Erythromycin, Roxithromycin, Azithromycin and Clarithromycin targeting the SARS-COV-2 spike protein: A molecular dynamics study

Eustacia Naidoo

2022

A research thesis submitted to the school of Health sciences, University of KwaZulu-Natal, Westville Campus, in fulfilment for the degree of Masters in Medical Sciences (Pharmaceutics)

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This is a thesis in which the chapters are written as a set of discrete research publications, with an overall introduction and final summary.

This is to certify that the contents of this thesis are the original research work of Miss Eustacia Naidoo. As the candidate's supervisor, I have approved this thesis for submission.

Supervisor: Dr Andile K.M Faya

Signed: Date: 27/07/2022

THESIS ABSTRACT

The Covid-19 global pandemic has taken the lives of many people and has left the health sector in ruins. There is a desperate need for the scientific and medical community to pose new strategies to combat this virus. One way is repurposing of already existing drugs for the safe treatment of Covid-19. In view of the above facts, the present research project work was planned and aimed to identify the mechanism of inhibition of macrolides by evaluating them *in silico* against the SARS-CoV-2 spike protein. We performed molecular dynamics and molecular docking studies on the following macrolide antibiotics: azithromycin, erythromycin, clarithromycin and roxithromycin, and we compared these with the results achieved from our controls (Ivermectin and Remdesivir). Our ligands were prepared using chemdraw and chimera software. Protein data bank was used to retrieve the four receptors: 3CLpro (6LU7), native human ACE2 (1R42), RdRp (6M71) and spike RBD-ACE2 (6LZG)). AutoDock Vina software was used to perform all docking experiments. Fitting analyses were performed using PyMOL and Biovia Discovery Studio. GROMACS was used for molecular dynamics studies to determine RMSD (Root Mean Square Deviation) and RMSF (Root Mean Square Fluctuation) values. We then went on to determine the intermolecular hydrogen bonding present, as well as the distances of the ligand whilst inside the pocket. Our findings revealed that all the macrolide antibiotics portrayed similar results with each other, as well as with each of our controls (Ivermectin and Remdesivir). Each of the macrolide antibiotics showed strong binding with each of the four receptors. However, azithromycin showed the greatest binding potential (-7.9 kcal/mol) relative to the two controls (Ivermectin (-10.4 kcal/mol) and Remdesivir (-8.5 kcal/mol)). Azithromycin-ACE2 complex showed the least deviation to the ACE2 protein and is therefore the most similar. The average RMSF values shows that there are potential interactions of azithromycin with the receptor protein (ACE2). Covid-19 emerged in 2019, and to this date, 2022, there is no known cures. There have been vaccines like Johnson and Johnson and Pfizer which have been tried and tested and has shown to reduce severity of disease and minimize fatality. However, due to the new emerging strains, there is a continuous need for new therapeutic interventions. Azithromycin has also showed similar results to both Ivermectin and Remdesivir (controls), showing great binding affinity for the ACE-2 receptor. Our analysis based on molecular dynamics simulation and MM-PBSA binding free energy calculation suggests that azithromycin, erythromycin, clarithromycin and roxithromycin could serve as SARS-CoV-2 inhibitors, hence an alternative solution to treat COVID-19 upon further clinical validation.

DECLARATION I - PLAGIARISM

I, Eustacia Naidoo, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original work.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been re-written, but the general information attributed to them has been referenced.
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5. This thesis does not contain text, graphics or tables copied from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the references section.

A detail contribution to publications that form part and/or include research presented in this thesis is stated (include published and submitted publications).

Signed:

DECLARATION II - LIST OF PUBLICATIONS

Eustacia Naidoo, Lucy Kiruri, Mbuso Faya (2022) *In Silico* evaluation of selected Macrolides as Sars-Cov-2 inhibitors: Journal of biomolecular structure and dynamics. Impact factor 3.392 (Under review)

Contribution:

- Eustacia Naidoo: lead author and performed all literature reviews, writing of the paper and summing the information, preparation and writing of the manuscript.
- Dr Lucy Kiruri: Assisted with the technical aspects of the manuscript
- Dr Andile K.M Faya: Supervisor

RESEARCH OUTPUT

LIST OF PUBLICATIONS

1. Submitted and under review: Journal of biomolecular structure and dynamics. Impact factor 3.392
   Submission number: 222823196

DEDICATION

- This thesis is dedicated to the Naidoo family: My mom, dad, sister, and boyfriend, as well as my beloved grandparents. I am grateful for all their love and support during my period of study. Their words of wisdom and encouragement have not gone unnoticed.

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- I'd like to thank all my colleagues who had a hand in making my research successful.
- Lastly, I'd like to thank UKZN College of Health Sciences department for the financial support during the course of my Masters degree.

LIST OF ABBREVIATIONS

AZM	Azithromycin
ACE-2	Angiotensin-Converting Enzyme 2
RdRp	RNA-dependent RNA Polymerase
M^{pro}	Viral Main Protease
RBD	Receptor Binding Domain
SARS	Severe Acute Respiratory Syndrome
PHEIC	Public Health Emergency of International Concern
M	Membrane protein
S	Spike protein
E	Envelope protein
rRNA	Ribosomal RNA
tRNA	Transfer RNA
ZAP	Zinc finger anti-viral protein
TNF	Tumour necrosis factor
QSAR	Quantitative Structure-Activity Relationships
MD	Molecular Dynamics
CLQ-OH	Hydroxychloroquine
PBP	Penicillin binding proteins
MMFF	Merc molecular force fields
GPU	Graphics processing unit
MCPB	Metal centre parameter builder
NRP1	Neuropilin-1
ARD	Acute Respiratory Disease
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
GROMACS	GROningen MAchine for Chemical Simulations
CTSB	Cysteine protease cathepsin B
CTSL	Cysteine protease cathepsin L
CNS	Central nervous system
TMPRSS2	Cellular transmembrane serine protease 2
CD147	Cluster of differentiation 147

AMBER	Assisted Model Building with Energy Refinement
MM-PBSA	Molecular Mechanics Poisson-Boltzmann surface area

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CHAPTER 1:
Thesis Introduction

CHAPTER 1

1. Introduction

1.1 Background & Rational

Covid-19 has plagued the world with a highly transmissible respiratory disease which emerged in December 2019 (Zheng, 2020). This respiratory disease was given the name "Coronavirus Disease 2019" (Covid-19), as it was first identified in Wuhan China (Yamin, 2020). Covid-19 is caused by a novel coronavirus that has great structural similarity to the virus that causes Severe Acute Respiratory Syndrome (SARS) yet still containing unique features (Fauci et al., 2020). Covid-19 virus has shown to take the lives of the elderly and people with underlying co-morbidities (Li et al., 2020). This outbreak has now spread worldwide since then (Mullol et al., 2020). On the 30th of January 2020, the World Health Organization (WHO) declared the Covid-19 outbreak a Public Health Emergency of International Concern (PHEIC) (Yan et al., 2020). The Covid-19 outbreak has posed critical challenges for the public health, research, and medical communities (Mbunge, 2020). Since the first Covid-19 outbreak, several experimental vaccines and treatments for Covid-19 have been repurposed, developed and tried, but they have not yet been fully tested for safety or effectiveness (Amawi et al., 2020). Scientists have been debating the origin of SARS-CoV-2 since its discovery. Some speculations include it being a by-product of manipulations in a laboratory (Ciotti et al., 2020).

Due to the health emergency posed and limited timeframe to generate a new drug, azithromycin has been repurposed to be used as a potential treatment (Pani et al., 2020). Macrolides are a class of antibiotics which include drugs like azithromycin, clarithromycin and erythromycin (Dinos, 2017a). Azithromycin is a broad spectrum macrolide antibiotic with a large volume of distribution and long half-life (Damle et al., 2020) Azithromycin has proven to have both immunomodulating and antiviral properties (Vázquez-Laslop and Mankin, 2018). In past *In-vitro* studies, azithromycin demonstrated to have the capacity to reduce pro-inflammatory cytokine production, reduce oxidative stress, as well as modulate T-helper functions. This particular macrolide antibiotic was associated with a reduction in ventilation days and mortality in other viral infections (Echeverría-Esnal et al., 2021). The simulations conducted in this research will allow us to understand the mechanism of inhibition of macrolides, by evaluating them against the spike protein of the SARS-CoV-2.

1.2 Problem identification

The coronavirus disease 19 (COVID-19) is a pathogenic viral infection caused by acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is highly transmissible, which emerged in Wuhan China,

and spread throughout the world (Shereen et al., 2020). Genomic analysis has revealed that SARS-like bat viruses has a phylogenetic relation to SARS-CoV-2, thus making bats a possible primary reservoir (Ye et al., 2020). The rapid human to human transfer has been confirmed globally, even though the intermediate source of origin and transfer to humans is unknown (Umakanthan et al., 2020)

There is a desperate need for the development of highly effective Covid-19 treatment and cure due to the high mortality rate of this disease. In this regard, however, there are additions of some promising chemical entities, such as macrolide antibiotics. These macrolides are currently given as a therapeutic measure to patients who have contracted Covid-19. Having these facts in mind, there is a compelling need for the analysis of the present macrolide antibiotics using molecular modelling studies.

1.3 Research Aims and Objectives

With the devastating impact of Covid-19 on the African continent as well as the biological threat it poses to the global community, it is apparent that the scientific community must devise new strategies, such as repurposing of already existing drugs for the safe and rational treatment of Covid-19. In view of the above facts, the present research project work was planned and the aims and objectives are as follows;

1.3.1 Aims

1. The Aim of this study is to identify the mechanism of inhibition of selected macrolides by evaluating them *in silico* (using molecular dynamics as well as molecular docking studies) against the SARS-CoV-2 spike protein. This paper focuses on the spike protein of SARS-CoV-2, as it is one of the biological characteristics that allow the COVID-19 virus to penetrate host cells and cause infection.

1.3.2 Objectives

1. To employ molecular docking approach in order to evaluate the macrolides binding affinity for the SARS-CoV-2 spike protein.
2. To use molecular dynamics to understand the physical movement of atoms between different macrolides, and their binding affinity to the SARS-CoV-2 active sites

1.4 Novelty and significance of study

Macrolide antibiotics belong to a large family of protein synthesis inhibitors which is of great clinical interest due to their pertinence to human medicine (Dinos, 2017b). This class of antibiotics are used to treat respiratory, skin, sexually transmitted, as well as mycobacterial infections (Patel and Hashmi, 2021).

