In silico and *in vitro* inhibition of quorum sensing and biofilm formation in *Chromobacterium violaceum* using semi-synthetic compounds

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Degree of Master of Science in Medicine by research only

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

Declaration

I, Madison Tonkin, declare that this dissertation is my own work. This dissertation is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Madison Tonkin (Signature of candidate)

28th day of May 2021 at 13:17

Dedication

This dissertation is dedicated to my parents, Ronnie and Simone Tonkin, my husband, Gregory Arendse and my sister, Taylor Tonkin.

PUBLICATIONS AND PRESENTATIONS

Publications

Tonkin, M., Khan, S., Wani, M.Y and Ahmad, A. Quorum Sensing – A Stratagem for Conquering Multi-Drug Resistant Pathogens. Accepted by *Journal of Current Pharmaceutical Design*. (**Appendix 7.1 – Review Article, In Press)**.

Tonkin, M., Khan, S., Wani, M.Y and Ahmad, A. Inhibition of Quorum Sensing and Biofilm Formation in *Chromobacterium violaceum* using Anti-Microbial and Drug Discovery Approaches. Submitted to *International Journal of Biological Macromolecules*. (**Appendix 7.2 – Research Article, Submitted)**.

Presentations

Poster Presentation: Tonkin, M., Khan, S. and Ahmad, A. *In silico* and *in vitro* inhibition of quorum sensing and biofilm formation in *Chromobacterium violaceum* using semi-synthetic compounds. This poster was presented at Faculty of Health Sciences Research Day and Postgraduate Expo, University of Witwatersrand held on October 15th, 2020. (**Appendix 7.3)**.

Poster Presentation and talk: Tonkin, M., Khan, S. and Ahmad, A. Targeting quorum sensing and biofilm formation in *Chromobacterium violaceum* with semi-synthetic compounds using *in silico* and *in vitro* techniques. This poster was presented virtually at e-Colloquium on "Emerging Trends in Health and Disease Research" held by Amity University, Haryana, India on 20 October 2020. The award of **Best Poster 2nd Position** was award. (**Appendix 7.4**).

ABSTRACT

Introduction

Over the past decades, the number of emerging drug resistant bacteria has increased at an alarming rate. Bacteria develop drug resistance through a variety of mechanisms with the prime contributor being the misuse and overuse of antibiotics. Adding to this, the development of new antibiotics with novel drug targets has decreased. Recently, researchers have identified biofilm and quorum sensing as emerging drug targets. Both biofilm and quorum sensing are inter-related processes and provide variety of opportunities for development of novel anti-bacterial drugs with different mechanisms of action. In this study, we used five novel imidazole derivatives (IMA-1 – IMA-5) to inhibit quorum sensing and biofilm formation in *Chromobacterium violaceum* using a variety of *in silico* and *in vitro* techniques.

Materials and Methods

In silico molecular docking was first performed using Maestro software to determine the binding scores of the compounds and interactions created when the compounds bind to target protein, CviR. Following molecular docking, molecular dynamic simulations were performed using Amber18 software to determine the stability, flexibility and solubility of the complexes formed with CviR and the compounds.

In vitro anti-bacterial activity was determined by calculating MIC and MBC, following the CLSI standard guidelines. To determine qualitative anti-quorum sensing activity of the test compounds, agar plate assay was employed. To further quantify anti-quorum sensing potential of imidazole derivatives, the percentage of violacein pigment was measured spectrophotometrically. To study the effect of imidazole derivatives on biofilm formation, standard crystal violet staining assay was performed on treated and untreated *C. violaceum*. Furthermore, the ability of the test compounds to inhibit quorum sensing genes was determined by performing RT-qPCR to calculate relative fold gene expression.

Results

Results from *in silico* molecular docking indicated that all the imidazole derivatives interacted with important amino acids in the binding pocket. The interactions formed between compounds and CviR included pi-pi stacking, salt bridge formation and hydrogen bond generation. Despite all the derivatives showed high binding energies (ΔG_{bind}), IMA-1 had the highest ΔG_{bind} at -34.44 kcal/mol with a binding score of -9.23 kcal/mol and IMA-5 had the lowest ΔG_{bind} at -30.74 kcal/mol with a binding score of -6.97 kcal/mol. Molecular dynamic simulation results

showed that all compounds formed stable complexes with the target protein, CviR. Results also indicated that all complexes do not disrupt the natural manner in which the natural CviR-HS-10 complex behaves in hydrophobic or hydrophilic environments and this indicates that the compounds do not change the natural state of the CviR once in complex.

In vitro susceptibility results indicated that all the compounds had anti-bacterial activity against *C. violaceum* with IMA-1 as the most effective derivative with an MIC and MBC values of 0.0488 and 0.0977 µg/ml, respectively. Anti-quorum sensing results indicated that IMA-1 was the most effective and IMA-5 showed the least promising anti-quorum sensing. The derivatives were tested to determine the ability to inhibit biofilm formation and it was found that IMA-1 is effective at disrupting mature biofilm. Results from gene studies revealed that treatment with all the derivatives were able to significantly reduce the expression of *CviI* gene, indicating that all the derivatives were able to disrupt quorum sensing at a gene level. Genes from the *VioA, VioB, VioC, VioD* and *VioE* operon were also tested, and it was found that treatment with all the compounds significantly reduce the expression of *VioB* and *VioD*. The compounds also disrupt the expression of *VioA* and *VioE* but not to the same extent.

Conclusion

This study targeted quorum sensing and biofilm formation using five newly synthesised imidazole compounds. Various *in silico* and *in vitro* studies revealed that the compounds caused inhibition of growth at higher concentrations and disrupted quorum sensing at lower concentrations. The results from the *in vitro* studies agree with the results of *in silico* studies in that the same trend was found where IMA-1 was seen as the most effective compound and IMA-5 as the least effective. This study provides data to support the novel ideas as to how the bacterial drug resistance could be evaded by developing molecules that could target quorum sensing and biofilm formation.

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge and thank my Heavenly Father for presenting me this opportunity to complete my Master's degree and providing me with the strength and determination to complete my research work. I give Him praise and glory for all this degree has achieved.

I would like to acknowledge my supervisor, Dr Aijaz Ahmad for all his support and allowing me to complete this degree under his guidance. Thank you, Dr Ahmad, for helping me publish my work and providing the opportunities to present this research at conferences.

I would like to acknowledge and thank my co-supervisor, Dr Shama Khan, for teaching me new techniques and introducing me to the field of bioinformatics. Thank you, Dr Khan, for your guidance, kindness, and support throughout this degree.

I would like to acknowledge and thank Dr Shabir Lone and Dr Musa Marimani for their assistance with laboratory techniques and helping me become acquainted to how the laboratory operates. Your time and energy spent with me during my degree has been greatly appreciated.

I would like to thank all the students and staff members of the Department of Clinical Microbiology and Infectious Diseases, University of Witwatersrand for allowing me to access the facilities needed to complete this research.

Finally, I would like to acknowledge and thank with all my heart my parents, sister and husband who have supported me throughout this degree and my academic life. I love you all and appreciate your unconditional support.

This research was supported by the South African National Research Foundation (NRF) Research Development Grant for Y-Rated Researchers (RDYR180418322304; Grant No: 116339), and National Health Laboratory Service Research Trust Grant, South Africa, (NHLSRT-1/2/17-1/9/19).

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LIST OF ACRONYMS, SYMBOLS AND ABBREVIATIONS

%	Percentage
<	Less than
±	Plus-minus
≤	Less than or equal
Mg	Microgram
µl	Microlitre
A. baumanii	*Acinetobacteria baumanii*
AHL	Acetyl homoserine lactone
Agr	Accessory gene regulator
AgrA	Accessory gene regulator A
AgrB	Accessory gene regulator B
AgrC	Accessory gene regulator C (receptor for *S. aureus*)
AgrD	Accessory gene regulator D
ATCC	American Type Culture Collection
Bp	Base pair
B. subtilis	*Bacillus subtilis*
CADD	Computer aided drug design
cDNA	Complementary deoxyribonucleic acid
CLSI	Clinical and Laboratory Standards Institute

ComD	Receptor used in quorum sensing in *S. pneumoniae*
ComP-ComA	Receptor used in quourum sensing by *B. subtilis*
Cqss	Receptor for quorum sensing in *V. cholerae*
CviI	Gene encoding homoserine lactone synthase in *C. violaceum*
CviR	Gene encoding receptor in *C. violaceum*
C. violaceum	*Chromobacterium violaceum*
Cq	Melting curve of amplicons
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
E. coli	*Escherichia coli*
G	Gram
GPU	Graphic processing unit
H_2O	Water molecule
IMA	Imidazole compound
K	Kelvin
Kb	Kilobase
Kcal	Kilocalorie
K. pneumoniae	*Klebsiella pneumoniae*
L	Litre

LB	Luria Bertani
LuxI	Synthase produced by Lux operon
LuxR	Receptor/regulatory proteins of the Lux operon
MBC	Minimum bactericidal concentration
MD	Molecular dynamics
MIC	Minimum inhibitory concentration
Ml	Millilitre
Mm	Millimetre
Mol	Mole xx
M. tuberculosis	*Mycobacterium tuberculosis*
MQSIC	Minimum quorum sensing inhibitory concentration
NaCl	Sodium Chloride
Ng	Nanograms
Nm	Nanometre
Ns	Nano seconds
P. aeruginosa	*Pseudomonas aeruginosa*
Pdb	Protein data bank format
PBS	Phosphate Buffer solution
PCR	Polymerase chain reaction
pH	Potential hydrogen

PQS	Pseudomonas quorum sensing system
Rgg	Family of transcription factors used for quorum sensing in Gram-positive bacteria
RhlR	Receptor for quorum sensing in *P. aeruginosa*
RMSD	Root mean structural dynamics
RMSF	Root mean structural fluctuations
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNPP	Family of transcription factors used for quorum sensing in Gram-positive bacteria
RoG	Radius of Gyration
Rpm	Revolutions per minute
RT-qPCR	Quantitative reverse transcription PCR
S. aureus	*Staphylococcali aureus*
S. nigricans	*Santiria nigricans*
S. pneumoniae	*Staphylococcali pneumoniae*
TAE	Tris acetate-EDTA buffer
USA	United States of America
UV	Ultraviolet
V	Voltage

V. cholerae	*Vibrio cholerae*
Xg	Gravity at the earth's surface
Å	Angstrom
β	Beta
˚C	Degrees Celsius
ΔCq	The difference between the average Cq of the house keeping gene subtracted from the Cq of target genes
ΔΔCq	The ΔCq of control cells subtracted from the ΔCq of the tested cells
ΔE_{vdW}	Free energy from van der Waals forces
ΔE_{ele}	Free electrostatic energy
ΔG_{gas}	Gas-phase energy
ΔG_{polar}	Polar contributions to solvation
$\Delta G_{nonpolar}$	Nonpolar contributions to solvation
ΔG_{sol}	Solvation free energy
ΔG_{bind}	Free binding energy

LIST OF AMINO ACIDS

Name	3 Letter Abbreviation	1 Letter Abbreviation
Alanine	Ala	A
Cysteine	Cys	C
Aspartic Acid	ASP	D
Glutamic Acid	GLU	E
Phenylalanine	PHE	F
Glycine	GLY	G
Histidine	HIS	H
Isoleucine	ILE	I
Lysine	LYS	K
Leucine	LEU	L
Methionine	MET	M
Asparagine	ASN	N
Glutamine	GLN	Q
Arginine	ARG	R
Serine	SER	S
Threonine	THR	T
Tryptophan	TRP	W
Tyrosine	TYR	Y
Valine	VAL	V

CHAPTER 1 BACKGROUND

1.1. Infectious Diseases

The human body consists of several mutualistic relationships with a variety of bacteria (Arleevskaya *et al.*, 2019; Hoffmann *et al.*, 2016). Several positive consequences result from such mutualistic relationships with beneficial bacteria that include development and continuous adjusting of various essential systems such as the immune system, detoxification in the liver, and development of the gastrointestinal tract (Arleevskaya *et al.*, 2019; Hoffmann *et al.*, 2016; Wiciński *et al.*, 2020). However, it is well known that not all bacteria are beneficial. Pathogenic bacteria can enter the human body resulting in a variety of infectious diseases and autoimmune diseases that can even result in mortality (Aminov, 2010; Yoo & Lee, 2016).

Infectious diseases have been problematic to human health for centuries and are among the leading cause of human mortality (Aminov, 2010; Bandow *et al.*, 2003). For a long time, there was no sufficient way of controlling the spread of infectious diseases, particularly bacterial infections (Ahmad *et al.*, 2015; Lewis, 2017). Thankfully, the development of antibiotics arose along with discovery of other control methods, such as vaccinations, to contain the spread of infectious diseases (Lewis, 2017; Yoo & Lee, 2016). Antibiotics have significantly contributed to the control of bacterial diseases and have aided in decreasing human mortality rates as they are effective in curing individuals of fairly common bacterial infections (Ahmad *et al.*, 2015; Aminov, 2010; Hoffman, 2001). This important research demonstrated that one drug could prevent numerous diseases and derivatives of that drug could be synthesised (Aminov, 2010).

This century, medicine has been acquainted with a new problem which is the exponential increase in the development of multi-drug resistant bacteria (Williams, 2017). Today, antibiotic drug development relies on the discovery of novel compounds that may be able to target structures such as receptor proteins, signalling molecules and the more detrimental and less common, but still effective targets of DNA or RNA (Aminov, 2010; Athamneh *et al.*, 2014). A large problem regarding antibiotics is development of drug-resistant bacteria (Aminov, 2010; Bandow *et al.*, 2003).

1.2. Antibiotics – An effective Method to Treat Bacterial Infections

Antibiotics have been studied since late 1800s which lead to the discovery of penicillin in early 1900s (Li & Webster, 2018). Penicillin was extremely beneficial to treat patients in World War II and lead to the development of various classes of antibiotics such as glycopeptides and aminoglycosides (Li & Webster, 2018). Many antibiotics have been developed from the natural environment such as plants or fungal species (Durand *et al.*, 2019). Although it appears odd to

gain understanding of how an antibiotic operates from a fungus, research has shown that microorganisms produce antimicrobial substances to protect themselves from other pathogens and can resist other antimicrobials (Durand *et al.*, 2019). With this protection, comes development of antimicrobial-resistant genes that microorganisms have to protect themselves from self-annihilation (Durand *et al.*, 2019). Antibiotics have a variety of effective mechanisms that are classified into five distinct groups. The first group includes non-ribosomal peptides that are derivative amino acids, the second group includes antimicrobials that are derived from acetyl coenzyme A, third group is comprised of hybrids of first and second groups. The fourth group consists of several carbohydrate units such as amine groups that make up an antibiotic, and finally, antibiotics can be comprised of various molecules such as alkaloids like metronidazole (Durand *et al.*, 2019).

In modern times, the medical world has greatly depended on antibiotics to prevent a variety of bacterial infections that once lead to fatality. Examples of these infections include urinary tract infections, meningitis, sepsis and pneumonia (Banin *et al.*, 2017). Antibiotics have also been vital in routine surgical procedures such as caesarean and organ transplants (Banin *et al.*, 2017; Durand *et al.*, 2019). However, many bacterial species are now developing resistance towards these antibiotics and this is becoming a global health problem. Table 1.1 represents classes of well-known antibiotics with examples of antibiotics in that class. The mechanism of action and examples of bacterial species that developed drug resistance towards antibiotics are also represented in Table 1.1.

Table 1.1: Currently known antibiotics with their mechanisms of action and bacterial species that have developed drug resistance towards these antibiotics.

Class of antibiotic	**Examples of antibiotics in that class**	**Mechanism of action**	**Bacterial species that developed drug resistance**	**References**
β-Lactams	Ampicillin, penicillin and cefazolin	Inhibits transpeptidases to prevent amino acid transfer of peptidoglycans.	*Staphylococci* species and *P. aeruginosa*	(Bassetti *et al.*, 2013; Hall & Mah, 2017; Kirui *et al.*, 2019; Lister *et al.*, 2009)

Lipopeptides	Daptomycin	Causes cell membrane to depolarise.	*K. pneumoniae, P. aeruginosa* and *A. baumanii*	(Hall & Mah, 2017; Velkov *et al.*, 2014)
Aminoglycosides	Kanamycin, amikacin and tobramycin	Interferes with protein synthesis by binding to the 16S rRNA of the 30S ribosomal subunit.	*P. aeruginosa* and *A. baumanii*	(Bassetti *et al.*, 2013; Hall & Mah, 2017; Lister *et al.*, 2009)
Macrolides	Azithromycin and clarithromycin	Inhibits peptide elongation by binding to the 23S rRNA of the 50S ribosomal subunit.	*Mycoplasma pneumoniae*	(Hall & Mah, 2017; Morozumi *et al.*, 2010)
Quinolones	Ciprofloxacin and norfloxacin	Prevents DNA replication by interacting with DNA gyrase and inhibits RNA polymerase.	*E. coli* and *P. aeruginosa*	(Hall & Mah, 2017; Kirui *et al.*, 2019; Lister *et al.*, 2009)

1.3. Antibiotic Resistance

The development of drug resistance has become a global concern and has resulted in the identification of twelve bacterial species that have been prioritised for research into how to treat such infections (Banin *et al.*, 2017). Bacterial species on this list include *Staphylococcus aureus* and *Klebsiella pneumoniae* (Banin *et al.*, 2017). Majority of research is dedicated to identifying new drug targets and designing new drug molecules to defeat multi-drug resistant bacteria. A variety of ways to develop novel antibiotics includes modifying and utilising already known structures to attack the bacteria (Banin *et al.*, 2017).

Antibiotics have been and are still considered the preferred method for treating bacterial infections (Abbas *et al.*, 2017). For many decades, antibiotics have been considered the most efficient way and the first line of defence against bacterial infections (Munguia & Nizet, 2017;

Zhuang *et al.*, 2019). However, this consideration has lost impact because over the past 10 years, there has been an alarming increase in drug-resistant bacterial species (Lewis, 2017; Soojhawon *et al.*, 2017; Williams, 2017). Multi-drug resistant bacteria are bacteria that can effectively proliferate despite various antibiotics administered to a patient (Muzondiwa *et al.*, 2020). *M. tuberculosis* is a well-known example of a multi-drug resistant pathogen (Muzondiwa *et al.*, 2020). In this study, drug resistance will be defined as any bacterial species that have the ability to overcome the effects of an antibiotic administered to kill the bacteria (Christaki *et al.*, 2020; Li & Webster, 2018).

Often, resistant bacteria develop into multi-drug resistance which can cause a patient to be more susceptible to infections and can lead to prolonged suffering (Giedraitienė *et al.*, 2011; Hoffman, 2001). Effects of bacterial resistance include increased cost and stay of hospitalisation, the need to isolate from people, infection could progress and lead to the need for intensive care and the patient can develop a greater predisposition to new infections (Li & Webster, 2018). The consequences of antibiotic resistance can be more serious in that drug-resistant bacteria can compromise the safety and efficacy of various surgeries such as organ transplants (Li & Webster, 2018). One of the largest reasons why bacteria have developed drug resistance is the overconsumption of antibiotics (Abbas *et al.*, 2017; Champalal *et al.*, 2018). Indeed research has discovered that there has been a dramatic increase in antibiotic consumption and this has guided development of numerous drug resistant bacteria, some developing multidrug resistance (Abbas *et al.*, 2017; Zhuang *et al.*, 2019). The current antibiotics are either bactericidal or bacteriostatic (Champalal *et al.*, 2018). The level of resistance, or how susceptible a bacterial species is to a particular drug, can be determined by identifying the minimal inhibitory concentrations (MIC) of that drug against the strain or species of bacteria (Morio *et al.*, 2017). The MIC is usually determined *in vitro* and terms the bacteria resistant to that drug (Morio *et al.*, 2017). A patient can become clinically resistant to a bacterial infection which is known to be exceptionally problematic as their treatment options are greatly reduced (Hall & Mah, 2017; Morio *et al.*, 2017). This usually develops in a clinical setting such as when the patient is being treated for an infection, however, the treatment is rendered ineffective and the infection persists – hence the patient is clinically resistant (Morio *et al.*, 2017). However, an additional issue that has added to the number of resistant bacteria, is the slow development of novel antibiotics that target the resistant pathogens (Abbas *et al.*, 2017; Munguia & Nizet, 2017). Certain antibiotics are now considered ineffective as represented in Table 1.1 (Zhuang *et al.*, 2019). Therefore, the conclusion made is that novel drug targets are

needed that do not disrupt bacterial growth but rather inhibit the ability of the pathogen to develop pathogenesis (Abbas *et al.*, 2017; Zhuang *et al.*, 2019).

1.3.1. Mechanisms of Drug Resistance in Bacteria

The mechanisms of drug resistance in bacteria have been well documented (Li & Webster, 2018; Swain *et al.*, 2020). Previous research has identified that bacteria develop drug resistance due to the improper use and abuse of antibiotics in humans (Hall & Mah, 2017). An example of a bacterium that has developed drug resistance due to the improper use of antibiotics is *M. tuberculosis*. This pathogen has developed a variety of genetic mutations due to inadequate routine intake of antibiotics during infection (Bechinger & Gorr, 2017; Swain *et al.*, 2020). It is important to note that each pathogen develops resistance through a different approach to a particular antibiotic (Li & Webster, 2018). Bacteria develop drug resistance through a variety of mechanisms (Li & Webster, 2018).

Currently, it has been identified that there are two broad classes of antibiotic resistance namely intrinsic resistance and acquired resistance (Li & Webster, 2018). Intrinsic resistance occurs when bacteria develop structural obstructions to prevent the antibiotic from having the desired effect. One method of this type of drug resistance occurs when bacteria have developed resistance in such a way that structurally, antibiotics are unable to infiltrate the outer layer of the bacteria. Another method of intrinsic resistance is when the bacteria expel the antibiotic through the use of efflux pumps (Li & Webster, 2018). Acquired resistance results from bacteria acquiring genetic abilities to adapt and tolerate antibiotics (Li & Webster, 2018). Indeed, bacteria can acquire drug resistance in various ways such as: developing structural implications so that the antibiotic has poor penetration into the bacterium, and this can lead to the minimization of the intracellular concentrations of the antibiotic; post-translational modification of the bacterial gene target and some bacteria are able to inactivate the antibiotic by the use of hydrolysis or modification of the antibiotic. All of these can lead to resistance (Hoffman, 2001). Which includes chromosomal mutations (Hoffman, 2001). However, the most effective method is the use of gene transferal where an antibiotic-resistant gene from a neighbouring bacterium enters a bacterium through horizontal gene transfer (Li & Webster, 2018).

Research has found that development of drug resistance can be caused by several genetic mutations that are transferable between bacteria (Hoffman, 2001). These mutations are chromosomal mutations and result in disruption of gene function or such mutations can result in structural damage of the chromosome itself (Hoffman, 2001). Drug resistance that occurs as a result of a genetic mutation is known as intrinsic resistance (Hoffman, 2001). Besides this

common mechanism, bacteria have also developed other defences against antibiotics. Some of these defences include proteolytic processing in which bacteria secrete proteases that break down antibiotics (Bechinger & Gorr, 2017; Christaki *et al.*, 2020). Bacteria can modulate their surface membrane charge, and this can reduce the adhesion ability of drug molecules to the bacteria, preventing the molecule from destroying the bacteria.

One characteristic that is vital for bacteria to attain for drug resistance to develop is persistence (Banin *et al.*, 2017). Persistence is a non-genetic mechanism of developing drug resistance whereby a bacterial population tolerates the effects of an antibiotic. An example of a mechanism adding to persistence is biofilm formation (Banin *et al.*, 2017). The phenomenon of persistence can add valuable insight into how drug resistance develops and can be treated. Another aspect of persistence is that bacteria have also developed toxin-antitoxin systems and these systems are well known to aid in evolutionary processes of antibiotic resistance by maintaining multi-drug resistance plasmids and this is known to be important in biofilm formation (Banin *et al.*, 2017).

Antibiotics typically target one or more of five vital processes in bacteria. These processes include cell wall synthesis, protein synthesis, RNA polymerase, DNA gyrase, membrane structure and the folate mechanism in bacteria (Li & Webster, 2018). Bacteria can therefore develop drug resistance in any one of these processes through pathways such as genetic mutation, transformation, coupling and, transduction. Different mechanisms are then expressed within these pathways to ensure drug resistance occurs. Such mechanisms include the use of efflux pumps and antibiotic modification (Li & Webster, 2018). *S. aureus* has developed drug resistance towards a variety of antibiotics through this complex pathway. Indeed, *S aureus mecA* gene encodes for a protein that has a low affinity for binding penicillin. With the variety of mechanisms that bacteria have developed in resisting antibiotics, it has highlighted the need for identifying novel methods of controlling bacterial infections.

The development of novel antibiotics should consider the following two aspects to achieve effective results of novel antibiotics that are designed to target novel ideas of drug development (Giedraitienė *et al.*, 2011). One aspect of antibiotic-resistant bacteria that has not been greatly explored is that of the biofilm (Stewart, 2002). The biofilm is bacteria's natural protection against antibiotics and other substances that may want to enter the cell (Stewart, 2002; Tung *et al.*, 2017). The biofilm also assists the bacterium in developing antibiotic resistance (Stewart, 2002; Tung *et al.*, 2017). Indeed, it has been found that bacterial species containing efficient biofilm formation can outcompete those that do not (Rutherford & Bassler, 2012; Stewart,

2002). Another novel aspect of antibiotic development is quorum sensing (Tung *et al.*, 2017; Williams, 2017).

1.3.2. Biofilm Formation

As of recent, a very important and disturbing facet of bacterial drug resistance is that some bacteria are able to develop a biofilm (Hassan *et al.*, 2019; Tung *et al.*, 2017). Biofilm is bacteria's natural self-produced defence mechanism and is formed when bacteria behave cooperatively (Hassan *et al.*, 2019; Høiby, 2017). Another description of biofilm is stalkless growth *in vivo* (Høiby, 2017). Bacteria that can form a biofilm tend to have a faster development of antibiotic resistance (Hassan *et al.*, 2019; Li & Webster, 2018; Tung *et al.*, 2017). This is as a result of several reasons such as the biofilm is impenetrable for certain antibiotics and some bacterial cells living within the biofilm matrix adopt the distinct biofilm phenotype whereas previously they showed a more planktonic phenotype (Li & Webster, 2018; Worthington *et al.*, 2012). Biofilm formation has shown to be especially concerning in clinical settings as this is a mechanism by which patients can develop a resistant infection once in a hospital (Stewart, 2002; Worthington *et al.*, 2012). The process of biofilm formation is controlled by another process known as quorum sensing (Saeki *et al.*, 2020; Di Somma *et al.*, 2020a).

