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CONTROLLING BIOFILM AND PERSISTER CELLS BY TARGETING CELL MEMBRANES

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Abstract

The rapid increase in antibiotic resistant infections and the slowing pace of antibiotic development emphasize the need for alternative therapeutic agents to cure infectious diseases especially those caused by multidrug-resistant (MDR) strains. Bacteria obtain resistance to antibiotics through multiple mechanisms. One of intrinsic mechanisms of drug resistance is persister formation, by which bacterial cells enter a metabolically inactive stage and become highly tolerant to essentially all antibiotics, even at the concentrations that are hundreds of times higher than the lethal dose required to kill normal planktonic cells of the same strain. Persister cells in biofilms are even more difficult to kill due to the presence of an extracellular matrix that can block or retard the penetration of antibiotics. Thus new antimicrobials that are effective against these drug tolerant cells are urgently needed for infection control.

In this study, we characterized the antimicrobial activities of newly designed synthetic peptides on *Escherichia coli* and *Pseudomonas aeruginosa* strains including regular planktonic cells and those in biofilms and at the persister stages. Our results revealed that 2D-24, an RW-rich dendrimeric peptide, can kill planktonic cells of both *P. aeruginosa* PAO1 and PDO300 (a mucoid strain) in a dose-dependent manner. Killing effect on biofilm and persister cells was observed at the concentrations without significant toxicity to IB3-1 cells originated from human lung tissues.

We also demonstrated that TN-5, a 1,3,5-triazine derivative, has antimicrobial effects on *E. coli* RP437, *P. aeruginosa* PAO1 and PDO300 cells, with a minimum inhibitory concentration (MIC) of 12.8 μM, and kills regular planktonic cells of both species dose dependently. TN-5 was also

found effective against persister and biofilm cells of both *E. coli and P. aeruginosa;* and the killing of biofilm cells of the mucoid PDO300 was enhanced by alginate lyase.

To understand the effects of AMP charge on the killing effects, we modified the net charge of calcitermin originated from human airway secretions, and tested the effects on *E. coli* and *P. aeruginosa* planktonic and persister cells at different pH values. The neutral derivative of calcitermin showed better killing effect on persister cells at pH 7.4.

Along with synthetic peptides, we also studied the membrane potential of persister cells with cell sorting and flow cytometry techniques using potentiometric dyes. Persister cells showed lower membrane potential along with lower efflux pump activities compared to normal cells. Based on these findings, we tested the hypothesis that persister cells can be effectively killed by antibiotics that are substrates of efflux pumps. Consistent with this hypothesis, erythromycin was found effective in killing persister cells of *E. coli* while normal cells are resistant to it. This higher killing activity of erythromycin was corroborated with higher erythromycin accumulation in persister cells based on the results of Mass Spectrometry analysis.

Key words: antibiotic tolerance, antibiotic resistance, antimicrobial peptides, membrane potential, persister cells, biofilm, killing, TN-5, 2D-24, *Escherichia coli*, *Pseudomonas aeruginosa*.

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Dedicated to my beloved wife and son,

Fatmagül and Ali Musa Bahar

CHAPTER 1

LITERATURE REVIEW

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1.1 Current situation of antibiotic resistance and associated challenges

Antibiotics have been used to treat infectious diseases for the last 70 years [1] with a great success in saving lives [2]. This achievement is considered as one of the most important breakthroughs in modern medicine [3]. However over-prescription and misuse of these drugs with unrestrained enthusiasm led to an unprecedented challenge to public health with the emergence of bacterial multidrug resistant strains [4]. Acquisition of antibiotic resistance is greatly aided by promiscuous transfer of conjugative plasmids, transposable elements and integron systems, among bacterial cells that are not necessarily related [5,6].

Antibiotic resistance has been recognized as a global threat in 2013 by Centers for Disease Control [7] and several strains are listed as primary targets of new therapeutics including *P. aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA). Infections caused by multidrug resistant bacteria are commonly associated with high mortality and health care costs [8] with 2 million people infected 23,000 and deaths annually in the U.S. alone [9], costing $20 billion to the healthcare system according to Infectious Disease Society of America [10]. Moreover, antibiotic-resistant infections cost $35 billion indirectly due to societal impacts. On average, each hospitalized patient with antibiotic resistant infection costs around $24,000 along with two times higher mortality rate than regular patients [8].

In addition to the increasing rate of the bacterial resistance, the number of bacterial species with antibiotic resistance has also been increasing. For example, the mortal sepsis rate caused by MRSA was 4% in the U.K. 1991; while this ratio increased to 37% in 1999 [11]. In a study by Kelman *et al.* in 2011, 69% of *S. aureus* isolates from meat products showed resistance to tetracycline and only 23% of the total isolates were found to susceptible to tetracycline,

penicillin, ampicillin, methicillin, erythromycin, clindamycin, gentamicin, chloramphenicol, oxacillin, cefoxitin, and quinupristin-dalfopristin antibiotics [12]. Another bacterial species causing serious infections is the opportunistic pathogen *Pseudomonas aeruginosa*. This bacterium can rapidly develop resistance to several classes of antibiotics through mobile genetic elements. In some cases, this bacterium was found to even develop resistance during antibiotic treatment of an infection [13].

Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa and Enterobacter, have been collectively named "the ESKAPE bugs" since they have remarkable capabilities to resist antibiotics and cause lethal infections [14]. Alarmingly, some infections are even resistant to all antibiotics that are currently available [15].

In this research, we focused on two intrinsic mechanisms of antibiotic resistance; biofilm formation and persistence. This rapid and alarming increase in antibiotic resistant infections and the slowing pace of new antibiotic development [16] emphasize the needs for novel alternative therapeutic agents to cure infectious diseases especially those caused by multidrug-resistant (MDR) bacteria.

Biofilms are multicellular structures of bacteria embedded in an extracellular polymeric substance (EPS) matrix [17]; and persister cells are metabolically inactive dormant phenotypic variants of bacterial cells that are highly tolerant to essentially all antibiotics [18]. These intrinsic tolerance mechanisms play major roles in the recalcitrance of chronic infections, such as the lung infections in cystic fibrosis patients [19].

1.2 Mechanisms of antibiotic; acquired vs. intrinsic

1.2.1 Acquired resistance

Acquired resistance is based on genetic elements that allow bacteria to survive the attack by a specific antibiotic and closely related agents [7,20]. Bacteria can acquire specific resistance genes via transformation (uptake of extracellular DNA elements), transduction (through viruses or phages) conjugation (horizontal gene transfer from another bacteria) or genetic mutation during growth [21].

The products of these genetic elements cause antibiotic resistance by degrading antibiotics, modifying the drug targets or quickly removing them from the cytosol. For example β-lactam antibiotics, such as penicillins are cleaved by β-lactamases in *P. aeruginosa* [22]. The widespread use of β-lactam antibiotics has caused the widespread of this gene among other bacteria such as *K. pneumoniae*, *Salmonella spp*. and *P. mirabilis*.

1.2.2 Intrinsic tolerance

Compared to acquired mechanisms that are against specific antibiotics, intrinsic mechanisms allow cells to tolerate a wide spectrum of different antimicrobials. Here we review three major examples; drug efflux, biofilm formation and persistence.

1.2.2.1 Bacterial transport proteins: efflux pumps and porins

Bacterial efflux pumps are substrate-specific protein transporters, which are located in the membrane and responsible for moving certain type of compounds, such as toxic metabolites and antibiotics, out of the cell [23]. These pumps are found in both Gram-negative and Gram-positive

bacteria and they can extrude virtually all clinically relevant antibiotics from cytosol to the external environment [24]. Efflux pumps require energy in the form of either ATP (originated from proton motive force) or ion gradient (Na^+ or H^+) across the cell membrane to extrude antibiotics [25].

There are five known classes of efflux pumps in Gram-negative bacteria [26] (Fig. 1.1): ATP-binding cassette family (ABC), multi antimicrobial extrusion protein family (MATE), small multidrug resistance family (SMR), resistance-nodulation-division (RND) family, and major facilitator superfamily (MFS) [26]. For example, 7 different efflux pumps have been identified in *P. aeruginosa* for transporting tetracycline, β-lactam, fluoroquinolones antibiotics, metal ions [27], small acylated homoserine lactones molecules [28], and quinolone signals [29].

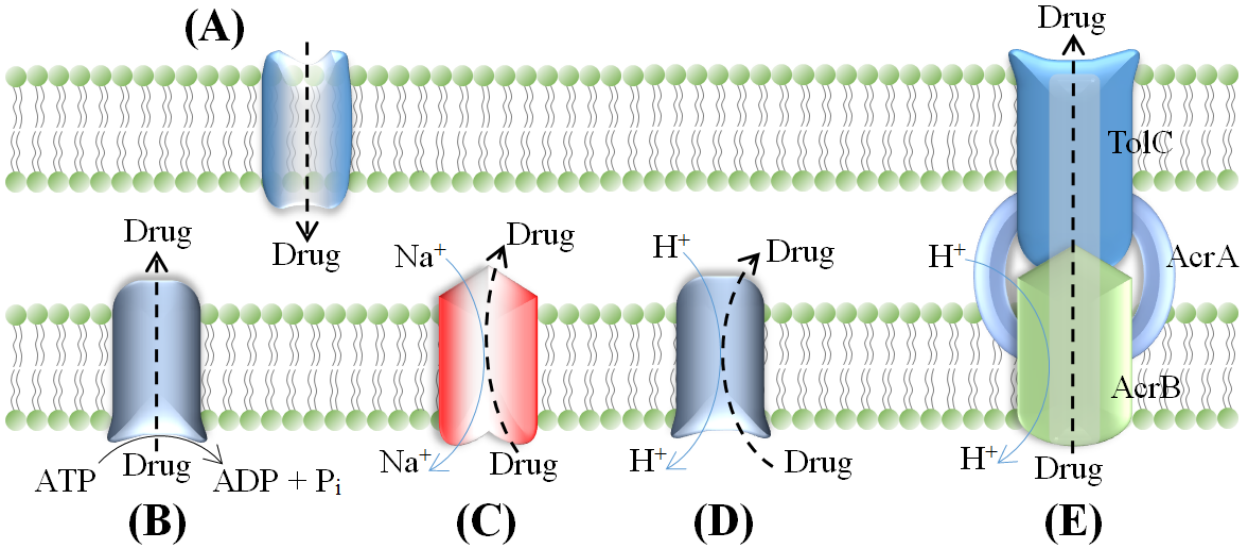

Figure 1-1. Schematic representation of membrane transport proteins causing antibiotic resistance. (A); porins, (B); ATP-binding cassette family and multidrug (ABC) family, (C); multi antimicrobial extrusion protein (MATE) family, (D); small multidrug resistance (SMR) family and major facilitator superfamily (MFS), (E); resistance-nodulation-division (RND) family.

The other type of transport proteins related to antimicrobial resistance is porin proteins mainly present in the outer membrane of Gram-negative bacteria. Porins play an important role in the semi-permeability of the membrane with unique channels for transporting small and charged molecules. These cylindrical shaped tubes are composed of β-strands while polar residues face inward into the aqueous channel and nonpolar residues face outward to interact with nonpolar lipid membrane [30].

1.2.2.2 Biofilm and persister formation

Biofilms are complex multicellular structures of bacteria [31] which protect bacteria against antimicrobial therapies and adverse environmental conditions. Extracellular DNA, polysaccharides, and fatty acids are some of the major components of the biofilm matrix macromolecules, which help to maintain the biofilm structure and facilitate the development of biofilm [32]. During biofilm formation, free swimming bacterial cells attach to a surface reversibly and form small cell clusters first. These small cell clusters then secrete polysaccharides and form the mature biofilm structure with large cell aggregates and water channels between cell clusters.

The multicellular biofilm structure protects bacterial cells from antimicrobials, immune factors and environmental toxins. For example, these biofilm cells exhibit up to 1000 higher tolerance to antibiotics compared to normal planktonic cells [33]; and biofilms are associated with more than 80% of human infections [34] with high mortality [35] such as cystic fibrosis [36].

In addition to the multicellular structure, biofilms also host a large number of persister cells which are phenotypic variants with inactive metabolism and thus high tolerance to antimicrobials

[37]. Persister cells are a small group of cells with no extra genetic modification for drug resistance [19]. Unbalanced toxins/anti-toxins production [38-41] and stress response and translation inhibition are known characteristics of persister cells [19,42]. These small group of cells can survive through the antibiotic course and form the normal population with similar percentage of persister cells again when the conditions turns back to normal for bacterial survival [43]. This repopulation ability provides higher level of antibiotic tolerance and chance to develop acquired resistance for bacterial cells [43]. Therefore, targeting persister cells will help for a better treatment of chronic infections caused by multidrug resistant bacteria [44].

Because of the essential functions of cell membranes, antimicrobial agents targeting cell membranes have good potential for persister controls. One class of such agents is antimicrobial peptides (AMP). AMPs are oligopeptides with a varying number (from five to over a hundred) of amino acids with a broad spectrum of targeted organisms ranging from viruses to parasites. Most of the AMPs are membrane-active agents, which are positively charged, and act on negatively charged phospholipid bilayer of bacterial membrane [45] where main energy synthesis and many other important function occurs. It has been reported that some AMPs can sensitize bacteria by depolarizing the membrane at sub-lethal concentrations [46] and resistance against such antibacterial agents is difficult to acquire [47].

1.3 Antimicrobial peptides

Due to rapid development of bacterial resistance to conventional antibiotics, antimicrobial peptides (AMPs), have received increasing attention as an alternative way of fighting against antibiotic resistance. AMPs are a growing class of natural and synthetic peptides with a wide spectrum of targets including viruses, bacteria, fungi, and parasites. In this section, we

summarize the major types of AMPs, their modes of action, and the common mechanisms of AMP resistance. In addition, we discuss the principles for designing effective AMPs and the potential of using AMPs to control biofilms and persister cells.

1.3.1 Sources and history of antimicrobial peptides

Historically AMPs have also been referred to as cationic host defense peptides [48], anionic antimicrobial peptides/proteins [49], cationic amphipathic peptides [50], cationic AMPs [51], host defense peptides [52], and α-helical antimicrobial peptides [53].

The discovery of AMPs dates back to 1939, when Dubos [54-55] extracted an antimicrobial agent from a soil *Bacillus* strain. This extract was demonstrated to protect mice from pneumococcal infection. In the following year, Hotchkiss and Dubos [56] fractionated this extract and identified an AMP which was named gramicidin. Despite some reported toxicity associated with intraperitoneal application [56], gramicidin was found effective for topical treatment of wounds and ulcers [57]. In 1941, another AMP, tyrocidine, was discovered and found to be effective against both Gram-negative and Gram-positive bacteria [58]. However, tyrocidine exhibited toxicity to human blood cells [59]. In the same year, another AMP was isolated from a plant *Triticumaestivum* [60], which was later named purothionin and found effective against fungi and some pathogenic bacteria [61].

The first reported animal-originated AMP is defensin, which was isolated from rabbit leukocytes in 1956 [62]. In the following years, bombinin from epithelia [63] and lactoferrin from cow milk [64] were both described. During the same time, it was also proven that human leukocytes contain AMPs in their lysosomes [65].

In total, more than 5,000 AMPs have been discovered or synthesized up to date [66]. Natural AMPs can be found in both prokaryotes (e.g., bacteria) and eukaryotes (e.g., protozoan, fungi, plants, insects, and animals) [67, 68-70]. In animals, AMPs are mostly found in the tissues and organs that are exposed to airborne pathogens; and are believed to be the first line of the innate immune defense [71,72] against viruses, bacteria, and fungi [68]. Thus, AMPs play an important role in stopping most infections before they cause any symptoms. For example, frog skin is the source of more than 300 different AMPs [67,73]. Most AMPs are produced by specific cells at all times, while the production of some AMPs is inducible. For example, using silk moth as a model system, Hultmark and colleagues [74] demonstrated that P9A and P9B can be induced in hemolymph by vaccination with *Enterobacter cloacae*. In another study [75], epithelial cells from different tissues of mice showed increased rate of mRNA transcription for defensin production after infection with *Pseudomonas aeruginosa* PAO1.

Several types of eukaryotic cells are involved in AMP production such as lymphs, epithelial cells in gastrointestinal and genitourinary systems [76,77], phagocytes [78], and lymphocytes of the immune system [68,79]. In addition to direct involvement in innate immunity, AMPs have also been found to influence host's inflammatory responses during an infection [80-82]. It is known that lipopolysaccharide (LPS) molecules, released from bacteria as a result of antibiotic treatment or host immunity, can induce AMP production in mammals [78]. For example, HEK293 cells produce defensin in response to LPS stimulation [83]. Some AMPs (e.g., CAP18 [84], CAP35 [85], and a lactoferrin-derivative [86]) can also block LPS-induced cytokine release by macrophages. Thus, these AMPs can reduce inflammatory response. In comparison, antibiotics do not have this type of regulation on inflammatory response of the host immune

system; and LPS secretion following antibiotic treatment might cause over-reaction of the host immune system. In some extreme cases, this can even lead to sepsis [78,87].

1.3.2 Structure and major activities of AMPs

Most AMPs reported to date can be characterized as one of the following four types based on their secondary structures: β-sheet, α-helix, extended, and loop. Among these structural groups, α-helix and β-sheet structures are more common [88]; and α-helical peptides are the most studied AMPs to date. In α-helix structures the distance between two adjacent amino acids is around 0.15 nm and the angle between them with regard to the center is around 100 degree from the top view (Fig. 1-2A). The best known examples of such AMPs are protegrin, magainin, cyclic indolicin, and coiled indolicin [53]. β-sheet peptides are composed of at least two β-strands with disulfide bonds between these strands [89].

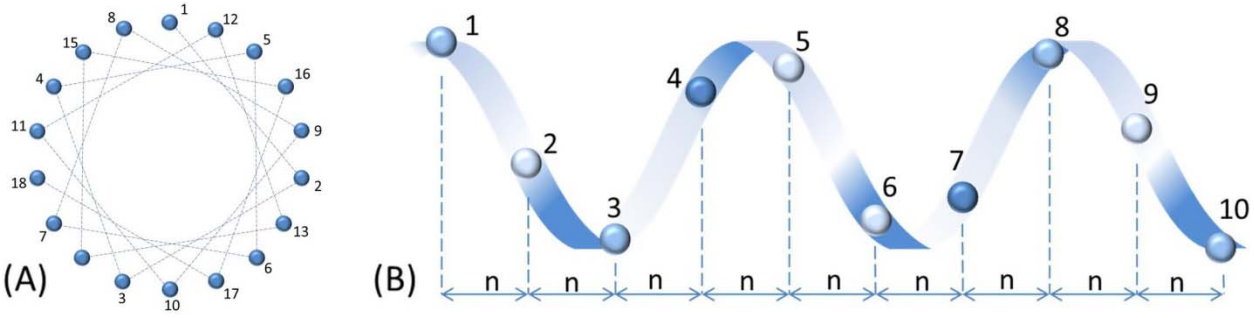

Figure 1-2. Schematic representation of an α-helical AMP. This figure assumes the same α-helix propensity for all amino acids in the peptide structure. (A) Helical wheel projection of the AMP (top view). The angle between two consecutive amino acids in the sequence is 100 degree. Dotted lines show two adjacent amino acids in the primary structure. (B) Side view of the peptide. The distance between two adjacent amino acids, "n", is 0.15 nm.

Some AMPs do not belong to any of these groups [90]. Some AMPs contain two different structural components [91]. Also, many peptides form their active structure only when they interact with the membranes of target cells. For example, indolicin shows globular and amphipathic conformation in aqueous solutions, while it is wedge-shaped in lipid bilayer mimicking environments [92]. This AMP also changes its conformation during interaction with DNA evidenced with decreased fluorescence intensity and a slight shift in the wavelength of maximum emission [93].

Unlikely antibiotics, which target specific cellular activities (e.g., synthesis of DNA, protein, or cell wall), AMPs target the lipopolysaccharide layer of cell membrane, which is ubiquitous in microorganisms. Having a high level of cholesterol and low anionic charge puts eukaryotic cells out of the target range of many AMPs [94].

Another important feature of AMPs is their rapid killing effect. Some AMPs can kill in seconds after the initial contact with cell membrane [95]. AMPs are also known to enhance the activities of antibiotics through synergistic effects. For example, the combination of penicillin with pediocin and ampicillin with nisin Z exhibited killing of *Pseudomonas fluorescens* with 13- and 155-fold lower minimum inhibitory concentration (MIC), respectively, compared to using antibiotics alone [96].

Because AMPs are made with amino acids, it is relatively easy to modify the structure (including library construction and screening) and immobilize AMPs on surfaces [97]. It is possible to make fully synthetic peptides by chemical synthesis [98] or by using recombinant expression systems [99,100]. These artificial sources of AMPs are useful for modification of existing AMPs and for

designing new synthetic AMPs. Such modifications have potential to change the targets of AMPs and improve the stability of AMPs against proteases [101].

Despite these advantageous features of AMPs, there are still some challenges to their applications, such as potential toxicity to humans [59,102,103], sensitivity to harsh environmental conditions (susceptibility to proteases and extreme pH [104,105]), lack of selectivity against specific strains [106], high production costs [107], folding issues of some large AMPs [108], reduced activity when used for surface coating [109], and bacterial resistance to some AMPs [110,111]. In the following section we will discuss the modes of actions of AMPs and the current efforts to address the above challenges.

1.3.3 Major categories of AMPs and mechanisms of action

1.3.3.1 Classification

In general, enzymatic mechanisms are not involved in the antimicrobial activities of AMPs [112]. For example, even though lysozyme is a monomeric peptide, it is not classified as an AMP because it is relatively large (148 aa) and kills bacteria through enzymatic activities by breaking 1,4-β-linkages in peptidoglycan chains [113]. Here, we categorize AMPs based on their target and mode of action. For natural AMPs, we will focus on those from eukaryotes, especially mammals.

1.3.3.1.1 Antiviral peptides

Antiviral AMPs neutralize viruses by integrating in either the viral envelope or the host cell membrane. Previous studies have shown that both enveloped RNA and DNA viruses can be targeted by antiviral AMPs [114,115]. AMPs can integrate into viral envelopes and cause membrane instability, rendering the viruses unable to infect host cells [116,117]. AMPs can also reduce the binding of viruses to host cells [118]. For example, defensins bind to the viral glycoproteins making herpes simplex viruses (HSV) unable to bind to the surface of host cells [119].

Besides disruption of viral envelopes and blocking viral receptors, some antiviral AMPs can prevent viral particles from entering host cells by occupying specific receptors on mammalian cells [120,121]. For example, heparan sulfate is important for the attachment of HSV viral particles to the host cell surface [122]. The heparan sulfate molecules are negatively charged glycosaminoglycan molecules [123]. Thus, some α-helical cationic peptides, e.g., lactoferrin [124], can prevent HSV infections by binding to heparan molecules and blocking virus-receptor interactions [125].

Compared to the above AMPs that target viral receptors on cell surface, some AMPs do not compete with viral glycoproteins for binding to the heparansulphate receptors on cell surface. Instead, these antiviral AMPs can cross the cell membrane and localize in the cytoplasm and organelles, causing changes in the gene expression profile of the host cells, which can help the host defense system fight against viruses or block viral gene expression. For example, NP-1, an AMP from rabbit neutrophils, prevents Vero and CaSki cell lines from infection by herpes simplex viruses type 2 (HSV-2). This AMP stops the viruses by preventing the migration of a

major viral protein, VP16, into the nucleus. This viral protein is required to form complexes with the host transcriptional factors to induce the expression of immediate early viral genes, which are required for the virus to defeat the first stage cellular response [126]. Thus, this AMP does not compete with viral particles to bind to the receptor on cell surface but it prevents cell-to-cell spread of viral particles [127].

1.3.3.1.2 Antibacterial peptides

Antibacterial AMPs are the most studied AMPs to date and most of them are cationic AMPs, which target bacterial cell membranes and cause disintegration of the lipid bilayer structure [128,129]. The majority of these AMPs are also amphipathic with both hydrophilic and hydrophobic domains. Such structures provide AMPs the capability to bind to lipid components (hydrophobic region) and phospholipid groups (hydrophilic region) [94].

Interestingly, researchers have demonstrated that some AMPs at low concentrations can kill bacteria without changing the membrane integrity. Instead of directly interacting with the membrane, these AMPs kill bacteria by inhibiting some important pathways inside the cell such as DNA replication and protein synthesis [130]. For example, buforin II can diffuse into cells and bind to DNA and RNA without damaging the cell membrane [131]. Drosocin, pyrrhocoricin, and apidaecin are other examples of such AMPs. These AMPs have 18–20 amino acid residues with an active site for their intracellular target [132,133].

In some cases, certain AMPs have been shown to kill antibiotic resistant bacteria. For example, both nisin (an AMP) and vancomycin (an antibiotic), can block cell wall synthesis. However, a

methicillin resistant *Staphylococcus aureus* (MRSA) strain was reported to be resistant to vancomycin, while it is still sensitive to nisin [134].

1.3.3.1.3 Antifungal peptides

Like antibacterial AMPs, antifungal peptides can kill fungi by targeting either the cell wall [135,136] or intracellular components [137]. However, bacterial membrane and fungi cell wall have different contents. For example, chitin is one of the major components of fungal cell walls and some of antifungal peptides are capable of binding to chitin [138-140]. Such binding ability helps AMPs to target fungal cells efficiently. Cell wall targeting-antifungal AMPs kill the target cells by disrupting the integrity of fungal membranes [141,142], by increasing permeabilization of the plasma membrane [143], or by forming pores directly [144].

Although the majority of antifungal AMPs have polar and neutral amino acids in their structures, [94] there does not appear to be a clear correlation between the structure of an AMP and the type of cells that it targets. For example, antifungal peptides have members from different structure classes such as α-helical (D-V13K [145] and P18 [146]), extended (indolicin [147]), and β-sheet (defensins [148]).

1.3.3.1.4 Antiparasitic peptides

Antiparasitic peptides are a smaller group compared to other three AMP classes. The first antiparasitic peptide reported is magainin, which is able to kill *Paramecium caudatum* [149]. Later, a synthetic peptide was developed against *Leishmania* parasite [150]. Another example of antiparasitic peptide is cathelicidin, which is able to kill *Caernohabditis elegans* by forming

pores in the cell membrane [151]. Even though some parasitic microorganisms are multicellular, the mode of action of antiparasitic peptides is the same as other AMPs. They kill cells by directly interacting with cell membrane [151].

1.3.3.2 Mechanism of action

As described above, AMPs kill cells by disrupting membrane integrity (via interaction with negatively charged cell membrane), by inhibiting proteins, DNA and RNA synthesis, or by interacting with certain intracellular targets. All AMPs known by the late-90s are cationic. However, the concept that AMPs need to be cationic was changed later with the discovery of negatively charged AMPs in 1997 [152]. For example maximin-H5 [153] from frog skin and dermicidin [154] secreted from sweat gland tissues of human are both anionic peptides.

Generally an AMP is only effective against one class of microorganisms (e.g., bacteria or fungi) [78]. However, there are exceptions and some AMPs are known to have different modes of action against different types of microorganisms. For example, indolicidin can kill bacteria, fungi, and HIV [116,155]. It exhibits antifungal activities by causing damages to cell membrane [147]. However, it kills *E. coli* by penetrating into the cells and inhibiting DNA synthesis [156]; and it shows anti-HIV activities by inhibiting HIV-integrase [157]. In comparison; some AMPs have the same mode of killing of different cell types. For example, PMAP-23 can kill both fungi and parasites by forming pores in their cell membranes [151,158].

One third of the total proteins of a bacterial cell are associated with the membrane and these proteins have many functions that are critical to the cell including active transport of nutrients, respiration, proton motive force, ATP generation, and intercellular communication [159]. The

function of these proteins can be altered with AMP treatment even if complete cell lysis does not occur. Therefore, AMPs' rapid killing effect does not only come from membrane disruption but can also come from inhibition of these functional proteins.

1.3.3.2.1 Membrane-active AMPs

Even if intracellular targets are involved, an initial cell membrane interaction with peptides is required for the antimicrobial activities of AMPs [160]; and this interaction determines the spectrum of target cells. Most membrane-active AMPs are amphipathic, which means that they have both cationic and hydrophobic faces. This feature ensures the initial electrostatic interaction with the negatively charged cell membrane and the insertion into membrane interior. The actions of AMPs do not stop after this initial interaction. The hydrophobic part of an AMP helps insert the AMP molecule into the cell membrane [161]. So the interaction mainly includes ionic and hydrophobic interactions. These interactions mostly depend on two properties, e.g., cationic state and hydrophobicity of the peptide. The major types of membrane-active AMPs and the mechanisms of their actions are summarized in Table 1-1 and Figure 1-3.

Table 1-1. The action mechanisms of membrane-active AMPs.

Interaction model	Mechanism	References
Carpet like (Detergent-like)	The peptide micelle touches the membrane first and coats a small area of the membrane. Then AMP molecules penetrate the lipid bilayer to let pore formation leaving holes behind.	[162-164]
Membrane thinning	AMPs insert themselves into only one side of the lipid bilayer. It can form a gap between lipid molecules at the chain region. This gap creates a force and pulls the neighboring lipid molecules to fill it.	[165-167]
Aggregate	AMPs stick to the membrane parallel to the surface. Then reorientation of AMPs occurs and they insert themselves into the membrane vertically to form sphere-like structures.	[162,168-170]
Toroidal pore	AMPs align perpendicularly into the bilayer structure with their hydrophobic regions associated with the center part of the lipid bilayer and their hydrophilic regions facing the pore.	[130,170]
Barrel-stave	Staves are formed first parallel to the cell membrane. Then barrels are formed and AMPs are inserted perpendicularly to the plane of the membrane bilayer.	[129,171,172]

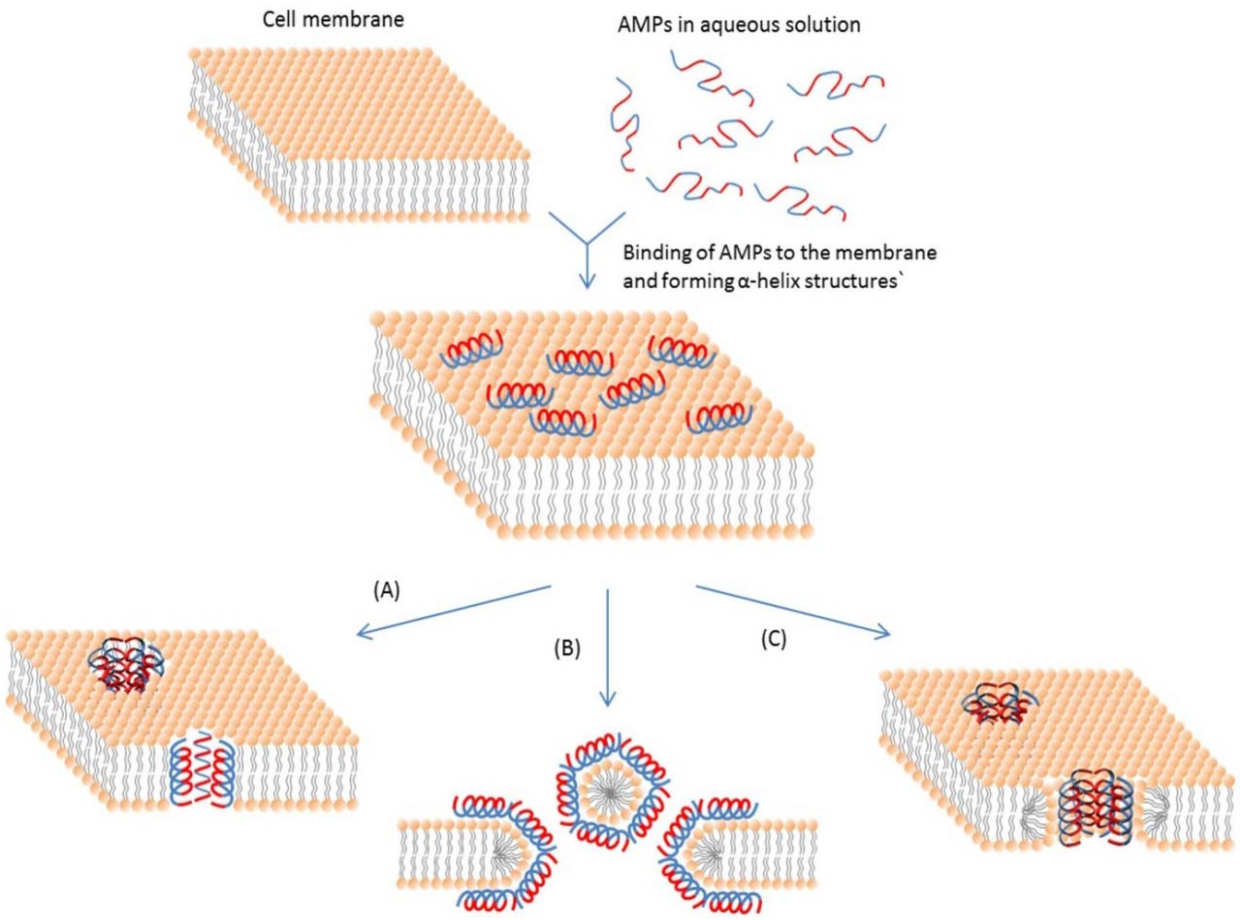

Figure 1-3. Schematic representation of some action mechanisms of membrane-active AMPs. (**A**) Barrel-Stave model. AMP molecules insert themselves into the membrane perpendicularly. (**B**) Carpet model. Small areas of the membrane are coated with AMP molecules with hydrophobic sides facing inward leaving pores behind in the membrane. (**C**) Toroidal pore model. This model resembles the Barrel-stave model, but AMPs are always in contact with phospholipid head groups of the membrane. The blue color represents the hydrophobic portions of AMPs, while the red color represents the hydrophilic parts of the AMPs.

1.3.3.2.2 Intracellularly active AMPs

In early AMP studies, permeabilization of bacterial cell membrane by AMP was thought as the primary mechanism of killing. It was suggested that AMPs should be used at concentrations high enough so that they can kill microorganisms by disrupting the membrane with sufficient channels and pores [173]. However, some AMPs were found to start membrane permeabilization at concentrations lower than their MICs, while others could only do so at concentrations higher than their MICs. The finding that some AMPs can kill their target cells without causing membrane permeabilization suggests that there may be other mechanisms of killing. Recently, intracellularly active AMPs have been shown to interact with targets inside the cells [174-176]. For example indolicin was shown to bind to DNA with a preferred sequence [93,177].