Since the outbreak of COVID-19 in 2019, there has been a great number of fatalities and thus a desperate need for therapeutic intervention (Khafaie and Rahim, 2020). Due to the lack of time, which is needed to generate a new drug for the treatment of COVID-19, scientists opted for a quick solution, known as drug repurposing (Kotecha et al., 2020).

Scientists have repurposed macrolide antibiotics for the therapeutic intervention of SARS-CoV-2 infections (Kotecha et al., 2020). Hospitals in and around South Africa began using azithromycin for the treatment of the chest-related symptoms seen with the SARS-CoV-2 infections (Adebisi et al., 2021). The exact mechanism by which azithromycin binds to and inhibits the spike protein of SARS-CoV-2 is unknown.

Therefore, we found it necessary to compare the following macrolide antibiotics: azithromycin, clarithromycin, erythromycin and roxithromycin, with the two controls: ivermectin and remdesivir. By conducting this research, we aim to provide new insights that will be useful in the future treatment of COVID-19 related symptoms which focuses on minimizing hospitalization and fatality rates.

1.5 Overview of thesis

This thesis is divided into five chapters. Brief explanation of chapter 1, 2, 3, 4 and 5 are explained below:

Chapter 1

Thesis introduction and novelty and significance of study

Chapter 2

Focuses on the historical background, Viral entry and life cycle, repurposing of already existing antibiotics, as well as the computational approaches used in this research.

Chapter 3

This chapter focuses on the methods used in this research. Molecular Docking, Molecular Dynamic (GROMACS engine is used) Simulations.

Chapter 4 (Research paper)

This chapter is presented in the required format of the journal and is the final version

Chapter 5

This chapter provides general conclusion of the overall thesis and future recommendations for future studies.

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CHAPTER 2:
Literature Review

CHAPTER 2

2. Introduction

2.1 Historical background

The cause of Coronavirus disease (Covid-19) is severe acute respiratory syndrome 2 (SARS-CoV-2) (Meehan et al., 2020). This disease has taken more than 6.33 million lives to date, worldwide. A vast majority of the patients presenting with this virus required hospitalization, or, had experienced severe life-threatening complications. Some of these life-threatening symptoms include Acute Respiratory Syndrome (ARDS) (Guven et al., 2021). ARDS may even result in many organs collapsing (Guven et al., 2021). Since SARS-CoV-2 was a new virus, there were very few antiviral drugs that were repurposed for the treatment of Covid-19 (eg. Remdesivir- which is an antiviral drug used for the treatment of hepatitis C) (Simonis et al., 2021). In order to generate/design prophylactic or possible therapeutic strategies for SARS-CoV-2 infections, more knowledge is needed on the underlying Covid-19 pathophysiology. In order for SARS-CoV-2 to infect a human host, it has to bind to the cell surface protein ACE-2 (angiotensin-converting enzyme 2) through the receptor binding domain (RBD) of its spike (S) protein (Wang et al., 2020b). TMPRSS2 (cellular transmembrane serine protease 2) is needed for the priming of the virus S protein (Bestle et al., 2020). It is also noted that entry into the cell may also depend on (although not likely essential) endosomal/lysosomal cysteine proteases cathepsin B and L (CTSB, CTSL). Since SARS-CoV-2 contains a unique (as compared to other coronaviruses) furin cleavage site in the S protein, it was recently found that furin protease may also be involved in the infection process (Johnson et al., 2020) (Figure 2.1). It was also noted that the cellular receptor Neuropilin-1 (which binds furin-cleaved substrates) potentiates the virus infectivity (Cantuti-Castelvetri et al., 2020). It also provides a pathway into the Central Nervous System (CNS). The putative alternative receptor (CD147) is expressed in high levels in the brain, and SARS-CoV-2 may possibly utilize this receptor to infect the cerebral nervous system (Qiao et al., 2020).

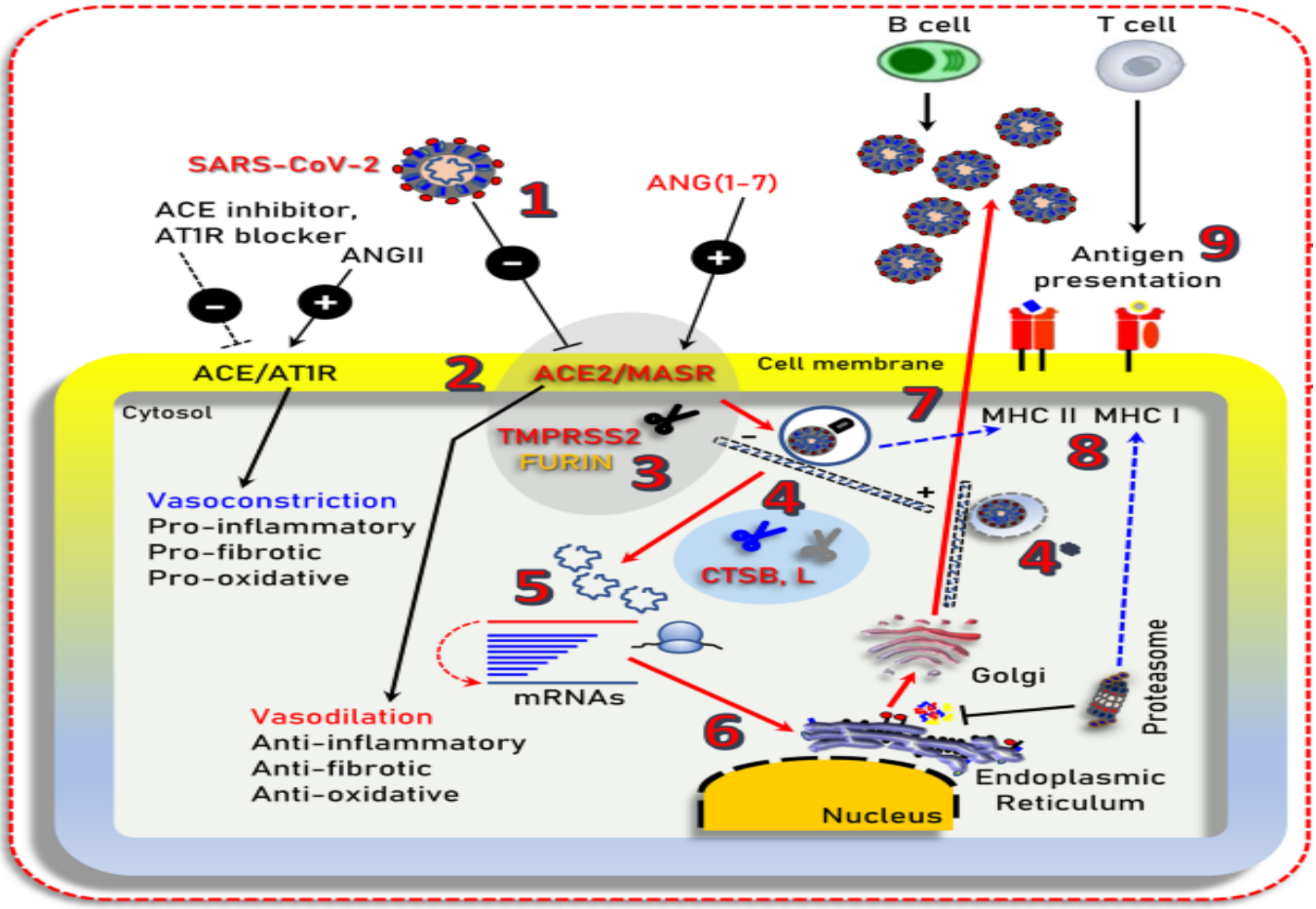

Figure 2.1. Illustration depicting the main cell signalling axes as well as other cellular components like furin and Transmembrane serine protease 2 which are involved in the SARS-CoV-2 infectivity, endocytosis and replication (Trougakos et al., 2021) [permission obtained].

2.2 Virus entry into host cells

The severity of infection and pathogenesis is determined by the binding of CoV to the host cell receptor, which is said to be the initial step of viral infection (Kirtipal et al., 2020). Viral entry into target cells is facilitated by the Spike (S) protein of the coronavirus (Hoffmann et al., 2020). This occurs when the S1 subunit of the S protein binds to a cellular receptor, which then facilitates the viral attachment to the surface target cells(Hoffmann et al., 2020). The functional role of the S2's subunit is the fusion of the virus to the host cell (Huang et al., 2020b). (Mishra and Nandi, 2021) states that the most critical factor for the viral entry, replication, maturation, and infection, is the binding of the Receptor-Binding Domain (RBD) of the S protein to the ACE-2 cell receptor. ACE2 receptors are widely distributed in the respiratory tract, such as in the epithelial cells of the trachea, bronchi, and alveoli (Liu et al., 2011) (Figure 2.2), they are also found in the alveolar monocytes and macrophages (Kuba et al., 2005). When the SARS-CoV-2 attacks these epithelial cells, mature virions are released to attack other new target

cells (Qinfen et al., 2004). There are also a variety of other susceptible targets to SARS-CoV-2, such as the endothelial cells of arteries and veins, cerebral neurons, immune cells, and tubular epithelial cells of the kidneys, as the ACE2 is also diffusely expressed in these cells (Gu and Korteweg, 2007, Guo et al., 2008). In anti-SARS CoV drugs studies, researchers have discovered small molecules which prevent SARS-CoV S-protein from binding to ACE-2 as well as the fusion with the host cell membrane, to avoid viral infection (Zhang et al., 2020).

