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LIPOSOMAL CLARITHROMYCIN DELIVERY FOR THE TREATMENT OF PSEUDOMONAL LUNG INFECTION IN CYSTIC FIBROSIS PATIENTS

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science (MSc) in Biology

The School of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

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School of Graduate Studies/École des études supérieures

Title of Thesis
Titre de la thèse: LIPOSOMAL CLARITHROMYCIN DELIVERY FOR THE TREATMENT OF PSEUDOMONAL LUNG INFECTION IN CYSTIC FIBROSIS PATIENTS

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.

Degree
Diplôme: Master of Science

Department/Program
Département/Programme: Chemical Sciences

Date of Defence
Date de la soutenance: October 11, 2013

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Abstract

The pulmonary infection with *Pseudomonas aeruginosa* is considered as one of the main causes of health deterioration in cystic fibrosis patients (CF). Efficient management of *P. aeruginosa* in CF remains difficult mainly with the emergence of multidrug-resistant strains leading ultimately to death. There is a pressing need for new approaches to control these Pseudomonal infections. Current studies on the antimicrobial efficacy of liposomal antibiotics have shown conflicting results. We sought to assess whether the incorporation of clarithromycin into liposomes could improve its antibacterial activity against clinical isolate of *Pseudomonas aeruginosa* from CF patients. Different formulations of liposomal clarithromycin were prepared, characterized and their antibacterial activities against resistant strains of *P. aeruginosa* were investigated. These formulations reduced the biofilm formation, the virulence factors production and the bacterial motilities compared to free drug. The therapeutic importance of liposome containing macrolides in the management of experimental pseudomonal lung infection in animals is warranted.

Keywords

Cystic fibrosis, liposomes, macrolides, *Pseudomonas aeruginosa*, virulence factors.

Acknowledgements

I would like to thank my supervisor, Professor, Abdelwahab Omri whose scholarly advice, help and constant encouragement have significantly contributed to the completion of this thesis work. I would like to thank my committee members Professor, Kabwe Nkongolo, and Professor, Leo G. Leduc for their critical input and suggestions for my thesis work. I wish to thank my external thesis committee member for his/her critical input for my work. I also wish to thank my fellow students (members of Dr. Omri's group) Moayad Alhariri and Ahad Hasanin for their invaluable input and for being a great source of support to me during my study. I also would like to acknowledge the generous financial support provided by the Ministry of Higher Education in the Kingdom of Saudi Arabia. Finally, I would like to thank my family and friends for being there for me throughout the entire Master program.

Dedication

I dedicate this work to my Father Mohsen Alhajlan, and Mother Aisha Almajed, who surrounded me with their endless support, encouragement, love and prayers. They have been a great source of motivation and inspiration.

Publication and Communication

1- Mai Alhajlan, Moayad Alhariri, Abdelwahab Omri. Efficacy and Safety of Liposomal Clarithromycin and Its Effect on *Pseudomonas aeruginosa* Virulence Factors. Antimicrobial Agents and Chemotherapy, 2013; 57(6):2694-2704.

2- M. Alhajlan and A. Omri. Enhanced Activity of Liposomal Clarithromycin against *Pseudomonas aeruginosa* Isolates from Patients with Cystic Fibrosis. Canadian Journal of Infectious Diseases and Medical Microbiology. Vol. 23 (Suppl B). Page 22B. 2012.

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List of Abbreviations

A549 :	Human Lung Carcinoma Epithelial Cell Line
AAV2 :	Adeno-Associated Vector 2
ABC :	ATP-Binding Cassette
Abelcet® :	Amphotericin B Injection (liposomal formulation by ENZON)
AmBisome® :	Amphotericin B (liposomal formulation by Astellas Pharma US, inc)
ANOVA :	Analysis of Variance
Arikace :	Amikacin (liposomal formulation by Transave, USA)
ATCC :	American Type Culture Collection
ATP :	Adenosine Triphosphate
BAL :	Broncho-Alveolar Lavage
Bcc :	*Burkholderia cepacia* complex
CaCl2 :	Calcium chloride
CAM :	Clarithromycin
CF :	Cystic Fibrosis
CFTR :	Cystic Fibrosis Transmembrane Conductance Regulator
CFU :	Colony Forming Unit
Chol :	Cholesterol
Cl^- :	Chloride
CYP3A4 :	Cytochrome P-450 system
DC-Chol :	3β-[N-(N', N'-dimethylaminoethane)-carbamoyl]-Cholesterol
DCP :	Dicetyl phosphate

DDAB:	Dimethyldioctadecyl-ammonium bromide
ΔF508 :	Delta Phenylalanine Amino Acid at Codon 508
DNA :	Deoxyribonucleic Acids
DOPE :	1,2-dioleoyl-sn-glycerol-phosphoethanolamine
DPPC :	Dipalmitoylphosphatidylcholine
DRV :	Dehydration-Rehydration Vesicles
eDNA :	Extrachromosomal DNA
EE :	Encapsulation Efficiency
ENaC :	Epithelial Na^+ Channels
ER :	Endoplasmic Reticulum
Free CAM :	Free Clarithromycin
IL-6 :	Interleukin 6
IL-8 :	Interleukin 8
Lipo-CAM :	Liposomal Clarithromycin
LPS :	Lipopolysaccharides
LTR :	Lower Respiratory Tract
LUV :	Large Unilamellar Vesicles
MAC :	*Mycobacterium Avium* Complex
MATE :	Multidrug and Toxic Efflux
MBC :	Minimum Bactericidal Concentrations
MBEC :	Minimum Biofilm Eradication Concentration
MF :	Major Facilitator

MIC :	Minimum Inhibitory Concentration
MLV :	Multilamellar Liposomes Vesicles
mRNA :	Messenger Ribonucleic Acid
MSD :	Membrane Spanning Domain
Na^+ :	Sodium
NBD :	Nucleotide-Binding Domain
NEG-Lipo-CAM :	Negatively charged Liposomal Clarithromycin
NEU-Lipo-CAM :	Uncharged Liposomal Clarithromycin
NPD :	Nasal Potential Differences
NTM :	Non-Tuberculosis Mycobacteria
OM :	Outer Membrane
OMPs :	Outer Membrane Proteins
OPS :	Octavalent O-Polysaccharide
PBS :	Phosphate-buffered saline
PC :	Phosphatidylcholines
PE :	Phosphatidylethanolamines
P-gp :	P-glycoprotein
PI :	Polydispersity Index
POS-Lipo-CAM :	Positively charged Liposomal Clarithromycin
PS :	Phosphatidylserines
R :	Regulatory domain
REV :	Reverse-phase Evaporation Vesicles

RNA : Ribonucleic Acids

RND : Resistance-Nodulation Division

SEM : Standard Errors of the Mean

SMR : Small Multi-drug Resistance

Sub-MIC : Sub-minimum inhibitory Concentration

SUV : Small Unilamellar Vesicles

CHAPTER ONE

1. General Review

1.1. Cystic Fibrosis

CF is the most common fatal inherited disorder that was first documented in the 1930s and is a challenging health problem still today (1-3). Although, CF can affect all races, the rate of occurrence among the Caucasian population (particularly among those of European descent) appears to be significantly higher, with 1 in 2,000 newborns being affected (2, 4). In Europe, the prevalence of CF among children and young adults is dramatically higher when compared to North American countries, the United States and Canada (4). CF arises as a result of a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (5). This protein regulates the production of digestive juices and mucus by acting as a channel to allow the movement of salt and water in and out of cells in the lungs and other tissues.

Although there is currently no cure for CF, therapeutics have been developed that can alleviate several of the symptoms associated with this disease.

1.1.1. Pathophysiology of cystic fibrosis

CF is considered a multi-system disease affecting the liver, sweat glands, pancreas, reproductive organs, intestines, sinuses, gastrointestinal tract and the lungs (3, 6). However, the most affected organs comprise the exocrine sweat glands, pancreas and the lungs (7, 8). The most debilitating aspect of CF is its effects on the lungs (7).

Mutations, that cause CF, affect the transport of sodium (Na^+) and chloride (Cl^-) ions across the epithelial membranes (8). The dysfunction of Cl^- ions transport and hyperabsorption of Na^+ give rise to the characteristic of salty skin phenotype among patients afflicted by CF (8-10).

This characteristic feature of CF led to the development of the salt skin diagnostic test, that involves placing a band on the child's forehead to measure Cl^- levels (7, 11).

The CFTR protein plays a critical role in the regulation and secretion of sweat, digestive enzymes and mucus (7, 12). With this disease, chloride secretion is reduced, which results in dehydration of water molecules from the airways (11). This dehydration causes thick and sticky mucus, invariably leading to dysfunction of cilia to cleanse the lungs (1) rendering them more susceptible to infections. Pulmonary infections cannot be cleared easily and they quickly develop to chronic infections, the main cause of morbidity and mortality in CF patients. Patients with CF have significant difficulties in breathing, often feeling as if they are drowning in their own mucus (11). The current hypothesis for the cause of CF lung disease suggests that CFTR dysfunction in the apical epithelial membranes results in an abnormal transport of Cl^-, Na^+ ions and H_2O leading to the depletion of airway surface fluid which is required for normal ciliary function (8-10). Once the disease has progressed to a point where the patient can no longer breathe unassisted, the only treatment that can be recommended to help alleviate this symptom is lungs transplantation.

1.1.2. Structure and function of CFTR

CF results from a mutation that affects the CFTR chloride channel, rendering it ineffective and unable to open. The CFTR functions as an ion channel that transports Cl^- ions across the cell membrane (13). When the transport of Cl^- ions is disrupted, the anion flow is decreased, resulting in the buildup of mucus in the lungs (14). The CFTR protein is primarily located in the apical membrane, where it provides a pathway for Cl^- ion movement across epithelia and regulates the rate of Na^+ ion flow.

The CFTR gene (Diagram 1) is located on the long arm of chromosome 7, spanning approximately 250-kb and containing 27 exons (15, 16). The CFTR gene encodes 1480 amino

acids, a protein product that comprises two repeated units of membrane spanning domains (MSDs) and nucleotide-binding domains (NBDs) (9). Each unit of MSDs consists of six hydrophobic transmembrane α-helices that form the anionic channel, while the NBDs interact with ATP (17). The two repeated units are linked by a single regulatory (R) domain that contains multiple consensus phosphorylation sites and many charged amino acids (17, 18). It separates the two MSDs and interacts physically with the NBDs. The R domain is unique for CFTR as it is not present in the other members of the ATP binding cassette (ABC) superfamily (18). The CFTR is classified as an ATP binding cassette transporter (ABC transporter); as the name suggests, the channel uses ATP binding to open and close the channel (13, 19).

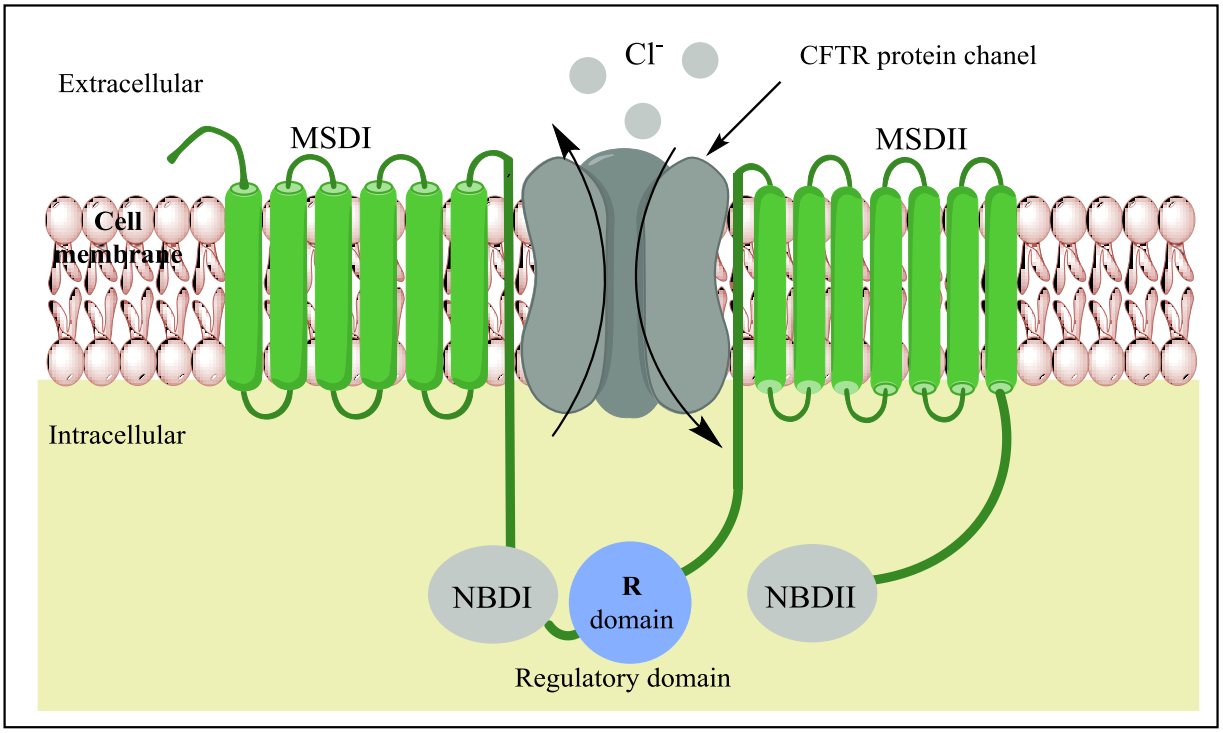

Diagram 1: Structure of cystic fibrosis transmembrane conductance regulator.

1.1.3. Classes of CFTR mutations

Since the discovery of the CFTR gene in 1989, more than 1,900 mutations have been identified (20-22). The most commonly studied mutations are the ΔF508 and G551D mutations. With the ΔF508 mutation, a phenylalanine amino acid is deleted from the NBDI sequence (23, 24). Interestingly, this deletion does not result in irregular protein folding as it was previously thought. Instead, the mutation causes the CFTR to be less soluble in H_2O. In addition, this mutation causes a chemical change in the CFTR protein, thereby making it a poor signal receptor. On the other hand, the G551D mutation prevents ATP from binding to the NBDs thereby making the channel gating slow and inefficient (13).

CFTR mutations are divided into 6 classes (Diagram 2) based on their effects on CFTR production and the level of residual CFTR function (20, 22, 25). Although not all mutations fit exclusively into one class, the system nonetheless is helpful towards determining which medication or course of treatment would prove beneficially for a particular mutation. The different CFTR mutations, that have been identified primarily, affect the translation of the CFTR gene product at different stages (11). Class I mutations result in the insertion of a premature stop codon, resulting in a truncated CFTR protein that fails to reach the cell membrane (26, 27). Class II mutations (e.g. ΔF508) result in defects of protein processing. The CFTR protein that fails to fold correctly, having no glycosylation on it, remains in the endoplasmic reticulum (ER) and eventually gets degraded before reaching the membrane (9, 27). Class III mutations are classified as gating mutations. The CFTR protein is transported to the cell membrane; however, the channel does not open properly and chloride transport cannot occur leading to a defect in chloride regulation of CFTR (11, 26). In class IV mutations, the CFTR protein reaches the cell membrane and is partially functional. Due to the decreased chloride conductance the chloride ion

fails to move through the channel effectively which results in conductance defect (11, 16, 28). Class V mutations result in splicing abnormalities in the CFTR protein, which reduce the number of functional CFTRs that reach the membrane; however, these proteins remain functional and are capable of effectively transporting Cl^- ions (9, 11). Finally, class VI mutations result in unstable CFTR protein with increased turnover rate (20, 22).