Biofilms are well described as surface-attached (in very limited cases, non-surface attached communities can also form biofilm) communities of bacteria (Baloyi *et al.*, 2019; Worthington *et al.*, 2012). The process of biofilm formation begins with the bacteria attaching themselves to a surface. This attachment signals to other cells to congregate on the surface (Remuzgo-Martínez *et al.*, 2015). Many different genes are involved in the attachment process (Remuzgo-Martínez *et al.*, 2015). This creates aggregation that leads to clumping which results in the bacteria producing and secreting polysaccharides that form an extracellular matrix - the biofilm layer (Baloyi *et al.*, 2019; Hassan *et al.*, 2019; Kirui *et al.*, 2019; Remuzgo-Martínez *et al.*, 2015). One particular study demonstrated that the use of nanotechnology has the potential to combat biofilms as it can remove bacteria from surfaces thus preventing formation of biofilm (Kirui *et al.*, 2019).

Bacteria undergo various phenotypic changes when biofilm formation is initiated and these phenotypic changes increase the chance of bacteria developing drug resistance (Worthington *et al.*, 2012). Such phenotypical changes include production of extracellular polymeric substances and significant upregulation of genes that encode for specialised efflux pumps which evict antibiotics from the bacterial cell (Worthington *et al.*, 2012). An additional phenotypic change is the rapid rate of genetic mutation. Research has identified that biofilm forming bacteria not only have a much higher expression fold of genes controlling biofilm formation but these genes

also mutate at an alarming rate compared to their planktonic counterparts (Worthington *et al.*, 2012). This creates difficulty in choosing effective treatment options because evolutionary survival pressures placed on the bacteria from antibiotics increases the mutation rate (Worthington *et al.*, 2012).

Research has identified that biofilm formation promotes the colonising ability of bacteria and this aids in development of persistent disease (Baloyi *et al.*, 2019). Infections that are biofilm related are often inconspicuous in nature and are well distributed throughout an infected patient (Ohrt-Nissen *et al.*, 2018). Patients who suffer from cystic fibrosis were among the first patients to discover housing biofilm forming bacteria in their lungs and it was determined that this finding significantly contributes to the persistent and chronic nature of the disease (Høiby, 2017; Rashmi *et al.*, 2018). Pathogens that are able to form biofilms have been documented for their resistance to numerous antimicrobial agents such as antibiotics (Baloyi *et al.*, 2019; Worthington *et al.*, 2012). A possible reason for such a high rate in development of drug resistance is because biofilm presenting pathogens enter a low metabolism state with decreased cell turnover and can therefore go undetected (Li & Webster, 2018; Ohrt-Nissen *et al.*, 2018). Previous research has suggested that the disruption or prevention of biofilm layer could attenuate the pathogenicity of bacteria. Therefore, biofilm has gained much interest as a novel drug target (Baloyi *et al.*, 2019; Ohrt-Nissen *et al.*, 2018).

Previously, research has suggested that the medium and the concentration of nutrients in the medium are vital for the formation of biofilm and can influence the quality of the biofilm produced (Remuzgo-Martínez *et al.*, 2015). These bacteria are able to essentially move to a more nutrient density location and this increases the strength of the biofilm and the bacterial proliferation (Nadell *et al.*, 2008). Indeed, research has found that the biofilm construct itself assists in the important functions of nutrient distribution and waste disposal of the cells living within it (Worthington *et al.*, 2012). From previous research, it has been found that planktonic bacteria are admirably adapted to uniform environments, whereas biofilm forming bacteria experience environments in a more gradient fashion thereby enabling them to physiologically adapt to undesirable environments (Hall & Mah, 2017). Although some studies have contradicted this idea by suggesting that certain bacteria in a hospital setting can proliferate and produce sufficient biofilm and consequentially survive a particularly hostile environment presented in a hospital (Espinal *et al.*, 2012). One study performed by Espinal et al. (2012) demonstrated how bacteria present in hospitals attach themselves to hospital equipment and are therefore able to survive in a more hostile environment. This finding presents the idea that although adequate nutrients are needed for bacterial proliferation, bacterial species are able to

adapt their biofilm in such a way to achieve persistent survival (Espinal *et al.*, 2012). This further enables the bacteria to develop strong antimicrobial drug resistance in harsher environments, and this poses a major clinical threat. The previously stated implies that bacteria are able to adapt to their environment and overcome a lack of important nutrients (Espinal *et al.*, 2012). These contradictions suggest disrupting biofilm formation or abolishing the biofilm effectively would result in bacteria being unable to survive harsh environments.

Biofilm presents itself as a barrier for known conventional antibiotics rendering them ineffective, hence biofilm can be a potential drug target (Kirui *et al.*, 2019; Ohrt-Nissen *et al.*, 2018). Research has shown that biofilms are present in 60%-80% of pathogenic bacteria (Chang *et al.*, 2019). A reason for such a high prevalence of biofilm forming bacteria is that bacteria that develop the biofilm phenotype, are able to outcompete planktonic bacteria from an evolutionary perspective. This could provide the clinical implication that a drug that targets biofilm may be used in conjunction with a known antibiotic (Bukhari & Aleanizy, 2019). Therefore biofilm could present itself as a potential drug target (Soojhawon *et al.*, 2017).

Seeing the importance of biofilms in drug development, researchers have sought options to treat bacterial biofilms (Di Somma *et al.*, 2020a). The first goal of eradicating biofilm is to prevent the adhesion process (Champalal *et al.*, 2018; Di Somma *et al.*, 2020a). Several ideas have been tested to determine how to effectively remove or disrupt adhesion (Bukhari & Aleanizy, 2019; Kirui *et al.*, 2019; Di Somma *et al.*, 2020b). Some of these include obstructing cellular receptors from recognising surfaces that they can adhere to (Di Somma *et al.*, 2020a). A recent study by Kirui and co-workers demonstrated the use of nanotechnology to remove the bacteria from surfaces, disrupting the biofilm and, therefore rendering the bacteria susceptible to drug treatment (Kirui *et al.*, 2019). Other research has also suggested that the disruption of biofilm causes bacteria to adapt to a more planktonic nature, thus allowing the bacteria to be more vulnerable to treatments (Bukhari & Aleanizy, 2019).

However, due to several of complications discovered during attempts to prevent adhesion, additional methods of removing or preventing biofilm formation were identified (Di Somma *et al.*, 2020a). One such strategy is the use of small molecules and synthetic peptides to mimic cell surface adhesins (Di Somma *et al.*, 2020a). Another approach is to utilise components of quorum sensing systems to prevent biofilm formation (Di Somma *et al.*, 2020a). This approach would include mimicking autoinducers secreted by the bacteria to prevent receptor activation and thus inhibit biofilm formation through the inhibition of quorum sensing (Di Somma *et al.*, 2020a). This approach is highly species-specific because each bacterial (or microorganism)

species produces and secretes its autoinducer (De Kievit & Iglewski, 2000; Di Somma *et al.*, 2020a).

1.4. Quorum Sensing

Quorum sensing has gained much interest in the past decade as it provides insights and reasons as to how bacteria and other microorganisms are able to launch a coordinated attack and overcome a host in a more efficient and collective manner (Banerjee & Ray, 2017; Deep *et al.*, 2011; Rocha-Estrada *et al.*, 2010). Quorum sensing is cell to cell communication used by bacteria and other microorganisms such as fungi (Ahmad *et al.*, 2015; Asfour, 2018; Castillo-Juárez *et al.*, 2015; Poli *et al.*, 2018). This communication allows the microorganisms to communicate significant information for their proliferation and virulence. This communication essentially enables microorganisms to behave in the same way as multicellular organisms (De Kievit & Iglewski, 2000; Rutherford & Bassler, 2012; Williams, 2017). Quorum sensing controls vital processes such as biofilm formation, development and management of virulence factors and provides the ability for microorganisms to coordinate or synchronise their behaviour (Castillo-Juárez *et al.*, 2015; Rutherford & Bassler, 2012; Singh & Bhatia, 2018). This activity is a major contributor to the bacteria becoming pathogenic (Banerjee & Ray, 2017; Rutherford & Bassler, 2012). As research was further developed regarding quorum sensing, it was revealed that bacteria have a distinct advantage in infecting a host as they are able to create a coordinated attack (Deep *et al.*, 2011; Martínez *et al.*, 2019). Literature has proven that this coordinated attack approach is the most effective way for pathogenic bacteria to infect a host (Martínez *et al.*, 2019).

The process of quorum sensing commences with a signal molecule, or peptide, released from the microorganism, and this signal molecule is detected in the cytoplasm by a receptor or regulatory protein. The autoinducer then binds to the receptor or regulatory protein, and the complex becomes activated to begin the transcription of genes that are regulated by quorum sensing (Banerjee & Ray, 2017). Previous research has identified that quorum sensing systems are crucial in the development of pathogenicity various microorganisms including bacterial species. Such a finding has highlighted the use of the quorum sensing system in drug development (Champalal *et al.*, 2018; Zhuang *et al.*, 2019). It has therefore been hypothesised, and clinically researched, that the inactivation of quorum sensing systems in a pathogenic bacteria could indeed achieve a decrease in pathogenicity and therefore infection (Zhuang *et al.*, 2019).

Quorum sensing is considered a sequential process that contains various components (Martínez *et al.*, 2019). Although these components can differ between species, the final result of the

production of autoinducers and a change in gene expression is achieved (Martínez *et al.*, 2019; Singh & Bhatia, 2018). The sequential process of quorum sensing begins with the production of autoinducers that are released into the cytoplasm and detected by receptors or transcriptional regulators (Zhu *et al.*, 2011). Once autoinducers are detected and bound to the receptor, a positive feedback loop is generated and quorum sensing commences and continues enabling the development of pathogenicity (Swem *et al.*, 2009; Zhu *et al.*, 2011). Quorum sensing is a cell density-dependent process (as illustrated through Figure 1.1), implying that a concentration of autoinducers must be reached for quorum sensing to be initiated (Zhu *et al.*, 2011).

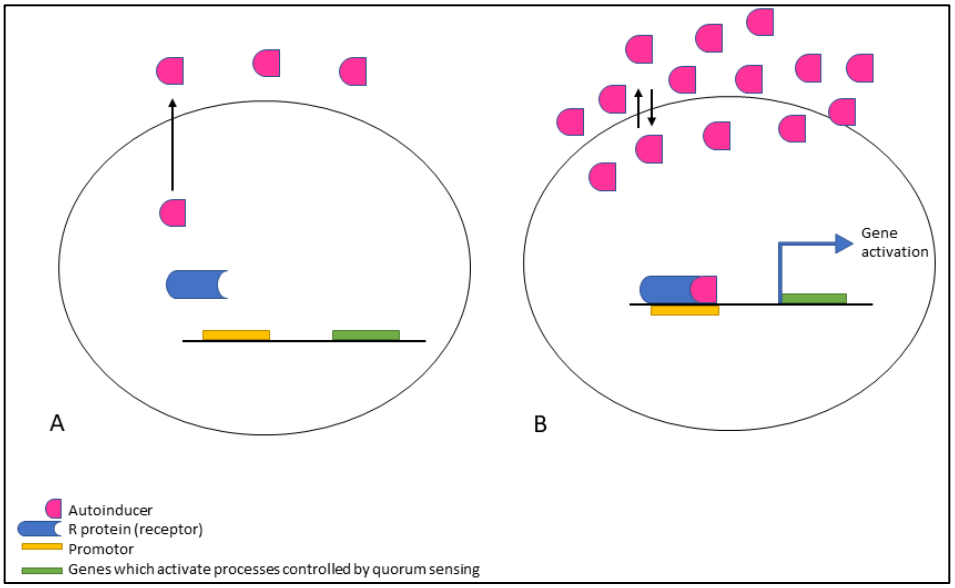


Figure 1.1: Quorum sensing mechanism – a cell density dependant action: Quorum sensing is a cell density dependent system. All bacteria produce autoinducers which accumulate in the extracellular matrix. If there is a low cell density, the autoinducer degrades and quorum sensing does not take place (A). However, if there is a high cell density, then many autoinducers will be present and accumulate in the extracellular matrix. The autoinducers are then able to bind to their respective receptors, or are detected by receptors, and this will cause a regulatory protein (R protein) to bind to the promoter on the DNA and activate transcription of genes. Image drawn using Microsoft PowerPoint and is published as such in Current Pharmaceutical Design (DOI: 10.2174/1381612826666201210105638).

It has been well documented in literature that quorum sensing is accountable for the development and regulation of virulence factors (Champalal *et al.*, 2018; Martínez *et al.*, 2019). Virulence factors aid bacteria in developing pathogenicity (Banerjee & Ray, 2017; Zhu *et al.*, 2011). Therefore, by targeting quorum sensing as a potential drug target, the pathogenicity of the bacteria may decrease. Consequently, the idea has been put forward that development of anti-virulence drugs, that also target the quorum sensing system, may be beneficial in the treatment of bacterial infections (Champalal *et al.*, 2018; Martínez *et al.*, 2019). Virulence factors have a conformational folding formation to be activated and this has been identified as

a potential drug target (Martínez *et al.*, 2019). Virulence factors are known to aid in secretion of toxins that consequentially establish the bacteria as pathogenic and host colonisation by evading the immune system response of the host (Martínez *et al.*, 2019). It has been suggested that by targeting virulence factors, biofilm formation will in turn also be disrupted and this can prove beneficial as then one potential drug could indeed have two different targets simultaneously (Martínez *et al.*, 2019).

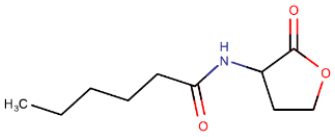
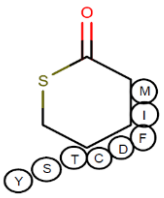
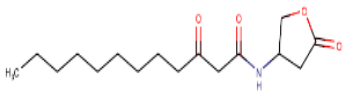
1.4.1. Autoinducers and Their Significance?

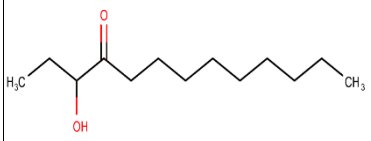
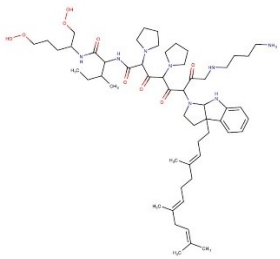
Signalling molecules, known as autoinducers activate quorum sensing (De Kievit & Iglewski, 2000; Poli *et al.*, 2018; Rutherford & Bassler, 2012). Autoinducers are able to cross the bacterial cell membrane and accumulate in the cytoplasm where they are detected by receptors (Poli *et al.*, 2018; Rutherford & Bassler, 2012). Autoinducers are key components of the architecture in quorum sensing systems (Martínez *et al.*, 2019; Singh & Bhatia, 2018). This component of the quorum sensing system can be targeted as a novel drug target due to the structures of the autoinducers being readily available and the structures therefore being able to be modified (Martínez *et al.*, 2019). The structure and occurrence of autoinducers is unique to each bacterial species because each bacterial cell produces autoinducers. This autoinducer is detectable by other bacterial cells within that same species (Banerjee & Ray, 2017; Kothari *et al.*, 2017; LaSarre & Federle, 2013). These molecules are secreted into the extracellular space and once a threshold has been reached, quorum sensing will be activated (Deep *et al.*, 2011; Rocha-Estrada *et al.*, 2010). Once the autoinducers' concentration surpasses a threshold, quorum sensing is activated and a positive feedback loop is created from the signal of autoinducers (Castillo-Juárez *et al.*, 2015; De Kievit & Iglewski, 2000; Rutherford & Bassler, 2012). Varying concentrations of autoinducers are known to activate different genes related to quorum sensing and this determines which collective behaviours are performed (Swem *et al.*, 2009). Autoinducer concentration is directly correlated to the population density of the bacterial population (Poli *et al.*, 2018).

Three different "classes" of autoinducers are used depending on bacterial species (Poli *et al.*, 2018). The first class consists of N-acyl-homoserine lactones (AHL). The majority of Gram-negative bacteria make use of these autoinducer types (Poli *et al.*, 2018). AHL are produced by Gram-negative bacteria and they are used to monitor the density of the colony to activate quorum sensing (Poli *et al.*, 2018). Oligopeptides are the second class of autoinducers and are habitually used by Gram-positive bacteria (Poli *et al.*, 2018). These oligopeptides are post-translationally altered to create functional peptides which are then able to determine the density of the population (Poli *et al.*, 2018). The final "class" consists of autoinducers that are universal

and can be utilised for interspecies communication. An example of such a molecule is known as Autoinducer 2 and this is known to be a very important autoinducer for cross-species communication (Poli *et al.*, 2018). Table 1.2 represents the structures of various autoinducers belonging to a variety of bacterial species, their structure and the unique receptor that they bind to.

Table 1.2: Bacterial species and their respective autoinducers

Bacterium Species	Gram Staining	Quorum sensing autoinducer	Autoinducer structure	Receptor	Reference
C. violaceum	Gram-negative	N-acyl-homoserine lactone (C10-HSL)		CviR	(Kothari, Sharma and Padia, 2017; Whiteley, Diggle and Greenber, 2017)
S. aureus	Gram-positive	Autoinducing peptide 1 (AIP1)**		AgrC	(Banerjee and Ray, 2017; Whiteley, Diggle and Greenber, 2017)
P. aeruginosa	Gram-negative	N-3-oxododecanoyl homoserine lactone		RhlR	(Banerjee & Ray, 2017; Williams

					et al., 2004)
V. cholerae	Gram-negative	Cholerae autoinducer-1 (CAI-1)		CqsS	(LaSarre & Federle, 2013; Ng *et al.*, 2010)
Bacillus subtilis	Gram-positive	ComX or CSF *	ComX*[1]: 	ComP-ComA	(Okada *et al.*, 2015; Verbeke *et al.*, 2017)
S. pneumoniae .	Gram-positive	CSP *	EMRLSKFFRDFILQRKK	ComD	(Banerjee & Ray, 2017; Verbeke *et al.*, 2017)

Classification of autoinducers produced by Gram-negative and Gram-positive bacterial species. HSL: Homoserine lactone. Agr: accessory gene regulator. ** Indicates that these autoinducers are linear peptide structures and the letters stand for each amino acid in the chain. *[1] amino acid chain: ADPITROWGD. *[2] amino acid chain: ERGMT. Both *[1] and *[2] are also linear signal peptides. * Indicates that this is also a linear signal peptide containing 17 amino acids and structure was not drawn. All structures drawn using MarvinStech tool, version 20.3 (https://chemaxon.com/products/marvin)

Gram-positive bacteria use autoinducing peptides to activate their quorum sensing (Rutherford & Bassler, 2012). These peptides contain hydrophobic motifs that have been found to be important for activity (Lyon & Muir, 2003). The signalling peptide is first produced in the nucleus as an oligonucleotide (Banerjee & Ray, 2017; Deep *et al.*, 2011; Rocha-Estrada *et al.*, 2010). The oligonucleotide is then post-translationally altered where it is cleaved so the functional signalling peptide is 10 to 20 amino acids in length (Banerjee & Ray, 2017). This

functional small peptide is transported out of the cell by an exporter protein (Banerjee & Ray, 2017). The peptides accumulate in the extracellular matrix and the concentration increases to reach a threshold (Banerjee & Ray, 2017).

Gram-negative bacteria produce AHL as their autoinducers (Champalal *et al.*, 2018). Once the extracellular concentration of autoinducers reach the threshold point, they enter the cell and activate transcription factors by binding to a cytoplasmic receptor (Banerjee & Ray, 2017; Rutherford & Bassler, 2012; Swem *et al.*, 2009). AHL have similar structures with oxidation state and chain length being the differing factors (Manner & Fallarero, 2018). The carbon chain is usually 4 to 14 carbons (Papenfort & Bassler, 2016; Sánchez-Sanz *et al.*, 2018). AHL in the different bacterial species have similar structures however they do differ in the oxidation state and carbon chain length (Manner & Fallarero, 2018; Sánchez-Sanz *et al.*, 2018). Often the autoinducers are synthesised from S-adenosylmethionine (Papenfort & Bassler, 2016). Some species of bacteria use analogues of AHL as their autoinducers and these are known as 3-oxo analogues (Papenfort & Bassler, 2016). Although analogues of AHL are used, all autoinducers in Gram-negative bacteria contain a lactone head group attached to an amide linker and a varying carbon chain is linked to the amide group. Research has shown that there are several types of autoinducers in Gram-negative bacteria (Sánchez-Sanz *et al.*, 2018). Some bacteria make use of analogues of AHL known as 3-oxo analogues. Research has shown that AHL, and analogues thereof, contain a lactone head group attached to an amide linker which is further attached to an alkyl chain varying in number of carbons (usually 4 to 14 carbons) (Papenfort & Bassler, 2016; Sánchez-Sanz *et al.*, 2018). The general structures of AHL are depicted in Figure 1.2.

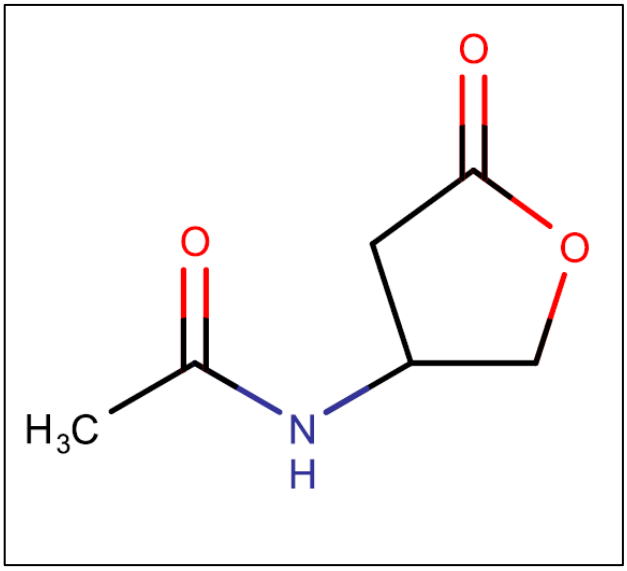


Figure 1.2: General structure of autoinducer N-acyl-homoserine lactone used by Gram-negative bacterial species.

The carbon chain of this autoinducer can vary in length. In this diagram, only 1 carbon is shown. The chain is usually between 4 and 14 carbons depending on the strain and species using the autoinducer. Single lines represent single bonds whereas two lines represent a double bond. C – carbon, N – nitrogen, H – hydrogen, O – oxygen.

1.4.2. Quorum Sensing in Gram-Positive Bacteria

The activation of quorum sensing in Gram-positive bacteria is dependent on density of autoinducers present in the extracellular matrix. Autoinducer concentration indicates whether the cell density is at the correct level for quorum sensing to initiate (Rocha-Estrada *et al.*, 2010). It is known that a high cell density is needed for quorum sensing pathways to be activated and this will result in the correct level of autoinducers present (Deep *et al.*, 2011). Therefore, it can be concluded that a high cell density is a requirement for quorum sensing (Banerjee & Ray, 2017; Deep *et al.*, 2011). This is illustrated in Figure 1.3.

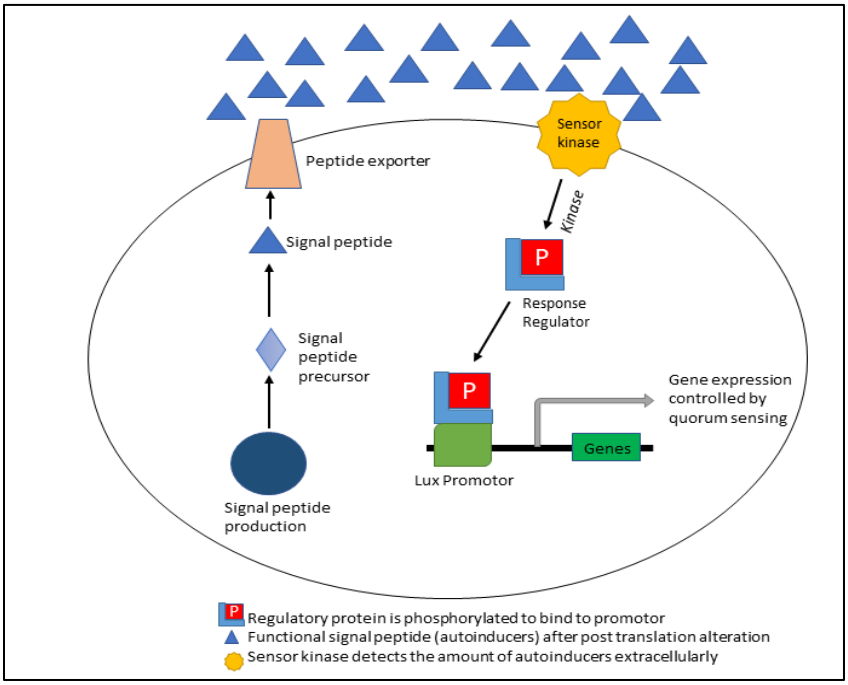


Figure 1.3: Quorum sensing in Gram-positive bacteria: Gram-positive bacteria generate oligopeptides which are then cleaved to form the signal peptide autoinducers. These functional peptides are transported out of the cell by the means of an exporter protein. They accumulate in the extracellular matrix where they are detected by a sensor kinase. The kinase phosphorylates (white P in red box) the regulator response protein which can then bind to the promoter so gene expression can occur. Image drawn using Power Point and is published as such in Current Pharmaceutical Design (DOI: 10.2174/138161282666201210105638).

Once the concentration has surpassed the threshold, autoinducers bind to a receptor on the cell membrane. The binding of the autoinducer consequently causes phosphorylation of a regulator protein that binds to the promotor region on the DNA and transcription of genes regulated by quorum sensing occurs (Banerjee & Ray, 2017). Previous research showed that once a high cell density is reached at a site of infection, proteins are produced from quorum sensing pathways. This is the favourable method of protein production over the secretion of surface proteins (Deep *et al.*, 2011).

It appears, from previous research, that the initiation pathway for Gram-positive bacteria contains two different components (Banerjee & Ray, 2017; Rocha-Estrada *et al.*, 2010). The first is the autoinducer which binds to its regulatory protein, as described above. The second is a receptor kinase which phosphorylates the regulatory protein and inevitably actives the protein (Banerjee & Ray, 2017; Rocha-Estrada *et al.*, 2010). The receptor kinase is a membrane-bound-two-component histidine kinase protein. Once an autoinducer has bound to the kinase, the phosphorylation of the regulatory protein occurs. The protein undergoes a conformational change where it bends to bind to DNA which then activates transcription factors (Banerjee & Ray, 2017). The transcription factors can belong to a variety of families, however, two of the

most common are the RNPP (Rap/NprR/PlcR/PrgX proteins that form a structural family) family or the Rgg (transcriptional regulators in low GC Gram-positive bacteria) family (Banerjee & Ray, 2017; Fleuchot *et al.*, 2011; Monnet & Gardan, 2015). The Rgg contains a pheromone-binding domain and the RNPP is considered a crucial element of control in many important processes including initiation of a pathogenic response and the formation of biofilm (Banerjee & Ray, 2017).

