin. PAN14 biofilms developed successfully on ECV304 cells for further assessment of the eradication effect of the combination therapy.

Conclusion: This study enabled us to assess the efficacy of combining Anidulafungin with certain antibacterial agents in eradicating pre-formed biofilms, and would therefore pave the way for further assessments of Anidulafungin as well as other Echinocandins *in vivo* and in potential clinical trials.

CONTENTS

ILLUSTRATIONS

Figure Page

TABLES

Table Page

CHAPTER I

INTRODUCTION

Several decades after the first patients were treated using antimicrobial agents, bacterial infections have again imposed a severe threat, as the quick emergence of resistant bacterial strains is occurring worldwide. The evolution of the resistance crisis is mainly driven by both the misuse and the overuse of antimicrobial agents (1). A recent review published on antimicrobial resistance (AMR) estimated that drug-resistant bacterial infections will result in a global mortality of 700,000 per year, and is expected to rapidly increase to reach about 10 million deaths by the year 2050, therefore exceeding the mortality rates of diabetes and cancer combined (2).

Among the most important pathogens that are capable of escaping the activity of antimicrobial therapy is *Pseudomonas aeruginosa*, characterized as an opportunistic gram-negative bacterium responsible for both acute and chronic infections. Its genome is relatively larger than that of other prokaryotes, and it also has several regulatory genes in its chromosome, contributing to the ability of the bacterium to adapt to various environmental conditions (3). The pathogenicity of *P. aeruginosa* infections depends on several factors, the most important of which is its ability to form a biofilm, defined as structured bacterial communities that can coat mucosal surfaces and invasive devices. This self-produced biofilm is considered as a survival strategy for *P. aeruginosa*, responsible for providing shelter against environmental assaults, increasing the level of antimicrobial resistance, and

rendering ineffective the bacterium clearance by the immune system. The formation of a biofilm is usually associated with chronic infections, where it causes persisting and recurring pathologies (4). *P. aeruginosa* is considered as a major nosocomial pathogen in patients with cystic fibrosis (CF), and one of the leading causes of chronic urinary tract infections (UTI) and pneumonia (5,6).

Despite current advances in antimicrobial therapy, high morbidity and mortality rates are being associated with *P. aeruginosa*. Standard antimicrobial therapy that is effective against planktonic bacterial cells (free-living) is found to be unsuccessful against biofilms. This unfavorable outcome is mostly due to the incomplete understanding of the pathogenesis of biofilm-associated infections, and therefore the failure to develop therapeutic strategies to prevent them (4). Hence, there's an urgent need to design new countermeasures and therapeutic approaches not only to inhibit the formation of *P. aeruginosa* biofilms but also to eradicate the pre-formed ones.

Previous research put forward the presence of 1,3β-D-glucan as a major component of *P. aeruginosa* biofilms, which is also found in a variety of fungal cell walls (7). Several studies at the department of Experimental Pathology, Microbiology and Immunology at the American University of Beirut (AUB) investigated the activity of an Echinocandin (antifungal) known as Micafungin, on inhibiting the synthesis of 1,3β-D-glucan, therefore disrupting the biofilm structure in case of *P. aeruginosa*, and exposing planktonic bacterial cells, which is a significant step in facilitating treatment (8). Bazzi et al. (2013) and Kissoyan et. al (2016) specifically targeted cyclic glucans through Echinocandins, paving the way for treating biofilm-associated infections. Results showed that following treatment with Micafungin, there was a decrease in biofilm formation as well

as in the mRNA transcription levels for the genes encoding the cell wall 1,3-β-D-glucan (8,9). Moreover, a study done by Rasheed (2016) evaluated the effect of Micafungin, singly or in combination with antibacterial agents, on *P. aeruginosa* biofilms. There was a decrease in total biofilm thickness, as measured by the confocal microscopy, following treatment (10). Another study by Issa (2019) investigated the combinatory effect of Anidulafungin, another Echinocandin, each with Levofloxacin and Amikacin in targeting pre-formed *P. aeruginosa* biofilms. Adding Anidulafungin to the antibacterial agents lead to a decrease in the minimum biofilm inhibitory concertation (MBIC) and the minimum biofilm eradication concentration (MBEC) values (11). Such studies shed light on the importance of considering various Echinocandins as a major part of therapy targeting biofilm-associated infections.

It would be intriguing to further investigate the potential inhibitory action of Echinocandins on pre-established biofilms. Moroeover, since it has been proven that anitimicrobial combinations generating synergy are a successful strategy in the fight against AMR (12), this study aims to investigate the effectiveness of the combination therapy that involves Anidulafungin with either Colistin, Gentamicin, or Ciprofloxacin in eradicating pre-formed *P. aeruginosa* biofilms in *in-vitro* and *ex-vivo* UTI models.

CHAPTER II

LITERATURE REVIEW

A. Pseudomonas Genus: Microbiological Characteristics, Species, and Habitat

Pseudomonas is a genus of bacteria belonging to the family Pseudomonadaceae. It includes more than 140 species, characterized as aerobic and motile gram-negative bacilli that can be cultured on most general-purpose media. *Pseudomonas* species normally inhabit soil, water, and vegetation. Moreover, they often colonize hospital food, sinks, taps, and respiratory equipment (13). Members of the *Pseudomonas* genus are considered highly adaptable, as proven by their successful colonization of various environments and their great deal of metabolic versatility and genetic plasticity (14). More than 25 species are associated with human beings, and these pseudomonads are known to cause opportunistic infections. *Pseudomonas aeruginosa* and *Pseudomonas maltophilia* account for approximately 80 percent of pseudomonads recovered from clinical specimens (13). Infections caused by *P. aeruginosa* are usually serious, especially in hospitalized patients suffering from cancer, cystic fibrosis (CF), and burns. With its striking fatality rate of 50%, *P. aeruginosa* has gained the most attention as an alarming nosocomial pathogen (13,14).

B. *P. aeruginosa*: Nosocomial Infections, Risk Groups, and Resilience

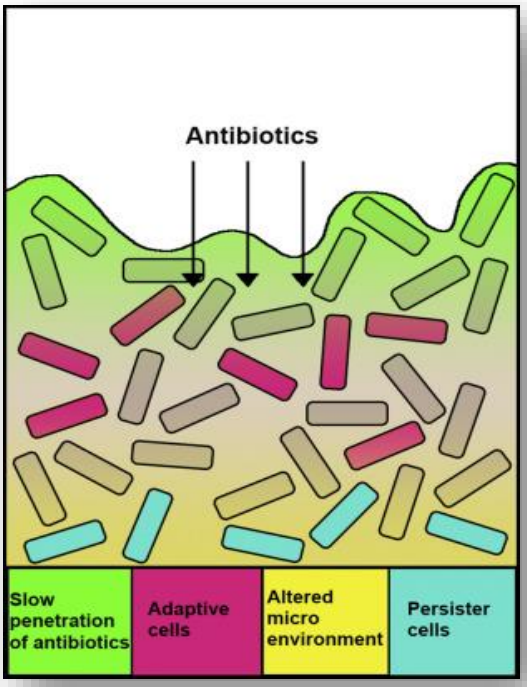
1. A Major Nosocomial Pathogen and its Risk Groups

P. aeruginosa is an opportunistic pathogen capable of causing a wide range of life-threatening acute and chronic infections. It is exceptional in its ability to infect several subpopulations of patients, mainly those with burns, CF, immunosuppression (especially granulocytopenia), and traumatic wounds (15,16). It is considered the leading cause of morbidity and mortality in CF patients, and one of the most important causative agents of nosocomial infections (17). More recently, *P. aeruginosa* was the most important isolate obtained from the wounds of 275 soldiers treated for combat injury (16). Moreover, ventilator-associated pneumonia and urinary catheter-related infections caused by *P. aeruginosa* are very common worldwide, and the persistence of such infections is mostly due to its high resilience and adaptive capacity (18,19).

2. Resilience

Multidrug resistance has increased dramatically over the years and is now recognized as a major threat worldwide (20). Strains of *P. aeruginosa* utilize their high levels of intrinsic, acquired, and adaptive resistance mechanisms to fight most antibiotics. To begin with, *P. aeruginosa* achieves intrinsic resistance through low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell, or the production of antibiotic-inactivating enzymes. Concerning the acquired resistance of *P. aeruginosa*, it can be achieved by either horizontal transfer of resistance genes or mutational changes. The adaptive resistance, which is a recently characterized mechanism, involves biofilm formation that serves as a diffusion barrier to prevent antibiotic entry to

the bacterial cells (21). A biofilm is characterized as an aggregate of structured bacterial microorganisms encased within an extracellular matrix that adhere and attach to mucosal surfaces and invasive devices (22). Fig. 1 shows the major mechanisms of biofilm mediated resistance. As shown in green, some antibiotics will be able to slowly penetrate the biofilm. Biofilm cells will express an adaptive stress response that permits their survival in harsh conditions (pink). An altered chemical microenvironment within the biofilm (shown in yellow) can induce slow bacterial growth, which in turn reduces antibiotic uptake. Eventually, persister cells (blue) will be formed, which are multidrug tolerant cells that form spontaneously within a biofilm (23).



Adapted from: Zheng Pang et al. (2018) Antibiotic resistance in *P. aeruginosa*: mechanisms and alternative therapeutic strategies

Figure 1. Mechanisms of biofilm-mediated antibiotic resistance

C. Pathogenesis of *P. aeruginosa*

P. aeruginosa has several virulence factors that contribute it its ability to infect and colonize a wide range of environments (24). These virulence factors can be classified into two types depending on whether they are produced in the case of acute or chronic infections (24,25). Acute infections spread quickly, and often cause tissue damage associated with high mortality rates, whereas chronic infections persist from weeks to years under intensive clinical care (26). The bacterium can usually choose what strategy to employ, as the phenotypic and the molecular mechanisms involved in each kind of infection vary widely (27). The transition from an acute to a chronic infection is, therefore, a result of various alterations in the bacterial cellular physiology responding to external stimuli (28).

1. Pathogenesis of an Acute Infection:

a. Adhesion and Colonization:

The colonization of *P. aeruginosa*, as well as the propagation of its infection, relies on a wide range of its virulence factors. Motility is a crucial feature that allows the bacterium to colonize and discover new niches (24). Type IV pili, responsible for the twitching motility and exploring new surfaces, plays a role in adherence and initial colonization on mucosal surfaces (29). Other adhesive filaments are the fimbriae that also play an important role in the pathogenesis of *P. aeruginosa*. Fimbrial structures facilitate bacterial attachment to the host tissues and promote the formation of biofilms (30). Bacterial pili and fimbriae are not only used for adherence, but they also provide the ability

to retract and migrate along a surface, allowing bacterial cells to escape from the surface when necessary (29). Flagellar motility is also a key feature of acute *P. aerugin*osa infections (26). Besides motility, flagella carry out several other functions that include attachment to host cells and activation of host inflammatory responses. Recently, flagella are being targeted by vaccine development, as intact flagella seem superior for the generation of immunity to *P. aeruginosa* (31).

b. Invasion and Tissue Damage:

The severity of *P. aeruginosa* infections is mainly due to its ability to secrete toxins and proteins that allow it to evade the host immune system and cause tissue damage (32). The most important of which are type 2 and type 3 secretion systems (T2SS and T3SS) responsible for the production of toxins and effector molecules. These are involved in evading the host phagocytic response and delivering effector proteins directly into the cytosol of eukaryotic cells (32–34). Specifically, most infections are associated with the production of T2SS dependent exoproteins such as the LasB elastase, a proteolytic enzyme that degrades collagen and non-collagen proteins, facilitating the spread of the infection by destroying the host physical barriers. Moreover, LasB elastase prevents the clearance of *P. aeruginosa* from the body by inhibiting the presentation of bacterial antigens to the immune system (32,35). Another T2SS dependent protein is the Type IV protease, whose major function is to degrade the host surfactant proteins A and D to prevent the association of the bacterium with the alveolar macrophages (32).

c. Dissemination:

The exact mechanism of dissemination in acute *P. aeruginosa* infections is poorly characterized. The bacterium uses T3SS such as ExoS, which is a GTPase activating protein (GAP) and an ADP ribosyltransferase (ADPRT) not only to inhibit phagocytosis but also to disseminate into the bloodstream (34). Also, two soluble proteins known as lectins (LecA and LecB) are found in the *P. aeruginosa* outer membrane and are both known to participate in the dissemination of the bacterium to the blood, facilitating its survival (36).