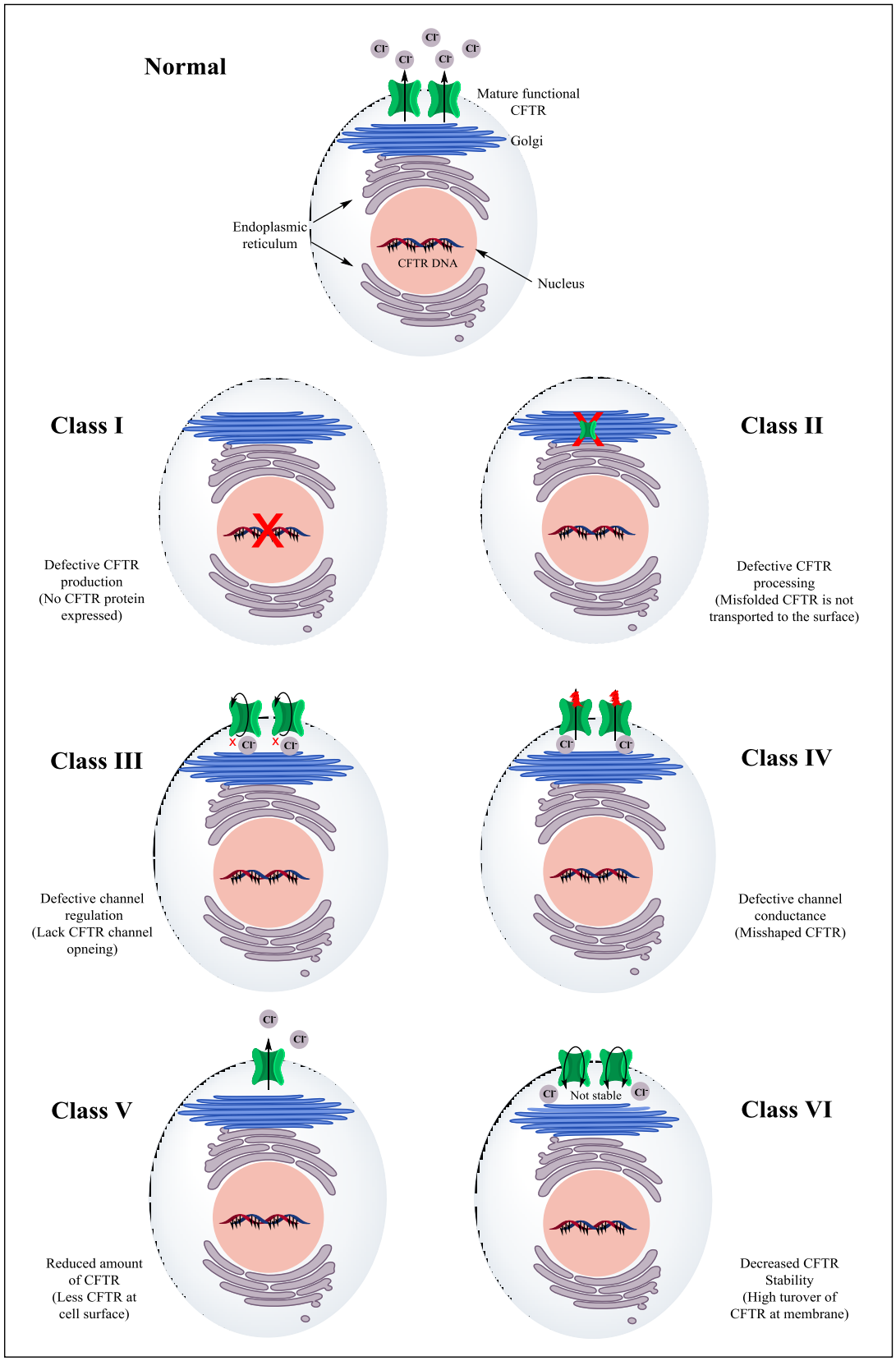

Diagram 2: Classification of CFTR mutations.

1.2. CF lung inflammation and infection

Patients with CF suffer from lung inflammation and are prone to infections. Numerous scientists have studied CF lung pathogenesis and have proposed several hypotheses of the cause. Firstly, lung inflammation is responsible for causing primary damage to lung parenchyma. Secondly, bacterial colonization of the mucus layer is at the root of inflammation and infection (29, 30). Finally, antimicrobial treatments prove ineffective in reducing the inflammation. Lung inflammation can be observed in patients with CF from a very early age (30). Studies have shown that young children with CF show signs of inflammation in their lungs even in the absence of bacterial infections (29). Lung biopsies show an absence of any pathogens which could be responsible for the inflammation, and therefore it is thought that the inflammation is a CF specific symptom (31). However, current tests do not detect mild viral infections, and therefore cannot be discounted as a cause of inflammation in patients with CF (31). Fluid biopsies taken from the lungs of patients with CF show increased concentrations of the cytokines interleukin (IL)-6 and IL-8 (32). These are produced by the respiratory epithelium cells to attract neutrophils and macrophages into the lung (33). The presence of neutrophils in the CF sputum is associated with neutrophilic inflammation and airway mucus obstruction. The airway surface fluid of patients with chronic airway diseases usually has a high concentration of neutrophil elastase, a serine protease, which is released by neutrophils. Neutrophil elastase can damage mucociliary clearance, thus stimulating mucin production, contributes to an overall increase in airway inflammation (34). This increase in inflammation causes direct mechanical damage to the lungs by alterations in mucociliary movements and by an autoimmune response against chronic airway bacterial infection, which causes enzymatic destruction of the lung parenchyma (35).

The environment in the lungs of CF patients is an ideal breeding ground for bacteria (36). The initial infection causes an imbalance in the sodium channel (ENaC) function, which results in a reduction of airway surface liquid volume (37). This leads to further increases in the levels of neutrophils and macrophages within the airways.

Patients with CF are prone to acute infections with bacteria, such as *Staphylococcus aureus* and *Haemophilus influenzae* (29). Also, many of these patients become chronically infected with another bacteria, *Pseudomonas aeruginosa,* and there are no treatments available with antibiotics against initial colonization (38). It is also possible that the infection and lung damage occur due to the presence of *P.aeruginosa* at bronchial tree (39). The majority of CF fatalities results from respiratory failure secondary to infection and inflammation (38, 39). Presently, modern advances in medical treatments have significantly reduced morbidity rates and the occurrence of severe acute infections that have contributed to the prolongation of life in patients diagnosed with CF. However, the constant state of inflammation and presence of chronic infections cause a slow buildup of damage to lung parenchyma (40).

1.3. Microbiology of lung infection in cystic fibrosis

From a very early age, patient affected by CF are more prone to contracting lung infections. Microbial cultures from the broncho-alveolar lavage (BAL) fluid of CF patients often show the presence of multiple microbial pathogens (41, 42). Most bacterial infections are caused by *S. aureus, H. influenzae, Burkholderia cepacia* and *P.aeruginosa* (43-45). Analysis of the BAL fluid has revealed a correlation between the presence of specific microorganisms and the age of the patients. For example, *S. aureus* is the most common pathogen found in CF children before the age of one, while *H. influenzae* and *P. aeruginosa* are the predominant species found in older

than one CF patients. *B. cepacia* on the other hand, is predominantly seen in adults and less in children (42).

1.3.1. *Staphylococcus aureus*

S. aureus is a Gram-positive, aerobic and non-motile coccus which is normally found on human skin (46, 47). This ubiquitous species causes infection in approximately 70% of children aged 6 to 10 (42). Studies have shown that infections with *S. aureus* and *P. aeruginosa*, alone or in combination, lead to varying degrees of morbidity in patients with CF. Fraunholz and Sinha, 2012, have delineated the mechanism by which this organism infects and survives within a host (48). The mechanism governing the entry of the pathogen into the host involves its ability to adhere to the host cell and the ability to alter host signaling pathways, apoptotic mechanisms and autophagy (46, 48, 49). *S. aureus* is usually the first organism to infect the lower airways of CF patients and typically precedes infection by *H. influenzae* and *P. aeruginosa*, respectively (50). In CF patients, *S. aureus* colonizes the lower respiratory tract, triggering an inflammatory cascade, which ultimately results in tissue damage (51). This compromised environment provides favorable conditions for other species such as *H. influenzae* and *P. aeruginosa* to flourish. *S. aureus* generates multilayered biofilms which contribute to antibiotic resistance, thereby making it difficult to eradicate the infection (42). Progress in CF research has shown that extrachromosomal DNA (eDNA) is a component of these biofilms, a feature that provides a new target for novel CF treatments. *In vitro* studies have shown some promising results by using a combination of DNase and antibiotic treatments (52) The DNase degrades the eDNA and weakens the biofilm, thereby removing the protective layer against antibiotics (53).

1.3.2. *Haemophilus influenzae*

H. influenzae is a Gram-negative, facultative anaerobic species which has been isolated from humans (54). This species is further categorized into six serotypes based on the nature of its outer oligosaccharide capsule (55). Some *H. influenzae* members do not contain this capsule and are classified as nonencapsulated or nontypeable *H. influenzae* (56). It has been found that the nonencapsulated form of *H. influenzae* predominates in the lower airways of CF patients with chronic lung infection (54). Interestingly, studies have shown that growth of nonencapsulated *H. influenzae* was inhibited by the mucoid *P. aeruginosa* and some *Proteus* species (57, 58).

H. influenzae is commonly found in the lungs of CF patients and some studies suggest that the colonization of *H. influenzae* in CF patients is comparable to that of *P. aeruginosa* (42). There is still much debate regarding the mechanism by which *H. influenzae* causes tissue damage in CF patients. In these patients, *H. influenzae* has been shown to colonize in the oropharynx, sputum and lower respiratory tract (LRT) (59). Infection of the LRT is not a common occurrence within the non-CF population (60). Once colonized in the LRT, *H. influenzae* disease progresses due to onset of inflammation (61). Increased the levels of leukocytes, particularly neutrophils, can be detected in the BAL of CF patients (42). Clinical consequences of *H. influenzae* infection in immunocompromised individuals, such as those afflicted with CF, include rhinosinusitis, otitis media and pharyngitis (62). The presence of biofilms also leads to early lung infections that compromise the tissue to subsequent and more severe infection by *P. aeruginosa* (62).

1.3.3. *Burkholderia cepacia*

The *Burkholderia cepacia* complex (Bcc) is a Gram-negative, non-spore forming, rod-like bacteria that occur naturally in plants, wet soil and any moist environment (63). This bacterium, previously known as *Pseudomonas cepacia*, is the primary causative agent of soft rot in onion

bulbs (64). The prevalence of *B. cepacia* in CF patients has been documented since 1970s and infections from this organism still persist today (44). It is estimated that nearly 40% of sputum cultures from CF patients contain *B. cepacia* (50). *B. cepacia* often precedes systemic bacterial infection, increases inflammatory mediators in the blood and decreases erythrocyte sedimentation rates (65). These symptoms are characteristic of the "cepacia syndrome", an often fatal condition (66). The compromised lung surface in CF patients tends to exacerbate the incidence and severity of this condition (50). Like other bacterial species, *B. cepacia* also damages the lungs cell surfaces (67), thus making it more susceptible to infection by other bacteria and/or viruses. *B. cepacia* tends to infect adults with CF and unfortunately, the morbidity associated with this organism exceeds that of *P. aeruginosa* (42).

B. cepacia infection mechanisms have been extensively studied. Similar to *P. aeruginosa, B. cepacia* also possesses the ability to form a glycoprotein capsule which protects it from host defence mediators (50, 66). Further, the Bcc group also exhibits intrinsic resistance to polymixins and most aminoglycoside and β-lactam antibiotics (68). In addition to this innate resistance, this bacterium can also acquire resistance to other kinds of antibiotics during *in vivo* treatment regimens (69). This feature makes the Bcc group especially dangerous. One method used by this organism to achieve antibiotic resistance is the production of inactivating enzymes, such as β-lactamase, a compound which has been shown to degrade β-Lactams class of antibiotics (70). In addition, *B. cepacia* also has active efflux pumps which efficiently pump out antibiotic agents that enter the cell. Furthermore, the bacteria can also alter receptors that the antibiotics target, thereby making the cells insensitive to the drugs (64). Apart from its pathogenic effects, *B. cepacia* can also make the lung surface more susceptible to infection by other bacterial species and viruses.

1.3.4. *Pseudomonas aeruginosa*

P. aeruginosa is the most common microorganism found in the sputum of patients with CF (44). Approximately 80% of patients over the age of 20 show *P. aeruginosa* colonization in their lungs (71, 72). Another common feature associated with this bacterial species is antibiotic resistance, which is one of the leading causes of chronic lung infections among CF patients (73). This virulent bacterium is classified as Gram-negative and creates biofilms in order to protect itself from host immune system and antibiotics (74). During the biofilm formations, the flagella and type IV pilus of *P. aeruginosa* are required for cell motility (75-77). Once bacteria gains access to the lung, this motile bacterium uses its flagella to attach into the host cell, causing infection (72, 74, 78). The altered surface membrane glycoproteins in the lungs of patients with CF act as anchoring points for the attachment of this bacterium (50). The pathogenesis of *P. aeruginosa* in the lungs of CF patients can be described as a two-phase mechanism. The first phase is characterized by the presence and isolation of the organism and its opposing antibodies, at this point having minimal effects on pulmonary function (79). The second phase is characterized by chronic infection, which arises as a result of a prolonged incubation of the pathogen within the host. At this point, the presence of the bacterium can be detected in the sputum of the affected individuals (80). During this chronic phase, the bacteria produce a polysaccharide, called alginate, which acts as an immunogen. Antibodies against alginate can be isolated from patients with CF (50). Long term infection by *P. aeruginosa* causes significant damage to the lung surface leading to airway obstruction and ultimately loss of pulmonary function (81). Previous studies have shown that *P. aeruginosa* produces a biofilm in the airways of the lungs of patients with CF (82, 83). The biofilm acts as a protective barrier for bacterial cells by limiting their exposure to antibiotics, thereby worsening the disease prognosis in CF

(72). Furthermore, biofilm contributes to the bacterial tolerance towards the host innate defense mechanisms (82, 84). These pathological changes seen in the CF lung are all associated with the mucoid nature of *P. aeruginosa* and its production of alginate (50, 81). In conclusion, all of these pathogens become chronic due to the resistance to commonly used antibiotic. Current research efforts focus on antibiotic therapy to develop effective treatments for cystic fibrosis.

1.4. Resistance mechanism of pathogens

1.4.1. Drug inactivation

Bacteria have evolved several mechanisms that make them resistant to antibiotics, thus limiting treatment options (85). Generally, bacteria acquire resistance to antibiotics by either (i) altering the antibiotic binding target or (ii) through the production of inactivating enzymes that decrease antibiotic activity (86).

Hydrolysis, group transfer and redox are other enzymatic strategies employed by bacteria to inactivate antibiotics. Amides are an example of enzymatic targets which are commonly found in antibiotics (86). Bacteria target the amides by cleaving hydrolytically susceptible chemical bonds, as a means of destroying antibiotic activity. In addition, resistance enzymes such as the phosphotransferases, the largest family of resistance enzymes, modify the antibiotics by causing structural alterations resulting in impaired target binding (86, 87). Bacteria can also produce and secrete enzymes that add acetyl groups to the antibiotic periphery which interferes with bacteria-antibiotic binding. Furthermore, oxidation is another method used for antibiotic detoxification (86). Finally, mutation or modification can also occur on the target sites of bacteria which prevent antibiotic binding to its ribosomal targets (88). Bacteria can acquire resistance to an antimicrobial agent through mutations by upregulating the production of enzymes that inactivate

the antimicrobial agent, such as erythromycin ribosomal methylase in staphylococci (88, 89). Mutations enable several bacterial species to quickly adapt to strains in their environment including antimicrobial agents (89).

1.4.2. Outer membrane permeability

The outer membrane offers strong permeability barrier to antibiotics that are effective against some bacteria. Gram-negative bacteria differ from Gram positives by structural differences of their cell wall. The Gram-negative bacterial membrane is composed of an inner layer of phospholipids and an outer layer of lipopolysaccharides (LPS) (90). In humans, LPS triggers an innate immune response which causes the release of cytokines thereby leading to inflammation. The structure of this membrane and the water-filled pore protein work together to derail the penetration and conduction of antibiotics across the bacterial cell membrane (89). The inner membrane restricts the penetration of hydrophilic substances which results in a perfect barrier. Gram-negative bacteria are sensitive to changes in the permeability properties of the lipid bilayer and the diffusion of porins which form channels in the cell membrane (90). These changes improve resistance to antibiotics through an adaptive counteractive mechanism. Studies have shown that changes in the proportions of porins found on the membrane could decrease cell envelope permeability to antibiotics. For example, low permeability of *P. aeruginosa* membrane often works in combination with efflux pumps in order to promote drug resistance (89).

Modifications of the lipid and protein composition of the outer membrane (OM) are used to enhance drug resistance in several bacterial species (90, 91). Other studies have also detected the presence of plasmids, transposons and bacteriophages that may promote antibiotic resistance (92, 93). Further, outer membrane proteins (OMPs) also undergo various adaptations depending on

specific microbial environmental conditions such as pH (91, 94). Therefore, OMPs play an important role in the adaptation of the bacteria to the host's biological environment.

1.4.3. Efflux systems

Bacteria, including *P. aeruginosa, E. coli, S. aureus,* often achieve drug resistance by increasing expression of efflux pumps (95, 96). Efflux pumps comprise a special type of protein that works to remove antibiotics from the bacterial cytoplasm by pumping them back into the external environment (95, 97). Macrolides and tetracycline have internal mechanisms of action and therefore efficiently pumping them out of the bacteria renders the antibiotic ineffective (88). Antibiotic extrusion proteins are commonly found in Gram-negative bacteria in addition to a number of eukaryotes (97). Efflux pumps typically work on one specific class of antibiotics; however, some pumps also show cross-reactivity to broader ranges of commercially available antibiotics (97, 98).

There are 5 major families of efflux pump transporters: (i) Major Facilitator (MF), (ii) Multidrug and toxic efflux (MATE), (iii) Resistance-nodulation division (RND), (iv) Small multi-drug resistance (SMR), and (v) ATP binding cassette (ABC). These efflux pump transporters exploit ATP hydrolysis and the proton motive force as their main energy source for displacement of antibiotics from bacterial cytoplasm to external environment (87, 95, 97, 99).

1.4.4. Biofilm

Biofilm refers to a community of microbes where cells are attached to each other on a surface (100-102). Microbes form these biofilms in response to a number of factors. For example, bacteria use the formation of biofilms to limit their exposure to toxic substances such

as antibiotics and/or antimicrobials (102-104). Biofilm matrix is composed of an assortment of exopolysaccharides, extracellular DNA (e-DNA) and proteins (101, 105).