Bacteria are pathogenic because of the virulence factors produced and this is controlled by quorum sensing (Champalal *et al.*, 2018; Deep *et al.*, 2011). The production of virulence factors is controlled by two signalling molecules: AgrB and AgrD. The genes found for these molecules are located on the *agr* locus which contains the *agrABCD* genes (Deep *et al.*, 2011). RNAII controls the transcription of these genes (Deep *et al.*, 2011). During quorum sensing, the *AgrC* gene is transcribed and the AgrC transducer is formed. This transducer is then phosphorylated in an automatic response to the autoinducer peptide (Deep *et al.*, 2011). Following this phosphorylation, the *AgrA* gene encodes the response regulator protein which is then transcribed and translated. The response regulator protein is then phosphorylated. This phosphorylation of the AgrA regulator results in RNAIII upregulating the expression of the agr locus. This amplifies the autoinducer production and causes a positive feedback loop of quorum sensing (Deep *et al.*, 2011).

1.4.3. Quorum Sensing in Gram-Negative Bacteria

Quorum sensing is also cell density-dependent in Gram-negative bacteria and uses small molecules as autoinducers (De Kievit & Iglewski, 2000; Lyon & Muir, 2003; Rutherford & Bassler, 2012). These small molecules are mostly AHL and they activate transcription factors once bound to a cytoplasmic receptor (De Kievit & Iglewski, 2000; Rutherford & Bassler, 2012). Gram-negative bacteria make use of the lux operon with its functional R protein (LuxI/LuxR), or its homologues, as its quorum sensing system (De Kievit & Iglewski, 2000; Rasmussen & Givskov, 2006; Ravichandran *et al.*, 2018a; Rutherford & Bassler, 2012). The quorum sensing system of Gram-negative bacteria is clearly illustrated in Figure 1.4 where is it shown that quorum sensing in Gram-negative bacteria is also a cell density-dependent process. The autoinducing molecules have become targets for drug development as anti-quorum sensing drugs could disrupt these molecules (Ahmad *et al.*, 2015).

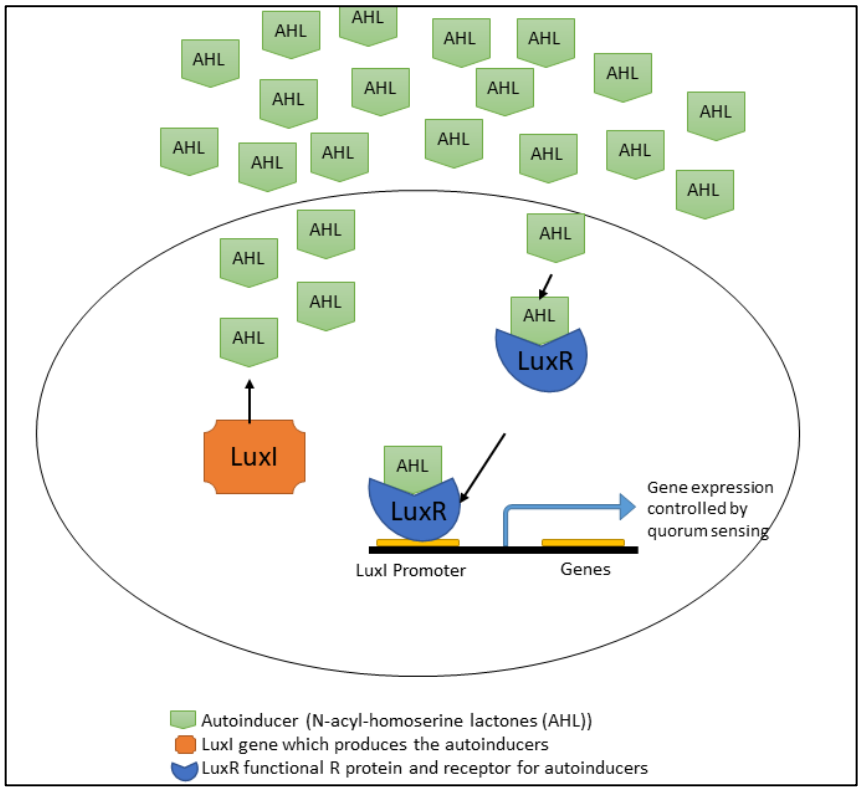


Figure 1.4: Quorum sensing in Gram-negative bacteria: The LuxI gene produces synthase enzymes which generate the autoinducers. Autoinducers are N-acyl-homoserine lactones (AHL). The AHL are able to exit the cell and accumulate extracellularly until a threshold is passed. Once the threshold is passed, the AHL bind to the LuxR receptor and this complex binds to the promoter region to activate transcription of genes regulated by quorum sensing.

It has been well documented that there are four basic aspects or components to having an effective quorum sensing pathway in Gram-negative bacteria (Papenfort & Bassler, 2016). The first component consists of autoinducers and analogues of autoinducers. The autoinducers are able to cross the cell membrane (Papenfort & Bassler, 2016). The second component only functions if the first is properly activated. The reason being is that the receptors (that are either located on the membrane or in the cytoplasm) of autoinducers make up the second component. The third component consists of genes that become activated from receptors binding to DNA. These genes control processes related to quorum sensing as well as the production of autoinducers to make a positive feedback loop and this is the fourth and final component (Papenfort & Bassler, 2016).

Literature has shown that quorum sensing systems that are dependent on AHL autoinducers for activation consist of two important protein families that are LuxI and LuxR families (Devescovi *et al.*, 2017). An enzyme known as an AHL synthase is typically what produces the autoinducer. This synthase belongs to the LuxI family of proteins (Devescovi *et al.*, 2017). The LuxR family proteins are responsible for sensing the autoinducer concentration and the density of the cells (Devescovi *et al.*, 2017). An AHL-binding regulator is vital for the activation of transcription

of target genes. This regulator is also part of the LuxR family (Devescovi *et al.*, 2017). This system is responsible for many communal behaviours such as the secretion of enzymes, the development of virulence, and biofilm formation (Devescovi *et al.*, 2017).

Receptors for Gram-negative bacteria are LuxR proteins, or homologues thereof (Manner & Fallarero, 2018). The receptors detect autoinducer signal and enter the cell. Due to quorum sensing being a cell density-dependent process, at a low concentration the autoinducers are degraded, however at a high cell density (this implies a high concentration of autoinducers) the autoinducer binds to the receptor to initiate quorum sensing pathways (Banerjee & Ray, 2017; Manner & Fallarero, 2018). The LuxR proteins contain two domains; an amino domain where the ligand binds and a carboxy domain where the receptor will bind to DNA (Papenfort & Bassler, 2016). A conformational change occurs in the receptor once the autoinducer binds to the correct binding site. The binding of autoinducer to receptor creates greater stability of the receptor (Papenfort & Bassler, 2016). The conformational changes result in the complex folding so it obtains the ability to bind to DNA and this results in the activation of genes (Swem *et al.*, 2009). Often these genes that are activated are responsible for the development and synchronisation of virulence factors. This enables the bacteria to become pathogenic (Banerjee & Ray, 2017; Manner & Fallarero, 2018). One other receptor type used by Gram-negative bacteria is similar to that of Gram-positive bacteria in that a histidine kinase receptor can be used (Papenfort & Bassler, 2016). This is also a two-component-membrane-bound histidine kinase which phosphorylates transcription factors to begin transcription of genes required for quorum sensing related pathways (Papenfort & Bassler, 2016).

1.4.4. Quorum Sensing as a Potential Drug Target

Using quorum sensing as a potential drug target could be beneficial as this prevents collective behaviours from taking place as the communication strategy used by bacteria will be inhibited. This can render bacteria non-pathogenic (Champalal *et al.*, 2018; Swem *et al.*, 2009). This is more beneficial as evolutionary survival pressures will not be placed on bacteria and drug resistance has a decreased risk of developing (Martínez *et al.*, 2019; Zhu *et al.*, 2011). Although quorum sensing has the potential to be such a beneficial therapeutic target, feasibility of the therapeutic option has proven to be challenging because many potential anti-quorum sensing inhibitors have been shown to be highly toxic *in vivo* (Martínez *et al.*, 2019; Zhu *et al.*, 2011). Many of the pathways form complicated interconnected networks and this provides the opportunity for a drug to attenuate one aspect of a separate pathway (such as virulence factor development) when it should rather inhibit quorum sensing (Champalal *et al.*, 2018; Martínez *et al.*, 2019). The idea to use quorum sensing as a potential drug target is to rather disrupt

communication between bacteria and not result in the death of the bacterial cells. However, some drugs that can target quorum sensing have the potential to kill the bacteria which can inevitably aid in the development of resistance (Martínez *et al.*, 2019). It is therefore vital for scientists to consider all aspects of drug design in the development of quorum sensing drug targets.

1.5. Quorum Sensing Inhibitors

The development of quorum sensing inhibitors has become increasingly popular in research (Anguige *et al.*, 2005; Rasmussen & Givskov, 2006). Quorum sensing inhibitors have been shown to not necessarily kill the bacteria but rather inhibit the communication mechanisms rendering collective activities and the development of virulence near impossible (Hossain *et al.*, 2017). This implies that the bacteria are less susceptible to developing drug resistance (Manner & Fallarero, 2018).

Research has shown that for a compound to be classified as an effective quorum sensing inhibitor, several criteria must be met (Kalia, 2013). These criteria include the compound must be a small molecule, the potential to disrupt gene expression must be present and a high level of specificity for a certain quorum sensing regulator or receptor must be evident. Ideally, the compound must contain minimal side effects (especially when disrupting gene function). The compound should be chemically stable and resistance to host degradation mechanisms is also considered important. The structure of the compound should mimic, or be similar to, that of the autoinducers, however, minor alterations are considered suitable (Kalia, 2013). Although many drugs have been developed and show anti-quorum sensing activity, no drug has yet been developed for consumption purposes as they have very high toxicity levels for humans and pharmacokinetic implications (Abbas *et al.*, 2017; Singh & Bhatia, 2018).

Quorum sensing inhibitors have been shown to have several mechanisms of action; they can degrade the autoinducer molecule enzymatically, inhibit synthesis of autoinducers, compete with autoinducers for binding sites on a receptor or inhibit binding of signal molecules to gene promotors (Asfour, 2018; Hossain *et al.*, 2017; Kalia *et al.*, 2019). These molecules have the ability to prevent bacteria from being or becoming pathogenic by disrupting the communication that is present with quorum sensing (Asfour, 2018; Hossain *et al.*, 2017; Ravichandran *et al.*, 2018a). This can prohibit bacteria from developing resistance as their growth is not inhibited but the ability to communicate is (Asfour, 2018; Kerekes *et al.*, in press; Sánchez-Sanz *et al.*, 2018). It has also been found that quorum sensing inhibitors can disrupt biofilm formation and interfere with the N-acyl homoserine lactones present in Gram-negative bacteria (Kerekes *et*

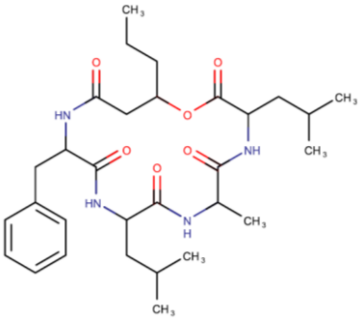
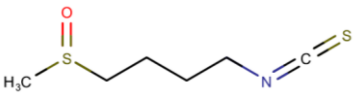
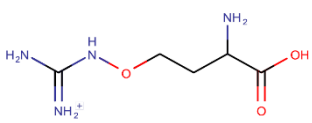
al., in press). Quorum sensing inhibitors can interact in competition for binding sites of receptors for autoinducers (Abbas *et al.*, 2017).

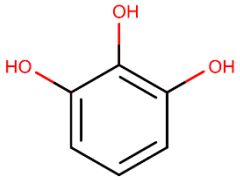
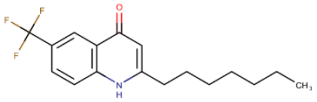
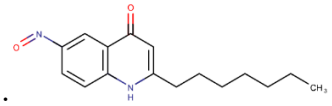
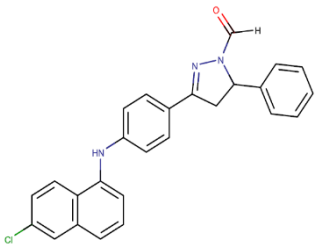
The approach of autoinducer production interference is based on inhibiting the activity of enzymes that are responsible for the autoinducer formation (Martínez *et al.*, 2019). This approach usually consists of creating analogues of the enzymes as the inhibitory molecules and these molecules are not bactericidal but rather inhibit quorum sensing (LaSarre & Federle, 2013; Martínez *et al.*, 2019). What has previously been shown is that these enzymes that create the autoinducers are not present in mammals and this implies that this method may be effective in preventing quorum sensing (LaSarre & Federle, 2013). However, there are certain drawbacks to this approach. One drawback in particular, is the fact that inhibitors have to travel intracellularly to the target site and overcome diffusion barriers of the cell (Martínez *et al.*, 2019). This must be kept in mind when new drugs are being developed.

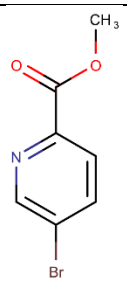
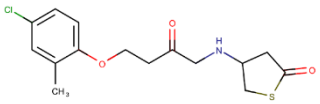
Some quorum sensing inhibitors have been designed as enzymes that target the autoinducer itself (LaSarre & Federle, 2013; Martínez *et al.*, 2019). The majority of these inhibitors target the AHL system and the enzymes include lactonases, acyl-homoserine lactone acetylases and acyl-homoserine lactone oxidoreductases (Martínez *et al.*, 2019). These enzymes destroy autoinducers by targeting the homoserine lactone ring and degrading the ring via hydrolysis of the ester bond (LaSarre & Federle, 2013; Martínez *et al.*, 2019). These enzymes are also able to modify the autoinducer instead of degrading them (Martínez *et al.*, 2019).

In an attempt to identify and distribute less toxic and more effective quorum sensing inhibitors, known drugs, which are FDA approved, are being screened for anti-quorum sensing properties (Abbas *et al.*, 2017). One such drug is metformin which is commonly used in diabetic patients. It was found that metformin can inhibit quorum sensing and virulence of some bacterial species (Abbas *et al.*, 2017). From this result, it is implied that this may be a beneficial strategy to find potential quorum sensing drugs. Other research indicates that use of home-grown natural materials may contain anti-quorum sensing properties and this can be beneficial as humans already consume these compounds, indicating toxicity levels may be adequate (Zhuang *et al.*, 2019). Other research has suggested that creating synthetic analogues of compounds or generating entirely new compounds, may be more beneficial in defeating quorum sensing (Singh & Bhatia, 2018). Table 1.3 represents a variety of potential anti-quorum sensing compounds that are from either a natural substance or are synthetic.

Table 1.3: Compounds with anti-quorum sensing property

Name of compound (*Class*)	**Origin**	**Source of Compound**	**Structure of Compound**	**Target**	**Reference**
Solonomide A (*Cyclodepsipeptide*)	Natural	Marine photo-bacterium		*S. aureus* virulence gene expression. Agr quorum sensing system.	(LaSarre & Federle, 2013; Mansson *et al.*, 2011)
Iberin (*Isothiocyanate*)	Natural	Horseradish		*P. aeruginosa* LasR, RhlR. The compound blocks expression of quorum sensing genes.	(Jakobsen *et al.*, 2012; Manner & Fallarero, 2018)
L-Canavanine (*Carboxylic acids*)	Natural	Alfalfa seeds		*C. volaceum* LuxR	(Keshavan *et al.*, 2005; LaSarre &

				Inhibits violacein production	Federle, 2013)
Pyrogallo (*Benzenetriol*)	Natural	Various fruits such as apricots		AI-2 analogue. Inhibits LuxN/LuxS quorum sesning system	(LaSarre & Federle, 2013; Ni *et al.*, 2008)
Compound 19 and 20 (*Alkylquinolone*)	Synthetic	Analogues of PQS	19:  20: 	*P. aeruginosa*. PqsR quorum sensing system. Reduce virulence factors	(LaSarre & Federle, 2013; Lu *et al.*, 2012)
Ia (*Alkylquinolone*)	Synthetic	Quinolone-derived		*P. aeruginosa*. PqsR quorum sensing system.	(Soukarieh *et al.*, 2018)

				The compound inhibits autoinducer production and reduces biofilm formation	
Compound 2a (*Pyridines, a carboxylic acid*)	Synthetic	Fusaric acid analogues		*E. coli.* LuxI-gfp system.	(Tung *et al.*, 2017)
4606-4237 (*Monocarboxylic acid*)	Synthetic	Synthesised analogues of the natural N-acyl homoserine lactone autoinducers		LuxN and LuxR type receptors, such as CviR in *C. violaceum*	(Swem *et al.*, 2009)

Structures of known quorum sensing inhibitors. Many of these compounds have been discovered for the use of bacterial infections. A variety of natural and synthetic compounds are shown in this table. An example of the respective bacterial species for which this compound can be used to treat, is shown in *italics* under the target column. The chemical group to which the compounds belong is shown in brackets and is *italicized* under the name of the compound. All structures were adapted from the respective references and drawn using MarvinSketch tool, version 20.3 (https://chemaxon.com/products/marvin). Table is published as such in Current Pharmaceutical Design (DOI: 10.2174/1381612826666201210105638).

A human pathogenic bacteria known as *Chromobacterium violaceum* has been shown to be promising with this treatment method of targeting quorum sensing and is widely used in research (Burt *et al.*, 2014; Kothari *et al.*, 2017; Rasmussen & Givskov, 2006).

1.5.1. Natural Quorum Sensing Inhibitors

Recently, researchers have been encouraged to consider and investigate natural sources as quorum sensing inhibitors (Baloyi *et al.*, 2019). Traditionally, natural compounds have been considered for their antibacterial activity, however, with the emergence of multidrug-resistant pathogens, the need for quorum sensing inhibitors has arisen to a large extent (Baloyi *et al.*, 2019). Research has proven that some plant extracts are able to interfere with the attachment process during biofilm formation as well as disrupt biofilm once it has already been established (Baloyi *et al.*, 2019). However, not all plant extracts are effective or considered to be beneficial quorum sensing inhibitors as some enhance bacterial proliferation instead of disrupting it (Baloyi *et al.*, 2019). This phenomenon could be explained by the number of nutrients that bacteria consider beneficial to their survival that is present in plant extracts (Baloyi *et al.*, 2019).

Natural compounds have been widely used to discover potential quorum sensing inhibitors (Manner & Fallarero, 2018). Many of these compounds are plant-derived such as phytochemicals, tannis, a compound found in garlic known as ajoene, horseradish produces a compound known as iberin which has been shown to have potential anti-quorum sensing activity (Manner & Fallarero, 2018). Often, these compounds are used by scientists to generate synthetic analogues of the compounds as some of these compounds have very high toxicity levels for humans (Burt *et al.*, 2014; Manner & Fallarero, 2018). Especially this is true for essential oils (Burt *et al.*, 2014).

Endophytes are the leading source for discovering natural compounds as potential drugs and are especially effective in targeting multidrug-resistant infections (Chatterjee *et al.*, 2020). However, many studies regarding endophytes have been done on plant pathogens and not many on human pathogens (Chatterjee *et al.*, 2020). One study conducted research using the plant *S. nigricans* for the phytochemicals that this plant can hold (Bodede *et al.*, 2018). It was found that this plant contains anti-pathogenic agents which reduce the concentration of virulence factors in bacteria (Bodede *et al.*, 2018). However, this study states that semi-synthetic compounds of these found phytochemicals would be more beneficial to humans as the toxicity levels may be lower (Bodede *et al.*, 2018).

It has been well documented in the literature that Chinese medicinal herbs have the potential to possess anti-quorum sensing properties (Wei *et al.*, 2020). Examples of such medicinal herbs

include the leaves of Folium, root bark from the *Cortex dictamni* and the root of *Solanum melongena* (Wei *et al.*, 2020). It was found that the effect of these medicinal herbs could be used to treat chronic and acute infections, however, if these infections contain a pathogen that is able to produce a biofilm then they are ineffective (Wei *et al.*, 2020). Results from studies have also shown Chinese medicinal herbs can inhibit transcription of the PQS quorum sensing system by specifically targeting the rhlRI regulator (Wei *et al.*, 2020). The medicinal herbs were then investigated chemically and it was found that acidic functional groups, such as acetic acid and propionic acid, are main components in the compounds (Wei *et al.*, 2020). This is beneficial to note as compounds can be screened for further research to identify if these functional groups exist in the compounds as this could indicate potential anti-quorum sensing activity.

Some researchers studied compounds that should be in foodstuffs that humans eat (Zhuang *et al.*, 2019). One such study looked at extracts of dried fruit (specifically from *Forsythia suspense*) as these compounds are also considered traditional Chinese medicine (Zhuang *et al.*, 2019). Previously, it was determined that this compound indeed has antibacterial activity and it has now been shown to inhibit quorum sensing (Zhuang *et al.*, 2019). It should therefore be considered to be developed into a pharmaceutical drug.

Recent research has shown that certain natural inhibitors are able to interact with the CviR transcription factor and therefore disrupt quorum sensing (Ravichandran *et al.*, 2018a). Some natural products, such as essential oils, have been shown to be effective quorum sensing inhibiting molecules (Burt *et al.*, 2014). However, these compounds have not been deemed fit for human consumption as they have high toxicity levels (Burt *et al.*, 2014).

1.5.2. Synthetic Quorum Sensing Inhibitors

Synthetic compounds have become increasingly popular as they are synthesised to be structural homologues of autoinducers or other quorum sensing proteins (Singh & Bhatia, 2018). Libraries of compounds that have different chemical classes are screened and *in silico* or synthetic methodology is used to identify potential compounds as quorum sensing inhibitors (Singh & Bhatia, 2018). These are known as leads. From the leads that are generated, the compounds are then tested in vitro to determine effectiveness of the compounds (Singh & Bhatia, 2018). Based on those results, some compounds are concluded to be ineffective or toxic and others can undergo further testing (Singh & Bhatia, 2018).

Synthetic compounds are generated using computer-aided drug design (CADD) (Zhuang *et al.*, 2019). These compounds are specifically designed to have a similar structure to the

autoinducers. If the compounds are similar in structure to the natural autoinducers, then they are able to compete for the binding site of the receptor (Zhuang *et al.*, 2019). CADD provides opportunity to test the probability of the compounds' effectiveness before *in vitro* or *in vivo* tests are conducted (Zhuang *et al.*, 2019). This is beneficial in drug development and discovery as it can save important cost resources as well as time.

Due to the level of toxicity of natural compounds, the need to develop synthetic compounds has arisen (Bodede *et al.*, 2018; Soojhawon *et al.*, 2017). These compounds are designed to be nonbiocidal and this is considered very beneficial for the generation of quorum sensing inhibitors (Bodede *et al.*, 2018). Often these compounds are generated in a library and are derivatives of furanones which are often halogenated (Chang *et al.*, 2019). These compounds have been shown to be effective in disrupting biofilm formation or disrupt biofilm once it has already been formed (Chang *et al.*, 2019). Some researchers suggest that compounds that have a furanone core structure seem to act more potently than those that have been generated without one (Chang *et al.*, 2019). Several different analogues have been generated of particular enzymes that are responsible for the generation of the autoinducers and may be effective inhibitors (Gutierrez *et al.*, 2009). One particular study generated transition states of one particular nucleosidase enzyme and it was found that the analogues could indeed inhibit quorum sensing (Gutierrez *et al.*, 2009).

One benefit of synthesising compounds, or altering known compounds to create analogues, is the ability to alter components of the compounds (Hossain *et al.*, 2017). Such components include the length of the acyl chain of the homoserine lactone autoinducer family and the addition of functional groups to the acyl chain (Hossain *et al.*, 2017). Literature has shown that substituting the 3-oxo group of an autoinducer with a methylene or hydroxyl group reduces the activity of the autoinducer (Hossain *et al.*, 2017). Another beneficial change that can be made is to add a phenyl group to the acyl terminus end as this encourages antagonist activity of the compound or it can lead to ineffective binding of the autoinducer to the receptor (Hossain *et al.*, 2017). These additions or changes are beneficial to consider for when the process of creating antagonists or inhibitors is in the beginning stages.

1.5.3. Imidazole Based Compounds

Imidazole molecules have been vital in the establishment of heterocyclic-based compounds and molecules (Slassi *et al.*, 2019; Zhang *et al.*, 2014). This important chemical class is more often than not the core component of a variety of chemical systems (Slassi *et al.*, 2019). A common feature of these compounds is the five-membered aromatic heterocyclic ring (Gaba & Mohan, 2016). This key characteristic has gained much interest for researchers to design and develop

imidazole-based drugs, or derivatives of compounds that could act as potential drugs (Slassi *et al.*, 2019; Zhang *et al.*, 2014). As of recent, many novel compounds that are imidazole based have been synthesised with proposed antifungal or antibacterial activity and this is very promising for the medical field in the area of drug development (Heerding *et al.*, 2001; Slassi *et al.*, 2019). Imidazole-based molecules have been shown to be significant in many bioactivities such as in the formation of proteins as imidazole is a vital component of histidine (Beheshti *et al.*, 2017; Zhang *et al.*, 2014).

One important structure that has been used in compound development is the imidazole ring (Beheshti *et al.*, 2017). The imidazole ring is in the manufacturing of novel drugs as the ring creates an electron-rich environment that expands the diversity of the drug to bind to many novel drug targets by forming numerous coordination bonds (Gaba & Mohan, 2016; De Vita *et al.*, 2017). Often, the imidazole ring is used as a skeleton molecule in drug design as this scaffold provides the desired structure for molecules to have a greater therapeutic potential (Gaba & Mohan, 2016). It is for this reason that many novel antifungals, antivirals and antihistamine drugs have been specifically designed with an imidazole backbone in the hope that these drugs will be more beneficial (Gaba & Mohan, 2016).

The imidazole ring contains two nitrogen atoms with one hydrogen atom bound to one of the nitrogen atoms and this provides the unique feature of this compound to behave in an amphoteric manner (Gaba & Mohan, 2016). The ability to form weak van der Waals forces is also considered an exceptional ability of compounds that are imidazole-based (Gaba & Mohan, 2016). What is mentioned above indicates that imidazole-based compounds are thus able to bind to a variety of therapeutic targets with many pharmacological activities and this is exceptionally beneficial in drug design and medicinal chemistry (Gaba & Mohan, 2016).

1.6. *Chromobacterium violaceum*

Chromobacterium violaceum (*C. violaceum*) is a large Gram-negative bacillus bacteria (Durán *et al.*, 2016; Kumar, 2012). *C. violaceum* has motility as it has either one or two large flagellum as well as lateral flagella (Kumar, 2012; Meher-Homji *et al.*, 2017). The bacterium survives and thrives in tropical areas where the water and soil are warm and rich in nutrients (McClean *et al.*, 1997; Meher-Homji *et al.*, 2017). *C. violaceum* is considered an opportunistic pathogenic bacteria to humans as it can accumulate in lesions of the skin and can result in lethal infections in other organs (Ghosh *et al.*, 2014; Kumar, 2012). Although this bacterium is considered pathogenic to humans, the number of cases is exceptionally small with only 200 recorded over a large variety of vast areas such as South-East Asia, India, the south-eastern regions of the

United States of America and Northern Australia (Durán *et al.*, 2016; Meher-Homji *et al.*, 2017). The low case number can be due to a variety of reasons such as poor diagnostic facilities (Ghosh *et al.*, 2014). Despite low prevalence rate of this pathogen, it is well known for having an exceptionally high mortality rate of 80% (Ghosh *et al.*, 2014). The most plausible explanation for such a high mortality rate is that *C. violaceum* is known to have developed drug resistance towards a variety of conventional antibiotics. This complicates treatment options in a significant way (Ghosh *et al.*, 2014).