2. Pathogenesis of a Chronic Infection:

During the transition from acute to chronic infection, *P. aeruginosa* adapts to the host environment undergoing multiple changes helping it to survive and evade the host defense mechanisms (37). The bacterium starts producing extracellular polysaccharides excessively, forms a biofilm, and upregulates the T6SS (33). On the other hand, it downregulates the expression of the flagellin as well as the T3SS. *P. aeruginosa* encodes three types of T6SS that have activity against both prokaryotes and eukaryotes. H1-T6SS grants *P. aeruginosa* a growth advantage by acting as a weapon to outcompete other bacteria that express the T6SS and that exist in the same ecological environment (38). H2-T6SS and H3-T6SS are both responsible for the host cell evasion as the H2 secretion machinery specifically promotes bacterial uptake into the epithelial cells. Moreover, H3 machinery is also important for *P. aeruginosa* internalization (38,39). Such mechanisms are observed in several biofilm-associated chronic infections like chronic urinary tract

infections (UTI), chronic CF lung infections, chronic wound infections, and medical devices associated infections (37).

D. *P. aeruginosa* Biofilm: Structure and Development

1. Structure

P. aeruginosa, which accounts for approximately 10% of the biofilm biomass, is responsible for the formation of Extracellular Polymeric Substances (EPS), which makes up to 90% of the biofilm. The EPS matrix is composed of several biomolecules, exopolysaccharides, extracellular DNA (eDNA), and polypeptides that form a highly hydrated polar mixture contributing to its overall structure and architecture of the biofilm (40). The major function of the EPS matrix is to allow microbes to function synergistically as a community by maintaining close contact and is responsible for adhesion to surfaces and cohesion in the biofilm (22).

Three polysaccharides determine the stability of the *P. aeruginosa* biofilm structure, which are alginate (Alg), Pel, and polysaccharide synthesis locus (Psl). Alg is an acetylated unbranched polysaccharide composed of non-repetitive monomers of b-1,4-linked L-guluronic and D-mannuronic acids (41). It is involved in the establishment of micro-colonies during the first stages of biofilm formation and is also responsible for the mechanical stability of mature biofilms. Its special physical and chemical properties function to protect *P. aeruginosa* cells by producing flat and more homogeneous biofilms (42).

The Pel polysaccharide is a glucose-rich matrix material whose exact composition is still unclear (40). It was first reported to be required for the formation of surface-associated biofilms (41). Psl, on the other hand, consists of a repeating pentasaccharide of D-mannose, L-rhamnose, and D-glucose (40). This polysaccharide plays a very important role in the attachment of *P. aeruginosa* cells to surfaces in microtiter trays and flow chambers. It has been shown to form a helical structure around the bacterium to increase cell-to-surface and cell-to-cell interactions, which are essential not only for the formation of biofilms but also for their maintenance (41).

Along with the major polysaccharides, 1,3-β-D-glucan is an accessory polysaccharide identified in the biofilm matrix of *P. aeruginosa*. It belongs to a family of cyclic β-(1→3)-linked glucans of 12 to 16 glucose residues with 30–50% of glucose units substituted by 1-phosphoglycerol at *O*-6 (43). Mah et al. (2003) revealed that the *ndvB* gene, which encodes a glycosyltransferase, is required for the biosynthesis of cyclic glucans in the *P. aeruginosa* biofilm matrix (7). In addition, several studies shed light on the significant relationship that exists between the *ndvB* gene and cyclic glucans with the biofilm's tolerance for aminoglycosides. They proved that the glucan enriched extract of *P. aeruginosa* PAN14 was capable of physically interacting with Tobramycin (Tb), an aminoglycoside antibiotic (7,43). Cyclic glucans are negatively charged due to the phosphoglycerol substitutions interacting with positively charged aminoglycosides. This interaction inhibits the former antibiotics from reaching their sites of action inside of the cell and exerting their activity. Finally, such results prove that biofilms are not simply a diffusion barrier for antibiotics, but rather that bacterial cells inside these microbial communities employ a mechanism of resistance to antimicrobial agents (7).

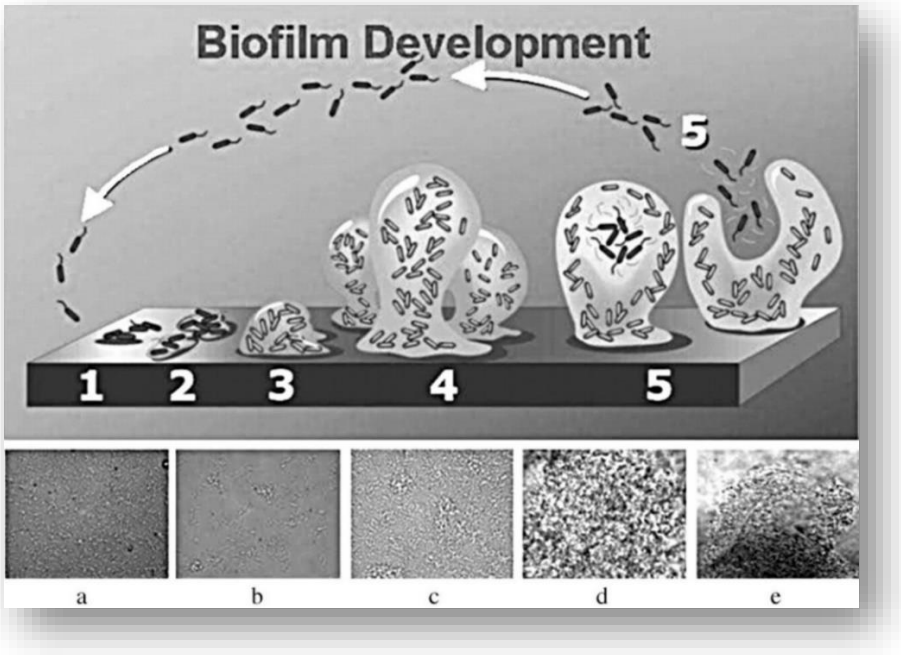
2. Development

The process of biofilm development is considered as an endless cycle. Different biofilm phenotypes are formed depending on *the different P. aeruginosa* strains and nutritional conditions found (40). For example, in a nutrient-rich medium, the bacterium tends to undergo a transition from the planktonic free-living form to the sessile surface attached form. Biofilms usually continue to develop in the presence of fresh nutrients, however, whenever these nutrients are deprived, biofilms detach from the surface and reverse back to the planktonic form. The following starvation response makes the bacterial cells search for another source of nutrients, explained by the adaptation mechanism that *P. aeruginosa* is known for (44).

a. Phenotypic Differentiation

Similar to other biological systems, the biofilm differentiation process that converts groups of adherent bacterial cells into a matrix enclosed community is perceived as a cycle of morphological changes. Initially, these individual adherent cells found on a surface are surrounded by a minimal amount of exopolymeric material and are therefore capable of moving independently (Fig.2 Stage 1). This stage is known as the reversible adhesion, as cells are not "committed" yet to the process of biofilm formation, and several individual cells leave the surface and go back to the planktonic form. Stage 2 shows the production of EPS which results in a firm and irreversible attachment (45). Davies & Geesey (1995) proved that the process of biofilm formation in *P. aeruginosa* is initiated by

a genetic event that involves the up-regulation of the cluster of genes that are responsible for Alg production following the cell's initial contact with the colonized surface (46). Bacterial microcolonies then expand leading to the formation of a more structured phenotypic architecture as shown in stage 3 (40). The biofilm then matures as shown in stage 4, and the bacteria will fill the non-colonized spaces to cover the whole surface. Finally, stage 5 shows the dispersion of bacterial cells from the biofilm to reenter the planktonic form again and spread to colonize new surfaces (45).



Adapted from Stoodley et al. (2002) Biofilms as Complex Differentiated Communities

The bottom panels "a- e" show the five stages of biofilm development as represented by a photomicrograph of *P. aeruginosa* when grown under a continuous flow of media.

Figure 2. Initial Processes in Biofilm Formation and Subsequent Structural Differentiation

E. Systems Regulating Biofilm Formation

Multiple regulatory systems govern the process of biofilm formation in *P. aeruginosa* (47). Any deficiency in the regulatory network that is essential for biofilm matrix formation results in modifications in the biofilm structure and architecture and, consequently, its protective role (40).

The most important regulatory systems are the following:

1. C-di-GMP Pool:

Cyclic dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger that plays an essential role in the molecular decision between the planktonic free-living and the sessile biofilm-associated forms. Specifically, high levels of c-di-GMP lessen both the expression and the activity of the flagella and stimulate the expression of adhesins and biofilm-associated exopolysaccharides, therefore facilitating the switch to the biofilm mode of growth (48). On the contrary, low c-di-GMP levels decrease the production of adhesins and exopolysaccharides, which weakens the biofilm and leads to its dispersal back to the planktonic form (47,48). Synthesis and degradation of c-di-GMP take place through the diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) that have an opposing activity. These enzymes contain sensory domains whose role is to sense and therefore respond to environmental cues (49). *P. aeruginosa* contains multiple DGCs and PDEs that cooperatively control the overall concentration of c-di-GMP and consequently modulate the production of EPS (50). C-di-GMP binds to and allosterically affects the activity of effector components that include various proteins as well as RNAs (48). For

example, SiaD and SadC are two DGCs that are stimulated by Psl to produce more of the molecule C-di-GMP, thus creating a positive feedback for biofilm formation (50,51). Another example is RoeA, a DGC that regulates biofilm formation by controlling polysaccharide production (50).

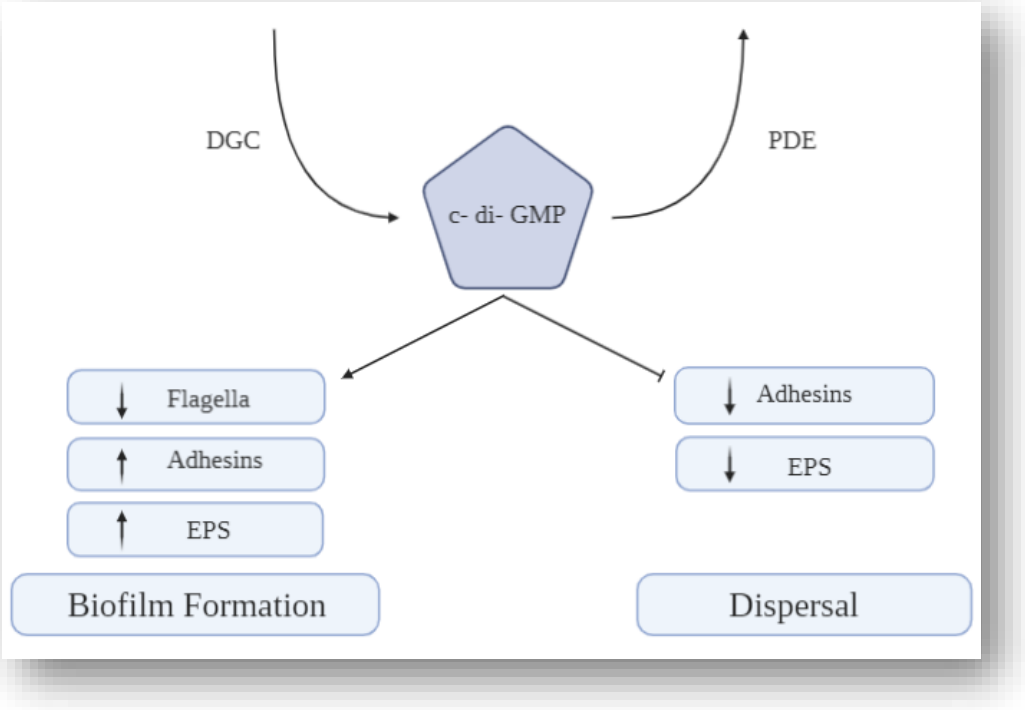


Figure 3. Schematic Diagram Representing the c- di- GMP Pathway

2. Quorum Sensing:

Quorum sensing (QS) is a cell-cell multicellular communication system in bacteria responsible for the production, release, and detection of molecules known as autoinducers (AIs) to control behaviors of the bacterial populations. QS provides a mechanism for bacterial cells to detect another's presence and to regulate gene expression in a cell density-

dependent manner (52). Many biological processes in bacteria are controlled by QS, including biofilm formation and antibiotics production (50). *P. aeruginosa* possesses two N-acyl homoserine lactone (AHL) based QS systems; Las and Rhl (53). AHLs are made up of fatty acids that vary in length and substitution, linked by a peptide bond to a homo-serine lactone moiety. Las and Rhl induce the production (via synthases known as LasI and RhlI) and the perception (via transcription factors known as LasR and RhlR) of the AIs *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) respectively. In general, signaling through QS is accomplished when a certain concentration of AHLs is produced, forming a complex with the transcriptional factors which enable binding to DNA and alter the expression of multiple virulence genes (54). Several genes are activated by these two interconnected AHL QS systems, including virulence factor genes as well as genes involved in biofilm formation (50). Specifically, the Las system controls the production of LasA (PA1871) and LasB (PA3724) elastases and exotoxin A (PA1148), involved in acute infection and host cell damage. On the other hand, the Rhl system induces the expression of genes encoding the production of rhamnolipids and suppresses the ones responsible for the function of the type III secretion system. The latter is considered as a major virulence determinant in human infections because it controls the release of several toxic proteins into the cytoplasm of eukaryotic cells (54).