Biofilm development (Diagram 3) involves different phases including attachment, formation of microcolony and macrocolony maturation, and detachment (101, 106). Biofilms incur resistance to antimicrobial/antibiotic agents by slowing or preventing their penetration beyond the film (107-109). Biofilm cells also express protective factors such as multidrug efflux pumps and stress-response regulators (84, 110, 111). Growth rates are significantly diminished in the center of the biofilms, as compared to the outermost portion of the biofilm, due to nutrient limitations (111). Increased resistance of *P. aeruginosa* against antimicrobial/antibiotic agents is attributed to its specialized biofilm matrix, which is called a mucoid *Pseudomonas* biofilm. This specialized biofilm has been shown to reduce antibiotic susceptibility by over 1,000 fold (101, 109). Cultures of *P. aeruginosa* biofilm show varying levels of oxygen, 50 to 90 μm between the outermost and innermost sections of the biofilm colony (101).

Biofilm secretes polymeric nutrients, metabolites and lyses as an antibiotic resistant adaptive mechanism (101, 112). This results in the formation of a complex biofilm matrix material and the formation of mushroom shaped macro-colonies of mature biofilm community (107). This resistance to antibiotics is attributed to the unique anatomical and biochemical features of biofilm, which are absent in other microbial cells. These features include the presence of the biofilm matrix, which forms a physical and chemical barrier against the antibiotics action on or inside the biofilm microbial cells (84). The mucoid-bearing biofilm demonstrates more than 1000 times reduced susceptibility to antibiotics than non-mucoid biofilm (101). It is also argued that the biofilm matrix involves retardation of the antibiotics action, which is mediated by a specialty antibiotic mutated gene (110). In conclusion, biofilms are responsible for

complications and fatalities associated with human diseases such as CF. The resistance properties of the biofilm make it extremely difficult to eradicate by antibiotic treatment alone.

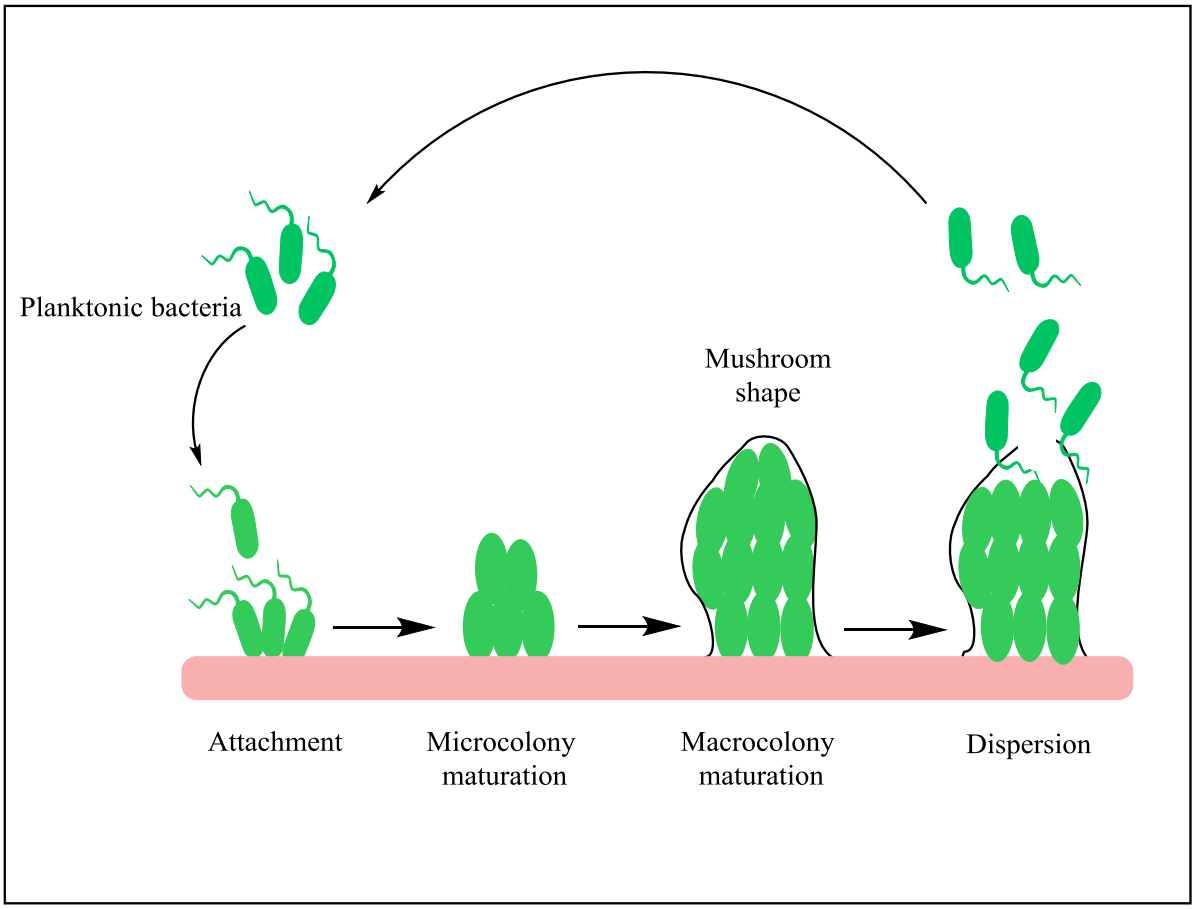

Diagram 3: Biofilm formation stages.

1.5. Treatment of pulmonary infections in CF

Once *P. aeruginosa* is established in the airways, it is difficult to eradicate from CF patients; however, aggressive treatment can delay the development of chronic infection. Novel approaches are required to improve quality of life of those afflicted by this disease and to also achieve further increases in life expectancy.

1.5.1. Antibiotics therapy

The commonly used treatment strategy in CF includes antibiotic therapy aimed at preventing, eradicating, or controlling respiratory infections (113). Over the years, a standard strategy, such as antipseudomonal drugs, has been used to enhance activity and reduce resistant organisms (114). Therefore, effective use of antibiotics holds great promise for improving the quality of life and reducing the impact of infection in patients with CF (39, 115). There are several classes of antibiotics with specific targets and/or functions that have been used as a treatment of CF.

1.5.1.1. Aminoglycosides

Aminoglycosides are the antibiotic of choice in patients suffering from CF with chronic *P. aeruginosa* infection (115). These antibiotics are mostly used to prevent infections against Gram-positive as well as Gram-negative bacterial pathogens (116, 117). They are also widely employed in order to manage pulmonary exacerbation in patients with CF who have developed chronic pulmonary infection with *P. aeruginosa* (116). These antibiotics inhibit bacterial protein synthesis by interfering with translation by causing misreading of the codons in the mRNA (118). They can also disrupt the translocation process by binding to the 30S ribosomal subunit of the bacterial ribosome (119) which results in bacterial death (118). Although aminoglycosides have been used to manage pulmonary infection caused by opportunistic pathogens such as *P. aeruginosa* with some success, drug cytotoxicity has limited their clinical application (115, 116). Amikacin, gentamicin and tobramycin are examples of aminoglycoside antibiotics, which are a choice in treatment of pulmonary infections caused by *P. aeruginosa* (119). However, the high

concentrations of aminoglycoside that required in treating pulmonary infection in CF are associated with toxicity risk (117, 119).

1.5.1.2. Macrolides

Macrolide antibiotics are characterized by the presence of a macrocyclic lactone ring (Diagram 4) to which one or more deoxy sugar moieties are attached (120, 121). Since their discovery in 1952, these antibiotics are effectively used to treat infections arising from Gram-positive (120) and Gram-negative bacteria pathogens (122, 123). The basic structure (Diagram 4) of the macrolides antibiotics is characterized by a lactone cycle with two osidic chains; and they are classified according to the number of lactone ring in the cycle: 14, 15 and 16 membered macrolides (123, 124). Macrolides exhibit both bacteriostatic and bactericidal property in susceptible bacteria according to antibiotic concentrations and bacterial sensitivity (125). Due to their broad spectrum property, macrolides have been the antibiotic of choice for those suffering from chronic pulmonary inflammatory syndromes including diffuse panbronchiolitis, CF lung infection, chronic obstructive pulmonary disease, and asthma (121, 125). Long-term use of macrolide at a low concentration has been found to be useful in patients with chronic airway disease (121, 126).

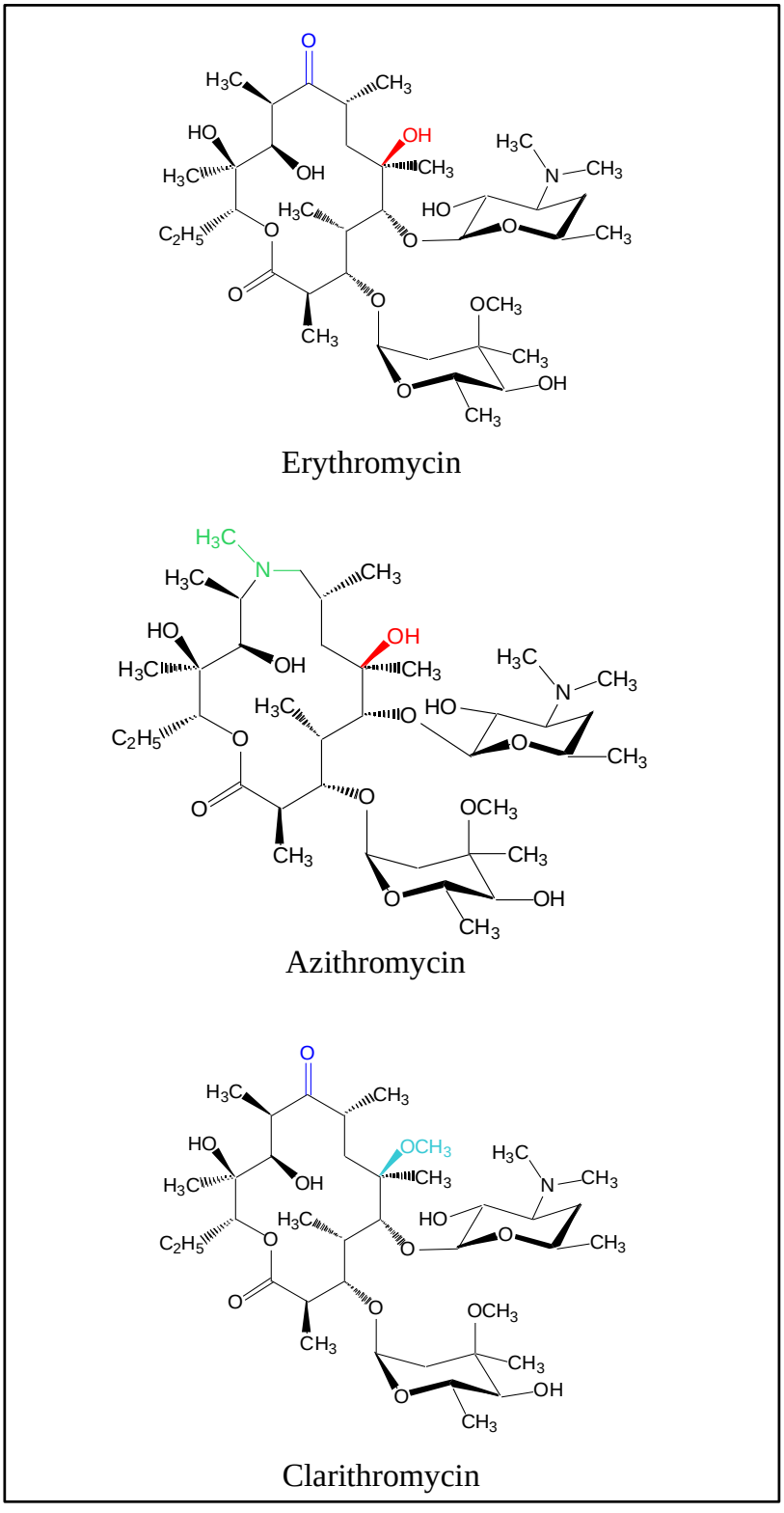

Diagram 4: Macrolide chemical structures.

1.5.1.3. Mechanism of action

Macrolides act by inhibiting bacterial protein synthesis directly (120). They bind to the P site of 50S ribosomal subunit (Diagram 5) and appear to inhibit the movement of the growing peptide chain (122). Moreover, these antibiotics have been shown to cause premature dissociation of peptidyl-tRNA from the ribosome (127). The antibiotic appears to prevent the movement of the growing peptide chain. They can also interfere with the bacterial protein synthesis by disturbing the binding of the peptide moiety of the peptidyl-tRNA (124).

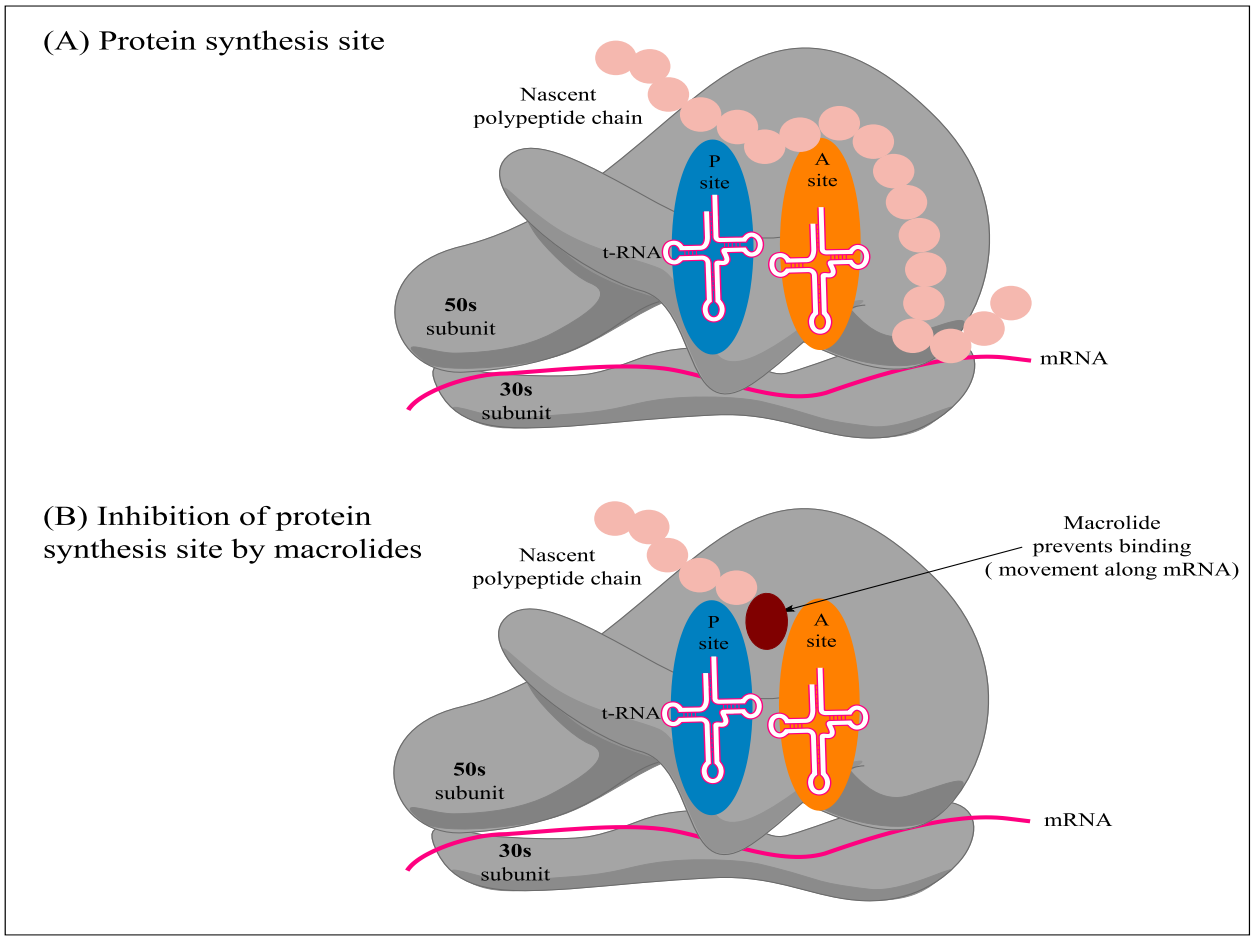

Diagram 5: Mechanism of action of macrolides.

1.5.1.4. Erythromycin

Erythromycin, the first member of macrolide antibiotics, has been in clinical use for more than four decades (124). Structurally, the antibiotic contains 14-membered lactone rings (124) and is derived from the bacterium *Streptomyces erythreus* (128). Erythromycin has been shown to effectively eradicate acute or chronic state of diphtheria (129). Furthermore, due to its close association with penicillin in terms of antimicrobial spectrum, it is often prescribed as an alternative to penicillin (130). The immunomodulatory effect of erythromycin has made it a good choice for treatment in CF lung infection and inflammation (131). It was demonstrated *in vivo* that an optimum dose of erythromycin would decrease sputum mucus secretion that binds to chloride channels in epithelial cells (132). In addition, erythromycin also reduces the production of pro-inflammatory cytokines and NF-κB activation in CF infections (131). Erythromycin shows potent anti-inflammatory effect and thus is widely used for treating chronic lower respiratory tract infections (131, 133). As well, it is the drug of choice in treatment of bronchiectasis and bronchiolitis which are characterized by chronic inflammation of respiratory bronchioles (134). Apart from that, treatment with erythromycin was effective even in the presence of erythromycin resistant bacteria such as *H. influenzae* (135, 136). A lower dose of erythromycin has significant clinical implications on patients with *P. aeruginosa* infection (136). Therefore, erythromycin might play an important role in the future development of novel antibiotic therapies against *P. aeruginosa* infection, especially in the cases of CF and bronchiectasis.