The method of contracting this bacterium is usually through a lesion on the skin that is in contact with soil or water that houses the bacterium (Meher-Homji *et al.*, 2017). Although the number of cases is scarce, the bacterial infection is known to be fairly severe and often a spectrum of other infections accompanies a *C. violaceum* infection adding to illness (Meher-Homji *et al.*, 2017). The infection from this bacterium has been known to cause septicaemia, urinary tract infections, wound infection and the swelling of the lungs or liver (Durán *et al.*, 2016; Hossain *et al.*, 2017).

The diagnosis of this infectious bacterial disease is done by two sets of blood cultures from the proposed infected patient. The smears are incubated and flagged for Gram-negative motile bacterium (Meher-Homji *et al.*, 2017). *C. violaceum* can be identified as oxidase-positive and forms beta-haemolytic colonies. The main identifying factor of these colonies is that they are deep purple in colour which displays the classifying characteristic of the bacterium (Meher-Homji *et al.*, 2017). Treatment for the bacteria is conventional antibiotics such as metronidazole in combination with others such as ceftriaxone (Meher-Homji *et al.*, 2017). However, the main antibiotic used is metronidazole, an imidazole-based antibiotic (Meher-Homji *et al.*, 2017). Other treatments can be used in conjunction with antibiotics depending on the level of severity and if there are any other complications. These include, use of ventilation equipment if breathing proves difficult as well as coagulation medication if there is any blood disorder present in the infected patient (Meher-Homji *et al.*, 2017).

C. violaceum is well known for the production of a natural purple pigment known as violacein which is a secondary bioactive metabolite (Kalia *et al.*, 2019; Kothari *et al.*, 2017; Kumar, 2012). Violacein is hydrophobic in nature and is of great biological and laboratory importance (Ballestriero *et al.*, 2014; Choi *et al.*, 2020). Violacein is known to possess many antimicrobial properties. Examples of this antimicrobial activity is that violacein can specifically behave as an antibacterial compound against Gram-positive bacteria, target parasites and it is even known to aid in cancer treatment in humans (Ballestriero *et al.*, 2014; Choi *et al.*, 2020). A variety of genes from the *vio* operon are activated during quorum sensing of *C. violaceum* to enable the

expression and production of violacein (Durán *et al.*, 2016). Research has found that the production of this pigment is regulated by quorum sensing (Champalal *et al.*, 2018; Kothari *et al.*, 2017). It is for this reason that *C. violaceum* has been widely used in research specifically relating to quorum sensing (Durán *et al.*, 2016; Hossain *et al.*, 2017). Indeed, for many researchers, this bacterium is used as a control organism to test a variety of compounds such as amino acids or small molecules for their potential at inhibiting quorum sensing (Champalal *et al.*, 2018; Durán *et al.*, 2016; Stauff & Bassler, 2011a).

1.6.1. Quorum Sensing System Used by *Chromobacterium violaceum*

Quorum sensing is known to have gained much attention recently and *C. violaceum* is more often than not, a preferred organism to test the inhibition of quorum sensing because the violacein pigment that is produced provides a visual representation of active quorum sensing (Ghosh *et al.*, 2014; Kerekes *et al.*, in press; Zhu *et al.*, 2011). It has been well established that *C. violaceum* is a Gram-negative bacterium and therefore must make use of a quorum sensing system which makes use of LUXI/R signalling cascade and uses AHL for their autoinducers (Champalal *et al.*, 2018; McClean *et al.*, 1997; Poli *et al.*, 2018). *C. violaceum* uses LuxI/LuxR homologues CviI/CviR as its quorum sensing system (Ravichandran *et al.*, 2018a; Stauff & Bassler, 2011a). This system is also responsible for violacein production (Evans *et al.*, 2018). LuxI-type protein, known as CviI in *C. violaceum*, is responsible for production of autoinducers. The LuxR homologue, *CviR*, is the gene that codes CviR receptor protein (Hossain *et al.*, 2017). This receptor is a transcription factor that, once bound to AHL, activates genes that are linked to important behaviours (Evans *et al.*, 2018; Stauff & Bassler, 2011a). This type of signalling cascade that makes use of LuxI/R proteins is crucial in the virulence of the bacterium (Hossain *et al.*, 2017; McClean *et al.*, 1997).

A vital aspect of *C. violaceum's* quorum sensing system is its ability to form a biofilm (Kamaeva *et al.*, 2014). This biofilm is a polymer matrix that clusters bacterial groups. Biofilm in *C. violaceum* is also well known for its role in virulence and provides an exceptional platform for the bacteria to develop drug resistance towards a variety of antibiotics (Kamaeva *et al.*, 2014). The hmsHFR-CV2940 operon produces a monomer of N-acetyl-D-glucosamine that initiates the polymerisation of polysaccharides (Kamaeva *et al.*, 2014).

As with all bacterial species, different strains can exist. The same holds with *C. violaceum*. The different strains tend to produce and secrete different autoinducers even though they all use CviI/CviR quorum sensing pathway or system and all autoinducers are still AHL with the differing factor being the carbon chain length (Devescovi *et al.*, 2017; Zhuang *et al.*, 2019). One strain, ATCC12472, is greatly used in research and this strain responds to and produces an

AHL known as N-decanoyl-L-homoserine lactone or C10-HSL (Devescovi *et al.*, 2017). It has been documented that this system of this strain is important for the development of virulence of the pathogen to be sustained (Devescovi *et al.*, 2017). In another study, it was found that strain ATCC31532 makes use of N-hexanoyl-L-homoserine lactone or C6-HSL (Devescovi *et al.*, 2017). The differences are displayed in Figure 1.5.

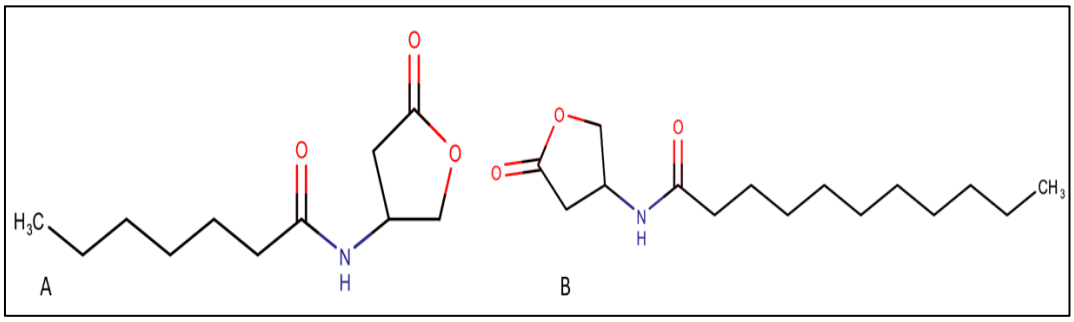


Figure 1.5: Two autoinducers used by *C. violceum* strains; C6-HSL and C10-HSL:
A: the autoinducer known as C6-HSL or N-hexanoyl-L-homoserine lactone. This autoinducer is used by the strain ATCC31532. This autoinducer has 6 carbon atoms as its carbon chain.
B: Autoinducer C10-HSL or N-decanoyl-L-homoserine lactone. This autoinducer is used by the strain ATCC12472. This autoinducer has a carbon chain length of 10 carbon atoms.
Single lines represent single bonds whereas two lines represent a double bond. Where no letters are present, assume carbon bonds. S – sulphur, C – carbon, N – nitrogen, H – hydrogen, O – oxygen. Both structures were drawn using MarvinSketch.

Quorum sensing pathway components in *C. violaceum* are regulated by *vio*A promoter (Devescovi *et al.*, 2017; Ravichandran *et al.*, 2018a). The process of violacein production is clearly illustrated in Figure 1.6. This promoter is part of a family of genes known as the *vioABCDE* genes. This specific promoter encodes for the production of violacein (Devescovi *et al.*, 2017; Ravichandran *et al.*, 2018a). Other genes in the family encode for the production of virulence factors and regulators needed for the uptake of autoinducers (Devescovi *et al.*, 2017). Previous studies have shown that the *vioA* promoter is responsible for positive regulation of the *CviR* gene (Devescovi *et al.*, 2017; Stauff & Bassler, 2011b). A recent study has shown that the promoter of the *vioABCDE* operon can be negatively regulated via a novel receptor known as VioS present in other microorganisms (Devescovi *et al.*, 2017). This repressor has also been shown to be important in the regulation of other quorum sensing related activities such as protease activity (Devescovi *et al.*, 2017). VioS has also been found in *C. violaceum* however, it has been found to rather positively regulate the *vioA* promoter in this bacterial species (Devescovi *et al.*, 2017). Although positive regulation of certain aspects can occur with the VioS repressor, it has been found that this repressor can also negatively regulate violacein production but it does so without influencing the overall quorum sensing system (Devescovi *et al.*, 2017).

Other research has found an efflux pump that has been shown to have a major role in antibiotic resistance of the *C. violaceum* (Evans *et al.*, 2018). This pump is known as the CdeAB-OprM pump and it is regulated by quorum sensing (Evans *et al.*, 2018). This pump seems to be structurally similar to that of RND pumps (Evans *et al.*, 2018). Mutations that occur in this pump are most common in the N-terminal DNA-binding domain and can influence the quorum sensing ability of the bacterium. Such influence consequently has an impact on development of drug resistance of the bacterium (Evans *et al.*, 2018).

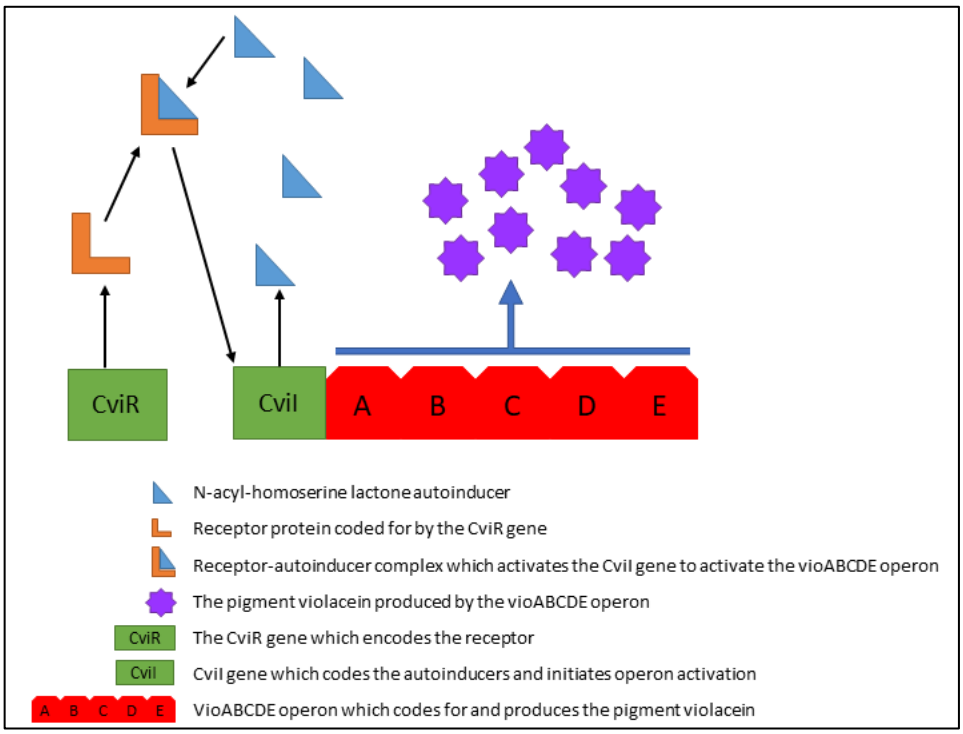


Figure 1.6: The production of the pigment violacein produced by *C. violaceum* using the *vio*ABCDE promoter: The *CviI* gene codes for the autoinducers of the bacterial species which is an acyl-homoserine lactone. The *CviR* gene codes for the receptor to which the autoinducer binds to. Once the autoinducer-receptor complex forms, this can result in the *CviI* gene to activate the *vioABDCE* operon. Within this operon, is the *vioA* promoter which enables the production of the pigment violacein, indicating that quorum sensing is indeed taking place. Image drawn using Microsoft Powerpoint.

As previously shown, the quorum sensing system of *C. violaceum* is comprised of a variety of facets that can be individually targeted to inhibit quorum sensing (Champalal *et al.*, 2018). Such facets include decreasing gene expression of genes that control quorum sensing, receptor production inhibition and competition of natural autoinducers (Champalal *et al.*, 2018). Previous studies have shown that quorum sensing inhibiting molecules used against *C. violaceum* have protected other organisms such as nematodes from infection (Ghosh *et al.*, 2014). Therefore, this research can be applied to prevent the development of drug resistance in a variety of pathogens.

From the previous literature regarding the development of drug resistance in bacteria, quorum sensing, and the link between the two, it is clear that this unprecedented problem needs to be addressed. Targeting bacterial resistance or bacterial infections by using novel drug targets such as biofilm and quorum sensing has been shown to be beneficial in reducing the risk of bacteria from developing drug resistance and targeting quorum sensing should reduce the pathogenicity in the organism. This literature search has also shown that the most effective anti-quorum sensing molecules tend to mimic the structure of autoinducers or are able to bind to the target site, and molecules that are imidazole-based are of vital importance in drug discovery. Taking all this information into consideration, this study was formed and conducted.

1.7. Problem Statement, Aim and Objectives

1.7.1. Problem Statement

The control of infectious diseases is still a pressing problem. With antibiotic resistant bacteria becoming more common and more problematic, novel ideas for drug discovery regarding antibiotics are crucial. Quorum sensing has become increasingly popular in this area of drug development. It has been previously suggested that the disruption or prevention of the biofilm layer could attenuate the pathogenicity of bacteria. Therefore, biofilms have also gained much interest as a novel drug target. Development of anti-quorum sensing compounds, which can also target biofilm formation, could be effective in controlling drug resistance in bacteria. These compounds can inhibit the ability of the bacteria to gain resistance by not posing a threat to their survival. Therefore, such compounds need to be developed and tested for their anti-quorum sensing and anti-biofilm activities, which will then be able to act as novel antibiotics.

1.7.2. Aim

The aim of this study was to determine or analyse *in silico* and *in vitro* inhibition of quorum sensing and biofilm formation in *Chromobacterium violaceum* using semi-synthetic, imidazole-based compounds.

1.7.3. Objectives

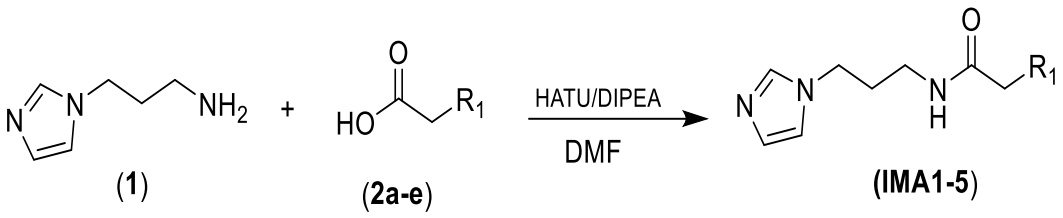
a. To use *in silico* docking and molecular dynamic simulations to screen five novel synthetic imidazole compounds and identify the most effective compounds with potential anti-quorum sensing activity.
b. To determine antibacterial activity of these imidazole compounds against *Chromobacterium violaceum.*
c. To determine anti-quorum sensing activity of these compounds using different *in vitro* assays.
d. To study the effect of these compounds on biofilm formation in *C. violaceum*.

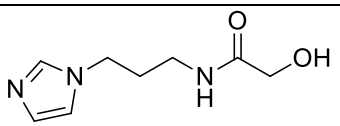
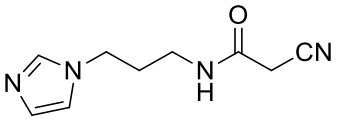
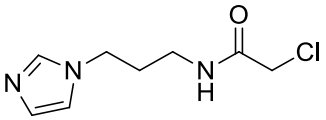
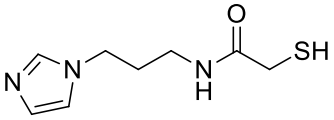
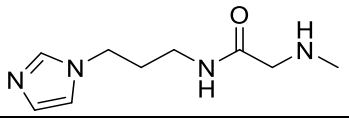
e. To study the effect of the most active compounds on expression of genes involved in quorum sensing.

2.1 Chemistry

2.1.1 Synthetic Compounds

Five newly synthesised imidazole compounds (IMA-1 – IMA-5) were synthesised by our collaborator, Dr. Mohmmad Y Wani, in the Department of Chemistry, University of Jeddah, Kingdom of Saudi Arabia. These compounds were synthesised by a coupling reaction between the amino group of the imidazole 1 (3-(1H-imidazol-1-yl)propan-1-amine) (1 mmol) and different carboxylic acid derivatives (2a-e) in equimolar ratios, using coupling agents HATU (1.5 mmol) and DIPEA (2.5 mmol) in DMF (see **Scheme 1**).

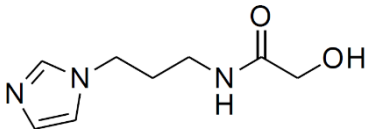
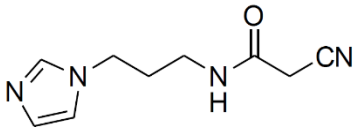
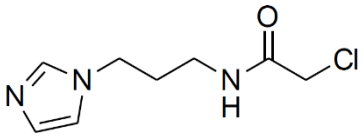
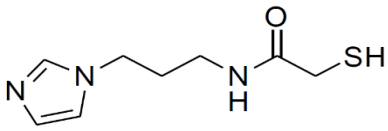
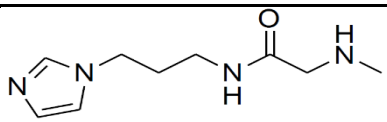


Compound	(2a-e) R_1	Structure
IMA-1	OH	
IMA-2	CN	
IMA-3	Cl	
IMA-4	SH	
IMA-5	$NH(CH_3)$	

Scheme 1. Synthesis of target imidazoleacetamide derivatives (IMA-1 – IMA-5).

The structures and names of these compounds are provided in Table 2.1. Stock solutions of 1:10 and 1:20 dilutions were made for these compounds using dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany). These stocks were kept in the fridge at 4 ºC and thawed when needed.

Table 2.1: Structures and IPUAC name of the 5 imidazole-based compounds used in this study.

Compound	Structure	Compound Name
IMA-1		2-hydroxy-N-[3-(1H-imidazol-1-yl) propyl] acetamide
IMA-2		N-(3-(1H-imidazol-1-yl) propyl)-2 -cyanoacetamide
IMA-3		2-chloro-N-[3-(1H-imidazol-1-yl) propyl] acetamide
IMA-4		N-[3-(1H-imidazol-1-yl) propyl]-2-sulfanylacetamide
IMA-5		2-amino-N-[3-(1H-imidazol-1-yl) propyl] acetamide

2.1.2 Ethics Clearance

In this study, existing stock culture of *C. violaceum* stored in the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand, was used. To use this isolate, ethics clearance (Ref.: W-CBP-200320-4) was obtained from the Human Research Ethics committee of University of the Witwatersrand, Johannesburg, South Africa, and performed according to the guidelines outlined in the Helsinki Declaration (**Appendix 7.1**).

2.2 *In silico* Studies

The idea of computer-based drug design (CADD) has become increasingly popular largely due to advancements in technology and the drastic increase in availability of molecular structures such as proteins. This has provided researchers with a unique opportunity to test multiple compounds to multiple targets with enhanced speed and expertise. CADD has also decreased time and costs spent on developing novel drugs.

The principle of Molecular Mechanics allows for reactivity, solubility, flexibility and stability of compounds and their complexes to be observed. This provides invaluable information regarding bonds formed between compounds and the target protein and offers the opportunity for the compound to be compared to the natural ligand from a binding energy perspective. In this study, molecular modelling was combined with molecular dynamic simulations to determine how compounds interact with the target protein (CviR in *C. violaceum*) in a variety of scenarios as well as competence of the test compounds at binding to the target protein. These methods provided a first impression as to how effective these compounds will be at inhibiting quorum sensing and biofilm formation in an *in vitro* setting.

2.2.1 *In silico* Molecular Docking

In silico docking is a method widely used in drug design and development. Molecular docking forms part of the molecular mechanics principle. This method has a conservative approach to predict optimal geometric conformation and binding energy of a compound to a protein in its respective binding site. In this study, noncovalent docking was used.

The three-dimensional structure of CviR protein was retrieved from the Protein Bank Database under the accession code 3QP4 (https://www.rcsb.org/structure/3QP4). The structure was modified by removing all water molecules and formatted correctly using Protein Preparation Wizard encoded in Schrödinger suite ("Protein Preparation Wizard | Schrödinger", s.a.). Protein preparation included adding in missing hydrogen or side chain atoms and a charge was added to the whole protein structure consistent to neutral pH taking into consideration the ionization states for each amino acid residue. The prepared protein was then subjected to energy minimization using OPLS-2005 force-field with an RMSD cut-off of 0.30 Å (Harder *et al.*, 2016).

Three-dimensional structures of the five newly synthesised compounds were sketched using MarvinSketch tool (Khan *et al.*, 2019). These compounds were then prepared using the LigPrep module implemented in the Schrödinger package ("LigPrep | Schrödinger", s.a.). Compound preparation included the addition of hydrogens, adjusting bond lengths and angles, correcting chirality and ionization states, adjusting tautomers, stereo chemistries and ring conformations. Partial charges were assigned to the compound structures using the OPLS-2005 force field. The compounds were also subjected to energy minimization until an average RMSD of 0.001 Å was reached. Epik ionization tool was used to set a neutral pH for the ionization state.

Molecular docking was performed using Glide docking tool of Maestro 11.6 with the default parameters on five novel compounds for this study. This method was performed by docking the compounds in the antagonist sites of the quorum sensing protein, CviR, and this prevented the autoinducers from binding to the target protein and thus quorum sensing was disrupted. Receptor grid was generated and the parameters for the gird box centre and size were X = 20.16, Y =16.24 Z = -8.36, respectively with a smaller binding box of dimensions with 23 x 23 x 23 Å. The grid box was centred on the central atoms of the known autoinducer inside the complex. The compounds were docked using the "Extra-Precision" mode (XP) protocol. Finally, all five compounds were analysed based on the docking and XP-G scores and successively imperilled to molecular dynamics simulation studies. Ideally, the compounds should all have negative binding affinities as the lower the binding affinity, the more effective the compound will be (Khan *et al.*, 2019). The complexes generated from molecular docking were then saved to be used in molecular dynamic simulations. The binding energies and bond interactions between the compound and CviR protein were noted.

2.2.2 Molecular Dynamic Simulations

Molecular dynamic simulations have been shown to be extremely beneficial in molecular biology as it classifies the dynamics of the atoms within compounds. This is important to determine how the compounds interact with the protein in a biological system by defining their flexibility, solubility, and stability within a complex. The more stable the carbon backbone of a compound is, the greater chance of success in an *in vitro* setting.

Molecular dynamic simulations enable analysis of how particles interact throughout an entire system in 100 ns. The analysis is done by the generation of a dynamical trajectory. This method is especially important in understanding the energies and structural dynamics of a biological system, such as a receptor interacting with a compound or protein. There are four main conditions of atoms in a biomolecular system which need to be taken into consideration: coordinates and acceleration of atoms as a group and individually. How the atoms interact with each other creates the trajectory and this is important to determine how compounds interact with the desired protein. The method for this study was adapted from Lone, Khan and Ahmad, 2019.

The compounds were exposed to Molecular Dynamic (MD) simulation using Graphics Processing Unit (GPU) version of PMEMD package in Amber-18. This was done to determine selectivity of the compounds' binding effect. To ensure compound stability, important termini residues were capped. The ANTECHAMER module was used to parametrise the compounds

as well as assign partial charges and atom types by implementing the general amber force field (Gupta *et al.*, 2020). The LEaP component of AMBER-18 was implemented and utilized for further processes. The first process was to add Cl^- and Na^+ counter ions to approve the system neutralisation before the production phase. A partial minimization of 1500 steps was achieved with 500 kcal/mol restraint potential gradient. Following this, a full minimization of 1000 steps was implemented by a conjugate gradient process by removing all the restraints that were previously applied. Each complex was gradually heated from 0 K to 300 K for 50 ps. This assured each simulation kept a stable volume and number of atoms. A potential harmonic restraint of 10 kcal/mol in combination with the collision frequency of 1 ps was implemented to the solutes in all systems (Roe and Cheatham, 2013). The equilibration of each system was performed by applying 500 ps equilibration step by ensuring a constant temperature of 300K. Isobaric-isothermal ensemble was used to keep a constant number of atoms and pressure within each system. The pressure was maintained at 1 bar on each system using Berendsen barostat. The production phase of 100 ns simulations was implemented on all the complexes by integrating the SHAKE algorithm to restrict hydrogen bonds(Roe & Cheatham, 2013). To confine hydrogen bonds, the SHAKE algorithm was implemented. The production phase was performed in runs of 50 ns which analysed the motions in the trajectory. Root Mean Structure Dynamics (RMSD), Root Mean Structure Fluctuation (RMSF), Radius of Gyration (RoG) and MMGBSA were calculated using CPPTRAJ and PTRAJ modules of Amber-18 package. All plots and visualizations of the trajectories were analysed using Origin data analysis tool and Maestro Schrödinger software.

2.2.2.1 Post-dynamic Trajectories Analyses

The coordinates of protein bound with ligands were saved after every 1 ps and trajectory curves of MD simulations were estimated using CPPTRAJ module (Roe & Cheatham, 2013) integrated into AMBER 18 package. The RMSD of C^{α} atoms, RMSF of each residue in the complex R_g, SASA, intramolecular and intermolecular hydrogen bond formation, distance correlation matrix and thermodynamic calculations of all the systems were calculated. We used Origin tool (Seifert, 2014) and VMD visualization tool (Humphrey *et al.*, 1996) for MD trajectories analysis.