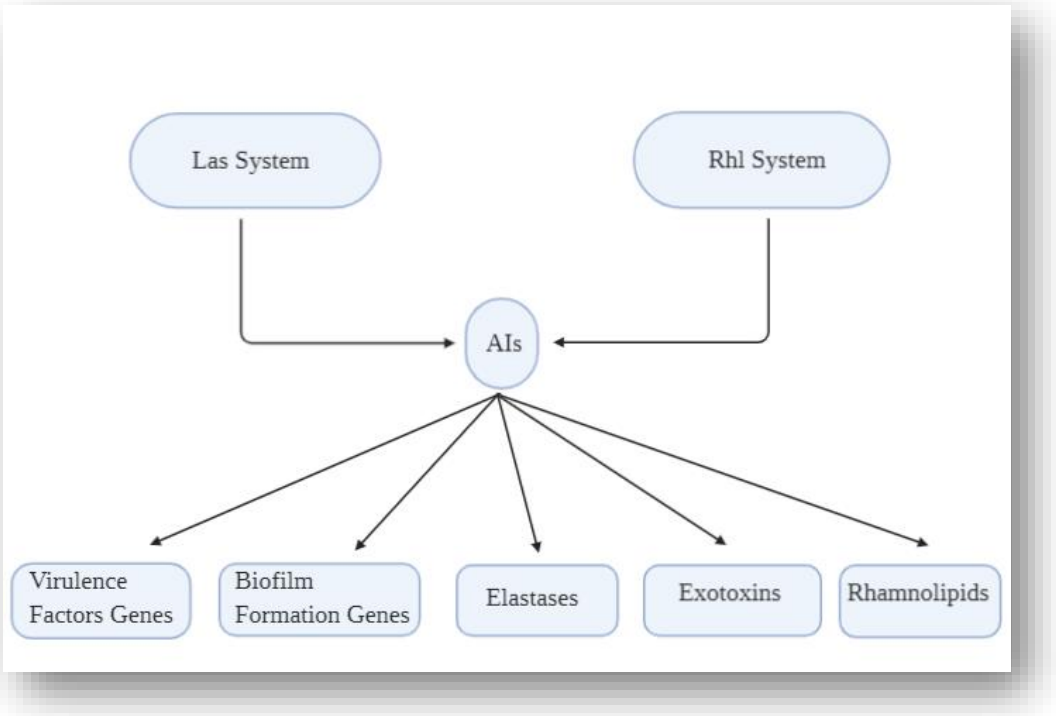
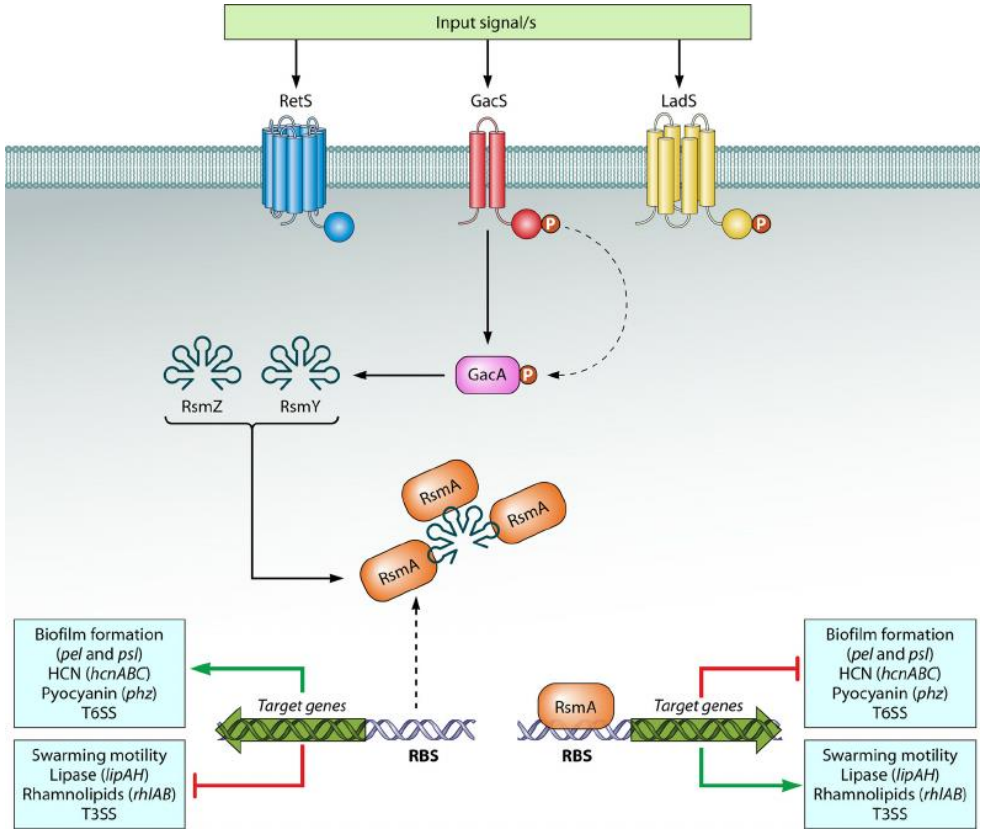


Figure 4. Schematic Diagram Representing the Quorum Sensing Pathway

3. The Gac/Rsm System:

P. aeruginosa controls its lifestyle (either planktonic or biofilm-associated) also via the two-component signal transduction systems. Such systems phosphorylate cascades that, in turn, induce conformational changes in regulatory proteins, therefore influencing gene expression. The best-studied one is the Gac/Rsm system due to the important role it plays in the microbe-host interactions (54). This system is composed of a transmembrane sensor kinase (known as GacS) that, upon autophosphorylation, transfers a phosphate group to the cognate regulator (GacA) responsible for upregulating the expression of the small regulatory RNAs (RsmZ and RsmY). RsmA is a small RNA binding protein that, once

activated, can stimulate the activity of genes involved in the formation of biofilm and suppresses genes involved in motility and virulence (50,54). Moreover, Andrew et al. (2004) showed that the histidine kinases RetS and LadS exert either a positive or a negative effect on the Gac/ Rsm system (55).



Adapted from Jmenez at al. (2012) The Multiple Signaling Systems Regulating Virulence in *P. aeruginosa*

Figure 5. Schematic Diagram Representing the Gac/ Rasm Pathway

F. Biofilm Antimicrobial Resistance Mechanisms

It has become clear that biofilms express distinctive properties that allow them to resist multiple antimicrobial agents. In fact, it has been observed that cells in a biofilm become 10- 1000 times more resistant than cells in their planktonic form (56,57). Multiple

mechanisms confer this increased multi-factorial resistance; the most important ones are explained below.

1. Antimicrobial Penetration Limitation:

The biofilm exopolysaccharide matrix, in addition to its scaffolding role, acts as a physical barrier that prevents antimicrobial agents from reaching bacterial cells embedded deep inside (56). As antimicrobials have to penetrate to reach their target site, certain characteristics of the cell envelope are found to be responsible for bacterial cell resistance (57). Biofilms serve as a selective barrier allowing the entry of some small molecules rather than others, and as the biofilm matures, the production of exopolysaccharides increases and diversifies, contributing to a more complex architecture. As a result, the polysaccharide composition leads to increased resistance contributing to the pathogenicity of the bacterium and causing devastating chronic infections (58). A study done by Tseng at al. (2013) revealed that non-mucoid *P. aeruginosa* biofilms could protect resident cells by limiting the penetration of tobramycin via ionic interactions with the biofilm (59). This result is explained by the ability of Tobramycin to be sequestered near the surface of the biofilm rather than penetrating it. Tobramycin is positively charged, therefore interacts ionically with the negatively charged components of the matrix, and the addition of an excess amount of cations increases its penetration into the biofilm. Results showed that increasing the penetration leads to a higher bacterial death rate, shedding light on the role of the limited penetration in protecting bacterial cells (59).

2. Stress Response, Slow Growth, and Metabolic Activity:

It is well known that when a bacterial cell culture is starved for a certain nutrient, its growth slows. This transition is generally associated with an increase in antimicrobial resistance (56). Since cells growing inside a biofilm are expected to have a limitation in the availability of some nutrients, it was suggested that this kind of physiological change is responsible for reduced metabolic activity, low rates of cell division, and increased resistance to antimicrobial agents (60). A study done by Evans et al. (1991) showed that in the case of *P. aeruginosa*, both the planktonic and the biofilm-associated bacterial cells were resistant to Ciprofloxacin at a slow rate of growth. However, as the growth rate increased, planktonic cells became susceptible to Ciprofloxacin, unlike biofilm-associated cells (61). Several other studies suggested that such mechanisms vary depending on the antimicrobial agent. For example, a slow growth rate in a *P. aeruginosa* biofilm accounted for increased resistance against Tetracycline, but not against Tobramycin (62).

3. Heterogeneity:

When studying biofilms, there's an important logical assumption that suggests that any given cell within a biofilm behaves differently when compared to other cells within the same biofilm, suggesting that every single cell will be growing at a variable rate. Several factors contribute to this heterogeneity, including nutrients gradient, signaling factors, and waste products (56). Recent advances in science led to the ability to visualize this heterogeneity. In a study done by Wentland et al. (1996), biofilm heterogeneity was assessed depending on the RNA to DNA content, where rapidly or slowly growing cells in

the biofilm were stained and identified using acridine orange. A change in color was obtained in different areas of the biofilm, corresponding to either a high or a low RNA content (63).

4. Multidrug Efflux Pumps:

Efflux pumps confer resistance to bacterial cells by pumping antimicrobial agents outside and away from their intracellular targets back into the extracellular space (64). *P. aeruginosa* has several multidrug efflux pumps that include MexAB-OprM, responsible for the planktonic cell resistance to antimicrobials (65). De Kievit et al. (2001) studied the contribution of multiple efflux pumps such as MexAB-OprM, MexCD-OprJ, MexEF-OprN to the *P. aeruginosa* biofilm resistance. Results showed that these characterized efflux pumps do not contribute to the antimicrobial resistance of *P. aeruginosa* biofilms (66). However, other studies shed light on the role of the MexAB-OprM mediated biofilm resistance to specific concentrations of ofloxacin. That was done by examining *P. aeruginosa strains, either overexpressing or lacking the efflux pump* MexAB-OprM. Resistance to ofloxacin was dependent on the expression of the efflux pump but only at a low concentration range (62).