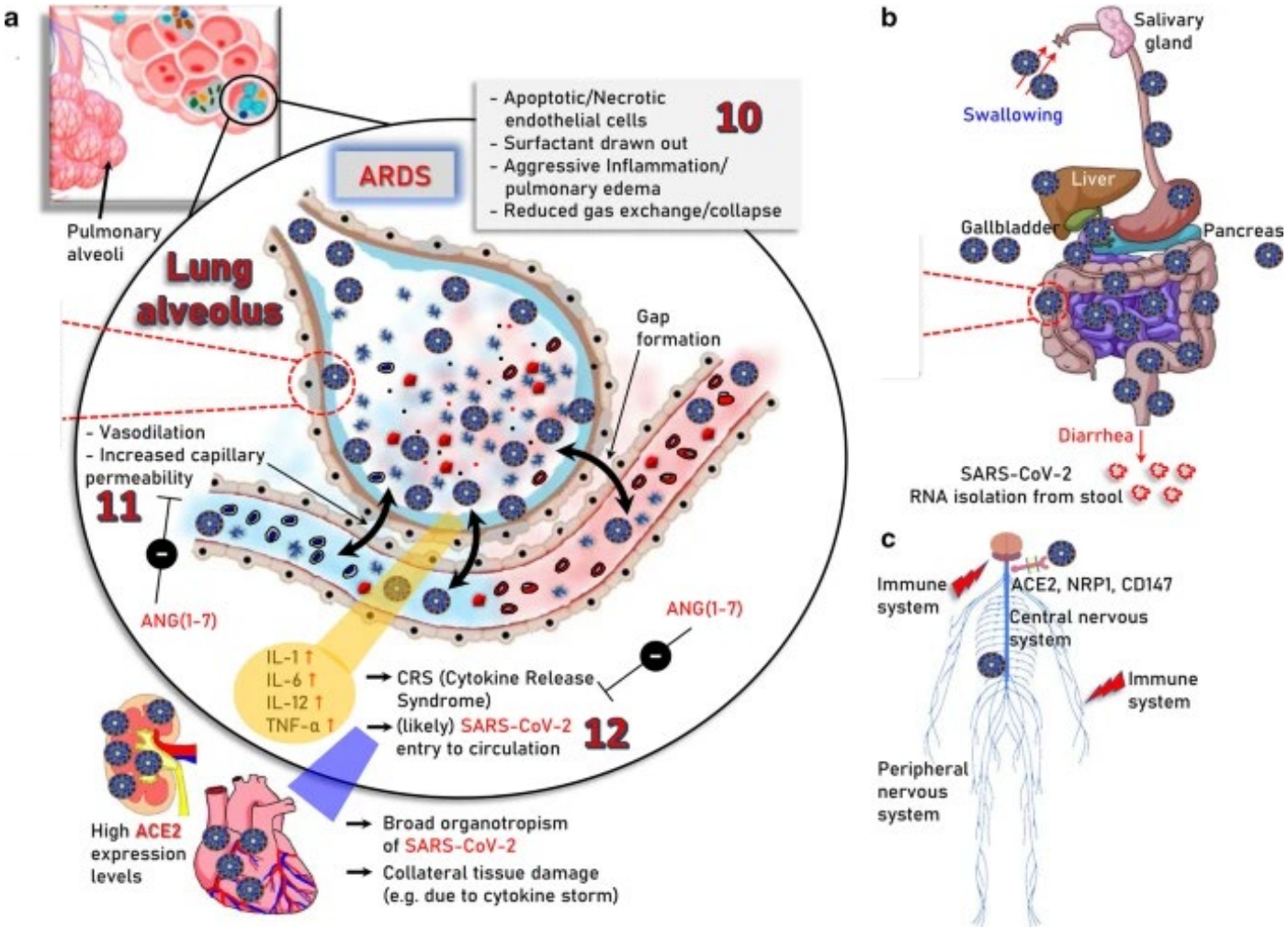

Figure 2.2. Illustrates the major infection routes and severe COVID-19 pathologies as well as the molecular pathways involved in the SARS-CoV-2 infection in humans (Trougakos et al., 2021) [permission obtained].

2.3 Cell entry receptors: Lessons from other respiratory viruses:

It was discovered that these viruses are subject to high rates of mutations, thus allowing them to change their binding affinity or specificity for a specific receptor (a low affinity interaction with the receptor

results in mild respiratory tract illness) (Scialo et al., 2020).

2.3.1 Life cycle of SARS-CoV-2 :

Step 1: This is where host cell recognition occurs. The spike proteins bind to specific host cell surface receptors (this is located in the host cell membrane) (Malik, 2020)

Step 2: There is one of two processes by which the virion enters: (2a) Membrane fusion- this is where the RNA genome of the virus gains access to the cytosol due to fusion of cellular membranes (Benton et al., 2020). (2b) Endocytosis- the receptor-bound virus is enveloped by the cell membrane and enters the cytosol within a vesicle (Glebov, 2020)

Step 3: Following either process, the viral RNA genome is released into the cytoplasm.

Step 4: This is where uncoating of the RNA occurs.

Step 5: A replicase gene on the RNA strand is translated into two replicase polyproteins. This occurs in the host cytoplasm (Kamitani, 2020).

Step 6: The polyproteins are further processed by the viral enzymes that break down proteins (proteinases) to yield individual replicase proteins.

Step 7: This allows for the production of full-length negative-stranded RNA, which will then serve as a template for positive-stranded virion genomic RNA.

Step 8: The full-length negative-stranded RNA is transcribed to produce mRNAs.

Step 9: These shorter mRNAs code for the structural proteins and non-structural accessory proteins, during translation.

Step 10: The newly produced plus-strand viral genomic RNA as well as non-structural and structural proteins are translocated to assembly sites between endoplasmic reticulum and golgi apparatus.

Step 11: Here, the virions assemble.

Step 12: Virions start to mature, bud off from the golgi membranes as vesicles.

Step 13: These vesicles are translocated to the host cell membrane.

Step 14: Here, they fuse with the host cell membrane and are released into the extracellular space. The host cell doesn't rupture in this process, and is termed "nonlytic exocytosis" (Frazão et al., 2020).

2.4 Structural considerations of SARS-CoV-2

The subfamily of coronaviruses belongs to *Coronavirinae, in the family of Coronoaviridae and the subfamily consists of four genera, namely: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus* (Wang et al., 2020a). The genome of CoVs is larger than any other RNA virus (Sofi et al., 2020). The capsid outside the genome is formed by the Nucleocapsid protein (N), the genome is further packed by an envelope (this is associated with three structural proteins : Membrane Protein (M), Spike protein (S) and Envelope protein (E)) (Brian and Baric, 2005). The spike glycoprotein trimmer (S) (that can be found in all CoVs) and the hemagglutinin-esterase (ES) (that's found in specific CoVs); are the two different types of spike proteins that have been discovered in CoVs (Belouzard et al., 2012). There are polybasic cleavage site (PRAR/S) between the subunits of the S protein (S1 and S2 subunits), these are found in some CoVs; including SARS-CoV and SARS-CoV-2. This allowed for digestion by host furin-like protease during the viral replication process (Bosch et al., 2003), and contributed in determination of host viral infectivity (Nao et al., 2017). Understanding the structural relations of SARS-CoV-2 is a step towards aiding researchers in targeting these structures as well as steps in their reproductive life cycle, for the therapeutic intervention of SARS-CoV-2, such as drug repurposing.

2.5 Repurposing of existing antibiotics against SARS-CoV-2

Drug repurposing, also known as drug repositioning, is an approach in drug discovery for which known, existing drugs are found new medical uses (Langedijk et al., 2015). Among others, antibiotics are one of the pharmacological agents being investigated as potential anti-SARS-CoV-2 activities (Glebov, 2020). These antibiotics are either used to treat bacterial infections co-existing with the covid-19 infection, or being used for its anti-viral properties (Yacouba et al., 2021a). Some of the antibiotics under investigation for repurposing include macrolide antibiotics, glycopeptides, tetracyclines, fluoroquinolones, aminoglycosides, cephalosporins:

2.5.1 Macrolide antibiotics (azithromycin, clarithromycin, roxithromycin and erythromycin)

Macrolides have a large molecular size and belong to a class of broad-spectrum antibiotics (Vázquez-Laslop and Mankin, 2018). Other examples include Erythromycin and Roxithromycin.

Other than antibacterial properties, macrolides also possess considerable antiviral properties (Poddighe and Aljofan, 2020). Azithromycin was shown to be frequently used for the treatment of Covid-19 infection. It is preferably used prior to the on-set of any major complications i.e. during the early stages of the disease (Echeverría-Esnal et al., 2021). Endosomal acidification and cleavage processes are required for the viral replication and infection (V'Kovski et al., 2021). Since Azithromycin is a weak base, it is proposed that it inhibits the acidification of the endosome during viral replication, by increasing the pH in the endosomal vesicles, in which it accumulates (Du et al., 2021) Azithromycin also has immunomodulatory and anti-inflammatory effects, it has thus been proposed as a treatment option for patients with viral infections or inflammatory basis (Parnham et al., 2014).

2.5.2 Glycopeptides (Teicoplanin)

This class of drug consists of Teicoplanin and Vancomycin. They are antibiotics with a large molecular weight (Zeng et al., 2016). Glycopeptides act by inhibiting the synthesis of peptidoglycan by binding to amino acids in the cell wall, thus preventing the addition of new units (Leclercq, 1999). Other than antibacterial activities, glycopeptides, specifically Teicoplanin, also exhibits great antiviral activities (Szűcs et al., 2020). It has been suggested that Teicoplanin be used in the treatment of Covid-19 as it is thought to potently block the entry of SARS-CoV-2 entry through the inhibition of the enzymatic activity of cathepsin L (Jean et al., 2020). Based on this information, authors have recommended the use of Teicoplanin in both the prophylaxis and management of patients infected with SARS-CoV-2 (Chien et al., 2021).

2.5.3 Tetracyclines (Doxycycline, Eravacycline)

Tetracyclines belong to a class of broad-spectrum bacteriostatic and lipophilic antibiotics (2012). This class of oral antibiotics has high tissue penetration in the lungs(Sodhi and Etminan, 2020) (Naline et al., 1991). Tetracyclines inhibit bacterial growth by inhibiting the translation process (Grossman, 2016). These drugs work by binding to bacterial ribosomes and interact with bacterial 16S ribosomal RNA (rRNA), this prevents the association of the aminoacyl-tRNA with the bacterial ribosome (Chukwudi, 2016). Tetracyclines, like Doxycycline have also have non-antibiotic effects, such as substantial anti-viral activities (Griffin et al., 2011). It was suggested that the anti-viral properties of Doxycycline may be due to up-regulation of Zinc finger anti-viral protein (ZAP), thus preventing viral RNA from accumulating in the cytoplasm (Gendrot et al., 2020). A common

complication of the Covid-19 infection is SARS-CoV-2-induced pneumonia and hyper-inflammation and the cytokine storm, however (Kim et al., 2021), Doxycycline also exerts anti-inflammatory effects in patients with viral infections, by inhibiting pro-inflammatory cytokines like IL-6 and Tumor Necrosis Factor (TNF)-α (McGonagle et al., 2020), thus making it a potential drug candidate for further study, for the treatment of SARS-CoV-2 infection (Chaves Filho et al., 2021).