2.2.2.2. Binding Free Energy Calculations

The relative binding free energies were computed using Molecular Mechanics/Generalized Born Surface Area (MM-GBSA) binding free energy method (Wang *et al.*, 2019). Water

molecules and counter ions were stripped using the CPPTRAJ module. The binding free energies (ΔG_{bind}) were calculated with the MM-GBSA method for each complex as below:

$$\Delta G_{bind} = G_{complex} - G_{protein} - G_{ligand} \quad (1)$$

The free energy term, ΔG_{bind} is calculated using the following equations:

$$\Delta G_{bind} = \Delta E_{gas} + \Delta G_{solvation} - T\Delta S \quad (2)$$

$$\Delta E_{gas} = \Delta E_{int} + \Delta E_{vdw} + \Delta E_{elec} \quad (3)$$

$$\Delta E_{int} = E\Delta_{bond} + \Delta E_{angle} + \Delta E_{torsion} \quad (4)$$

$$\Delta G_{solvation,} = \Delta G_{polar} + \Delta G_{nonpolar} \quad (5)$$

$$\Delta G_{nonpolar} = \gamma \Delta SASA + \beta \quad (6)$$

The TΔS is change of conformational entropy on ligand binding which was not considered here as it has been found that calculations of entropic contribution are often neither reasonable nor necessary when taking binding affinities of ligands (**Eq. 2**). The gas phase energy (ΔE_{gas}) is the sum of the internal (ΔE_{int}), van der Waals (ΔE_{vdW}) and Coulombic (ΔE_{elec}) energies, (**Eq. 3**). The internal energy (ΔE_{int}) is energy associated with vibrations of bonds and bond angles and rotation of single bond torsional angles (**Eq. 4**). The solvation free energy ($\Delta G_{solvation}$) is the pattern of polar (ΔG_{polar}) and nonpolar ($\Delta G_{nonpolar}$) contributions (**Eq. 5**). The polar solvation contribution is estimated using the Generalized Born (GB) solvation model with the dielectric constant 1 for solute and 80.0 for the solvent. Conversely, the nonpolar free energy contribution was assessed using **Eq. 6**, where the surface tension proportionality constant, γ and the free energy of nonpolar solvation of a point solute, β, were set to 0.00542 kcal mol^{-1} Å^{-2} and 0 kcal mol^{-1}, respectively (Gohlke *et al.*, 2003). The SASA is calculated by linear combination of pairwise overlap (LCPO) model (Weiser *et al.*, 1999).

2.2.2.3.Dynamic Cross-Correlation Matrix

The dynamic cross-correlation matrix (DCCM) analysis was assessed to determine the deviations and movements in Cα backbone atoms. Factors for i and j cross-correlation Cα atoms are shown underneath:

$$\mathbf{C_{ij}} = \frac{< \mathbf{\Delta ri}.\mathbf{\Delta rj} >}{(< \mathbf{\Delta r_i^2} >< \mathbf{\Delta r_j^2} >)^{\frac{1}{2}}} \quad (7)$$

where $\Delta r_{i,j}$ is movement of i^{th} and j^{th} atom average point and angle braces specified over complete curves. All correlated movements are symbolized by $C_{ij} = 1$, however, $C_{ij} = -1$ denoted highly anti-correlated movements over time. The deviation of movements from 1 to -1 describe that i and j movements are correlated and anti-correlated respectively. The DCCM was assessed using CPPTRAJ module employed in Amber18 and all matrices schemes using Origin software.

2.3 *In vitro* studies

2.3.1 Bacterial Strain and Growth

The bacterial strain used in this study was *Chromobacterium violaceum* ATCC12472. This strain of *C. violaceum* is known as a bioreporter strain and thus is beneficial to use in various *in vitro* assays. This strain was stored at -80 ºC in 70% glycerol (Merck, Darmstadt, Germany) in the Department of Clinical Microbiology and Infectious Diseases at the University of the Witwatersrand. New glycerol stocks were made at the beginning of this study for the purpose and duration of this study. This ensured that the bacteria were able to propagate at the desired rate for the experiments conducted in this study.

The primary bacterial culture used in all *in vitro* assays was cultured using various sized Eldemire flasks (Schott Duran, Germany). Bacteria require a significant amount of oxygen to grow, therefore flask size was chosen based on volume that was going to be cultured multiplied by 10. The desired volume of LB broth (**Appendix 7.6**) (0.01% peptone powder (Merck, Darmstadt, Germany), 0.005% yeast extract (Merck, Darmstadt, Germany), 0.005% NaCl (Merck, Darmstadt, Germany)) was added to the flask and a scrape of bacterial glycerol stock was added to the flask. The culture was set to propagate under conditions of 200 g agitation for 24 hours and 30 ºC using a shaking incubator (Series 25 incubator shaker, New Brunswick, Scientific co inc., Edison, New Jersey, USA).

2.3.2 Determination of Antibacterial Activity

The newly synthesised imidazole compounds were first tested for their antibacterial activity. This was done by determining minimum inhibitory and bactericidal concentrations of all the compounds. This provided insight into the strength of the compounds as antibacterial agents and provided appropriate concentrations to use for the remainder of *in vitro* studies.

2.3.2.1 Determination of Minimum Inhibitory Concentrations (MIC)

To obtain the MIC values of the compounds, 12 dilution factors were used for each compound to ensure that the exact MIC values were determined. The stock of 12.5 µg/ml for each compound was used for this assay.

The MIC of all imidazole compounds (IMA-1 - IMA-5) were determined by broth microdilution assay following the approved guidelines of Clinical and Laboratory Standards Institute (CLSI, 2015).

For bacterial growth, a loop full of *C. violaceum* were streaked on LB agar plates and grown overnight to obtain a pure colony. From the agar plate, pure colonies were picked and added to 10 ml of LB media in a 100 ml Eldemire flask. The bacterial inoculum was then set to propagate for 24 hours at 30 ºC at an agitation of 200 g. Following the incubation, a suspension of 1.5×10^8 bacterial cells was prepared. To prepare cell suspension, the bacteria were transferred into a 15 ml centrifuge tube (Lasec, South Africa) and centrifuged at 3000 g for 10 minutes. The supernatant was discarded and 1 ml of 8.5% saline solution (**Appendix 7.6**) was added to wash the cells. The pellet was then resuspended and centrifuged once again at 3000 g for 10 minutes. The supernatant was discarded and 1 ml of 8.5% saline solution was added to cells and the pellet was resuspended. To obtain the correct cell suspension of 1.5×10^8, 10 ml of saline solution was added to a glass tube and 100 µl of cells were added and mixed. A Siemens Microscan Turbidity Meter (Merk, Millipore, Darmstadt, Germany) was used to measure the cell suspension.

To perform the MIC assay, 100 µl of LB media was added to each well of a 96 well microtiter plate (Thermo Fischer Scientific, MA, USA). To the first well of each row in the 96 well plate, 100 µl of test compounds (12.5 µg/ml) were added to achieve concentrations ranging from 3.125 to 0.00153 µg/ml. A serial twofold dilution was performed by first mixing the compounds with the Luria Bertani (LB) media and then transferring 100 µl of the solution to the well below it and mixed well. This occurred for the remainder of the wells until the last well, where 100 µl was finally discarded. Lastly, 100 µl of the 1.5×10^8 *C. violaceum* cell suspension was added to each well except the sterility control. The plates were then covered in aluminium foil and incubated at 30 ºC for 24 hours. Following incubation, the results were read and the lowest with no visible bacterial growth was considered as MIC. For each experiment, positive (ampicillin, 0.2 µg/ml), negative (1% DMSO), sterility (cell free) and growth (compound free) controls were included. All experiments were performed in triplicate

2.3.2.2 Determination of Minimum Bactericidal Concentrations (MBC)

This assay was performed following the results for the MIC assay and was used to determine the lowest concentration of the compound that killed the bacteria. This method was adapted from Ahmad et al., 2015 and the CLSI standard method. The MBC values for IMA-1 – IMA-5 were evaluated by sub-culturing 10 µl from wells with no turbidity on LB agar plates (0.01% peptone powder (Merck, Darmstadt, Germany), 0.005% yeast extract (Merck, Darmstadt,

Germany), 0.005% NaCl (Merck, Darmstadt, Germany) and 0.015% agar powder (Sigma-Aldrich, MO, USA)). Results were recorded after incubating plates at 30 °C for 24 hours.

2.3.3 Anti-Quorum Sensing Studies

The ability of test compounds to inhibit quorum sensing was determined both qualitatively and quantitatively. The quantitative assay was performed as a preliminary test to screen if imidazole compounds were able to inhibit quorum sensing. Based on these results, quantitative assay was performed to determine the extent of anti-quorum sensing activity of imidazole compounds.

2.3.3.1 Qualitative Anti-Quorum Sensing Assay

The efficiency of the test compounds to inhibit quorum sensing against *C. violaceum* was determined qualitatively by means of agar plate method (Ahmad *et al.*, 2015). *C. violaceum* was cultured for 24 hours in 10 ml of LB broth at 30 ˚C in a shaking incubator for 24 hours. A 20 µl of the suspension of 1.5×10^8 cells per ml was spread on LB agar plates and allowed to dry at room temperature. Sterile filter disks (Munktell, Ahlstram) were placed at equidistant points on the bacteria spread layer, to ensure that the compounds and bacteria could be in contact with each other. To the filter disk, 10 µl of the compounds at concentrations of MIC were placed and allowed to absorb at room temperature. Three controls were used in this experiment: 1% eugenol as a positive control, 1% DMSO as a negative control and one filter disk with nothing on it as a sterility control. All the plates were then incubated at 30 ºC for 24 hours. Following the incubation, the plates were observed for the clear and turbid zones around the disk. The clear zone of inhibition indicates the anti-bacterial activity of the compounds, whereas the zone of turbidity is an indication of the anti-quorum sensing activity of the compounds. The diameter of the zones was measured in mm alongside a violet background of bacterial growth. The measurement for each zone started from the centre point of each filter disk and included the disk as part of the measurement.

2.3.3.2 Quantitative Anti-Quorum Sensing Assay

To further quantify the anti-quorum sensing potential of imidazole compounds (IMA-1 – IMA-5), the percentage of violacein pigment was measured in treated and untreated *C. violaceum* as described previously (Ahmad *et al.*, 2015) with minor adjustments. A primary culture of *C. violaceum* was prepared by inoculating pure colonies of bacteria in LB broth for 24 hours in a shaking incubator set at 30 ºC at 200 g agitation. Following the incubation,10 ml of cell suspension of 1.5×10^8 colony forming units per ml (CFU/ml) was prepared using turbidometer in a 50 ml centrifuge tubes (Lasec, South Africa). To this, various concentrations (MIC, MQSIC, and ½ MQSIC) of the test compounds were added. Three controls were also included in this test: eugenol as a positive control (10%), 1% DMSO as a negative control and a growth

control (200 µl of 1.5 x 10^8 CFU was placed into 4.8 ml of LB broth). Eugenol was used as a positive control instead of a known antibiotic as there is no known antibiotics with anti-quorum sensing capabilities, whereas eugenol has been well reported to have anti-quorum sensing activity (Sybiya Vasantha Packiavathy *et al.*, 2012). The tubes were incubated for 24 hours in a shaking incubator set at 30 ºC and agitation of 200 g. Following the incubation, 1 ml of each tube was transferred to a 1.5 ml microcentrifuge tube (Lasec, South Africa) and centrifuged (Eppendrof 5417C, Merck, Darmstadt, Germany) at 12 000 g for 10 minutes to obtain a pellet containing the bacterial cells with the violacein pigment. The supernatant was discarded, and the pellet was washed with 1 ml of PBS. The tubes were centrifuged again at 12 000 g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 1 ml of 100% DMSO to solubilise violacein pigment. Each tube was vortexed for 30 seconds to ensure the total pigment was solubilised. The tubes endured a final centrifugation at 12 000 g for 10 minutes which pelleted out any remaining bacterial cells. 200 µl of the remaining supernatant containing violacein was transferred to a 96 well plate and results were read using a microplate reader set at an absorbance of 585 nm (SpectraMax iD3, Separations, Molecular Devices, San Jose California, USA). The percentage of violacein inhibition was then calculated using the formula from Packiavathy et al., 2012 where the control value was the value of the pure growth control used in this assay:

$$\text{Percentage of violacein inhibition} = \frac{\text{Control OD585 nm} - \text{Test OD585 nm}}{\text{Control OD585 nm}} \times 100$$

This assay was performed in singlet and repeated 4 times and results were displayed as mean ± standard deviation.

2.3.4 Biofilm Inhibition Assay

To determine how effective the test imidazole compounds (IMA-1 – IMA-5) are at inhibiting biofilm formation, a standard crystal violet staining method was used (Ravichandran *et al.*, 2018a). A primary culture of *C. violaceum* was prepared as described above. To provide enough space for the cells to form biofilms, this assay was done in a 6 well plate (Cellstar, Greiner Bio-one, Kremsmünster, Austria) with the final volume of 1 ml in each well. LB broth was added to each well and different concentrations of the test compounds (MIC and 2 × MIC) were added. The plates were then incubated in a stationary fashion for 24 hours at 30 ºC. Following the incubation, plates were carefully removed from the incubator to avoid disturbing the biofilm. The media was removed, and wells were washed with 1 ml of PBS (pH 7.4) to remove any planktonic cells which could influence results of this experiment. The PBS was then removed and 1 ml of 0.1% crystal violet solution was added to the wells. The crystal violet solution was set to incubate for 20 minutes at room temperature to ensure adequate staining of the biofilm.

Followed by the short incubation, the crystal violet was carefully removed, and the wells were washed twice with 1 ml of sterile distilled water. This was done to effectively remove any additional crystal violet solution, which could influence results. Following the washing, the wells were left to dry at room temperature and then 1 ml of 95% ethanol (Sigma-Aldrich, MO, USA) was added to the wells to solubilise the crystal violet. The plates were then read at an absorbance of 470 nm using a microplate reader (SpectraMax iD3, Separations, Molecular Devices, San Jose California, USA). In this assay, a growth control and 1% DMSO as a negative control and 0.2 µg/ml of ampicillin as a positive control were also included. Each experiment was performed in duplicate, and the assay was repeated three times and results were displayed as mean ± standard deviation.

2.3.5 Gene Expression

To study the effect of imidazole compounds (IMA-1 – IMA-5) on expression of quorum sensing genes, RT-qPCR was done. Gene expression of *CviI*, *vioA*, *vioB*, *vioD* and *vioE* was determined in *C. violaceum* treated with MQSIC values of the test compounds and gene expression were compared to untreated *C. violaceum*. A housekeeping gene, *16S,* was also used as a control in this study.

2.3.5.1 RNA Extraction

C. violaceum cells treated with MQSIC values of test compounds were used for total RNA extraction using RNA extraction kit Quick-RNA Fungal/Bacterial Miniprep kit (Zymo Research, CA, USA), following manufacturer's instructions. *C. violaceum* was cultured at 30 ºC for 24 hours in a shaking incubator with treatment of the five compounds and an untreated control. One ml of the culture was then transferred to microcentrifuge tubes and centrifuged for 15 minutes at 2200 g. The supernatant was discarded, and cells were resuspended in 800 µl of RNA lysis buffer and was then transferred to ZR BashingBead lysis tube. Each lysis tube was thoroughly vortexed for 2 minutes and then centrifuged at 3000 g for 1 minute. After centrifugation, 600 – 800 µl of the supernatant was transferred to a Zymo-Spin IIICG Column that is placed in a collection tube. The complex was again centrifuged for 30 seconds at 3000 g. To the flow-through, 1 ml of 95 – 100% ethanol was added to each tube. This mixture was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 12 000 g for 1 minute and the flow-through was discarded. This was done to ensure the RNA was bound to the filter. Each sample was then treated with DNase 1 Reaction Mix, which was comprised of 5 µl of Dnase1 and 75 µl of DNA digestion buffer. Eighty µl of this reaction mix was then added directly to the column matrix and left to incubate at room temperature for 15 minutes. Following the incubation, 400 µl was added to each column and centrifuged for 1 minute at

12 000 g. RNA wash buffer was added to the column (700 µl) and centrifuged at 12 000 g for 1 minute. The flow-through was discarded and a final 400 µl was added to the column and centrifuged for 2 minutes at 12 000 g to ensure adequate washing was done. The column was placed in a new RNA free tube and 52 µl Dnase/Rnase-Free water was added directly to the column matrix and a final centrifugation was performed for 30 seconds at 12 000 g. The RNA was stored at -70˚C for future use. The integrity and concentration of RNA was determined via Nanodrop 2000 spectrophotometer (Thermo Scientific) (**Appendix 7.9**).

2.3.5.2 cDNA Synthesis

The total RNA extracted was used to synthesise cDNA by performing reverse transcription. This was performed using iScript cDNA Synthesis Kit (Bio-Rad, California, USA) following the manufacturer's instructions. Each reaction was made up of 4 µl of 5X iScript Reaction Mix, 1 µl iScript Reverse Transcriptase, 1 µg RNA template and nuclease free water to make a final volume of 20 µl. An Eppendorf Thermomixer (Sigma-Aldrich, MO, USA) was used for heating and cooling conditions. Tubes containing reaction mixtures were left at room temperature for 5 minutes to complete the priming phase of the reaction. The tubes were then transferred to the thermomixer and heated to 46 ˚C for 20 minutes for reverse transcription to take place. Reverse transcription was inactivated by heating the tubes to 95 ˚C for 1 minute. The concentration and purity of cDNA was determined by Nanodrop 2000 spectrophotometer (**Appendix 7.9**). cDNA samples were stored at -20 ˚C or used immediately.

2.3.5.3 cDNA Quality and Primer Assessment

The quality of cDNA was assessed by means of conventional polymerase chain reaction (PCR). Conventional PCR was also used to determine the integrity of the primers (for genes *CviI*, *vioA*, *vioB*, *vioD*, *vioE* and *16S* (Anatech, Randburg, South Africa)) that will be used for RT-qPCR as well as identify the correct annealing temperature. Primer sequences used are displayed in Table 2.2. Each reaction mixture consisted of 12.5 µl PCR Master Mix, 0.5 µl forward primer, 0.5 µl reverse primer, 9.5 µl nuclease free water per reaction. From the reaction mixture, 23 µl aliquots were added to PCR tubes. To this, 2 µl of cDNA (200 ng/µl) was added to make a final reaction volume of 25 µl. A gradient thermocycler was used to determine the ideal annealing temperature. The thermocycler conditions were 95 ˚C for 3 minutes as the initial melting step, 95 ˚C for 30 seconds, 52 – 54 ˚C for 30 seconds at annealing temperature and 72 ˚C for one minute. This was repeated for 35 cycles. A final extension of 72 ˚C for 10 minutes was performed and the thermocycler held at 4 ˚C once completed. The PCR products were run on a 2% Agarose gel stained with ethidium bromide (Merck, KgaA, Darmstadt, Germany). The gel was placed in a gel electrophoresis tank and covered with 1X TAE buffer (**Appendix 7.8**). To

each sample, 3 µl of loading dye (Thermo Fischer Scientific, MA, USA) was added and a final 10 µl of each sample was loaded to the wells. A 50 bp ladder (Thermo Fischer Scientific, MA, USA) was included to ensure the correct size of the PCR product was obtained. A nuclease free water control was also included. The gel ran for 1 hour at 100 V and visualised using ChemiDocXRS gel documentation system (Bio-Rad, California, USA) under ultraviolet light.

Table 2.2: List of primers used for RT-qPCR.

Gene	Forward primer	Reverse primer
CviI	5'-GGATCCCCGTAGGCAAAGAACTAA-3'	5'-GAATTCTTGTGTCTGAACGCCA-3'
VioA	5'-GGATCCGCCCAAAGCCAGACTA-3'	5'-GAATTCTGAACGGCACGATTGA-3'
vioB	5'-GGCCTATTACGAGCTGGTCT-3'	5'-TGGGCCAGGTATTTGAGGAA-3'
vioD	5'-GCCGCAACAAGTACATCTGG-3'	5'-AACACCTTGGCGACGTATTC-3'
vioE	5'-TGGAAGCAGAAGGTGGCC-3'	5'-GCGGCGTCCAGGTACAAC-3'
16S	5'-GCGCAACCCTTGTCCTTAGTT-3'	5'-TGTCACCGGCAGTCTCCTTAG-3'

2.3.5.4 Real Time PCR

Using the synthesised cDNA, primers, and annealing temperature of 54 ˚C, real time PCR was performed using SYBER Green Master Mix (Thermo Fischer Scientific, MA, USA) and the Light Cycler Nano Real-Time PCR System (Roche, Basel, Switzerland). One master mix was made for each gene (*CviI*, *vioA*, *vioB*, *vioD*, *vioE* and *16S*) and 18 µl aliquots were added to real time PCR tubes. Each master mix consisted of 10 µl SYBER Green Master Mix, 0.5 µl forward primer, 0.5 µl reverse primer and 7 µl nuclease free water. To the 18 µl aliquots, 2 µl of cDNA (100 ng/µl) was added. The cycling conditions used were uracil-DNA glucosylase activation at 50 ˚C for 2 minutes. A dual-lock DNA polymerase step for 2 minutes at 95 ˚C followed the activation step. For 40 cycles of denaturation at 95 ˚C for 15 seconds, annealing at 54 ˚C for 15 seconds and extension at 72 ˚C for 1 minute. Once the conditions were completed, the thermocycler held at 4 ˚C. A no template control was included for each gene and every run to ensure no contamination was present in the PCR. Results were quantified by analysing the melting curves produced by each PCR product amplification using the following formula:

$$2^{-\Delta\Delta Cq}$$

Where ΔCq is the difference between the average Cq of the house keeping gene subtracted from the Cq of target genes and ΔΔCq was the ΔCq of control cells subtracted from the ΔCq of the

tested cells. Results were displayed in graphical form and statistical analysis (Two-Way ANOVA) was performed.

2.4 Statistical Analysis

Quantitative anti-quorum sensing assay, biofilm inhibition assay and real time PCR were all performed in triplicates and displayed in graphical form as mean ± standard deviation (SD) except for real time PCR results where only means are displayed. The statistical test of two-way ANOVA was performed to determine statistical difference between treatment and non-treatment group as well as the difference between each concentration used. The statistical test was performed using GraphPad PRISM Software Version 5.04 (GraphPad Software Inc., San Diego, California, USA) and a p-value of < 0.05 was considered statistically significant.

3.1 *In silico* Results

3.1.1 Molecular Docking

Molecular docking was performed by docking the test compounds to the antagonistic binding site of the target CviR protein. Molecular docking of all five compounds was performed using XP mode implemented in Maestro (version 12.5). The binding scores of the compounds were obtained by subtracting the energies of the entire system, subtracting the energies of the compound and protein, and then docking the test compounds to the binding site. The resulted binding energies for all the test compounds can be seen in Table 3.1. Results specified that IMA-3 had the most negative binding score (-9.28 kcal/mol) with IMA-1 having the second most negative binding score of -9.23 kcal/mol indicating both of these compounds show the greatest potential to be effective quorum sensing inhibitors. Results also indicated that IMA-2 has the weakest binding score (-6.74 kcal/mol). All the binding energies of the compounds fall in this range of -9.28 to -6.74 kcal/mol. These results do provide a clear indication of how effectively the compounds interact with the target protein and how strong their binding is. Hence all five compounds were used in further analysis.

The resulting binding interactions of all the compounds are shown in Figure 3.1 (A-E). Table 3.1 shows which binding site residues the compounds interact with. Due to the aromatic ring that is characteristic of imidazole-based compounds, it means that all the compounds have the potential to create pi-pi stacking with amino acid residues that contain an aromatic ring such as tyrosine (Tyr) and tryptophan (Trp). Results from molecular docking do indeed show that all compounds created pi-pi stacking. As seen in Figure 3.1A, IMA-1 created pi-pi stacking between the aromatic ring of the compound and amino acid residues Tyr80 and Tyr72. Similar interactions can be seen in Figures 3.1B and E where IMA-2 and IMA-5 had the same interactions with the CviR protein and formed pi-pi stacking with Trp76. Figures 3.1C and D also show that IMA-3 and IMA-4 both created pi-pi stacking with Trp76.

Other interactions were also made between the compounds and the protein which can also be seen in Figure 3.1 (A-E). Results from Figure 3.1A reveal that IMA-1 generated a pi-cation interaction between Tyr72, Tyr80 and the nitrogen from the aromatic ring of the compound. Hydrogen bonds were also formed between Trp76 of the protein and double-bonded oxygen of IMA-1. Figures 3.1B and E showed that IMA-2 and IMA-5 interacted extensively with residue Asp89 as two separate interactions were created. These interactions were salt bridge formation with the aromatic ring and H-bond formation with the hydrogen in the carbon chain. Both compounds also had additional H-bond formation with the polar Ser147 and the oxygen

molecule in the carbon chain of both compounds. Figures 3.1C and D also revealed that IMA-3 and IMA-4 interacted in a similar manner to that of IMA-2 and IMA-5 with the target protein and formed salt bridges with Asp89. H-bond formation also occurred between the polar Ser147 and the oxygen in the carbon chain of both compounds.

Although the compounds create similar interactions with the same amino acids and form similar bonds, the conformation in which they bind to the protein differs extensively. This is especially true for IMA-1 as both the OH and double-bonded oxygen are in a downward-facing direction compared to that of IMA-3 (Figure 3.1 C) where the double-bonded oxygen is in the upper left corner of the complex. This conformation enables IMA-3 to form a hydrogen bond with the polar Ser147, whereas IMA-1 is unable to form a hydrogen bond with this residue due to the distance between the oxygen molecule of the compound and the hydrogen of Ser147. IMA-2 and IMA-4 have similar conformations within the binding pocket. It is therefore important to explore aspects of the formed complex such as flexibility, solubility and stability as this will provide more insight into why the binding energies are so close together and the significance of the interactions formed. All the compounds had significant binding scores and ideal interactions and hence were considered for extended structural investigations.

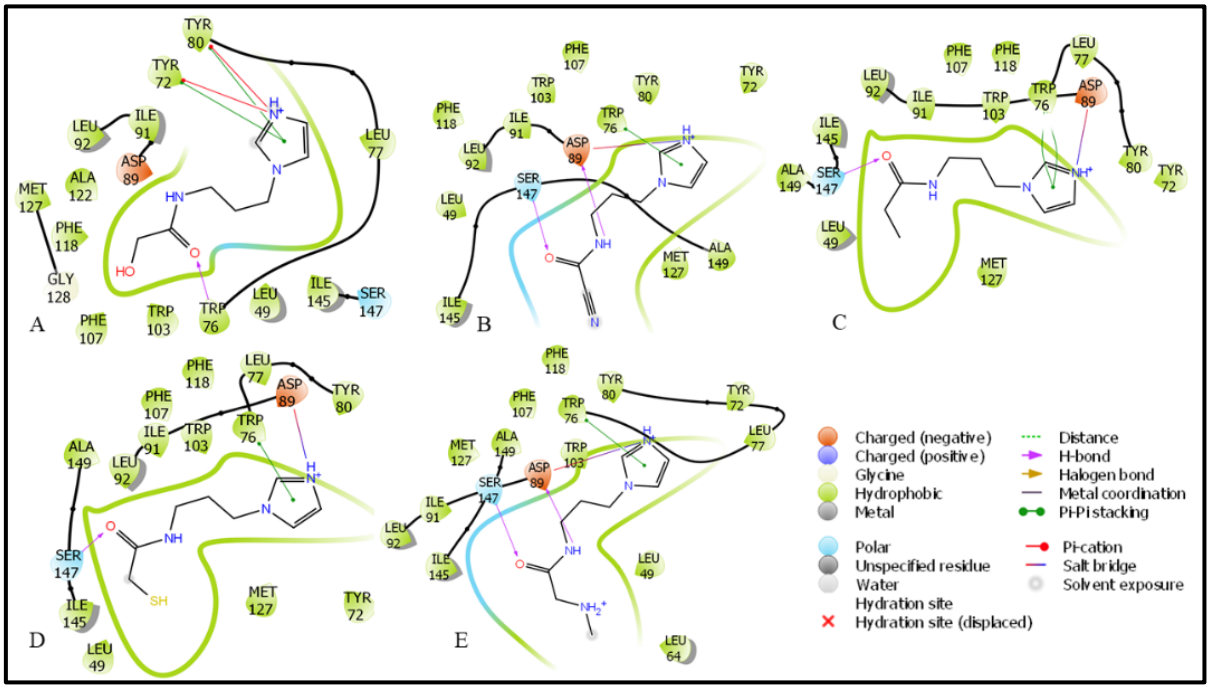


Figure 3.1: **Interactions between test compounds and the allosteric site of CviR protein from molecular docking results.** A. IMA-1 compound; B. IMA-2 compound; C. IMA-3 compound; D. IMA-4 compound and E. IMA-5 compound, respectively.