1.5.1.5. Azithromycin

Azithromycin has been produced by chemical modification of erythromycin (Diagram 4); it has a 15-membered macrocyclic lactone ring structure (137). This antibiotic exhibits bacteriostatic activity against Gram-positive bacteria although many Gram-negative rods, such as *P. aeruginosa*, are inherently resistant to azithromycin (137, 138). It is one of the most potent drugs for the treatment of upper and lower respiratory tract infections (138, 139). Earlier studies also suggested that long term use of azithromycin in low doses is beneficial in CF patients harboring chronic *P. aeruginosa* infection (138, 140). Apart from that, azithromycin shows anti-pseudomonal activity through inhibition of a pseudomonal quorum sensing-system (105, 140). It suppresses virulence factor production, reduces oxidative stress response and interferes with biofilm formation, but has no influence on its proliferation (138, 141, 142). *In vitro* study, using low doses of azithromycin, has shown to improve many key parameters of lung functions in CF patients (143). Due to its antagonistic role in the quorum-sensing system, azithromycin administration results in improved clearance of *P. aeruginosa* biofilms along with a marked inhibition in alginate production, all of which reduces disease severity (142, 144). Furthermore, it has been demonstrated that Sub-MICs of azithromycin can inhibit the production of several QS-related virulence factors of *P. aeruginosa* (141, 142). Recent *in vivo* studies with sputum samples from CF patients showed similar results with the findings of clinical studies that azithromycin treatment improved lung function in CF patients (142). Prolonged use of azithromycin has been shown to improve clinical outcomes in patients with chronic inflammatory lung diseases, such as CF (144). However, azithromycin use has been reported to be associated with and increased infection rate of non-tuberculosis mycobacteria (NTM) (145).

In vitro studies have confirmed azithromycin as the most potent anti-inflammatory drug, followed by roxithromycin, clarithromycin and erythromycin respectively (146).

1.5.1.6. Clarithromycin

Clarithromycin is a relatively new class of macrolide antibiotic which was introduced around 1991 (135). It has a 14-member ring (147) structure like erythromycin (Diagram 4) but contains a methylated hydroxyl group at position 6 with a lactone ring attached to two sugar moieties (135). Clarithromycin contains several important pharmacokinetics properties; for example, unlike erythromycin, it is acid stable and has a half-life twice that of erythromycin (148). Moreover, the antibiotic is readily absorbed and widely diffused into most tissues with high concentrations especially in lungs (149). In addition, it is extensively metabolized in the liver by hepatic cytochrome P-450 enzymes (135, 150). Some of the usage of clarithromycin includes pharyngitis, tonsillitis, acute bacterial exacerbation of chronic bronchitis, and pneumonia (148, 150, 151). The antibiotic has been reported to be effective against respiratory tract infections (135) as well as skin infections in clinical studies. Clarithromycin inhibits bacterial growth by interfering with its protein synthesis even at a sub inhibitory concentration level (152). Apart from the bacteriostatic activity, clarithromycin also exhibits bactericidal activity (153). Moreover, the antibiotic has been shown to down-regulate inflammatory responses and is associated with immunological changes (147). Clarithromycin exhibits anti-inflammatory effect through inhibition of the neutrophil migration and pro-inflammatory cytokines (154) which are responsible for the increased phagocytosis and natural killer cell activity (151). In addition, the antibiotic might play an important role in the suppression of inflammation by inhibiting neutrophils and reducing the level of several cytokines (134). Lower level of cytokines has been observed both in plasma and sputum in clinical studies (155).

Furthermore, multiple studies suggest that, clarithromycin may regulate mucus glycoprotein formation by inhibiting the expression of transcriptional factors of mucus glycoproteins (156, 157). Even in studies with AIDS patients and *Mycobacterium avium* complex infections, clarithromycin has been found to be effective and safe (152, 158). Despite its several remarkable benefits, clarithromycin resistance is increasingly being reported in clinical isolates of pathogens and therefore, an approach using a combination of clarithromycin and other antibodies is currently being tested (159). For instance, a combination of clarithromycin/tobramycin has been successfully reported to eliminate *P. aeruginosa* biofilm formation in CF patients suffering chronic infection (153, 160). In addition, when used with amikacin, clarithromycin was able to improve amikacin activity through a reduction of bacterial adherence to the biofilm (161). However, one of the limitations associated with clarithromycin is that prolonged usage can result in gastrointestinal irritation and hepatotoxicity, especially among elders (152). Clarithromycin uses cytochrome P-450 system (CYP3A4) both as a substrate and an inhibitor (150, 162). Further studies have also found that clarithromycin is associated with high probability of digoxin toxicity and the risk is four times greater than with erythromycin or azithromycin (163, 164). Also, clarithromycin has been found to inhibit P-glycoprotein (P-gp) resulting in an increase in digoxin level (164). This poses a higher risk of overt toxicity and due to this its application has been limited. All these warrant further studies to explore more viable combinatorial antibiotic therapies. For instance, recent studies have shown that liposome mediated drug delivery is more efficient than combination therapy in free form in reducing drug toxicity (165). Due to this limitation, an appropriate drug delivery system such as liposomes is needed to overcome drugs' toxicity.

1.6. Liposomes as drug delivery system

Drug carrier systems, such as polymer micelles, nanoparticle dispersions consisting of small particles, nanocrystals, and liposomes were shown to have great promise as drug delivery systems (166-168). Bangham first characterized liposomes in the early 1960's as bilayer vesicles of phospholipids in aqueous solutions (169, 170). Liposomes were considered as potential drug delivery systems to modify drug biodistribution and pharmacokinetics with the purpose of reducing the drugs' toxicity by their accumulation at the target tissue (170-172). The application of liposomes as potential drug delivery systems has been under investigation since the early 1970's (173). Liposomes can be filled with drugs, and used to treat several diseases including cancer and fungal infection (171). Among the various drug carriers, only a few make it to the stage of clinical trials. However, liposome showed a strong potential for effective drug delivery to the site of action (170). The liposome-loaded drugs can be delivered by various routes such as intravenous, oral inhalation, local application, and ocular (168, 172, 174). Liposomes, as a drug delivery system, were used in medicine due to their unique properties of encapsulating both hydrophobic and hydrophilic drugs (175). The hydrophilic drugs are held in the center of the liposomal vesicle, whereas hydrophobic drugs can be incorporated into the membrane bilayers for transport (171, 175).

1.6.1. Liposomal structure

Liposome (Diagram 6) contains one or more concentric lipid bilayers with a hydrophilic head group and a hydrophobic tail enclosing an internal aqueous volume (176, 177). The hydrophilic tails organize themselves to face each other in the bilayer and the lipids' headgroups

are exposed to the aqueous phase (176, 178). Different phospholipids (Diagram 7) can be used in fabricating the liposomal vesicles such as phosphatidylcholines (PC), phosphatidylethanolamines (PE) and phosphatidylserines (PS) are commonly used as phospholipids for liposome preparation (176, 179). Cholesterol can be added to the bilayer mixture to reduce the permeability of liposomal membrane and increase the stability (180). Liposomes can have different net charges depending on the phospholipids' content such as dipalmitoylphosphatidylcholine (DPPC) neutral, dimethyldioctadecyl-ammonium bromide (DDAB) cationic charges, and dicetyl phosphate (DCP) anionic charges (181).

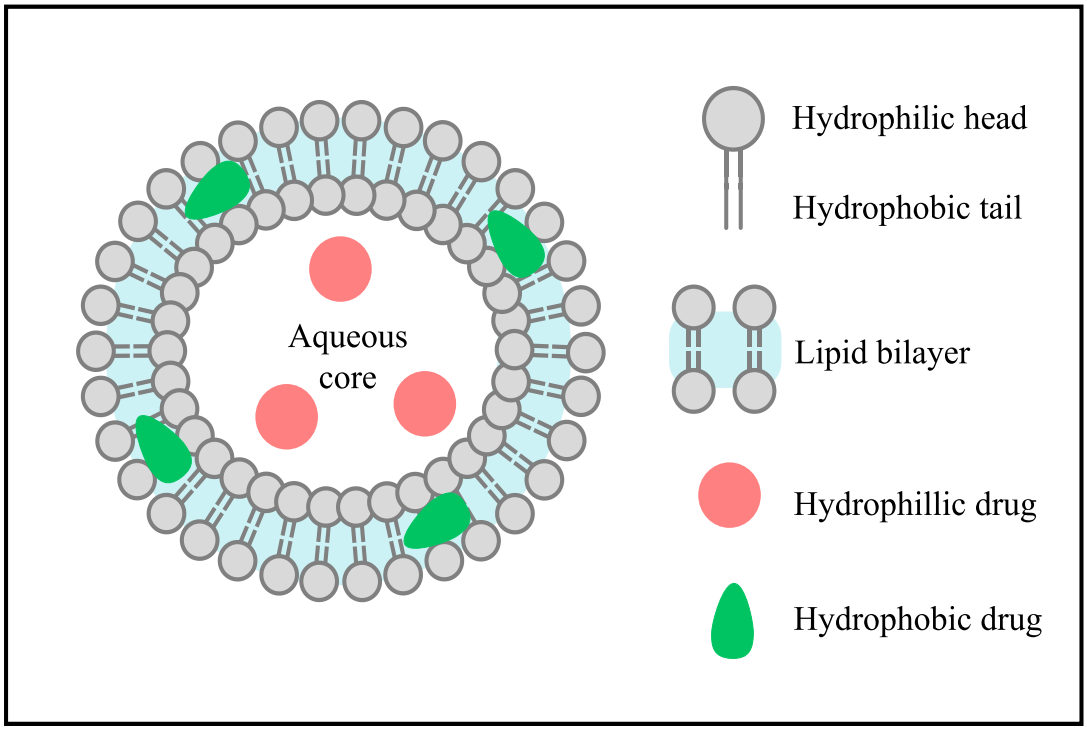

Diagram 6: Structure of liposome.

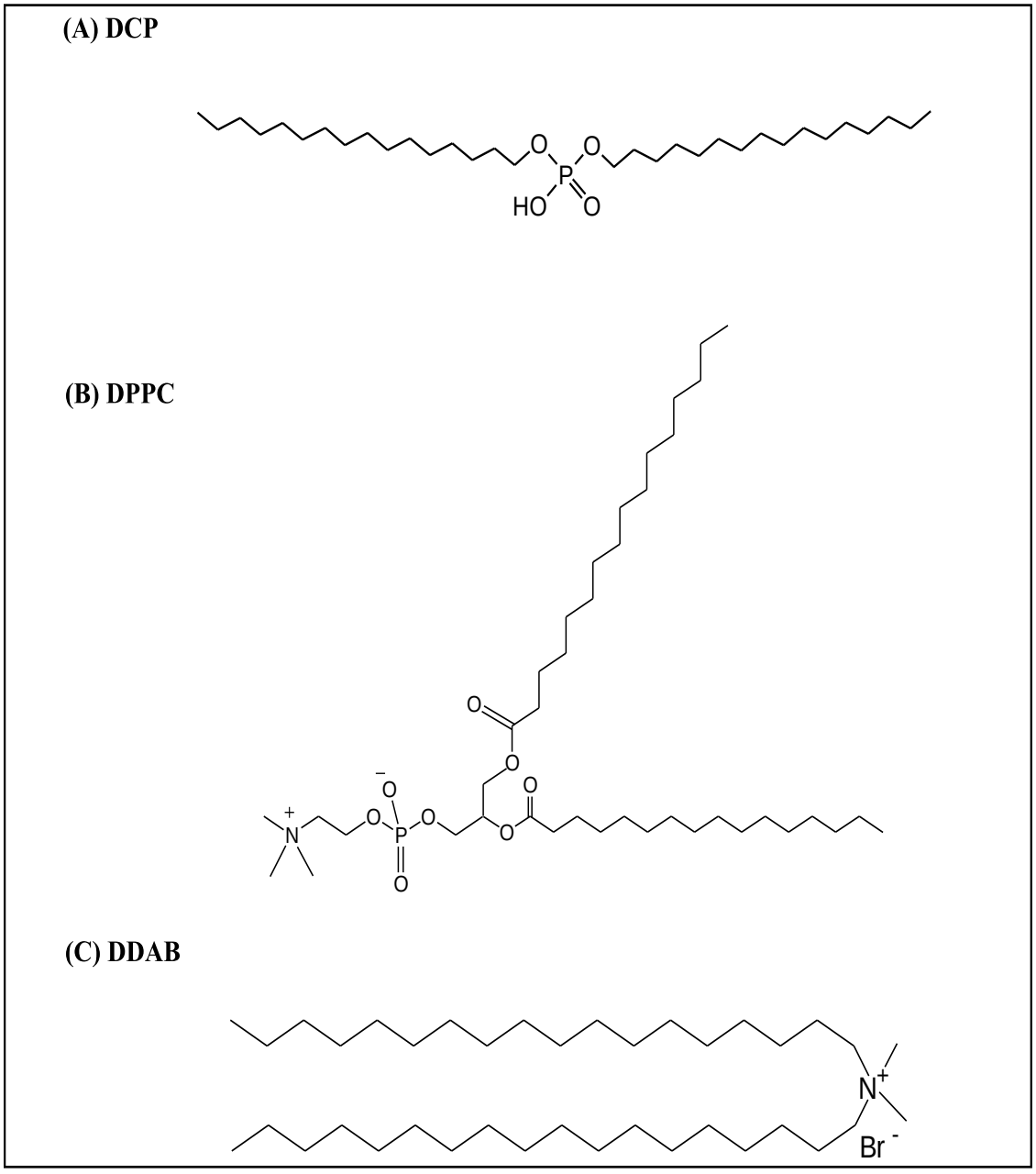

Diagram 7: Examples of phospholipids structure: (A) DCP-Dicetyl phosphate, (B) DPPC-Dipalmitoylphosphatidylcholine, and (C) DDAB- Dimethyldioctadecyl-ammonium bromide.

1.6.2. Liposomal preparation and characterization

Different types of liposomes can be prepared using various methods, implying that there are several operating mechanisms in liposome formation (Diagram 8). The first method used to prepare liposomes is the Bangham method (179, 181, 182), which is the most commonly used for the preparation of Multilamellar Liposomes vesicles (MLV) (183). Briefly, lipids are distributed onto a thin film on a round bottom flask, dissolving the lipids in an organic solvent such as choloroform, and later evaporating that solvent (184). Then the film is hydrated by adding an aqueous solution under vortexing conditions and this results in liposome formulation (183, 185). However, the major limiting factors of the Bangham method are its low encapsulation efficiency, low internal volume, and heterogeneous size distribution (185,186). A Reverse-phase evaporation vesicles (REV) method has been developed to increase the entrapped efficiency (187) through the addition of several phospholipids and cholesterol to organic solvents followed by the removal of the solvent via reducing pressure with a rotary evaporator. Next, lipids are redissolved in organic phase, followed by adding aqueous solution. The mixture is then sonicated to produce inverted micelles. The organic solvent is removed and system is kept under continuous nitrogen until the mixture becomes a homogeneous dispersion (188). However, this method is not safe for pharmaceutical purposes due to the usage of a large quantity of organic solvent, which is not suitable for the encapsulation of the fragile molecule (179, 182) Another technique used is the dehydration-rehydration vesicle (DRV) method, which has been reported to encapsulate large amounts of aqueous materials. This method was generally used to obtain small unilamellar vesicles (SUV) (189). A lipid mixture was dissolved in chloroform/methanol and dried to a film in a round bottom flask by using a rotary evaporator. Next, the drug was added to the film (169, 186). This technique is simple; however, it results in a larger size of liposomes

(186). Moreover, liposomes can be stored in a lyophilized state which improves shelf-life stability of liposomes as drug carriers (189). Due to the high encapsulation efficiency and stability of liposomal formulation, the DRV technique was used to prepare our formulations in this study.