Zhang and Mah (2008) identified an efflux pump that is more highly expressed in *P. aeruginosa* biofilm cells rather than planktonic cells, which is the PA1875-1877 biofilm-specific multidrug efflux pump. Deletion in the genes encoding this pump, PA1874 to PA1877 (PA1874-1877) genes increased in the *P. aeruginosa* sensitivity against a subset of antibiotics including Gentamicin, Tobramycin, and Ciprofloxacin. Moreover, expressing

these genes in planktonic cells resulted in increased resistance to antimicrobial agents. Their significant discovery of combining the *ndvB* gene mutation with the PA1874-1877 gene deletion resulted in a *P. aeruginosa* mutant strain that is considered more sensitive to antimicrobial agents than the single mutant strain, suggesting that *ndvB* and PA1874-1877 contribute to two distinct mechanisms of biofilm specific resistance (67).

G. Optional Therapies that Target Biofilms

Due to the multiple tolerance mechanisms, *P. aeruginosa* biofilms are considered highly resistant to antibiotic therapies (68). In addition, this bacterium has the extraordinary ability to develop tolerance to a wide range of available antimicrobial agents (69). Because of this, several potential drugs and therapies are under trial for the treatment of biofilm-associated *P. aeruginosa* infections.

1. Targeting Cellular Adherence to Surfaces:

Silver, as well as its compounds, are known to have a strong inhibitory effect and a broad-spectrum antimicrobial activity for bacteria, viruses, and fungi (70). When compared to other metals, silver has the ability to exhibit higher toxicity to microorganisms and lower toxicity to mammalian cells (71). Lately, research on silver nanoparticles (also known as SNPs) has been given a lot of attention, because they appear to have a significant role in inhibiting the growth and reproduction of bacteria. SNPs are suggested to damage the structure of the bacterial membrane, as well as decrease the activity of several membranous enzymes, causing eventual bacterial cell death (72). In the case of *P. aeruginosa*, the major

problem is the ability of its biofilm to attach to different types of surfaces, which is considered as a major step in the initiation of infections (68). In that sense, it's essential to try to render such surfaces unfavorable for biofilm attachment. Since silver is considered antimicrobial and somehow not toxic for mammalian cells, its use in developing prosthetic devices with surfaces non-adherent to bacterial biofilms would be of considerable value. Ahearn et al. (1995) studied the effect of certain silver preparations on the adherence of several bacteria such as *P. aeruginosa* to solid surfaces. Results showed that silver-coated surfaces that released silver ions are biocidal and inhibit bacterial attachment (73). On the other hand, it has been proved that bacteria can acquire resistance to silver and that silver resistance determinants are directly spread among the clinically important bacteria (74).

2. Enhancing the Dispersal of Cells:

Enhancing the dispersal of biofilm cells is considered as a promising strategy that is being studied as a biofilm specific therapy. This strategy aims to disrupt the architecture of the biofilm, releasing planktonic cells (60). Santiago et al. (2016) investigated the effect of combining EIP (escapin intermediate products) with H_2O_2 on bacterial biofilms, using *P. aeruginosa* as a model. Results obtained suggested that this combination affects biofilms by destabilizing its matrix and interfering with its attachment to surfaces. Hence, EIP combined with H_2O_2 is a potential therapy for treatment, as these two substances work synergistically to disperse the biofilm of *P. aeruginosa* (75). The disadvantage of this approach is that it leads to the release of large amounts of planktonic cells, which is

dangerous, and therefore the dispersal agents must be accompanied also with an antibiotic for a more effective killing (60).

3. Quorum Sensing Inhibitors:

Quorum quenching refers to the use of certain molecules that would inhibit signaling via quorum sensing pathways (60,76). QS inhibitors are considered an important therapy used against biofilm-associated infections because they deny bacterial cells their complete protection from antimicrobial agents that is provided by the biofilm (77). Such strategy is accomplished by multiple ways that include inhibiting QS molecules synthesis, reducing their availability, or blocking their reception. In the case of *P. aeruginosa*, the most important approach is targeting AHLs, the major QS molecules (60). AHLs can be biologically inactivated by either enzymatic degradation or antibody-mediated inactivation of the QS molecule (77). For instance, Migiyama et al. (2013) evaluated the efficacy of AiiM, an AHL lactonase enzyme, in a mouse model of acute pneumonia caused by *P. aeruginosa*. The study supported the potential of AHL lactonases in therapy, after proving that AiiM can attenuate the virulence of *P. aeruginosa* by disturbing the QS signaling pathways. However, the drawback, in this case, is the way of administration of the lactonase overexpressing plasmid to mice, which renders this approach applicable only to experimental studies, rather than therapy (78).

4. Combination Therapy:

Therapy that involves combinations of antibiotics is considered more effective in killing *P. aeruginosa* biofilm-associated infections than the use of single antibiotics (79). These combinations often involve different classes that demonstrate synergy as they prolong the life of one another and delay the development of resistance (79). In their study on killing the *P. aeruginosa* associated infections in rats and patients with CF, Herrmann et al. (2010) found that using Colistin-Tobramycin combination is much more effective than the use of these respective antibiotics alone. Indeed, *in vitro* experiments demonstrated that combining Colistin with Tobramycin significantly decreased bacterial cell counts. Similar results were reported in a rat lung infection model as well as in patients suffering from CF (79). In another study by Pamp et al. (2008), combining either Colistin with Ciprofloxacin or Tetracycline resulted in complete eradication of all *P. aeruginosa* biofilm cells (80).

H. The Role of Anidulafungin in Targeting Biofilms

Anidulafungin (commercially available as Ecalta) is classified as a cyclic lipopeptide Echinocandin derivative that has an antifungal activity. This drug works by inhibiting the activity of the enzyme 1,3-β-D-glucan synthase involved in the synthesis of the fungal cell wall, leading to cell lysis and, therefore, death (81). Anidulafungin exhibits good fungicidal activity against *Candida* species and *Aspergillus* (81,82). In a study by Rosato et al. (2013), the activity of Anidulafungin was tested using an *in vitro* model of *C. albicans* and *C. tropicali*. Results showed that Anidulafungin has a strong inhibitory activity against both the planktonic and the biofilm cells of both species (83). Another

study done *in vivo* demonstrated the efficacy of Anidulafungin against mature biofilms of *C. albicans* in a rat model of catheter-associated candidiasis. Fragments of catheters were coated with *C. albicans* then implanted subcutaneously. Biofilms were formed, and the rats were given daily injections of Anidulafungin for a week. Catheters that were retrieved from treated rats showed a decrease in fungal cell number when compared to catheters taken from untreated rats. These results show that Anidulafungin is promising as a treatment option for *C. albicans* biofilms (84).

Echinocandins are also under ongoing research for their potential activity to target preformed biofilms of *P. aeruginosa*. Bazzi et al. (2013), Kissoyan et al (2016), and Rasheed (2016) assessed the effect of an Echinocandin known as Micafungin on the formation of *P. aeruginosa* biofilms (8–10). Issa (2019), on the other hand, studied the effect of Anidulafungin with antibacterial agents on pre-formed *P. aeruginosa* biofilms (11). Results showed a significant phenotypic reduction in biofilm formation, as well as a decrease in the mRNA transcription levels of the *ndvB* gene, encoding for the production of 1,3-β-D-glucans. Furthermore, confocal microscopy images showed a decrease in biofilm thickness upon the addition of Micafungin. In conclusion, Echnocandins are considered effective for disrupting the biofilm structure in *P. aeruginosa*, exposing the core planktonic bacterial cells, and facilitating treatment (8–10).

CHAPTER III

MATERIALS AND METHODS

A. Source of Bacterial Isolate

The *P. aeruginosa* PAN14 clinical strain used in this study is a strong biofilm forming clinical strain previously isolated from a deep tracheal aspirate of a patient with a nosocomial infection. The isolate was stored in Brucella broth at -80°C.

B. Bacterial Identification

The isolate's identity was confirmed based on colonial morphology, production of the characteristic green pigment, pyoverdine, and the fruity smell when cultured on Luria Bertani (LB) agar. Furthermore, biochemical profiling tests, including the oxidase test and API NE kit (bioMerieux.SA 69820, Marcy l'Etoile-France), were performed. Finally, its susceptibility against the most recommended antibiotics, according to the Clinical and Laboratory Standards Institutes (CLSI) guidelines was performed using the disc diffusion method.

C. Source of Drugs

The drugs used for screening were provided from American University of Beirut Medical Center (AUBMC) pharmacy:

• Anidulafungin (Ecalta, Pfizer, Belgium)

• Colistin Sulfate (Sigma)

• Gentamicin Sulfate Salt (Sigma)

• Ciprofloxacin (Ciprolon, HIKMA Pharmaceuticals, Jordan)

• Ceftazidime (Sigma)

• Cefepime (Qpime, Gulf Pharmaceutical Industries, U.A.E)

• Tazocin (Wyeth Lederle S.p.A., Italy)

D. Susceptibility Assessment of Colistin, Gentamicin, Ciprofloxacin, Ceftazidime, Cefepime and Tazocin on *P. aeruginosa* PAN14 Planktonic Cells

Serial broth microdilution assay was performed to determine the minimal inhibitory concentration (MIC) of several antimicrobial agents on PAN14 planktonic cells.

1. Materials

• 96-well polystyrene microtiter plates (Costar® Inc, NY, USA)

• Colistin Sulfate (Sigma)

• Gentamicin Sulfate Salt (Sigma)

• Ciprofloxacin (Ciprolon, HIKMA Pharmaceuticals, Jordan)

• Ceftazidime (Sigma)

• Cefepime (Qpime, Gulf Pharmaceutical Industries, U.A.E)

• Tazocin (Wyeth Lederle S.p.A., Italy)

• Optical density reader DENSIMAT (bioMerieux. SA 69820, Marcy l'Etoile-France)

• PAN14 culture on LB agar (Luria-Bertani, Pronadisa, Conda, Madrid, Spain)

• Cation-Adjusted Mueller-Hinton Broth (CAMHB) (BBL^{TM}, BD, Frankin Lakes, NJ, USA).

2. Protocol

• An aliquot of 90 µL of CAMHB broth was added to all the wells of 96-well microtiter plates. Then, serially diluted Colistin, Gentamicin, Ciprofloxacin, Ceftazidime, Cefepime or Tazocin were added into the broth containing wells.

• Few PAN14 colonies were inoculated into CAMHB and the bacterial suspension turbidity was adjusted to 0.5 McFarland using the optical density reader. The bacterial suspension was then diluted with CAMHB to obtain a final concentration of 5×10^6 CFU/mL.

• 10 µL aliquots of the bacterial suspension were added into the wells. Positive and negative controls were run and antimicrobial agents' treated wells were repeated in duplicates. The positive control included bacteria and CAMHB while sterile CAMHB broth stood as the negative control.

• Finally, the plates were covered and incubated overnight on the shaker at 37°C.

The protocol above was adapted from CLSI (85).

E. Minimum Biofilm Inhibitory Concentration (MBIC) and Minimal Biofilm Eradication Concentration (MBEC) for Colistin, Gentamicin, Ciprofloxacin, Ceftazidime, Cefepime and Tazocin

Minimum Biofilm Inhibitory concentration (MBIC) corresponds to the minimal antimicrobial concentration at which there is no observable growth of the adherent

microcolonies, while MBEC corresponds to the minimal concentration to eradicate the adherent biofilm microcolonies.

The MBIC for the above antimicrobial agents towards PAN14 biofilms was performed next.