Table 3.1: Docking score and interacting binding site residues of five newly synthesised imidazole compounds.

Compounds	Docking Score (kcal/mol)	Interacting Binding Site Residues
IMA-1	-9.23	Tyr72, Tyr80, Trp76
IMA-2	-6.74	Trp76, Asp89, Ser147
IMA-3	-9.28	Trp76, Asp89, Ser147
IMA-4	-9.16	Trp76, 89, Ser147
IMA-5	-6.97	Trp76, Asp89, Ser147

3.1.2. Post-Dynamics Trajectory Analysis

Molecular dynamic simulations allow for the complex between the compound and target protein to be tested in a variety of scenarios that are simulated. Simulations include the structural integrity of the complex, the solubility of the complex in solvents such as water, the level of flexibility in the carbon backbones in the compounds, the strength and consistency of hydrogen bond formation, and how the molecular structure of the complex is altered. All these simulations allow for a greater understanding of how the compounds bind to the CviR protein and how this binding could influence the biological process that cascades from this binding. Complexes that were used for molecular dynamic simulations came from the results obtained from the molecular docking studies.

3.1.2.1.Comparative Structural Dynamics

The binding of small molecules to receptors can influence cascading biological processes and structural integrity of the protein (Lone *et al.*, 2019). Therefore, it is crucial to evaluate structural changes associated with binding of these small molecular compounds. Molecular dynamic simulations were conducted using Amber18 software to determine how binding of the compounds influence the structural dynamics of the CviR protein as well as the stability of the formed complex.

The root-mean-squared dynamics (RMSD) of Cα atoms were calculated to determine the stability, efficiency and consistency of the simulated CviR protein in complex with the five compounds. The five complexes tested were IMA1-CviR IMA-2-CviR, IMA-3-CviR, IMA-4-CviR and IMA-5-CviR including apo-CviR as positive control (Figure 2A). The RMSD parameter is proposed to calculate anomalies in the conformational stability of macromolecules from the carbon backbone structure. The RMSD results are illustrated in Figure 3.2. Results

indicated that IMA-5-CviR complex significantly altered protein conformation and presented more instability compared to the other four complexes. Great stability was shown for IMA-1 and IMA-3 complexes with IMA-1-CviR displaying a similar pattern to that of Apo-CviR. This signifies that this complex has remarkably similar stability to that of the natural ligand complex. IMA-2-CviR and IMA-4-CviR significantly differ from the control pattern with very few irregularities. From these results, it is evident that two complexes (IMA-1-CviR and IMA-3-CviR) are most similar to that of the natural complex and can therefore be effective at inhibiting quorum sensing. The RMSD analysis also suggests that any further calculations achieved on the produced trajectories for all the complexes were reliable.

The RMSD results reinforce what the molecular docking results revealed as the pattern of stability of the complexes is like that of binding energies. The compounds which had parallel interactions (such as IMA-2 and IMA-4) also showed a similar pattern of stability. Results also show that IMA-1 and IMA-3 both have the most effective binding energies and from these results, they have a stability pattern most similar to that of Apo control. However, all compounds show sufficient stability in complex with CviR and this insinuates that *in vitro* these compounds will be stable and may indeed inhibit quorum sensing.

To further validate the impact that the compounds have on the quorum sensing system of *C. violaceum*, residual fluctuations of Cα backbone atoms were calculated by determining the RMSF of each complex formed and trajectories throughout 100 ns of MD simulations was produced. Results were analysed and displayed in Figure 3.2B. As illustrated in Figure 3.2B, all five complexes showed similar fluctuations to each other and an apo-CviR control. The compounds showed particularly good stability from the 75^{th} to 100^{th} residue. Previous research has determined that between these residues lies the binding pocket (Gnanendra *et al.*, 2012). This means that the carbon backbone of all the compounds is stable in the binding pocket of the CviR protein and can therefore be effective at inhibiting quorum sensing as they are able to effectively bind to the CviR protein and all complexes are stable. From these results, it is reasonable to assume that the compounds can indeed compete with the natural ligand to bind to CviR as there is stability in all complexes. At some residues, the compounds show greater stability than the apo control indicating that these compounds have the potential to out-compete the natural ligand. The prominent binding of these compounds at the active site might be correlated with the structural inactivation of CviR protein.

Another important finding molecular dynamic simulation provides is the ability to determine how novel compounds alter molecular structure of the formed complex. This is done by computing Radius of Gyration (Rg) over a period of 100 ns of MD simulations. The

computations of Rg allow for the investigation of the overall compactness and flexibility of the complexes. Ideally, the complexes should be compact as this indicates that the molecular structure is not significantly altered to that of the natural complex. The results for Rg are shown in Figure 3.2C which revealed that over the period of 100 ns, all complexes prevailed sufficient stability, and were therefore in a compact conformation from 38 ns. However, for the first 20 ns all complexes had increased flexibility and this finding suggests that the compounds may need time to effectively bind to the CviR protein and for the complex to gain sufficient stable conformation.

IMA-1-CviR complex displayed significant flexibility for the first 38 ns but the remainder of the 100 ns, it is very stable. This complex's structural conformation is most similar to that of the Apo-CviR indicating that it may behave most similarly to that of the natural complex. Results revealed that IMA-2-CviR exposed the fewest number of irregularities from 20 ns time point. This reveals that IMA-2 establishes a more compact complex once IMA-2 has bound itself to the CviR protein. Rg of IMA-4-CviR complex reveals a similar pattern to that of IMA-2-CviR complex. This is to be expected as results from Figure 3.2A and B revealed that IMA-2 and IMA-4 are similar in structural dynamics of the complexes. IMA-3-CviR exhibited greater flexibility between 50 and 70 ns indicating that the complex can obtain a compact conformation. However, IMA-4-CviR complex can adjust conformation to have more flexibility and adjust to become once again compact. Similarly, IMA-5-CviR showed greater flexibility for a short 4 ns (Between 68 ns and 72 ns, Figure 3.2C) indicating that the complex gains elasticity for a brief moment and then regains a compact conformation. These results signify that the five compounds do not alter the structural integrity and molecular structure of the five complexes formed with the CviR protein.

The final aspect of comparative structural dynamics that was considered was hydrophobic or hydrophilic nature of the complexes. This was determined by simulating a hydrophobic environment and seeing how amino acid residues bound to the compounds behave. This simulation made use of the carbon backbone of the compounds and the duration of the simulation was 100 ns. Figure 3.2D shows that all complexes showed consistent rapid SASA values throughout 100 ns. This analysis reveals that the compounds retain resident hydrophobic or hydrophilic integrity. Results indicated that the compounds, when in complex with the CviR protein, will not change the hydrophobic or hydrophilic nature of the natural complex. This is reassuring as it confirms that these compounds could indeed behave as effective quorum sensing inhibitors.

Overall, the comparative structural dynamics reveal that IMA-1 is the compound that is most indistinguishable from that of the natural ligand in a complex with the CviR protein and IMA-5 is shown to have the most irregularities when in complex with CviR. Nevertheless, the results are promising as it provides in-depth detail that the compounds are structurally sound and will form adequate complexes that are significantly stable in a variety of environments. This insinuates the conclusion that the compounds are able to be potential quorum sensing inhibitors.

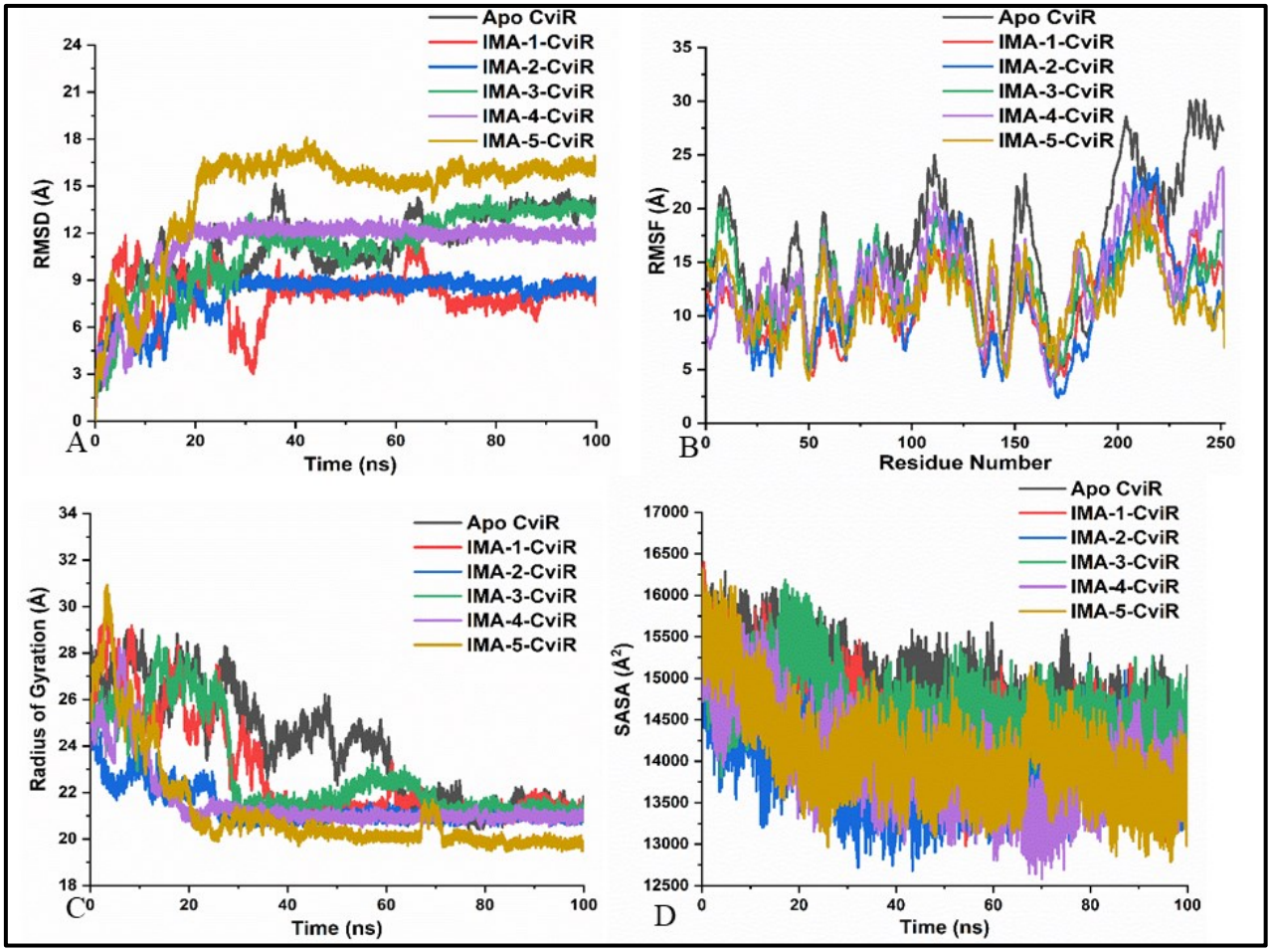


Figure 3.2: Comparative structural dynamics of the five test compounds in complex with CviR: Structural dynamics of CviR-IMA complexes. (A) RMSD, (B) RMSF, (C) Rg and (D) SASA values across the carbon backbone in Å of CviR-IMA-1, CviR-IMA-2, CviR-IMA-3, CviR-IMA-4, CviR-IMA-5 and apo CviR; in Å across the carbon backbone of all conditions calculated during 100 ns of MD trajectories.

3.1.2.2. Intermolecular and Intramolecular Hydrogen Bond Analysis

Hydrogen bonds are strong interactions formed between hydrogen and either oxygen, nitrogen, chlorine or fluorine atoms. These bonds are crucial in complexes as they provide structural integrity to a complex (Gupta *et al.*, 2020). Therefore, all complexes formed in this study underwent hydrogen bond analysis. The analysis was performed on both hydrogen bonds

formed intermolecularly and intramolecularly to provide an indication of the number of hydrogen bonds formed between the compound and CviR protein (intermolecular hydrogen bonds) as well as bonds formed between the atoms of the compound itself (intramolecular hydrogen bonds).

The number of hydrogen bonds formed over a 100 ns period was calculated to confirm the overall structural integrity, stability, and strength of the interactions from molecular docking (Figure 3.1). Results displayed in Figure 3.3A showed that all compounds had consistent hydrogen bond formation between their atoms revealing that the compound was free to interact with CviR protein, instead of creating interactions between itself.

The results in figure 3.3B specify that IMA-2 generated up to 3 additional intermolecular hydrogen bonds in antagonistic binding pocket between 15 ns and 35 ns. IMA-5 was shown to generate the fewest hydrogen bonds in the binding pocket as on average 0-1 intermolecular hydrogen bonds were formed. This confirms previous results that IMA-5 is the least stable of the five test compounds and forms a weaker complex. IMA-1 consistently generated between 1 and 2 intermolecular hydrogen bonds throughout the 100 ns period (Figure 3.3B). This result indicates that this complex has the fewest number of fluctuations in bond formation and is thus the most stable. IMA-1-CviR is also the only complex in which there are no significant fluctuations in hydrogen bond formation (from either 0 to 1 or 2 to 3 bonds formed). IMA-4-CviR shows great instability over the 100 ns period as there are fluctuations of bond formation of 0 to 2 throughout 100 ns. These results provide indications that IMA-1 forms the strongest complex with the CviR protein, while IMA-4 and IMA-5 may create a weaker complex. These results are confirmation of what is presented in Figure 3.2 in that IMA-1 is indeed a significant compound to be considered as a quorum sensing inhibitor.

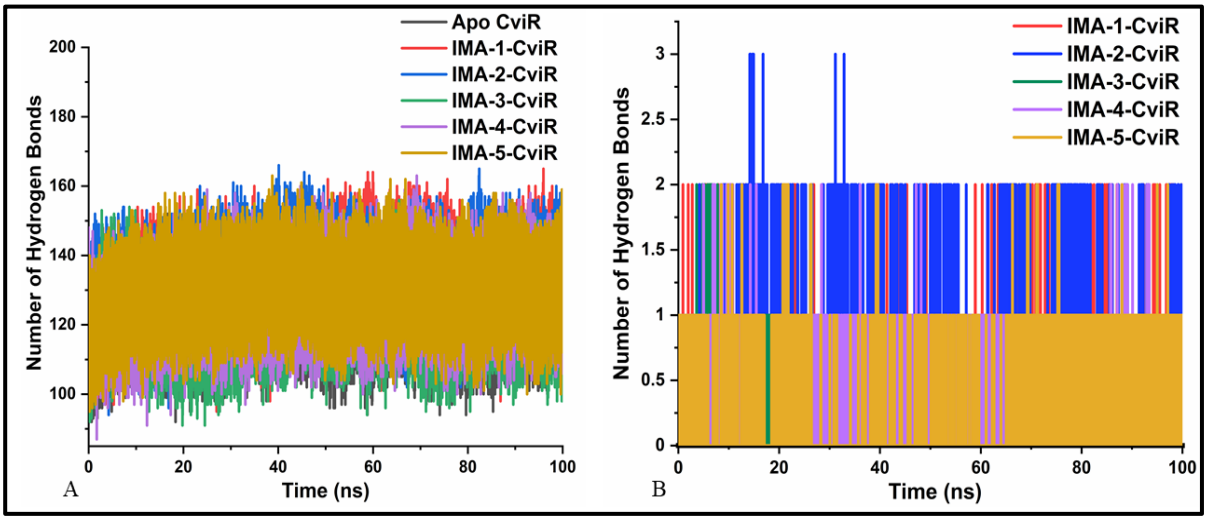


Figure 3.3: Hydrogen bond analysis of the five test compounds in complex with CviR: A intramolecular hydrogen bonds in IMA-1-CviR, IMA-2-CviR, IMA-3-CviR, IMA-4-CviR, IMA-5-CviR complexes and Apo-CviR complex as a control for a period of 100 ns of simulations. B intermolecular hydrogen bonds of IMA-1-CviR, IMA-2-CviR, IMA-3-CviR, IMA-4-CviR and IMA-5-CviR complexes over a period of 100 ns of simulations.

3.1.2.3. Dynamic Cross-Correlation Matrix Analysis

The final aspect of simulations that was examined was the dynamic cross-correlation matrix. Dynamic cross-correlation provides an overall picture of the similarities in the movement of amino acids of CviR. All the complexes formed were compared to the Apo-CviR control. Two major groups were created from the CviR protein, one comprised of positive movements and the other negative movements of the amino acid residues. Movements of amino acids from the five complexes that had the same movement as the control were considered to have a positive correlation are depicted in red in Figure 3.4. Those amino acids which had differing motions were considered to have negative correlation are portrayed as black in Figure 3.4. Diverse dynamics among IMA-1-CviR, IMA-2-CviR, IMA-3-CviR, IMA-4-CviR, IMA-5-CviR and Apo-CviR complexes were measured by plotting dynamic cross-correlation matrix for the correlated and anti-correlated motions of amino acid residues. These findings are vital as it is well known that the binding of a ligand to a protein causes a conformational change, but the change may not be to such a great extent that the protein can no longer bind to the DNA.

Results are displayed in Figure 3.4 where 3.4A indicates that apo-CviR complex is comprised of both negative and positive correlations. It was observed that IMA-1-CviR complex exhibited an overall more positive correlation as residues 200 - 240 and 100 – 160 revealed less negative correlation than that of the apo-CviR (Figure 3.4B). However, the positive correlation in the binding pocket (residues 80 – 120) displayed a different pattern of positive correlation, including a weaker positive correlation at amino acid residue 100. Nevertheless, results from

Figure 3.4 provide promising assumptions that IMA-1 is a great contributor to the structural dynamics of this complex.

IMA-2-CviR complex displayed a correlation pattern remarkably like that of the Apo control and IMA-1-CviR with noteworthy stronger positive correlation at residues 80 – 120 (binding pocket residues) and 200 – 240 (Figure 3.4C). However, a more negative correlation overall can be seen, especially at residues 120 and 220. Observations made in Figure 3.3 are indeed in agreement with the hydrogen bond results and comparative structural dynamics. Cross-correlation matrix analysis of IMA-3-CviR (Figure 3.4D) revealed a significant loss of positive correlation in the complex. Noteworthy losses are seen among amino acids 80 – 160 and this reinforces previous results (Figures 3.2 and 3.3) as to why this complex has less structural integrity. IMA-3-CviR displays an overall decrease in negative correlation compared to that of the apo control. This decrease in negative correlation provides an indication that the whole complex is more stable and structurally intact compared to that of the other complexes. The important observation is that there is still a significant correlation in the binding pocket of the complex indicating that the compound provides structural integrity once bound.

The dynamic cross-correlation seen in IMA-4-CviR reveals that the pattern of negative correlation significantly differs from what can be seen in the other three complexes and Apo control (Figure 3.4E). Results also showed a positive correlation in the binding pocket with a positive correlation pattern like that of Apo control. Noteworthy changes are among residues 80-120 and 200-240 as more negative correlation can be seen in this box compared to that of the other complexes. However, results do indeed agree with results from molecular docking and structural dynamics as Figure 3.4E shows a structure of sufficient integrity. Results for the IMA-5-CviR complex (Figure 3.4F) exposes a great increase of negative correlation across all residues involved in the binding pocket. This complex also reveals no significant increase in positive correlation. These results indicate that IMA-5 may not be as important as the other compounds for the overall structural integrity of the protein and may decrease the stability of the CviR protein. This result provides in depth clarifications for previous results that indicate that the IMA-5-CviR complex is the least stable complex.

The results from Figure 3.4 indicate that the compounds are important contributors to the overall dynamics of the complexes formed with CviR. It has been established that the binding of ligands is important to alter the conformation of the CviR protein to enable the protein to bind to the DNA. These results clearly show that these compounds will be able to bind to the CviR protein and thus inhibit quorum sensing.

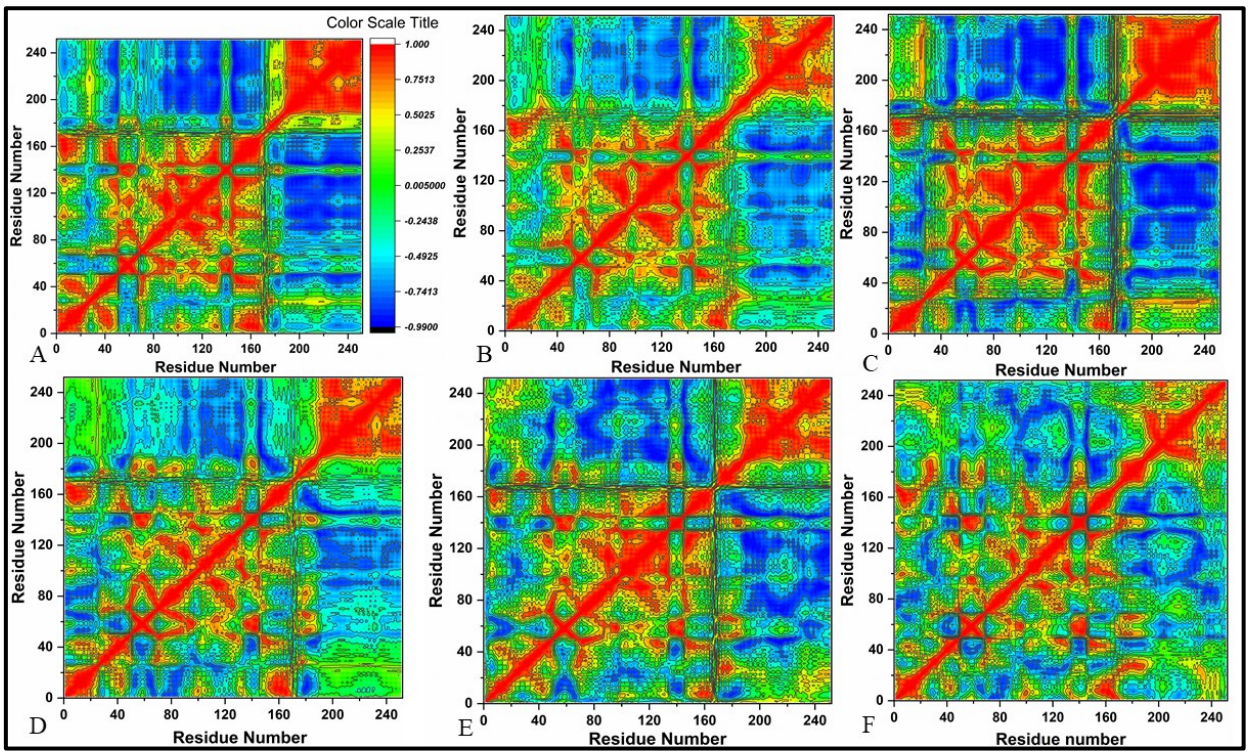


Figure 3.4: Dynamic cross-correlation matrix analysis of the five test compounds in complex with CviR: Dynamic cross-correlation matrix analysis. A apo-CviR complex, B IMA-1-CviR complex, C IMA-2-CviR complex, D IMA-3-CviR complex, E IMA-4-CviR complex and D IMA-5-CviR complex. Values near to 1 (shown as red) indicate high correlation and values near -1 (shown as black) indicate anticorrelation between pairs of residues.

3.1.2.4.Thermodynamics Free Binding Energy Analysis

The molecular interactions of residues in the binding pocket contribute significantly to stability, binding affinity and selectivity of the ligand. The thermal energy of the compound is a significant contributor to the structural stability of the compound bound in the active site (Gupta *et al.*, 2020; Lone *et al.*, 2019). The effect of ligand's binding free energy to the overall binding free energy of the complex is directly related to the structural stability of ligand in the active pocket of its target enzyme. It was therefore pivotal to assess the binding affinity of IMA-1, IMA-2, IMA-3, IMA-4, and IMA-5 to the CviR protein. The MM/GBSA approach was used as it is a standard technique to calculate the free binding energy of small molecules to biological macromolecules such as the CviR protein (Lone *et al.*, 2019). The results are displayed in Table 3.2.

The free binding energy (ΔG_{bind}) of the IMA-1-CviR complex was proven to be the highest at -34.44 kcal/mol. This complex also showed to have the weakest ΔE_{vdW} of -26.75 kcal/mol which indicates that this complex consists of stronger bonds to that of IMA-5-CviR as this complex was found to have the highest ΔE_{vdW} of -28.85 kcal/mol. Calculations also revealed that the

IMA-5-CviR complex had the lowest binding affinity of -30.74 kcal/mol. These binding energies are indicative of the effectiveness of the test compounds binding to CviR. These results reinforce the significance of previous results found in Figures 3.2 and 3.3.

Table 3.2: MM/GBSA-based binding free energy profile of IMA-1-CviR, IMA-2-CviR, IMA-3-CviR, IMA-4-CviR and IMA-5-CviR. Results are shown in kcal/mol.

Complex	ΔE_{vdW} (kcal/mol)	ΔE_{ele} (kcal/mol)	ΔG_{gas} (kcal/mol)	ΔG_{polar} (kcal/mol)	$\Delta G_{nonpolar}$ (kcal/mol)	ΔG_{sol} (kcal/mol)	ΔG_{bind} (kcal/mol)
IMA-1-CviR	-26.75	-40.49	-67.24	37.16	-4.36	32.80	-34.44
IMA-2-CviR	-31.68	-38.07	-69.76	40.85	-4.49	36.36	-33.39
IMA-3-CviR	-28.79	-39.11	-67.91	41.23	-4.27	36.95	-30.95
IMA-4-CviR	-28.77	-37.02	-65.80	39.20	-4.35	34.84	-30.95
IMA-5-CviR	-28.85	-32.02	-60.87	34.23	-4.10	30.13	-30.74

3.2. *In vitro* Results

3.2.1. Anti-Bacterial Activity

The compounds were first assessed for their ability to inhibit growth in *C. violaceum* strain ATCC12472. The minimum inhibitory concentration (MIC) is the lowest concentration of compound needed to inhibit the growth of *C. violaceum*.