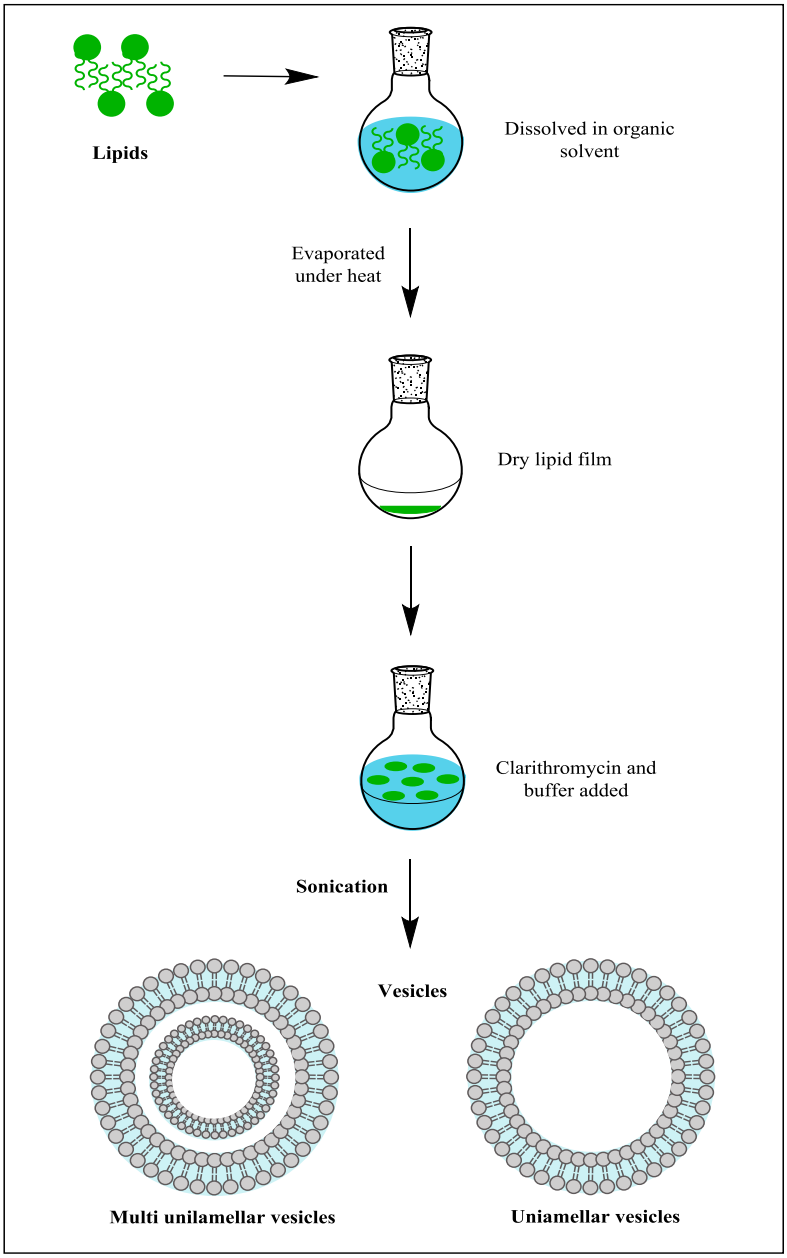

Diagram 8: Preparation of liposomes.

1.6.3. Stability of liposomes

Liposome stability is one of the most important issues in liposome applications and this mainly depends on the physicochemical properties of the lipid membrane (phospholipids) (190). The stability of a liposome is usually enhanced by cholesterol incorporation (191), especially when using unsaturated phospholipids in liposomal formulation. The stability of liposome can be divided into physical, chemical, and biological. The physical stability of the liposomes depends on its size distribution due to aggregation/fusion of liposome bilayers or leakage of encapsulated material (189). Moreover, the temperature of stored liposomes is an essential perquisite for physical stability (192). Chemical stability of the lipids during storage is another point of concern. It can be determined by monitoring the oxidation of the unsaturated fatty acid chains or the hydrolysis of the lipids (192, 193). Oxidation of phospholipids is more likely due to the unsaturated fatty acyl chains, whereas incorporation of cholesterol and antioxidants to the liposome formulation can usually protect the phospholipids from oxidation (180, 189, 191). Hydrolysis of phospholipids detaches the hydrophobic chains of ester bonds, which might increase permeability of the phospholipids (189, 194, 195). The biological stability of liposomes can be increased by covering liposomes with inert hydrophilic polymers (193). In addition, the properties of liposomes, such as size, net surface charge, hydrophobicity, fluidity, and packing of the lipid bilayers would limit the stability of liposomes (190). Shelf-life stability of liposomes can be enhanced by optimizing the size distribution, pH, the addition of antioxidants and lyophilization.

1.6.4. Liposomal toxicity

Liposomal toxicity is an essential issue in liposomal drug enterprise. Some drugs are associated with toxic side effects such as poor penetration, solubility and stability of the drug after uptake (196). The toxicity of liposomal formulation depends on the net charge of lipids and the properties of the charged lipids (193, 197). For instance, by using cationic lipids in liposomal formulation such as dioleoylphosphatidylethanolamine (DOPE) and dimethyldioctadecyl-ammonium bromide (DDAB) the cell proliferation would be affected (198, 199). Moreover, cationic liposomes can be used effectively as carriers for pulmonary delivery of an anionic material and successfully used to deliver DNA inside mammalian cells (173, 193, 198). Despite the negatively charged liposomes have a shorter half-life in the blood than neutral liposomes, positively charged liposomes have been found to be toxic and were quickly removed from circulation (197, 198). Taken together, the choice of main lipids and charged component in formulation of the liposomes has to be considered to reduce the liposomal toxicity (173, 193, 200).

1.6.5. Applications of liposomes

Liposomes have been studied for many years as carrier systems for drugs. At this time, several liposomal drugs have been approved for clinical use and numerous others are in clinical trials. Liposomal formulation containing amikacin currently undergoes phase III clinical trial for the treatment of pseudomonal lung infections.

Entrapment of drugs into liposomes has several advantages such as: (1) increased circulation lifetime, (2) enhanced deposition in the infected tissues, (3) reduced toxicity of the encapsulated agent, and (4) improved pharmacokinetic of drugs (173, 193). Liposomes have an

ability to accommodate drugs in target tissues with different physicochemical properties such as size, surface charge, permeability and stability (172, 173, 176). Liposomes can be distinguished based on the number of lamellae they possess, such as small unilamellar vesicles (SUV), or large unilamellar vesicles (LUV), or multilamellar vesicles (MLV) (172, 181). SUVs are usually smaller than 50 nm while LUVs are usually large than 50 nm; MLVs usually range from 500 to 10,000 nm (176, 181). Moreover, the ranges in diameter from 10,000 to 1,000,000 nm are called giant liposomes (176). For instance, the diameter size of liposomes, < 200 nm or smaller, can exhibit circulation half-lives of several hours and accumulate at active sites (176). In addition, liposomes can be loaded with polar and nonpolar substances which cross different hydrophobic barriers to deliver the entrapped substances into the hydrophobic environment. Liposomes have the ability to target specific cells for drug delivery by fusing with the lipid bilayer of the cell membrane.

1.6.6. Liposomes in infectious diseases

The robust treatment of several infectious diseases requires an intense and enduring antibiotic treatment. One of the methods by which one can potentiate the action of antimicrobial drugs is through the use of liposomes as drug delivery vehicles (201). Liposomes have been shown to boost the distribution of the drug in the target tissue with minimal toxic side effects. Furthermore, liposomes can effectively be modified to potentiate the efficacy of the drug by altering the charge, surface properties, fluidity and the composition of the lipid layers (201). Previous studies have shown that liposomes can fuse to the membranes of Gram-negative bacteria by exploiting their structural similarity. This feature allows liposomes to be effective antimicrobial agents. For instance, it has been demonstrated that when macrolide antibiotics were encapsulated within liposomes, they showed enhanced bactericidal activity compared to

non-encapsulated control groups (202). Presently, several liposomal formulations of antibiotics, including Arikace (Liposomal-Amikacin), have been approved for phase II and III clinical trials in treating *P. aeruginosa* infection in the lungs of CF patients (172). Other liposomal formulations such as AmBisome® (Amphotericin B), Abelcet® (Amphotericin B Injection), and Amphotec® (Amphotericin B) are being employed to treat fungal infections such as aspergillosis (172). Finally, liposomal formulations are also being employed in the treatment of cancer (168, 172, 203, 204). Doxil® and Myocet® are two examples of liposomal formulations being used as anti-cancer therapeutics in the treatment of breast cancer and Kaposi's sarcoma (168, 172, 205). Taken together, using liposomes as drug delivery vehicles may pave the way towards developing more advanced and powerful therapeutic measures against infectious diseases and cancer.

1.7. Objective of the thesis

This thesis aimed to demonstrate the bactericidal activities of macrolide antibiotics and liposomal macrolides against *P. aeruginosa,* which causes morbidity and mortality in CF patients. New liposomal formulations with different charges of lipids compositions were characterized to determine whether the use of liposomal delivery system would enhance antibacterial activity of clarithromycin against resistant strains of *P. aeruginosa*.

CHAPTER TWO

2. Efficacy and Safety of Liposomal Clarithromycin and Its Effect on *Pseudomonas aeruginosa* Virulence Factors

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Running title: Liposomal clarithromycin

Published in Antimicrobial Agents and chemotherapy; April 2013; 57(6):2694-2704

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Abstract

We investigated the efficacy and safety of liposomal clarithromycin formulations with different surface charges against clinical isolates of *Pseudomonas aeruginosa* from the lungs of cystic fibrosis (CF) patients. The liposomal clarithromycin formulations were prepared by the dehydration-rehydration method, and their sizes were measured using the dynamic-light-scattering technique. Encapsulation efficiency was determined by microbiological assay, and stabilities of the formulations in biological fluid were evaluated for a period of 48 h. The MICs and minimum bactericidal concentrations (MBCs) of free and liposomal formulations were determined with *P. aeruginosa* strains isolated from CF patients. Liposomal clarithromycin activity against biofilm-forming *P. aeruginosa* was compared to that of free antibiotic using the Calgary Biofilm Device (CBD). The effect of subinhibitory concentrations of free and liposomal clarithromycin on bacterial virulence factors and motility on agar were investigated on clinical isolates of *P. aeruginosa*. The cytotoxicities of the liposome preparations and free drug were evaluated on a pulmonary epithelial cell line (A549). The average diameter of the formulations was >222 nm, with encapsulation efficiencies ranging from 5.7% to 30.4%. The liposomes retained more than 70% of their drug content during the 48-h time period. The highly resistant strains of *P. aeruginosa* became susceptible to liposome-encapsulated clarithromycin (MIC, 256 mg/liter verses 8 mg/liter; $P<0.001$). Liposomal clarithromycin reduced the bacterial growth within the biofilm by 3-4 log units ($P<0.001$), significantly attenuated virulence factors production, and reduced bacterial twitching, swarming, and swimming motilities. The clarithromycin-entrapped liposomes were less cytotoxic than the free drug ($P<0.001$). These data indicate that our novel formulations could be a useful strategy to enhance the efficacy of

clarithromycin against resistant *P. aeruginosa* strains that commonly affect individuals with cystic fibrosis.

2.1. Introduction

Cystic fibrosis (CF) is a fatal inherited disease that is common among the Caucasian population and affects 30,000 and 3,000 newborns/year in the United States and Canada, respectively (1, 2). Cystic fibrosis is a multiorgan disease affecting the liver, pancreas, gastrointestinal tract, and lungs; however, pulmonary injury is the main cause of death among CF patients (3-5).

The underlying molecular mechanism of CF is mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene located on chromosome 7 (4, 6). The CFTR molecule is a 1,480-amino-acid membrane-bound chloride channel (7). The structure and function of the channel in CF are compromised by over 1,800 types of mutations (2). The most prevalent mutation, delta F 508, is a deletion of phenylalanine at position 508 and is responsible for 70% of CF cases worldwide (6, 7). The CFTR glycoprotein regulates salt and water transport across epithelial cells (8, 9). Accumulation of the chloride ion inside the cells carrying defective CFTR protein results in dehydration of the epithelial lining fluid and overproduction of thick and sticky mucus (10). The condition, in part, provides a suitable environment for microbial growth, including bacteria, such as *Staphylococcus aureus, Haemophilus influenzae, Burkholderia cepacia and Pseudomonas aeruginosa* (11). *P. aeruginosa*, however, persists in the lungs of over 80% of adults suffering from CF and causes recurrent infection and inflammation (1, 4, 12).

P. aeruginosa is a ubiquitous aerobic Gram-negative bacterium that affects individuals with compromised immune system and has a high intrinsic resistance to most antibiotics (13, 14). *P. aeruginosa* possesses a large array of virulence factors, such as flagellum, pili, elastase,

chitinase, lipase, and proteases (15-17). The flagellum and pili can bind to the overexpressed asialoganglioside (GM1) in CF epithelial cells and help bacteria to twitch, swarm, and swim toward nutritional signals, as well as in biofilm formation (18-21). Elastase, chitinase, lipase, and proteases can cause degradation and damage of elastin, collagen, and immunoglobulins, affecting the alveolar epithelial permeability (22).

There are several molecular mechanisms by which bacteria, including *P. aeruginosa*, resist the action of macrolide, including target site modification by methylation and/or mutation that prevents the binding of antibiotics to their target molecules and inactivation of the drugs or efflux (23-25).

Forming biofilm is one of the strategies for bacteria to evade chemotherapy, as well as the host immune response (26). Biofilm formation, however, starts with attachment of the microorganism to a surface, followed by a production of extracellular matrix composed of polysaccharides and proteins, which mediate bacterial attachment during the initial biofilm community formation process (27-29). Biofilm protects bacteria from phagocytosis, opsonization by antibodies, and their removal by the ciliary action of tracheal epithelium (30, 31). Furthermore, the extracellular polymeric matrix delays diffusion of some antibiotics into the community (27, 32), and thus, bacteria might be expose to a drug concentration below the MIC, leading to increased mutation and resistance of the bacteria (33). *P. aeruginosa* in biofilms was found to be resistance to macrolide due to mutation in *nfxB*, which encodes the negative transcriptional regulator protein NfxB for the efflux pump, leading to increased expression of the efflux pump MexCD-OprJ and resistance of *P. aeruginosa* to macrolide (34).

Pseudomonal lung infections are treated with antibiotics, such as aminoglycosides and macrolides, to reduce infection (35, 36). Macrolide antibiotics are usually characterized by a

large lactone ring within their structure (23). They are classified according to the number of lactone ring components: 14-membered (erythromycin and clarithromycin [CAM]), 15-membered (azithromycin) (37), and 16-membered (roxithromycin) (17) groups. Macrolides are effective against most aerobic and anaerobic Gram-positive organisms and many Gram-negative bacteria (35, 37). They are used for treating respiratory tract and soft tissue infections (23, 38). Macrolides, such as clarithromycin, inhibit protein synthesis in bacteria by reversibly binding to the 50S ribosomal subunits (38). Clarithromycin is also known as the most effective chemotherapy against *Mycobacterium avium* complex (MAC) (39). The effective doses of oral clarithromycin are 200 to 500 mg/ml in adult humans; long exposure and high doses are required for treating chronic respiratory *P. aeruginosa* infection (40, 41). A group of investigators reported the beneficial effect of clarithromycin on treatment of biofilm-associated chronic respiratory *P. aeruginosa* infection in a murine model (26). Clarithromycin, however, is a known inhibitor of the hepatic microsomal cytochrome CYP3A4 (42), which has a significant role in metabolizing macrolides. The loss of CYP3A4 catalytic ability resulted in elevated serum drug levels and hepatotoxicity (43). Due to the high resistance of *P. aeruginosa* to most antimicrobial agents, including macrolides (35), and the appearance of toxicity of some drugs (44), there is a strong demand for novel drugs as well as new and safe delivery systems, such as liposomes, to combat *P. aeruginosa*-induced chronic infection (45).

Liposomes are round vesicles consisting of one or more phospholipid bilayers surrounding an aqueous solution space (46, 47). Hydrophobic drugs, such as macrolides, can be entrapped in the lipid bilayers of the biocompatible and biodegradable liposomes, while hydrophilic drugs can be incorporated in to their aqueous compartments (36, 48, 49). Liposomes, as a drug carrier system, have the ability to improve antibiotic therapy by decreasing antibiotic

toxicity and enhancing bactericidal efficacy through fusion with the bacterial membrane (48, 50-52). Liposomes have the ability to protect their loads from the host cellular elements and the action of bacterial enzymes (50, 53-55). Another study indicated that the liposomal formulation was effective in enhancing polymyxin B antimicrobial activity against Gram-negative bacteria compared to the free drugs (56). A previous *in vivo* study performed in a rat model demonstrated that liposomal tobramycin administered intratracheally improved the pharmacokinetic parameters and significantly reduced *P. aeruginosa* bacteria after multiple treatments (57). Another study showed that liposome encapsulated clarithromycin significantly increased the uptake of human macrophages to the encapsulated agent and reduced *Mycobacterium avium* complex infection compared to the free drug (58). Furthermore, combination therapy using liposomal amikacin in the initial phase of chemotherapy in *M. avium* infection enhanced the efficacy of clarithromycin/ethambutol regimen (59).

The aim of this work was to investigate whether the lack or the type of surface charges in liposomal formulations containing clarithromycin; negatively charged liposomal clarithromycin (NEG-Lipo-CAM), positively charged liposomal clarithromycin (POS-Lipo-CAM), and uncharged liposomal clarithromycin (NEU-Lipo-CAM); would enhance clarithromycin antimicrobial activity. We also evaluated clarithromycin cell toxicity and measured the liposomal formulation size, stability, and antibacterial activity (MIC and minimum bactericidal concentrations [MBC]) *in vitro*. Furthermore, we investigated the formulations' ability to prevent biofilm formation, virulence factors production, and motility of clarithromycin-resistant strains of *P. aeruginosa*.