2.5.4 Fluoroquinolones (ciprofloxacin, moxifloxacin and levofloxacin)

Fluoroquinolones are a class of broad-spectrum synthetic antibiotics. They inhibit DNA replication and transcription, by inhibiting the activities of prokaryotic DNA gyrase – topoisomerase II and IV (which is essential for DNA synthesis) (Egorov and Sazykin Iu, 2000). This class of drugs also exhibit potential anti-viral properties against both DNA and RNA viruses (Leyva-Ramos and Hernández-López, 2017). Ciprofloxacin and Moxifloxacin exert their effects by inhibiting SARS-CoV-2 replication (Marciniec et al., 2020). Fluoroquinolones also have immune-modulatory activity, and is thus effective in the treatment of the infamous cytokine storm syndrome seen in patients with the Covid-19 infection (Karampela and Dalamaga, 2020).

2.5.5 Aminoglycosides

Aminoglycosides possess antibacterial properties. It interferes with the protein synthesis process by specifically binding to the aminoacyl site of the 16S ribosomal RNA (rRNA), which is within the 30S ribosomal subunit (Cavallo and Martinetto, 1981). This class of antibiotics also have proven non-antibacterial properties, including anti-viral properties (Cimolai, 2021). The clinical use of aminoglycosides in the treatment of patients infected with SARS-CoV-2 was discouraged. This was due to the adverse impact of the SARS-CoV-2 infection on olfaction, which counteracts the known ototoxicity that is associated with the use of aminoglycosides (Ahmed et al., 2021).

2.5.6 Cephalosporins (Cefuroxime)

Cefuroxime is a broad spectrum, second-generation cephalosporin (Olarte-Luis et al., 2018). Authors in a recent review have shown in-silico evidence of potential action of cefuroxime against

three of SARS-CoV-2 proteins, one of which is ACE2-spike protein complex (Durojaiye et al., 2021). However, no human clinical trials or in-vitro studies have been conducted to prove these findings.

One of the therapeutic strategies employed to combat covid-19 infections is the repurposed use of antibiotics (Yacouba et al., 2021b). The abovementioned antibiotics are either being used to target and inhibit the viral properties, and/or treatment of co-existing bacterial infections (Yacouba et al., 2021b). The use of some of these antibiotics have shown promising results, however, remain highly controversial due to great possibility of antibiotic resistance emergence (Narendrakumar et al., 2021).

2.6 Computational approaches:

2.6.1 Virtual screening

This approach consists of a number of computational methods, to identify potential hit candidates after analysing large databases (Walters and Namchuk, 2003) (Figure 2.3). Due to the ever-present availability of massive computational resources, virtual screening is now commonly used to identify the drug potential of novel targets, where there is often an unknown binder (Forli, 2015). Computational studies allows us to obtain a detailed interpretation of results, which would not be achievable using only experimental methods. In a study by (Kandeel and Al-Nazawi, 2020), the Main protease (M-pro) of the covid-19 crystal structure was targeted using virtual screening approaches, by using FDA approved drug datasets. The significance of this study was to use already approved safe, and marketed drugs as the potential treatment for covid-19. After the virtual screening of M-pro was conducted, it was concluded that systemically acting drugs, as well as a set of antimicrobials and antivirals, were able to bind more potently to M-pro, as compared with curcumin (known inhibitor of M-pro) (Zahedipour et al., 2020). They proposed an alternative treatment for covid-19, by finding new potential M-pro inhibitors of the SARS-CoV-2 virus (Dai et al., 2020). They used: 1) Databases that were previously built from docking compounds with M-pro and 2) databases that were built from compounds with known anti-SARS activity.

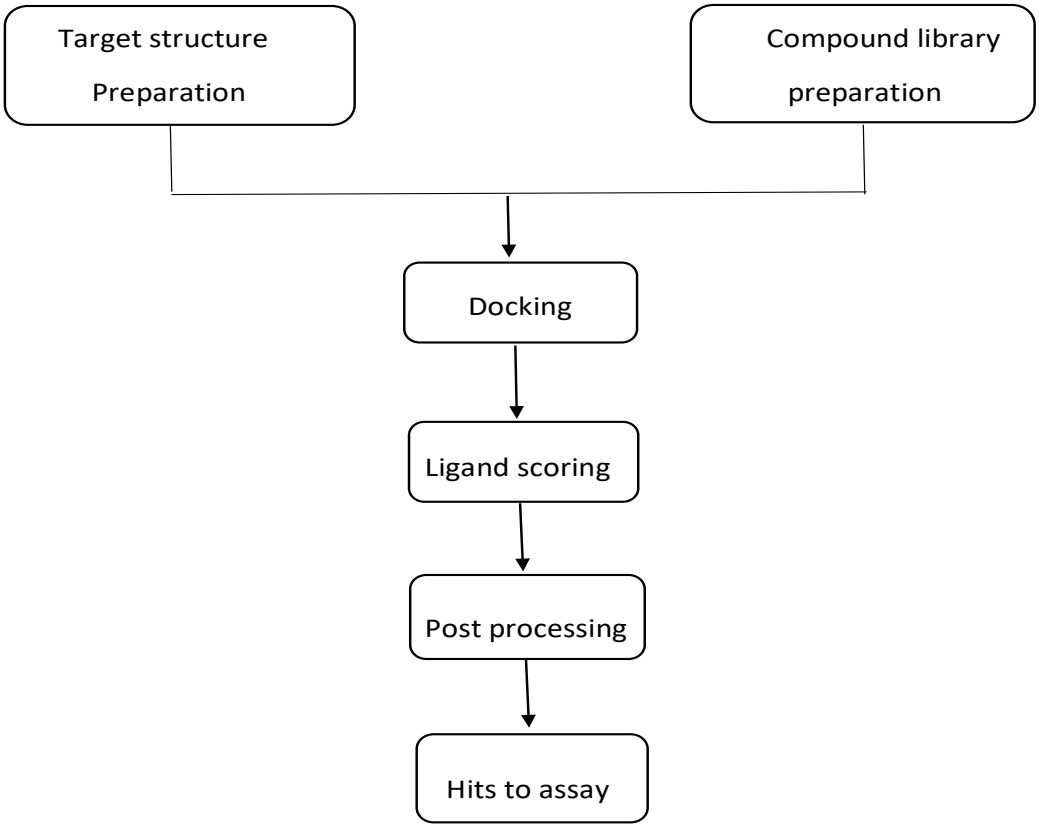

Figure 2.3. Virtual screening in discovery process from target structure and compound library preparation till hits assay is achieved. [prepared by author]

2.6.1.1 Pharmacophore based approaches

Pharmacophore-based techniques have been successfully used in carrying out tasks such as virtual screening, lead optimization and de novo design (Horvath, 2011). These techniques have thus become an integral part of many computer-aided drug design studies and workflows (Baig et al., 2016). Pharmacophores are simplistic and abstract in nature and is therefore easy to comprehend and perfectly suited for computer processing (Seidel et al., 2019). Pharmacophore models provide an abstract description of essential non-bonded interactions that occur between small-molecule ligands and macromolecule targets (Gao et al., 2010). In (Arun et al., 2021) study, the phase module of Schrodinger suite was used to carry out the pharmacophore based virtual screening. This was carried out to generate a group of drugs with the desired molecular features for optimal binding to M-pro. The fitness scores were then used to select the best hits. Similarly, in (Kumar et al., 2020) study, based on the crystal structure of SARS-CoV-2 co-crytsallized with alpha-ketoamide 13b and non-covalent inhibitor X-77, two structure based pharmacophore models were developed. Since natural products have been previously reported to possess significant anti-CoV activity (Shahzad et

al., 2020), the virtual screen carried out against the natural product databases could result in the identification of putative active compounds against SARS-CoV-2. The pharmacophoric features of the standard serine protease inhibitor camostat mesylate were selected for pharmacophore-based screening in (Rahman et al., 2020) study. The lead compounds with the best docking scores were identified by docking the pharmacophore-based screened library compounds with the target protein.

2.6.1.2 Structure based approaches

Great interest in the development of methods for structure-based function prediction has been brought about due to the increasing number of protein structures determined by structural genomic projects. These methods are classed into two groups (Gherardini and Helmer-Citterich, 2008): 1) Some use a comparative approach. This is done by identifying the presence of structural motifs associated with a known biochemical function. 2) Alternatively, only physicochemical properties are used to identify functional patches on the surface of a protein. In the study carried out by (Choudhary et al., 2020), structure based virtual screening was used against ACE-2 receptor and S-RBD. A template of the S-protein of SARS-CoV-2 was used to model the spike protein S-RBD, this, together with the crystal structure of ACE-2 receptor protein, was used in this study. The specific residues of ACE-2 and S-RBD were targeted, and the top hit compounds were then selected and further analyzed by AutoDock Vina. This was done to identify specific interactions involved in binding of molecules to targets. Structure-based approaches have also been used in the discovery of new chemotherapeutic agents, as it is cost and time-efficient way of discovering new drugs (Craig and Eakin, 2000). The use of structural information as well as structure-based drug design approaches was highlighted by (van Montfort and Workman, 2009). This was used in the discovery of small molecule inhibitors for cancer drug targets and this research provides an outlook for future exploitation of structural information in the future of cancer drug discovery. A general depiction of the structure based virtual screening is highlighted in Figure 2.6.

2.6.1.3 Ligand based approaches

This type of approach relies on the knowledge of molecules that bind to a biological target of interest, and is used in the absence of the receptor 3D information (Tresadern and Bemporad, 2010). The most common techniques used for ligand-based drug design are pharmacophore and Quantitative Structure-Activity Relationships (QSAR) (Macalino et al., 2015) as illustrated in figure 2.4 below. QSAR is a computational method to determine the correlation between the chemical structures of compounds and the biological process or a particular chemical (Yang et al., 2021). Different restrictions required to generate a reliable QSAR model include (Cherkasov et al., 2014) (Melo-Filho et al., 2014):

(a) The bioactivity data should be acquired from a common experimental protocol and should be of minimum twenty compounds with activity, such that potency values can be compared.
(b) With regards to training and test sets, there should be a proper selection of compounds.
(c) In order to avoid over-fitting, molecular descriptors for the ligands should have no autocorrelation.
(d) To determine applicability and predictivity, internal and/or external validation should be used to validate the model.

Once a dataset with a good range of potency becomes available, QSAR models can be used if the models are robust enough for prediction purposes (Verma et al., 2010). The same concept is applied when the target is well known with many compounds already known in public literature/databases, then machine-based learning models can also be attempted (Yu and MacKerell, 2017).
There is an assumption that exists, that compounds with similar structures, display a similar biological response and target interaction (Philip et al., 2007). In order to generate a reliable ligand-based screening model, the compound set should include at least four orders of magnitude i.e a wide concentration range (Melo-Filho et al., 2014).