Some AMPs can inhibit DNA and protein synthesis [178,179]. One example of this is PR-39, an AMP from pig intestines, which kills bacteria in a non-lytic process by acting like a proteolytic agent and stopping protein and DNA synthesis [180]. Similar to PR-39, indolicin does not lyse cells directly. It enters the cytoplasm and kills bacterial cells by targeting DNA synthesis [156] [178]. Also, some human immune system derived AMPs such as tPMP-1 and aHNP-1 inhibit DNA and protein synthesis within an hour after they enter the cells [181]. Apidaecin is another protein synthesis blocking AMP which lacks pore forming ability. This AMP is only effective against Gram-negative bacteria. It is suggested that this AMP is actively transported with a transporter protein and then it blocks protein synthesis with a series of molecular interactions with different targets [182].

Some AMPs can also inhibit proteases of microbes. For example, histatin 5 stops the periodontal tissue destruction by inhibiting a protease from *Bacteriocides gingivalis* [183]; and eNAP-2 has

anti-protease activities against microbial serin proteases [184]. Interestingly, there are some intracellular AMPs which can only kill cells at certain growth stages. For example, diptericin is only effective against actively growing bacterial cells, suggesting it may interact with certain specific metabolic pathways during bacterial growth [185,186].

Among these intracellularly active AMPs, some of them have multiple targets. For example seminalplasmin inhibits RNA polymerase and can stop RNA synthesis completely at concentrations lower than many other antibacterial agents [187]. On the other hand, the same AMP can activate an autolysin protein inside the target cells leading to autolysis [188,189].

Inhibition of intracellular pathways by AMPs [173,178] suggests that there might be mechanisms of cellular uptake of AMPs. Two such mechanisms have been reported: direct penetration and endocystosis [161]. According to Jones [190], cellular uptake of AMPs can take place through endocytosis, which includes macropinocytosis and receptor mediated endocytosis. In macropinocytosis, the cell membrane folds inward and forms vesicles with the help of dynamin proteins. These vesicles are called macropinosomes and they are like small cells with only a membrane around them [161]. In receptor mediated endocytosis, a part of the membrane is coated with clathrin or caveolin proteins followed by pit formation. Later, these pits bud from the membrane to inner side of the cell and form vesicles [190,191].

1.3.4 Designing new synthetic AMPs: major factors to consider

To date, no data have been reported to demonstrate a clear relationship between the structural groups of an AMP and its mode of action, the degree of activity, or the host range. Even the AMPs with very similar structures can have drastically different mechanisms of action and the

range of targeted cells [94]. For example, buforin targets DNA and RNA; while magainin 2, an AMP with similar structure, targets the cell membrane causing cell lysis [192,193]. Although a structure-based precise prediction of activity, mode of action, and host range may not be possible, certain general design principles have been proposed by previous studies. The AMP structure is certainly important, while the size, charge, hydrophobicity, amphipathicity, and solubility are all crucial physiochemical properties for their antimicrobial activities and target specificity of AMPs [194]. Changing these features will help to modify the activity and target spectrum of AMPs.

1.3.4.1 Important physiochemical properties of AMPs

1.3.4.1.1 Length

The length of an AMP is important to its activity because at least 7–8 amino acids are needed to form amphipathic structures with hydrophobic and hydrophilic faces on opposite sides of a peptide molecule. The size for an AMP to transverse the lipid bilayer of bacteria in the barrel-stave model should be at least 22 amino acids for α-helical AMPs, while eight amino acids are needed for β-sheet AMPs [195]. Besides the effects of length on its 3D structure and mode of action, the length of an AMP may also affect its cytotoxicity. For example, a shortened melittin with 15 residues at its C-terminal [196] and a shorter derivative of HP(2-20) [197] exhibited at least 300 times less toxicity to rat erythrocytes and human erythrocytes, respectively, compared to their original forms. Therefore, the length of AMP should be taken into consideration when designing new synthetic peptides with low toxicity.

1.3.4.1.2 Net charge

The net charge of known AMPs, which is the sum of all charges of ionizable groups of the peptide, varies from negative to positive and it is the main factor for the initial interaction with negatively charged cell membranes. By changing the net charge of an AMP, its antimicrobial and hemolytic activities can be altered to achieve selective killing of microbes with no or minimized effects on host cells. For example, increasing positive net charge of V13K from +8 to +9 resulted in higher hemolytic activity, while decreasing the net charge to lower than +4 abolished its activity against *P. aeruginosa* [145].

1.3.4.1.3 Helicity

Helicity represents the ability of an AMP to form spin structure. It is less important for the activity of an AMP compared to other factors discussed above. However, it is important for determining the toxicity on eukaryotic cells [53]. Reducing helicity by incorporating D-amino acids into the primary sequence has been shown to lower the hemolytic effect, while the antimicrobial effect was retained [198]. For example, Papo *et al*. [101] modified some α-helical peptides by replacing 35% of the L-amino acids with D-amino acids and found that this modification eliminated the hemolytic activity. Besides, these new synthetic AMPs are not sensitive to proteases. Therefore, incorporating D-amino acids to change helicity is a useful strategy for designing new synthetic peptides with less hemolytic activity and enhanced stability against proteolytic cleavage. Another important factor associated with the helicity of AMP is the helix propensity of each amino acid in the primary sequence. For example, proline and glycine have lower helix-forming propensities compared to other amino acids [199]. Thus, these residues

are not preferred when designing α-helical AMPs. In addition, peptides should be flexible enough to change their conformation during the membrane insertion process [94].

1.3.4.1.4 Hydrophobicity

Hydrophobicity has also been shown to influence the activity and selectivity of AMP molecules. Almost 50% of amino acids in the primary sequence of natural AMPs are hydrophobic residues [194]. In most cases, increase in hydrophobicity on the positively charged side of an AMP below a threshold can increase its antimicrobial activity [53], while decreasing hydrophobicity can reduce antimicrobial activity [200]. There appears to be an optimal hydrophobicity for each AMP, beyond which its activity decreases rapidly [176]. Therefore, when designing new synthetic peptides, the hydrophobicity should be selected within an optimal window. Some previous studies have shown that hydrophobicity is also critical for determining the range of target cells of an AMP. Increasing the hydrophobicity of an AMP can change the range of targets [201,202]. For example, magainin is an AMP that is only effective against Gram-negative bacteria. However, some synthetic analogs with higher hydrophobicity can also kill some Gram-positive bacteria and eukaryotic cells [203].

1.3.4.1.5 Amphipathicity

Amphipathicity is another important property of AMPs to ensure their activity and interaction with microbial membranes. Fernandes-Vidal *et al*. [204] showed that amphipathicity is more important than hydrophobicity for binding to microbial membranes. Because amphipathicity of AMPs is required for a strong partition into the membrane interface, priority should be given to the amphipathic structure when designing synthetic AMPs for specific target cells.

1.3.4.1.6 Solubility

Since AMPs should act on or enter through lipid membranes, they need to be soluble in aqueous environments. If AMP molecules aggregate, it will lose its ability to interact with the cell membrane. For example, a hybrid synthetic AMP composed of cecropin and melittin has a tendency to form dimers. Substituting a Lys (L) residue on the non-polar face of this hybrid AMP prevents dimerization and leads to reduced hemolytic activity. Losing dimerization ability makes this AMP more effective for its incorporation into microbial membranes [205]. This example demonstrates the importance of solubility and the value of structural optimization.

1.3.4.2 The relationship between physiochemical properties of AMPs

As discussed above, many factors affect the activities of AMPs and some interactions exist between these factors. In AMP design, these properties need to be considered together since changing one of these parameters to get a desired modification of an AMP may alter other parameters. Even a simple change in primary sequence can affect many other physicochemical parameters which are often vital for the activity of an AMP and the range of target cells [206]. Predicting the results of an AMP modification or the function of a synthetic peptide beforehand is still an unmet challenge. Application of molecular simulation to analyze the details of the folding of AMP molecules and interaction with target cells [207,208] may be a promising approach to improve current trial and error methods.

1.3.4.3 AMP modifications

While most of AMPs are directly synthesized in their active forms, post-translational modification of certain AMPs is necessary for their functions. Naturally forming AMPs are processed with different post-translational modifications such as phosphorylation [209], addition of D-amino acids [210,211], methylation [212], amidation [213], glycosylation [214], formation of disulphide linkage [215], and proteolytic cleavage [71,216]. In some cases, these posttranslational modifications might be important for designing new synthetic AMPs. Even though recombinant cell systems can be used to produce these synthetic peptides with post-translational modifications, incorporation of unnatural amino acids may require chemical synthesis [107].

1.3.4.3.1 Modification of AMPs with covalent bonds

Covalent modification can have profound effects on the structure and function of an AMP. Even a single disulfide bond can change the antimicrobial effect of an AMP. For example, protegrin missing a disulphide bond becomes inactive against HSV [217]; while adding disulphide bond in sakacin P resulted in higher antimicrobial activities [91]. In another study, a disulfide bond was added in CP-11, a derivative of indolicidin [218], and a Trp-Trp cross-link was added in indolicin [219]. These modified structures of indolicidins showed higher protease stability with no change in antimicrobial activity. However increase in stability does not always lead to better AMPs. For example, Houston *et al*. [220] introduced a covalent bond to form a lactam bridge between Gln (G) and Lys (L) residues in two α-helical AMPs, e.g., cecropin and mellitin. This modification helped AMPs to form more stable α-helix structures but decreased the antimicrobial activity of both.

1.3.4.3.2 Modification of AMPs by changing amino acid content

Alteration of amino acid content is one of the most studied strategies of AMP modification. Most of these studies focus on certain amino acids since their physiological characteristics play important roles in the activity and target spectrum of AMPs. For example proline content in the primary sequence of an AMP has been found to affect its ability to penetrate cell membranes. Higher proline content reduces the capability of CP26 to permeabilize *E. coli* cell membrane [221]. This effect might be because of proline's low propensity to form α-helical structures. Thus changes in the proline content may lead to alterations of α-helical posture of an AMP.

Changing amino acid content can also affect cytotoxicity. In a study by Nell *et al*. [222], LL37, a human AMP, was modified by removing neutral amino acids Asn (A) and Gln (E), and adding more positively charged residues (two Arg (R) units) into the primary sequence. The new synthetic peptide showed less cytotoxic effects on eukaryotic cells. This peptide was named P60.4 and has been successfully used in nasal applications against MRSA [223]. Another strategy to improve AMP stability is to include D-amino acids in the sequence because they are more tolerant to proteases [224,225].

1.3.4.3.3 Modification of AMPs by amidation

With new developments in peptide synthesis, it is possible to incorporate special chemical groups or unnatural molecules into AMPs. One of these modifications is the addition of amide groups at the end of the peptides. In 2011, Kim *et al.* [226] modified PMAP-23 with amidation at the carboxyl end and found that this derivative of PMAP-23 orients perpendicularly inside the bacterial membrane while original PMAP-23 orients parallel to the membrane. This modification

resulted in almost 10 fold higher cellular uptake, faster interaction with Gram-negative bacteria cell membrane, and deeper insertion into the inner membrane than the original PMAP-23. This carboxyl-end amidated synthetic peptide also showed better membrane-permeabilization in liposome release tests [226]. Therefore amidation of carboxyl end has good potential to improve the function of synthetic AMPs.

C-terminal modifications can also affect the stability of AMPs. In a previous study by Berthold *et al.* [227], the C-terminal amide group of Api88 was replaced by a free acid. This modification did not change its antimicrobial activity, but resulted in a 15 times more stable Api88 derivative against proteases in blood serum. Replacing Arg-17 of this AMP with L-ornithine or L-homoarginine gave 35 times higher proteolytic stability than the original Api88. However, the latter modification decreased the antimicrobial activity by eight fold [227].

1.3.4.3.4 Modification of AMPs with unnatural amino acids

A number of studies on synthetic peptides have attempted to incorporate unnatural amino acids into the primary sequence [146,228,229]. β-didehydrophenylalanine is an unnatural amino acids and is used to provide better folding properties for AMPs [228]. It is widely used in medicinal chemistry to alter the native bioactive AMPs [230]. Incorporation of β-didehydrophenylalanine in the primary sequence of VS1 resulted in higher stability against proteases. Researchers have also been able to introduce antifungal activities to some AMPs by incorporating undecanoic acid and palmitic acid into their primary sequence [146,231].

1.3.4.3.5 Modification of AMPs with computer-assisted methods

The use of computer-assisted methods in AMP research has been increasing significantly [232-237]. Estimating the structure of an AMP based on its primary sequence [238], then predicting potential mechanism of action and activity is becoming easier with the help of computational approaches [239]. These types of artificial AMP design strategies hold potential for developing new synthetic peptides against antibiotic-resistant superbugs [237]. Several databases about AMPs have been created and can be accessed to compare currently available AMPs. One of the latest AMP databases, LAMP (linking antimicrobial peptides), currently has 3904 natural and 1643 synthetic peptides [66].

1.3.4.4 New AMP design by homology modeling

Most studies about AMPs to date are inspired by natural AMPs. For example, Tossi *et al.* [240] designed some synthetic peptides by identifying the common amphipathic structure of 87 different natural α-helical AMPs. These natural AMPs are composed mainly of cecropins, magainins, brevinins, and cathelicidin peptides sourced from insects, amphibian, and mammals. This synthetic peptide study focused on the first 20 amino acids in each sequence because the N-terminal region was shown to be necessary for antimicrobial activities [241,242]. The synthetic peptides designed based on this strategy are able to transform into α-helical structures from random structures with the addition of trifluoroethanol in aqueous environments. These synthetic AMPs exhibited antimicrobial activities against Gram-positive and Gram-negative bacteria, including some drug resistant strains. In addition, these synthetic AMPs showed low toxicity to some eukaryotic cell lines [240].

Designing synthetic AMPs by homology modeling within the same class might also provide a better understanding of activity-structure relationship. Important elements from the same AMP class may be identified using this approach to help design better molecules. Storici *et al.* [242] showed that 20 residues (named PMAP-36) of an antibacterial peptide from pig bone marrow cells are sufficient for related antibacterial activity. The AMP with these 20 residues was chemically synthesized and showed the capability to form α-helix in the presence of trifluoroethanol. This short synthetic peptide was found to induce permeabilization of the inner membrane of *E. coli* ML35 at concentrations lower than 50 μM; while even at 100 μM it did not cause any permeabilization to human erythrocytes [242]. In another study of homology modeling, arenicin, protegrin, and thanatin were used as templates to generate three synthetic peptides: AMP72, AMP126 and AMP2041. These new synthetic AMPs showed lower cytotoxicity compared to the original AMPs and exhibited dose dependent antimicrobial activities (0.17 to 10.12 μM) against Gram-negative bacteria [243].

It is also possible to broaden the target spectrum of an AMP by homology modeling. For example, normally lactoferrampins are not effective against *E. coli* O157. A conserved sequence, which corresponds to an α-helical region, among these AMPs was found, by aligning multiple sequences with ClustalW analysis. This common region was modified by inserting GKLI sequence into its primary sequence, and the new synthetic peptide showed activities against *E. coli* O157 with a more stable structure compared to other lactoferrampins [244].

1.3.5 New targets of AMPs: biofilms, persister cells, and drug resistance bacteria

Because AMPs can directly target bacterial cells, they have potential to control antibiotic tolerant cells. Here we review some recent work on biofilms and persister cells. Biofilms are immobile bacterial populations attached to surfaces such as human tissues and medical implants. Biofilm formation on implant surfaces is a serious problem since every year more than $3 billion is spent to treat implant-associated biofilm infections in the U.S. alone [245]. With cells protected by an extracellular matrix, biofilms are highly tolerant to antimicrobials [246] and are a major cause of chronic infections; e.g., approximately 80% of human bacterial infections are associated with biofilms [247]. In addition to the protection by the extracellular matrix [248], biofilm associated antibiotic resistance is also attributed to the slow growth of biofilm cells [249]. Even though some antibiotics have been shown to effectively penetrate biofilm matrix [250], they are not effective against these slowly growing cells, especially the dormant subpopulation known as persister cells [251-253]. Since most AMPs target cell membrane, they may be more effective against these dormant cells compared to antibiotics.

1.3.5.1 Biofilm control

The first obstacle of using AMPs against biofilms is the possible electrostatic interaction between cationic peptides and negatively charged biofilm matrix [254]. Such interactions may retard or prevent AMPs from reaching biofilm cells. Previous studies have investigated the effects of some AMPs on biofilm inhibition and killing of bacterial cells in established biofilms. The second type of study is especially important since treatment of mature biofilms is highly challenging [252]. In a study by Singh *et al.* [255], lactoferrin was found to block biofilm

formation of *P. aeruginosa* at concentrations lower than those required to kill the planktonic cells. Also, LL-37, a human cathelicidin AMP, was shown to prevent *P. aeruginosa* biofilm formation at the concentration of 0.5 μg/mL, which is below its MIC (64 μg/mL). This AMP also showed activity against preformed (2-days old) *P. aeruginosa* biofilms; e.g., it reduced the biofilm thickness by 60% and destroyed microcolony structures of the treated biofilms [256]. In another study, a derivative of LL-37 was found effective against both Gram-positive and Gram-negative bacteria. Despite its weak antimicrobial activity against planktonic cells, this AMP inhibited biofilm formation of *P. aeruginosa*, *Burkholderia cenocepacia*, and *Listeria monocytogenes* with more than 50% reduction in biofilm mass compared to untreated controls [257]. The same study showed that this inhibition is due to decrease in swarming and swimming motilities, increase in twitching motility, and repression of some biofilm genes.

In addition to free AMPs, surface coating with AMPs has also been pursued since surface modifications with AMPs might help reduce device associated infections [258-261]. Many AMPs have been tested for their inhibitory effects on biofilm formation on implant surfaces. For example, Tet-20, a synthetic peptide (KRWRIRVRVIRKC), tethered on an implant surface exhibited broad antimicrobial activities both *in vivo* (rats) and *in vitro*. It is able to stop biofilm formation and appears to be non-toxic to eukaryotic cells [258]. In another study, histatin 5 and lactoferrin were used to coat Ti surfaces covered with an anchor peptide minTBP (RKLPDAP), which helps binding of AMP to Ti surfaces. The conjugates of both AMPs resulted in higher binding efficiency to Ti surfaces than AMPs alone and *Porphyromonas gingivalis* showed less ATP activity and reduced biofilm formation on coated surfaces [262].

In addition to naturally existing AMPs, some synthetic AMPs were also used to treat biofilms. A synthetic histatin analogue dhvar4 was tested against oral flora on hydroxyapatite disks and this

AMP reduced the number of viable biofilm cells by 1.5 log compared to the control [263]. MUC7, a native saliva AMP from humans, and its modified forms, MUC17 12-mer-L and 20-mer, showed inhibitory effects on *S. mutans* biofilm formation [264]. In another study, a derivative of LL-37 which is an AMP from human innate immune system, cleared *P. sinusitis* biofilms *in vivo* (New Zealand rabbits). However, it also led to some toxicity and proinflammation in the sinuses [265].

As discussed above, the extracellular matrix of a biofilm is thought to form a diffusion barrier against certain AMPs [246]. It is known that this negatively charged barrier protects the cells inside from positively charged antimicrobial agents and the alginate in biofilm matrix can reduce the diffusion of antimicrobial agents [266]. Thus, it is important to obtain AMPs that can diffuse into biofilms and kill biofilm cells. Recently a synthetic peptide, $(RW)_{4D}$ dendrimer [267] was demonstrated to inhibit planktonic growth and biofilm formation of *E. coli* dose dependently. This AMP inhibited biofilm formation by 93.5% at 40 μM. This dendrimer did not detach preformed biofilms, but was able to kill most of the cells residing in mature biofilms dose dependently [268]. Later, $(RW)_n$-NH_2 based AMPs with different chain length (where n = 2, 3, and 4) were compared for their effects on *E. coli* RP437 biofilms. The chain length was found to be important to the activity of these peptides. Longer peptides, $(RW)_3$-NH_2 and $(RW)_4$-NH_2, showed significant inhibition of planktonic growth (36% reduction in growth rate) while a shorter peptides $(RW)_2$-NH_2 did not cause a clear inhibition at concentrations up to 200 μM. This length-activity relation was also found for biofilm inhibition. *E. coli* biofilm surface coverage and the viability of biofilm cells were reduced significantly by the longer peptides (95% inhibition of biofilm growth by 200 μM $(RW)_3$-NH_2 and 84.4% inhibition of biofilm growth by 200 μM $(RW)_4$-NH_2). Preformed biofilms were also tested with these peptides. However, the

treatment of preformed biofilms with these peptides did not show the same length-activity relationship. Interestingly, 200 μM $(RW)_3$-NH_2 showed significant killing of biofilm cells while 200 μM $(RW)_4$-NH_2 showed strong biofilm dispersion (91.5% reduction in biofilm surface coverage at 200 μM) with no apparent killing effect on biofilm cells. Although 200 μM $(RW)_4$-NH_2 did not kill biofilm cells directly, the detached biofilm cells were killed by this peptide effectively [269].

AMPs have also been tested against the biofilms of drug resistant bacteria. In a study by Okuda *et al.* [270], nisin A and lacticin Q were tested against mature biofilms of a MRSA strain, *S. aureus* MR23. Nisin A at 40 μM was found to kill more than 95% biofilm cells while lacticin Q at 80 μM killed around 90% of the biofilm cells. In another study, GL13K derived from human parotid secretory protein (PSP) killed 99.9% of 24 hour biofilm cells of *P. aeruginosa* when it was added at 100 μg/mL for a two hour treatment [271].

NRC-16, a synthetic peptide, was tested against biofilm formation of three *P. aeruginosa* strains and compared with antibiotics such as ampicillin, chloramphenicol, and ciprofloxacin. NRC-16 showed biofilm inhibition at 8 μg/mL, which is 64 fold less than the antibiotic concentrations required to kill these *P. aeruginosa* strains [272].

There are also some AMPs that can sensitize biofilm cells to other antimicrobial agents. For example, lactoferrin does not kill *S. epidermidis* or affect its growth. However treatment of *S. epidermidis* biofilms on contact lenses with lactoferrin and vancomycin together showed a 2 fold decrease in both MBC (minimal bactericidal concentration) and MIC of biofilm cells compared to the treatment with vancomycin alone [273].

1.3.5.2 Persister control

Persister cells can be found in almost any microbial populations [19]. However, membrane integrity is essential for the survival of bacteria irrespective of the metabolic stage of the cell and cell membrane is a major target of AMPs. Thus, AMPs may have good potential to kill persister cells. In a recent study, a synthetic cationic peptide, $(RW)_4$-NH_2, was found to kill more than 99% of *E. coli* HM22 persister cells in planktonic culture. Besides, this synthetic peptide reduced the number of persister cells in mature biofilms by up to 98% at 40 μM. More interestingly, the combination of this peptide with oflaxacin (5 μg/mL) resulted in complete eradication of viable cells in *E. coli* HM22 biofilms including persister cells [274]. Thus, the combination of conventional antibiotics with AMPs may offer a synergy to control drug tolerant infections.

1.3.6 Resistance to antimicrobial peptides

There are mainly two different types of resistance mechanisms against AMPs: constitutive resistance and inducible resistance [275]. The inducible resistance mechanisms include substitution [276], modification [277], and acylation [278] of the membrane molecules, activation of some proteolytic enzymes [279] and efflux pumps [280], and modifications of intracellular targets [281]. The constitutive resistance mechanisms include electrostatic shielding [282], changes in membrane potential during different stages of cell growth [283], and biofilm formation [275]. These resistance mechanisms are illustrated in Figure 1-4.

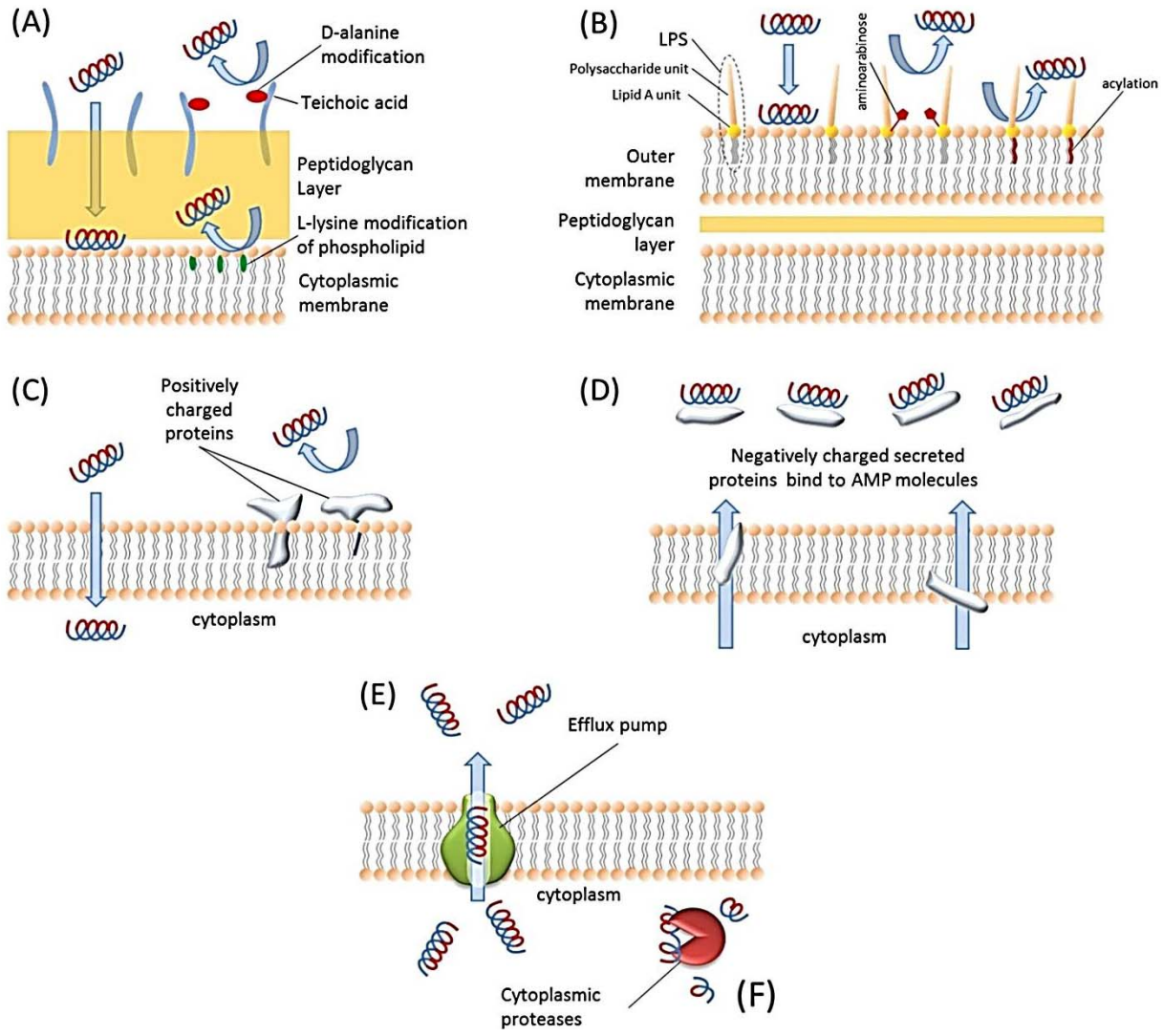

Figure 1-4. Schematic representation of AMP resistance mechanisms. (**A**) Gram-positive bacteria resist AMPs via teichoic acid modification of LPS molecules and L-lysine modification of phospholipids. (**B**) Gram-negative bacteria resist AMPs by modifying LPS molecules with aminoarabinose or acylation of Lipid A unit of LPS molecules. (**C**) Bacteria express some positively charged proteins and integrate them in the membrane so positive charges repulse each other and bacteria can resist such AMPs. (**D**) Bacteria produce negatively charged proteins and secrete them into extracellular environment to bind and block AMPs. (**E**) The intracellular AMPs are extruded by efflux pumps. (**F**) The AMPs inside the cell are degraded by proteases.

For example the activity of some AMPs against *S. aureus* can be inhibited by adhesin molecules on the cell surface of this bacterium. These adhesin molecules are polymeric substances and stay on the cell surface after secretion [284]. Since adhesin is a positively charged polymer, it can form a repulsive barrier against positively charged AMPs. *Salmonella typhimurium* also has a membrane bound lipid A modification system, which defends themselves against AMPs from the host [285]. In this system, PhoQ is a membrane bound sensor kinase and PhoP is intracellular response regulator. PhoQ is activated in the presence of high level positive charges outside the cells. It then phosphorylates the PhoP causing up-regulation of some genes including those related to AMP resistance. This system is not active when the extracellular level of Ca^{2+}, Mg^{2+}, or Mn^{2+} ions is low since divalent cations interact with PhoQ and change its conformation [110]. Although bacteria have diverse mechanisms for resistance to AMPs, it is encouraging to notice that the general lipid bilayer structure of bacterial membranes makes it hard to develop a complete resistance against AMPs. Also, the resistance against AMPs reported to date is not as strong as those against antibiotics and it only covers a limited number of AMPs.

1.4 Outlook

The urgent need to obtain new antimicrobials has been driving AMP research. With rapid growth in related knowledge and lead compounds, more AMPs may enter clinical tests and treatment in the near feature. However, infection control by AMP is still hindered by several challenges including low specificity, high manufacturer cost, potential toxicity to animal cells, and lack of a robust guideline for rational design.

As we have seen from synthetic and modified AMP studies, it is easy to change characteristics of an AMP with even small modifications. However, predicting the results of these changes is still

challenging. Thus, there is a need to understand the effects of structural modifications on the physiochemical characteristics of AMPs as well as their target spectrum and activity. Recently, these types of studies have been increasing and computational approaches have been involved in AMP research. These efforts will help to better understand the mode of action of AMPs and predict their activities.

Another understudied area is using AMPs to control antibiotic resistant bacteria, biofilms, and persister cells. These targets are highly resistant to traditional antibiotics and play important roles in infections. Since AMPs target cell membrane, they have good potential in such applications. On the other hand, because AMPs have not been well studied for biofilm and persister control, there might be some existing natural AMPs that are effective against these targets with potential synergy with antibiotics. Applying AMPs with biofilm matrix degrading enzymes might also be a good strategy to eliminate biofilms. Further development in this area and AMP research in general will benefit from close collaboration of different disciplines and new tools that can decipher the structure-function relationship and more efficiently synthesize and modify AMP molecules.

To gain better fundamental understanding of the characteristics and antimicrobial activities of AMPs we have tailored different design approaches to develop better AMPs. Among these approaches, a dendrimeric peptide with repetitive functional groups on a branched core (2D-24, Chapter 2), a triazine derivative (TN-5, Chapter 3), which is a relatively new source for antimicrobial agent design, and a native human originated AMP with its neutral charged derivative (calcitermin, Chapter 5) were studied. The majority of AMPs targets cell membrane, disrupting its integrity and causing membrane depolarization. Having antimicrobial agents active on cell membrane holds a great potential with low risk for bacterial resistance since membrane

structure is present in all microorganisms and changing the content or structure of this lipid bilayer can be lethal to bacteria. To better understand the membrane function and antimicrobial susceptibility, a new strategy to target cell membrane of persister cells was also evaluated with the antibiotic erythromycin (Chapter 4), which is the substrate of efflux pump AcrAB.

1.5 References

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CHAPTER 2

SYNTHETIC DENDRIMERIC PEPTIDE ACTIVE AGAINST BIOFILM AND PERSISTER CELLS OF *PSEUDOMONAS AERUGINOSA*

This chapter has been published as below with minor modifications. Ali Adem Bahar, Zhigang Liu, Filbert Totsingan, Carlos Buitrago, Neville Kallenbach, Dacheng Ren. Synthetic dendrimeric peptide active against biofilm and persister cells of *Pseudomonas aeruginosa*. Applied Microbiology and Biotechnology 2015, 99, 8125-8135.

2.1 Abstract

Antimicrobial dendrimeric peptides (AMDP) are a relatively new class of agents displaying repetitive functional groups on a branched core. Previous work done by Liu *et al*. in 2007 has investigated the length requirement for antimicrobial activity of peptides consisting of repeated arginine (R) and tryptophan (W) side chains and found that even short linear RW repeats are active, providing a starting point for a de novo design of multivalent structures. In this study, we tested a new synthetic dendrimer, 2D-24, for its antimicrobial activity against *Pseudomonas aeruginosa*, including the wild-type PAO1 and its mucoid mutant PDO300. This synthetic AMDP was found to kill planktonic cells of both PAO1 and PDO300 in a dose-dependent manner, with nearly complete killing of both strains observed when treated with 50 μM of this agent. In addition to planktonic cells, 2D-24 was also found to kill biofilm cells of both strains in a dose-dependent manner. For example, treatment with 30 μM 2D-24 led to 94.4 ± 1.4 and 93.9 ± 4.2 % killing of PAO1 and PDO300 biofilm cells, respectively. Furthermore, 2D-24 was effective in killing multidrug-tolerant persister cells of PAO1 and PDO300. While higher concentrations of 2D-24 were required to kill persister cells, combinations of 2D-24 with ciprofloxacin, tobramycin, or carbenicillin showed synergistic effects on killing persister cells of both strains. Based on hemolysis assays using a co-culture model of PAO1 and human epithelial cells, 2D-24 was found to kill *P. aeruginosa* cells at concentrations that are not toxic to mammalian cells.

2.2 Introduction

Bacteria have evolved diverse mechanisms to survive from the attack of antibiotics. One such mechanism involves persister cell formation, by which a small population in a bacterial culture enters an inactive stage and exhibits high tolerance to antibiotics and other forms of stress [1]. Consistently, persister cell formation has been implicated in chronic infections by *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* [2,3].

In addition to persister formation, bacteria can also evade antibiotics by forming multicellular structures, known as biofilms, in which bacterial cells adhere to a surface and secrete an extracellular matrix that protects them from environmental stresses [4]. Due to limited mass transfer [5], biofilm cells grow slowly with reduced metabolism and exhibit high tolerance to antimicrobial agents [6,7]. It is estimated that 80 % of bacterial infections in humans involve biofilms [8]. Thus, biofilms present a serious complication associated with chronic infections especially in patients with compromised immune systems [9-12].

To address the challenge of drug-resistant infections, it is important to develop new antimicrobials that are effective against biofilms and persister cells. Antimicrobial peptides (AMP) are promising alternative antibiotics due to their rapid killing effects, low frequency of resistance development, and broad spectrum of target microbes [13]. Thousands of AMPs have been identified to date [14], and many synthetic analogs have been developed and tested against infectious microorganisms including protozoa, fungi, bacteria, and viruses [15-19]. However, most AMPs are not appropriate for therapeutic use due to cytotoxicity, high MIC (minimum inhibitory concentration) values, in vivo inactivation by proteases, and/or high production cost [20]. Therefore, it is important to develop new AMPs to overcome these challenges. One such

strategy is to modify the AMP molecules by amidation of carboxyl ends [21], cyclization, covalent modification with disulfide bonds [22], and/or alteration of amino acid content such as adding prolines [23]. Modifications of this kind can affect critical physiochemical properties of natural AMPs and thus the target spectrum, cytotoxicity to eukaryotic cells, antimicrobial activity, and stability against proteases [13].