2.2. Materials and methods

2.2.1. Chemicals and media

Clarithromycin was obtained from Sigma-Aldrich (Oakville, ON, Canada). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Other chemicals such as didodecyldimethylammonium bromide (DDAB), dicetyl phosphate (DCP), Triton X-100, trypan blue, elastin-Congo red, chitin azure, and agarose, were also obtained from Sigma-Aldrich (Oakville, ON, Canada). For antibiotic susceptibility test, Mueller-Hinton agar, trypsin-EDTA, and Cell Titer Blue Cell Viability Assay kit were purchased from Fisher Scientific (Ottawa, ON, Canada).Tryptic soy agar, tryptic soy broth, Luria-Bertani (LB) broth, and Luria-Bertani agar were purchased from Becton Dickinson Microbiology Systems (Oakville, ON, Canada). Cationic-adjusted Mueller-Hinton broth for culturing microorganisms was purchased from BD (Franklin Lakes, NJ). Normal pooled plasma was purchased from Precision Biologic (Dartmouth, NS, Canada). ABt medium consisted of 27 mM $(NH_4)_2SO_4$, 30 mM $Na_2HPO_4 \bullet 2H_2O$, 20 mM KH_2PO_4, 47 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM $FeCl_2$, 0.5% (wt/vol) glucose, 0.5% (wt/vol) Casamino Acids, and 0.00025% (wt/vol) thiamine.

2.2.2. Microorganisms

A laboratory strain of *Bacillus subtilis* (ATCC 6633) was used as an indicator organism for clarithromycin activity. Laboratory strains of *P. aeruginosa* (ATCC 10145 and ATCC 25619) and clinical isolates of *P. aeruginosa* (PA-M13639-1, PA-M13641-2, PA-1, PA-11, PA-12, PA-13572, and PA-M13640) were purchased from PML Microbiologicals (Mississauga, ON, Canada) or obtained from the Clinical Microbiology Laboratory of Memorial Hospital (Sudbury,

ON, Canada). All strains were stored at -80°C in cationic-adjusted Mueller-Hinton broth supplemented with 10% glycerol.

2.2.3. Liposomes preparation

Clarithromycin was encapsulated into liposomes composed of different lipids (DPPC, DDAB, and DCP) and cholesterol (CHOL). The positively charged liposomal formulation was composed of DDAB, DPPC, and cholesterol in a ratio of 4:2:1; the negatively charged liposomal formulation was composed of DCP, DPPC, and cholesterol in a ratio of 4:2:1; and the uncharged liposomal formulation was composed of DPPC and cholesterol in ratio of 6:1. The liposomal formulations were prepared by the dehydration-rehydration method (60, 61). Briefly, the lipids were dissolved in the chloroform-methanol solution (2:1 [vol/vol]). A rotary evaporator (Rotavapor; BÜCHI Labortechnik AG) was used to evaporate the organic solvent (62). Once a thin, dry lipid layer was formed, clarithromycin solution (1 mg/ml) was added, followed by a series of sonication using the Sonic Dismembrator (FS20H; Fisher Scientific, Ottawa, Canada) (45).

2.2.4. Microbiological assay

After preparation of the liposomes, the mixture was centrifuged at 16,000 × *g* for 20 min at 4°C. Triton X-100 was added to the pellet to release the drug, as previously reported (60, 63). The concentrations of clarithromycin incorporated into liposomes were measured by agar diffusion assay (56). A *B. subtilis* laboratory strain (ATCC 6633) was used as the indicator organism for clarithromycin activity, as recommended by the Clinical and Laboratory Standards Institute (CLSI). *B. subtilis* was cultured overnight in cationic Mueller-Hinton broth, and a bacterial solution was prepared equivalent to a 0.5 McFarland standards ($1.5 \times l0^8$

CFU/ml) (60). The cells were added to an agar solution at 41°C and quickly poured into a sterile glass plate (440 mm × 340 mm) to form a thin layer of agar and bacteria. Wells 5 mm in diameter made with a well puncher were filled with 25 µl of samples or standard solutions (62), and the plate was incubated for 18 h at 37°C (47). After the incubation period, inhibition zones in the plate were measured in triplicate. The averaged values for each triplicated sample were used to analyze the encapsulation efficiency of the liposomal formulations for clarithromycin.

The sensitivity of the assay was 0.002 mg/liter. The quantifiable limit for clarithromycin was 0.002 mg/liter. At concentrations from 0.002 to 0.0125 mg/liter, the coefficients of variation ranged between 1 and 2%. Over the same concentrations, the intraday coefficients of variation ranged between 2 and 3%. For 10 samples of spiked clarithromycin, the standard curve linearity extended over the range of 0.002 to 0.0125 mg/liter and gave a correlation coefficient greater than 0.99. The concentration measurements are the means of at least three independent experiments, with each experiment measured in triplicate..

2.2.5. Encapsulation efficiency determination

The encapsulation efficiency of liposomal clarithromycin was determined as the percentage of clarithromycin entrapped in the liposomes relative to the initial total amount of the drug in solution (54). The concentration of the entrapped clarithromycin was determined by the microbiological assay outlined above (62).

2.2.6. Size determination and polydispersity index

The polydispersity index (PI) and the mean diameter of liposomes were determined by using a Submicron Particle Sizer Model 270 (Nicomp, Santa Barbara, CA) (47, 54, 64).

2.2.7. Stability of liposomal clarithromycin

The stability of liposomal clarithromycin was assessed in phosphate-buffered saline (PBS) at 4°C and 37°C. The stability of liposomal clarithromycin was determined as the percentage of retention of the initial encapsulated drug after a period of time under different conditions (54, 65). Briefly, liposomal clarithromycin was suspended in PBS and incubated in water bath shaker with mild agitation at 100 rpm (Julabo SW22 Incubator Shaker; Labortechnik, Seelbach, Germany). After incubation times of 0.25, 0.5, 1, 3, 6, 12, 24, and 48 h (64), samples were centrifuged at 18,300 × *g* for 15 min at 4°C to remove the released drugs (52, 66). The supernatants of the liposomal samples were collected, and 25 µl was transferred into holes on a plate containing agar prepared with an appropriate bacterial culture (*B. subtilis* ATCC 6633). The plates were then incubated at 37°C for 18 h, and the inhibition zones were measured. Free-clarithromycin concentrations were also determined by agar diffusion assay (60).

2.2.8. MICs and MBCs

A broth dilution method was used to determine the MICs of liposomal clarithromycin. Overnight cultures of the clinical strains of *P. aeruginosa* were diluted in cationic Mueller-Hinton broth to achieve 0.5 McFarland standards (56). The bacterial cell population were then exposed to several dilutions of liposomal or free clarithromycin ranging from 0.031-256 mg/liter and thoroughly mixed with Mueller-Hinton agar. The plates were incubated for 18 h at 37°C (45). For MBC assays, bacterial suspensions were mixed with subinhibitory concentrations, MICs, and two times the MICs of free CAM or Lipo-CAM, and the plates were incubated for 24 h at 37°C (63). Broth medium alone and free clarithromycin bacterial cultures were used as negative and positive controls, respectively.

2.2.9. Bactericidal activity of liposomal clarithromycin against *P. aeruginosa* in biofilm (MBEC)

In order to assess the minimum biofilm eradication concentration (MBEC), *P. aeruginosa* strain PA-13572 was allowed to form a biofilm in a Calgary biofilm plates (Innovotech, Edmonton, AB, Canada) as previously reported (67). Briefly, strain PA-13572 (1.5×10^6 CFU/ml; 24 ml) was added to the Calgary biofilm device plate. The plates were placed in an incubator shaker rotating at 50 rpm (Innova 4000 Incubator Shaker; New Brunswick scientific, NJ) at 37°C for 4 days, ensuring equal distribution of medium in the troughs and adhesion of PA-13572 to the pegs (fresh broth was added every 24 h to remove the nonadherent bacteria). After 5 days, the biofilm on the pegs were washed twice with medium, and a sterile forceps were used to transfer the biofilm pegs into microcentrifuge tubes containing 1 ml of PBS. The pegs were then sonicated for 1 min to detach and disperse bacteria, followed by vortexing for 2 min. the bacterial suspension was subjected to 10-fold serial dilution for a bacterial count to serve as the control. Aliquots of 100 µl of each dilution were plated on Mueller-Hinton agar and incubate for 24 h at 37°C. The rest of the pegs were then submerged in a 96-well plate containing 200 µl of different dilutions of free CAM, NEG-Lipo-CAM, POS-Lipo-CAM, and NEU-Lipo-CAM. The plate was incubated at 37°C for 24 h. The peg lid was washed with medium twice, and the pegs were removed and transferred to microcentrifuge tubes, sonicated, and serially diluted (10-fold) for bacterial counts (CFU) after 24 h of incubation at 37^{o}C.

2.2.10. Virulence factors assays

To test which concentrations below the MIC are subinhibitory, free or liposomal formulations (NEG-Lipo-CAM, NEU-Lipo-CAM and POS-Lipo-CAM) at concentration of 1/2

to 1/8 the MIC were introduced into *P. aeruginosa* PA-13572. The bacterial growth was repetitively monitored (optical density at 600 nm [OD_{600}]) up to 8 h. For experiments involving lipase, chitinase, elastase, and protease assays, *P. aeruginosa* PA-13572 was cultured in ABt medium for 18 h at 37°C (68), and then the cell density of bacteria in a 100-ml flask was adjusted to match 0.5 McFarland standard (OD_{600}=0.132) following incubation for 1 h at 37°C. When the bacterial cell density doubled to an OD_{600} of 0.26 (67), the cells were exposed to equal volumes of free and liposomal formulations at 1/8 the MIC. After 24 h of incubation, bacterial concentrations were measured at OD_{600} and the suspension was centrifuged for 15 min (16,000 × *g* at 4°C) and filtered sterilized (0.22 μm) for biochemical assays.

2.2.10.1. Lipase assay

The reaction mixture of the lipase assay consisted of 0.6 ml of 10% Tween 20 in Tris buffer; 0.1 ml of 1 M $CaCl_2$, 0.6 ml of filtered supernatant, and 1.6 ml of double-distilled water; we used medium alone (blank) as a control (68, 69). The reaction mixtures were incubated at 37°C for 24 h (69) with agitation at 200 rpm (Innova 4000 Incubator Shaker; New Brunswick scientific, NJ). Lipase uses Tween 20 as a substrate and converts it into fatty acid and alcohol. The resulting fatty acid bound to the calcium and formed an insoluble complex the absorbance of which was measured in a spectrophotometer at 400 nm (67, 70). Lipase experiments were done three times in triplicates.

2.2.10.2. Chitinase assay

Insoluble chitin azure (5 mg) was properly mixed with 1 ml of filtered suspended supernatant or medium alone (blank) in 1 ml of PBS. The reaction mixture was incubated for 24 h at 37°C. Chitinase breaks chitin azure and produces a blue compound whose absorbance was

determined at 290 nm. Experiments were performed three times with two replicates. The resulting data were normalized by dividing the optical density by cell density (OD_{600}) (68, 71). The experiment was repeated three times in triplicates.

2.2.10.3. Elastase assay

Insoluble elastin-Congo red (20 mg) (72) was mixed with 1 ml of PBS and 1 ml of filtered suspended supernatants or medium alone (blank) as a control (68). The mixture was incubated for 24 h at 37°C with agitation at 200 rpm (Innova 4000 Incubator Shaker; New Brunswick scientific, NJ). Elastase breakdown of insoluble elastin–Congo red produced a red compound whose absorbance was measured at OD_{459} (73) after centrifugation at 16,000 × *g*. All these experiments were repeated at least three times in triplicates (68).

2.2.10.4. Protease assay

Filtered supernatants or medium alone (100 µl) was transferred into the wells of a petri dish containing 2% agarose and 2% skim milk, following incubation for 48 h at 37°C. Zones of clearance due to the proteolytic activity of protease could be easily observed (74) and were measured in (mm) by using digital calipers (67, 68). All these experiments were repeated at least three times in triplicates.

2.2.10.5. Effect of liposomal clarithromycin on *P. aeruginosa* motility

The motility of *P. aeruginosa* was investigated by methods described previously (67). Briefly, *P. aeruginosa* PA-13572 grown overnight was diluted to 1.5×10^8 CFU/ml, and 1 µl was inoculated onto a 3-mm depth of ABt-agarose plates containing a subinhibitory concentration of free or liposomal clarithromycin (1/8 the MIC). Inoculation into the bottom of

ABt medium with agarose (1% [wt/vol]) was used for twitching, and point inoculation onto the medium with agarose (0.3% [wt/vol]) was used for swimming and swarming (0.5% [wt/vol]). After 12 h of incubation at 37°C, swimming and swarming diameters were measured. For twitching at the agarose-petri dish interface, after 24 h of incubation at 37°C, the medium was gently removed and the petri dish was air dried. A 1% crystal violet solution was used to stain the Petri dish for 10 min. The Petri dish was rinsed, and the crystal violet-stained twitching pattern was measured. All experiments were performed in three independent experiments in triplicates.

2.2.11. Determination of the liposomal clarithromycin cytotoxicity

The viability of cells was determined by Cell Titer Blue assay. The cells were seeded into 24- well plates (75) at a density of 5×10^5 cells/ml and left to adhere to the surfaces of the wells overnight (50). The cell culture medium was then replenished with 500 µl of fresh media containing free clarithromycin or liposomal clarithromycin at four different concentrations (2×, 1.5×, 1×, and 0.5× the MICs) and transferred into the 24-well plates. In this assay, different liposomal formulations of clarithromycin-positively charged liposomal clarithromycin (DPPC, DDAB, and CHOL), negatively charged liposomal clarithromycin (DPPC, DCP, and CHOL), and uncharged liposomal clarithromycin (DPPC and CHOL) were used. The concentrations of free or liposomal clarithromycin that had been introduced to the A549 cells were calculated based on the MIC value for each formulation, as stated above. All of these liposomal preparations were exposed to these concentrations for three different periods - 24, 48, and 72 h - following incubation in 5% CO_2 at 37°C. The wells containing only cell culture medium without the drug were used as controls (50, 51). Once the required exposure period was over, they were removed, and the cells were washed once with PBS and subsequently with cell culture medium

to remove any residual Lipo-CAM or free-CAM. Then, the existing medium was replaced with 500 µl of fresh media, and 100 µl of resazurin dye was added to each of 24-well plates. The cells were then incubated overnight in the dark at 37°C in 5% CO_2 (50). The absorbance was measured at 570 nm, using 600 nm as a reference wavelength in a spectrophotometer. A blank well containing Cell Titer Blue reagent without cells was used as a reference (75). All experiments were performed in independently three experiments in triplicates.

2.2.12. Data analysis

The data are represented as mean ± standers errors of the mean (SEM) of three independent experiments. For comparisons of multiple groups, one-way analysis of variance (ANOVA) was performed using GraphPad Prism, followed by a post-*t* test. *P* values of *<0.05*, *<0.01,* and*<0.001* were considered statistically significant.

2.3. Results

2.3.1. Encapsulation efficiency and size

The percent encapsulation efficiency (EE %), size, and size distribution for liposomal formulations are summarized in Table 1. A PI<0.1 indicates a homogenous population. The results are given as means ± SEM of three separate experiments

Table 1: Encapsulation efficiencies, particle sizes, and polydispersity indexes of macrolide antibiotics.

Liposomal Clarithromycin	**Size(nm) (mean ± SEM)**	**PI (mean ± SEM)**	**EE% (mean ± SEM)**
Neutral DPPC- CHOL	221.60 ± 14.98	0.776 ± 0.004	15.96 ± 0.05
Negative DPPC -DAP-CHOL	199.63 ± 6.87	0.786 ± 0.024	30.37 ± 0.10
Positive DPPC-DDAB-CHOL	169.20 ± 16.75	0.518 ± 0.035	5.70 ± 0.01

2.3.2. Stability of liposomal clarithromycin

The stability of liposomal clarithromycin was evaluated in PBS at 4°C (storage temperature) and at 37°C (body temperature) for a study period of 48 h. It was evident from the data that the liposomal clarithromycin stored at 4°C was more stable than that incubated at 37°C. The negatively charged liposomal clarithromycin retained 94.15% ± 0.34% of the drug at 4°C, and its retention rate was 92.03% ± 0.78% at 37°C. Antibiotic retention of the positively charged liposomes followed the same pattern, though the retention rates were much lower (60.13% ± 0.92% at 4°C and 53.00% ± 0.95% at 37°C). The negatively charged liposomes formulation was significantly more stable than the positively charged formulation (P<0.001). The uncharged liposomal clarithromycin showed more stability at a lower temperature and retained more antibiotics than the liposomes at 37°C (95.07%± 0.005% versus 91.00% ± 0.76%, respectively). There was no significant difference between the stabilities of negatively

charged liposomes and uncharged liposomes; however, the uncharged formulation was significantly more stable than the positively charged formulation ($P<0.001$).