(Yang et al., 2021) explains how ligand-based virtual screening would be conducted. It is either based on active compounds with no target information or target protein-related compounds. The output offers suggestions in choosing promising molecules for further experimental evaluation, by using the ranked 2/3D molecular similarity for all involved pairs of database ligands and input molecules.

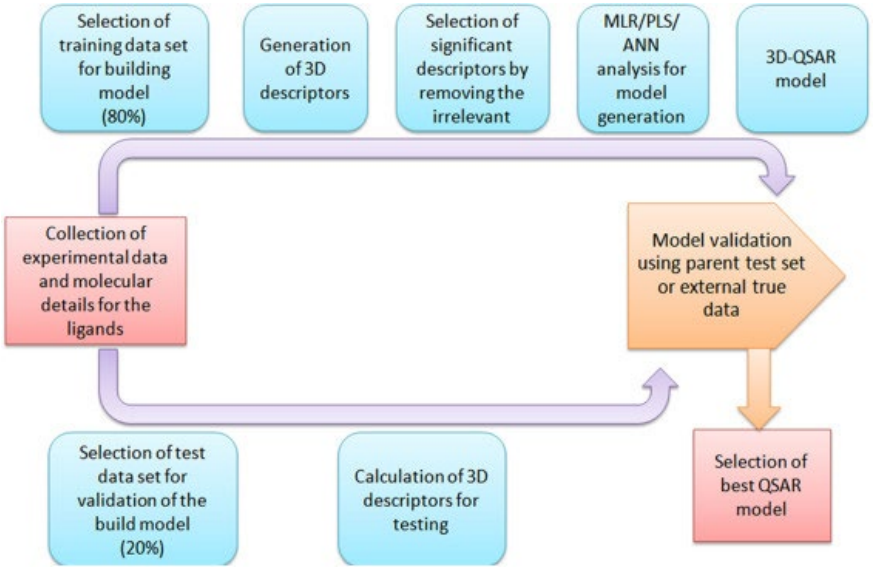

Figure 2.4. Illustrates ligand-based virtual screening from collection of experimental data till model validation and selection of best Quantitative Structure-Activity Relationship model (Ajjarapu et al., 2022) [Permission obtained].

2.7 Docking and molecular dynamics

In order to understand protein-ligand interaction systems in a virtual manner, computer-based drug discovery methods have been implemented (Singh, S et al., 2022). Both molecular dynamic and molecular docking studies are vital toolboxes for *in silico* drug discovery.

Molecular docking is an *in silico* method used to predict molecular interactions, it does this by foreseeing binding modes of small compounds or macromolecules in contact with a receptor (Pinzi and Rastelli, 2019). Using particular scoring functions, compounds are ranked according to a hierarchy (Santos et al., 2019). Molecular docking plays a critical role in the drug discovery and development process. This approach will assist to design a dosage form in the most cost effective and time saving manner (Tejinder et al., 2019).

Molecular Dynamics (MD) is a technique that should be done together with molecular docking. Another way to conduct these studies would be to do docking of compounds onto the active site of the target and from those that produce the best docking scores, you can run molecular dynamics to see how well/poorly the ligand stays bound to the active site.

(Santos et al., 2019) study was aimed at improving the drug discovery process by focusing on protocols that offer the docking-MD combination. In (Shree et al., 2020) study, six phytochemicals were identified using molecular dynamics, from medicinal plants, with potential inhibition and high affinity towards

SARS-CoV-2-Mpro. It works by obstructing further translation of viral protein that assists in damaging the host's vital organs. These phytochemicals could potentially be repurposed for use against Covid-19. Structural stability was displayed by the studied complexes during the trajectories analysis, whilst carrying out the MD runs (Shree et al., 2020). A similar study was carried out by (Yu et al., 2020). The optimized model was used as the docking target, AutoDock Vina software was used in all docking experiments. It was concluded that Luteolin may be used to select new compounds that bind specifically to the SARS-CoV-2 main protease sites.

2.8 *In silico* studies evaluating the interaction of antibiotics on the SARS-CoV-2 proteins

Over the years, the use of antibiotics to treat a viral infection has been discouraged due to the rise of antibiotic resistance and loss of effectiveness (Levy, 1998). Moreover, viruses have different structures and replicate in a different way as compared to bacteria (Rogers, 2010). Antibiotics inhibit particular bacteria by targeting the growth machinery in bacteria (Gottlieb and Shaw, 2013) and most doctors prescribe anti-viral drugs to shorten the infection and to prevent complications. However, in the case of macrolides - in addition to their antibacterial activity, they possess anti-viral activity (Sybilski, 2020) (Bayarski, 2006). They have shown (in this study as well as previous studies mentioned below) to improve clinical course of viral respiratory infections, at least through indirect mechanisms relying on some variable immunomodulatory and/or anti-inflammatory effects.

In the vicinity of the primary coronavirus receptor, angiotensin-converting enzyme-2 (ACE-2), Hydroxychloroquine (CLQ-OH) molecules are shown to saturate virus attachment sites on the gangliosides (Maisonnasse et al., 2020). When taken concurrently, AZM is directed against the virus, whilst CLQ-OH is directed against cellular attachment cofactors, thus proving that these drugs are competitive inhibitors of SARS-CoV-2 attachment to the host cell membrane (Braz et al., 2020).
In our study, AZM exhibited strong binding with each of the four receptors under investigation, demonstrating great binding potential in biological processes relating to the viral replication of SARS-CoV-2. The macrolide antibiotics under investigation were able to firmly bind to the structures that are responsible for the replication processes of SARS-CoV-2. Therefore, these drugs are potential candidates for inhibiting and reinforcing processes, showing promising results in clinical trials. Since Azithromycin is a weak base, it is proposed that it inhibits the acidification of the endosome during viral replication, by increasing the pH in the endosomal vesicles, in which it accumulates (McDonald and Pruul, 1991). Azithromycin also has immunomodulatory and anti-inflammatory effects, it has thus been proposed as a treatment option for patients with viral infections or inflammatory basis (Parnham et al.,

2014).

Cefpiramide is a third generation antibiotic, and is able to interact with penicillin binding proteins (PBP) and inhibits peptidoglycan formation (Yilmaz and Paterson, 2017). It is used for the treatment of severe infections caused by susceptible bacteria such as *P.aeruginosa* (Drigues et al., 1986) , it also has an inhibitory role on community acquired pneumonia (Ramirez and Anzueto, 2011). In (BANK et al., 2020) study, it was found that Cefpiramide might interact with the S protein of SARS-CoV-2. Likewise, the macrolide antibiotics under investigation in our study, were able to target the points of binding interaction between ACE2 and the spike protein of the SARS-CoV-2. Each of the four macrolides showed strong binding interactions with each of the four receptors.

Antibiotics with known anti-viral activity were repurposed as potential inhibitors of SARS-CoV-2 M^{pro}, with the aid of molecular simulation approaches (Bharadwaj et al., 2020). Based on the docking scores and molecular dynamic simulation, four antibiotics (doxycycline, minocycline, demeclocycline and tetracycline) could be used as potential SARS-CoV-2 M^{pro} inhibitors. These can be further evaluated for in vitro SARS-CoV-2 M^{pro} inhibition and viral infection as monotherapy or combinational therapy against SARS-CoV-2 infection.

2.9 The need for such antibiotic evaluations

As seen above, antibiotic evaluation studies have been carried out by multiple authors. Due to the emergence of covid-19 and the urgent need for treatment, the science community has repurposed the use of antibiotics for SARS-CoV-2 infections. These surveillance studies are important as they provide vital information which would allow health care professionals to identify trends in viral incidence and antimicrobial resistance, as well as emergence of new strains of the virus at both national and global levels. It helps the medical community keep up with viable treatment options that are available.

2.10 Conclusion and future perspectives

New and re-emerging disease outbreaks, such as COVID-19, as well as the fragile state of the healthcare system of most African countries, can potentially paralyze our health systems. Due to the state of urgency, computer-aided drug design approaches have to be implemented to identify and repurpose drugs that are potential inhibitors of SARS-CoV-2. This is done by the application of *in silico*

drug design programmes. The fundament of controlling the spread of infection in the future, involves the design or repurposing of effective drugs that would potentially reduce morbidity and mortality rates, thus putting an end to the devastating pandemic.

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CHAPTER 3:
Methods

CHAPTER 3

3. Methods

This chapter briefly explains molecular modelling, as well as the molecular docking and molecular dynamic simulations performed in this study. Chapter 4 (Research paper) further highlights and explains these techniques.

3.1 Virtual screening and molecular docking

3.1.1 Ligand preparation

The 2D and 3D structures of the select drugs (azithromycin, clarithromycin, erythromycin, roxithromycin, remdesivir and ivermectin) were built, then Gasteiger charges were added. Universal force field (UFF) was used to minimize these structures energy (by using Avogadro to employ steepest descents minimization). Open Babel was sued to convert these optimized structures to pdbqt file format. The next step was screening by docking using AutoDock Vina.

3.1.2 Protein preparation

RCSB protein data bank is where we obtained our protein structures from, with the following PDB IDs: 3CLpro (6LU7), native human ACE2 (1R42), RdRp (6M71) and spike RBD-ACE2 (6LZG). Before converting the protein structures to pdbqt format (which is required by autodock vina for docking calculations), Gasteiger charges and missing hydrogen at pH 7.4 were added. In order to assess whether our method could reproduce experimental binding modes, the co-crystalized ligands were extracted from the crystal binding pose and were redocked to the putative binding pocket. All redocked ligands gave acceptable RMSD values of RMSD values of ≤3Å (which was found to be reliable and able to reproduce the experimental binding mode). Blind screening was done against the SARS-CoV-2 targets. The compounds with lower binding free energy of -7.9 kcal/mol were promising and chosen for further experimentation and similarity search. For the purpose of this study, the following ligand criteria was set: since the inhibition constant is set at Ki=3.03 uM, any ligands with binding free energy of ≤7.9 kcal/mol (which is similar to the inhibition constant), is considered to be a potential or probable candidate. In order to repurpose those select drugs (against SARS-CoV-2 proteins), similarity search had to be carried out.

MD consists of 4 continuous steps that are repeated a number of times, until a trajectory is generated (Figure 3.1).