Following the recommended CLSI protocol, the MIC for all five novel imidazole compounds was determined. Results are displayed in Table 3.3. These results were visually observed and the last well with no turbidity was noted as the MIC for that compound.

Every compound had an initial concentration of 3.125 µg/ml in the first well of the serial dilution. The MIC values ranged from 0.0488 µg/ml to 1.563 µg/ml (Table 3.3), with IMA-1 having the lowest MIC value and IMA-5 with the highest MIC value. This indicates that IMA-1 has potential to be the most effective compound as an antibiotic, as less compound is needed to inhibit growth of *C. violaceum*. The MIC results show a similar pattern to *in silico* results in this study where it was clearly shown that IMA-1 showed greatest stability and docking score when bound to the CviR protein. On the basis of MIC values, order of potency of the imidazole compounds was IMA1> IMA2> IMA3> IMA4> IMA5.

Following MIC determination, minimum bactericidal concentrations (MBCs) were also determined and were observed to follow the same trend as that of the MICs, with IMA1 having the lowest MBC value of 0.0977 and IMA5 having the highest MBC value of 1.563 (Table 3.3). MBC values are generally one or two folds higher than MIC values except that of IMA-5, where both MIC and MBC values were same. A noteworthy observation is that the compounds IMA-2 and IMA-3 have the same MBC value (0.39 µg/ml) in spite of the compounds having differing MIC values. The same was seen for IMA-4 and IMA-5, where the MBC value is 1.563 µg/ml. From these results it can be concluded that these compounds, despite inhibiting growth of the pathogen at different concentrations, were equally able to kill the pathogen at similar concentrations. The similar MBC results can also be corelated to chemical structural similarities between these compounds.

Furthermore, in the same assay minimum quorum sensing inhibitory concentration (MQSIC) values were determined by reading concentrations of wells where turbid colourless growth was observed. As expected, MQSIC values also follow the same trend as that of MIC and MBC values, where IMA-1 has the highest anti-quorum sensing potential with MQSIC of 0.0244 µg/ml (Table 3.2). Based on MQSIC values, the order of anti-quorum sensing potential of these compounds was IMA1> IMA2> IMA3> IMA4> IMA5. MQSICs for all the imidazole compounds were one-fold lower than their MIC values.

Table 3.3: Minimum inhibitory concentration (MIC), minimum quorum sensing inhibitory concentration (MQSIC), minimum bactericidal concentration (MBC) of five newly synthesised imidazole compounds.

Compounds	MIC (µg/ml)	MQSIC (µg/ml)	MBC (µg/ml)
IMA-1	0.0488	0.0244	0.0977
IMA-2	0.0977	0.0488	0.39
IMA-3	0.195	0.0977	0.39
IMA-4	0.781	0.39	1.563
IMA-5	1.563	0.781	1.563

3.2.2. Qualitative Anti-Quorum Sensing

Quorum sensing is cell to cell communication used by bacteria to initiate numerous processes that enable bacteria to behave collectively (Castillo-Juárez *et al.*, 2015; Rutherford & Bassler, 2012). Quorum sensing has been hypothesized to be a novel drug target for microorganisms that have developed drug resistance to a variety of antimicrobe drugs (Asfour, 2018). Therefore, five newly synthesised imidazole compounds were tested for their ability to inhibit quorum sensing.

Qualitative anti-quorum sensing assay was carried out using agar plate method and diameters of the clear and turbid zones produced were measured in mm (Table 3.4). For each compound, the respective MIC value was used to determine the zones of turbidity and zones of inhibition. Eugenol (10%) was used as the positive control, 1% DMSO was used as the negative control and a sterile control was included. All the test compounds showed good anti-bacterial and anti-quorum sensing activities against *C. violaceum.* This is evident from clear and turbid zones formed around sterile discs, respectively (Table 3.4). It was observed that IMA-1 created the largest zones of inhibition and turbidity of all compounds. This observation provides the indication that this compound has the greatest potential to inhibit growth as well as quorum sensing in *C. violaceum*. Conversely, IMA-5 was able to produce the smallest zones of inhibition and turbidity, and this further confirms that IMA-5 is the least active compound in the tested imidazole series to inhibit growth and quorum sensing in *C. violaceum*. Furthermore, these results reinforce what was concluded from molecular docking and dynamic simulation results conducted in this study.

Table 3.4: Zones of turbidity (ZoT) representing qualitative anti-quorum sensing activity and zones of inhibition (ZoI) representing qualitative anti-bacterial activity of the five imidazole compounds at their respective MICs. All the diameters of the zones are calculated in mm.

	ZoT (mm)	ZoI (mm)
IMA-1	42	37
IMA-2	37	31
IMA-3	23	20
IMA-4	22	17
IMA-5	13	9

3.2.3. Quantitative Anti-Quorum Sensing

To verify the results from qualitative anti-quorum sensing assay, the anti-quorum sensing activity of the test compounds was tested quantitatively by determining the percentage of violacein produced in treated and untreated samples. Violacein is the pigment produced by *C. violaceum* when quorum sensing is active. This pigment is a well-known characteristic of this bacterium and provides a visual representation of quorum sensing. Therefore, quantifying this pigment after treatment with the five test compounds is beneficial as it provides a clear indication of how active quorum sensing is in *C. violaceum* after treatment with the test compounds.

After treating cells with MQSIC and sub-MQSIC values, it was observed that for all test compounds the effect on quorum sensing was concentration dependent, with higher inhibition of violacein at the higher concentrations (Figure 3.5). All the compounds displayed varying levels of effective anti-quorum sensing capabilities at their respective MQSIC values. Percentages of quorum sensing inhibition ranged from 14 % to 89 %, when cells were treated with different concentrations of test compounds. At the MQSIC values, all the compounds significantly inhibited the violacein production (54% to 89%), whereas at sub-MQSIC very least effect on quorum sensing was observed. From results displayed in Figure 3.5, IMA-1 was the most effective compound at inhibiting quorum sensing by 89% at its MQSIC. IMA-5 showed the least effective quorum sensing inhibition as this compound was only able to inhibit violacein production by 54.43%. These results are in agreement with *in silico* results where IMA-1 showed to be the most stable compound with the greatest potential and IMA-5 was predicted to be the least effective compound.

IMA-1 being the most active compound while inhibiting the growth and quorum sensing of *C. violaceum,* effected the highest violacein suppression at MQSIC (89%), ½ MQSIC (76%) and

¼ MQSIC (41%) (Figure 3.5). Similarly, compounds IMA-2, IMA-3, IMA-4 and IMA-5, showed significant violacein inhibition at MQSIC with percentages of inhibition at 76%, 54%, 47% and 46% respectively. These results are encouraging as these compounds are able to inhibit quorum sensing effectively at MQSIC concentration. This implies that these compounds can also be used at lower concentrations compared to their MICs, without the concern for increased risk of the development of bacterial drug resistance in patients.

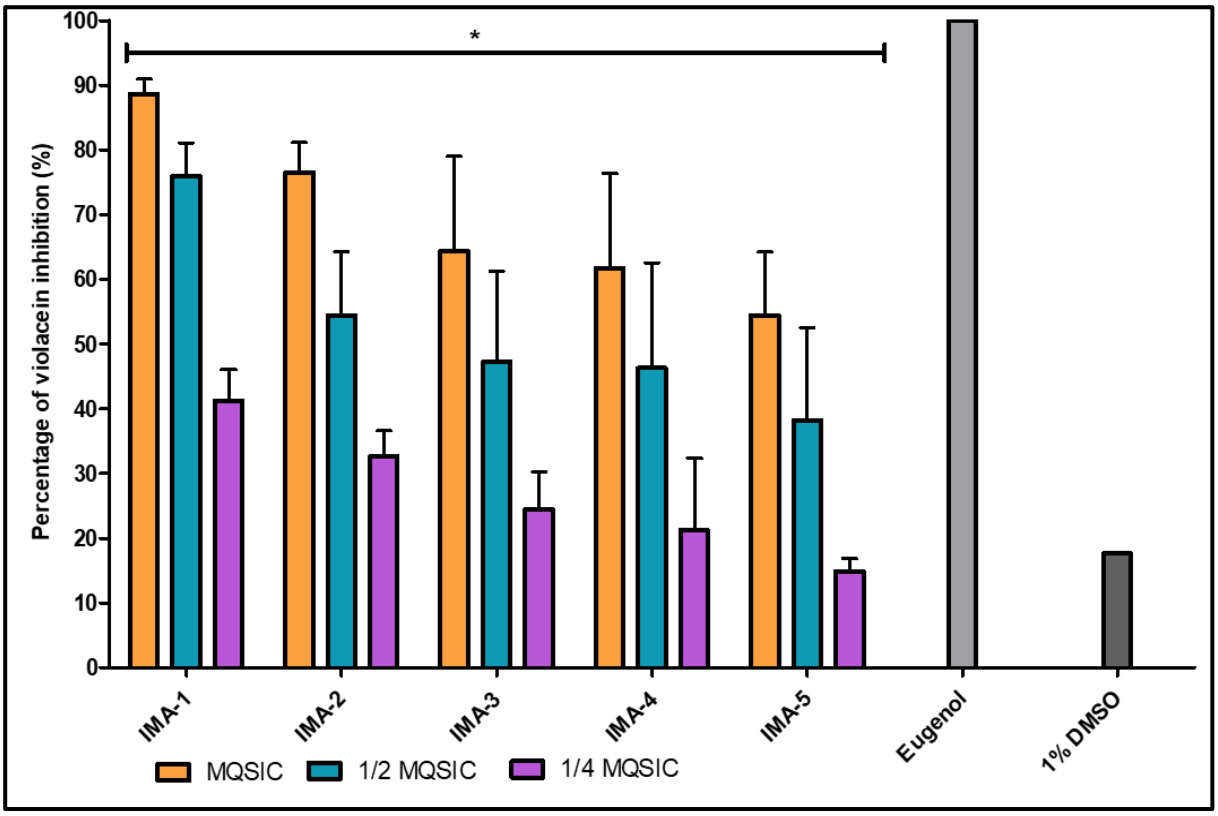


Figure 3.5: Percentage of violacein pigment inhibition indicating the inhibition of quorum sensing by different imidazole compounds. *C. violaceum* cells were incubated with MQSIC, ½ MQSIC and ¼ MQSIC values of test compounds for 24 h. Growth bar indicates untreated cells, accepted as 0% inhibition, while as eugenol and 1% DMSO represents positive and negative controls, respectively. Data are presented from three independent experiments using means ± S.D. *$p<0.05$, two-way ANOVA.

3.2.4. Biofilm Formation

Biofilm has been reported as an emerging drug target in order to control drug resistant bacteria. Both biofilm formation and quorum sensing are interlinked processes (Di Somma *et al.*, 2020), and therefore, it was vital to test how the five imidazole compounds influence biofilm formation in *C. violaceum*. The effect of the test compounds on biofilm formation was determined by staining the biofilm with crystal violet. Crystal violet specifically stains cellular structures, such as the cell membrane, that contain polysaccharides and biofilm are rich in polysaccharides.

Growth was set to 100% as there was nothing preventing the formation of biofilm in these untreated cells. All test compounds displayed significant inhibition in biofilm formation at MIC values (Figure 3.6). As expected, IMA-1 had the lowest percentage of biofilm formation (29.61%), following the same trend as in susceptibility and anti-quorum sensing assays (Figure 3.6). IMA-2, IMA-3 and IMA-4 also had significant inhibition in biofilm formation by 36.2%, 46% and 47.9% respectively, at MIC values. IMA-5 was the least active compound to show inhibition of biofilm formation, which is in line with results obtained in previous sections. At sub-inhibitory concentrations, none of the compound showed strong biofilm inhibition. This indicates that although these compounds may have the potential to be effective anti-bacterial and anti-quorum sensing compounds, they may not be effective at inhibiting biofilm formation at lower concentrations.

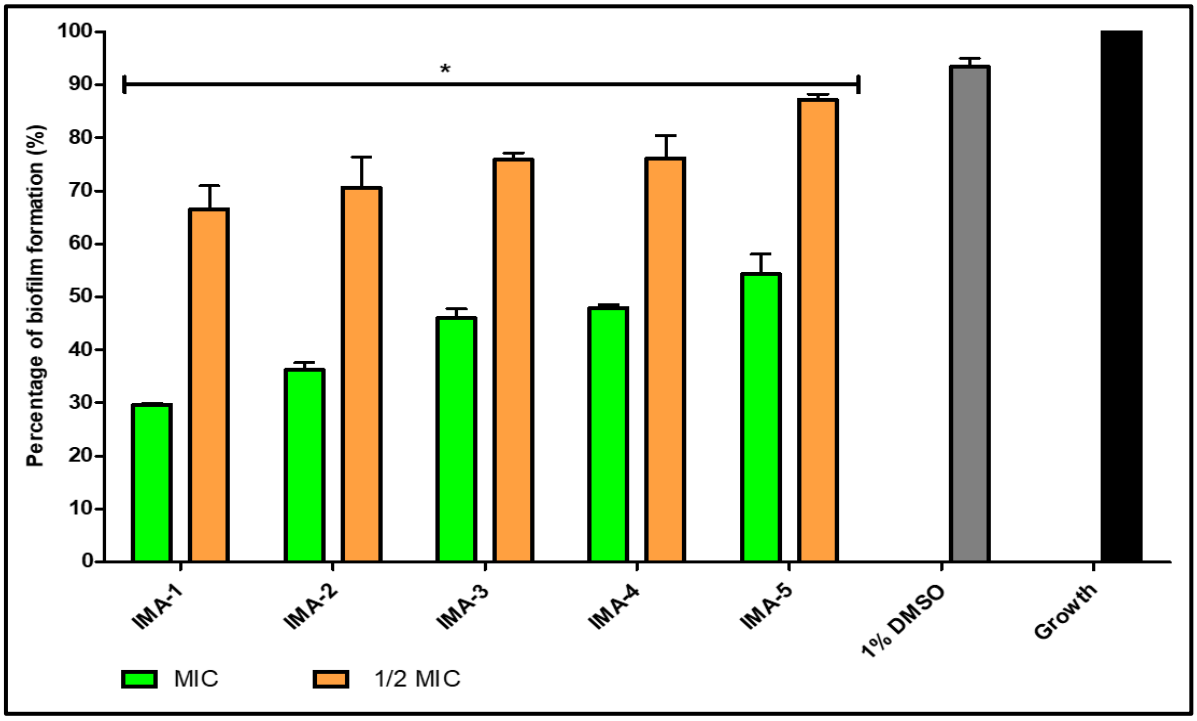


Figure 3.6: Percentage of biofilm formation in treated and untreated *C. violaceum*. The results were standardised and displayed as a percentage of formation compared to that of untreated (growth) control. Data are presented from three independent experiments using means ± S.D. *p<0.05, two-way ANOVA.

3.2.5. Gene Expression

To further test the ability of these derivatives (IMA-1 – IMA-5) to inhibit quorum sensing, related gene expression was determined after *C. violaceum* cells were treated with MQSIC values of the test compounds. Gene expression was the final aspect tested in this study using total RNA from treated and untreated cells. The RNA can be used to determine how compounds effect the gene expression. Several genes are involved in the quorum sensing system of *C.*

violaceum, such genes are *CviI*, *VioA*, *VioB, VioD* and *VioE* and these genes were tested for their expression in this study. In addition, the housekeeping gene *16S* was also included as a control in every set of experiments. It was vital to see if the compounds can affect quorum sensing at gene level, as they were significantly effective in inhibiting quorum sensing at a protein level.

The RNA was extracted from treated and untreated *C. violaceum* cells and converted to cDNA via reverse transcriptase. Performing conventional PCR was a vital step as it confirmed that the primers for each gene were of adequate usage and provided the annealing temperature for real-time PCR. Once cDNA integrity was confirmed, RT-qPCR was conducted using SYBR Green Master Mix to accurately quantify the amplicons produced. Results are displayed in Figure 3.7 and are shown as relative fold change in expression levels compared to the untreated control.

In comparison to untreated cells, a significant decrease in gene expression was observed when cells were exposed to imidazole compounds, with IMA-1 being the most active compound followed by IMA-2, IMA-3, IMA-4 and IMA-5 (Figure 3.7). All test compounds at their MQSIC values downregulated expression of all target genes, with a fold range of 1.5 to 6.3-folds, 1.6 to 5.1-folds, 1.3 to 3.3-folds, 1.2 to 3.1-folds, and 1.1 to 2.0-folds by IMA-1, IMA-2, IMA-3, IMA-4 and IMA-5 respectively, compared to control (untreated cells) that were set to 1.0 (Figure 3.7). *CviI* gene is well known for encoding the autoinducers in *C. violaceum*. Therefore, downregulation of this gene would imply that quorum sensing is inhibited, and upregulation of this gene would imply that more autoinducers are then available to bind to the receptor protein CviR. Results shown in Figure 3.7 indicate that treatment with IMA-1, IMA-2, IMA-3 and IMA-4 all significantly decrease the expression of this gene upon treatment. IMA-1 had the greatest decrease in gene expression of *CviI* where the fold change decreased from 1 to 0.16. IMA-2, IMA-3 and IMA-4 had decreases in expression from 1 to 0.20, 0.38, 0.41, respectively. This result provides a clear indication that the compounds inhibited quorum sensing as these four compounds significantly decrease expression of *CviI* implying that the number of autoinducers generated and present significantly decreases with treatment of these compounds. IMA-5 was the only compound with weaker effect on expression of this particular gene compared to other compounds.

Genes *VioA*, *VioB*, *VioD* and *VioE* are all part of the *vioABCDE* promotor that becomes activated once CviR complex (with autoinducer bound) binds to DNA. This promotor is known for initiating several processes linked to quorum sensing including production of the violacein pigment. Figure 3.7 illustrates expression fold change in *VioA*, when *C. violaceum* is treated with the five test compounds, result in least decrease in expression levels. On the other hand,

downregulation of gene expression for *VioD* was the most significant among all the genes. Also, the expression fold change in *VioB* and *VioE* are significantly downregulated when cells were treated with these compounds.

These results are promising as they demonstrate that the compounds are effective in inhibiting quorum sensing at a gene level. Although not all genes had significant changes in expression, it is important to note that none of the compounds caused an upregulation of any of the genes tested. IMA-1 is the most promising compound at downregulating gene expression because it had the greatest downregulation of all the genes compared to the other compounds. This observation is in agreement with previous results illustrated in this study that IMA-1 is the most effective compound of the five test compounds. The results from this experiment also confirm previous results and conclusions regarding IMA-5 that it is the weakest compound, although still effective to a certain extent.

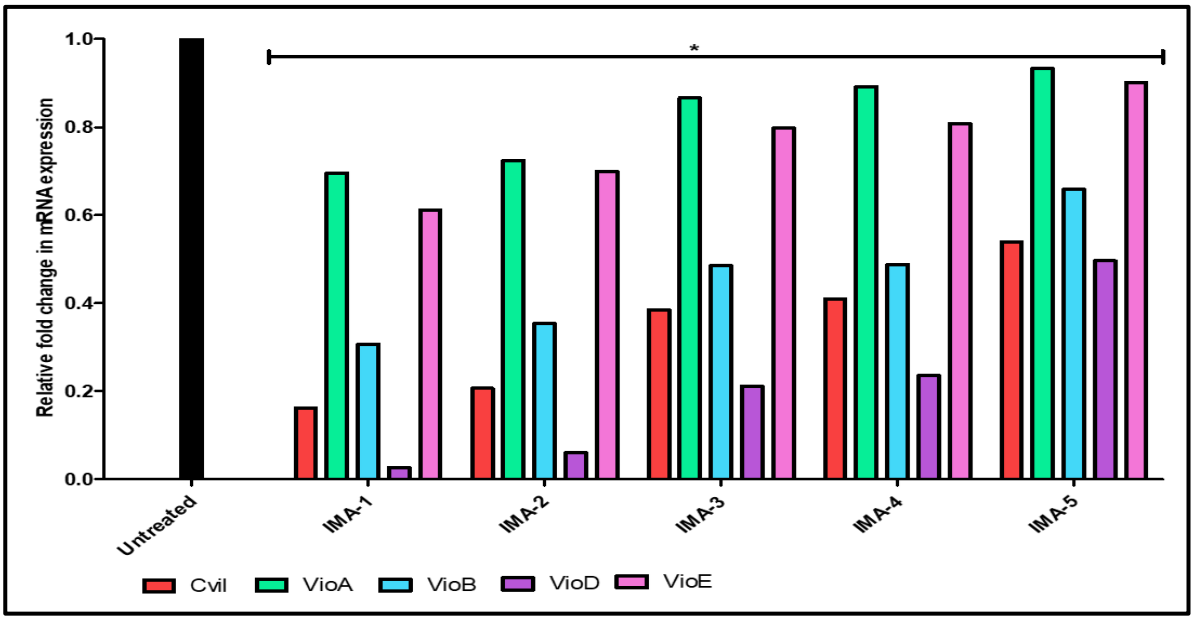


Figure 3.7: Relative gene expression of quorum sensing genes in *C. violaceum* when exposed to MIC values of imidazole compounds. The gene expression was normalised to the housekeeping genes and the expression is compared to that of untreated cells. Data are presented from three independent experiments using means. *p<0.05, two-way ANOVA.

CHAPTER 4 DISCUSSION

The development of drug resistance in bacteria has become an increasing problem in clinical and non-clinical settings for the past few decades. Besides several reasons for drug resistance, one of the major reasons is overuse of traditional antibiotics with limited drug targets. This has created an urgent need to identify novel drug targets to combat drug resistant bacteria (Yoo & Lee, 2016; Zhuang *et al.*, 2019). Two such emerging drug targets have been identified as biofilm and quorum sensing (Ahmad *et al.*, 2016). Biofilm has been established as very problematic in clinical settings due to biofilm enabling bacterial communities to attach themselves to surfaces and thrive in hostile environments (Hassan *et al.*, 2019). Quorum sensing is cell to cell communication used by bacteria and is characterised as a cell density dependant process, (Zhao *et al.*, 2017) which allows the bacteria to behave as a multicellular organism and attack a host cohesively (Asfour, 2018).

Autoinducers are small molecules produced by bacteria that are transported extracellularly. The autoinducers indicate a high cell density as they accumulate extracellularly and once the concentration of autoinducers has reached a threshold, quorum sensing is activated (Papenfort & Bassler, 2016). Previous studies have indicated that quorum sensing in *C. violaceum* is vital for the development of pathogenesis in this bacterium and therefore anti-quorum sensing compounds have potential to decrease pathogenicity of this bacterium and possibly many other bacterial species (Stauff & Bassler, 2011). Potential anti-quorum sensing compounds have been known to mimic the structure of autoinducers (Deryabin & Inchagova, 2017). In this study, five novel imidazole-based compounds were tested for their anti-bacterial and anti-quorum sensing activities using different *in silico* and *in vitro* approaches.

Previous research has shown that the most effective antibiotic against *C. violaceum* is imidazole-based synthetic compounds, which have been also been found to inhibit biofilm in a variety of bacterial species (Meher-Homji *et al.*, 2017; Steenackers *et al.*, 2011). The imidazole aromatic ring present in the compounds is known to provide an opportunity for a variety of drug targets to be used as it creates an electron-rich environment (Heerding *et al.*, 2001; Slassi *et al.*, 2019). The imidazole compounds used in this study are therefore hypothesized to possess anti-bacterial as well as anti-quorum sensing activities against *C. violaceum*. Results from *in silico* studies provide preliminary indications of how effective the compounds were at binding to the target protein (CviR) and how the compounds interacted or behaved with the protein.

Molecular docking results revealed that IMA-1 and IMA-3 had the most effective binding scores. Molecular docking results also offered the interactions that the compounds created with

the amino acid residues of the protein. Previous research has indicated that amino acids Tyr72, Trp76, Tyr80 and Asp89 are among the highly conserved amino acids in the CviR protein within the binding pocket (Gnanendra *et al.*, 2012). Of these conserved sites, Trp72, Tyr80 and Asp89 are known to be crucial for autoinducer binding (Gnanendra *et al.*, 2012). The results of this study indicated that all the compounds interacted favourably with the vital amino acid residues from the CviR protein in autoinducer binding (Figure 3.1). A previous study tested natural compounds as potential anti-quorum sensing compounds and found that their compounds had the same interactions with the same amino acids used in this study (Ravichandran *et al.*, 2018b). Another study tested imidazole molecules, similar to the ones used in this study, against LuxR quorum sensing system (Sabbah *et al.*, 2011). This study produced similar results to the results generated in this study (Sabbah *et al.*, 2011). It is advantageous to see that the compounds used in this present study show similar interactions with what has been shown in previous studies.

In silico results are evident of the imidazole ring this being of vital importance as interactions such as pi-pi stacking and pi-cation formation occur due to the ring (Liao *et al.*, 2013). Advantageously, the results from this study have the same interactions in previous studies as it indicates that the compounds are able to interact with the important conserved amino acids in the binding site (Liao *et al.*, 2013). This indicates that the compounds could indeed mimic the autoinducer binding and prevent quorum sensing. Results also indicated a lack of Van der Waals interactions and several hydrogen bond formations. This is confirmed in Table 3.2 where the energy of Van der Waals forces is the energy that contributes least to the free binding energy. This presents the notion that the compounds create strong bonds with CviR and therefore form a stable complex.

The stability of complexes formed with IMA-1, IMA-2, IMA-3, IMA-4, IMA-5 and CviR was determined by performing molecular dynamic simulations. Comparative structural dynamics indicated that all formed complexes are stable in nature with IMA-1-CviR confirmed as most stable with the greatest free binding energy. This result adds to the integrity of the formed complex between IMA-1 and CviR. Previous research indicated that once an autoinducer binds to CviR, a conformational change occurs, and greater stability is achieved (Papenfort & Bassler, 2016). Results from this study do indeed indicate the same finding as structural changes are observed and greater stability of the complex is achieved with all compounds (Figures 3.1-3.3). These results provide important indications of how the compounds will behave in an *in vitro* system. Based on the promising results from *in silico* studies, *in vitro* studies were conducted to further test how the compounds influence growth and quorum sensing in *C. violaceum*. The

bioreporter strain ATCC12472 was used in this study as it has been widely used in research and uses CviI/CviR homologues for quorum sensing (Devescovi *et al.*, 2017). All the five imidazole compounds were tested for their anti-bacterial and anti-quorum sensing activities in *C. violaceum*.

For susceptibility, MICs and MBCs were determined for all the compounds and *in vitro* results clearly replicate *in silico* findings, with IMA-1 being the most active compound and IMA-5 being the least active compound. Besides being significantly effective against the sessile cells, these compounds were found to have high anti-bacterial activity against biofilm formation. Previous studies have reported the anti-bacterial activity of different types of synthetic and semi-synthetic compounds against sessile bacteria and biofilm formation (De Lima Pimenta *et al.*, 2013). In our previous study, we reported high anti-bacterial and anti-quorum sensing activities of citral and its semi-synthetic derivatives against *C. violaceum* (Batohi *et al.*, 2021). Furthermore, imidazole and benzimidazole have been reported to occupy central position in drug development due to the nitrogen-containing heterocycles rings (Gaba & Mohan, 2016). Due to the versatile nature of imidazole compounds, their clinical application is diverse as is evident from their anti-bacterial, anti-fungal, anti-viral anti-parasitic, anti-cancer, and other therapeutic uses (Gaba & Mohan, 2016). Results of this study could add to findings on the use of imidazole compounds in treating both planktonic and biofilm bacterial cells.