2.3.3. MICs and MBCs

The MICs of liposomal clarithromycin against *P. aeruginosa* strains were significantly lower than those of free clarithromycin, as illustrated in Table 2. The experiments were done with two highly clarithromycin-resistant mucoid and nonmucoid clinical strains of *P. aeruginosa*. As demonstrated in Table 2, the MICs of free clarithromycin for all *P. aeruginosa* strains were ≥ 256 mg/liter compared to 64 mg/liter for negatively charged liposomal clarithromycin. The MICs for uncharged liposomal clarithromycin against *P. aeruginosa* strains were reduced from over 256 mg/liter for free clarithromycin to 32 mg/liter, whereas positively charged liposomal clarithromycin was effective at 8 mg/liter. The MIC against one of the resistant strains (PA-1) was 4 mg/liter for positively charged liposomal clarithromycin compared to 16 mg/liter for uncharged liposomal clarithromycin and 32 mg/liter for negatively charged liposomal clarithromycin. The difference between the MICs and MBCs of liposomal clarithromycin and free clarithromycin against *P. aeruginosa* strain was remarkable (2 to 4 versus 256 to 512 mg/liter, respectively). The MBCs of free clarithromycin against *P. aeruginosa* strains were ≥ 512 mg/liter compared to 64 mg/liter for NEG-Lipo-CAM and NEU-Lipo-CAM. The positively charged liposomal clarithromycin was bactericidal at 16 mg/liter (Table 2).

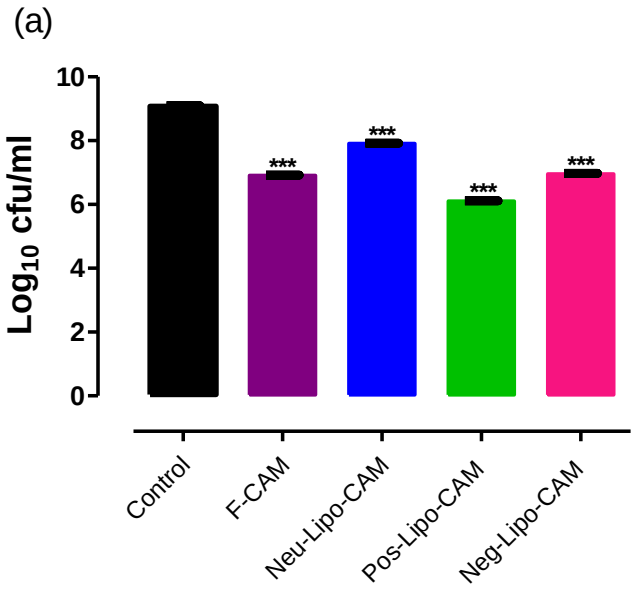
Table 2: Antimicrobial activities of free and liposomal clarithromycin on *P. aeruginosa* strains

Bacterial strain	Description[a]	Antimicrobial activity (mg/liter)							
		NEU-Lipo-CAM		NEG-Lipo-CAM		POS- Lipo-CAM		F-CAM	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ATCC 10145	Non Muc	32	> 64	64	> 64	8	16	>256	512
ATCC 25619	Non Muc	32	64	64	> 64	8	16	256	512
PA-M13639-1	Muc	32	64	64	64	8	> 64	256	512
PA-M13641-2	Muc	32	> 64	64	> 64	8	16	256	512
PA-1	Non Muc	16	> 64	32	32	4	8	>255	512
PA-11	Non Muc	32	> 64	64	> 64	8	16	>256	512
PA-12	Non Muc	32	> 64	64	> 64	8	16	>256	512
PA-13572	Non Muc	32	64	64	64	8	16	>256	512
PA-M13640	Non Muc	32	64	64	64	8	16	256	512

[a] Non Muc, non mucoid ; Muc, mucoid.

2.3.4. Liposomal clarithromycin bactericidal activity on *P.aeruginosa* biofilm

POS-Lipo-CAM and NEG-Lipo-CAM completely eradicated *P. aeruginosa* 13572 in the biofilm, whereas, NEU-Lipo-CAM and free CAM reduced bacterial numbers in biofilm communities by 5 and 3 log units (CFU/ml), respectively, at 128 mg/liter (Fig. 1). POS-Lipo-CAM still proved to have a potent effect on bacteria in biofilms: it was able to completely eliminate the bacteria within the biofilm at a lower concentration of 64 mg/liter. The other formulation could only decrease *P. aeruginosa* at 64 mg/liter by 3 log units for free and negatively charged liposomes and 4 log units for uncharged liposomes compared to the control. It was also observed that the liposomal formulations and free clarithromycin at 32 mg/liter reduced bacterial counts by 2 log units for free CAM and NEG-Lipo-CAM and 3 log units for POS-Lipo-CAM compared to the control.



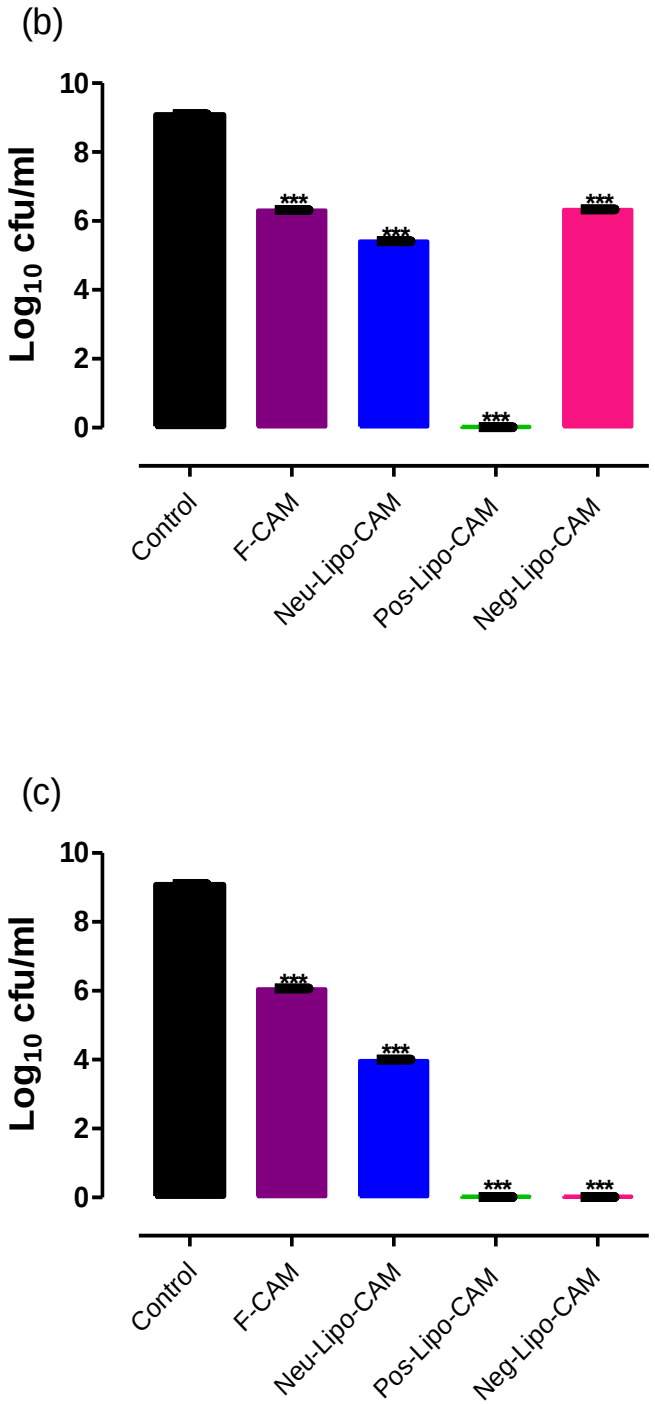
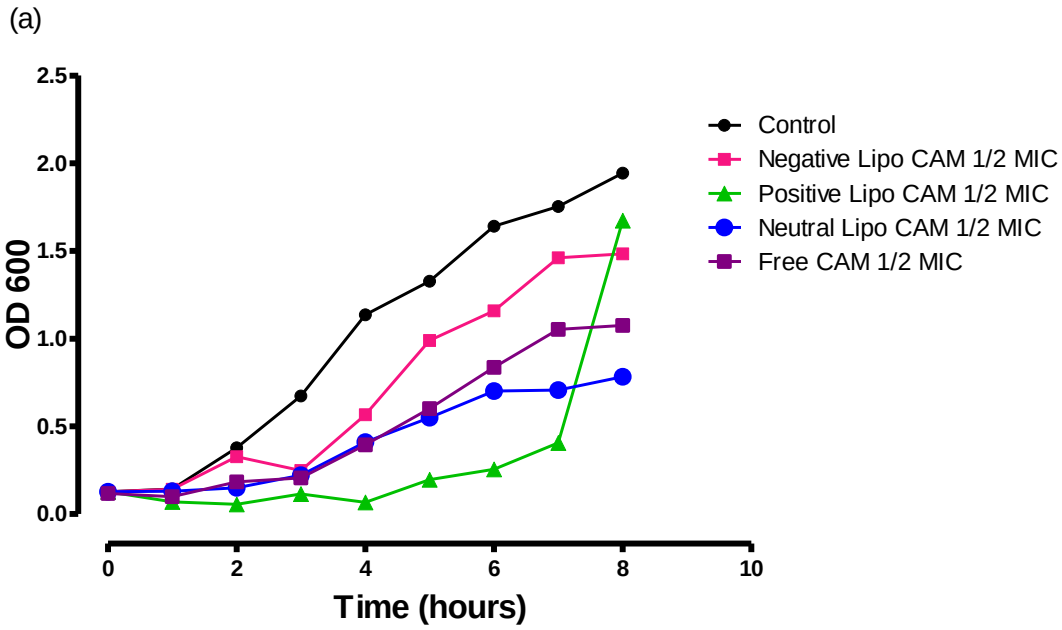


Figure 1: Liposomal clarithromycin activity on *P.aureginosa* PA-13572 biofilm (MBEC assay). Free (F-CAM) or liposomal formulations were introduced to mature biofilm at concentrations of 32 mg/liter (a), 64 mg/liter (b), and 128 mg/liter (c). Untreated biofilm acted as control. The data

represent three independent experiments in triplicate and are shown as means ± SEM. *P* values were considered significant compared with the control: ***,*P<0.001*.

2.3.5. Effect of subinhibitory concentration of free and liposomal clarithromycin on growth of *P. aeruginosa*

Both free and liposomal clarithromycin affected the growth of *P. aeruginosa* PA-13572 (Fig. 2a and b). Subinhibitory concentrations (1/8 the MIC), however, did not inhibit bacterial growth (Fig. 2c). For this reason, all experiments involving virulence factors were performed using subinhibitory concentrations of 1/8 the MIC.



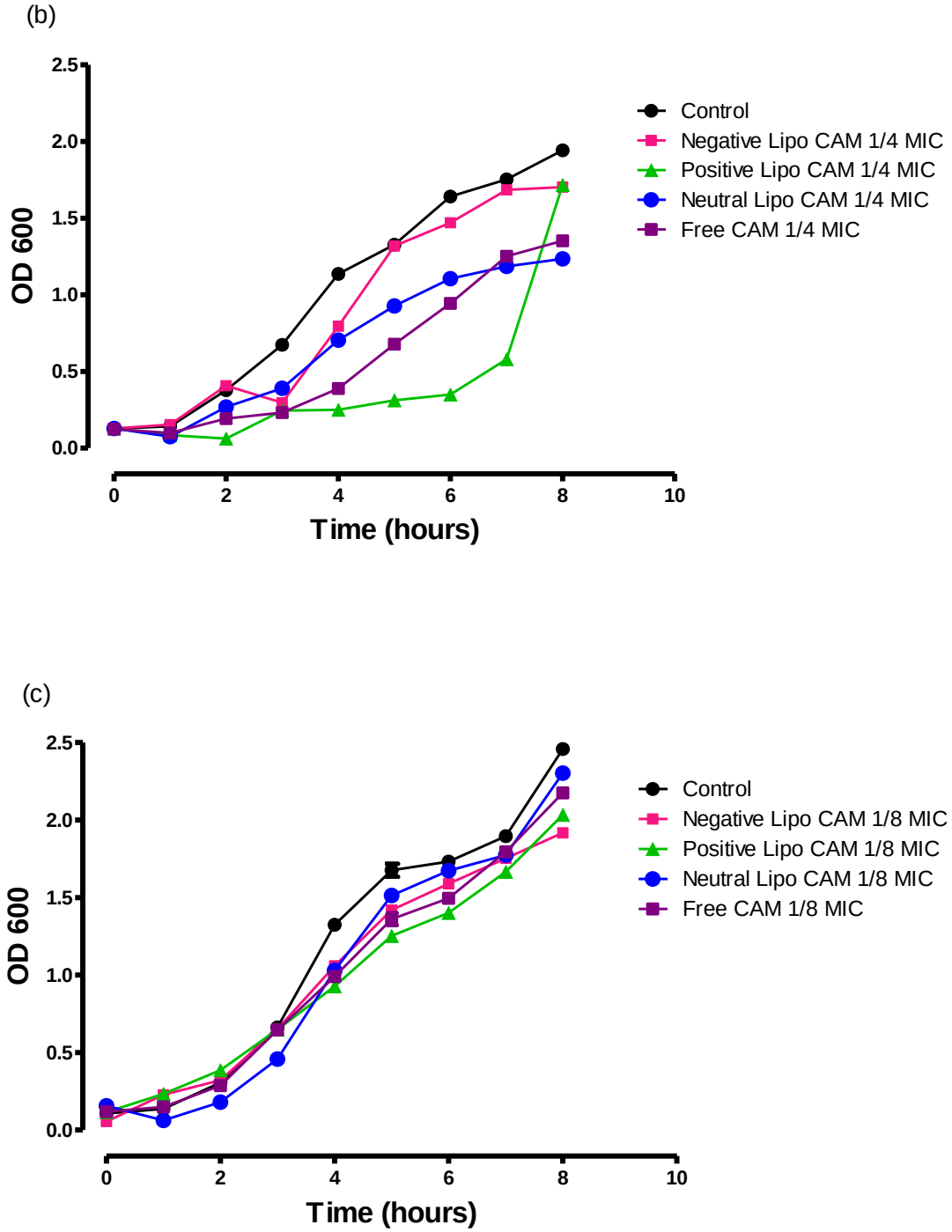


Figure 2: Effect of subinhibitory concentrations of liposomal clarithromycin formulations on growth of PA-13572 at 1/2 the MIC (a), 1/4 the MIC (b), and 1/8 the MIC (c). Experiments were tested three times in triplicate, with means shown (the error bars were deleted for clarity of the

graphs). Shown are control (black), negatively charged liposomal clarithromycin (pink), positively charged liposomal clarithromycin (green), uncharged liposomal clarithromycin (blue) and for free clarithromycin (purple).

2.3.6. Effect of liposomal clarithromycin on bacterial virulence factors

The levels of the lipase, chitinase, elastase, and protease in the free- or liposomal-clarithromycin-treated PA-13572 cultures were measured at 1/8 the MIC. Positively charged liposomal clarithromycin attenuated lipase production significantly compared to the control ($P<0.001$), while uncharged liposomal CAM and negatively charged liposomal CAM were ineffective (Fig. 3a). Chitinase production in the supernatant was evaluated by quantifying the release or breakdown of chitin azure (see Materials and Methods). Liposomal CAM (uncharged and positively charged) reduced chitinase production. However, the negatively charged liposomal CAM reduced chitinase productions significantly ($P<0.001$) (Fig. 3b). All liposomal formulations reduced elastase and protease production significantly at 1/8 of the MIC ($P<0.05$) compared to the control (Fig. 3c and d, respectively). There were no significant differences in the attenuation of elastase and protease production between the free-clarithromycin and liposomal-clarithromycin formulations.