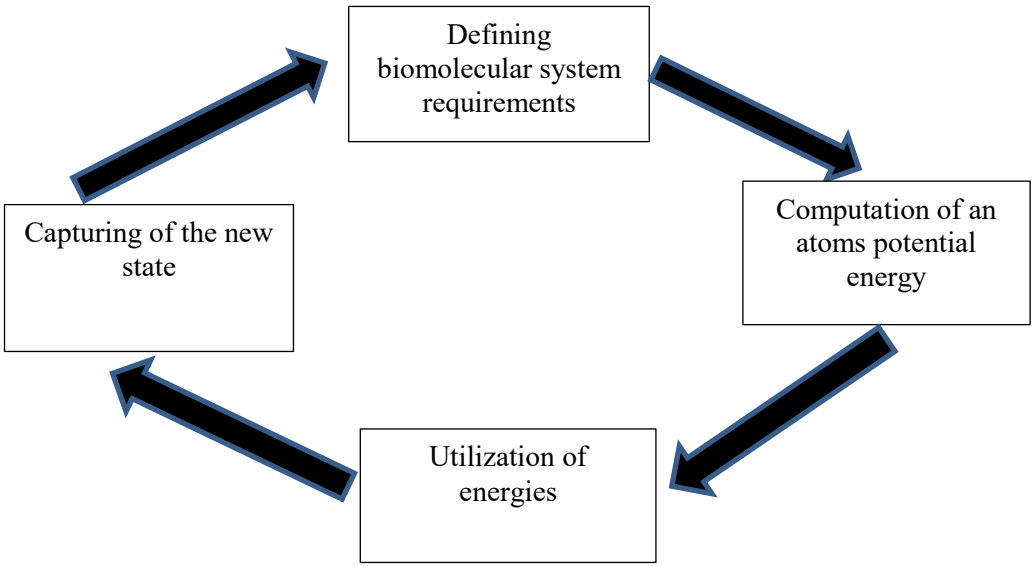

Figure 3.1 The cycle of molecular dynamics steps [Prepared by Author].

The steps are elaborated below:

1. The coordinates, acceleration and bond characteristics of each atom are defined.
2. The potential energy of each atom is computed.
3. These energies are then used to solve the equation of motion.
4. The new state of the system will need to be captured and saved, the coordinates of the atoms will change.
5. The cycle starts over from step 1

Quantitative analysis of the systems' time-evolution can continue, once the trajectory is completely generated.

3.1.3 system stability:

3.1.3.1 Root Mean Square Deviation (RMSD):

Is a trajectory defined as:

$$\mathbf{RMSD} = \left(\frac{\sum_N (\mathbf{R_i} - \mathbf{R_1^0})^2}{\mathbf{N}}\right)^{\frac{1}{2}}$$

Where R_i is the expected values
R_o is the observed values
and n is the total number of values

3.1.3.2 Root Mean Square Fluctuation (RMSF):
The following calculation is applied:

$$\mathbf{sRMSF} = \frac{(\mathbf{RMSF_i} - \overline{\mathbf{RMSF}})}{\boldsymbol{\sigma}\ (\mathbf{RMSF})}$$

Where: the average RMSF is subtracted from $RMSF_i$ (which is the RMSF of the i^{th} residue). This value is then divided by the RMSF's standard deviation, to obtain the RMSF value.

3.1.3.4 Thermodynamic energy calculations:

Free energy calculations in molecular dynamic simulations are now used in many areas of research such as solvation thermodynamics, protein folding and molecular recognition (Hansen, N. and W. F. van Gunsteren, 2014).
The Molecular Mechanics/Generalized Born Surface Area method (MM/GBSA) methods are very popular approaches which have been adopted in order to estimate the free energy of the binding of small ligands to biological macromolecules (Genheden, S. and U. Ryde, 2015) and are considered to be the most precise and well-structured method (Homeyer and Gohlke, 2012). The free binding energy formula can be represented as:

$$\Delta \mathbf{G_{bind}} = \mathbf{G_{complex}} - \mathbf{G_{receptor}} - \mathbf{G_{ligand}}$$

$$\Delta \mathbf{G_{bind}} = \mathbf{E_{gas}} + \mathbf{G_{sol}} - \mathbf{TS}$$

$$\mathbf{E_{gas}} = \mathbf{E_{int}} + \mathbf{E_{vdw}} + \mathbf{E_{ele}}$$

$$\mathbf{G_{sol}} = \mathbf{G_{GB/PB}} + \mathbf{G_{SA}}$$

$$\mathbf{G_{SA}} = \boldsymbol{\gamma} \mathbf{SASA}$$

Here, Egas symbolizes gas-phase energy consisting the internal energy
Gsol is the solvation free energy that was computed from the polar states, non-polar states

GSA is the non-polar solvation energy
T and S signifies the total entropy of the temperature and solute respectively
γ - coefficient which is related to surface area
SASA- solvent accessible surface area

Results and discussion for MM-PBSA calculation follow in chapter 4.

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CHAPTER 4: Research Paper

CHAPTER 4

Molecular Docking, Molecular Dynamics, and MM-PBSA Approach of some selected Macrolides as SARS-CoV-2 inhibitors

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Abstract

Covid-19 is a highly transmissible, pathogenic, viral, respiratory disease which results from contact with the acute respiratory syndrome coronavirus 2 (SARS-CoV-2). There is a desperate need for the development of highly effective Covid-19 treatment and viable therapies due to the high hospital admissions and death rate of this disease. In this regard, however, there are additions of some promising chemical entities, such as Macrolides, teicoplanin, clarithromycin, doxycycline etc. Macrolides are currently being explored in South African hospitals and are given as a therapeutic measure to patients who have contracted Covid-19. There is a compelling need for the analysis of the present macrolide antibiotics using molecular modelling studies. Therefore, this study analysed macrolide antibiotics, which could be repurposed, to identify their mechanism of inhibition, by evaluating them against the spike protein of SARS-CoV-2. Very similar results were seen among the macrolide antibiotics in the Molecular Docking studies, however, AZM showed the greatest binding potential with all receptors under investigation: –7.9 kcal/mol (ACE2), –7.9 kcal/mol (RdRp), –6.9 kcal/mol (Mpro) and – 6.4 kcal/mol (RBD). AZM was then compared with the controls. The binding energies of all three were fairly similar, with all being above -6.0 kcal/mol, making them possible drug candidates for the treatment of SARS-CoV-2. Based off the Molecular Dynamics studies, we gained more information on the profile of each drug bound to the ACE-2 receptor. The results shown between AZM and ACE-2 were similar to those seen between the controls and ACE-2, further highlighting the fact that AZM would make a great potential candidate drug against SARS-CoV-2.

Key words: AZM (Azithromycin), ACE-2 (Angiotensin-Converting Enzyme 2), RdRp (RNA-

dependent RNA Polymerase), M^{pro} (Viral Main Protease), RBD (Receptor Binding Domain)

4.1 Introduction

As of December 2019, a global health threat, an ongoing outbreak has plagued the world and taken the lives of many people (Zheng, 2020). This respiratory disease was first discovered in December 2019 in Wuhan China, and was thus given the name Covid-19 (Coronavirus Disease-19) (Yamin, 2020). The virus that causes SARS (Severe Acute Respiratory Disease) has shown to have great structural resemblance to the Covid-19 virus (Fauci et al., 2020). This virus has proven to cause higher morbidity and mortality among the elderly, as well as patients with co-morbidities (Li et al., 2020). The very first confirmed covid-19 case was identified in Wuhan and reported on the 31st of December 2019. On the 13th of January 2020, the first case outside China was reported, in Thailand (Hui et al., 2020). This outbreak has now spread to more than 50 countries since then (Mullol et al., 2020). On the 30th of January 2020, the World Health Organization (WHO) declared the Covid-19 outbreak a Public Health Emergency of International Concern (PHEIC) (Yan et al., 2020). As of the last recorded update on 24th April 2021, there were 146 million active cases, (Mishra and Nandi, 2021)84 million recoveries and 3, 09 million deaths (Baud et al., 2020) . This respiratory disease outbreak has brought about great challenges for the health and research communities (Mbunge, 2020).

Since the first Covid-19 outbreak, several experimental vaccines and treatments for Covid-19 have been repurposed, developed and tried, but challenges still remain regarding the safety profile as well as the efficacy(Amawi et al., 2020). Azithromycin has been proposed as a potential clinical treatment for this respiratory disease (Pani et al., 2020). Drug repurposing, also known as drug repositioning, is an approach in drug discovery for which known, existing drugs are found new medical uses (Langedijk et al., 2015). Antibiotics are one of the drug classes that are being considered as potential treatment due to their inhibitory effects on the SARS-CoV-2 virus (Glebov, 2020). These antibiotics are either used to treat secondary bacterial infections caused by covid-19 or used for its anti-viral properties (Yacouba et al., 2021a). Macrolides include drugs like Azithromycin, Clarithromycin and Erythromycin(Dinos, 2017a). Azithromycin is a broad spectrum macrolide antibiotic with a long half-life and a very large volume of distribution (Damle et al., 2020) Azithromycin has proven to have both immunomodulation as well as anti-viral properties (Vázquez-Laslop and Mankin, 2018). In past *In-vitro* studies, Azithromycin was demonstrated to have the capacity to reduce pro-inflammatory cytokine production, reduce oxidative stress, as well as modulate T-helper functions (Parnham et al., 2014). In March 2020, an online survey was conducted over 6000 physicians in 30 different countries, which showed that Azithromycin was the second most commonly prescribed treatment for Covid-19 (Shehab et al., 2020).

Forty one percent of the respondents reported that they had either prescribed Azithromycin for the treatment of Covid-19 or have seen it prescribed for this indication (Linka et al., 2021). This particular macrolide antibiotic was associated with a reduced ventilation days and reduced mortality in other viral infections (Echeverría-Esnal et al., 2021). Antiviral effects are most likely due to changes in mammalian cellular functions (and not by Azithromycin binding directly to the viral targets), which will disturb the mechanisms by which viruses survive, reproduce and spread (Tyteca et al., 2002). Additionally, during early phases of infection In-vitro, Azithromycin blocked the internalization of influenza virus by host cells – this was then translated to an influenza mouse model in which Azithromycin caused a reduction of viral load after a single administration intranasally (Tran et al., 2019). Similarly, Azithromycin could potentially interfere with the binding of the virus to the ACE2 receptor, thus inhibiting the entry of SARS-CoV-2, but this has only been proposed using quantum mechanical energetics modelling (Venditto et al., 2021). Virus entry and replication within host cells takes place when the Receptor-Binding Protein (RBP) of the virus' spike (S) protein, binds to the cell surface of the Angiotensin-Converting Enzyme 2 (ACE2) receptor (Murgolo et al., 2021). The viral main protease was recently shown to mirror a fitting target for drugs that work by inhibiting viral replication (Cui et al., 2020). This study aimed to evaluate the molecular interactions of Azithromycin, Erythromycin, Clarithromycin and Roxithromycin through Angiotensin-Converting Enzyme 2 (ACE2), RNA-dependent RNA polymerase (RdRp), viral main protease (M^{pro}) and the Receptor Binding Domain (RBD) using molecular docking (Autodock Vina software). We thus also aim to provide results that can be useful in other studies intending to use these drugs' mechanisms of action in the treatment approaches to covid-19. The simulations conducted in this research will allow us to understand the mechanism of inhibition of macrolides, by evaluating them against the spike protein of the SARS-CoV-2.