1. Materials

• 96-well polystyrene microtiter plates (Costar® Inc, NY, USA)

• Colistin Sulfate (Sigma)

• Gentamicin Sulfate Salt (Sigma)

• Ciprofloxacin (Ciprolon, HIKMA Pharmaceuticals, Jordan)

• Ceftazidime (Sigma)

• Cefepime (Qpime, Gulf Pharmaceutical Industries, U.A.E)

• Tazocin (Wyeth Lederle S.p.A., Italy)

• Optical density reader DENSIMAT (bioMerieux. SA 69820, Marcy l'Etoile- France)

• PAN14 culture on LB agar (Luria-Bertani, Pronadisa, Conda, Madrid, Spain)

• LB broth (Luria-Bertani, Pronadisa, Conda, Madrid, Spain)

• Cation-Adjusted Mueller-Hinton Broth (CAMHB) (BBL™, BD, Frankin Lakes, NJ, USA)

• 0.9% saline (NaCl)

2. Protocol

• An overnight culture of PAN14 in LB broth was adjusted to reach a turbidity of 0.5 McFarland, then 100 μL aliquots of the bacterial suspension were added to the wells of 96 well-microtiter plates.

• The plates were incubated for 24 h at 37°C without shaking to allow bacterial adherence.

• The wells were washed three times with 0.9% saline (NaCl) under aseptic conditions to remove planktonic bacteria and left to air dry for 15 minutes.

• 100 μL of appropriate two-fold dilutions of the antimicrobials in Mueller–Hinton broth were transferred into the emptied and air-dried wells with established biofilms.

• The microtiter plates were incubated for 18–20 hours at 37 ºC, followed by analysis to determine their respective MBIC where there was no observable growth in the wells.

• To determine the MBEC, 1μL was taken from the treated wells and plated on LB agar. The MBEC corresponded to the absence of growth on the plates.

• Each antibiotic was performed in duplicates; negative and positive controls were also run, and the experiment was repeated twice. The positive control consisted of 100 μL aliquots of untreated bacterial suspension.

The protocol used above was adapted from Reiter et al. (2012) with modifications (86).

F. Minimum Biofilm Inhibitory Concentration (MBIC) and Minimal Biofilm Eradication Concentration (MBEC) for all Antimicrobials in Combination with Anidulafungin

Minimum Biofilm Inhibitory concentration (MBIC) corresponds to the minimal antimicrobial concentration at which there is no observable growth of the adherent

microcolonies, while MBEC corresponds to the minimal concentration to eradicate the adherent biofilm microcolonies.

The impact of combining Anidulafungin on the MBIC and MBEC of all antimicrobials towards PAN14 biofilms was assessed next.

1. Materials

• 96-well polystyrene microtiter plates (Costar® Inc, NY, USA).

• Aniulafungin (Ecalta, Pfizer, Belgium)

• Colistin Sulfate (Sigma)

• Gentamicin Sulfate Salt (Sigma)

• Ciprofloxacin (Ciprolon, HIKMA Pharmaceuticals, Jordan)

• Ceftazidime (Sigma)

• Cefepime (Qpime, Gulf Pharmaceutical Industries, U.A.E)

• Tazocin (Wyeth Lederle S.p.A., Italy)

• Optical density reader DENSIMAT (bioMerieux. SA 69820, Marcy l'Etoile France)

• PAN14 culture on LB agar (Luria-Bertani, Pronadisa, Conda, Madrid, Spain)

• LB broth (Luria-Bertani, Pronadisa, Conda, Madrid, Spain)

• Cation-Adjusted Mueller-Hinton Broth (CAMHB) (BBL^{TM}, BD, Frankin Lakes, NJ, USA).

• 0.9% saline (NaCl).

2. Protocol

• An overnight culture of PAN14 in LB broth was adjusted to reach a turbidity of 0.5 McFarland, then 100 µL aliquots of the bacterial suspension were added to the wells of a 96 well-microtiter plate.

• The plates were incubated for 24 h at 37°C without shaking to allow bacterial adherence.

• The wells were washed three times with 0.9% saline (NaCl) under aseptic conditions to remove the planktonic bacteria and are then left to air dry for 15 minutes.

• 50 µL of appropriate two-fold dilutions of the antimicrobials in Mueller Hinton broth were transferred into the emptied and air-dried wells with established biofilms. 50 µL of each 3.3 mg/mL and 1.65 mg/mL Anidulafungin were added to the antimicrobial containing wells of each plate.

• The microtiter plates were incubated for 18–20 hours at 37 ºC, followed by analysis to determine their respective MBIC where there was no observable growth in the wells.

• To determine the MBEC, 1µL was taken from the treated wells and plated on LB agar. The MBEC corresponded to the absence of growth on the plates.

• Each antimicrobial agent was performed in duplicates; negative and positive controls were also run, and the experiment was repeated twice. The positive control consisted of 100 µL aliquots of an untreated bacterial suspension.

The protocol used above was adapted from Reiter et al. (2012) with modifications (86).

G. Scanning Electron Microscopy of Biofilms Grown on Catheter Sections under Static Conditions

PAN14 biofilms were formed on catheter sections, then subjected to Scanning electron microscopy to examine their morphology.

1. Materials

• 25-well polystyrene microtiter plates (Costar® Inc, NY, USA)

• All silicone Foley catheter 12Fr/Ch (Tenso Med®)

• PAN14 culture on LB agar (Luria-Bertani, Pronadisa, Conda, Madrid, Spain)

• LB broth (Luria-Bertani, Pronadisa, Conda, Madrid, Spain)

• 10X Phosphate Buffer Saline (PBS)

• 0.1% Safranin

• Metal coating system (Q150T ES; Quorum Technologies)

• Scanning Electron Microscope (SEM) (MIRA3 LMU with OXFORD EDX detector by TESCAN)

2. Protocol

• An overnight culture of *P. aeruginosa* PAN14 was diluted to 1:50 in LB broth, and 200 µL aliquots of the liquid culture were added to 1cm catheter sections placed in the wells of a 25-well plate.

• The plate was incubated for 48 hours at 37◦C without shaking, and the media was then slowly removed without disturbing the biofilms, and fresh LB broth was added every 24 hours.

• The media was removed, and the catheters were then rinsed once with Phosphate Buffer Saline (PBS) and left to air dry for 15 minutes at room temperature. The sections were then stained with 0.1% safranin for 5 minutes, then washed once with PBS and allowed to dry for 15 minutes at room temperature.

• After drying, the catheters were further sectioned and adhered to double-sided carbon adhesive tape to aluminum stubs.

• The sections were coated with 15-17 nm thick platinum using a metal coating system (Q150T ES; Quorum Technologies) and examined by SEM for the presence of biofilm structures.

• The negative control consisted of catheter sections not subjected to PAN14 bacteria.

This protocol was adapted from Nair et al. (2016) with modifications (87).

H. Scanning Electron Microscopy of Biofilms Grown on Catheter Sections under Flow

PAN14 biofilms were also grown on catheter sections under dynamic conditions in a flow cell inspired system (88), then the SEM was used to examine morphological changes that took place at the level of the biofilm after being subjected to a series of antimicrobial agents and combinations.

The flow system model was also used in previous studies by Rasheed and Issa done in the bacteriology lab. However, in this study, several modifications in its set-up have been made.

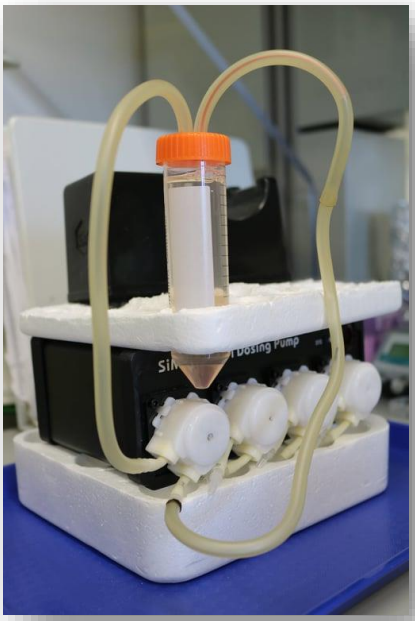


Figure 6. The flow System

1. Materials

• Pump (Simalai Wi-Fi dosing pump)

• Autoclavable connecting tubes

• 50ml tubes (Falcon® Conical Centrifuge Tubes)

• LB broth (Luria-Bertani, Pronadisa, Conda, Madrid, Spain)

• PAN14 culture on LB agar (Luria-Bertani, Pronadisa, Conda, Madrid, Spain)

• All silicone Foley catheter 12Fr/Ch (Tenso Med®)

• 5mL Falcon® Round-Bottom Polystyrene tubes

• Aniulafungin (Ecalta, Pfizer, Belgium)

• Colistin Sulfate (Sigma)

• Gentamicin Sulfate Salt (Sigma)

• Ciprofloxacin (Ciprolon, HIKMA Pharmaceuticals, Jordan)

• Ceftazidime (Sigma)

• Cefepime (Qpime, Gulf Pharmaceutical Industries, U.A.E)

• Tazocin (Wyeth Lederle S.p.A., Italy)

• Artificial urine (prepared in the lab using the following ingredients: distilled water, urea, sodium chloride, potassium chloride, sodium phosphate, creatinine, and albumin)

pH and Specific gravity (SG) of urine were measured using Combur7 Test® strips (pH= 5 to 7 and SG= 1.015 to 1.025)

2. Protocol

a. Setting up the Flow System

• A 50 mL Falcon tube was filled with variable concentrations of LB broth and artificial urine. Two holes were made on the cover of the tube.

• A sterile catheter was placed inside an autoclaved connecting tube that links the 50ml Falcon with the pump's input port.

• The export port of the pump was connected to the 50 mL Falcon tube with an autoclaved connecting tube through the other hole.

• The pump was turned on to allow the media and artificial urine to flow through the system.

b. Inoculation of the Bacteria

• An overnight culture of *P. aeruginosa* PAN14 was diluted to 1:50 in LB broth.

• 2 ml aliquots were added to the 50 mL Falcon tube containing variable concentrations of media and artificial urine.

• The system was left running for 48 hours with change of media every 24 hours.

• After 48 hours, the pump was turned off and the catheter was retrieved, sectioned and transferred into 5 ml tubes containing 0.5ml of the treatment in 0.5 ml of LB broth. The treatment conditions included each antibacterial agent alone, in combination with Anidulafungin, and Anidulafungin alone.

• The tubes were incubated at 37°C under static conditions for 24 hours and 48 hours. Then the catheter sections were processed for SEM following the steps in the previous section to check for the formation of biofilms (Section G).

Overall treatment conditions were performed and repeated in duplicates.

Pan14 biofilms grown on catheter sections were also stained with Crystal Violet. Catheters were washed 3 times with 0.9% NaCl, stained with Crystal Violet for 15 minutes, then washed again and allowed to air dry.

I. *P.* aeruginosa PAN14 Biofilm Growth on ECV 304 Epithelial Cell Line

1. Materials

• ECV304 Epithelial cells (Takahashi K et al. Spontaneous transformation and immortalization of human endothelial cells) (89)

• Roswell Park Memorial Institute Medium (RPMI) (Sigma)

• Fetal Bovine Serum (FBS)

• L- glutamine

• Penicillin

• 48-well polystyrene microtiter plates (Costar® Inc, NY, USA).

2. Protocol

a. Static Co-culture Biofilm Model

• Cells were grown in RPMI medium supplemeted with 10% fetal bovine serum, 2mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C and 5% CO_2-95% air for 4- 7 days to form a confluent monolayer before inoculation with bacteria. The medium was changed every two days. 0.5 mL medium was added per well in 48-well plates. These conditions have been shown to lead to formation of a confluent monolayer.

• *P. aeruginosa* was grown in 5 mL LB for 18 hours at 37°C on an incubator shaker at 200 rpm. Under these conditions, *P. aeruginosa* cultures typically reached a density of $5x10^9$ CFU/ mL.

• For bacterial inoculation, the medium was removed from the ECV304 cells, and RPMI supplemented with 10% fetal bovine serum, 2mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin was added. Confluent ECV304 monolayers were inoculated with *P.*

aeruginosa at a multiplicity of infection of approximately 30:1 relative to the number of cells originally seeded. This equates to 1.2 X 10^7 CFU/mL in 0.5 mL MEM/well for 48-well plates.

• Plates were incubated for 1 hour at 37°C and 5% CO_2-95% air.

• Following the 1 hour incubation, the supernatant was removed and replaced with RPMI medium supplemented with 10% fetal bovine serum and 2mM L-glutamine only.