Biofilm formation has caused incredible amounts of worry and panic in clinical and non-clinical settings (Chang *et al.*, 2019; Di Somma *et al.*, 2020a). The process of biofilm formation is controlled by quorum sensing and therefore has the potential of becoming an emerging drug target (Soojhawon *et al.*, 2017; Williams, 2017). In this study, despite having high anti-bacterial activity against biofilm formation at inhibitory concentrations, it was evident that none of the test compounds at sub-inhibitory concentrations have a significant effect of biofilm formation. These results are in contradiction to previous findings, where essential oils and their individual components, such as carvacrol, were reported to be effective at inhibiting biofilm formation in *C. violaceum* at very low concentrations with overnight treatment (Burt *et al.*, 2014). However, results in another study were in congruent with this study's findings, where natural compounds were reported effective against *C. violaceum's* ability to form biofilm using overnight treatment at high concentrations only (Ravichandran *et al.*, 2018b). There are also studies reporting that imidazole-based compounds that contain chlorine groups are 10 times more effective at inhibiting biofilm (Steenackers *et al.*, 2011). Research also found that imidazole-based compounds with methyl groups improve biofilm inhibition to a lesser extent (Steenackers *et al.*, 2011). As the imidazole compounds in this study also contain such functional groups it can

be concluded that these compounds may be better suited to inhibit biofilm formation. Another novel aspect of this study is characterisation of the link between biofilm formation and quorum sensing, which has not yet been well established (Kamaeva *et al.*, 2014). Therefore, results from this study are exceptionally promising as they provide a link between the compounds used in this study and how they affect both quorum sensing and biofilm formation.

Despite numerous studies reporting anti-bacterial and other therapeutic applications of imidazole compounds, there is a clear gap of applying these compounds as anti-quorum sensing agents (Heerding *et al.*, 2001). The clinically available classical antibiotics aim to treat the symptoms by either inhibiting growth of a pathogen (bacteriostatic) or by killing the pathogen (bactericidal). However, targeting quorum sensing is advantageous as it blocks communication required for the physiology and pathogenicity of bacteria, without placing any survival pressures on the pathogen and therefore have potential to bypass drug resistance. All imidazole compounds at their sub-inhibitory concentrations were able to target quorum sensing at the varying levels. These results are congruent with our previous findings, where different semi-synthetic derivatives were reported to possess anti-quorum sensing activity at lower concentrations and anti-bacterial activity at higher concentrations (Ahmad *et al.*, 2015; Batohi *et al.*, 2021). Previous studies have tested novel compounds, both natural and synthetic, to be potential anti-quorum sensing compounds (Deryabin & Inchagova, 2017; Ghosh *et al.*, 2014; Di Somma *et al.*, 2020a). There are also studies where anti-quorum sensing properties of fruit extracts were determined to reduce percentage of violacein production (Zhu, He and Chu, 2011). Similar to our findings, a previous study on citral and its derivatives to inhibit quorum sensing both qualitatively as well as quantitively were reported (Batohi *et al.*, 2021). In these studies, results presented the same trend that was shown in this present study and this is beneficial as it validates the results from this present study. The results from the quantitative anti-quorum sensing assay in this study are also in line with similar studies performed with natural and synthetic compounds (Ghosh *et al.*, 2014; Zhu *et al.*, 2011). IMA-1 to IMA-4 produced significant anti-quorum sensing activity at MQSIC levels, whereas IMA-5 was the only compound to show least inhibition of quorum sensing at MQSIC levels. However, this result is to be expected as it was seen from *in silico* studies that IMA-5 has the weakest binding score and is less stable as a complex, rendering it the least effective compound out of the five. IMA-5 also produced the smallest zones of inhibition and turbidity during the qualitative anti-quorum sensing assay (as seen in Table 3.4). Furthermore, it can be seen from the results that these compounds at MQSIC were also able to inhibit biofilm formation to some extent,

indicating that the mechanism of these compounds to inhibit biofilm formation can also be linked to quorum sensing inhibition.

Following the effect at protein level, all compounds were studied for gene expression studies on genes involved in the quorum sensing process of *C. violaceum*. Gene expression results indicated that treatment with all compounds, significantly downregulates the expression of the tested quorum sensing genes at varying levels (Figure 3.7). The genes *VioA*, *VioB*, *VioD* and *VioE* are pivotal in the process of violacein production and the pigment violacein is an indication of active quorum sensing (Gnanendra *et al.*, 2012; Ravichandran *et al.*, 2018a; Valones *et al.*, 2009). All tested imidazole compounds down regulated these genes at varying levels, which are then transcribed at protein level as well. Previous studies have also reported down regulation of quorum sensing genes, specifically *CviL, VioA, VioB, VioD* and target genes *VioE* in *C. violaceum* upon treatment with different compounds (Devescovi *et al.*, 2017; Liu *et al.*, 2013).

VioA gene is known to regulate autoinducer synthesis as it encodes for the autoinducer itself (Burt *et al.*, 2014). Therefore, the concentration of autoinducers produced by *C. violaceum* is significantly lower when treated with all compounds, except IMA-5, and thus less autoinducers are present to bind to the CviR protein and initiate quorum sensing. The qPCR results from this study have shown that treatment with all test compounds, have slightly less impact on expression of *VioA* and *VioE* (Figure 3.7). It has been established that *VioA* is pivotal in catalysing the oxidation of tryptophan and this begins violacein synthesis (Kothari *et al.*, 2017). One previous study concluded that treatment of *C. violaceum* with the natural peptide amino acid lysine inhibited quorum sensing but resulted in significantly increased expression of *CviI* and *VioA* (Champalal *et al.*, 2018). This is contradictory to our present study, however many other studies were conducted where *CviI* and *VioA* were not significantly downregulated upon treatment with a variety of compounds, despite these compounds showed anti-quorum sensing properties (Batohi *et al.*, 2021; Burt *et al.*, 2014; Ravichandran *et al.*, 2018a)

Treatment with all compounds significantly down-regulate *VioB* and research has indicated that inhibition of *VioB* leads to total depletion of violacein intermediates and thus prevents production of violacein further (Kothari *et al.*, 2017). From what is said above, results from this study indicate that the five newly synthesised semi-synthetic compounds can inhibit violacein production as the majority of genes involved in violacein production are significantly downregulated. The genes *VioB* and *VioD* are also pivotal in the process of violacein production and the pigment violacein is an indication of active quorum sensing (Kothari *et al.*, 2017). Both

these genes are significantly downregulated by all the test compounds at varying levels, which was directly transcribed at the protein level of quorum sensing inhibition.

From the results of this study, it is clear that the five newly synthesised imidazole compounds can inhibit violacein production as majority of the genes involved in violacein production were inhibited. This provides adequate indication that these compounds can indeed be potential anti-quorum sensing compounds because the production of violacein pigment is directly related to quorum sensing in *C. violaceum* (Kothari *et al.*, 2017; Ravichandran *et al.*, 2018a).

CHAPTER 5 CONCLUSION

Drug resistance is one of the biggest clinical problems of current times. The abuse and misuse of antibiotics lead to evolutionary survival pressures placed on bacteria resulting in genetic mutation and phenotypic development of characteristics such as biofilm formation. Two new drug targets have been identified as biofilm and quorum sensing. Compounds that can target quorum sensing have been hypothesised to not pose the same evolutionary survival pressures on the bacteria and thereby bypasses the risk of developing drug resistance.

This study targeted quorum sensing and biofilm formation using five newly synthesised imidazole compounds against *C. violaceum*. Various *in silico* and *in vitro* studies revealed that the compounds caused growth inhibition at higher concentrations and quorum sensing at lower concentrations. Furthermore, it was also noted that these compounds at higher concentrations can target biofilm formation in *C. violaceum* to varying levels depending upon the compound and concentration used. In addition, the effect of the compounds on the quorum sensing related genes revealed that these compounds downregulated different quorum sensing genes to various extents. This study provides a data to support novel ideas as to how bacterial drug resistance could be evaded by developing molecules that can target quorum sensing and biofilm formation.

5.1. Limitations of This Study

a. Only one strain of *C. violaceum* (strain ATCC12472) was used to test the five test compounds in this study. It would have been beneficial if another *C. violaceum* strain was included that had different properties to the strain used in this study. For example, strain ATCC31532 has a slightly different autoinducer (C6-HSL) to strain ATCC12472 (C10-HSL).
b. All *in vitro* work was performed as overnight treatment and not performed as exposure time intervals. Exposure time intervals would have provided more accurate representation of how the compounds were able to disrupt biofilm formation. This is especially true with the concentrations that were used in the biofilm assay. A more accurate representation of the anti-quorum sensing properties and gene inhibition would have also been shown had exposure been used instead of overnight treatment.
c. The gene CviR that regulates the expression of the cytoplasmic receptor was not tested in this study and it would have been beneficial to determine if the compounds affect this aspect of the quorum sensing system in any way.
d. No specific genes known to assist biofilm formation were tested in the gene expression studies. Therefore, this study provides little insight into how the compounds affect biofilm formation of *C. violaceum*.

e. It was well established that the compounds in this study were specifically designed to disrupt quorum sensing in Gram-negative bacteria. It would have been advantageous to include Gram-positive bacterial species.

5.2. Future Recommendations

a. This study concluded that the five newly synthesised compounds inhibit quorum sensing. Therefore, it would be beneficial to test this *in vivo* to determine how effective these compounds are at disrupting a live infection.
b. Future studies could use a known drug resistant Gram-negative bacterial species such as *P. aeruginosa* to test if these compounds would be effective at treating drug resistant bacteria.
c. *P. aeruginosa* makes use of a different quorum sensing system to *C. violaceum* that is still a homologue of the LuxI/LuxR system. However, the receptors and autoinducers for this system differ greatly from the CviI/CviR system and it would be advantageous to determine the variety of bacterial species the compounds could be effective on.
d. To further test the universality of these compounds, a fungal species such as *Candida* species could be used to determine if the compounds are able to disrupt quorum sensing in resistant and susceptible fungal infections.
e. The mechanisms of anti-bacterial and anti-quorum sensing in this present study were not determined. It would also be beneficial to determine how the compounds disrupt the biofilm.

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7.1. Review article: In Press

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Current Pharmaceutical Design, 2021, *27*, 000-000 1

REVIEW ARTICLE

Quorum Sensing – A Stratagem for Conquering Multi-Drug Resistant Pathogens

Madison Tonkin[1], Shama Khan[1], Mohmmad Younus Wani[2] and Aijaz Ahmad[1,3,*]

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ARTICLE HISTORY

Received: July 05, 2020
Accepted: October 25, 2020

DOI:
10.2174/1381612826666201210105638

Abstract: Quorum sensing is defined as a cell to cell communication between micro-organisms, which enables micro-organisms to behave as multicellular organisms. Quorum sensing enables many collaborative benefits such as synchronisation of virulence factors and biofilm formation. Both quorum sensing, as well as biofilm formation, encourage the development of drug resistance in micro-organisms. Biofilm formation and quorum sensing are causally linked to each other, playing a role in the pathogenesis of the micro-organisms. With the increasing drug resistance against the available antibiotics and antifungal medications, scientists are combining different options to develop new strategies. Such strategies rely on the inhibition of the communication and virulence factors rather than on killing or inhibiting the growth of the micro-organisms. This review encompasses the communication technique used by micro-organisms, how micro-organism resistance is linked to quorum sensing, and various chemical strategies to combat quorum sensing, thereby, drug resistance. Several compounds have been identified as quorum sensing inhibitors and are known to be effective in reducing resistance as they do not kill the pathogens but rather disrupt their communication. Natural compounds have been identified as anti-quorum sensing agents. However, natural compounds have several disadvantages. Therefore, the need for the development of synthetic or semi-synthetic compounds has arisen. This review argues that anti-quorum sensing compounds are effective in disrupting quorum sensing and could, therefore be effective in reducing micro-organism drug resistance.

Keywords: Quorum sensing, multi-drug resistance, quorum sensing inhibitors, biofilms.

1. INTRODUCTION

Infectious diseases are one of the leading causes of human mortality [1]. Micro-organisms such as bacteria and fungi are well known for causing infectious diseases. However, not all species of these micro-organisms are considered pathogenic and lead to human diseases [2, 3]. A novel concern is the dramatic increase in the number of invasive bacterial or fungal infections in recent times. This rise in infection number may be due to several factors, one of them being drug resistance [4, 5]. In the past few decades, pathogenic bacteria, as well as pathogenic fungi, have been reported to develop resistance at an alarming pace [1, 6]. This has highlighted several major concerns, such as the development of drug-resistant infections in a clinical setting. This has emphasised that previously determined effective antibiotics are losing their effectiveness. Another concern is the dramatic increase in the number of pathogens that have acquired novel drug resistance [4, 7]. Increasing drug resistance among fungal pathogens is gaining concern as the number of available antifungal drugs is limited. Biofilms are known to be among the key mechanisms for drug resistance. Biofilms are multicellular, a surface-bound natural defence mechanism self-produced by micro-organisms. This protective layer is impenetrable to the current anti-microbial drugs, meaning the current drugs are unable to reach the micro-organisms located within the biofilm [8, 9].

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In view of these concerns, scientists are combining different strategies to prevent the creation of drugs that are microbiocidal or microbial static, with the hope of decreasing drug resistance. One such strategy, which is being widely researched, is quorum sensing. Quorum sensing is believed to greatly discourage the development of drug resistance in micro-organisms. Novel compounds areneeded to be identified to effectively target the quorum sensing cascades and biofilm formation. Research has shown that biofilm formation is a key group behaviour that is controlled by quorum sensing and the link between quorum sensing and biofilm formation is vital in the pathogenesis of micro-organisms [10].

Quorum sensing is a cell to cell communication used by many micro-organisms for intraspecies and interspecies communication [6, 11]. Various processes are regulated by quorum sensing, which assists micro-organisms in becoming pathogenic. These processes include the synchronisation of behaviours, the expression of virulence factors, the ability of the bacteria to attach to the surfaces, and the excretion of nutrient-sequestering compounds [10, 12]. Autoinducers are small molecules or peptides produced by the micro-organisms and are pivotal in initiating quorum sensing [12]. Autoinducers accumulate extracellularly, and once a certain threshold is reached, they bind to a receptor, which activates genes to initiate quorum sensing [13]. Targeting quorum sensing is beneficial as inhibiting quorum sensing will not have microbicidal or micro-static results but rather inhibit the ability of the micro-organisms to act collectively. This is hypothesised to reduce the ability of the micro-organisms to develop drug resistance [6, 14].

Several natural, semi-synthetic, and synthetic compounds, nanoparticles, peptides, *etc.,* have been identified as potential drugs

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7.2. Research Article: Submitted

Applied Microbiology and Biotechnology

Quorum Sensing and Biofilm Disrupting Potential of Imidazole Derivatives in Chromobacterium violaceum Using Anti-Microbial and Drug Discovery Approaches

--Manuscript Draft--

Manuscript Number:	AMAB-D-21-00226
Full Title:	Quorum Sensing and Biofilm Disrupting Potential of Imidazole Derivatives in Chromobacterium violaceum Using Anti-Microbial and Drug Discovery Approaches
Article Type:	Original Article
Section/Category:	Applied microbial and cell physiology
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Funding Information:	National Research Foundation (RDYR180418322304) — Dr Aijaz Ahmad
	University of the Witwatersrand, Johannesburg (AZMD019) — Dr Aijaz Ahmad
Abstract:	Populations of drug resistant bacteria have increased at an alarming rate in the past few decades. The major reason for increasing drug resistance is the lack of new antibiotics and limited drug targets. It has therefore been a vital task to develop new antibiotics with different drug targets. Two such targets are biofilm formation and quorum sensing. Both biofilm and quorum sensing are inter-related processes and play a major role in physiological and pathogenesis processes. In this study, five novel imidazole derivatives (IMA-1–IMA-5) were synthesised and tested for their anti-bacterial and anti-quorum sensing activities against Chromobacterium violaceum using different in silico and in vitro techniques following the standard protocols. In silico results revealed that all compounds were able to effectively bind to and interact sufficiently with the target protein CviR. In silico results also revealed that the compounds generated favourable structural dynamics implying that the compounds would be able to effectively bind to CviR and inhibit quorum sensing. Susceptibility results revealed that IMA-1 is the most active of all the derivatives against both planktonic cells and biofilms. Qualitative and quantitative evaluation of anti-quorum sensing activity at sub-inhibitory concentrations of these compounds also revealed high activity for IMA-1. Down regulation of most of the quorum sensing genes when cells were treated with the test compounds affirmed the high anti-quorum sensing activities of these compounds. The results from this study are promising and urges on

7.3. Abstract for Poster Presentation

Faculty of Health Sciences Research Day and Postgraduate Expo, University of Witwatersrand

In silico and *in vitro* inhibition of quorum sensing and biofilm formation in *Chromobacterium violaceum* using semi-synthetic compounds

Madison Tonkin[1], Dr Shama Khan[1], and Dr Aijaz Ahmad[1, 2].

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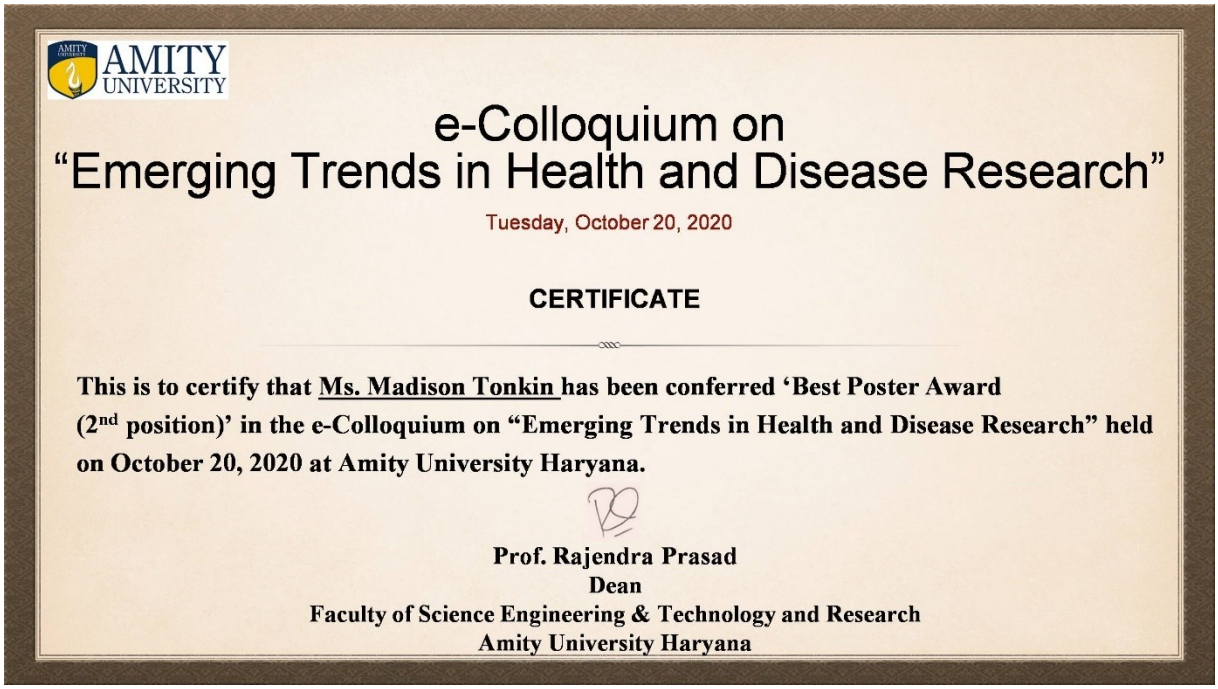
[2]Infection Control, Charlotte Maxeke Johannesburg Academic Hospital, National Health Laboratory Service, South Africa

Infectious diseases have been problematic to human health for centauries and are the leading cause of human mortality. As of recent, one of the biggest problems facing the medical field is antibiotic resistant bacteria. Bacteria have developed resistance from the exploitational use of antibiotics and therefore new drugs and drug targets will need to be identified. One such emerging drug target is quorum sensing, which controls many important aspects of bacteria such as communication, production of virulence factors and formation of biofilms. This study aims to determine the antibacterial and anti-quorum sensing of five newly synthesised imidazole amide compounds using a bioreporter species of *Chromobacterium violaceum*. *In silico* docking results have provided which compounds have the best biding affinity for the target protein and therefore are the most effective at inhibiting quorum sensing. These results directly correlate with the MIC results as it was shown in both methods that compound 1 was the most effective compound. Anti-quorum sensing activity of the compounds was determined by both the agar plate method and the broth dilution method. In both results, MIC and MQSIC concentrations effectively inhibited quorum sensing while concentrations sub MQSIC did not. Compounds 4 and 5 were the most effective at inhibiting quorum sensing. Biofilm formation was tested by means of crystal violet and at MIC concentrations, the compounds are effective at inhibiting biofilm formation. There is a gradual decrease of biofilm formation at sub-MIC concentrations. These results are promising as it shows that the 5 newly developed compounds could indeed act as quorum sensing inhibitors and this is promising for the development of novel antibiotics targeting new targets such as quorum sensing and biofilm formation. The next step in this research is to determine if these compounds have any genetic impact on quorum sensing.

7.4. Abstract for Poster Presentation and Mini Talk:

e-Colloquium on "Emerging Trends in Health and Disease Research" held by Amity University, Haryana, India on 20 October 2020.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e-Colloquium on
"Emerging Trends in Health and Disease Research"

Tuesday, October 20, 2020

CERTIFICATE

This is to certify that Ms. Madison Tonkin has been conferred 'Best Poster Award (2nd position)' in the e-Colloquium on "Emerging Trends in Health and Disease Research" held on October 20, 2020 at Amity University Haryana.

Prof. Rajendra Prasad
Dean
Faculty of Science Engineering & Technology and Research
Amity University Haryana

Targeting quorum sensing and biofilm formation in *Chromobacterium violaceum* with semi-synthetic compounds using *in silico* and *in vitro* techniques

Madison Tonkin[1], Dr Shama Khan[1], and Dr Aijaz Ahmad[1, 2].

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[2]Infection Control, Charlotte Maxeke Johannesburg Academic Hospital, National Health Laboratory Service, South Africa

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by both the agar plate method and the broth dilution method. In both results, MIC and MQSIC concentrations effectively inhibited quorum sensing while concentrations sub MQSIC did not. Compounds 4 and 5 were the most effective at inhibiting quorum sensing. Biofilm formation was tested by means of crystal violet and at MIC concentrations, the compounds are effective at inhibiting biofilm formation. There is a gradual decrease of biofilm formation at sub-MIC concentrations. These results are promising as it shows that the 5 newly developed compounds could indeed act as quorum sensing inhibitors and this is promising for the development of novel antibiotics targeting new targets such as quorum sensing and biofilm formation. The next step in this research is to determine if these compounds have any genetic impact on quorum sensing.

7.5. Ethical Clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG | HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Ref: W-CP-200320-4

20 March 2020

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator:	**Miss Madison Tonkin (student no 1358899) and Dr Shama Khan**
Supervisor:	**Dr Aijaz Ahmad**
Faculty:	Health Sciences
School:	Therapeutic Sciences
Department:	Clinical Microbiology and Infectious Diseases
Project title:	**In silico and in vitro inhibition of quorum sensing and biofilm formation in chromobacterium violaceum using semi-synthetic compounds.**
Reason:	For an in vitro laboratory study for MSc in Medicine, not part of a project that already has ethics approval. Using in vitro using bacteria cultures purchased from ATCC and strain of bactria stored at the Department of Microbiology and Infectious Diseases.

Dr Clement Penny

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi,Iain Burns & Mapula Ramaila

7.6. Recipe for LB Broth and LB Agar Plates

LB Broth

LB broth was used for all in vitro experiments.

10 g peptone powder (Merck)

5 g yeast extract

5g NaCl

The powdered reagents were added to a sterile bottle. Deionised, sterile water was added to the bottle to reach a final volume of 1 L. The bottle was autoclaved at 121 ˚C for 20 minutes. Once sterile, the bottle of LB broth was kept at 4 ˚C and allowed to come to room temperature before use.

LB Agar Plates

10 g peptone powder (Merck)

5 g yeast extract

5g NaCl

1.5 g agar

The powdered reagents were added to a sterile bottle. Deionised, sterile water was added to the bottle to reach a final volume of 1 L. The bottle was autoclaved at 121 ˚C for 20 minutes. The LB agar was cooled to 45 ˚C and poured into petri dishes. The LB agar was left to set under a UV light to ensure sterility.

7.7. Recipe for 8.5% Saline Solution

An 8.5% saline solution was used for the duration of this study when making a 0.5 McF solution. To make the 8.5% saline solution, 8.5 g of NaCl was added to 1 L of ultra-pure water in a sterile bottle. The bottle was autoclaved and kept at 4 ˚C until needed.

7.8. Recipe for Agarose Gel and TAE Buffer

1 x TAE Buffer

A 50 x TAE buffer was prepared.

242 g Tris base

15.5 g EDTA

57 ml glacial acetic acid

All the reagents were added to a sterile bottle and dissolved in 1 L of ultra-pure water. The assay required 1 x TAE buffer to be used, therefore, the 50 x TAE buffer was diluted by adding 20 ml of the 50 x TAE buffer to 980 ml ultra-pure water and mixed well.

2% Agarose Gel

To determine if the primers were functional, conventional PCR was performed and the products were run on a 2% agarose gel.

For the PCR products, a gel of 100 ml was made by adding 2 g agarose powder to 100 ml 1 x TAE buffer. To this, 3 µl of ethidium bromide was added. The solution was microwaved until the agarose had completely melted. The gel was poured into a tray with a comb and set to solidify at room temperature.

7.9. Purity and Integrity of RNA and cDNA

The quality and concentration of RNA was measured following RNA extraction via nanodrop. This was used in cDNA synthesis. The generated cDNA was also measured for integriy and concentration using nanodrop because the cDNA was used in RT-qPCR.

Table 7.1: RNA and cDNA integrity, purity and concentration following RNA extraction and cDNA synthesis.

Treatment of *C. violaceum*	RNA Purity A260/A280	RNA Concentration (ng/µl)	cDNA Purity A260/A280	cDNA Concentration (ng/µl)
IMA-1	2.04	132.7	1.81	1964.9
IMA-2	2.06	76.0	1.77	2538.3
IMA-3	1.81	108.6	1.79	1916.6
IMA-4	1.95	83.3	1.78	2090.3
IMA-5	2.00	336.7	1.81	2036.1
Untreated	2.07	48.4	1.78	2284.4

7.10. Turnitin Report

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