4.2 Methods

4.2.1 Ligand preparation

The ligands azithromycin, erythromycin, clarithromycin and roxithromycin were selected for binding with various target protein of Covid-19. The structures of these compounds were downloaded from PubChem database (http://pubchem.ncbi.nlm.nih.gov/) as SDF file formats. This was followed by energy minimization using MMFF94 force fields (parameters: 500 steps of steepest descent algorithm, convergence criterion of 10e-7). The optimized structures were converted into PDB files using Avogadro software ready for screening by docking with AutoDock Vina. We also had two FDA approved drug molecules for docking analysis

(Remdesivir and Ivermectin).

4.2.2 Receptor preparation

The Prime Module of Schrodinger Suite was used to prepare the receptor proteins. 3D structures of target proteins, viz., 3CLpro (6LU7), native human ACE2 (1R42), RdRp (6M71) and spike RBD-ACE2 (6LZG) were downloaded from Protein Data Bank (PDB) . The ligands were removed from each of the receptors and the final, cleaned structure was saved in PDB format. The proteins were prepared for docking by removing all the water molecules (as it is not involved in the binding, and is best to remove it before the water molecules in the pocket distort the pose search) and adding hydrogens.

4.2.3 Molecular Docking

Docking calculations were validated by first extracting a co-crystallized inhibitor and then redocking it to the putative binding pocket. This was done to evaluate whether our docking method can duplicate experimental binding modes. In all cases, we had RMSD values of ≤ 3 Å which were found to be well-grounded and acceptable in reproducing experimental binding mode. After validating the docking protocol, the four macrolides were blindly screened against four SARS-CoV-2 protein targets. The antibiotics with lower binding energy of ≤ -7.5 kcal/mol were selected for further analysis using molecular dynamics.

4.2.4 Molecular Dynamics

Following the docking studies, all the drugs with lower binding energies from the docking experiment were subjected to all-atom MD simulation to evaluate time-lapse stability using GROMACS (Version 2020.4). The CHARMM27 protein-lipid parameter was used to generate topologies and was set for the proteins. The CHARMM General Force Field (CGenFF) parameter was set for the macrolides. All systems were centralized in the cubic box solvated with TIP3P water model. The systems went through energy minimization to resolve bad contacts and clashes using 5,000 steps of steepest descent method. Further, the systems were subjected to two steps of equilibrations, first 100 ps of NVT ensemble using the V-rescale thermostat and 500 ps of NPT ensemble using Parrinello-Rahman barostat at 1 bar of isotropic pressure. MD production was then carried out for 100 ns for each system without position

restraints. Similarly, the pressure (1 bar) and temperature (300 K) were maintained using Parrinello-Rahman barostat and V-rescale thermostat. Particle Mesh Ewald (PME) method was applied to treat long-range electrostatic interactions with a cutoff distance of 1.1 nm for both electrostatic and van der Waals interactions. An integration of time step 2 fs was used and all bond lengths constrained using LINCS algorithm. Structural and conformational analysis was then performed using modules implemented in GROMACS package.

4.3 Results and Discussion

4.3.1 Evaluation of fitting score (Binding affinities)

Figure 4.1 below shows the binding affinities/ fitting scores for ACE2, RBD, RdRp and their respective ligands.

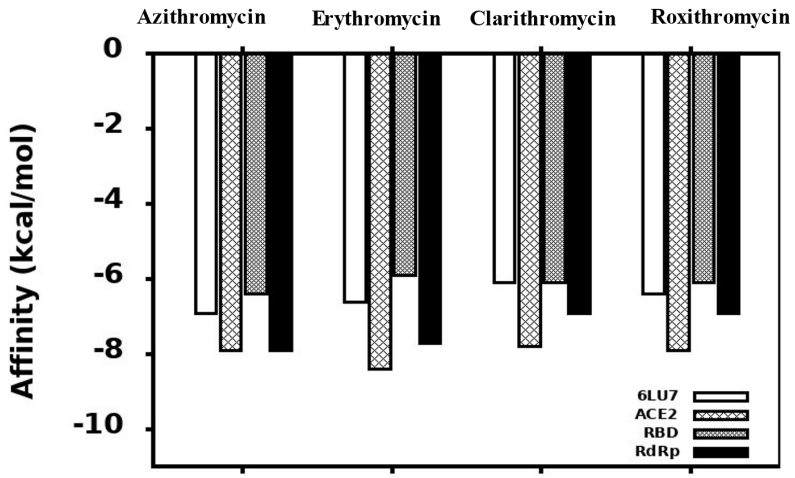

Figure 4.1. Graphical representation of binding energies (in kcal/mol) of molecular docking between the ligands [Azithromycin, Erythromycin, Clarithromycin and Roxithromycin] and targets [angiotensin-converting enzyme 2 (ACE2), RNA-dependent RNA polymerase (RdRp), viral main protease (M^{pro}) and the receptor-binding domain (RBD) calculated by AutoDock Vina software.

(a) Azithromycin

This study has shown that azithromycin shows strong binding energies of –7.9 kcal/mol (ACE2), –7.9 kcal/mol (RdRp), –6.9 kcal/mol (Mpro) and –6.4 kcal/mol (RBD). Furthermore, slightly similar results were obtained for the other antibiotics: erythromycin showed the following interactions –8.4 kcal/mol (ACE2), –7.7 kcal/mol (RdRp), –6.6 kcal/mol (Mpro) and –5.9 kcal/mol (RBD). roxithromycin showed the following interactions –7.8 kcal/mol (ACE2), –6.9 kcal/mol (RdRp), –6.1 kcal/mol (Mpro) and – 6.1 kcal/mol (RBD). clarithromycin showed the following interactions –7.9 kcal/mol (ACE2), –6.9 kcal/mol (RdRp), –6.4 kcal/mol (Mpro) and –6.1 kcal/mol (RBD).

In the case of AZM, its mechanism of antiviral action remains unclear, however, a great number of human *in vitro* and *in vivo* studies show us evidence of antiviral activity of macrolide antibiotics against a wide range of viruses and their viral families (Oliver and Hinks, 2021).

AZM exhibited great binding potential with each of the four receptors (Figure 4.2). This shows us that AZM has the potential to bind to and inhibit the viral replication of SARS-CoV-2. The macrolide antibiotics under investigation were able to rigidly bind to the structures that are responsible for the replication processes of SARS-CoV-2. Therefore, these drugs are potential candidates for inhibiting processes, showing favourable results in clinical trials.

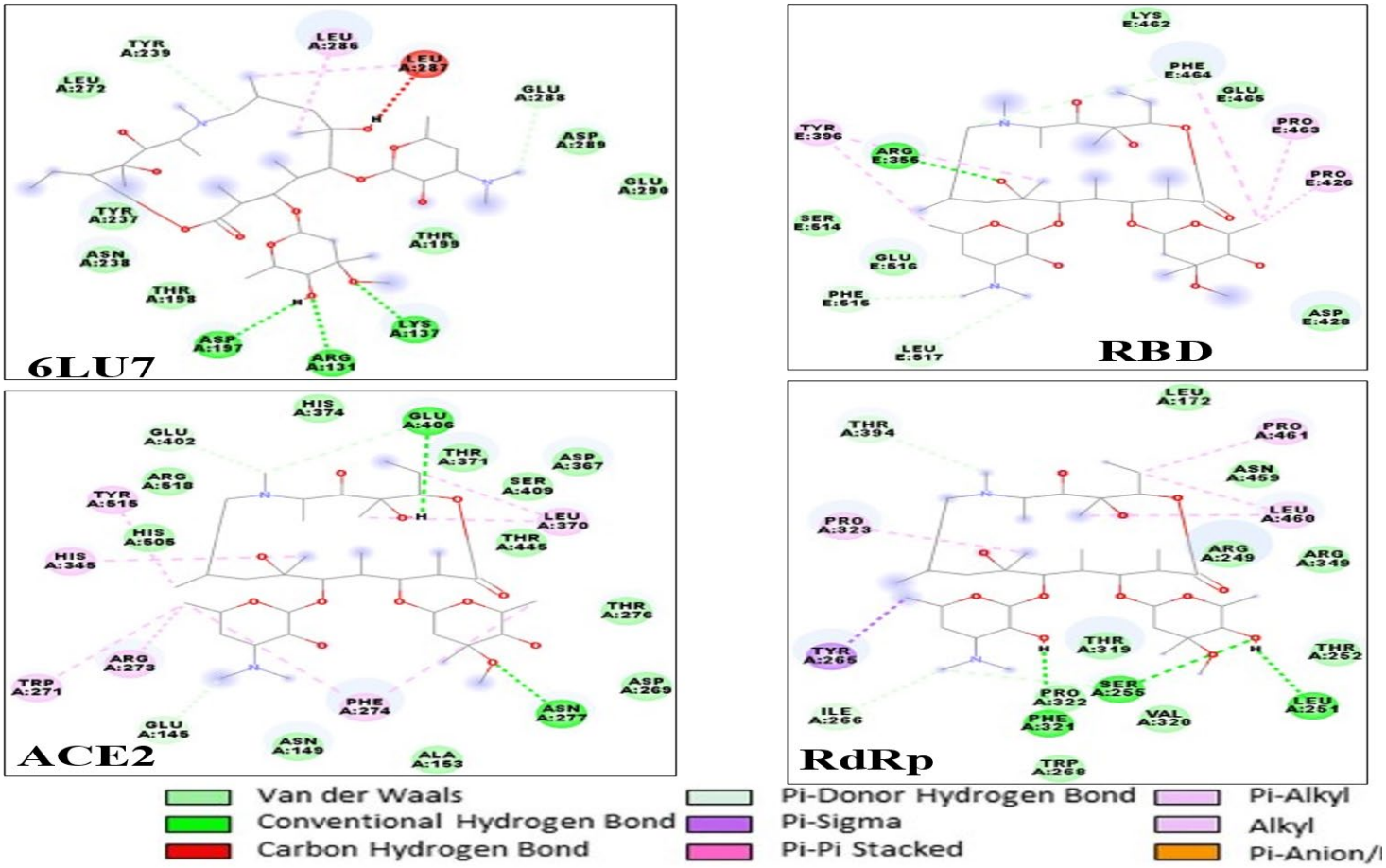

Figure 4.2. Interactions between azithromycin and angiotensin-converting enzyme 2 (ACE2), RNA-dependent RNA polymerase (RdRp), viral main protease (Mpro) and the receptor-binding domain (RBD).The dark green dashed lines represent the H-bonds, whilst other types of intermolecular forces are represented by the other dashed lines.