• Plates were incubated at 37°C and 5% CO_2-95% air for 18 hours

• Cells were then processed for staining (Section J) and SEM following the steps in the previous section to check for the formation of biofilms (Section G).

This protocol was adapted from Moreau-Marquis et al. (2010) with modifications (90).

J. Microtiter Plate Assay for Detection of PAN14 Biofilms on ECV304 Epithelial Cell Line

1. Materials

• 96-well polystyrene microtiter plates (Costar® Inc, NY, USA).

• 48-well polystyrene microtiter plates (Costar® Inc, NY, USA).

• Optical density reader DENSIMAT (bioMerieux. SA 69820, Marcy l'Etoile France).

• 0.9% saline (NaCl).

• 1% Crystal Violet

• 95% ethanol

2. Protocol

• After growing *P. aeruginosa* PAN14 biofilms on the ECV304 cells following the Static co- culture Biofilm Model (Section I), the culture medium was removed from each well by pipetting and the cells were washed away three times using 0.9% saline (NaCl).

• The 48-well microtiter plates were left to air dry at room temperature for about an hour to allow the fixation of the biofilms.

• One hundred fifty microliter of 1% Crystal Violet (CV) were to the wells and left at room temperature for 15 to 20 minutes.

• CV was removed by pipetting and washed by adding and removing 0.9% (NaCl) to the wells.

• 200 μL of 95% ethanol was added to the wells and left for 20min.

• 120 μL from each well was transferred to a 96-well microtiter plate.

• The absorbance was then measured using BIO-TEK ELx800 Automated Microplate Reader at 630nm.

• The experiment was performed twice, the average optical density of the replicates was calculated and the p value was determined using the student t-test.

This protocol was adapted from adopted from Imquestbio (2016) with modifications (91).

CHAPTER IV

RESULTS

A. Susceptibility Profile of *P. aeruginosa* PAN14 Planktonic Cells

Based on the MIC assay, PAN14 planktonic cells displayed susceptibility to six different antimicrobial agents. Results showed that this strain had an MIC value of ≤0.5 μg/mL for Gentamicin, 4 μg/mL for Tazocin, ≤1 μg/mL for both Ceftazidime and Cefepime, 0.5 μg/mL for Colistin, and 0.125 μg/mL for Ciprofloxacin. Results for all antimicrobials tested are summarized in Table 1, and the breakpoints used to define PAN14 planktonic cells as susceptible or resistant are according to CLSI (2020) guidelines (85).

Drug	MIC (μg/mL)
Gentamicin	≤0.5
Tazocin	4
Ceftazidime	≤1
Cefepime	≤1
Colistin	0.5
Ciprofloxacin	0.125

Table 1. MIC values of antimicrobials against *P. aeruginosa* PAN14 planktonic cells

B. The Impact of Anidulafungin on the Minimal Biofilm Inhibitory and Eradication Concentration of Gentamicin, Colistin, and Ciprofloxacin

Based on the MBIC assay, the MBIC values of Gentamicin, Colistin, and Ciprofloxacin were 4 μg/mL, 16 μg/mL, and 0.125 μg/mL respectively. Combining Anidulafungin at concentrations of 3.3 μg/mL and 1.65 μg/mL to Gentamicin resulted in a

2-folds decrease in the MBIC value to 2 µg/mL as shown in Table 2 and Figure 1. No change was reported in the MBIC values in case of Colistin and Ciprofloxacin when combined with Anidulafungin.

The MBEC values of Gentamicin, Colistin, and Ciprofloxacin were 16 µg/mL, 128 µg/mL and 1 µg/mL respectively. As reported in Table 2 and Figure 1, the MBEC value of Gentamicin was 4-folds higher than its MBIC value, and 8-folds higher in case of Colistin and Ciprofloxacin. With respect to Colistin, there was a 2- fold decrease in the MBEC value which dropped to 64 µg/mL when combined with Anidulafungin at a concentration of 3.3 mg/mL. As for the addition of Gentamicin and Ciprofloxacin each with AFG at 1.65 µg/mL, there was a 2-fold decrease in both their MBEC values reaching 8 µg/mL and 0.5 µg/mL respectively. However, Colistin showed no change in its MBEC value when combined with Anidulafungin at a subclinical dosage. (n is the number of times the experiments were repeated).

Drug	MBIC (µg/mL)	MBEC (µg/mL)
Gentamicin	4	16
Colistin	16	128
Ciprofloxacin	0.125	1
Gentamicin + AFG (3.3mg/mL)	2	16
Gentamicin + AFG (1.65mg/mL)	2	8
Colistin + AFG (3.3mg/mL)	16	64
Colistin + AFG (1.65mg/mL)	16	128
Ciprofloxacin + AFG (3.3mg/mL)	0.125	1
Ciprofloxacin + AFG (1.65mg/mL)	0.125	0.5

Table 2. MBIC and MBEC values for Gentamicin, Colistin, and Ciprofloxacin alone and in combination with Anidulafungin (n= 2)

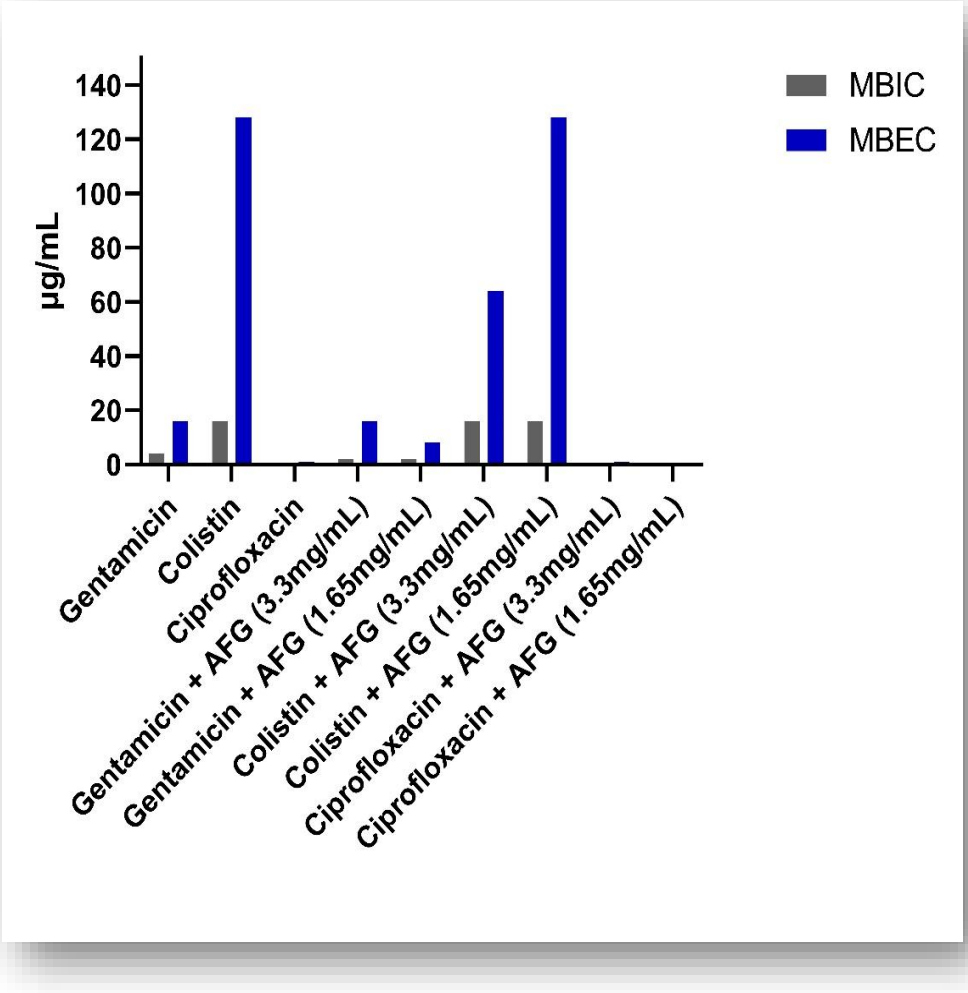


Figure 7. MBIC and MBEC values of Gentamicin, Colistin, and Ciprofloxacin alone and in combination with Anidulafungin

C. The Impact of Anidulafungin on the Minimal Biofilm Inhibitory and Eradication Concentration of Tazocin, Ceftazidime, and Cefepime

When tested alone, the MBIC of Tazocin was at a high value of 4096 µg/mL as reported in Table 3. The same value was obtained after combining Tazocin with Anidulafungin at both concentrations of 3.3mg/mL and 1.65 mg/mL. Similarly, both Ceftazidime and Cefepime had an MBIC value of >1024 µg/mL when tested alone and when coupled with different concentrations of Anidulafungin.

With respect to the MBEC of Tazocin, a value of >4096 μg/mL was obtained when testing it alone and when combined with 3.3mg/mL or 1.65 mg/mL of Anidulafungin. A similar result was reported in the case of Ceftazidime and Cefepime, where the MBEC value was >1024 μg/mL when the antimicrobials were tested alone and in combination with Anidulafungin.

In conclusion, Tazocin, Ceftazidime and Cefepime had high MBIC and MBEC values, and combining them with Anidulafungin didn't have any effect on the results. This reflects the fact that these antimicrobials are ineffective against biofilms, and are therefore not considered in future experiments.

Drug	MBIC (μg/mL)	MBEC (μg/mL)
Tazocin	4096	>4096
Ceftazidime	>1024	>1024
Cefepime	>1024	>1024
Tazocin + AFG (3.3mg/mL)	4096	>4096
Tazocin + AFG (1.65mg/mL)	4096	>4096
Ceftazidime + AFG (3.3mg/mL)	>1024	>1024
Ceftazidime + AFG (1.65mg/mL)	>1024	>1024
Cefepime + AFG (3.3mg/mL)	>1024	>1024
Cefepime + AFG (1.65mg/mL)	>1024	>1024

Table 3. MBIC and MBEC values for Tazocin, Ceftazidime, and Cefepime alone and in combination with Anidulafungin (n=2)