Antimicrobial dendrimeric peptides (AMDP) are a relatively new class of AMPs with repeating functional groups linked to a multivalent scaffold. The Kallenbach group has investigated the length requirement for antimicrobial activity of peptides only consisting of amino acid arginine (R) and tryptophan (W) repeats. Even short repeats of RW (trimers, for example) are active, providing a starting point for a de novo design of minimal structures [24]. Further studies found that dendrimeric displays of di- or tripeptides of arginine and tryptophan can render the AMPs more potent and less cytotoxic than natural AMPs [25]. Recently, the Ren lab showed that some synthetic AMPs with RW repeats are effective against planktonic and biofilm cells of *Escherichia coli* [26,27] including persister cells [28]. To further evaluate the potential of AMDPs as more effective antibacterial agents, the agent 2D-24, containing RWR and RTtbR(2) tripeptide branches (Fig. 2-1), was tested in this study for its antimicrobial activities against planktonic, biofilm, and persister cells of the wild-type *P. aeruginosa* PAO1 and its mucoid mutant PDO300.

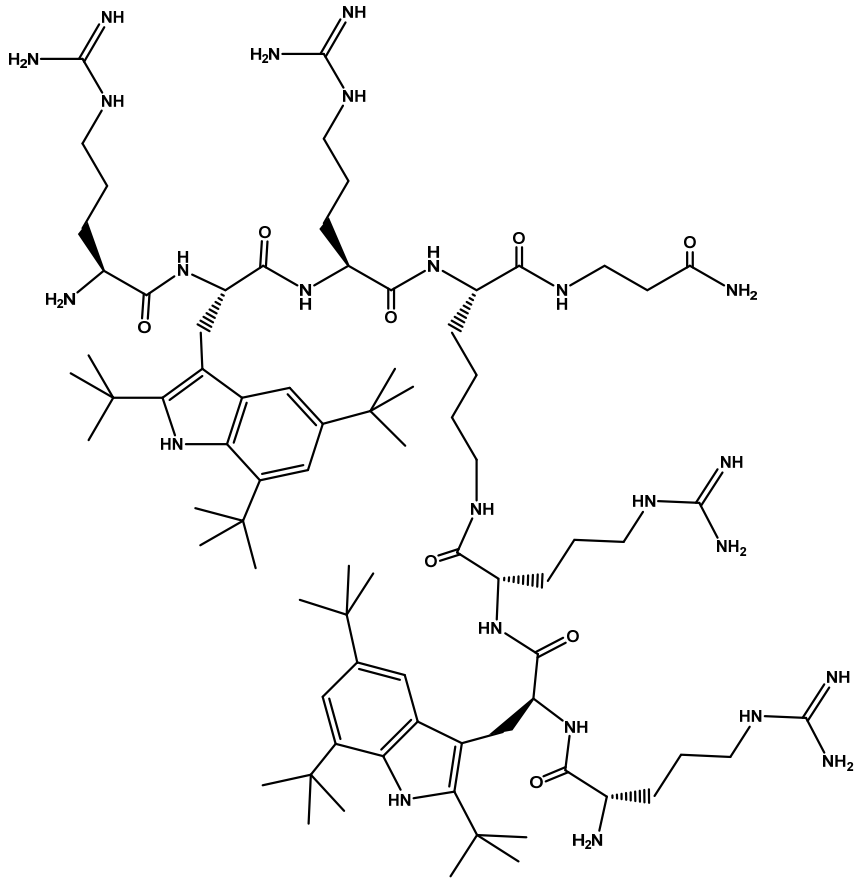

Figure 2-1. Structure of 2D-24

P. aeruginosa is a Gram-negative bacterium with exceptional capabilities to adapt to different living environments [29]. It is a well-known opportunistic human pathogen [30], causing serious infections in patients with comprised immunity or with cystic fibrosis [31,32]. *P. aeruginosa* can acquire antibiotic tolerance through biofilm and persister formation [33,34]. In addition, during chronic colonization in the lungs of cystic fibrosis patients, *P. aeruginosa* commonly acquires mutations that lead to a mucoid phenotype overproducing the polysaccharide alginate and therefore acquires a higher-level antibiotic tolerance [35-37]. With its strong clinical relevance, *P. aeruginosa* is a good model bacterium for studying antibiotic resistance and for testing new antimicrobials.

2.3 Materials and methods

2.3.1 2D-24 Synthesis

The synthesis of 2D-24 (50-μmol scale) was performed in the lab of Kallenbach at New York University.

2.3.2 Bacterial strain and growth media.

P. aeruginosa PAO1 (ATCC BAA-47, henceforth PAO1) and *P. aeruginosa* PDO300 (*mucA22* mutant of *P. aeruginosa* PAO1, constructed based on a clinical isolate of *P. aeruginosa* FRD1 from cystic fibrosis patient [38]; henceforth, PDO300 [39]) were routinely cultured in Lysogeny broth (LB) medium [40] containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl at 37°C with shaking at 200 rpm. To minimize the difference in persistence between overnight cultures by freeze and thaw, all overnight cultures were inoculated with single-use glycerol stocks prepared from the same overnight culture of desired bacteria.

2.3.3 Effects of 2D-24 on planktonic cells

Exponential cultures were prepared by inoculating 3 mL LB medium with an overnight culture to an optical density at 600 nm (OD_{600}) of 0.01 and incubated at 37°C with 200 rpm shaking until OD_{600} reached 0.5. Then, different concentrations of 2D-24 were added. The control and treated samples were incubated for 3 h at 37°C with 200 rpm shaking. Then, the cells were washed three times with phosphate buffered saline (PBS, pH 7.4). The viability of each sample was determined by counting colony-forming units (CFUs) using a drop-plate method as described

previously [28]. Briefly, each sample was diluted in a 10× series with 0.85 % NaCl solution. The CFU was determined by loading 10 µL of each diluted sample on a LB agar plate and counting the CFUs after incubation at 37°C overnight.

2.3.4 Effect of 2D-24 on biofilms

For each treatment, three separate polished (using 1000 grit sand paper, 3 M, St. Paul, MN, USA) sterile 2 cm × 1 cm 316-L stainless steel coupons were placed in a petri dish containing 20 mL LB medium. To initiate biofilm formation, the medium was inoculated with an overnight culture to OD_{600} of 0.01. These coupons were incubated for 24 h at 37°C without shaking. The coupons were then washed gently with PBS three times and placed in a 12-well plate (one coupon in each well) with 2 mL PBS in each well and 2D-24 supplemented at concentrations of 0.1 to 30 µM. After incubation for 3.5 h, biofilm coupons were washed with PBS and transferred to 15-mL sterile conical tubes with 3 mL PBS buffer in each. The samples were gently sonicated for 4 min in a sonication bath (Branson B200 Ultrasonic, Danbury, CT, USA) and vortexed for 15 s (this condition was confirmed not to kill the cells; data not shown). The cells, thus detached from coupons, were plated using the drop-plate method [28] to determine the CFUs.

2.3.5 Persister isolation and killing by 2D-24

Persister cells of PAO1 and PDO300 were isolated by killing normal cells in overnight cultures with 200 µg/mL ciprofloxacin (Cip) for 3.5 h at 37°C, as described previously [41,42]. Longer treatment, 6.5 h, was also tested to confirm the treatment time was sufficient (no additional killing with extended time) as described in (Pan *et al*. 2012). After antibiotic treatment, surviving persister cells were washed twice with PBS buffer to remove any remaining antibiotic. The

collected cells were resuspended in 3 mL PBS supplemented with 2D-24 at different concentrations and incubated for 3.5 h at 37°C with 200 rpm shaking. After treatment, the cells were washed with PBS and plated to determine the viability by counting CFU as described above.

2.3.6 Fluorescence microscopy

To corroborate the CFU results, the LIVE/DEAD BacLight™ bacterial viability kit (Life Technologies Inc., Carlsbad, CA, USA) was used to determine the viability of biofilm cells of PAO1 and PDO300 on 316-L stainless steel coupons. The 24-h *P. aeruginosa* biofilms grown on these surfaces were washed gently with 0.85 % NaCl (*w*/*v*) solution three times to remove planktonic cells. Then, 1 µL of 20 mM propidium iodide and 1 µL of 3.34 mM SYTO 9 stains were added in 1 mL PBS to stain each biofilm sample for 10 min in the dark. The stained biofilm samples were examined with an Axio Imager M1 fluorescence microscope (Carl Zeiss Inc., Berlin, Germany). At least five randomly selected spots were examined for each sample.

2.3.7 Synergistic effects between 2D-24 and antibiotics

Three different antibiotics were tested including Cip (a fluoroquinolone targeting DNA gyrase) [43], tobramycin (Tob, an aminoglycoside targeting translation by binding to ribosome subunits) [44], and carbenicillin (Car, a carboxypenicillin targeting bacterial cell wall synthesis) [45]. Normal and persister cells of PAO1 and PDO300 were treated with antibiotic alone, 2D-24 alone, or a combination of an antibiotic and 2D-24 to assess any synergistic effects in bacterial killing. The viability of bacterial cells was determined using the drop-plate method as described above.

2.3.8 Cytotoxicity and antimicrobial effects in co-cultures

The cytotoxicity of 2D-24 on eukaryotic cells was evaluated using IB3-1 cells and LIVE/DEAD® viability/cytotoxicity kit (Invitrogen, NY, USA). IB3-1 cells were grown in LHC-8 basal medium (Invitrogen, NY, USA) supplemented with 10 % (*v*/*v*) fetal bovine serum (FBS) in flasks pre-coated with 100 μg/mL bovine serum albumin (BSA) and 30 μg/mL collagen at 37°C with 5 % CO_2. All treatments were performed in antibiotic-free medium. To test the cytotoxicity, IB3-1 [46] (a compound heterozygote bronchial epithelial cell line from a CF patient) cells were seeded into black-sided clear-bottom 96-well plates (5×10^4 cells per well) and incubated until the cell density reached 1×10^6 cells per well. Then, 2D-24 was added at concentrations of 1 to 32 μM. After treatment for 24 h, EthD-1 and calcein AM were used to assess the viability of IB3-1 cells. The optimal concentrations of EthD-1 (1 μM) and calcein AM (2 μM) were determined prior to the treatments to label live and dead IB3-1 cells distinctively. After staining for 30 min at 37°C, samples were washed with PBS and analyzed using a fluorescence microplate reader (model FLx800, Bio-Tek Instruments, Winooski, VT, USA). To test the effects of 2D-24 on PAO1 in co-cultures, PAO1 cells (4×10^6 cells/mL) were added either with 2D-24 together or at 4 h prior to 2D-24 treatment (20 h after the inoculation of IB3-1 cells). The viability of IB3-1 cells was determined as described above. To compare the effects on bacteria, PAO1 cells were treated in the same medium (LHC-8 basal medium) but in the absence of IB3-1 cells. The viability of PAO1 cells was determined by counting CFU using the drop-plate method as described above. All treatments were tested in triplicate.

2.3.9 Statistical analysis

The data from CFU experiments were analyzed with one-way ANOVA followed by Tukey test using SAS version 9.3 (SAS Institute, Cary, NC, USA). Differences with $p < 0.05$ were considered to be statistically significant.

2.4 Results

2.4.1 Antimicrobial effects of 2D-24 on planktonic cells

First, the effects of 2D-24 on normal planktonic cells were evaluated by treating cells in exponential phase cultures with different concentrations of 2D-24. As shown in Fig. 2-2, 2D-24 was effective in killing both PAO1 and PDO300 planktonic cells in a dose-dependent manner. For example, 5 μM 2D-24 was found to kill 53.3 ± 3.3 % PAO1 ($p < 0.001$) and 40.3 ± 6.2 % PDO300 ($p < 0.005$) cells, respectively. The killing efficiency increased with the concentration of 2D-24. At 20 μM, 79.9 ± 4.7 % ($p < 0.001$) and 84.6 ± 7.1 % ($p < 0.001$) killing was obtained for PAO1 and PDO300, respectively. No viable cells of either strain were found after treatment with 50 μM 2D-24, suggesting that complete killing is possible. Interestingly, while the mucoid strain PDO300 is more tolerant to antibiotics than the wild-type PAO1 [37], 2D-24 exhibited similar activities in killing PAO1 and PDO300.

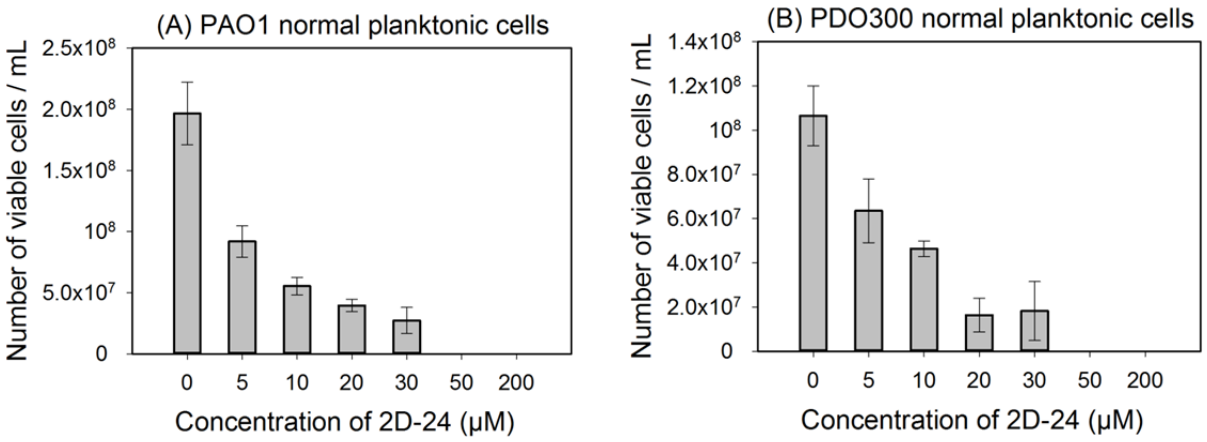

Figure 2-2. Effects of 2D-24 on planktonic cells of PAO1 (A) and PDO300 (B). The dendrimer 2D-24 was added at different concentrations to exponential cultures, and the viability was determined by counting CFUs

2.4.2 Antimicrobial effects of 2D-24 on biofilm cells

In addition to planktonic cells, 2D-24 was also found to kill PAO1 and PDO300 biofilm cells effectively (Fig. 2-3). At 5 μM, 2D-24 is slightly more effective against PAO1 than PDO300. For example, 2D-24 exhibited 55.8 ± 6.5 % killing ($p < 0.001$) of PAO1 biofilm cells, while the killing of PDO300 biofilm cells with the same concentration of 2D-24 was 31.6 ± 8.8 % ($p < 0.05$). The activity increased with 2D-24 concentration for both strains, e.g., 2D-24 killed 87.8 ± 3.1 % ($p < 0.001$) and 94.4 ± 1.4 % ($p < 0.001$) of PAO1 biofilm cells when treated at 20 and 30 μM, respectively. Similar activities against PDO300 biofilms were observed, e.g., 2D-24 exhibited 81.7 ± 3.5 and 93.9 ± 4.2 % killing of PDO300 biofilm cells when added at 20 and 30 μM, respectively ($p < 0.001$ for both). Since the mucoid strain PDO300 is tolerant to multiple antibiotics [47,48], the finding that 2D-24 is equally effective in killing PAO1 and PDO300

biofilms is encouraging. The effective killing of biofilm cells, especially those of PDO300, suggests that 2D-24 is able to penetrate the biofilm matrix and the alginate layer of the mucoid strain.

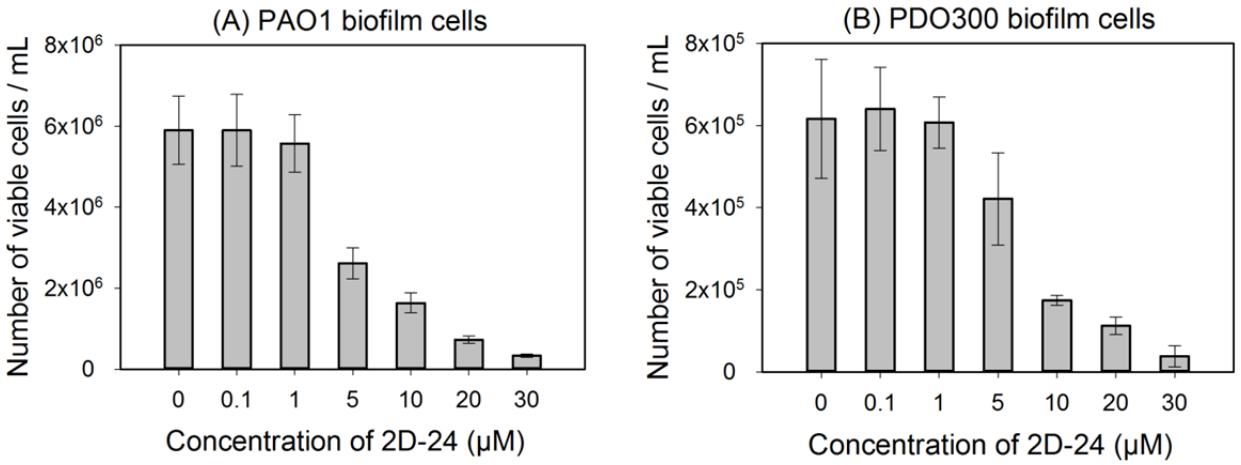

Figure 2-3. Effects of 2D-24 on PAO1 (A) and PDO300 (B) biofilm cells. The biofilms were cultured for 24 h in LB medium before treatment with 2D-24 for 3.5 h in PBS

2.4.3 LIVE/DEAD staining of biofilms

To corroborate the CFU results of biofilm killing, the control and 2D-24-treated biofilm samples were analyzed using LIVE/DEAD staining. Consistent with CFU data, the LIVE/DEAD images also showed dose-dependent killing of biofilms cells by 2D-24. While the cells in untreated control were healthy (green), killing was observed (red) with addition of 2D-24 and the effects were dose dependent (Fig. 2-4).

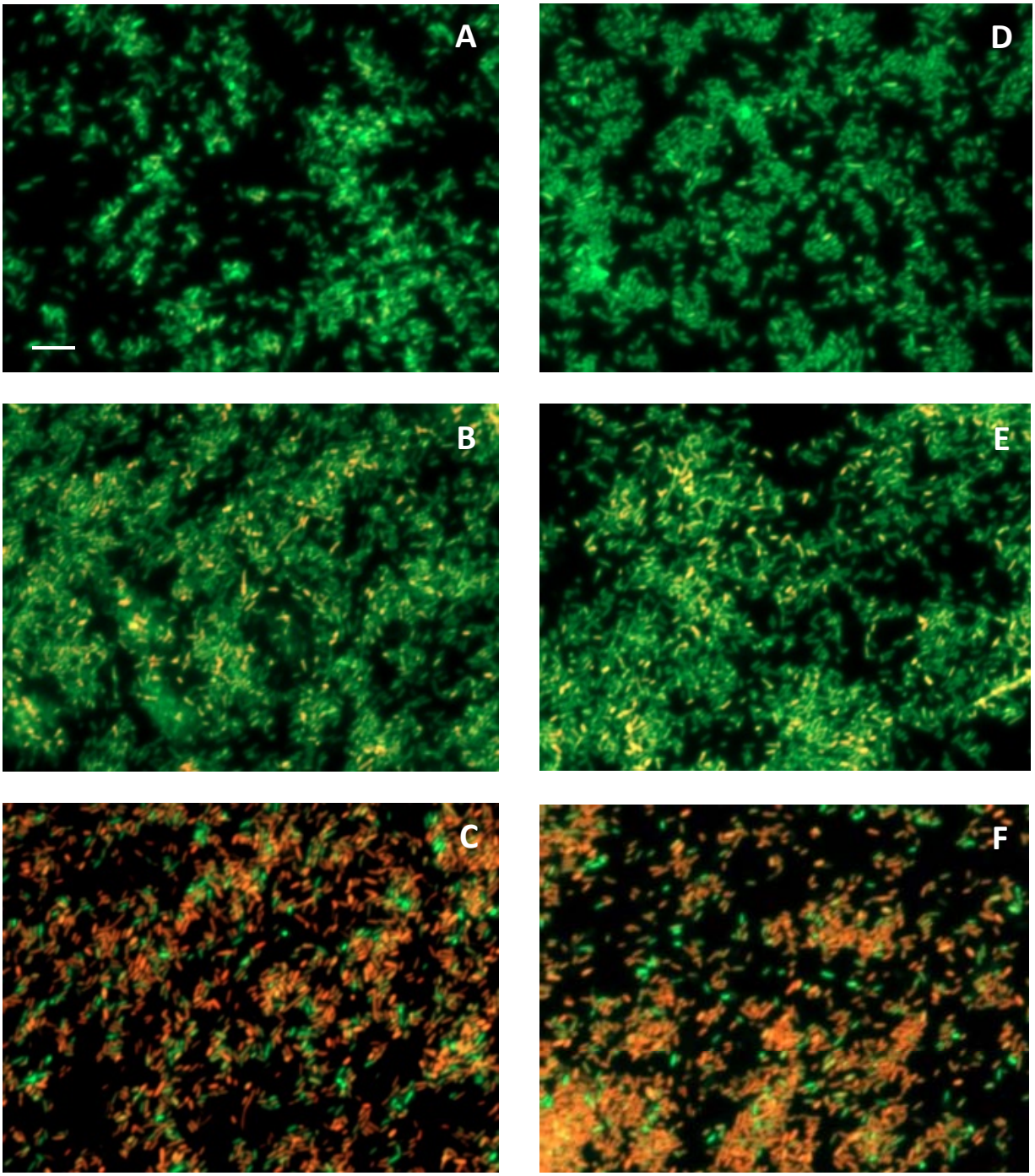

Figure 2-4. LIVE/DEAD images of PAO1 and PDO300 biofilms. A, B, C: PAO1 biofilms treated with 0, 10, and 30 μM 2D-24, respectively. D, E, F: PDO300 biofilms treated with 0, 10, and 30 μM 2D-24, respectively. *Bar* = 10 μm

2.4.4. Antimicrobial effects of 2D-24 on persister cells

Because persister cells are highly tolerant to antibiotics [49], we were motivated to also test 2D-24 against persister cells of PAO1 and PDO300 (Fig. 2-5). It was found that 2D-24 is effective in killing persister cells of both strains, although higher concentrations of 2D-24 than those required to kill normal cells are needed. For example, 200 μM of 2D-24 killed 68.7 ± 6.7 % ($p < 0.001$) of PAO1 and 89.0 ± 3.2 % ($p < 0.001$) of PDO300 persister cells. At concentrations below 50 μM, 2D-24 exhibited a stronger killing effect on PAO1 persister cells compared to PDO300. For example, at 30 μM, 2D-24 showed 30.3 ± 6.1 % ($p < 0.05$) killing of PAO1 persister cells, while the same condition did not show any significant killing ($p = 0.08$) of PDO300 persister cells. Interestingly, there was a sharp increase in the susceptibility of PDO300 persister cells between 30 and 50 μM of 2D-24; the killing increased from 10.71 ± 6.2 to 87.6 ± 3.3 % ($p < 0.05$) when 2D-24 was added at 30 and 50 μM, respectively. These results suggest that a threshold may exist for the activity of 2D-24 to kill PDO300 persister cells effectively.

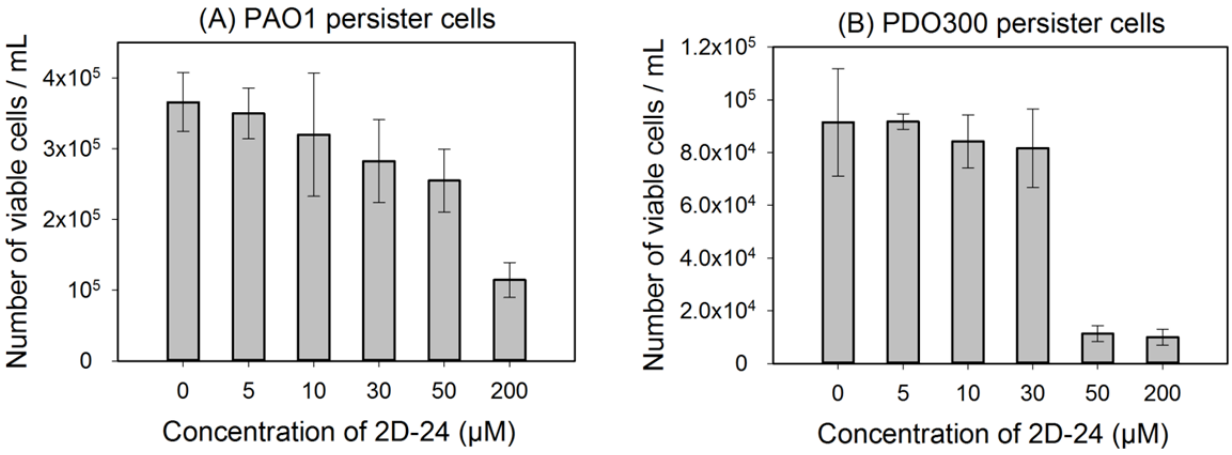

Figure 2-5. Effects of 2D-24 on persister cells of PAO1 (A) and PDO300 (B). Persister cells were isolated by killing normal cells in overnight cultures with 200 μg/mL Cip for 3.5 h

2.4.5 Synergistic effects between 2D-24 and antibiotics

Since some membrane-active AMPs can synergize with traditional antibiotics in bacterial killing [50], we also tested if the activity of 2D-24 can be enhanced through synergy with antibiotics. To achieve this, 1 μg/mL Cip, 1.5 μg/mL Tob, and 0.75 μg/mL Car (which can kill ~0.5 log of normal planktonic cells) were tested in the presence and absence of 2D-24. As shown in Figure 2-6, treatment with 1.5 μg/mL Tob or 5 μM 2D-24 alone killed 0.4 log (59.2 ± 0.7 %) and 0.3 log (54.1 ± 1.7 %) of normal planktonic PAO1 cells, respectively. In comparison, co-treatment with both agents caused 2.3 logs of killing (99.6 ± 0.6 %). Similar results were obtained for Cip and Car. Thus, there is indeed a synergy between 2D-24 and these three antibiotics. Co-treatments of normal planktonic cells of PDO300 with antibiotics and 2D-24 gave similar results. Thus, 2D-24 and the tested antibiotics show synergy in killing both strains.

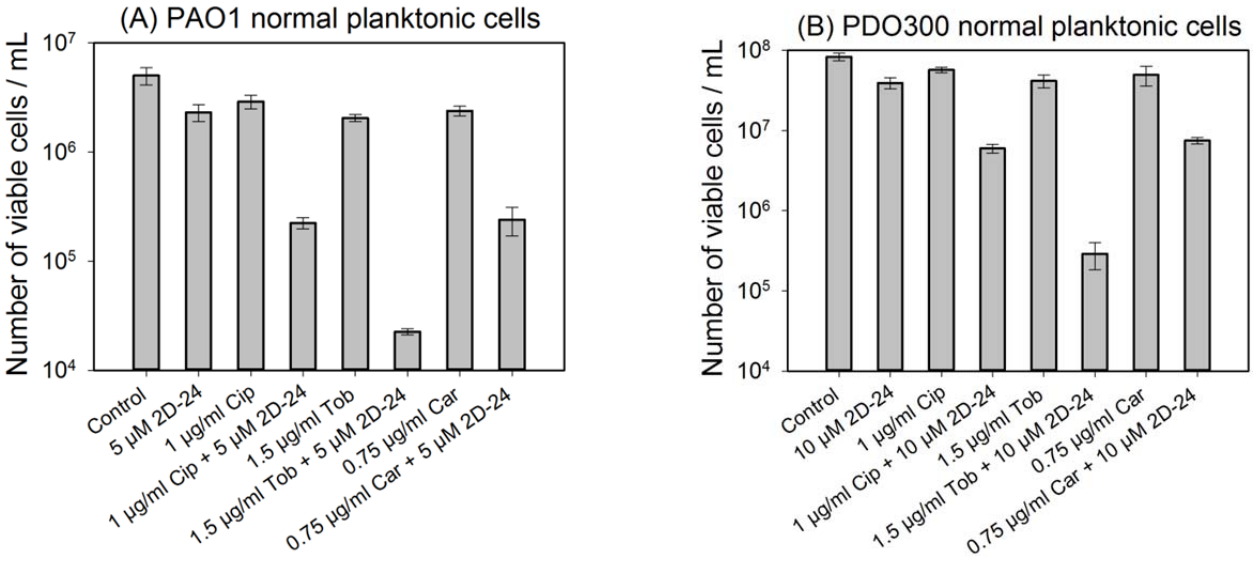

Figure 2-6. Co-treatment of planktonic PAO1 (A) and PDO300 (B) cells with 2D-24 and antibiotics. Cip, Tob, and Car antibiotics were tested on exponential cultures ($OD_{600} = 0.5$). Antibiotics or 2D-24 alone were used as controls

Previously, we reported that some membrane-active AMPs, containing tryptophan and arginine repeats, synergize with ampicillin in killing *E. coli* persister cells [28]. Thus, we tested if there is also a synergy in killing persister cells with 2D-24 and antibiotics. The results showed that there are synergies in killing persister cells, especially the co-treatment with 2D-24 and Tob (Fig. 2-7). For example, Tob (100 μg/mL) or 2D-24 (30 μM) alone killed <1.5 % and 0.1 log (20.7 ± 1.4 %) of PAO1 persister cells, respectively. In comparison, co-treatment with these two agents led to 0.58 log (73.8 ± 3.8 %) killing of persister cells. Similar results were obtained for PDO300 persister cells.

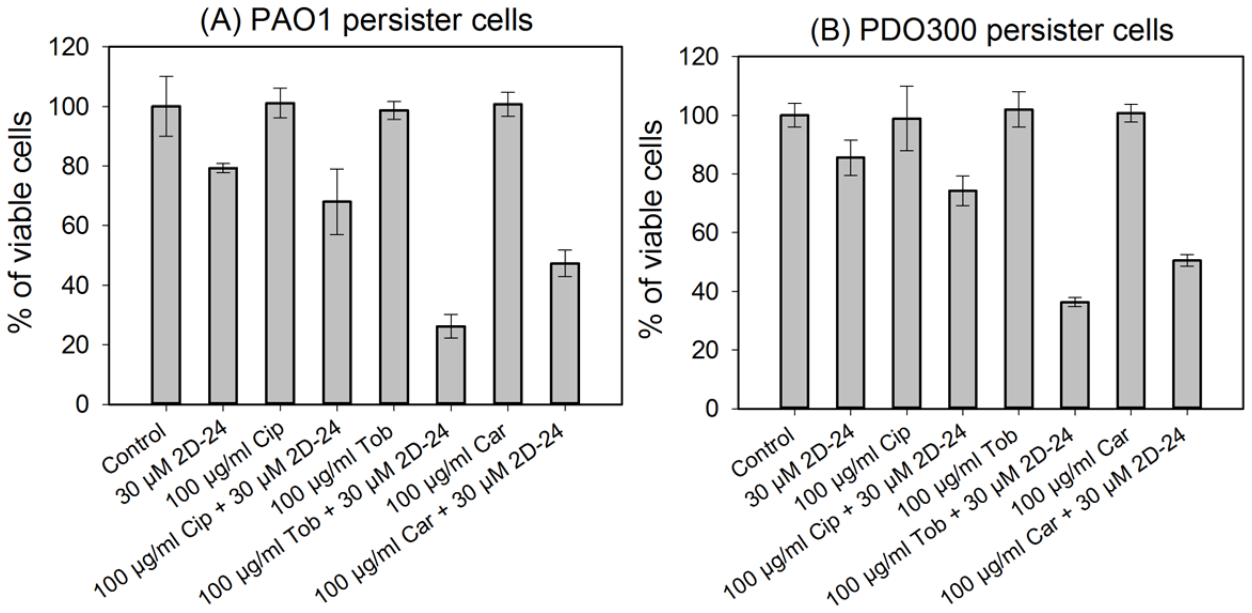

Figure 2-7. Co-treatment of PAO1(A) and PDO300 (B) persister cells with 2D-24 and antibiotics. Cip, Tob, and Car antibiotics were tested in the presence and absence of 30 μM 2D-24. The persister cells were isolated from overnight cultures by killing normal cells with 200 μg/mL Cip for 3.5 h. Antibiotics were tested at different concentrations (1–1000 μg/mL tested), but only the results of 100 μg/mL are shown. The CFU data of untreated controls in each run were normalized as 100 % for the convenience of data comparison across the samples

2.4.6 Cytotoxicity of 2D-24 on IB3-1 lung epithelial cell line in a co-culture with PAO1

It is encouraging to find that 2D-24 is effective against the biofilm and persister cells of *P. aeruginosa*. To determine if 2D-24 is safe to mammalian cells at the effective concentrations, we further tested the cytotoxicity of 2D-24 on the IB3-1 lung epithelial cell line. The results showed that 2D-24 is not toxic to IB3-1 cells at concentrations up to 32 μM after treatment for 24 h (Fig. 2-8), e.g., no significant change in viability was found compared to the 2D-24-free control ($p = 0.28$). In comparison, when PAO1 cells were treated by 2D-24 at the same concentration in the same medium, significant killing was observed. For example, 84.9 ± 9.5 % of PAO1 cells were killed by 16 μM 2D-24. Consistently, 2D-24 was found to protect IB3-1 epithelial cells from the infection of PAO1 in a co-culture model. In the absence of 2D-24, only 45.7 ± 2.6 % of IB3-1 cells remained viable after 24 h incubation with PAO1. In comparison, the presence of 2D-24 increased the viability of IB3-1 cells in a concentration-dependent manner. For example, 94.7 ± 2.7 and 82.6 ± 5.2 % of IB3-1 cells remained viable when 16 μM 2D-24 was added along with the inoculation of PAO1 and at 4 h after inoculation, respectively.