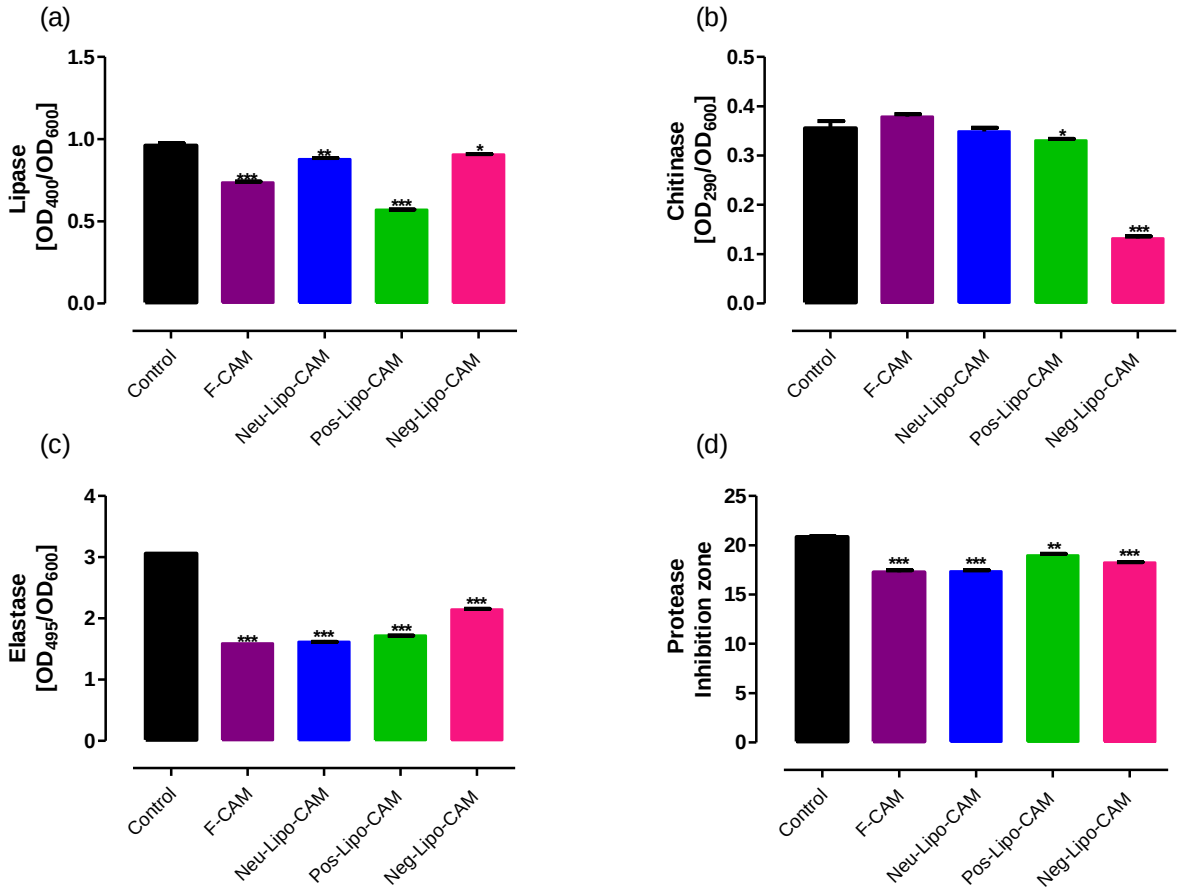


Figure 3: Effects of subinhibitory concentrations (1/8 the MIC) of free and liposomal clarithromycin on PA-13572 virulence factors production. (a) Lipase. (b) Chitinase. (c) Elastase. (d) Protease. The results represented the mean ± SEM of three independent experiments in triplicates. For lipase, chitinase, and elastase experiments, the results were normalized by dividing the OD by the OD_{600} (cell density). *P* values were considered significant compared with the control: ***, *P<0.001;* **, *P<0.01;* *, *P<0.05*.

2.3.7. Effect of liposomal clarithromycin on bacterial motility

We examined bacterial motility, including twitching, swarming, and swimming, in the presence of subinhibitory concentrations of either free or liposomal clarithromycin with different surface charge (neutral, positive, and negative). Liposomal-loaded clarithromycin significantly reduced twitching of *P.aeruginosa* PA-13572 compared to free-clarithromycin and control groups ($P<0.001$) (Fig. 4a). However, positively charged liposomes exhibited more reduction in the twitching motility of *P.aeruginosa* ($P<0.01$ and $P<0.001$ compared to negatively charged and uncharged liposomal formulations, respectively). For swarming, liposomes (neutral, positive, and negative) were able to reduce swarming of *P.aeruginosa* PA-13572 at the subinhibitory concentration compared to the free-clarithromycin and control groups (Fig. 4b). However, positively charged liposomes reduced swarming more significantly than uncharged and negatively charged liposomes ($P<0.001$). Liposomal formulations attenuated the swimming activity of *P.aeruginosa* compared to the free-clarithromycin and control groups (Fig. 4c). However, positively charged liposomes reduced swimming activity significantly *(*$P<0.001$) compared to neutral and negatively charged liposomes.

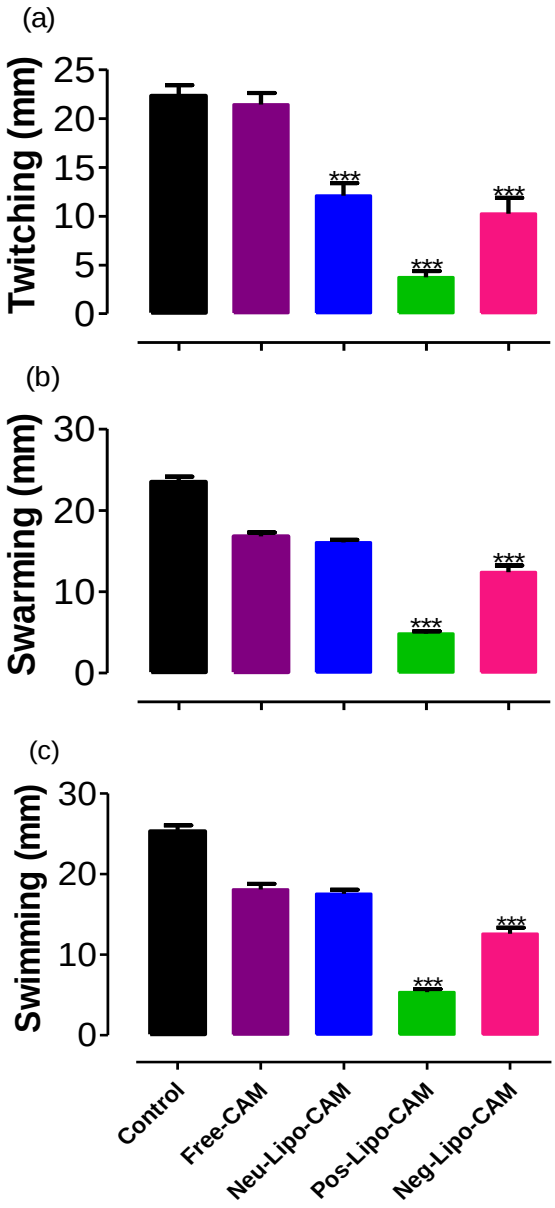


Figure 4: Effect of a subinhibitory concentration of liposomal clarithromycin on *P. aeruginosa* motility. Free or liposomal clarithromycin at 1/8 the MIC was added to agarose plates, and motility was examined. Twitching (1% agarose [wt/vol]) (a), swarming (0.5% agarose [wt/vol]) (b), and swimming (0.3% agarose [wt/vol]) (c) were measured with digital calipers. *P* values were considered significant compared with the control and between groups: ***, $P < 0.001$.

2.3.8. Toxicity of liposomal clarithromycin

When lung cells were incubated for 24 h and exposed to 2× the MIC of treatments, cell viabilities were 100% for NEG-Lipo-CAM, 99% for NEU-Lipo-CAM, 1% for POS-Lipo-CAM, and 20% for free CAM. Following a 48-h period, the viabilities of lung cells exposed to treatment formulations at 2× the MIC were 97% for NEG-Lipo-CAM, 95% for NEU-Lipo-CAM, 1% for POS-Lipo-CAM, and 5% for free CAM. After 72 h of incubation, at 2× MIC, the percentages of cell viability were 93% for NEG-Lipo-CAM, 98% for NEU-Lipo-CAM, 0% for POS-Lipo-CAM, and 1% for free CAM (Table 3).

Table 3: Cell viability of epithelial lung cells exposed to free or liposomal clarithromycin formulations

Time (h)	Cell viability (%)							
	Free CAM		**NEG-Lipo-CAM**		**NEU-Lipo-CAM**		**POS-Lipo-CAM**	
	1×MIC	**2× MIC**	**1×MIC**	**2× MIC**	**1× MIC**	**2× MIC**	**1× MIC**	**2× MIC**
0	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 00	100 ± 00	100 ± 0.00	100 ± 0.00
24	98 ± 2.08	21 ±7.60^{b}	99 ± 1.04	100 ± 0.63^{a,b}	100 ± 0.40	99 ± 0.40^{a,b}	0 ± 0.06^{a,b}	1 ± 0.04^{a,b}
48	95 ± 6.14	5 ±0.41^{b}	97 ± 1.38	97 ± 1.79^{a,b}	96 ± 2.15	95 ± 3.38^{a,b}	0 ± 1.29^{a,b}	1 ± 1.97^{a,b}
72	97 ±1.57	1 ±1.48^{b}	96 ± 3.67	93 ± 9.04^{a,b}	93 ± 3.14	98 ± 3.26^{a,b}	0 ± 0.20^{a,b}	0 ± 0.02^{a,b}

^{a}P values were considered significant compared with free clarithromycin (***P*<0.001).**

^{b}P values were considered significant for free or liposomal clarithromycin compared to the control (***P*<0.001**

2.4. Discussion

In this study, we established that macrolide antibiotics can be efficiently encapsulated in liposomes composed of DPPC-cholesterol, DPPC-DDAB-cholesterol, or DPPC-DCP-cholesterol. Our data show that the efficiency of encapsulation of clarithromycin by negatively charged liposomes was significantly higher than that of other formulations due to attractive interaction with positively charged clarithromycin (76). These results were in agreement with a study that showed that an electrostatic attraction between the negatively charged drug ciprofloxacin and positively charged lipids increases the percent encapsulation efficacy compared to a negatively charged drug (77).

The size of a liposomal formulation is a determining factor in terms of intended anatomical target. The particle diameter of negatively charged liposomal clarithromycin was larger than that of the positively charged formulation. This is due to the inclusion of charge, which results in an increased space between the adjacent bilayers. The phenomenon can be explained by the attraction of the drug to negatively charged particulates and pushing the phospholipid head group apart (54, 77).

An additional factor that must be considered in the development of an effective drug delivery system is the carrier stability. The liposome's stability was found to be largely independent of temperature, although liposomal formulations were more stable at 4°C. In order to enhance the stability further, we chose to incorporate cholesterol in our formulations. Cholesterol reduces the bilayer permeability of the liposomal membrane, allowing greater drug retention at higher temperatures (64, 66). We found that liposomes composed of DPPC-CHOL and DCP-DPPC-CHOL retained more drug in PBS than liposomes composed of DDAB-DPPC-CHOL at the end of a 48-h experimental period. This may be due to the electrostatic repulsion

that occurs between the drug and the positively charged liposomes, which results in a higher rate of drug release and a change in the phase transition temperature (77). This may also be explained by highlighting important factors that contribute to liposome stability, such as the formulation of the saturated neutral phospholipids with different acyl chain lengths and the transition temperature of the phospholipids (78). An earlier report from our laboratory confirmed the notion that the higher transition temperatures of liposomes are more stable due to an increase in the acyl chain length of constituent lipids; this may explain the better stability of liposomes in formulations containing DPPC (64).

We have shown that the liposomal clarithromycin formulations enhance clarithromycin antimicrobial activity against a resistant clinical strain of *P.aeruginosa.* This is in agreement with previous reports indicating that liposomal formulations are highly effective against most strains of bacteria compared to free drug (52). The MICs and MBCs of liposomal clarithromycin were less than those of free clarithromycin. In most cases, the MIC of free clarithromycin was 256 mg/liter, which is consistent with a previous study (78). To our knowledge, our formulations are the first liposomal carriers that enhance clarithromycin antibacterial activity against antibiotic-resistant clinical strains of *P. aeruginosa*. Positively charged liposomal clarithromycin was highly effective against *P. aeruginosa* strains, reducing the MIC from a resistant level of 256 mg/liter to a sensitive level of 8 mg/liter. It is possible that electrostatic attraction and fusion occurring between positively charged liposomal clarithromycin and the cell membrane of *P. aeruginosa* enhances its activity *in vitro*. Similar results were obtained in previous studies ,which showed other formulation of positively charged liposomes (PC-DOPE-DOTAP [phosphatidylcholines- dioleoyl-glycero-phophoethsnolamine - dioleyloxy trimethyl ammonium-propane] and PC-CHOL-DOTAP)

exhibited better antimicrobial efficiency against *P. aeruginosa* than other formulation of liposome (79).

Clarithromycin in its free form is known to be a bactericidal enhancer in the treatment of *P. aeruginosa* biofilm (80, 81). A pervious study offered two explanations for the eradication of membranous structures of biofilms after treatment with clarithromycin: destruction of polysaccharide glycocalyx by clarithromycin and inhibition of *de novo* polysaccharides synthesis (80). In terms of the improved efficacy of our liposomal formulations, a continuous contact with the target and slow antibiotic release may accelerate biofilm penetration by the drug (50). Positively charged liposomal clarithromycin affects biofilm more than other formulations, as it completely eradicates the biofilm community at lower concentrations than the others. This might be due to the attraction between the opposite charges of the bacterial membrane and the liposomal formulation. This allows better penetration of the liposomes into the biofilm and release of antibiotics within the community (79). Different electrical charges affect biofilms differently: negatively charged liposomal clarithromycin eradicated biofilms completely at high concentration, while uncharged liposomal clarithromycin produced an acceptable reduction of the biofilm community. This is in agreement with our earlier findings on the efficacy of uncharged liposomal antibiotic formulations (65, 82). Thus, formulations containing a variety of liposomes are superior in biofilm eradication and can be utilized to overcome bacterial resistance to antibiotics.

Previous studies demonstrated that macrolides at subinhibitory concentration inhibited bacterial motility, which contributes to biofilm formation, by affecting the gene expression responsible for producing flagella and preventing proper assembly of type IV pili on the surface of bacteria (30, 83), thereby causing a reduction of bacterial motility, including twitching,

swarming, and swimming. Here, we demonstrated that encapsulation of clarithromycin into liposomes resulted in improving the efficacy of clarithromycin in inhibiting *P.* aeruginosa motility. The enhanced activity of liposomal formulations might be attributed to fusion of liposomes to the bacterial cell wall (66) so that a high concentration of antibiotic can be delivered directly to the bacterial cytoplasm, allowing inhibition of flagellar and type IV pilus activities (30, 35). Furthermore, we have noted improved efficacy of positively charged liposomal formulation, which could be explained by the interaction of the positively charged liposomes surface with the negatively charged bacterial cell wall, which attracts a high concentration of liposomes-loaded clarithromycin to fuse with the bacterial cell membrane (79).

A subinhibitory concentration of macrolides might reduce the production of virulence factors and host tissue damage (84, 85). A previous study demonstrated the effect of subinhibitory concentration on reducing virulence factor using azithromycin (86). Wozniak and Keyser showed that a sub-MIC level of clarithromycin inhibited the formation of the biofilm matrix and the production of proteases (30, 87). A sub-MIC level of clarithromycin, however, is less effective than azithromycin in reducing the production of elastase and lipase (88). Considering these reports, a combination of the antitwitching property of clarithromycin, which might be enhanced when encapsulated in novel liposomal formulations, with the bactericidal properties of other drugs may prove more effective in treating chronic bacterial infections.

Exposure of A549 human lung cells to free clarithromycin reduced cell viability by 99% at 2× the MIC after 72 h of treatment. After the same period of treatment, negatively charged and uncharged liposomal clarithromycin at similar concentrations protected the cell against clarithromycin toxicity. These and other published data supports our hypotheses that liposomal

formulations protect host cells from toxic drugs. It was also shown in previous work that the liposomal formulation reduced the toxicity of antibiotics for cell line A549 for different formulations of liposome (DPPC: dimyristoyl glycerol-phosphglycerol [DMPG]) (50, 51, 53). In contrast, positively charged liposomal clarithromycin at a low concentration decreased the viability of A549 cells. This phenomenon has since been observed by others for positively charged liposomes containing DDAB lipid (89). It is possible that the positively charged lipid DDAB had an adverse effect on cell proliferation (51, 90, 91).

In conclusion, these data indicate that negatively charged liposomal clarithromycin successfully reduced clarithromycin toxicity, greatly affected biofilm community members, and improved clarithromycin activity against highly resistance *P.* aeruginosa. Future experiments will assess the efficacy of these liposomal clarithromycin formulations in animal models.

Acknowledgements

This work was supported by the Ministry of Higher Education of the Kingdom of Saudi Arabia.

We have no conflicts of the interest to declare.

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CHAPTER THREE

Conclusion and future work

Antibiotic therapy has prolonged the life span of CF patients by preventing and eradicating the lung infection and inflammation. Encapsulation of drugs with liposomes is also known to be associated with improved activity against resistant strains. We have developed new drug formulations with different charges of lipids in an attempt to enhance clarithromycin bactericidal activity. These charged liposomal clarithromycin formulations were tested to prove their efficacy including stability, MIC, MBC, MBEC, virulence factor production, motilities, and toxicity in comparison to free drug form.

Our data indicate a steady drug release from liposomal clarithromycin formulations whereas positively liposomal clarithromycin failed to do so. The MICs and MBCs of clarithromycin liposomal formulations were less than that of the free drug. Negatively charged liposomal clarithromycin greatly reduced the biofilm community and enhanced clarithromycin activity against highly resistant *P. aeruginosa strains*. These formulations, therefore, have potential to overcome bacterial resistance to antibiotics. Liposomal clarithromycin would improve the efficacy of clarithromycin in inhibiting the motility at sub-inhibitory concentration. The liposomal formulations (negative and uncharged) protected the A549 lung epithelial cells against clarithromycin toxicity whereas positively charged liposomal clarithromycin decreased the viability of A549 cells.

Future work in our laboratory would study the mechanism of liposome bacterium interactions by transmission electron microscopy (TEM), flow cytometry, lipid mixing assay, and fluorescence activated cell sorter (FACS). Interaction of free CAM and Lipo-CAM with bacterial membrane will also be investigated by using immunocytochemistry. Also, we will address the mechanism of action of virulence factor production by using specific inhibitors and

may be helpful for the development of novel therapies for *P. aeruginosa* caused pulmonary diseases. Quorum sensing (QS) and β-glycosidase assay will be examined after exposure to different charged liposomal clarithromycin. Finally, the efficacy of these formulations will be evaluated in animal models of chronic pulmonary infection with *P. aeruginosa*.

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