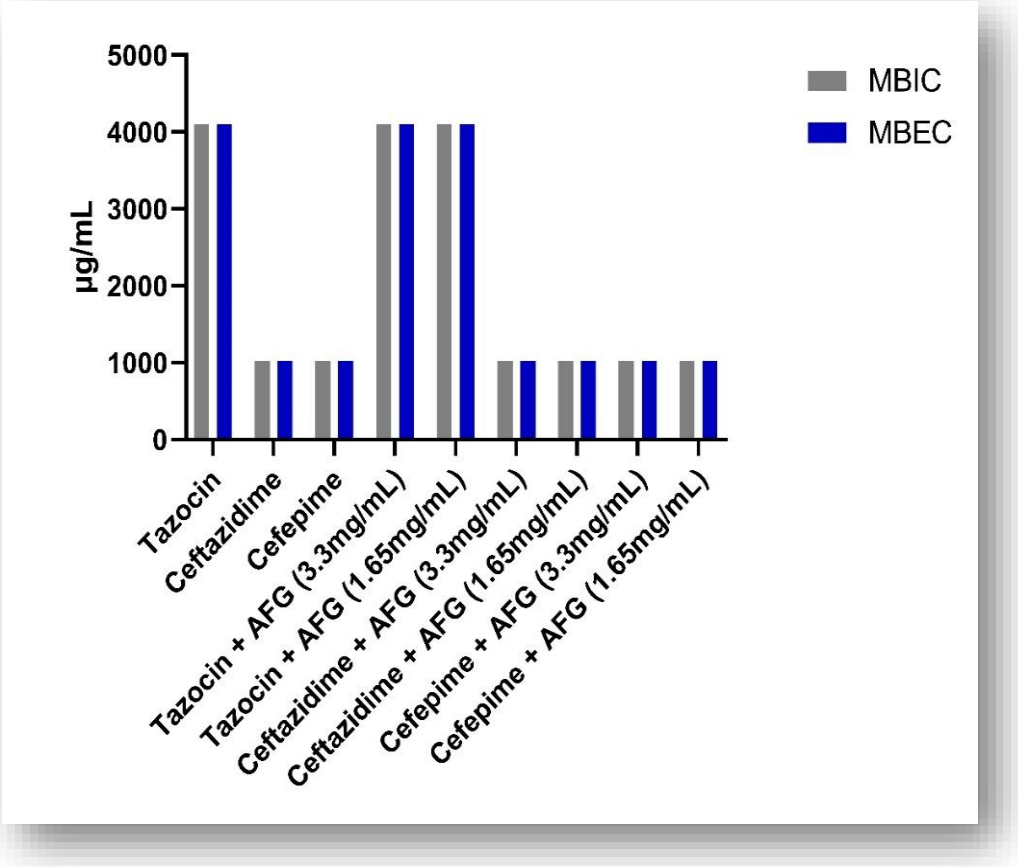
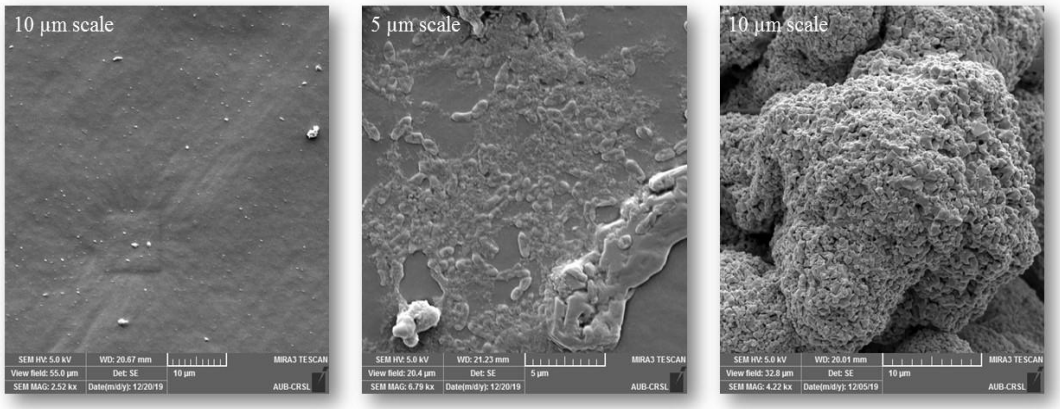


Figure 8. MBIC and MBEC values of Tazocin, Ceftazidime, and Cefepime alone and in combination with Anidulafungin

D. Scanning Electron Microscopy Images of PAN14 Biofilms on Catheter Sections

Scanning Electron Microscopy (SEM) was performed to visualize in detail the morphology of the biofilms grown under different conditions, as well as the changes that took place in the biofilms' architecture upon treatment. To achieve that, biofilms were grown on silicon catheters in a 25 well plate under static conditions, and in a flow system under the continuous run of LB broth. The aim behind using a flow system was to investigate the efficacy of biofilm formation in this *in vitro* model, which is considered to mimic the conditions found inside the human body to a certain extent.

Biofilms grown under either static or flow conditions both showcased distinct aggregates with rough surfaces. However, compared to the biofilms formed under stagnant conditions, the flow system grown biofilms had a more defined architecture, and showed a much developed and intricate 3D structure (Figure 3. b, c). Moreover, SEM images showed a substantial less biofilm biomass on the catheters obtained under static conditions than the ones obtained under flow.



a) Negative control b) Static- 100% LB broth c) Flow- 100% LB broth

Figure 9. SEM images of PAN14 biofilms grown under static and flow conditions in 100% media

Based on these results, the flow system was considered as a suitable model and biofilms were grown on catheters under variable flow conditions and altered concentrations of media and artificial urine. Increasing the amount of artificial urine with respect to media affected the overall architecture of the biofilms, as observed in Figure 4. b. In 80% artificial urine and 20% media, there was a decrease in the biofilms' population, in terms of

thickness and 3D structure compared to the biofilms obtained under equal concentrations of artificial urine and LB broth. In addition, SEM images obtained in both cases showed a decrease in the total biofilm biomass when compared to the ones obtained when biofilms were grown under flow in 100% of media (Figure 3. c).

a) Flow- 50% LB broth with 50% artificial urine b) Flow- 20% LB broth with 80% artificial urine

Figure 10. SEM images of PAN14 biofilms grown under flow in different concentrations of media and artificial urine

E. Crystal Violet Stain of PAN14 Biofilms Grown on Catheter Sections Under Flow Conditions in 100% Media

Catheters obtained from the flow system retained the purple color of Crystal Violet, corresponding to the presence of biofilms, compared to the negative control, a catheter section not subjected to *P. aeruginosa*.

Figure11. Catheter Sections Stained with Crystal Violet Stain (A is a negative control)

F. Scanning Electron Microscopy Images of PAN14 Biofilms on Catheter Sections Following Treatment

After growing PAN14 biofilms on catheters under flow conditions using 50% LB broth and 50% artificial urine, the catheters were sectioned and subjected to different treatment conditions. Shown in Fig. 5. b is a 3D structure of a biofilm after 24 hours of treatment with Colistin. The architecture of the biofilms didn't seem to be affected following treatment with Colistin alone, as there are no reported changes in the biofilms' structure when compared to that obtained under the same conditions but before treatment (Figure 4. a). In addition, using Anidulafungin alone didn't have the ability to eradicate the pre-formed biofilms, as Figure 5. d shows a 2D structure of PAN14 biofilms coating a catheter section. On the other hand, there was a complete eradication of the pre-formed biofilms when the catheter section was subjected to a combination of Colistin and Anidulafungin for 24 hours in comparison to using either monotherapeutic agents alone

(Figure 5. c). The eradication is further elucidated when compared to the negative control, a catheter section not subjected to PAN14 and treated exactly like the samples.

Following 48 hours of treatment with only Colistin, there was an increase in the total biofilms' biomass, as they showed a much developed 3D structure than the biofilms obtained after only 24 hours (Figure 5. f), adding to the fact that Colistin alone doesn't have any effect in eradicating the pre-formed biofilms on its own. Moreover, Anidulafungin alone did not have any impact on the biofilms even after 48 hours, as shown in Figure 5. h. Combining Colistin with Anidulafungin produced the same result as that at 24 hours post treatment, showing complete eradication of the pre-formed biofilms after 48 hours in comparison to the negative control and when both drugs were used alone (Figure 5. g).

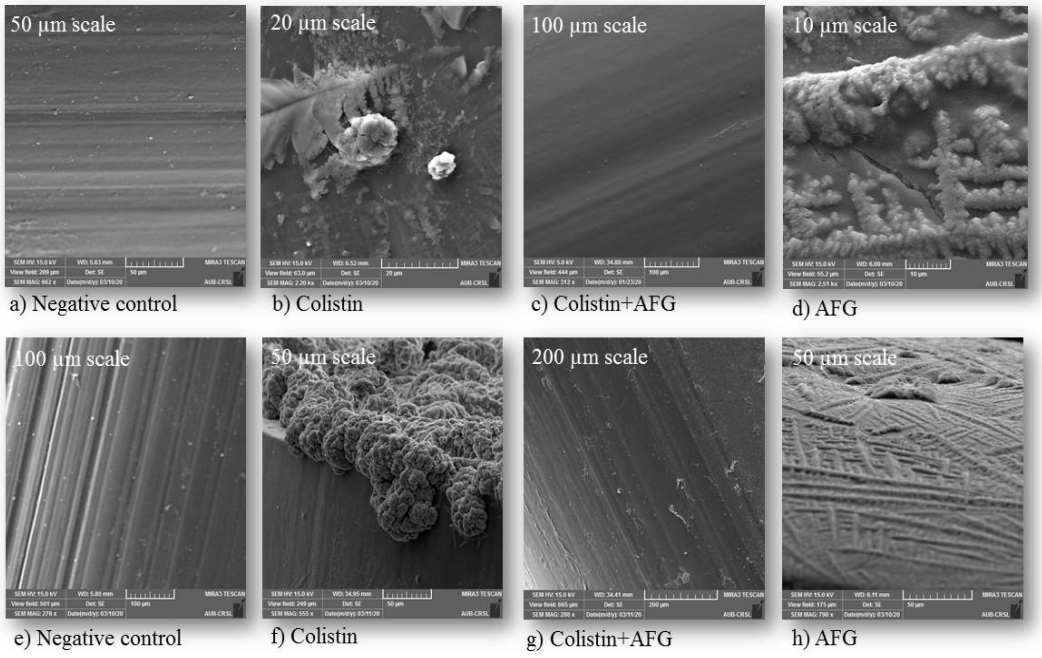


Figure 12. SEM images of PAN14 biofilms grown under flow in 50% media and 50% artificial urine following the first treatment options (upper row is after 24 hours and lower row is after 48 hours of treatment)

Similar results were obtained upon using a different antimicrobial agent, Gentamicin. Treating the catheter grown biofilms with Gentamicin alone didn't have any effect on the pre-formed biofilms after either 24 or 48 hours, as observed in Figure 6. b, f. Moreover, when the obtained catheters were treated with Anidulafungin alone for 24 and 48 hours, biofilms persisted and SEM images showed sections of biofilm coated catheters (Figure 6. d, h). Following the combination of Gentamicin with Anidulafungin, there was a complete eradication of the pre-formed biofilms after both time intervals (Figure 6. c, g), similar to what was obtained after combining Colistin with Anidulafingin.

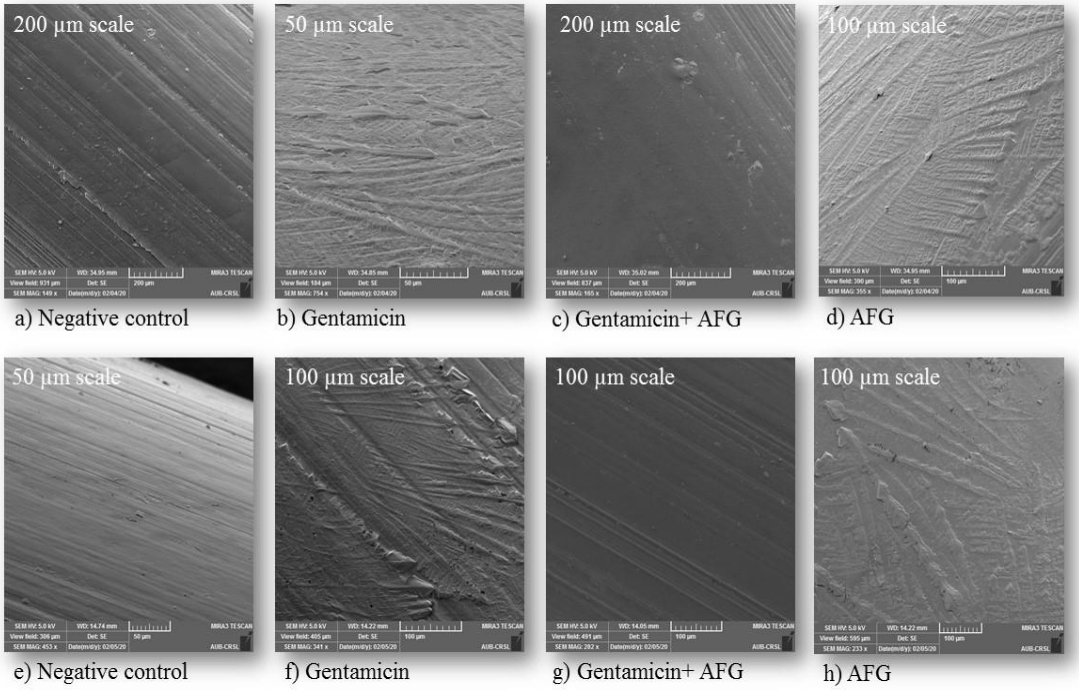


Figure 13. SEM images of PAN14 biofilms grown under flow in 50% media and 50% artificial urine following the second treatment options (upper row is after 24 hours and lower row is after 48 hours of treatment)

The usage of Ciprofloxacin alone didn't have an effect on the catheter grown biofilms neither after 24 nor after 48 hours (Figure 7. b, f). A similar result was observed

upon treating the biofilms with Anidulafungin alone (Figure 7. d, h). Surprisingly, combining Ciprofloxacin with Anidulafungin was not able to eradicate the pre-formed biofilms, contrary to when Anidulafungin was combined with either Colistin or Gentamicin (Figure 7. c, g).