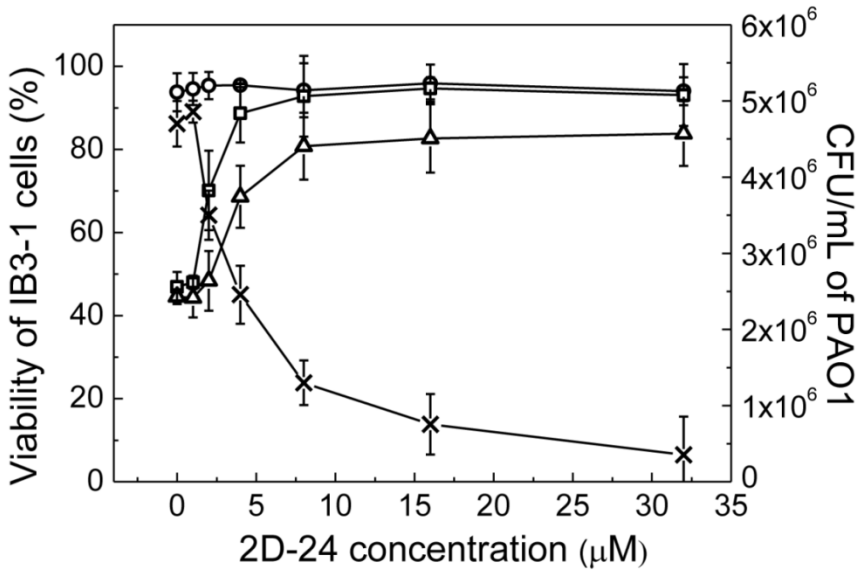

Figure 2-8. Cytotoxicity of 2D-24 on IB3-1 cells and the effects in a co-culture model. Different 2D-24 concentrations, from 1 to 32 μM, were tested on IB3-1 cells alone (*circles*), added together with PAO1 inoculation (*squares*), or 4 h after PAO1 inoculation (*triangles*). Besides, PAO1 cells alone in LHC-8 medium with no IB3-1 cells (*crossings*) were treated with the same concentrations of 2D-24 to evaluate bacterial killing. The viability of IB3-1 cells was determined using the LIVE/DEAD staining kit by following the manufacturer's protocol. The viability of PAO1 was determined by counting CFUs

2.5 Discussion

Although more than 5000 AMPs have been identified to date [51], most previous studies of their activities have focused on activities against normal planktonic bacterial cells [13]. In comparison, the effects of AMPs on biofilm cells have been less studied, and few reports exist regarding their effects on persister cells. In this study, a new synthetic AMDP, 2D-24, was tested

against planktonic and biofilm cells, including persister cells of *P. aeruginosa* PAO1 and PDO300. Significant killing effects were observed for all the cell types tested.

Most synthetic AMPs are designed based on the characteristics of natural AMPs. In general, they are positively charged, amphiphilic molecules [52,53] and act on negatively charged cell walls and membranes, impairing the membrane potential and leading to cell death [13]. AMPs have a wide spectrum of target microbes. It has been postulated that the effectiveness of AMPs on bacteria depends on the peptide/lipid ratio during treatment [54]. In this study, the killing of PDO300 persister cells increased drastically when the concentration of 2D-24 reached 30 μM, which indicates that the killing may be dependent on peptide/lipid ratio as well. However, the biofilm and normal planktonic cells of PDO300 and all types of PAO1 cells tested here did not show a threshold concentration of 2D-24 for killing. It will be helpful to further investigate if persister formation affects the interaction between 2D-24 and the cell membrane of this mucoid strain.

Biofilms are composed of bacterial populations that include cells at different stages embedded in a complex extracellular matrix [55]; cells in biofilms can be up to 1000 times more tolerant to antibiotics than their planktonic counterparts [56]. Intriguingly, 2D-24 appeared to be equally effective against normal planktonic cells and biofilm cells of PAO1 and PDO300. This finding suggests that 2D-24 can penetrate biofilm matrix and is effective in killing biofilm cells. In comparison, persister cells tolerated higher concentrations of 2D-24. Overall, concentrations higher than 20 μM of 2D-24 were found sufficient to kill more than 80 % of both planktonic and biofilm cells of PAO1 and PDO300, while concentrations higher than 50 μM are required to achieve a similar level of killing of persister cells of these strains. Interestingly, when treated with higher concentrations of 2D-24, persister cells of PDO300 were slightly more susceptible

than PAO1 persister cells. For example, 50 and 200 μM 2D-24 exhibited 19.8 and 57.3 % more killing of PDO300 persister cells compared to PAO1, respectively.

Mucoid conversion with overproduction of alginate is thought to play an important role in chronic infections by *P. aeruginosa* [57] since mucoid strains are generally more tolerant to antibiotics than the wild-type strain [47,48]. It is interesting that the killing effects of 2D-24 on biofilm cells of PDO300 and PAO1 were similar. This suggests that 2D-24 is able to penetrate alginate and interact with PDO300 cellular targets.

Besides the killing of *P. aeruginosa* cells by 2D-24 alone, intriguing synergistic effects were observed when the cells were treated with 2D-24 and Cip, Tob, or Car, which are antibiotics targeting DNA, protein, and cell wall synthesis, respectively. Similar synergistic effects were observed for killing both PAO1 and PDO300 normal planktonic and persister cells (Figs. 2-6 and 2-7). Among these antibiotics, the strongest synergy was observed for Tob, while Cip exhibited lower synergy. This is probably because the persister cells had been exposed to Cip during persister isolation. The mechanism of synergistic killing of bacteria by 2D-24 and antibiotics is unknown. Most AMPs target cell membrane and thus alter the membrane potential [13]. We speculate that membrane disruption, depolarization, or permeabilization by 2D-24 can lead to reduced membrane potential along with lower energy production. This may favor the penetration of cell membrane by some antibiotics, which is consistent with a recent study by Schmidt *et al*. [58] showing a peptide-tobramycin conjugate -Pentobra- can effectively enter the cells of *P. aeruginosa*, *E. coli*, and *Staphylococcus aureus*. The peptide domain of this molecule is rich in arginine and tryptophan residues (RQIKIWFQNRRW), similar to the amino acid content of 2D-24.

We speculate that the increase in intracellular antibiotic concentration along with membrane damage by 2D-24 may render the persister cells unable to wake up, leading to cell death. Alternatively, it may also be possible that the stress response to 2D-24 treatment could activate certain cellular activities targeted by antibiotics and thus increase the antibiotic susceptibility of persister cells. It will be interesting to test the effects of 2D-24 on the integrity of treated cells and compare the penetration of antibiotics through cell membrane in the presence and absence of 2D-24.

It is generally appreciated that AMPs and analogs can have more than one inactivating target in bacteria. Even though persister cells are known to be latent and different from normal active cells, they still need to maintain intact cell membrane structures. While the cell surface and membrane are generally implicated in the antibacterial action by AMPs, we still lack a complete understanding of the detailed mechanism(s) involved. Similar to bactericidal antibiotics, we recently reported that a different AMDP analog, $(RW)_{4D}$, generates hydroxide radicals in target bacterial cells via a Fenton reaction [59]. It will be interesting to study the interaction of 2D-24 with cellular targets among regular planktonic, persister, and biofilm cells of *P. aeruginosa*.

In addition, we obtained data showing that 2D-24 is effective in killing *P. aeruginosa* at concentrations not toxic to IB3-1 epithelial cells. Similar to the uninfected control, the viability of IB3-1 cells increased to near 90 % when 2D-24 is added into the culture medium together with PAO1 at inoculation. This finding is intriguing since cytotoxicity is a major challenge in bacterial control with AMPs. Further in vivo tests will help evaluate the clinical potential of this new dendrimer.

In summary, a new antimicrobial dendrimer 2D-24 was tested for its effects on the biofilm and planktonic cells (including persister cells) of PAO1 and its mucoid mutant PDO300. Similar killing activities were observed for regular planktonic and biofilm cells of both strains, and this agent was also found to kill persister cells of both strains. Synergy between 2D-24 and antibiotics in killing *P. aeruginosa* was also observed. Further testing using co-cultures of PAO1 and human IB3-1 cells demonstrated that 2D-24 is effective against bacteria at concentrations nontoxic to mammalian cells. Thus, our in vitro data provide encouraging evidence for potential application of 2D-24 in treatment of chronic infections caused by *P. aeruginosa*. In addition to the investigation of mechanistic aspects of 2D-24 action, animal studies will be needed to demonstrate its efficacy in vivo.

2.6 Acknowledgments

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CHAPTER 3

CONTROLLING PERSISTER AND BIOFILM CELLS OF GRAM-NEGATIVE BACTERIA WITH A NEW 1,3,5-TRIAZINE DERIVATIVE

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3.1 Abstract

Infections caused by multidrug-resistant bacteria have been on the rise. This important issue presents a great challenge to the healthcare system and creates an urgent need for alternative therapeutic agents. As a potential solution to this problem, antimicrobial peptides (AMPs) have attracted increasing attention due to their broad spectrum of targeted microbes. However, most AMPs are expensive to synthesize, have relatively high cytotoxicity to mammalian cells, and are susceptible to proteolytic degradation. In order to overcome these limitations, novel synthetic AMPs are desired. Using 1,3,5-triazine (TN) as a template, several combinatorial libraries with varying cationic charge and lipophilicity were designed and screened by the Kallenbach lab. From this screening, TN-5 was identified as a potent lead. In the present study, this compound was tested for its antimicrobial activities on *Escherichia coli* and *Pseudomonas aeruginosa*. In addition to regular planktonic cells, the effects on biofilms and persister cells (metabolically inactive and antibiotic tolerant subpopulation) were also investigated. TN-5 was found to have a minimum inhibitory concentration (MIC) of 12.8 μM for both species and kill regular planktonic cells of both species dose dependently. TN-5 is also effective against persister cells of both *E. coli* and *P. aeruginosa*. The killing of biofilm cells of the mucoid *P. aeruginosa* PDO300 was enhanced by alginate lyase.

3.2 Introduction

Since the discovery of penicillin in 1928 [1] and the achievement of economical production of this antibiotic in the 1940s [2], the use of antibiotics has been a crucial step in controlling infectious diseases with numerous lives saved [3]. However, the emergence and spread of antibiotic resistant microorganisms have rendered many antibiotics ineffective [4]. Such rapid development of multidrug resistant bacteria coupled with the insufficient investment in antimicrobial research has led to a concerning decline in effective therapies against bacterial infections, which presents a serious public health problem [5].

In addition to antibiotic resistance based on drug resistance genes [6], bacteria also exhibit high level antibiotic tolerance by forming persister cells (dormant subpopulation of phenotypic variants [7] and biofilms (surface attached structures with bacterial cells embedded in an extracellular matrix secreted by attached cells [8]). Persister cells and biofilms are not based on drug resistance genes; however, they allow bacteria to survive the treatment with potent antibiotics and facilitate the development of drug resistant strains through mutation and horizontal gene transfer [9].

According to the U.S. Centers for Diseases Control and Prevention, 80% of all infections in developed countries are associated by biofilms [10]. The biofilm matrix serves as a protective barrier, making bacterial cells more tolerant to antibiotics as well as host defense [11]. Biofilms are also enriched in persister cells. Thus, even if an antibiotic can penetrate the biofilm matrix, it might only kill normal cells within a biofilm population. After the course of antibiotic treatment, persister cells revive and repopulate the biofilm, which in turn causes an infection to relapse [10].

An additional concern with biofilms of *Pseudomonas aeruginosa* is their ability to convert to mucoid variants [12]. A mucoid strain is characterized by overproduction of the exopolysaccharide alginate, with increased tolerance to some antibiotics [13] and phagocytosis by human macrophages [14], as well as enhanced protection from dehydration [15]. In cystic fibrosis patients, the leading cause of mortality is a respiratory failure due to chronic lung infection with *P. aeruginosa* strains that undergo mucoid conversion [12]. Mucoid isolates typically coincide with persistent chronic infection in cystic fibrosis patients.

The challenges of drug tolerant infections have created an urgent need for new antimicrobials and treatment strategies. Recent research has shown the great potential of antimicrobial peptides (AMPs) as a class of powerful agents against both Gram-positive and Gram-negative bacteria [16,17]. A wide range of AMPs are naturally produced by the innate immune system of multicellular organisms in response to infections [18]. These AMPs are a unique group of molecules with a varying number of amino acids (generally from 12 to 50), including positively charged residues (such as arginine, lysine, or histidine) and a large proportion of hydrophobic residues. In humans, AMPs are found mainly in the tissues and organs that are exposed to airborne pathogens [19].

The structure and charge of an AMP play a major role in the mechanism of its actions. AMPs are generally cationic molecules with both hydrophobic and hydrophilic faces [20]. This amphipathic characteristic helps these molecules to integrate into the lipid bilayer membranes [21]. Membrane integrity disruption (via interaction with negatively charged cell membrane), inhibition of macromolecule (protein, DNA and RNA) synthesis, or interaction with certain intracellular targets are thought to be the primary mechanisms in AMP lethality [22]. Positively charged side chains in AMPs enable an initial nonspecific electrostatic interaction with the

negatively charged cell membrane. This process is followed by the insertion of AMP molecule into cell membrane with the help of hydrophobic residues [23]. For example, in the AMPs LL-37 and β-defensin, the cationic face is positioned on the opposite side of the hydrophobic face, which helps the penetration into the membrane [20].

AMPs afford promising candidates for novel therapeutic agents and complement traditional antibiotic therapies because of some unique advantages, including broad-spectrum activity (antibacterial, antiviral, and antifungal), less resistance by microbes, and related broad anti-inflammatory activities [24]. A number of AMPs and derivatives have been developed as therapies for infectious diseases such as oral mucositis [25], pulmonary infections associated with cystic fibrosis [26], and some sexually transmitted diseases [27]. AMPs can be used alone, in co-treatment with antibiotics, or as stimulators for immune system and toxin inhibiting agents in septic shocks [24].

Despite the aforementioned advantages, wide applications of AMPs are still limited by several factors. They are generally expensive to synthesize [28], vulnerable to proteolytic degradation upon intravenous administration [29] and sensitive to environmental factors such as salt concentration, pH, and the presence of plasma and serum proteins [30]. Another challenge is that some AMPs are cytotoxic to host cells [31].

In order to overcome these challenges, a number of synthetic AMPs and AMP-mimetics have been developed. Based on the concept that cationic charge, size, and lipophilicity are recognized major factors determining the antibacterial activity of AMPs, recently, the Kallenbach lab designed and screened several combinatorial libraries based on 1,3,5-triazine as a scaffold. Several lead compounds with good antimicrobial activity and low hemolytic activity were

identified from the screening of hundreds of triazine compounds [32]. With further structure activity relationship analysis, the compound TN-5 was identified as a potent antimicrobial compound (the screening results will be published elsewhere). In this study, TN-5 (Fig. 3-1) was tested for its antimicrobial activity on *E. coli* and *P. aeruginosa*, including regular planktonic cells, persister cells, and biofilms.

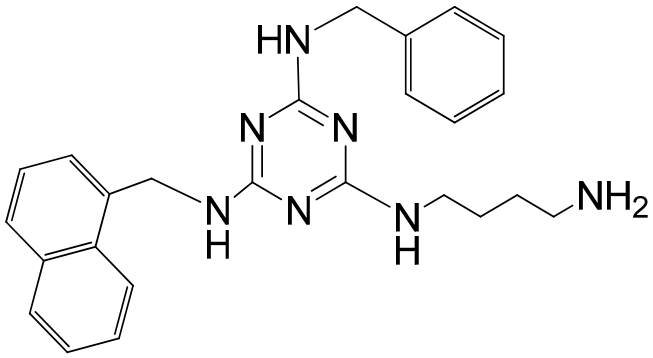

Figure 3-1. Chemical structure of TN-5.

3.3 Material and methods

3.3.1 Chemical synthesis of TN-5

TN-5, N2 - (4-Aminobutyl) - N4 - benzyl - N6 – naphthalenemethyl - 2,4,6 – triamino - 1,3,5-triazine, white solid, was synthesized by our collaborator in Kallenbach lab at New York University, using an orthogonal synthetic approach based on cyanuric chloride stepwise reaction with naphthalenemethylamine, benzylamine and Boc-1,4-diaminobutane as documented previously [32].

3.3.2 Bacterial strains and growth media

E. coli RP437 [33] was provided by Dr. John S. Parkinson at the University of Utah. *P. aeruginosa* PAO1 [34] and *P. aeruginosa* PDO300 [12] were obtained from Dr. Matthew Parsek at the University of Washington. All strains were routinely grown in Lysogeny broth (LB) containing 10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone with pH 7.0. To ensure consistent experimental conditions throughout this study, all overnight cultures of a particular strain were started with single-use glycerol stocks originating from the same culture. Each experimental condition was tested with three independent cultures (three biological replicates).

3.3.3 Determining Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of TN-5

TN-5 was tested at different concentrations against *E. coli* and *P. aeruginosa* strains to determine MIC and MBC values following a previously described protocol [35] with slight modifications. MIC is referred to the concentration of an antimicrobial agent, which inhibits the visible growth of a given bacterium completely by checking with unaided eye. MBC is referred to the minimum concentration of an antimicrobial agent required to completely kill all the tested bacterial cells of a particular strain by checking colony formation on the plates after treatment. Briefly, exponential cultures of bacterial samples were used to inoculate test samples with a cell density of $5x10^5$ colony forming unit (CFU)/mL. TN-5 was tested at concentrations from 0.2 μM to 96 μM, increasing logarithmically. Cultures grown without antimicrobial and sterile LB medium were used as positive and negative controls, respectively. All samples were adjusted to a final volume of 3 mL and incubated for 16-18 h at 37°C. After incubation, the concentrations with no visible growth were assigned by visual check to determine MIC. MBC value of TN-5 was

identified by spreading TN-5 (0.2 μM to 96 μM) treated cells (overnight in LB) on LB agar plates and checking for growth after 24 h. Three independent cultures for each concentration were tested for MIC and MBC tests.

3.3.4 Effects on planktonic cells

To examine the antimicrobial activity of TN-5, the planktonic growth of *E. coli* RP437, *P. aeruginosa* PAO1 and PDO300 with TN-5 added at different concentrations was examined. Overnight cultures of each strain were grown in 50 mL LB medium for 12-16 h at 37°C. Subcultures were then prepared by inoculating LB medium with overnight cultures to an optical density at 600 nm (OD_{600}) of 0.01 and harvested when the OD_{600} reached 0.4-0.5. These exponential cultures were washed with phosphate buffered saline (PBS) three times. Cell pellets were washed with fresh PBS and resuspended in 20 mL PBS buffer. For the TN-5 treatment, 3 mL of each sample was taken and mixed with TN-5 at different concentrations. The samples were incubated for 3, 6, 12 and 24 h at 37°C with shaking at 200 rpm, and washed three times with PBS. Then a serial dilution of each sample was performed and the cells were spread on LB agar plates for counting CFU. The amount of DMSO (solvent to dissolve TN-5 in stock solutions) was adjusted to be the same for all samples to eliminate any solvent effect. Each condition was tested with three independent cultures.

3.3.5 Persister isolation and treatment

To isolate persister cells, overnight cultures of *P. aeruginosa* PAO1 and *P. aeruginosa* PDO300 strains were treated with 200 μg/mL ciprofloxacin (Cip) while exponential cultures of *E. coli* RP437 were treated with 100 μg/mL ampicillin (all for 3 h at 37˚C) to kill normal cells as

described previously [36-38]. Then the persister cells were harvested by centrifugation, washed with PBS three times to remove remaining antibiotic, and resuspended in 20 mL PBS. Aliquots (1 mL) of each sample were supplemented with TN-5 at different concentrations. Three replicates were tested for each condition and all samples were incubated for 3 h at 37°C with shaking at 200 rpm. No change in persister cell number was found before and after incubation for 3 and 6 h in PBS without TN-5 (regardless the presence of antibiotics; Fig. 3-2). After TN-5 treatment, the samples were washed with PBS three times and plated to count CFU as described above. Each condition was tested with three independent cultures.

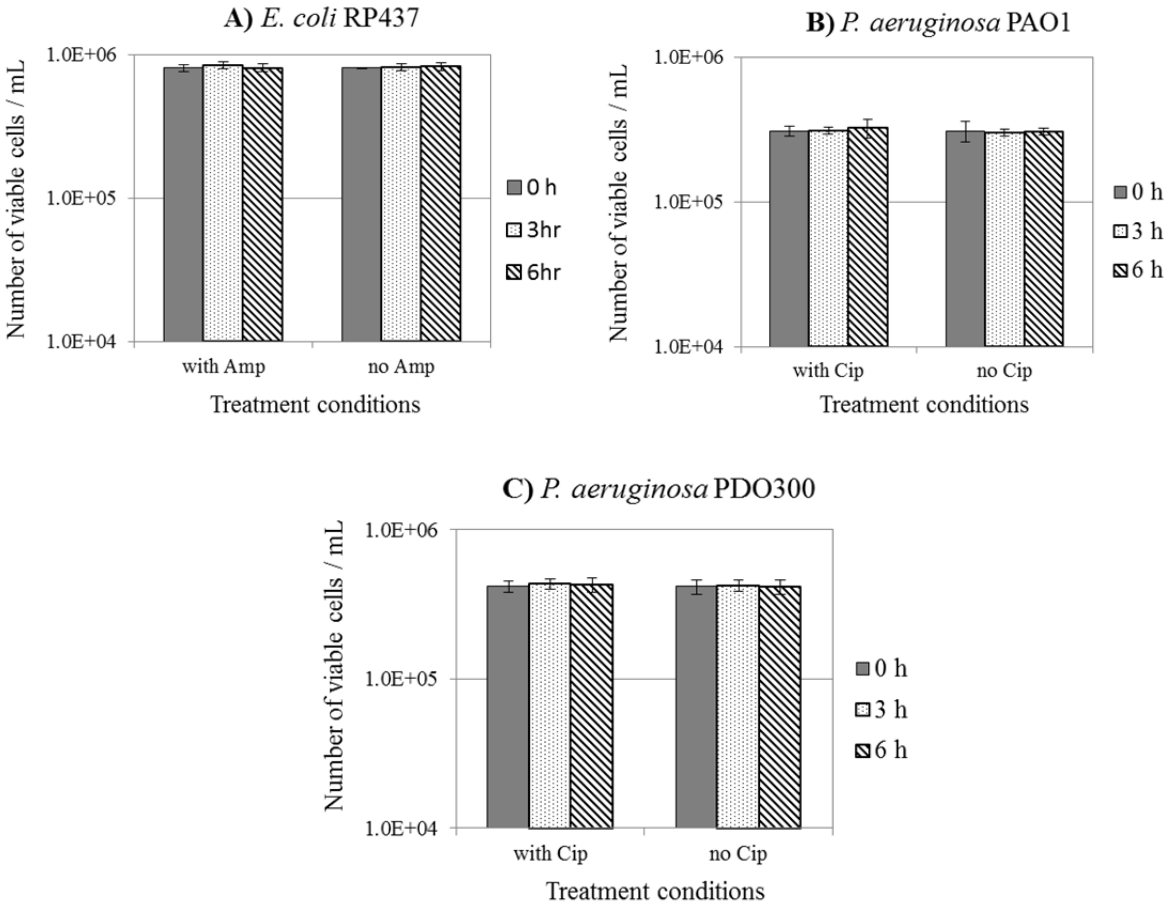

Figure 3-2. The isolated persister cells remained persistence after incubation in PBS. After isolation, the persister cells were incubated with or without antibiotic for 3 and 6 h. The persister

cells of *E. coli* RP437 (A), *P. aeruginosa* PAO1 (B), and *P. aeruginosa* PDO300 (C) were studied. Ampicillin at 100 μg/mL and ciprofloxacin at 200μg/ml were used to treat *E. coli* and *P. aeruginosa* persister cells, respectively.

3.3.6 Biofilm experiments

To study the effects of TN-5 on biofilm cells of *E. coli* and *P. aeruginosa*, biofilms were grown on 2 cm x 1 cm 316L stainless steel coupons. Each coupon was polished with 220 Grit sandpaper (3M, Sandblaster, St. Paul, MN) on both sides and sterilized with 70% ethanol for at least 15 min. The coupons were then dried in a 50°C oven for 15 min. Sterilization was confirmed by incubating three coupons in LB medium for 24 h at 37°C and checking the turbidity of the cultures. To culture biofilms, sterilized coupons were transferred into new sterile plates containing 20 mL LB medium. To initiate biofilm formation, each sample was inoculated to OD_{600} of 0.01 with an overnight culture. The coupons were incubated for 24 h at 37°C without shaking to grow biofilms. After 24 h incubation the coupons were washed gently with PBS three times to remove planktonic cells. Coupons were then placed in 12 well plates separately, each including a different concentration of TN-5 in 2 mL PBS buffer. The coupons were incubated for 3 h at 37°C with no shaking followed by washing with PBS three times. Each coupon was then transferred into a 15 mL sterile conical test tube containing 3 mL PBS. Samples were gently sonicated for 4 min in a water sonication bath (Branson B200 Ultrasonic, Danbury, CT, USA) and then vortexed for 15 s. Three replicates were tested for each condition. The cells in the suspension were then spread on LB agar plates to count CFU as described above. Each condition was tested with three independent cultures.

Besides treating established biofilms, the capability of TN-5 to prevent biofilm formation was also evaluated by adding TN-5 prior to the inoculation of biofilm cultures. After incubation for 24 h, the coupons were washed gently and the cells were removed from coupon surface by sonication and vortex for CFU count as described above.

3.3.7 Co-treatment of alginate lyase and TN-5 on *P. aeruginosa* biofilm cells

To determine the contribution of a biofilm degrading agent on killing by TN-5, we repeated biofilm killing experiments in the presence of 50 μg/mL alginate lyase during TN-5 treatments. The same protocols were used for *P. aeruginosa* PAO1 and PDO300 biofilms. Each condition was tested with three independent cultures.

3.3.8 Statistical analysis

The data from CFU experiments were analyzed with one-way ANOVA followed by Tukey test using SAS version 9.4 (SAS Institute, Cary, NC, USA). Differences with $p<0.01$ were considered as statistically significant.

3.4 Results

3.4.1 MIC and MBC values of TN-5

TN-5 was found to completely inhibit the growth of *E. coli* RP437, *P. aeruginosa* PAO1, and *P. aeruginosa* PDO300 at the concentration of 12.8 μM (MIC, Table 1). The MBC value was found to be higher than 96 μM for all three strains.

Table 3-1. MIC and MBC values of TN-5 on bacterial strains used in this study (based on three biological replicates).

	E. coli RP437	*P. aeruginosa* PAO1	*P. aeruginosa* PDO300
MIC (μM)	12.8	12.8	12.8
MBC (μM)	> 96	> 96	> 96

3.4.2 Antimicrobial effects of TN-5 on planktonic cells

To further study the killing activity of TN-5, exponential cultures were used to test the effects of TN-5 on the viability of planktonic cells. TN-5 was found effective in killing all bacterial strains tested in this study (*E. coli* RP437, *P. aeruginosa* PAO1 and PDO300) dose dependently in 3 h (Fig. 3-3). Longer incubation times (up to 24 h) did not cause additional killing (Fig. 3-4). For *E. coli* RP437, TN-5 showed 20.3±2.5% (p=0.07), 55.6±8% (p<0.001), 84.6±8.5% (p<0.001), and 99.9±0.1% (3.4 log; p<0.001) killing of the total population (>99% as normal cells) at concentrations of 5, 20, 50, and 100 μM, respectively (Fig. 3-3A). These results show that TN-5 is highly effective against normal planktonic cells of *E. coli* RP437.

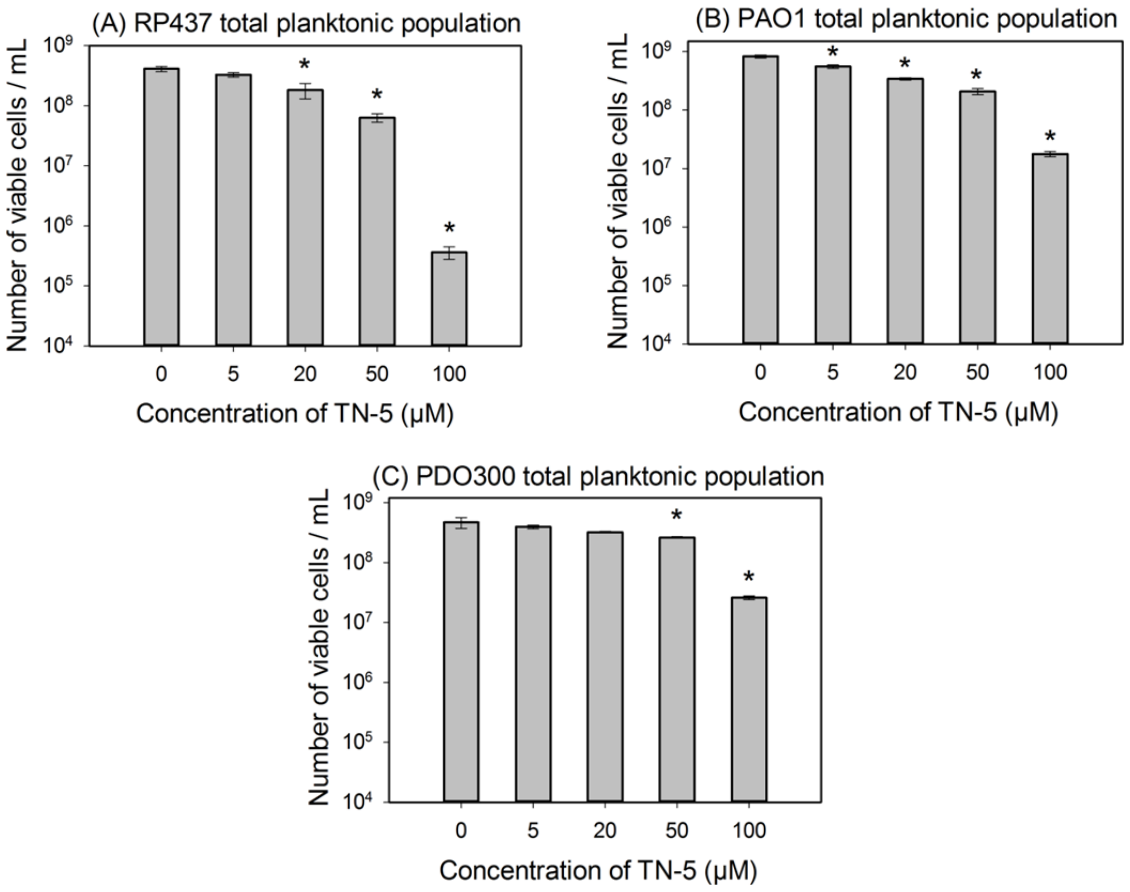

Figure 3-3. Effects of TN-5 on planktonic cells of *E. coli* RP437 (A), *P. aeruginosa* PAO1 (B), and *P. aeruginosa* PDO300 (C). TN-5 was added in exponential phase cultures at different concentrations and the viability after treatment was determined by counting CFU. All significant differences (compared to the TN-5 free control) with $p<0.01$ are marked with an asterisk

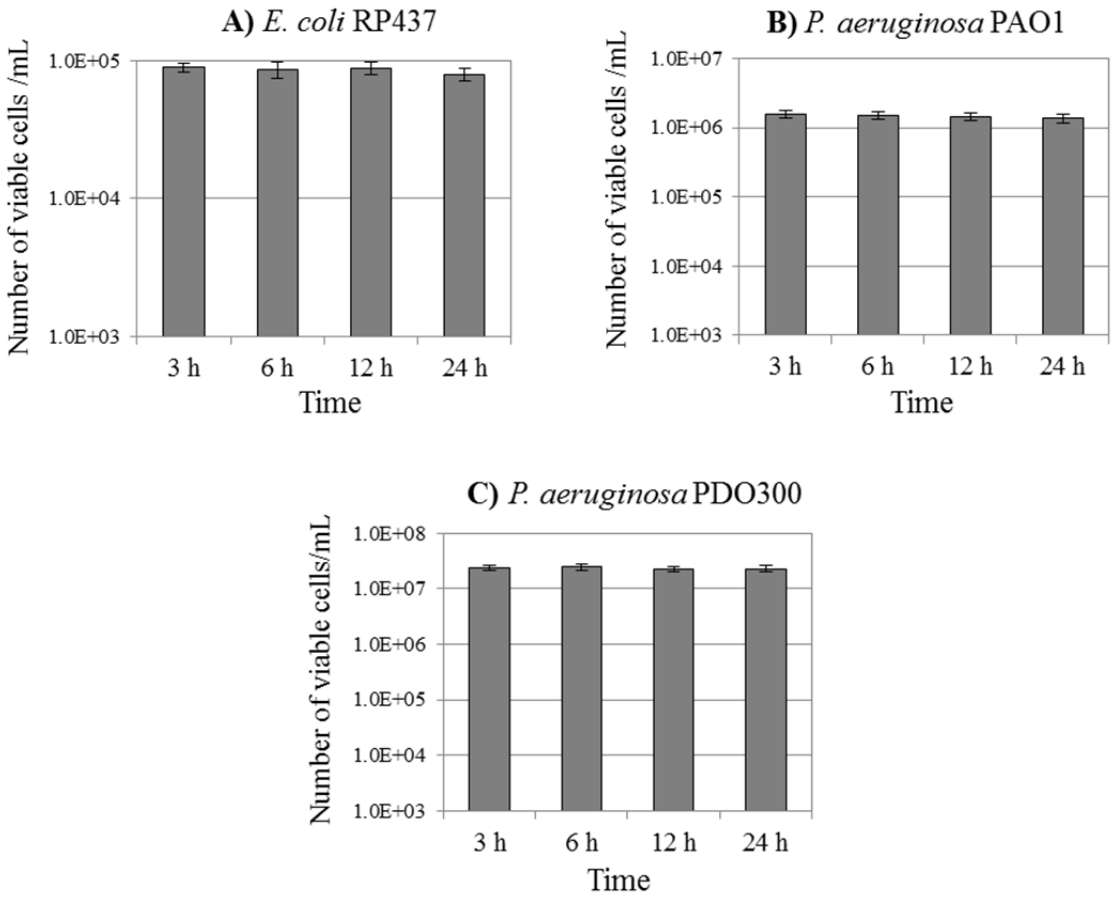

Figure 3-4. Extended treatment time did not increase the killing of planktonic cells of *E. coli* and *P. aeruginosa* by TN-5. Each sample was treated with 100 μM TN-5 for 3, 6, 12, or 24 h. Three independent replicates were tested for each condition. *E. coli* RP437 (A), *P. aeruginosa* PAO1 (B), and *P. aeruginosa* PDO300 (C) were studied.

Significant killing effects were also observed on planktonic cells of *P. aeruginosa* PAO1 and PDO300 strains. For example, killing of 22.7±6.7% (p<0.001), 61.8±4.5% (p<0.001), 74.7±11.8% (p<0.001), and 97.8±10% (1.6 log; p<0.001) of *P. aeruginosa* PAO1 was observed when TN-5 was added at 5, 20, 50, and 100 μM respectively (Fig. 3-3B). For *P. aeruginosa* PDO300, significant killing was observed at 50 and 100 μM with 44.1±3.1% (p<0.001) and 94.1±6.8% (1.23 log; p<0.001) of the total population (>99% as normal cells) killed,

respectively (Fig. 3-3C). These results show that TN-5 is also effective against normal planktonic cells of *P. aeruginosa*.

3.4.3 Antimicrobial effects of TN-5 on persister cells

TN-5 was found to kill persister cells of *E. coli* RP437, *P. aeruginosa* PAO1 and *P. aeruginosa* PDO300 (Fig. 3-5). The killing of *E. coli* RP437 persister cells was 33.8±0.8% ($p<0.001$), 43.8±2.8% ($p<0.001$), and 96.3±3.0% (1.35 log; $p<0.001$) when TN-5 was added at 50, 100, and 200 μM, respectively (Fig. 3-5A).

Similarly, TN-5 was also able to reduce the viability of *P. aeruginosa* PAO1 persister cells where 79.6±2.9% ($p<0.001$) and 89.9±4.5% ($p<0.001$) of cells were killed by 100 μM and 200 μM of TN-5, respectively (Fig. 3-5B). In comparison, TN-5 was less effective on persister cells of the mucoid strain *P. aeruginosa* PDO300, with only 33.2±5.6% ($p<0.001$) and 36.4±5.2% ($p<0.001$) killed by 100 μM and 200 μM of TN-5, respectively (Fig. 3-5C). This is likely due to the presence of alginate on the surface of the mucoid *P. aeruginosa* PDO300 cells [39], which may reduce the penetration by TN-5.

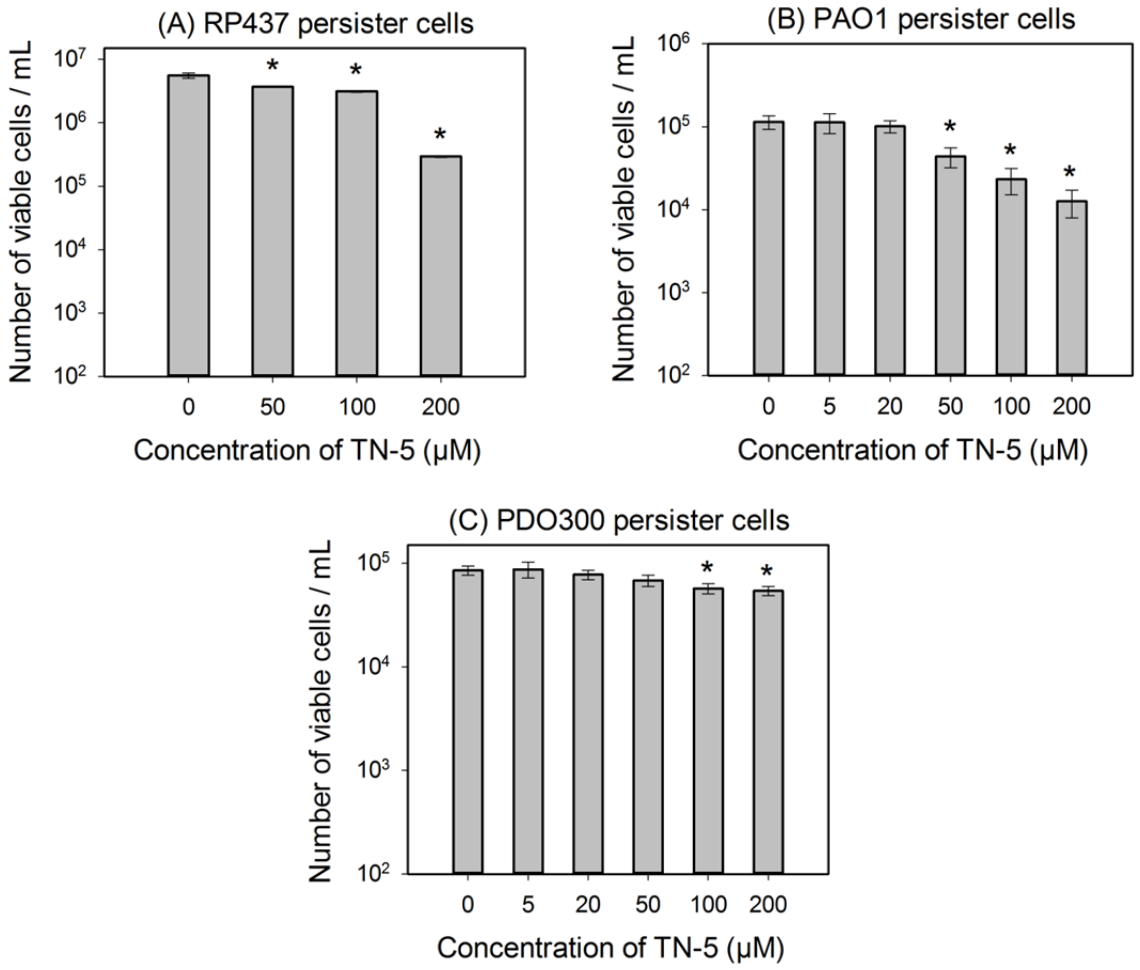

Figure 3-5. Effects of TN-5 on persister cells of *E. coli* RP437 (A), *P. aeruginosa* PAO1 (B), and PDO300 (C). The persister cells were isolated by treating exponential cultures of *E. coli* RP437 with 100 μg/mL ampicillin and overnight *P. aeruginosa* cultures with 200 μg/mL ciprofloxacin (both for 3 h). All significant differences (compared to the TN-5 free control) with $p<0.01$ are marked with an asterisk.

3.4.4 Antimicrobial effects of TN-5 on biofilm cells

TN-5 exhibited effective and dose dependent killing of *E. coli* RP437 biofilms. Killing of 78.5±30.2% (p<0.001) and 98.9±9.6% (1.24 log) (p<0.001) of *E. coli* RP437 biofilm cells was achieved when TN-5 was added at 100 and 200 µM, respectively (insignificant at 50 µM) (Fig. 3-6). In addition to the effects on established biofilms of *E. coli* RP437, TN5 effectively prevented biofilm formation of all the strains used in this study. Complete biofilm inhibition of *E. coli* RP437 and *P. aeruginosa* (both PAO1 and PDO300) was achieved with 10 and 20 µM TN-5, respectively (Fig. 3-7). However, 100 µM TN-5 alone did not show significant killing of cells in established biofilms of *P. aeruginosa* PAO1 or PDO300 (p>0.1) (Fig. 3-8A and 3-8C).

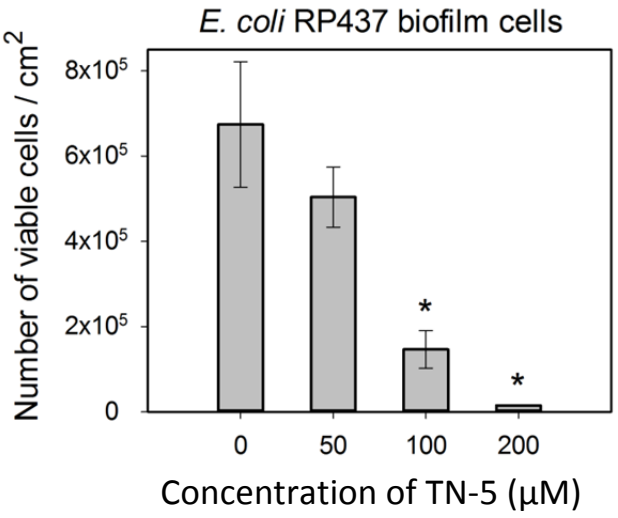

Figure 3-6. Effects of TN-5 on *E. coli* RP437 biofilms. The biofilms were cultured for 24 h in LB on stainless steel coupons prior to treatment with TN-5. All significant differences (compared to the TN-5 free control) with p<0.01 are marked with an asterisk. Note: Data are plotted in linear scale.

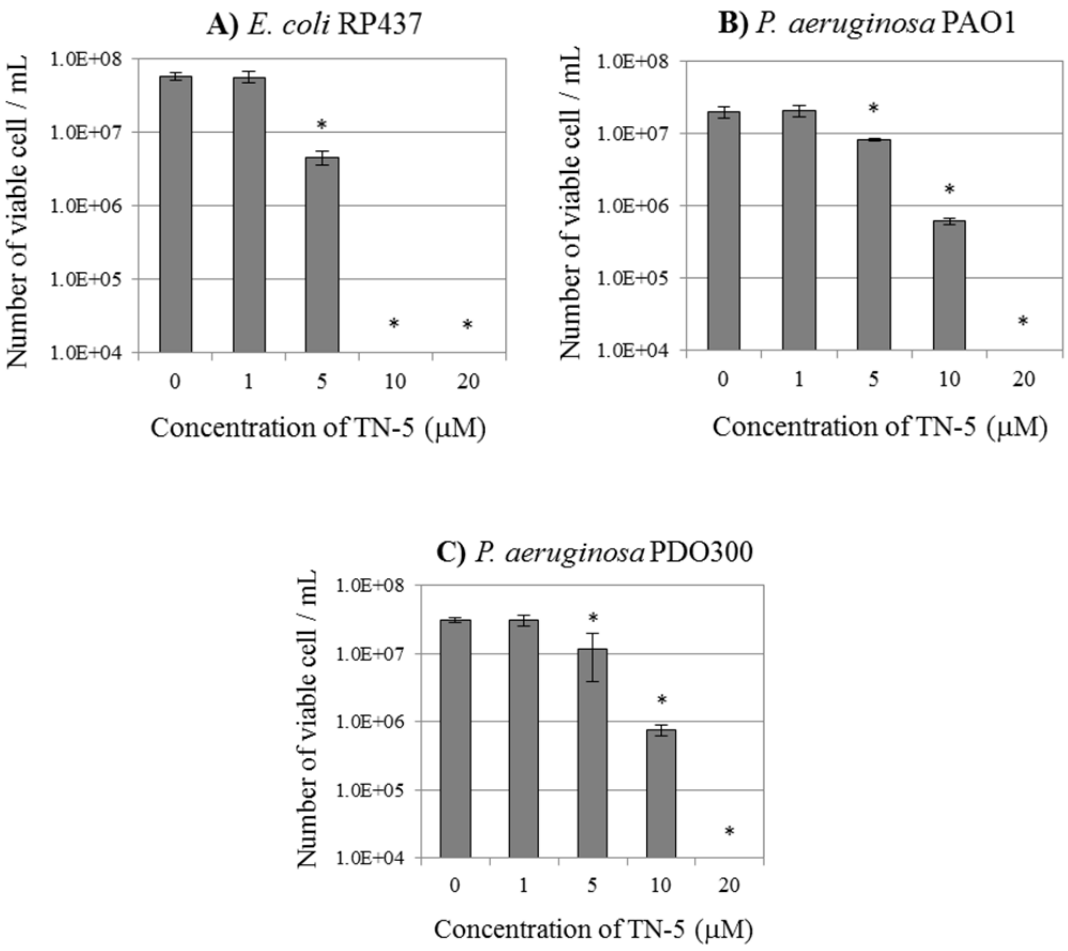

Figure 3-7. Effects of TN-5 on biofilm formation of *E. coli* RP437 (A), *P. aeruginosa* PAO1 (B), and *P. aeruginosa* PDO300 (C). Initial biofilm cultures were supplemented with TN-5 at different concentrations and incubated for 24 h at 37˚C. Then coupons were sonicated and vortexed to count CFU in biofilms. Three independent replicates were tested for each condition. All significant differences (compared with the TN-5 free control) with $p<0.01$ are marked with an asterisk.

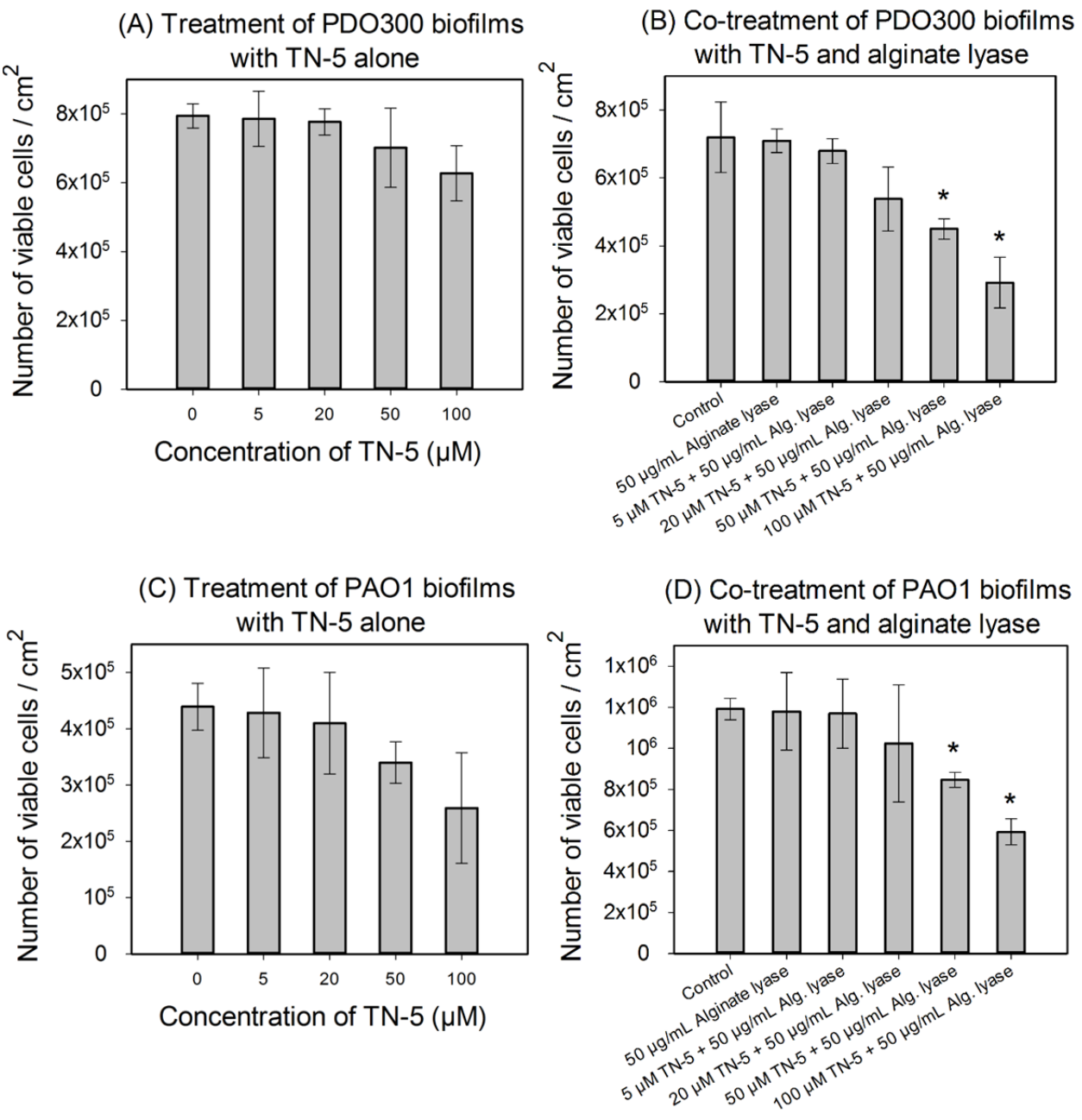

Figure 3-8. Effects of TN-5 alone and co-treatment with alginate lyase on *P. aeruginosa* PDO300 (A&B) and PAO1 (C&D) biofilms. The biofilms were grown for 24 h and treated with TN-5 alone or in combination with alginate lyase for 3.5 h. All significant differences (compared with the TN-5 free control) with $p<0.01$ are marked with an asterisk. The co-treatment with TN-5 (at 50 or 100 μM) and 50 μg/mL alginate lyase caused significant killing than the control (with

no TN-5 and alginate lyase) for both *P. aeruginosa* PDO300 and PAO1, but only significantly increased the killing by TN-5 alone for *P. aeruginosa* PDO300. Note: Data are plotted in linear scale.

We speculated that the lack of antimicrobial effects against *P. aeruginosa* biofilms is because of the presence of biofilm matrix. Alginate is a major component of biofilm matrix of the mucoid *P. aeruginosa* strains [13,39]. Thus we tested the concurrent treatment of *P. aeruginosa* biofilms with alginate lyase and TN-5. As shown in Fig. 3-8B and 3-8D, alginate lyase itself showed no effect on the viability of biofilm cells (p=1). However, the combination of TN-5 with alginate lyase increased the killing of PDO300 biofilms; e.g., addition of 50 μM alginate lyase increased the activity of 50 μM TN-5 on *P. aeruginosa* PDO300 biofilms from no significant killing (p=0.58) to 36.5±4.6% (p<0.005) and that of 100 μM TN-5 from insignificant killing (p=0.12) to 57±9.6% (p<0.005), respectively (Fig. 3-8A vs. Fig. 3-8B). The addition of alginate lyase did not show the same effects on *P. aeruginosa* PAO1 biofilm cells; e.g., 50 μM alginate lyase did not increase the effects of 50 and 100 μM TN-5 (p>0.1) (by comparing the % of killing based on corresponding data between Fig. 3-8C & Fig. 3-8D). This is likely because alginate is not a major component of its biofilm matrix.

3.5 Discussion

AMPs have been proposed as a promising source of new antimicrobial agents [40-43]. Different strategies have been tested to achieve effective killing of microbes while maintaining low hemolytic activity. In some of these studies, Trp (W) and Arg (R) containing 1,3,5-triazine structures [32] and dendrimeric peptides [44] have been used as AMP templates and lead compounds with antimicrobial activities and low toxicity to red blood cells have been identified.

In this study, one of the triazine-1,3,5 derivatives, TN-5, was tested on Gram-negative bacteria *E. coli* and *P. aeruginosa*. TN-5 was found to be effective against both species. The killing of persister and biofilm cells is of particular interest because these cells are difficult to eliminate and conventional antibiotics are generally ineffective. Our results show that TN-5 alone is effective against persister and biofilms of *E. coli* RP437 with up to 2-log killing achieved at 200 μM. It is also interesting that TN-5 caused more killing of biofilm cells than planktonic persister cells of *E. coli*. For example, 100 μM TN-5 killed 43.8±2.8% and 78.5±30.2% of persister and biofilm cells of *E. coli* RP437, respectively. This finding suggests that TN-5 can penetrate the biofilm matrix of *E. coli*.

Comparable effects were not observed for *P. aeruginosa* biofilms, however, possibly due to the difference in biofilm matrices. With a thick layer of alginate, biofilms of mucoid bacteria have high-level tolerance to some antimicrobials [13]. Thus, breaking down alginate in the biofilm matrix could be essential for certain antimicrobials to kill biofilm cells, especially the agents that can be absorbed or neutralized by the matrix components. Consistently, we found that the killing of *P. aeruginosa* PDO300 biofilm cells was enhanced by alginate lyase. It is worth noting that TN-5 is effective against *P. aeruginosa* PDO300 persister cells, but not its biofilm cells. Thus, it will be interesting to study if and how TN5 interacts with alginate directly and if there is any difference in the amount and structure of alginate between biofilm matrix and the surface of mucoid cells. In comparison, the killing of *P. aeruginosa* PAO1 biofilms was not enhanced by alginate lyase. This is consistent with the report [45] that alginate is not the primary component of *P. aeruginosa* PAO1 biofilm matrix. It will be interesting to test other matrix degrading enzymes, such as DNase.

In a previous study performed in our lab, Trp- and Arg- containing synthetic antimicrobial peptides were shown to inhibit 95.0 ± 1.1% of biofilm formation at 200 μM [46]. Intriguingly, TN-5 inhibited biofilm formation of both *E. coli* and *P. aeruginosa* completely at 20 μM (Fig. 3-7). Therefore, TN-5 may also be an important antimicrobial agent for biofilm control.

The mechanism of bacterial killing by TN-5 or in fact any AMP deserves more study. The positive charge of the arginine (R) side chain can help an AMP to interact with negatively charged lipopolysaccharides in Gram-negative bacterial walls [47]. Tryptophan (W) residues favor location below the head groups of bilayers, and are assumed to provide lipophilic interaction sites which cause membrane disruption [48]. Thus, RW mimicking TN-5 might target the negatively charged bacterial cell membrane. Another observation is that TN-5 mode of action occurs within the first 3 h (Fig. 3-2).

E. coli is generally more susceptible to antibiotics than *P. aeruginosa*. Among 47 antimicrobial agents tested in an early study [49], only tobramycin showed the same MIC value (0.5 μg/mL) for *E. coli* (NCTC 10418 and ATCC 25922) and *P. aeruginosa* (NCTC 10662 and ATCC 27853) reference strains. The other antimicrobial agents tested all showed higher (up to 500 fold) MIC values for *P. aeruginosa* compared to *E. coli* [49]. Interestingly, TN-5 showed the same MIC value (12.8 μM) for both species in our study, which also indicates possible membrane targeting activities.

Overall, this study shows that the triazine derivative TN-5 is a promising lead compound for developing new synthetic AMPs. It is encouraging that TN-5 is effective against both *E. coli* and *P. aeruginosa* at different growth stages. Persister cells of *Pseudomonas* strains, especially those of the mucoid strains, are highly resistant to antibiotic treatments. Because most AMPs kill

microbes through mechanisms that differ from those of conventional antibiotics, e.g. by targeting cell membranes, the killing effect of TN-5 on persister cells might be increased by synergy with some antibiotics if TN-5 can be effectively delivered to target cells. We assume that triazines are not susceptible to proteolytic degradation since there is no specific motif to trigger cleavage. Future experiments with mammalian cells and animal models are needed to evaluate the potential to use TN-5 and antibiotics together as a novel therapy for chronic infections involving biofilms and persister cells.

3.6 Acknowledgements

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CHAPTER 4

CONTROLLING PERSISTER CELLS BY TARGETING MEMBRANE POTENTIAL DEPENDENT ANTIBIOTIC EFFLUX

4.1 Introduction

Persister cells were first defined in 1944 when Bigger showed surviving *Staphylococcus* after penicillin treatment. Re-inoculation of surviving cells gave rise to colonies, the majority of which can again be lysed by penicillin [1]. Forming metabolically inactive cells is one of the major strategies of bacterial survival in harsh conditions such as antibiotic treatments and host's immune reactions. These dormant persister cells are highly tolerant to antibiotics and thus serve as a reservoir for the population to reestablish after the treatment leading to recalcitrance of chronic infections [2].

To date, the mechanism of persister formation and physiological characteristics of persister cells are still not fully understood. Shah *et al*. [3] labeled *E. coli* ASV cells with a unstable GFP to follow cell growth activities with fluorescence under the control of ribosomal *rrnB*P1 promoter. Fluorescence-activated cell sorting (FACS) based on GFP gave two distinct populations; the bright normal cells and dim persister cells. Visualization with microscopy and further antibiotic treatment of these two populations revealed that persister cells are shorter than normal cells. Persister also exhibits a gene expression profile with increased level of SOS stress response, phage-shock and heat and cold-shock genes [4].

Previous efforts to determine the genes specifically responsible for persister formation by screening knock-out mutant libraries have been largely unsuccessful [5,6]. Increasing evidence indicated that there are redundant pathways leading this dormancy stage. Some best characterized systems associated with persistence include toxin/antitoxin (TA) modules and the SOS response system. For example, persister cells have increased level of TisB protein, which decreases proton motive force and the ATP level and thus causes the cells to enter dormancy [2].

Essentially all bacterial populations include a small portion of persister cells [7]. These cells have low cellular energy due to their dormancy. Normal bacterial cell membrane has relatively high transmembrane potential, between –120 to –200 mV, which is important to ATP production and provides energy for many cellular processes [8]. Thus we hypothesize that persister cells have lower membrane potentials than normal cells and this temporary metabolic downshift might inactivate efflux based antibiotic resistance mechanism leading to intracellular accumulation of antimicrobials and kill persister cells during wakeup.

Bacterial cell membrane is a semipermeable phospholipid bilayer with embedded proteins. This membrane plays an important role in the growth and stress response of bacteria. It is also the part of cell that antibiotics must penetrate to take action. The membrane permeability is affected by its composition [9]. Transporters, ion channels, ATP synthases, receptors, antigens and some signaling proteins are found in the membrane and represent about one third of the total proteins in a cell. These proteins are responsible for many important biological functions such as cell–cell contact, surface recognition, signaling, enzymatic activities, and transporting substances across the membrane [10]. An important function of cell membrane is to generate proton motive force, which requires the activities of ATP synthases, motility, DNA repair, and synthesis of macromolecules such as polysaccharides, proteins, nucleic acids, and numerous other processes in all living cells. Therefore, disturbance of membrane potential may have profound effects on the physiology and viability of bacterial cells.

Energy-dependent drug efflux systems embedded in cell membranes play an important role in bacterial drug resistance [11,12], which requires proton motive force generated with appropriate membrane potential [13]. Most efflux pumps are proton pumps with a broad spectrum of target substrates [14]. For example, *E. coli* cells are resistant to erythromycin molecules via such efflux

pumps [15] and this activity depends on the energy produced by ATP synthase [16]. Some efflux pumps and their substrate antibiotics are summarized in Table 4-1.

Table 4-1. Bacterial efflux pumps and substrate antibiotics

Efflux family	Efflux pump	Resistance against	Ref
RND	AcrAB	Eyrthromycin, tetracycline, chloramphenicol, clarithromycin, ampicillin, nalidixic acid, and rifampin	[17, 18]
ABC	YbjYZ	Clarithromycin, azithromycin, erythromycin	[15, 19]
MFS	MdfA	Rifampin, tetracycline, chloramphenicol	[20]
MATE	NorM, YdhE	Norflaxacin, fluoroquinolones, rhodamine 6G, acriflavine, berberine, novobiocin, enoxacin, and tetraphenylphosphonium chloride	[21-23]
SMR	EmrE	Tetracycline and tetraphenylphosphonium	[24]

It has been reported that efflux pump mutants of *Mycobacterium* accumulate more ethidium bromide (EtBr) inside the cells and consequently higher susceptibility to antimicrobials; such as rifampicin, ethambutol, ciprofloxacin, streptomycin, amikacin, clarithromycin and erythromycin [25]. All these antimicrobial agents act on energy-dependent metabolic pathways such as RNA synthesis (rifampicin), cell wall synthesis (ethambutol [26]), protein synthesis (streptomycin, amikacin, clarithromycin, and erythromycin [27-30]), and function of DNA gyrase [31].

Efflux pumps have a wide variety of substrate antibiotics. For example, erythromycin, tetracycline and chloramphenicol, which target protein synthesis by binding and inhibiting ribosome complex, are all substrates of RND efflux family. These pumps are widespread especially in Gram-negative bacteria and are active against many different antibiotics [32].

To test if persister cells have lower membrane potentials than normal cells, we characterized the membrane potential and efflux activities of normal and persister cells of *E. coli* and compared the killing effects of erythromycin, chloramphenicol and tetracycline on normal and persister cells of *E. coli*. These antimicrobial agents were chosen among ribosome targeting antibiotics. Under normal conditions *E. coli* efflux pumps use these antibiotics as substrate [18, 33-36]. However this efflux pump activity requires membrane potential and proton motive force. Therefore lower membrane potential may result in accumulation of antibiotics in treated cells.

Erythromycin, which is a macrolide antibiotic, generally shows bacteriostatic effect by inhibiting protein synthesis. Since it targets the ribosome complex, the production of all the peptides including the ones essential to bacteria survival is inhibited. Erythromycin is used to treat several serious bacterial infections, such as pneumonia, bronchitis, diphtheria, pertussis, rheumatic fever, and epithelial infections. In 1986 no clinical isolate was found resistant to erythromycin [37]; however, a study in 2009 [33] showed that the majority of bacterial strains among 190 isolates collected from 5 different countries have MIC values higher than 128 μg/mL, indicating a rapid development of resistance. Erythromycin susceptibility can be restored with an AcrAB-TolC efflux pump inhibitor in *E. coli* [18]. Therefore, we hypothesize that lower membrane potential is expected to inhibit the activity of efflux pumps and increase the susceptibility of *E. coli* cells to erythromycin.

4.2 Materials and Methods

4.2.1 Efflux activity

The activity of efflux pumps was monitored with EtBr, by measuring its accumulation inside the cells. EtBr gives strong fluorescence when interacting with nucleotides [38] and can be pumped out by efflux systems of *E. coli* in the presence of proton motive force across cell membrane. Thus, the accumulation of EtBr inside the cells is a cumulative result of membrane permeability and efflux activity [39].

E. coli Top10 pBAD *hipA*, a strain with inducible persister formation (engineered by Jing Wang in Ren lab) was used to test efflux activities. This strain can form large number of persister cells upon induction of *hipA* gene expression by 0.2% arabinose. *E. coli* Top10 pBAD *hipA* culture was grown in Lysogeny broth (LB) containing 10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone with pH 7.0.

To induce persister formation, O/N culture of *E. coli* Top10 pBAD *hipA* was sub-cultured with a starting cell density of 0.01 at OD_{600} and incubated till the OD_{600} reached 0.15-0.2. Then the culture was supplemented with 0.2% arabinose and incubated for another 3 h at 37˚C with shaking at 200rpm to induce the persister formation. Additionally, 50 µg/mL tetracycline was added and incubated for 0.5 h following arabinose induction for further persister formation. After induction with arabinose and tetracycline, the culture was harvested by centrifuging at 10,000g for 8 minutes and resuspended in PBS by gently vortexing for 15 seconds. These cells were washed again with PBS and used for EtBr staining and persister count.

A portion of each induced and uninduced sample was taken and used to determine the persistence level by treating with 100 μg/mL ampicillin and counting CFU. The rest of each sample was treated with 25μg/ml EtBr to evaluate membrane permeability by measuring fluorescent signal from EtBr-nucleic acid complex of the cells. Following EtBr treatment, the cells were washed with PBS to get rid of excess EtBr signal in extracellular media and 200 μL cell suspension from each treatment was plated in a clear bottomed black walled 96 well plate for measuring fluorescence using a microplate reader (model FLx800, Bio-Tek Instruments, Winooski, VT, USA) with excitation at 360 nm and emission at 590 nm in PBS. Each experimental condition was tested with three independent cultures (three biological replicates).

4.2.2 Flow-cytometer compensation and analysis of EtBr treated samples

Flow cytometry was used to corroborate the results following persister induction and EtBr staining. To compare with the results of the wild-type *E. coli* K12, we also tested a *ΔacrB* mutant strain obtained from the Keio collection [40]. This mutant lacks efflux pump activity regardless the membrane potential. The exponential cultures of *E. coli ΔacrB* were stained with 25μg/ml EtBr for 10 minutes. Then excess EtBr dye was washed away with PBS. The changes in fluorescence intensity were determined using flow cytometry (Fig. 4-1).

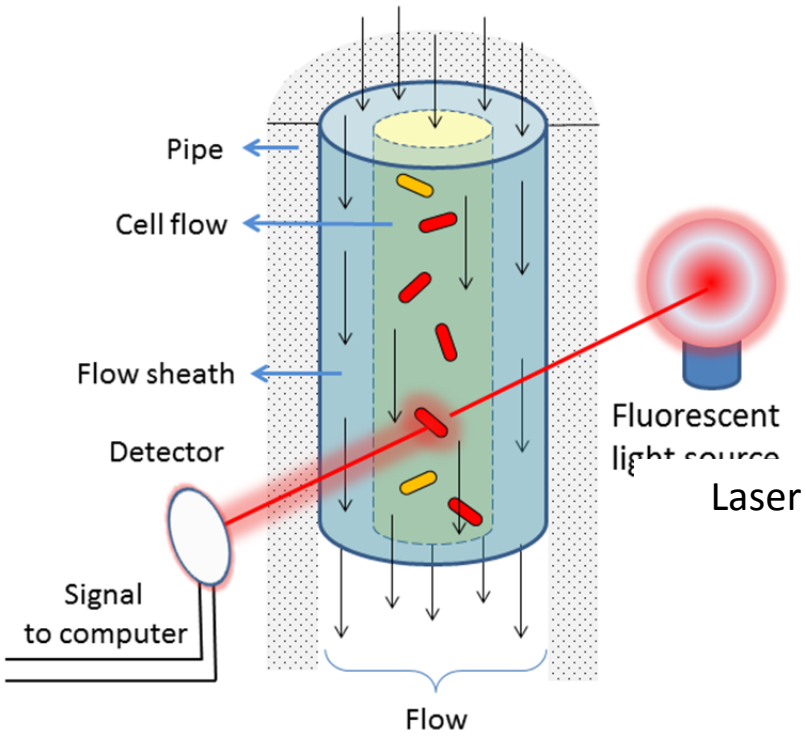

Figure 4-1. The principle of flow cytometry. The cells are dispersed in a stream of fluid so that they can individually travel through fine tubing and detected. The signal from each event is recorded by a computer and analyzed.

To test EtBr staining of persister cells with flow cytometry, an exponential culture of *E. coli* Top10 pBAD *hipA* was induced with 0.2% arabinose as described previously and compared with an uninduced control by staining with EtBr and analyzing with flow cytometry. In addition to the efflux mutant and high persistence strain, exponential culture of *E. coli* ASV strain was also used with flow cytometry to test if there is a higher EtBr accumulation in persister cells (dim population).

4.2.3 Comparing membrane potential of persister and normal cells with potentiometric dye and flow cytometry

To corroborate the EtBr results and further understand if persister formation affects membrane potential, we used a potentiometric dye, JC-1, to compare the membrane potential of normal planktonic cells and persister cells. JC-1 stains mitochondria and bacterial cells based on membrane potential [41]. This dye can give two different colors; red fluorescence when the dye molecule aggregate and green fluorescence from free dye molecules. With a high membrane potential, more JC-1 molecules can aggregate on the membrane and emit red fluorescence. In comparison, when the membrane potential lowered; less JC-1 accumulates in the membrane. This leads fluorescence dye in cytoplasm to give a green color. Thus a shift in red/green fluorescence can indicate the change in membrane potential.

To perform this experiment, a high persistence strain, *E. coli* Top10 pBAD *hipA*, was grown exponentially at 37°C by shaking with 200 rpm till OD_{600} reached 0.2-0.3. Then the cells were washed with centrifugation and resuspended in PBS. Ten μL JC-1 dye was added per 100 – 300 μL cell sample. After gentle mix by slightly tapping the tubes, cell and potentiometric dye mixture was incubated at 37°C for 15 minutes in dark. After incubation, the cells were centrifuged and excess JC-1 dye was washed away. Immediately after washing, samples were analyzed with flow cytometry by monitoring green and red fluorescence.