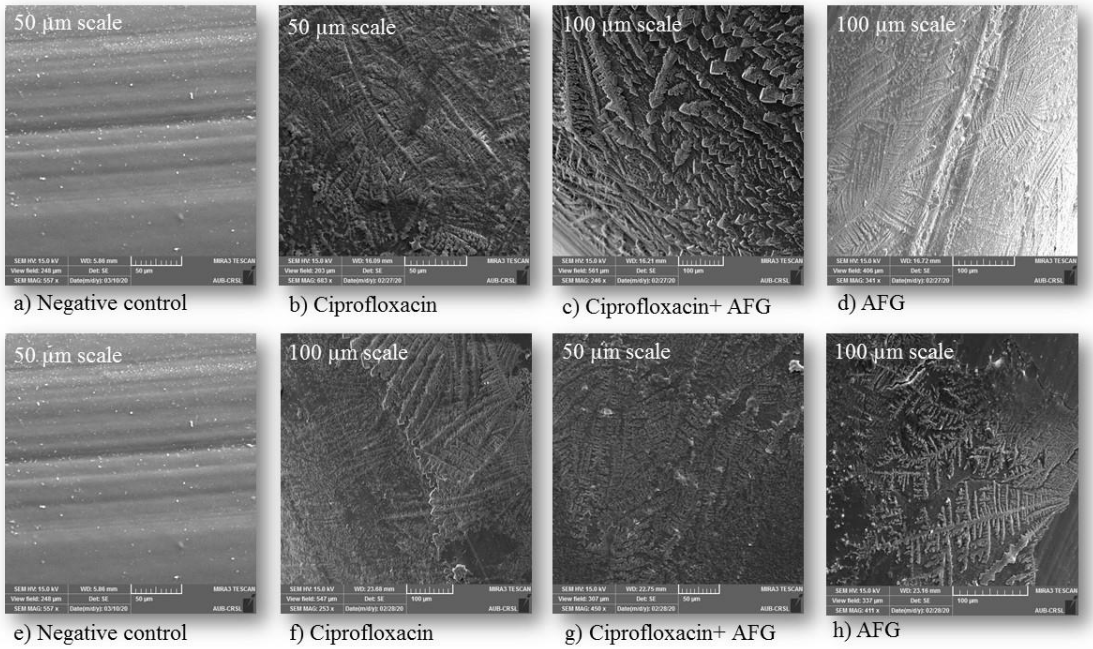


Figure 14. SEM images of PAN14 biofilms grown under flow in 50% media and 50% artificial urine following the third treatment options (upper row is after 24 hours and lower row is after 48 hours of treatment)

G. Biofilm Formation on ECV304 Epithelial Cell Line

Results show that the wells containing infected cells have a significantly higher OD compared to the wells containing only cells without PAN14 (P-value<0.0001). This indicates that the PAN14 biofilms were capable of efficiently growing on the ECV304 cell line.

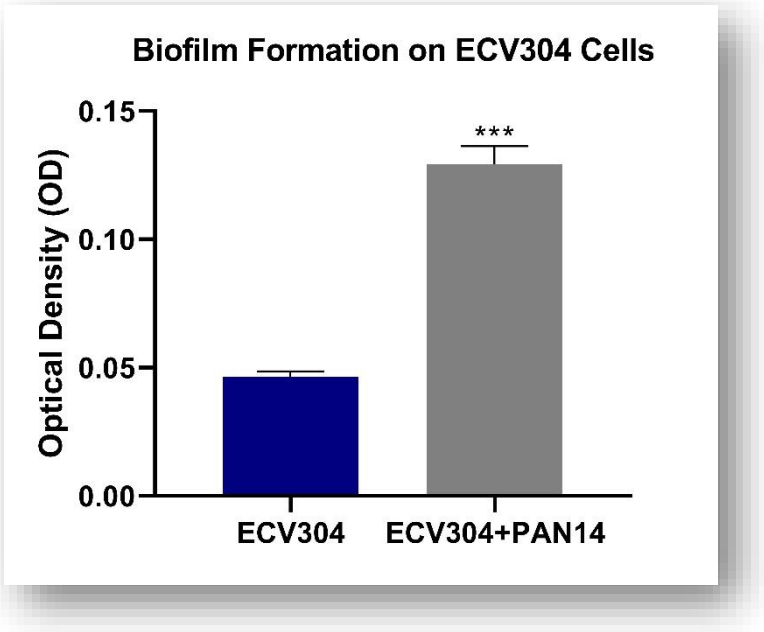
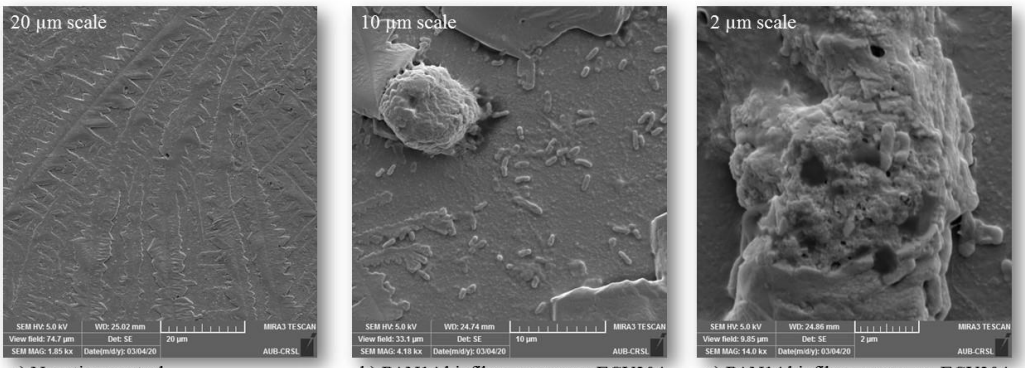


Figure 15. Biofilm formation on ECV304 cells

Moreover, SEM images of the biofilms show their well-developed and intricate 3D structure with several bacterial cells dispersed in the surroundings (Figure 9. b), compared to the negative control containing only the ECV304 cells. Based on these results, the ECV304 cell line was considered as a good model for PAN14 biofilm growth, and can therefore be used to study the efficacy of combination therapies in eradicating biofilms in an *ex-vivo* model.



a) Negative control

b) PAN14 biofilms grown on ECV304 cells

c) PAN14 biofilms grown on ECV304 cells

Figure 16. SEM images of PAN14 biofilms grown on ECV304 cells

CHAPTER V

DISCUSSION

The limited biofilm specific therapies and the increased resistance that these biofilms confer against the conventional antimicrobial agents put forward the importance of finding new strategies for targeting biofilm-associated infections (92). Echinocandins exert an anti-biofilm effect on many pathogenic fungi including Candida species by inhibiting the synthesis of 1, 3-β-D- glucan, which is a critical biofilm component. Since their major target is glucan synthase, an enzyme absent in mammalian cells, Echinocandins have a favorable safety profile (93). Bazzi et al. (2013) shed light on the inhibitory effect of Micafungin, which belongs to the family of Echinocandins, on the formation of biofilms by several PAN14 clinical isolates *in vitro* (8). Moreover, many studies stressed on the role of the ndvB gene encoding the synthesis of cyclic glucans in mediating increased biofilm antimicrobial resistance (7,9,43). Rasheed (2016) and Issa (2019) each studied the effectiveness of combining different Echinocandins with antibacterial agents in inhibiting *P. aeruginosa* biofilm formation (10,11). All these findings paved the way for our study, to investigate the potential effect of another Echinocandin known as Anidulafungin in combination with either Colistin, Gentamicin, or Ciprofloxacin in eradicating pre-formed *P. aeruginosa* PAN14 biofilms in *in-vitro* and *ex-vivo* UTI models.

It is well established that *P. aeruginosa* PAN14 planktonic cells are less resistant than their biofilm-associated counterparts (56,57). This is further noted while studying the

susceptibility profile of PAN14 planktonic cells (Table 1), where they displayed sensitivity to six different antibacterial agents belonging to several families. Moreover, the MBIC value for Colistin showed 32-fold increase compared to its corresponding MIC performed on planktonic cells. A similar result was obtained in the case of Gentamicin, where its MBIC value was 8-fold more than its corresponding MIC. Hence, these findings come in accordance with the fact that biofilms play a significant role in the emergence of resistant bacterial strains.

When added to Colistin and Ciprofloxacin, Anidulafungin did not have an impact on their MBIC values, and therefore on inhibiting the growth of biofilm-associated cells. On the other hand, combining Anidulafungin to Gentamicin resulted in a 2-fold decrease in its MBIC value, shedding light on the inhibitory effect that Echinocandins have against biofilms. This effect is further observed in the MBEC values upon the addition of Anidulafungin to Gentamicin and Ciprofloxacin at a subclinical concentration, and to Colistin at a clinical concentration. Moreover, the MBEC value of Gentamicin alone was 4-fold higher than its MBIC value, and 8-fold higher in the case of Colistin and Ciprofloxacin. This suggests that a higher concentration of the antibacterial agents is required for the complete eradication of biofilm cells. As for Tazocin, Ceftazidime, and Cefepime, their MBIC and MBEC values were very high, and no decrease was reported upon combining them with different concentrations of Anidulafungin.

In order to evaluate the effect of combining Anidulafungin with either Colistin, Gentamicin, or Ciprofloxacin on pre-formed *P. aeruginosa* PAN14 biofilms in an *in-vitro* UTI model, biofilms were grown on catheter sections under either static or flow conditions. SEM images revealed that biofilms grown in the flow system had a more defined 3-D

architecture compared to the ones grown under static conditions. Therefore, the flow system was established as a suitable model in this case, and PAN14 biofilms were then grown under variable concentrations of media (LB broth) and artificial urine. Increasing the amount of artificial urine to 80% lead to a decrease in the overall biofilms' population, compared to the ones obtained from the flow system under 50% media and 50% artificial urine. Moreover, catheter sections obtained from the flow system under 100% media were stained with Crystal Violet, where they retained a purple color indicating the presence of biofilms. Hence, the combination therapy was tested on PAN14 biofilms grown on catheter sections in a flow system under equal concentrations of media and artificial urine.

Adding either Colistin, Gentamicin, or Ciprofloxacin alone didn't have any effect on the pre-formed PAN14 biofilms neither after 24 nor after 48 hours. A similar result was obtained upon adding Anidulafungin alone. However, the combination therapy that included Anidulafungin each with Colistin or Gentamicin lead to the complete eradication of the pre-formed biofilms after 24 hours of treatment. This shows that such combinations display synergy, as adding the antifungal to either antibacterial agents produces a combined effect that is potent and greater than the sum of their separate effects. On the other hand, a synergistic effect was not observed upon combining Anidulafungin with Ciprofloxacin, as SEM images revealed catheter sections coated with biofilms following 24 and 48 hours of treatment. Finally, SEM images revealed that PAN14 biofilms were able to grow significantly on ECV304 epithelial cells, and can therefore be used as an *ex-vivo* model to further study the effect of the combination therapy.

In conclusion, results showed that *P. aeruginosa* PAN14 planktonic cells were susceptible to Colistin, Gentamicin, Ciprofloxacin, Tazocin, Ceftazidime, and Cefepime.

PAN14 biofilms, however, displayed resistance to multiple mono-therapeutic agents, which is explained by the significant increase in the MBIC and MBEC values of the antibacterial agents. Furthermore, the combination therapy that involves Anidulafungin with either Colistin or Gentamicin was able to completely eradicate PAN14 pre-formed biofilms, contrary to the effect of either antifungal or antibacterial agents alone, or Anidulafungin combined with Ciprofloxacin. Subsequently, we envision to validate further the action of the combination therapy in the *ex-vivo* model, and study the effect of the combination therapy at the molecular level. We are also aiming to use rat models that would help us test the combinatorial effect in *in-vivo* pneumonia and urinary tract infection models.

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