4.2.4 Activity of efflux substrate antibiotics on persister cells

Persister cells of *E. coli* HM22 and Top10 pBAD *hipA* strains were isolated as described previously. Isolated persister cells were treated with erythromycin, tetracycline and

chloramphenicol for 3.5 h at 37°C with 200 rpm shaking. After the treatment, surviving persister cells were washed twice with PBS buffer to remove any remaining antibiotic. The collected cells were resuspended in PBS plated to determine the viability by counting CFU as described previously.

4.2.5 Erythromycin accumulation of persister cells

To verify if persister cells did accumulate more erythromycin than normal cells, we conducted an experiment to treat the cells with erythromycin first and compared the antimicrobial activities of the lysates from these cells. We also directly measured the intracellular concentration of erythromycin using mass spectrophotometry (MS).

Briefly persister cells were isolated from exponential cultures of *E. coli* HM22 by treating with 100μg/mL Amp for 3.5 hours to kill normal cells. The CFU of normal and persister cell culture samples were normalized based on OD_{600} readings. Both samples were treated with 100 μg/mL erythromycin in PBS for 10 minutes. Then cells were collected by centrifugation and supernatant samples were stored at -20˚C till MS analysis and killing experiment. The cell pellets were sonicated (5 cycles at 100% amplitude with 30 s treatment for each cycle in ice-water) and pipetted up and down to disrupt the cell membrane. Then, cell lysates were filtered through 0.2 μm filters and sent for MS analysis to Dr. Myriam Cotton at Hamilton College, Clinton, NY. A ^{13}C labeled erythromycin was used as an internal standard to verify the molecular weight of erythromycin used in this study. The samples were also tested on fresh planktonic cultures of *E. coli* HM22 for their killing activity and compared to that of 50 μg/ml erythromycin.

4.3 Results

4.3.1 Efflux activity of normal and persister cells

The induction of persister formation with arabinose was successful and the persister population was 0.16% in uninduced sample, and 17% with induction with arabinose and Tet. The isolated persister cells were treated with EtBr solution to see if persister cells could accumulate more EtBr intracellularly due to lower activity of efflux pumps. As expected, it was found that the induced sample had 29.9±1.6% more EtBr signal than uninduced sample after 10 minutes of incubation (Fig. 4-2).

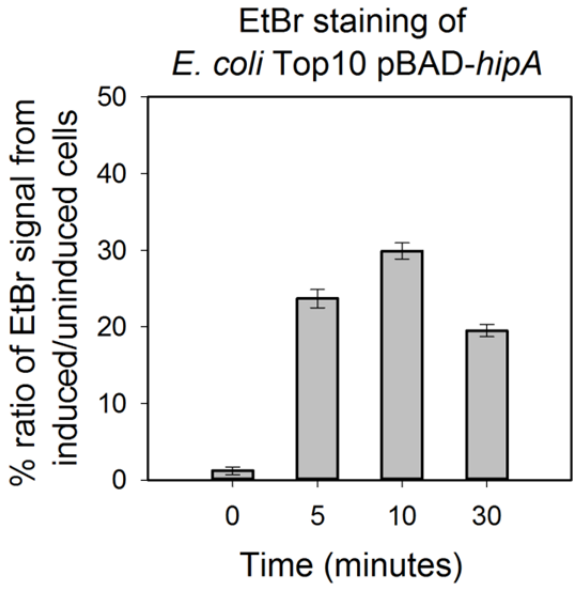

Figure 4-2. Ratios of EtBr signal of induced cells / uninduced cells. EtBr signals were measured using a fluorescence microplate reader (model FLx800, Bio-Tek Instruments, Winooski, VT, USA) with excitation at 360 nm. The ratios; 1.12±0.8%, 24.3±3.2%, 29.9±1.6%, and 18.9±1.5% at 0, 5, 10, and 30 minutes after adding EtBr are shown.

4.3.2 Flow cytometry analysis of efflux activity with EtBr staining

The results show that adding EtBr caused *E. coli ΔacrB* cells, which have no efflux activity, to shift from low red fluorescence (less than 10^3) to high red fluorescence area (higher than 10^3) (Fig. 4-3). This finding proves that EtBr accumulates inside the cells if the efflux activity is not present, and demonstrates that flow cytometry analysis with EtBr staining is effective in membrane potential studies.

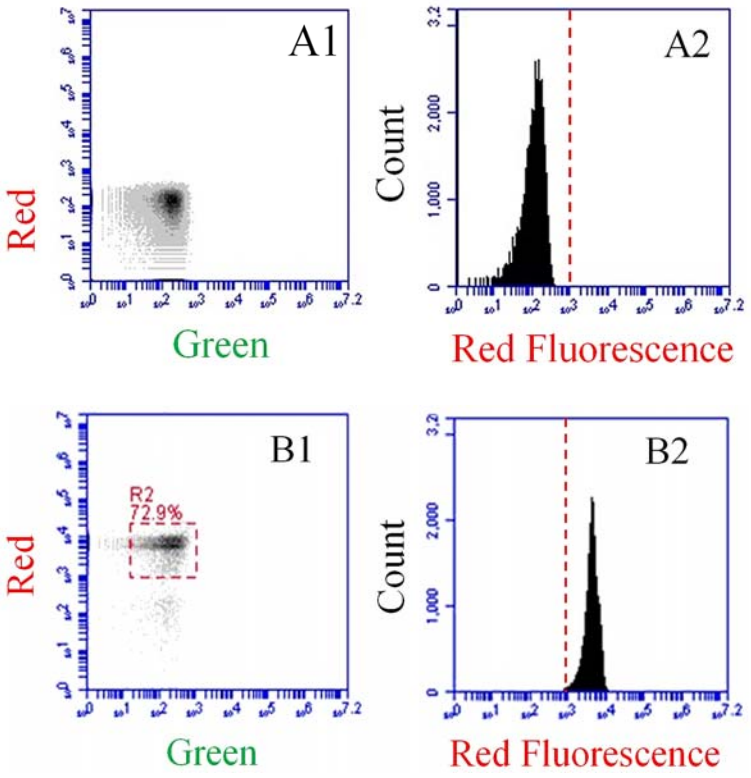

Figure 4-3. Flow cytometry data showing fluorescent signals of unstained and EtBr stained *E. coli* Δ*acrB* cells. The cells were analyzed for their green (FL1) and red (FL2) fluorescence. A1) and A2) are unstained samples while, B1) and B2) are EtBr stained samples.

4.3.3 Persister induction

E. coli Top10 pBAD *hipA* (Fig 4-4 A1 and 4-4 A2) cells that were not induced for persister formation did not show significant fluorescent signal after incubation with EtBr for 10 min (Fig. 4-4 B1 and 4-4 B2). In contrast, 13% of the cells in arabinose induced sample (circled with red dotted line) showed more than 10 fold higher red fluorescent signal than the rest of the population and uninduced cells. Consistently, 18% of cells in this sample were confined to be persister cells based on CFU results. This finding shows that the persister population of *E. coli* Top10 pBAD *hipA* strain has lower efflux activity compared to normal cells, which is consistent with the results of *E. coli* Δ*acrB* efflux mutant. Since the efflux pump activity depends on membrane potential, these results suggest that persister cells have lower membrane potential than normal cells.

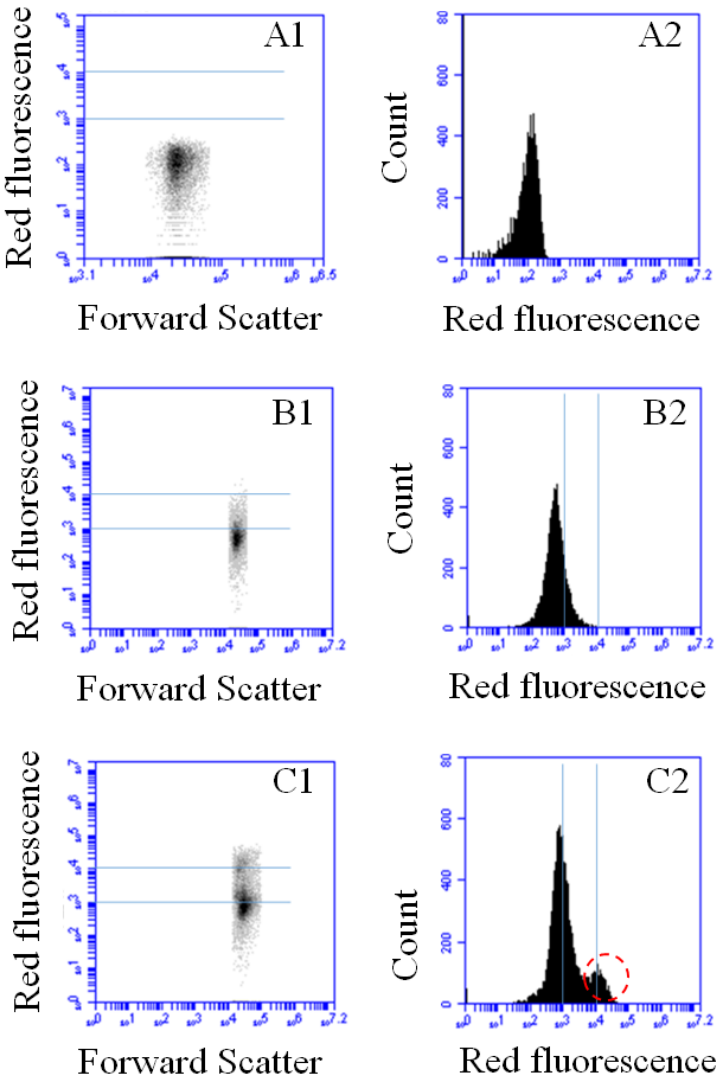

Figure 4-4. Persister formation led to increase in intracellular concentration of EtBr. A1 and A2; no induction and no staining, B1 and B2; without induction of persister formation, but with EtBr staining, C1 and C2; with induction of persister formation and EtBr staining. *E. coli* Top10 pBAD *hipA* was used in all the tests.

In addition to high level persistence strain, *E. coli* ASV which only has around 0.1% persister ratio was also tested with flow cytometry for their dim color compared to green active exponential cells with no staining. This small number of persister cells showed up as a distinct

population plot in cytometry analysis (Fig. 4-5). This strain is only green when the cells are active, and persister cells did not show any green color.

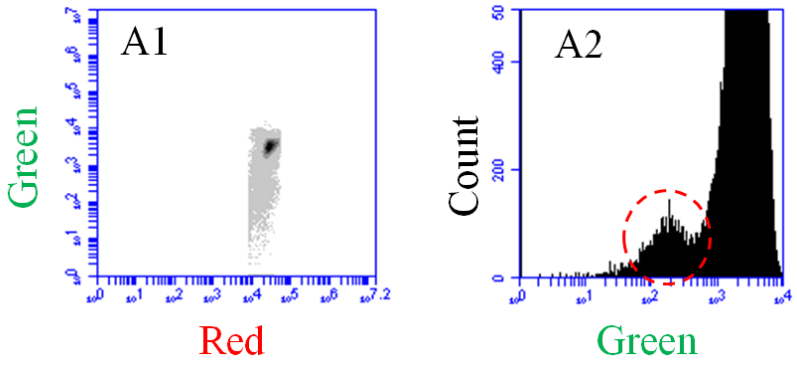

Figure 4-5. Flow cytometry analysis of *E. coli* ASV strain. Red circle shows the dim persister population.

4.3.4 Comparing membrane potential of persister and normal cells with potentiometric dye and flow cytometry

We further tested membrane potential using, *E. coli* Top10 pBAD *hipA* with and without the induction of persister formation with 0.2% arabinose. Fig. 4-6 shows that the induced persister formation led to decrease in red fluorescence, while the level of green fluorescence (stains all cells) did not change. This reduced level of red fluorescent suggest that less JC-1 dye molecules accumulated on the membrane due to lower membrane potential.

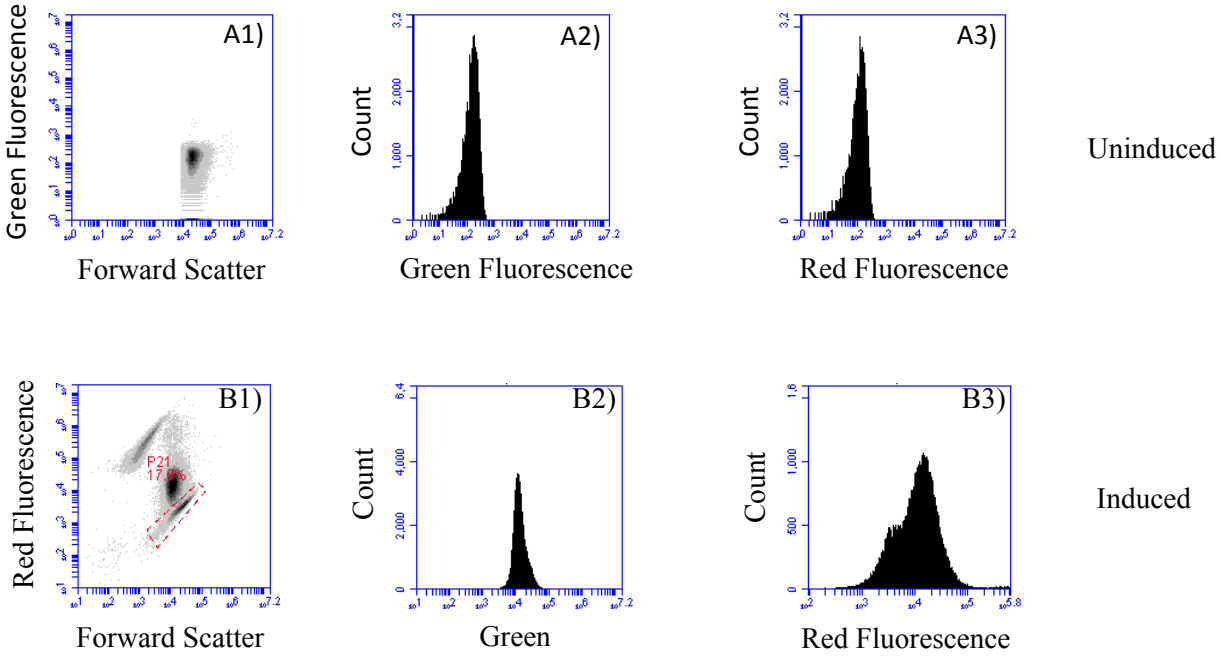

Figure 4-6. Potentiometric staining and flow cytometry results of uninduced (A1-A3) and induced (B1-B3) *E. coli* Top10 pBAD *hipA* cells. A2) and B2) show the signal of green fluorescence while A3) and B3) show the signal of red fluorescence.

4.3.5 Activity of efflux substrate antibiotics on persister cells

Among tested antibiotics 50µg/ml erythromycin killed 78.2% of *E. coli* HM22 persister cells after 3 h (Fig. 4-7B) while the same concentration has less than 30% killing on normal cells (Fig. 4-7A). This higher killing effect of erythromycin on persister cells could only be repeated when PBS is used as washing and treatment buffer. This indicates presence of some ions helped erythromycin effect to kill persister cells.

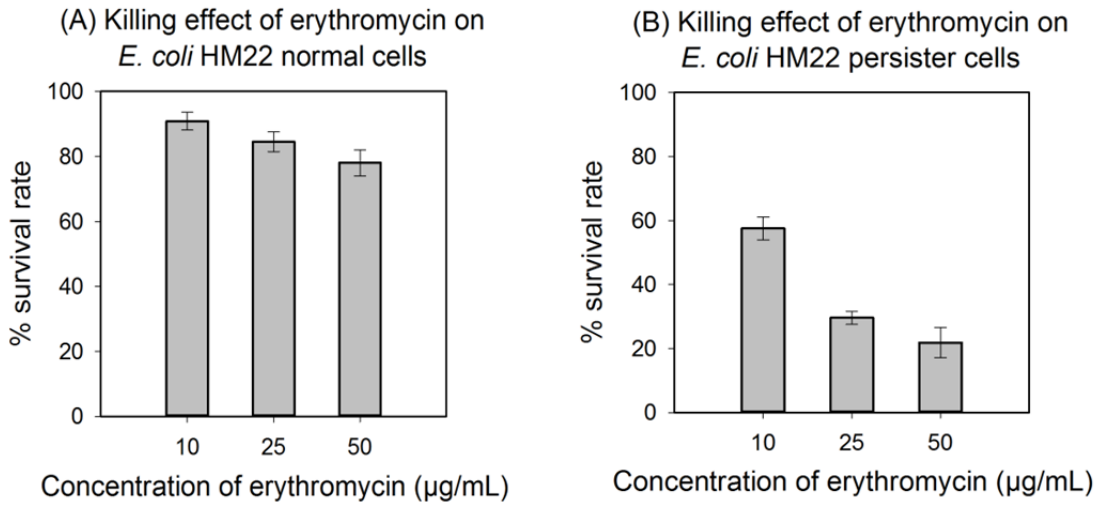

Figure 4-7. Effects of erythromycin on the viability of *E. coli* normal (A) and persister (B) cells. The number of cells before treatment was normalized as 100%.

4.3.6 Intracellular accumulation of erythromycin

As shown in Fig. 4-8, lysate from erythromycin treated persister cells exhibited higher killing activity on *E. coli* HM22 cells than that from erythromycin treated normal cells. As negative controls, cell lysates without pretreatment with erythromycin did not cause any significant change in cell viability (less than 4%). The killing by the lysate of persister cells treated with erythromycin pellet was 18.1 ± 3.6%, similar to while it was 19.4 ± 4.7% by adding 50 μg/mL erythromycin directly. This suggests that persister cells accumulated a high concentration of erythromycin.

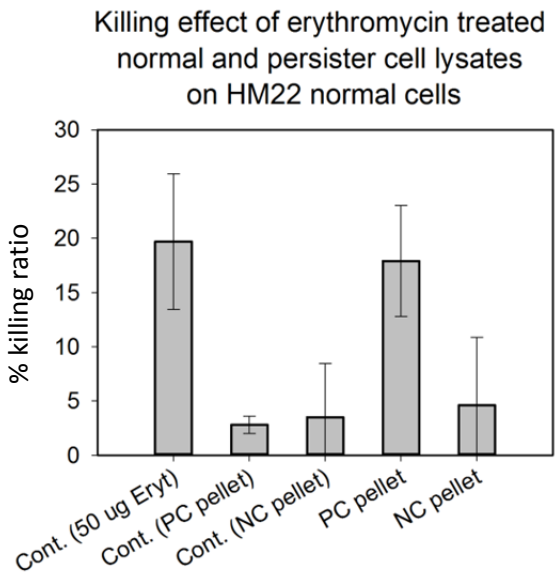

Figure 4-8. Antimicrobial effects of cell lysates from erythromycin treated normal & persister cells of *E. coli* HM22. NC; normal cells, PC; persister cells. In total 8 types of samples were tested; NExEr (normal cells extracellular erythromycin, Fig. 4-9), NInEr (normal cells intracellular erythromycin, Fig. 4-10), PExEr (persister cells extracellular eryhtromycin, Fig. 4-11), PInEr (persister cells intracellular eryhtromycin, Fig. 4-12), NEx13 (normal cells extracellular ^{13}C labeled eryhtromycin, Fig 4-13), NIn13 (normal cells intracellular ^{13}C labeled eryhtromycin, Fig 4-14), PEx13 (persister cells extracellular ^{13}C labeled eryhtromycin, Fig 4-15), PIn3 (persister cells intracellular ^{13}C labeled erythromycin, Fig 4-16).

Consistent with the CFU results, MS analysis revealed that persister cell pellet had relatively higher erythromycin accumulation compared to normal cells. As shown in Table 4-2, the extracellular erythromycin levels were significantly higher (at least 14 fold) than the intracellular erythromycin level in normal cells. This result confirmed that normal cells can actively pump out erythromycin. In contrast, this ratio becomes much lower (1.6) for persister cells (8.7 fold decrease). This finding suggests that persister cells can not actively pump the erythromycin out.

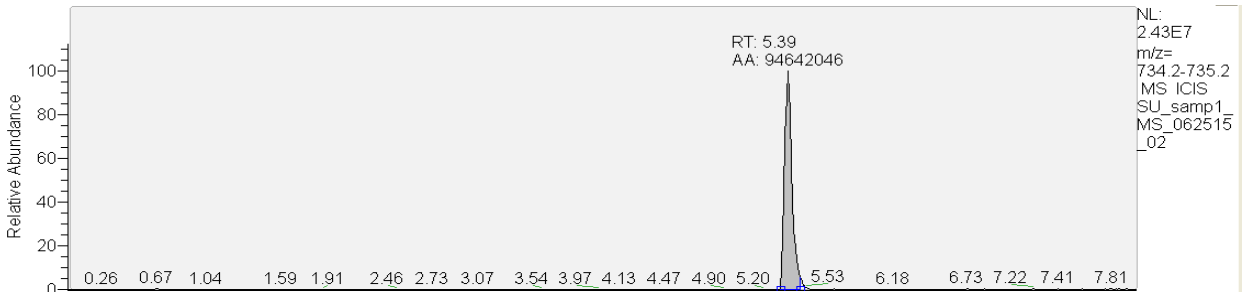

Figure 4-9. Mass Spectrophotometry analysis of erythromycin level in the extracellular supernatant of normal cell samples

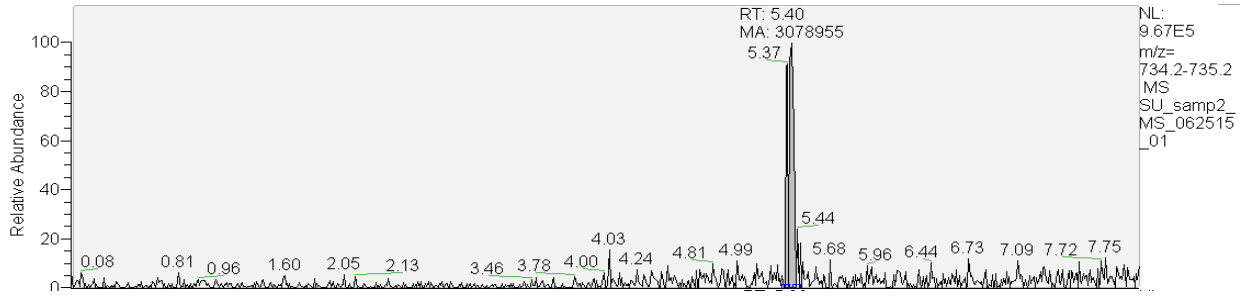

Figure 4-10. Mass Spectrophotometry analysis of intracellular erythromycin level in normal cell samples

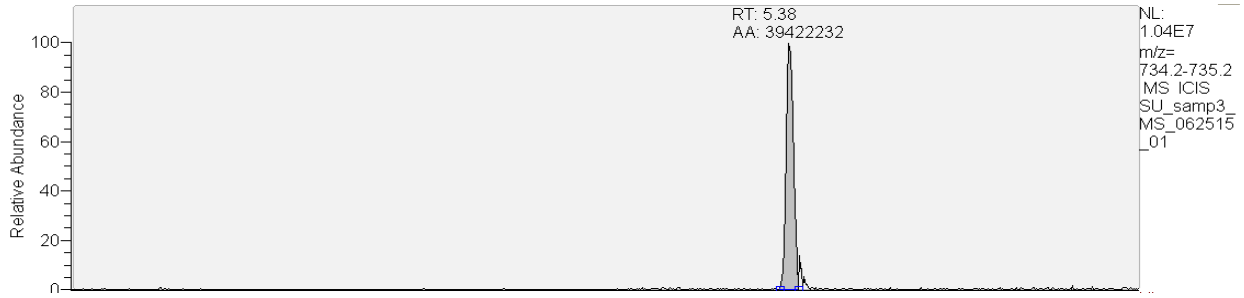

Figure 4-11. Mass Spectrophotometry analysis of erythromycin level in the extracellular supernatant of persister cell samples

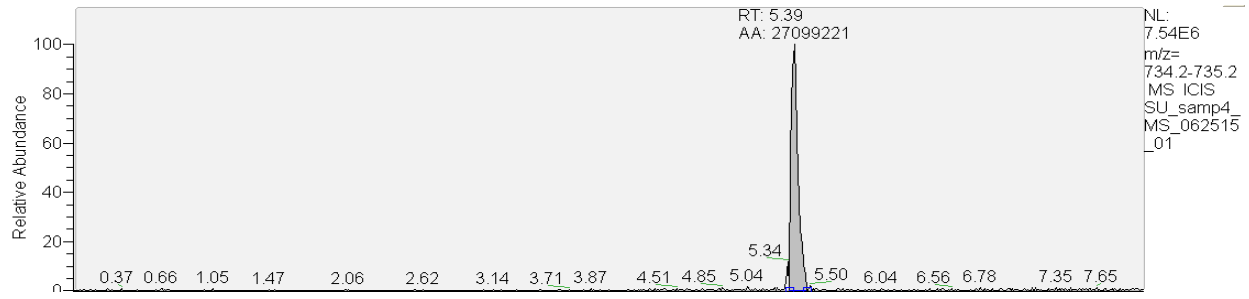

Figure 4-12. Mass Spectrophotometry analysis of intracellular erythromycin level in persister cell samples

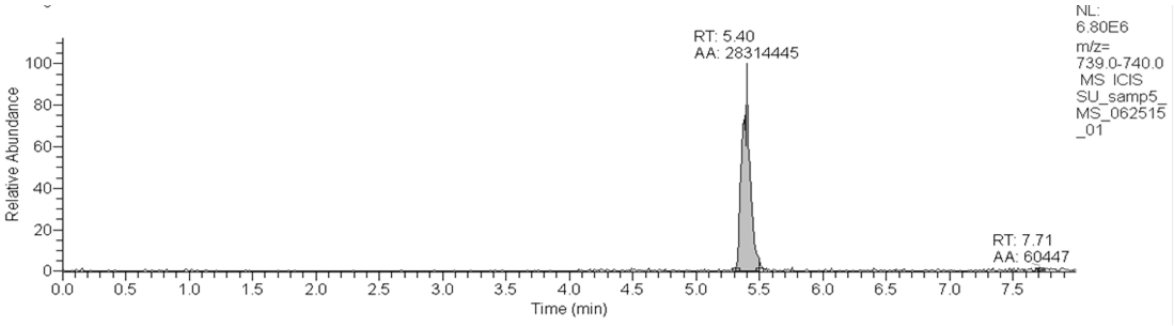

Figure 4-13. Mass Spectrophotometry analysis of ^{13}C labeled erythromycin level in the extracellular supernatant of normal cell samples.

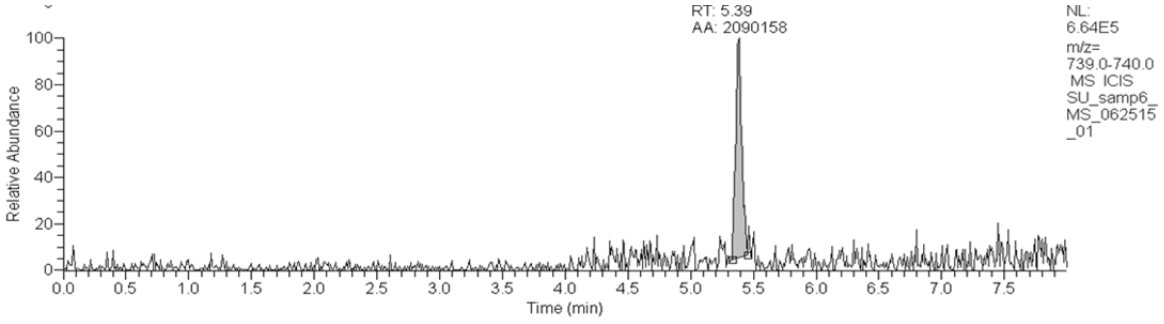

Figure 4-14. Mass Spectrophotometry analysis of intracellular ^{13}C labeled erythromycin level in normal cell samples

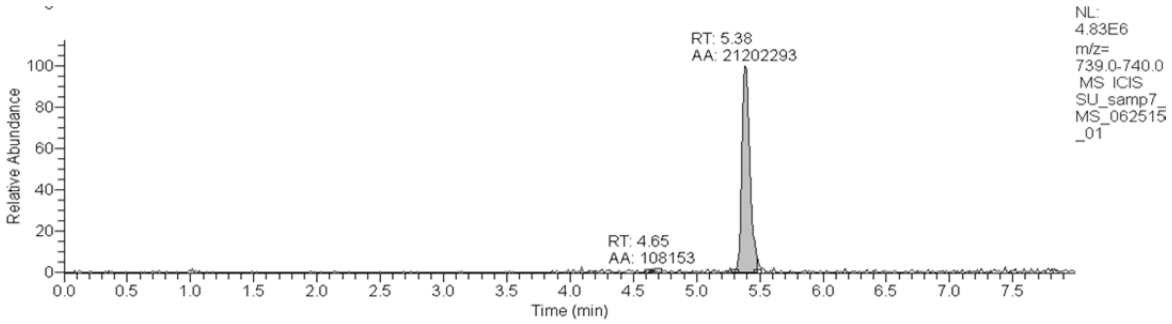

Figure 4-15. Mass Spectrophotometry analysis of ^{13}C labeled erythromycin level in the extracellular supernatant of persister cell samples

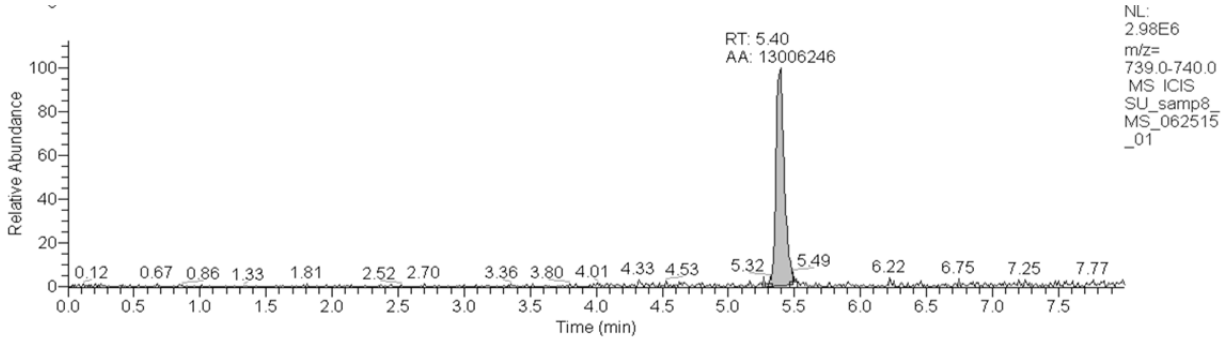

Figure 4-16. Mass Spectrophotometry analysis of intracellular ^{13}C labeled erythromycin level in persister cell samples

Table 4-2. Ratios of intracellular vs extracellular concentration of erythromycin. The concentrations were determined using MS analysis.

Relative ratio between	**Value (fold)**
NExEr/NInEr	30.2
NEx13/NIn13	14
PExEr/PInE	1.4
PEx13/PIn13	1.6

4.4 Discussion

Normal *E. coli* cells pump EtBr out by AcrAB-TolC efflux pumps [42]. Erythromycin is also a substrate of this efflux pump [18]. Consistently *E. coli ΔacrB*, which has a nonfunctional AcrAB-TolC pump, showed high level of EtBr accumulation in flow cytometry experiments (Fig. 4-3B1, 4-3B2).

Having more EtBr accumulation in persister cells (Fig 4-2) suggests that persister formation does cause lower efflux activities since inactive efflux pumps cannot extrude EtBr. The slight decrease in decrease in signal level after 10 minutes can be explained by cell damage or death due to the toxic effects of prolonged EtBr treatment on *E. coli* cells [43].

Since the frequency of persister formation is generally low [2], we tested with a high persistence strain, *E. coli* Top10 pBAD *hipA*, using flow cytometry to determine efflux activities of persister cells. As seen in Fig. 4-4, EtBr signals of the population shifted to red fluorescence and a population of persister cells was distinguished with even higher red signal. These data also support the low efflux activities in persister cells, as a result of dormant nature of persister cells.

These efflux activity results were corroborated with a potentiometric dye, JC-1, which can directly label the potential of a cell membrane [44,45]. When the arabinose induced *E. coli* Top10 pBAD *hipA* was stained with JC-1 dye and analyzed using flow cytometry, a small subpopulation with low red color was detected. The amount of cells in this population correlates well with the persistence level, further support that persister cells have lower membrane potentials than normal cells.

In parallel to membrane potential experiments, we also studied the effect of some ribosome targeting antibiotics, which are also the substrates of the AcrAB-TolC pump efflux system [18], [33]. Erythromycin, tetracycline and chloramphenicol are all substrates of *E. coli* AcrAB-TolC pumps [18, 33-36]. Because persister cells have reduced membrane potential & efflux activity, we expect these antibiotics can accumulate more intracellularly in persister cells. Interestingly, erythromycin showed higher killing effect on persister cells than normal cells. Since efflux pump activity is essential for the resistance to erythromycin [46], we speculated that this higher killing activity against persister cells may be caused by inactive efflux pumps due to low membrane potential of these cells which led to accumulation of more erythromycin molecules in persister cells.

It has been reported that tetracycline uptake occurs with both energy dependent and independent mechanisms [47]. *E. coli* cells in exponential cultures are susceptible to tetracyline while the persister cells are tolerant. This energy dependent cellular uptake of tetracycline might explain why normal cells are more susceptible compared to dormant persister cells, which is different from the results of erythromycin.

As illustrated in Fig. 4-17, erythromycin molecules diffuse into the cells [48], regardless of the metabolic stage. This antibiotic kills bacterial cells by binding to ribosomal complex and inhibiting protein synthesis [49]. In active cells, efflux proteins can pump erythromycin out before they reach and bind to the target. Persister cells have lower membrane potential, and thus inactivate efflux systems. Therefore more erythromycin will accumulate in the cells and target the ribosome complex during wakeup (on agar plates), leading to bacterial killing.

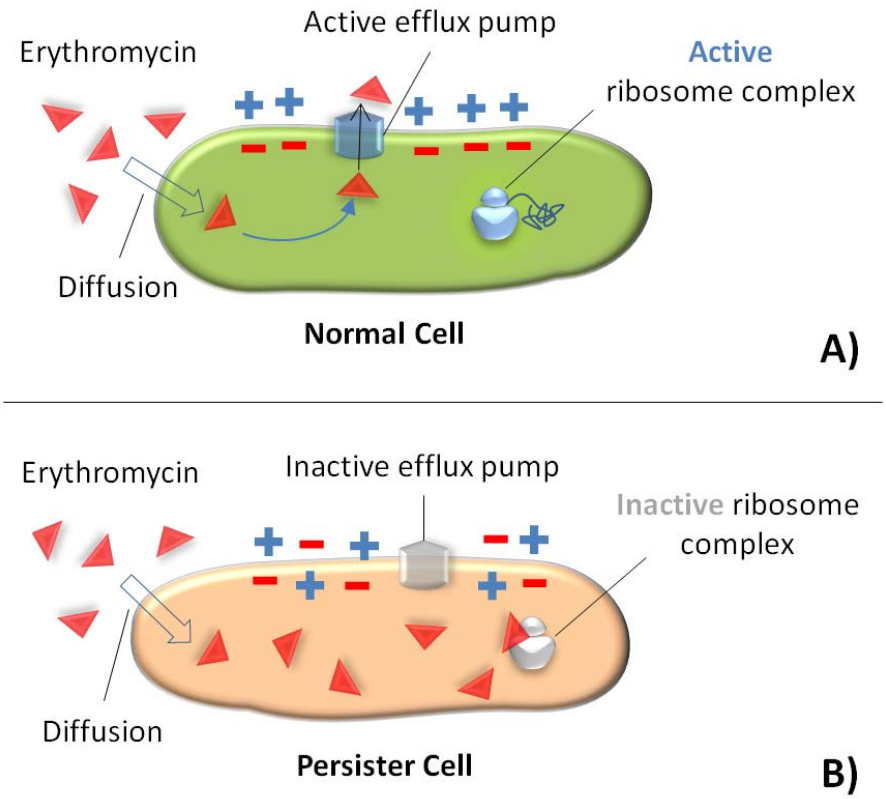

Figure 4-17. Schematic of possible effects of erythromycin on normal (A) and persister (B) cells

Normally *E. coli* is resistance to erythromycin and susceptible to tetracycline. However, we have observed higher killing with erythromycin on *E. coli* persister cells while the cells were not affected with tetracycline treatment. Erythromycin is a bacteriostatic antibiotic [50] which means it requires bacterial activity to inhibit and eventually kill the bacteria. The activity of erythromycin also requires the presence of K^+ ions for binding to ribosome [49,51,52]. Consistently, when 0.85% NaCl solution rather than PBS was used to wash the cells, persister killing by erythromycin was not observed. Erythromycin forms a very stable complex with its target ribosome. The dissociation constant of eryhtromycin and ribosomes is between 1.1×10^{-7} and 3.4×10^{-7} M [53]. In comparison, the dissociation constant between tetracycline and its target

is $1x10^{-6}$ [54], a magnitude higher than erythromycin. Collectively, the differences in binding strength may explain why the other two antibiotics did not show similar effects as eryhtromycin since their diffusion is faster than wake up upon removal of antibiotics.

In the case of chloramphenicol, the binding is even weaker than tetracycline; e.g; the dissociation constant is $2x10^{-6}$ [55]. Besides, chloramphenicol uptake by Gram negative bacteria is suggested by means of an energy-dependent processes [56]. These two reasons might explain why chloramphenicol could not show killing effect on persister cells. These results revealed that there are weaknesses of persister cells, which can be targeted by antimicrobials to achieve killing of this dormant population.

4.5 References

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CHAPTER 5

NEW AMP DERIVATIVES OF CALCITERMIN AND THEIR EFFECTS ON *PSEUDOMONAS AERUGINOSA* and *ESHERICHIA COLI*

5.1 Introduction

The growing interest in AMPs, also defined as natural microbicidals [1], as alternative antimicrobial agents to current antibiotics is also favored by their low propensity to induce drug resistance [2]. Native AMPs can serve as a source of antimicrobial agents for treatment of different infectious diseases. However, clinical use of native AMPs have issues such as potential toxicity to human cells and proteolytic degradation. Thus many studies have been conducting to search for better AMPs by re-designing native antimicrobial peptides recently [3]. For example, dermicidin secreted from epihelia can be further improved by removing 23 amino acid from N-terminal end to have 2-fold increase in killing activity against *E. coli* [4] and this new derivative of dermicidin can be more effective in skin infections. This will also adress concerns related to the stability of AMPs. Efforts have also been made to generate and screen AMP libraries based on native AMPs to study a large number of candidates simultaneously. This allows identification of important features in AMP structures and physiochemical properties required for specific activities of AMPs [1].

AMPs from different sources identified to date have provided important information for designing new sythetic AMPs with improved antimicrobial effects, target spectrums, and stabilities under different physiological conditions along with reduced cost due to short amino acid sequences [3].

We hypothesized that some human originated natural AMPs can be further improved to kill bacterial persister cells by and optimizing their physiochemical characteristics. Specifically, we studied the effects of the net charge of an AMP on planktonic & persister cells of *E. coli* and *P. aeruginosa*.

Among these AMPs we selected calcitermin, which has 15 amino acid in length with a net charge of "+1" and an α-helical structure [5], to study the effects of AMP net-charge on the persister cells of *P. aeruginosa* PAO1 (from cystic fibrosis patients) and *E. coli* HM22. The antimicrobial effects of native calcitermin on *E. coli* and *P. aeruginosa* has been reported previously [6] and its short length is considered a favorable characteristics for low cost manufacturing.

Both the amino acid sequence and local pH can affect the net charge of an AMP [7]. Therefore by adding more negatively charged amino acids into the sequence of calcitermin, we designed neutral and negatively charged calcitermin derivatives with the help of Iterative Threading ASSEmbly Refinement (I-TASSER) Protein Structure & Function Predictions which is available online [8].

5.2 Materials and Methods

5.2.1 Selecting the candidate AMP to study

To choose the AMP, 277 human originated AMPs chosen from two antimicrobial peptide databases, Antimicrobial Peptide Database (APD2) and Linking Antimicrobial Peptide (LAMP) which has 5547 AMPs in total, were compared for their given physiochemical characteristics. Human originated peptides were selected to minimize cytotoxicity in human body. Some of these AMPs are shown in (Table 5-1). Calcitermin was selected due to its short size (low production cost), relatively high hydrophobicity (for amphipathic structure and antimicrobial activity), relatively low boman index (for less interaction with other proteins), positive charge, origin tissue (airway secretions), and α-helical structure which is important to target cell membrane.

Table 5-1. Selected human originated AMPs with some of their important physiochemical characteristics, sources in the body and activities. The data is collected from APD2 and LAMP libraries. G(+); Gram positive bacteria, G(-); Gram negative bacteria.

Access number	Name	Source	Sequence	Length	Net charge	Hydrophobicity	Bowman index	Structure	Activity
AP00309	KS-27	Skin	KSKEKIGKEFKRIVQRIKDFLRNLVPR	27	7	33%	3.38	Unknown	G(+), G(-),
AP00481	Kaliocin-1	Lactoferrin	FFSASCVPGADKGQFPNLCRLCAGTGENKCA	31	1	45%	0.99	Unknown	N/A
AP00625	KR-20	Sweat	KRIVQRIKDFLRNLVPRTES	20	4	35%	3.68	Helix	G(+), G(-), Antifungal,
AP00626	KS-30	Sweat	KSKEKIGKEFKRIVQRIKDFLRNLVPRTES	30	6	30%	3.47	Helix	G(+), G(-),
AP00627	RK-31	Sweat	RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	31	7	29%	3.83	Helix	G(+), G(-), Antifungal,
AP00628	LL-23	Skin	LLGDFFRKSKEKIGKEFKRIVQR	23	5	34%	3.01	Helix	G(+), G(-), Antifungal,
AP00629	LL-29	Skin	LLGDFFRKSKEKIGKEFKRIVQRIKDFLR	29	6	37%	2.95	Helix	G(+), G(-),
AP00765	Salvic	Saliva	MHDFWVLWVLLEYIYNSACSVLSATSSVSSRVLNR SLQVKVVKITN	46	2	50%	0.7	Unknown	N/A
AP00798	Hst1	Saliva	DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNY LYDN	38	0	10%	4.29	H Rich	Antifungal
AP00799	Histatin 2	Saliva	RKFHEKHHSHREFPFYGDYGSNYLYDN	27	0	14%	3.53	H Rich	Antifungal
AP00801	Histatin 6	Saliva	DSHAKRHHGYKRKFHEKHHSHRGYR	25	6	8%	5.21	H Rich	G(+), G(-), Antifungal,
AP00802	Histatin 7	Saliva	RKFHEKHHSHRGY	13	3	7%	5.07	H Rich	Antifungal
AP00803	Histatin 9	Saliva	RKFHEKHHSHRGYR	14	4	7%	5.78	H Rich	G(+), G(-), Antifungal,
AP01407	SgI-29	Sperm	HNKQEGRDHDKSKGHFHRVVIHHKGGKAH	29	4	17%	3.74	H Rich	G(+), G(-),
AP02017	hGAPDH	Placenta	GKVKVGVNGFGRIGRLVTRAAFNSGKVDIVA	31	5	45%	1.07	Unknown	Antifungal

AP02230	HMGN2	Leukocyte	PKRKAEGDAKGDKAKVKDEPQRRSARLSAKPAPP KPEPKPKKAPAKKGEKVPKGKKGKADAGKEGNNP AENGDAKTDQAQKAEGAGDAK	89	12	21%	3.2	Unknown	G(-), Antifungal, Antiviral
AP02343	Beta 2-globulin	Amnio	IQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDIE VDLLKNGERIEKVEHSDLSFSKDWSFYLLYYTEFTP TEKDEYACRVNHVTLSQPKIVKWDRDM	99	-2	31%	2.31	Beta	G(+), G(-),
AP00176	Neutrophil peptide-1	Neutrophil	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	30	3	53%	1.07	Beta	G(+), G(-), Antiviral, Antifungal, Antiparasitic, Cancer cells
AP00179	Neutrophil peptide-4	Neutrophil	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRV	33	4	51%	1.4	Beta	G(+), G(-), Antiviral, Antifungal,
AP00180	Defensin 5	Skin	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR	32	4	40%	2.6	Beta	G(+), G(-), Antifungal
AP00181	Defensin 6	Skin	AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL	32	2	40%	1.71	Beta	Antiviral, Antifungal,
AP00192	Hepcidin 20	Blood	ICIFCCGCCHRSKCGMCCKT	20	3	60%	0.46	Beta	Antifungal,
AP00193	LEAP-1	Liver	DTHFPICIFCCGCCHRSKCGMCCKT	25	2	52%	0.89	Beta	G(+), G(-), Antifungal
AP00196	hBD-26	Skin	WYVKKCLNDVGICKKKCKPEEMHVKNGWAMCG KGRDCCVPAD	42	4	42%	1.58	Bridge	G(-),
AP00197	hBD-27	Skin	QLKKCWNNYVQGHCRKICRVNEVPEALCENGRYC CLNIKELEAC	44	2	43%	2.08	Bridge	G(-),
AP00283	Beta defensin 3	Skin	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTR GRKCCRRKK	45	11	33%	2.87	Helix and beta	G(+), G(-), Antiviral, Antifungal, Chemotactic,
AP00307	Buforin I	Stomach	AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLL RKGNY	39	12	28%	3.08	Unknown	G(+), G(-), Antifungal
AP00310	LL-37,	Neutrophil skin, sweat, lung,	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	6	35%	2.99	Helix	G(+), G(-), Antiviral, Antifungal, Antiparasitic, Spermicidal, Chemotactic, Cancer cells

AP00334	Cathepsin	Neutrophil	IIGGR	5	1	40%	0.64	Unknown	G(+), G(-),
AP00433	Dermicidin	Sweat	SSLLEKGLDGAKKAVGGLGKLGKDAVEDLESVGK GAVHDVKDVLDSV	47	-2	38%	1.11	Helix	G(+), G(-), Antifungal
AP00449	MSH	Brain	SYSMEHFRWGKPV	13	1	30%	2.01	Unknown	G(+), Antifungal, Antiviral,
AP00451	hBD-1	Keratinocyte skin, platelets	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCC K	36	4	36%	1.3	Helix and beta	G(+), G(-), Antiviral, Cancer cells
AP00504	MUC7 20	Saliva	LAHQKPFIRKSYKCLHKRCR	20	7	35%	3.16	Helix	G(+), G(-), Antifungal
AP00505	Histatin 5	Parotid secretion	DSHAKRHHGYKRKFHEKHHSHRGY	24	5	8%	4.81	Helix, H-rich	G(+), G(-), Antiviral, Antifungal, Enzyme inhibitor
AP00509	Calcitermin	Airways secretions	VAIALKAAHYHTHKE	15	1	46%	0.89	Helix	G(-), Antifungal
AP00520	Histatin 3,	Saliva, parotid secretion	DSHAKRHHGYKRKFHEKHHSHRGYRSNYLYDN	32	5	9%	4.72	H-rich	G(+), G(-), Antifungal
AP00523	Histatin 8	Saliva	KFHEKHHSHRGY	12	2	8%	4.25	H-rich	G(+), G(-), Antifungal
AP00524	Defensin 2	Skin, lung, trachea epithelia, and uterus,	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTK CCKKP	41	7	36%	0.9	Helix and beta	G(+), G(-), Antiviral, Antifungal, Chemotactic,
AP00624	ALL-38,	Sperm	ALLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTE S	38	6	36%	2.87	Helix	G(+), G(-),
AP00800	Histatin 4,	Saliva	RKFHEKHHSHRGYRSNYLYDN	21	3	9%	4.84	H-rich	Antifungal,
AP00675	Defensin 4,	Skin	FELDRICGYGTARCRKKCRSQEYRIGRCPNTYACCL RKWDESLLNRTKP	49	7	32%	3.35	bridge	G(+), G(-),
AP00780	lactoferricin	Lactoferrin	GRRRRSVQWCAVSQPEATKCFQWQRNMRKVRGP PVSCIKRDSPIQCIQA	49	10	36%	3.14	Helix	G(+), G(-),
AP00811	LEAP-2	Liver	MTPFWRGVSLRPIGASCRDDSECITRLCRKRRCSLS VAQE	40	4	40%	2.94	Helix and beta	G(+), G(-), Antifungal
AP00833	Drosomycin	Skin	CLAGRLDKQCTCRRSQPSRRSGHEVGRPSPHCGPS RQCGCHMD	43	5	25%	3.58	Bridge	Antifungal,

AP00857	Catestatin,	Brain	SSMKLSFRARAYGFRGPGPQL	21	4	33%	1.98	Unknown	G(+), G(-), Antifungal, Chemotactic, Antioxidant,
AP01161	Granulysin,	Cytolytic t cells	GRDYRTCLTIVQKLKKMVDKPTQRSVSNAATRVC RTGRSRWRDVCRNFMRRYQSRVTQGLVAGETAQQ ICEDLR	74	11	33%	3.5	Helix	G(+), G(-), Antifungal, Antiparasitic, Cancer cells
AP01315	hBD-28	Skin	ARLKKCFNKVTGYCRKKCKVGERYEIGCLSGKLCC AN	37	8	40%	1.91	Beta	G(+), G(-),
AP01372	CXCL14,	N/A	SKCKCSRKGPKIRYSDVKKLEMKPKYPHCEEKMVI ITTKSVSRYRGQEHCLHPKLQSTKRFIKWYNAWNE KRRVYEE	77	13	27%	3.03	Unknown	G(+), G(-), Antifungal
P01373	TC-1	Blood	AELRCMCIKTTSGIHPKNIQSLEVIGKGTHCNQVEVI ATLKDGRKICLDPDAPRIKKIVQKKLAGDES	68	6	38%	1.72	Helix and Beta structure	G(+), G(-), Antifungal
AP01374	TC-2	Blood	NLAKGKEESLDSDLYAELRCMCIKTTSGIHPKNIQS LEVIGKGTHCNQVEVIATLKDGRKICLDPDAPRIKK IVQKKLAGDES	83	2	36%	1.87	Unknown	G(+), G(-), Antifungal
AP01408	SgII	Sperm	KQEGRDHDKSKGHFHMIVIHHKGGQAHHG	29	2	20%	2.85	H-Rich	G(+), G(-),
AP01471	Substance P	Blood	RPKPQQFFGLM	11	1	36%	1.57	Helix	G(+), G(-), Antifungal
AP01472	Neurotensin,	Brain	ELYENKPRRPYIL	13	3	23%	3.16	Unknown	G(+), G(-), Antifungal
AP01473	Bradykinin	Brain	RPPGFSPFR	9	2	22%	2.92	Unknown	G(+), G(-), Antifungal
AP01474	Neuropeptide Y	Brain	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQR Y	36	1	25%	3	Helix	G(+), G(-), Antifungal
AP01475	DEFB120	Pooled fetal lung, testis, b-cell	ECWMDGHCRLLCKDGEDSIIRCRNRKRCC	29	2	41%	3.66	Bridge	G(+), G(-), Antifungal
AP01476	Calcitonin	Thyroid	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGS KAF	37	4	43%	1.08	Helix	G(-), Antifungal
AP01477	Vasoactive polypeptide	Intestine	HSDAVFTDNYTRLRKQMAVKKYLNSILN	28	4	35%	2.48	Unknown	G(+), G(-), Antifungal
AP01479	Adrenomedullin	Skin	YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTD KDKDNVAPRSKISPQGY	52	6	28%	2.6	Helix	G(+), G(-),
AP01494	GHH20	Skin	GHHPHGHHPHGHHPHGHHHPH	21	0	0%	2.7	H-Rich	Antifungal,

AP02071	TCP	Skin	NLPIVERPVCKDSTRIRITDNMFCAGYKPDEGKRGD ACEGDSGGPFVMKSPFNNRWYQMGIVSWGEGCDR DGKYGFYTHVFRLKKWIQKVIDQFGE	96	2	33%	2.15	Unknown	G(+), G(-),
AP02072	Elafin	Skin	AQEPVKGPVSTKPGSCPIILIRCAMLNPPNRCLKDT DCPGIKKCCEGSCGMACFVPQ	57	3	42%	0.94	Beta	G(+), G(-), Antiviral, Antifungal, Enzyme inhibitor
AP02073	Abeta40	Brain	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLM VGGVV	40	-3	42%	0.98	Helix	G(+), G(-), Antiviral, Antifungal
AP02075	Abeta42	Brain	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLM VGGVVIA	42	-3	45%	0.77	Helix	G(+), G(-), Antiviral, Antifungal
AP02076	RNase 5	Liver, intestine, skin	QDNSRYTHFLTQHYDAKPQGRDDRYCESIMRRRGP TSPCKDINTFIHGNKRSIKAICENKNGNPHRENLRIS KSSFQVTTCKLHGGSPWPPCQYRATAGFRNVVVA CENGLPVHLDQSIFRRPRP	125	11	28%	2.99	Helix and Beta structure	G(+), Antifungal
AP02077	RegIII alpha	Islets, intestine	EEPQRELPSARIRCPKGSKAYGSHCYALFLSPKSWT DADLACQKRPSGNLVSVLSGAEGSFVSSLVKSIGNS YSYVWIGLHDPTQGTEPNGEGWEWSSSDVMNYFA WERNPSTISSPGHCASLSRSTAFLRWKDYNCNVRLP YVCKFTD	149	1	33%	1.77	Helix and Beta structure	G(+),
AP02078	Psoriasin	Skin, tounge	MSNTQAERSIIGMIDMFHKYTRRDDKIDKPSLLTM MKENFPNFLSACDKKGTNYLADVFEKKDKNEDKK IDFSEFLSLLGDIATDYHKQSHGAAPCSGGSQ	101	-1	32%	2.3	Helix	G(-), Chemotactic
AP02079	RNase 7	Skin, urinary tract	KPKGMTSSQWFKIQHMQPSPQACNSAMKNINKHT KRCKDLNTFLHEPFSSVAATCQTPKIACKNGDKNC HQSHGAVSLTMCKLTSGKYPNCRYKEKRQNKSYV VACKPPQKKDSQQFHLVPVHLDRVL	128	16	32%	2.16	Helix and Beta structure	G(+), G(-), Antifungal
AP02080	CCL20	Skin	SNFDCCLGYTDRILHPKFIVGFTRQLANEGCDINAII FHTKKKLSVCANPKQTWVKYIVRLLSKKVKNM	69	8	43%	1.34	Helix and Beta structure	G(+), G(-), Antifungal, Antiparasitic,
AP02081	CXCL1	Bone marrow	ASVATELRCQCLQTLQGIHPKNIQSVNVKSPGPHCA QTEVIATLKNGRKACLNPASPIVKKIIEKMLNSDKS N	73	6	38%	1.51	Helix and Beta structure	G(+), G(-), Chemotactic
AP02082	CXCL2	Bone marrow	APLATELRCQCLQTLQGIHLKNIQSVKVKSPGPHCA QTEVIATLKNGQKACLNPASPMVKKIIEKMLKNGK SN	73	8	39%	1.17	Helix and Beta structure	G(+), G(-), Antiparasitic, Chemotactic,
AP02083	CXCL3	Bone marrow	ASVVTELRCQCLQTLQGIHLKNIQSVNVRSPGPHCA QTEVIATLKNGKKACLNPASPMVQKIIEKILNKGST N	73	6	39%	1.27	Unknown	G(+), G(-), Chemotactic

AP02084	CXCL9	Bone marrow	TPVVRKGRCSCISTNQGTIHLQSLKDLKQFAPSPSCE KIEIIATLKNGVQTCLNPDSADVKELIKKWEKQVSQ KKKQKNGKKHQKKKVLKVRKSQRSRQKKTT	103	20	28%	2.7	Unknown	G(+), G(-), Antiparasitic, Chemotactic,
AP02085	CXCL10	Bone marrow	VPLSRTVRCTCISISNQPVNPRSLEKLEIIPASQFCPR VEIIATMKKKGEKRCLNPESKAIKNLLKAVSKERSK RSP	77	11	36%	2.25	Helix and Beta structure	G(+), G(-), Antifungal, Antiparasitic,
AP02086	CXCL11	Bone marrow	FPMFKRGRCLCIGPGVKAVKVADIEKASIMYPSNN CDKIEVIITLKENKGQRCLNPKSKQARLIIKKVERKN F	73	11	41%	1.83	Helix and Beta structure	G(+), G(-), Chemotactic
AP02087	CXCL12	Stomach	KPVSLSYRCPCRFFESHVARANVKHLKILNTPNCAL QIVARLKNNNRQVCIDPKLKWIQEYLEKALNK	68	9	42%	1.93	Helix and Beta structure	G(+), G(-), Antiviral
AP02088	CXCL13	Stomach	VLEVYYTSLRCRCVQESSVFIPRRFIDRIQILPRGNG CPRKEIIVWKKNKSIVCVDPQAEWIQRMMEVLRKR SSSTLPVPVFKRKIP	87	11	41%	2.1	Unknown	G(+), G(-), Chemotactic
AP02089	XCL1	Stomach	VGSEVSDKRTCVSLTTQRLPVSRIKTYTITEGSLRA VIFITKRGLKVCADPQATWVRDVVRSMDRKSNTR NNMIQTKPTGTQQSTNTAVTLTG	93	9	32%	2.41	Beta	G(+), G(-), Chemotactic
AP02090	I-309,	Lymphocyte	KSMQVPFSRCCFSFAEQEIPLRAILCYRNTSSICSNE GLIFKLKRGKEACALDTVGWVQRHRKMLRHCPSK RK	73	10	41%	2.25	Helix and Beta structure	G(+), G(-), Chemotactic
AP02091	MCP-2,	Macrophage	PDSVSIPITCCFNVINRKIPIQRLESYTRITNIQCPKEA VIFKTKRGKEVCADPKERWVRDSMKHLDQIFQNL KP	75	6	37%	2.27	Helix and Beta structure	G(-), Chemotactic
AP02092	Eotaxin,	Eosinophil	GPASVPTTCCFNLANRKIPLQRLESYRRITSGKCPQ KAVIFKTKLAKDICADPKKKWVQDSMKYLDQKSP TPKP	74	11	33%	2.03	Helix and Beta structure	G(+), G(-), Chemotactic
AP02093	MCP-4,	Macrophage	QPDALNVPSTCCFTFSSKKISLQRLKSYVITTSRCPQ KAVIFRTKLGKEICADPKEKWVQNYMKHLGRKAH TLKT	75	11	36%	1.89	Helix and Beta structure	G(-), Chemotactic
AP02094	TARC,	Platelet	ARGTNVGRECCLEYFKGAIPLRKLKTWYQTSEDCS RDAIVFVTVQGRAICSDPNNKRVKNAVKYLQSLER S	71	6	36%	2.42	Helix and Beta structure	G(+), G(-), Chemotactic
AP02095	PARC,	Platelet	AQVGTNKELCCLVYTSWQIPQKFIVDYSETSPQCPK PGVILLTKRGRQICADPNKKWVQKYISDLKLNA	69	5	37%	1.39	Unknown	G(+), G(-), Chemotactic
AP02096	MIP-3	Leukocyte	GTNDAEDCCLSVTQKPIPGYIVRNFHYLLIKDGCRV PAVVFTTLRGRQLCAPPDQPWVERIIQRLQRTSAK MKRRSS	77	7	37%	2.23	Unknown	G(-), Chemotactic

AP02097	TECK,	Leukocyte	QGVFEDCCLAYHYPIGWAVLRRAWTYRIQEVSGSC NLPAAIFYLPKRHRKVCGNPKSREVQRAMKLLDAR NKVFAKLHHNTQTFQAGPHAVKKLSSGNSKLSSSK FSNPISSSKRNVSLLISANSGL	127	15	37%	1.81	Unknown	G(+), G(-), Chemotactic
AP02099	SLC,	Leukocyte	SDGGAQDCCLKYSQRKIPAKVVRSYRKQEPSLGCSI PAILFLPRKRSQAELCADPKELWVQQLMQHLDKTP SPQKPAQGCRKDRGASKTGKKGKGSKGCKRTERS QTPKGP	111	16	27%	2.65	Helix and Beta structure	G(+), G(-), Chemotactic
AP02158	MDC,	Leukocyte	GPYGANMEDSVCCRDYVRYRLPLRVVKHFYWTSD SCPRPGVVLLTFRDKEICADPRVPWVKMILNKLSQ	69	4	40%	1.85	Unknown	G(+), G(-), Chemotactic
AP02182	SLPI	Saliva, Airway	SGKSFKAGVCPPKKSAQCLRYKKPECQSDWQCPG KKRCCPDTCGIKCLDPVDTPNPTRRKPGKCPVTYG QCLMLNPPNFCEMDGQCKRDLKCCMGMCGKSCV SPVKA	107	12	34%	1.87	Beta	G(+), G(-), Antiviral, Antifungal, Enzyme inhibitor,
AP02184	UBI 1-59	Small intestine	KVHGSLARAGKVRGQTPKVAKQEKKKKKTGRAK RRMQYNRRFVNVVPTFGKKKGPNANS	59	19	25%	3.28	Unknown	G(+), G(-),
AP02185	RNase 8	Urinary tract	KPKDMTSSQWFKTQHVQPSPQACNSAMSIINKYTE RCKDLNTFLHEPFSSVAITCQTPNIACKNSCKNCHQ SHGPMSLTMGELTSGKYPNCRYKEKHLNTPYIVAC DPPQQGDPGYPLVPVHLDKVV	127	4	31%	1.7	Unknown	G(+), G(-), Antifungal
AP02186	ECP	Eosinophilic leukocytes	RPPQFTRAQWFAIQHISLNPPRCTIAMRAINNYRWR CKNQNTFLRTTFANVVNVCGNQSIRCPHNRTLNNC HRSRFRVPLLHCDLINPGAQNISNCTYADRPGRRFY VVACDNRDPRDSPRYPVVPVHLDTTI	133	13	36%	2.71	Helix and Beta structure	G(+), G(-), Antiviral, Antiparasitic, Mammalian cells,
AP02187	hPF4	Platelet	EAEEDGDLQCLCVKTTSQVRPRHITSLEVIKAGPHC PTAQLIATLKNGRKICLDLQAPLYKKIIKKLLES	70	3	40%	1.46	Unknown	Antiparasitic, Antimalarial,
AP02195	DEFB114	Gingival cells	DRCTKRYGRCKRDCLESEKQIDICSLPRKICCTEKL YEEDDMF	43	0	32%	3.58	Bridge	G(+), G(-), Antifungal
AP02196	Alarin	Brain	APAHRSSTFPKWVTKTERGRQPLRS	25	5	24%	3.47	Unknown	G(-),
AP02231	CXCL6	Bone marrow	GPVSAVLTELRCTCLRVTLRVNPKTIGKLQVFPAGP QCSKVEVVASLKNGKQVCLDPEAPFLKKVIQKILD SGNKKN	77	8	41%	1.14	Unknown	G(+), G(-), Antiparasitic, Chemotactic,
AP02257	Kinocidin	Saliva, milk, epithelial cells/mucosal tissues	MQQRGLAIVALAVCAALHASEAILPIASSCCTEVSH HISRRLLERVNMCRIQRADGDCDLAAVILHVKRRRI CVSPHNHTVKQWMKVQAAKKNGKGNVCHRKKH HGKRNSNRAHQGKHETYGHKTPY	127	16	39%	2.3	Unknown	G(+), G(-), Antifungal, Antiparasitic, Chemotactic,

AP02337	CCL27	Brain	PPSTACCTQLYRKPLSDKLLRKVIQVELQEADGDC HLQAFVLHLAQRSICIHPQNP	56	1	41%	1.57	Helix and Beta structure	Antifungal, Chemotactic
AP02409	Chemerin	Epidermis, skin,	ELTEAQRRGLQVALEEFHKHPPVQWAFQETSVESA VDTPFPAGIFVRLEFKLQQTSCRKRDWKKPECKVR PNGRKRKCLACIKLGSEDKVLGRLVHCPIETQVLRE AEEHQETQCLRVQRAGEDPHSFYFPGQFAFS	137	3	36%	2.38	Unknown	G(+), G(-), Antifungal, Chemotactic,
AP02425	hIAPP,	Islets	KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNT Y	37	3	37%	1.48	Helix	G(+), G(-),
AP02451	KDAMP 10	Cornea	RAIGGGLSSVGGGSSTIKY	19	2	26%	0.55	Unknown	G(-),
AP02452	Lysozyme	Secretions and tissues	KVFERCELARTLKRLGMDGYRGISLANWMCLAKW ESGYNTRATNYNAGDRSTDYGIFQINSRYWCNDGK TPGAVNACHLSCSALLQDNIADAVACAKRVVRDP QGIRAWVAWRNRCQNRDVRQYVQGCGV	130	8	40%	2.28	Helix	G(+), G(-), Antifungal

5.2.2 Designing new calcitermin derivatives with different net charges

We used I-TASSER to design neutral and negatively charged derivatives of calcitermin based on the original amino acid sequence; VAIALKAAHYHTHKE. This prediction method helped us to keep α-helical structure of calcitermin during designing process. First, positively charged Lys (K), at position 14, residue was replaced with Gly (G) to obtain a neutral AMP derivative VAIALKAAHYHTH*G*E (0). Then Ala (A), at position 8 was replaced with a negatively charged Glu (E) for AMP derivative with a net charge of "-1", VAIALKA*E*HYHTH*G*E. Finally, an Ala (A) at position 4 residue was replaced with Glu (E) to obtain an AMP with a net charge of "-2", VAI*E*LKA*E*HYHTH*G*E. Each amino acid change was individually confirmed to ensure no change in the amphipaticity and the α-helical structure. The helical wheel projection model was used to retain protect amphiphilic topology during design studies. These derivatives were also checked for their net charges at slightly acidic conditions (pH 5.5) to mimic the CF airways.

5.2.3 Killing effects of calcitermin and its derivatives on planktonic cells at pH 7.4 and pH 5.5

To examine the antimicrobial activity of calcitermin and its neutral derivative, the planktonic cultures of *E. coli* HM22, and *P. aeruginosa* PAO1 were treated. The pH values 5.5 and 7.4 were studied to resemble the nasal airway conditions in cystic fibrosis patients and healthy individuals respectively. Overnight cultures of each strain were grown in 50 mL LB medium for 12-16 h at 37°C. Subcultures were then prepared by inoculating LB medium with overnight cultures to an optical density at 600 nm (OD_{600}) of 0.01 and harvested when the OD_{600} reached 0.4-0.5. These exponential cultures were divided into two sample groups; to be washed with PBS (pH 7.4) and

PBS (pH 5.5 with HCl), and resuspended in the same buffer after washing. For the treatment with calcitermin and its neutral derivative, 3 mL of each sample was taken and mixed with different concentrations of calcitermin. The samples were incubated for 3 h at 37°C with shaking at 200 rpm, and washed three times with PBS at pH 7.4 and pH 5.5. Then a serial dilution of each sample was prepared to obtain the concentrations from 1 to 100μg/mL. The cells were then washed again and spread on LB agar plates for counting CFU. Each condition was tested with three independent cultures.

5.2.4 Persister isolation and calcitermin treatment at pH 7.4 and pH 5.5

To isolate persister cells, overnight cultures of *P. aeruginosa* PAO1 and exponential cultures of *E. coli* HM22 strains were treated with 200 μg/mL ciprofloxacin (Cip) and 100 μg/mL ampicillin respectively for 3 h at 37˚C to kill normal cells as described previously [9,10]. Then the persister cells were harvested by centrifugation, washed with PBS at different pH values (pH 7.4 and pH 5.5 with HCl) three times to remove remaining antibiotic, and resuspended in 20 mL PBS at pH 7.4 and 5.5. The collected cells were resuspended in 3 mL PBS supplemented with calcitermin at different concentrations (from 1 to 100μg/mL). Three replicates were tested for each condition and all samples were incubated for 3 h at 37°C with shaking at 200 rpm. After calcitermin treatment, the samples were washed with PBS three times and plated to count CFU using drop plate method.

5.3 Results

5.3.1 New calcitermin derivatives designed with I-TASSER prediction method

Neutral and negatively charged AMP derivatives of calcitermin were successfully designed by replacing amino acids guided by helical wheel projections (Fig. 5-1). The amphiphilic topology and the helical structure of each derivative were tested with I-TASSER prediction method. Lys (K), which is a positively charged amino acid at position 14, was replaced with Gly (G), a neutral amino acid, and the net charge of the peptide changed from "+1" to "0" without altering the 3D structure of calcitermin. Besides, changing positions 4 and 8 to Glu (E) was found to protect the α-helix conformation (Fig 5-1) but changed the net charge of calcitermin to negative. Besides, prediction analysis with I-TASSER suggested a candidate binding region for Ca^{2+} ions (Fig. 5-2). This binding region is surrounded by three His (H) at positions 9, 11, 13 and one Tyr (Y) 12. In derivative design this Ca^{2+}ion binding region was successfully retained in all calcitermin derivatives.

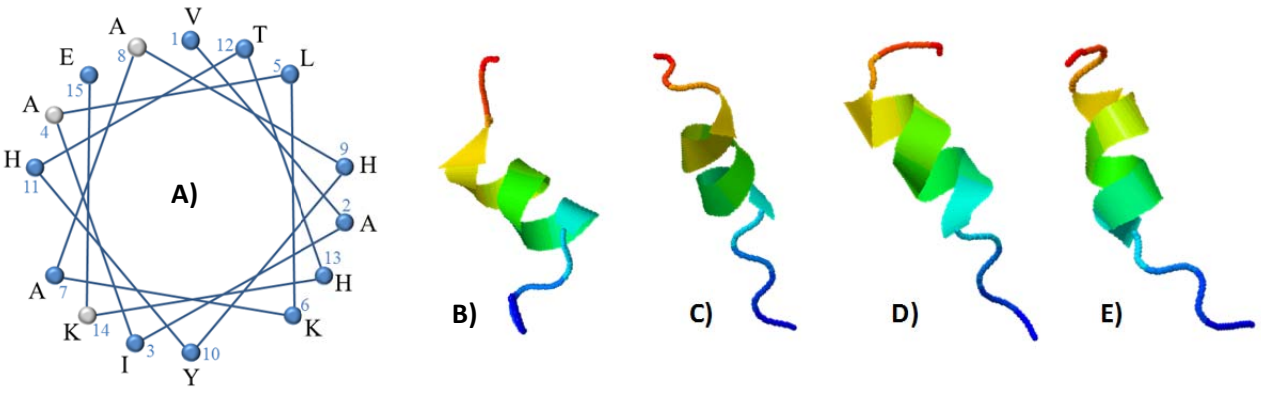

Figure 5-1. A) Helical wheel projection of calcitermin. Modified amino acid positions are colored gray. B) Native calcitermin VAIALKAAHYHTHKE (+1), C) neutral derivative

VAIALKAAHYHTHGE (0), D) negatively charged VAIALKAEHYHTHGE (-1), E) negatively charged VAIELKAEHYHTHGE (-2). Modified amino acids are underlined along the new net charges are shown in parenthesis.

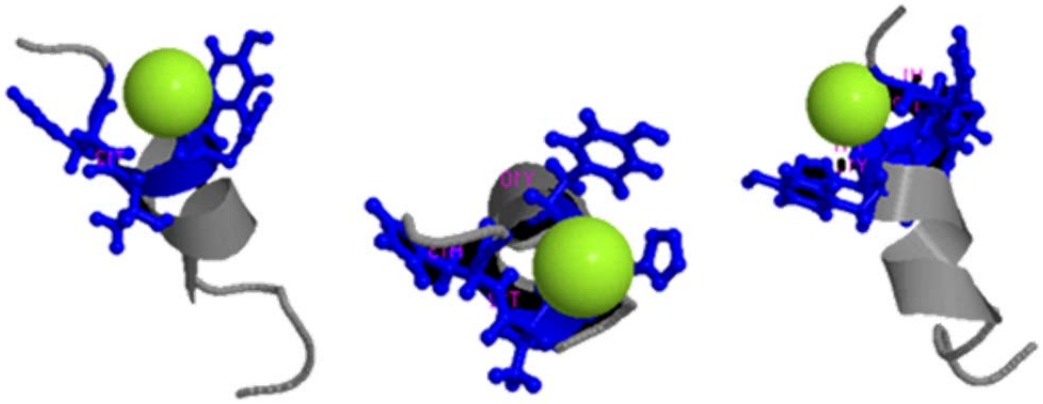

Figure 5-2. Predicted Ca^{2+} binding site on the native calcitermin from different angles. Green sphere represent Ca^{2+} ion. The blue part, which shows Ca^{2+} ion binding pocket, is rich with amino acids carrying carbon rings in their structures.

5.3.2 Killing effects of calcitermins on planktonic cells

Native calcitermin is effective in killing PAO1 and HM22 cells at pH 5.5 dose dependently. However, no significant killing effect was observed when pH in calcitermin treatment elevated to pH 7.4 (Fig. 5-3). This is consistent with the in vivo observation that pH is 5.5 in chronic disease condition. Calcitermin showed significant killing on both PAO1 and HM22 planktonic cells at pH 5.5 when its concentration is increased to 10μg/mL and higher.

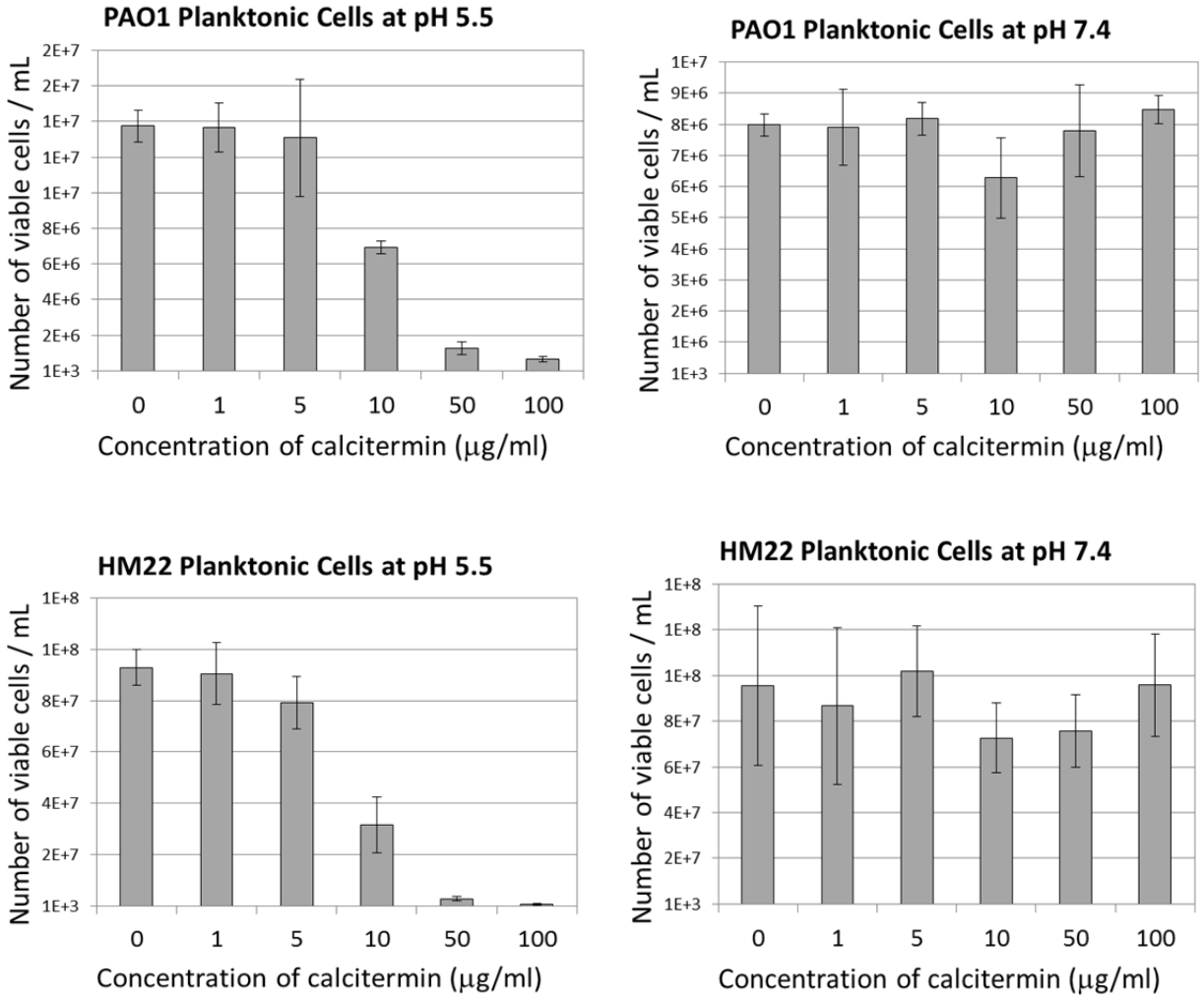

Figure 5-3. Antimicrobial effect of native calcitermin on the planktonic cells of *P. aeruginosa* PAO1 and *E. coli* HM22 strains. Both strains were tested at pH 7.4 and pH 5.5.

The killing effect of neutral calcitermin derivative on planktonic cells was found different than the native calcitermin. Neutral calcitermin derivative showed comparable killing with a dose dependence when the experiment pH was adjusted to pH 7.4 (Fig. 5-4). However, this killing was not as significant as native calcitermin.

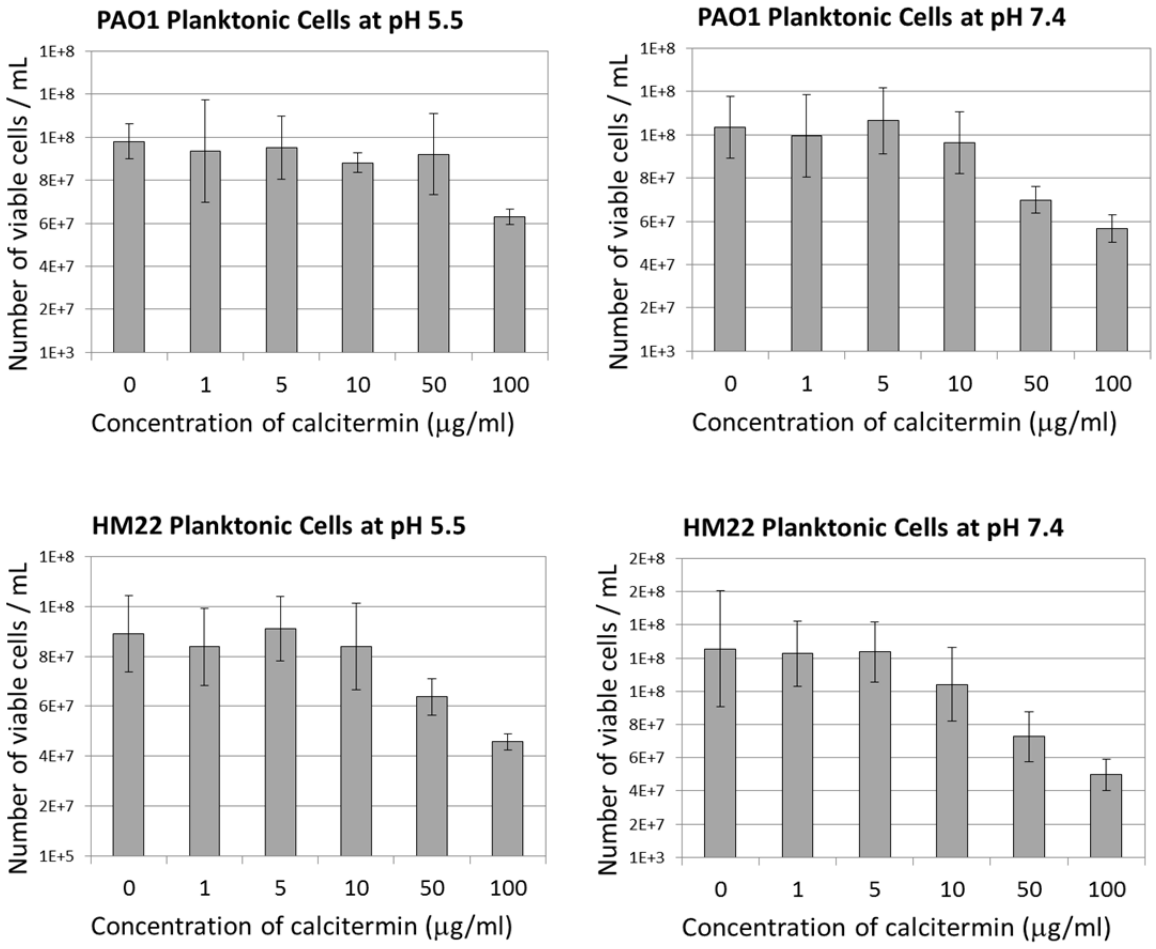

Figure 5-4. Antimicrobial effects of neutral calcitermin derivative on planktonic cells of *P. aeruginosa* PAO1 and *E. coli* HM22 strains. Both strains were tested at pH 7.4 and pH 5.5.

5.3.3 Killing effects of calcitermins on persister cells

Even though the total killing is less than normal planktonic cells, native calcitermin was found effective against the persister cells of both strains and the effect was stronger at pH 5.5 than pH 7.4 (Fig 5-5). On the other hand, neutral derivative of calcitermin showed stronger effect on both *P. aeruginosa* PAO1 and *E. coli* HM22 persister cells at pH 7.4 than pH 5.5 (Fig. 5-6).

The killing effect of the neutral derivative on persister cells are rather potent, e. g., 83.35±5.2% and 96.54±2.3% of *P. aeruginosa* PAO1 and *E. coli* HM22 persister cells were killed at 100 µg/mL respectively.

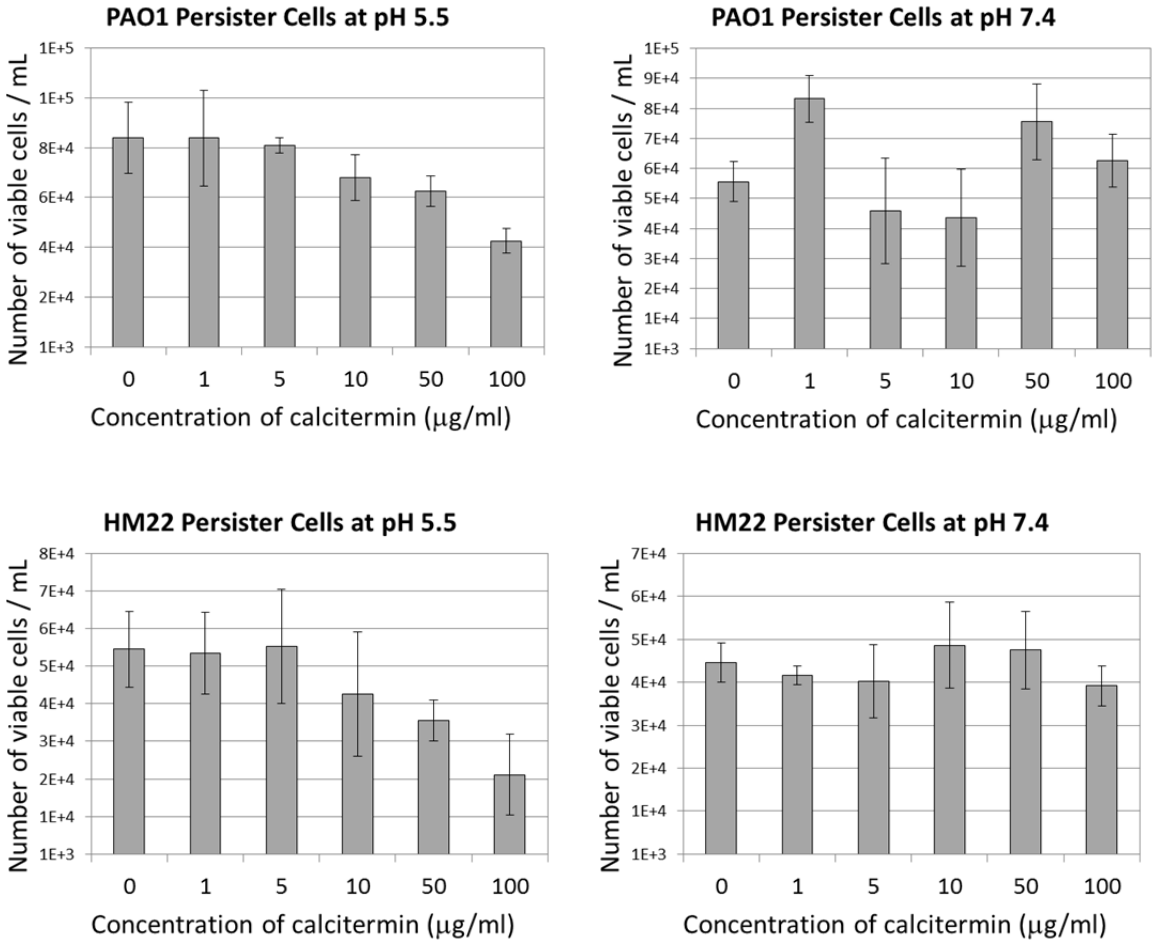

Figure 5-5. Antimicrobial effects of native calcitermin on persister cells of *P. aeruginosa* PAO1 and *E. coli* HM22 strains. Both strains were tested at pH 7.4 and pH 5.5.

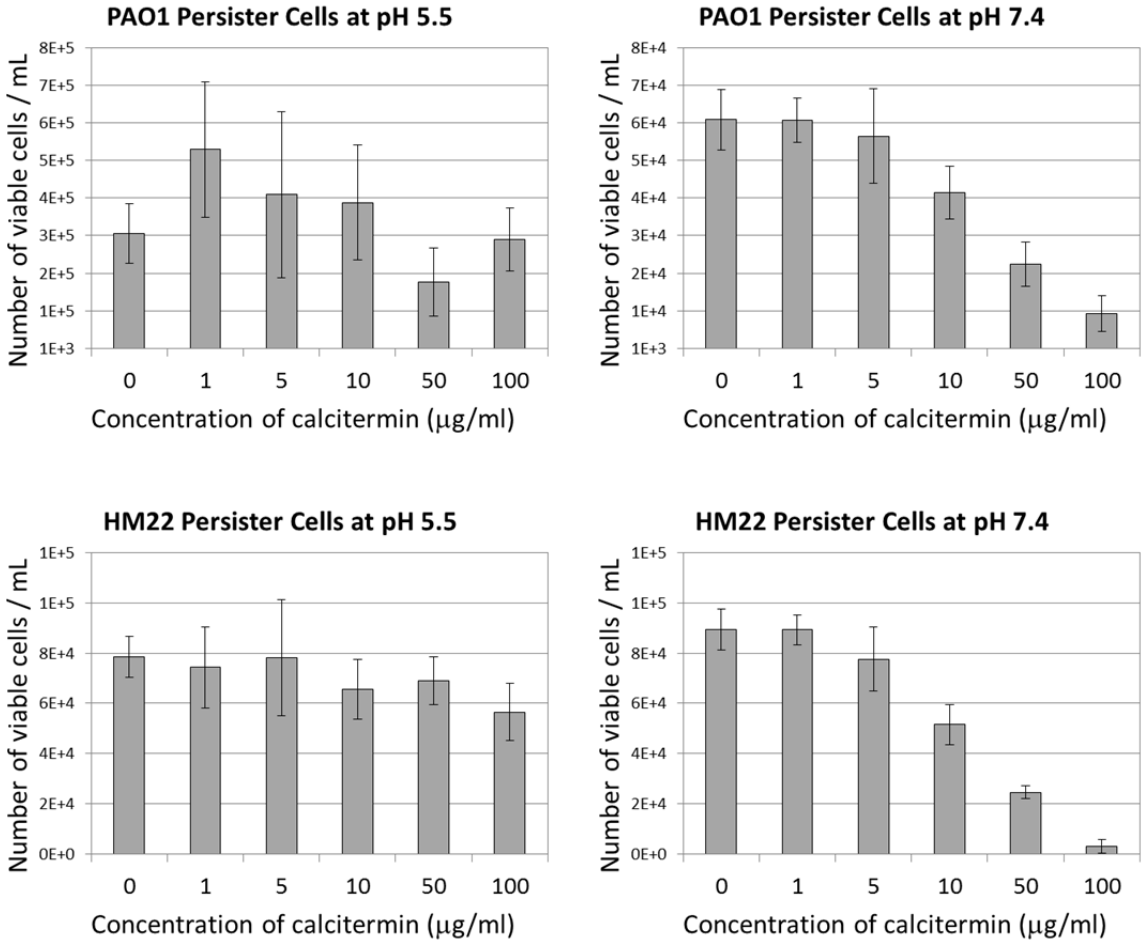

Figure 5-6. Antimicrobial effects of neutral calcitermin derivative on persister cells of *P. aeruginosa* PAO1 and *E. coli* HM22 strains. Both strains were tested at pH 7.4 and pH 5.5.

5.4 Discussion

The amphiphilic topology and α-helical structure were successfully maintained for the new calcitermin derivatives by using I-TASSER prediction method. These two physiochemical features of this AMP are required for insertion into bacterial cell membrane and for the antimicrobial effects [2]. To protect α-helical structure, amino acid changes were made based on their α-helix formation propensities. Prediction studies showed that replacing Gly (G) at position

14 did not cause any change in the on α-helix structure. This amino acid was chosen to change the net charge of new calcitermin derivative since there are only two amino acids with negatively charged side chains: Gly (G) and Ala (A). These two amino acids are also members in the family of high helix forming propensity amino acids, while the others are with Met (M), Leu (L) and Lys (K) [11]. This high helix forming propensity of Gly (G) might have helped the unimpaired α-helix structure of new derivatives. However, replacing positions 4 and 8 with Ala (A) caused deterioration of the α-helix structures for new designs even though Ala (A) is a high helix forming propensity amino acid.

Another important factor for the antimicrobial effects of calcitermin is the binding of Ca^{2+} ions, which were also demonstrated by I-TASSER predictions (Fig 5-2). The calcitermin sequence is equivalent to 15 amino acid carboxyl end of a calgranulin, S100 [5] which are also called calcium binding proteins (CaBP) [12]. S100 proteins, involved in Ca^{2+} homeostasis [13], are also found in vertebrates and characterized by two calcium-binding sites with α-helix structures [14]. Ion binding regions are known to be important for the function of many peptides [15]. Calcitermin derivatives designed with I-TASSER were shown to preserve Ca^{2+} ion binding pocket. Ca^{2+} ions are also known as important for the integrity of membrane lipid bilayer structure [15]. This Ca^{2+} ion binding capacity of calcitermin might affect stability of the membrane since Ca^{2+} ions are important for membrane integrity. Therefore, calcitermin derivatives might still hold potential to bind Ca^{2+} ions and be effective on the membrane stability.

The amino acids in Ca^{2+} binding pocket of calcitermin have either 5 carbon ring as in His (H) or 6 carbon ring as in Tyr (Y). Some Ca^{2+} binding proteins are known His (H) rich peptides [16].

This suggests that having three His (H) amino acids close to each other within a 5 amino acid region is important for the Ca^{2+} binding ability of calcitermin. During new derivative design studies these amino acids involved in Ca^{2+} binding pocket were retained to protect the Ca^{2+} binding capacity of the peptide.

It was shown that antimicrobial activity of calcitermin is enhanced in acidic environment (pH 5.4) [5]. In addition, the native calcitermin, which is positively charged, showed significant killing of *P. aeruginosa* PAO1 and *E. coli* HM22 cells in exponential cultures only at pH 5.5, but not effective at pH 7.4 (Fig 5-3). For example, the killing on *P. aeruginosa* PAO1 exponential cultures by ratios with 10, 50, and 100 μg/mL calcitermin was 49.63±6.24%, 90.72±7.6%, and 95.27±3.28%, while it showed slightly higher killing effect on *E. coli* HM22 exponential cells, e.g., 66.08±12.07%, 97.07±2.31%, and 99.46±3.98% at the same concentrations. Similar to planktonic cells, native calcitermin showed comparable effects on persister cells; better killing at pH 5.5 while no clear effect was observed at pH 7.4 (Fig 5-5). For example at 50 and 100 μg/mL, calcitermin was able to kill 25.29±3.57% and 48.82±3.6% *P. aeruginosa* PAO1 persister cells respectively. Persister cells of *E. coli* HM22 showed similar susceptibility to native calcitermin and the killing was 34.1±3.8% and 60.13±9.7% at 50 and 100 μg/mL respectively. Even though it is less effective than killing normal planktonic cells, the killing effect of native calcitermin on persister cells holds a potential, which might be further improved via co-treatment with other antibiotics. Besides comparable killing effects of native calcitermin on the normal and persister cells of both strains, a general mechanism of action might be cell membrane targeting since *E. coli* is more susceptible to antibiotics in general [17].

The neutral calcitermin derivative did show strong killing effect on normal planktonic cells of *P. aeruginosa* PAO1 and *E. coli* HM22 at pH 7.4 compared native calcitermin. However, this new derivative showed similar effects at pH 7.4 and pH 5.5. For example, 100 μg/mL neutral calcitermin derivative killed 35.71±3.52% and 45.41±5.71% of normal planktonic PAO1 cells at pH 5.5 and pH 7.4 respectively. Similarly, the neutral calcitermin derivative showed slightly better killing effect on *E. coli* HM22 planktonic cells at pH 7.4 compared to pH 5.5; e.g., 28.33±5.97% and 48.62±2.96% with pH 5.5, and 42.18±8.72% and 60.46±6.3% with pH 7.4, at 50 and 100μg/mL, respectively. The trend of less killing effect at lower pH diminished when the neutral calcitermin derivative was tested on persister cells of *P. aeruginosa* PAO1 and *E. coli* HM22. However, a significantly higher killing effect with pH 7.4 on persister cells compared to planktonic cells of both strains was observed when treated with neutral calcitermin derivative. For example, 10, 50, and 100 μg/mL neutral calcitermin showed 31.3±8.23, 62.22±7.1%, and 83.35±5.2% killing of PAO1 and 42.37±5.08%, 72.58±1.92%, and 96.54±2.3% killing of HM22 respectively.

Persister cells provide a reservoir for recurring infections [18]. As an effective AMP at pH 7.4, which is the pH level of nasal airways in healthy people, calcitermin might be able to eliminate persister cells before the infection becomes chronic. Another important outcome from this study is the finding that the neutral derivative is more effective on persister cells than its positively charged native form. We expect that other AMPs can be engineered following this principle to achieve better control of chronic infections. Besides, the higher killing effect of neutral calcitermin derivative on persister cells might be further improved by combining it with the native form or via co-treatment with other antimicrobial agents so that both normal and persister cells can be eliminated.

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CHAPTER 6

CONCLUSION AND FUTURE WORK

As alternative antimicrobials, AMPs have advantages since these compounds attack bacterial membranes and thus have a broad spectrum of targeting microbes. Cell membrane is present in all bacteria with a relatively simple bilayer structure. The functions of membrane embedded proteins that are essential for the survival of bacteria and the rapid action of AMPs leave little room for bacteria to develop resistance. These properties led to growing interest in AMP research and some novel lead compounds have shown great potential for infection control. However, the lack of a methodology for rational AMP design, high production cost, susceptibility to proteolytic degradation, potential toxicity to eukaryotic cells and low specify for a given bacterial species are the major challenges in AMP development which need to be addressed in new design studies. Therefore, more research on the mechanisms of AMP killing an the design of synthetic AMPs are required.

Up to date, most of the AMP studies focused on planktonic cells. Treatment of biofilms and persister cells, that are highly resistant to traditional antibiotics, with AMPs is still an understudied area. Through collaboration with the Kallenbach lab at New York University, we tested the newly designed synthetic AMPs for their activities against bacterial cells at different physiological stages including normal planktonic cells, persister cells and biofilms.

RW rich peptides are known to be effective on bacterial cells. Here we demonstrated that 2D-24, a new RW rick dendrimer with a branched core is effective on multidrug-tolerant persister cells of *P. aeruginosa* PAO1 and PDO300 in a dose dependent manner with no toxicity to eukaryotic cells at the effective concentrations. Besides, changing the net charge of calcitermin from +1 to neutral resulted in a better killing of persister cells at neutral pH. Triazine derivatives were also proven effective on normal planktonic and persister cells as well as biofilm cells (in the presence

of alginate lyase). These results show that AMPs can be engineered with optimized structure for better killing of multi-drug tolerant bacteria at different physiological stages. It is also possible to achieve synergy with conventional antibiotics in bacterial killing with lowered cytotoxicity to eukaryotic cells.

The structure-function relation of effective AMP derivatives at different pH values and cellular stages might be studied to further develop AMPs for different clinical applications.

In addition to membrane targeting AMPs, we also tested some antibiotics that are substrates of efflux pumps on persister cells. Erythromycin was found effective on *E. coli* HM22 persister cells, although the normal planktonic cells are resistant to this antibiotic. This antibiotic is a known substrate of the efflux pump AcrAB and these pumps are active in normal planktonic cells. For persister cells, these efflux pumps are inactive and erythromycin molecules can accumulate in cytoplasm. The strong binding between erythromycin and bacterial ribosome may lead to killing of persister cells during wake-up process.

Human originated AMPs are a great source for developing new control agents to treat different infections. Different physiological conditions of different tissues might limit or hinder the effectiveness of a particular AMP for certain infections. Therefore, designing different AMP structures specific for different tissues will be a useful strategy for future studies.

In addition to the bacterial killing, the diverse antimicrobial and structural properties of AMPs make them a convenient tool for other biological applications such as drug delivery vehicles. The close interaction between AMP with membrane can be used to deliver other therapeutic agents through the membrane. For example chloramphenicol requires active transport for cellular

uptake. These transport mechanism are expected to be inactive in persister cells. Using AMP-chloramphenicol hybrids to deliver these drugs to persister cells may lead to enhanced antimicrobial activity. Thus, understanding the biological and structure properties of AMPs and their interaction with bacterial membranes is important for developing peptide-based therapeutics.

Curriculum Vitae

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Worked on "Developing a selection method with RNA aptamers, Apta-CHIP, which binds to specific factors and proteins as an alternative way to low molecular weight targets"

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Peer Reviewed Publications

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Conference Presentations

"Investigation of Bacterial Flora and Insecticidal Activity *Oberea linearis*". **Bahar A A**, Demirbag Z; **18th National Biology Congress**, Adnan Menderes University, Aydın / TURKEY (June 20-30, 2006).

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"Chitinase Activity Determination by Using PCR and M9-CAD Methods Among East Black Sea Region Pest". **Bahar A A**, Sezen K, Nalcacioglu R, Demirbag Z; **19th National Biology Congreess**, Karadeniz Technical University, Trabzon / TURKEY (23-27 June 2008).

"Chitinase Profile and Effectiveness of Pathogenic Bacteria Originated from Coleopteran Pests in Black Sea Region". **Bahar A A**, Demirbag Z, Sezen K, Nalcacioglu R; **60st International Symposium on Crop Protection**, Gent / BELGIUM (May 20-23, 2008).

"Chitinase Profile and Insecticidal Effects of Bacteria Originated from Hazelnut Pests". **Bahar A A**, Demirbag Z, Sezen K, Nalcacioglu R; **41th Annual Meeting of the Society for Invertebrate Pathology**, University of Warwick, Coventry / UNITED KINGDOM (August 3-7, 2008).

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"Controlling *Pseudomonas aeruginosa* PAO1 and PDO300 Strains with a Synthetic Antimicrobial Peptide, 2D-24" **Bahar A A**, Totsingan F, Liu Z, Kallenbach N, Ren D; **Nunan Research Day 2014,** Syracuse, New York / USA (Apr 4, 2014).

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Project title: Investigating membrane potential of persister cells to target them with native and synthetic antimicrobial peptides.

Project title: Utilization of Next-Generation Sequencing with Tn-Kan mutation library kit to test the individual contribution of each gene to the fitness of a bacterial population.

Project title: Utilization of Phage Display system with whole bacterial cell surface to study specific surface proteins.

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Cell Biology: Transient transfection; Human and insect cell cultures maintenance; Electroporation; Eukaryotic cell culture development; Differential fluorescent staining of bacterial and mammalian co-cultures; Biofilms.

Protein Techniques: Large scale protein production with bacteria and eukarya; Western-Blot; Affinity chromatography; Biotinylated antibody application; Prosieve marker application; Protein quantification; Amino and carboxyl oligopeptide labeling; Column purification; Antimicrobial Peptide detection; Antimicrobial activity test of synthetic antimicrobial peptides; ELISA immunoprecipitation.

Detection: Bradford & Lowry; Molecular bacterial and virus identification; Bacterial pathogeny; Signal transduction; effects of new therapeutic agents; Plasmid detection; Micoplasma detection; Bacteriocin quantification; Nanodropper analysis of protein, RNA and DNA content; Qubit.

DNA: CHIP; DAPI; Genomic DNA isolation from different biological sources (from bacteriophage to mammalian organs); Hybridization; Southern Blotting; RFLP; DIG labeling; Transposon mutagenesis; DNA sonication, junction repair, tailing, adapter adding, and end repairing; DNA sample preparation for different sequencing purposes; Plasmid mapping; Restriction site detection; Phage isolation and amplification.

RNA: RNAi and SiRNA; RT (Reverse Transcriptase) PCR; Q-PCR (Quantitative PCR; Real Time); RNA isolation from bacteria eukarya; cDNA synthesis; Dot-Blot.

Molecular Kits: DNeasy Blood & Tissue Kit; End-It™ DNA End-Repair Kit; QIAquick PCR Purification Kit; SuperTaq™ Plus Polymerase (Cloned); MinElute PCR Purification Kit; Quick Ligation™ Kit.

Microscopy: GFP; 3D Z-stack imaging; Scanning electron microscopy; Transmission electron microscopy.

Bioinformatics: Mass DNA data analysis from Next Generation Sequencing; BLAST; 16SrDNA sequence analysis; Primer design; Peptide modification; Peptide structure predictions.

Cell sorting & Flow cytometry: Bacterial viability analysis at individual cell level; Compensation; Troubleshooting; Nozzle size selection; PSI selection.

Teaching and Mentoring

Lab Manager, 2010 –2014
Biofilm Laboratory, Biomedical and Chemical Engineering Department, Syracuse University.

Trained new graduate students on basic laboratory regulations, and aseptic techniques.

Trained lab members on following topics; DNA isolation, PCR primer design, Quality check of primer sets, DNA annealing, DNA alignment & Clustal analysis, Real-time PCR, Melting curve analysis, Agarose gel and western blot imaging and analysis, Basic trouble shooting for DNA studies, DNA recombination studies.

NSF REM Mentoring, Summer 2011, 2011, 2012, 2013, 2014
Biomedical and Chemical Engineering Department, Syracuse University.

Trained a high school student, 3 high school teachers and 4 undergrad students to manage complex science projects and helped them to gain experience on how to design a scientific experiment and data reporting.

Biotechnology Workshop Committee Chair, June 2008
Biology Department, Karadeniz Technical University.

Organized "National Biotechnology Spring Course" with undergrad students to help them for their future career, by showing how to choose trend topics on science and how to see opportunities in their future careers in molecular biology and microbiology fields.

Graduate Teaching Assistant, 2003-2008
Biology Department, Karadeniz Technical University.