(b) Erythromycin

Erythromycin showed the following interactions with their receptors: –8.4 kcal/mol (ACE2), –7.7 kcal/mol (RdRp), –6.6 kcal/mol (Mpro) and – 5.9 kcal/mol (RBD). Similar to AZM, Erythromycin showed good binding energies with their receptors. This shows that Erythromycin would be able to rigidly bind to the structures which play a role in the replication processes of SARS-CoV-2. Erythromycin has also shown a great number of hydrogen bonds present when bound to each receptor (Figure 4.3), which makes the molecules "stickier", such that greater energy is required to separate them, this would also result in the Van der waals dispersion forces becoming greater.

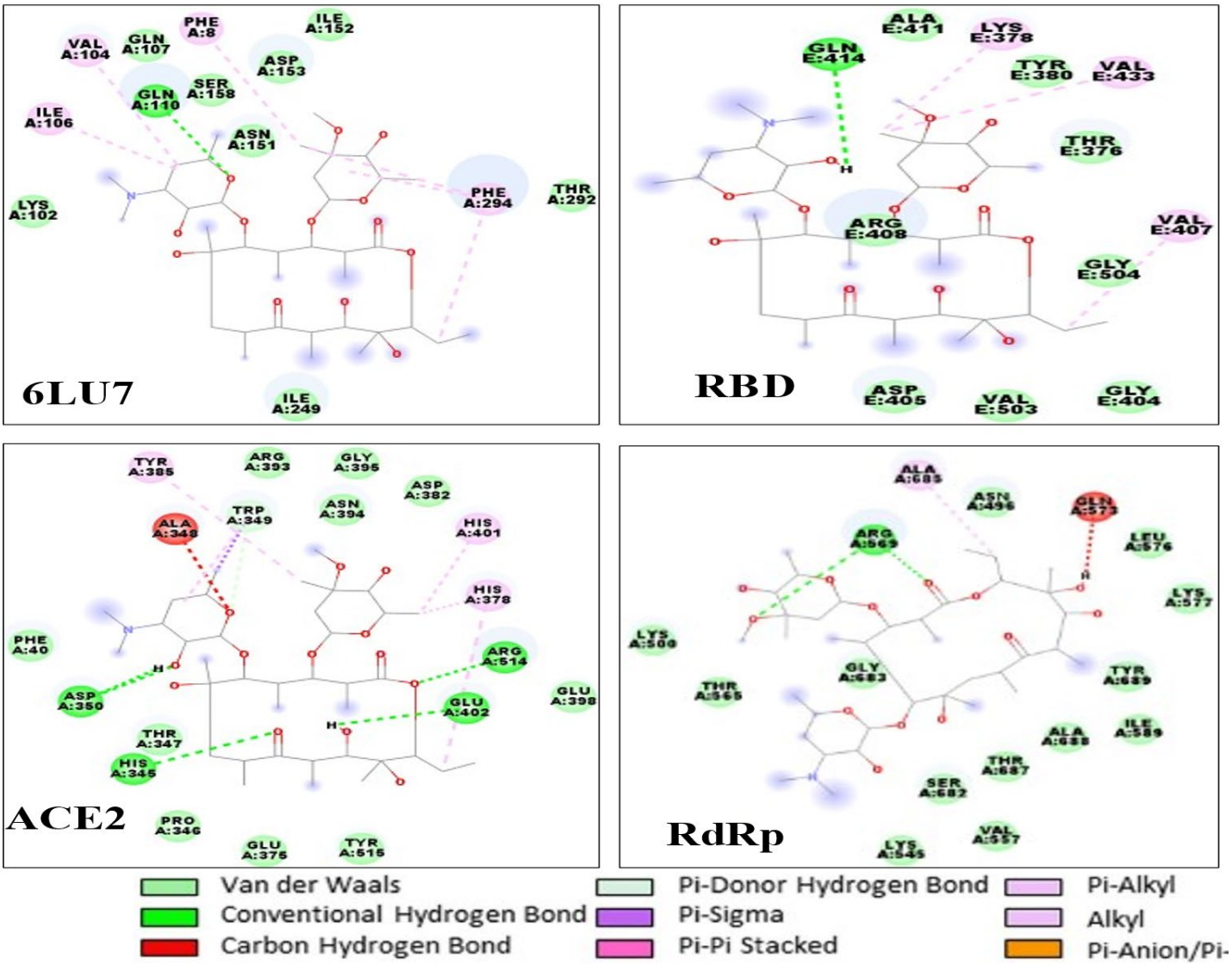

Figure 4.3. Interactions between Erythromycin and angiotensin-converting enzyme 2 (ACE2), RNA-dependent RNA polymerase (RdRp), viral main protease (Mpro) and the receptor-binding domain (RBD). The dark green dashed lines represent the H-bonds, whilst other types of intermolecular forces are represented by the other dashed lines.

(c) Clarithromycin

Clarithromycin showed the following interactions with each of the four receptors: –7.9 kcal/mol (ACE2), –6.9 kcal/mol (RdRp), –6.4 kcal/mol (Mpro) and – 6.1 kcal/mol (RBD). Similarly, Clarithromycin also has a great number of hydrogen bonds (Figure 4.4), and thus, van der waals interactions, indicating a good binding potential with each of its receptors. The presence of non-covalent interaction between aromatic rings is vital for its role in biological recognition as well as the organization of biomolecular structures (Li et al., 2013).

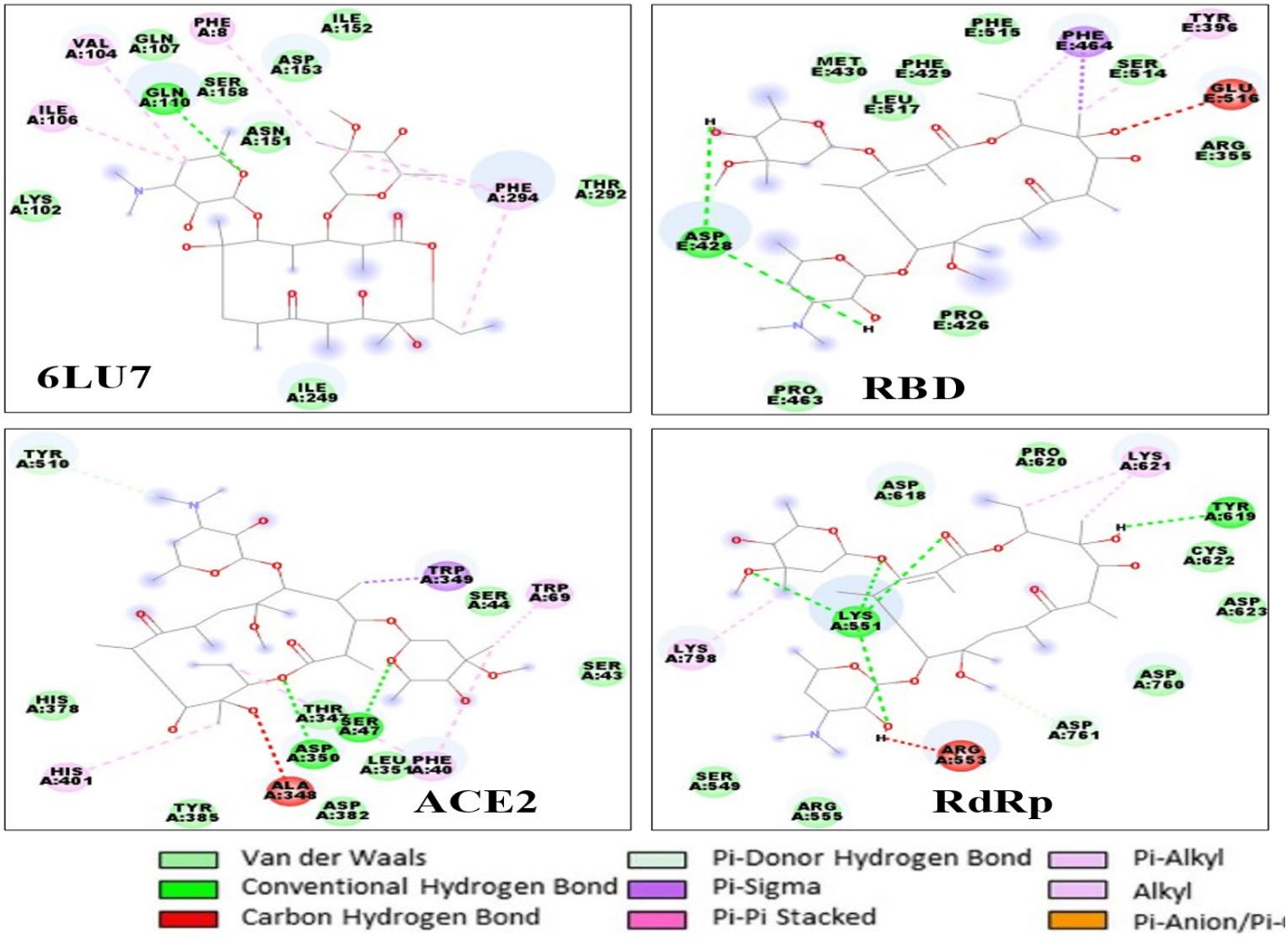

Figure 4.4. Interactions between clarithromycin and angiotensin-converting enzyme 2 (ACE2), RNA-dependent RNA polymerase (RdRp), viral main protease (Mpro) and the receptor-binding domain (RBD), The dark green dashed lines represent the H-bonds, whilst other types of intermolecular forces are represented by the other dashed lines.

(d) Roxithromycin

Roxithromycin showed the following interactions with each of their receptors: –7.8 kcal/mol (ACE2), –6.9 kcal/mol (RdRp), –6.1 kcal/mol (Mpro) and – 6.1 kcal/mol (RBD). Similar to the other macrolide antibiotics under investigation, Roxithromycin also has a great number of hydrogen bonds (Figure 4.5), and thus, van der waals interactions, indicating a good binding potential with each of its receptors. The presence of non-covalent interaction between aromatic rings is vital for its role in biological recognition as well as the organization of biomolecular structures (Li et al., 2013).

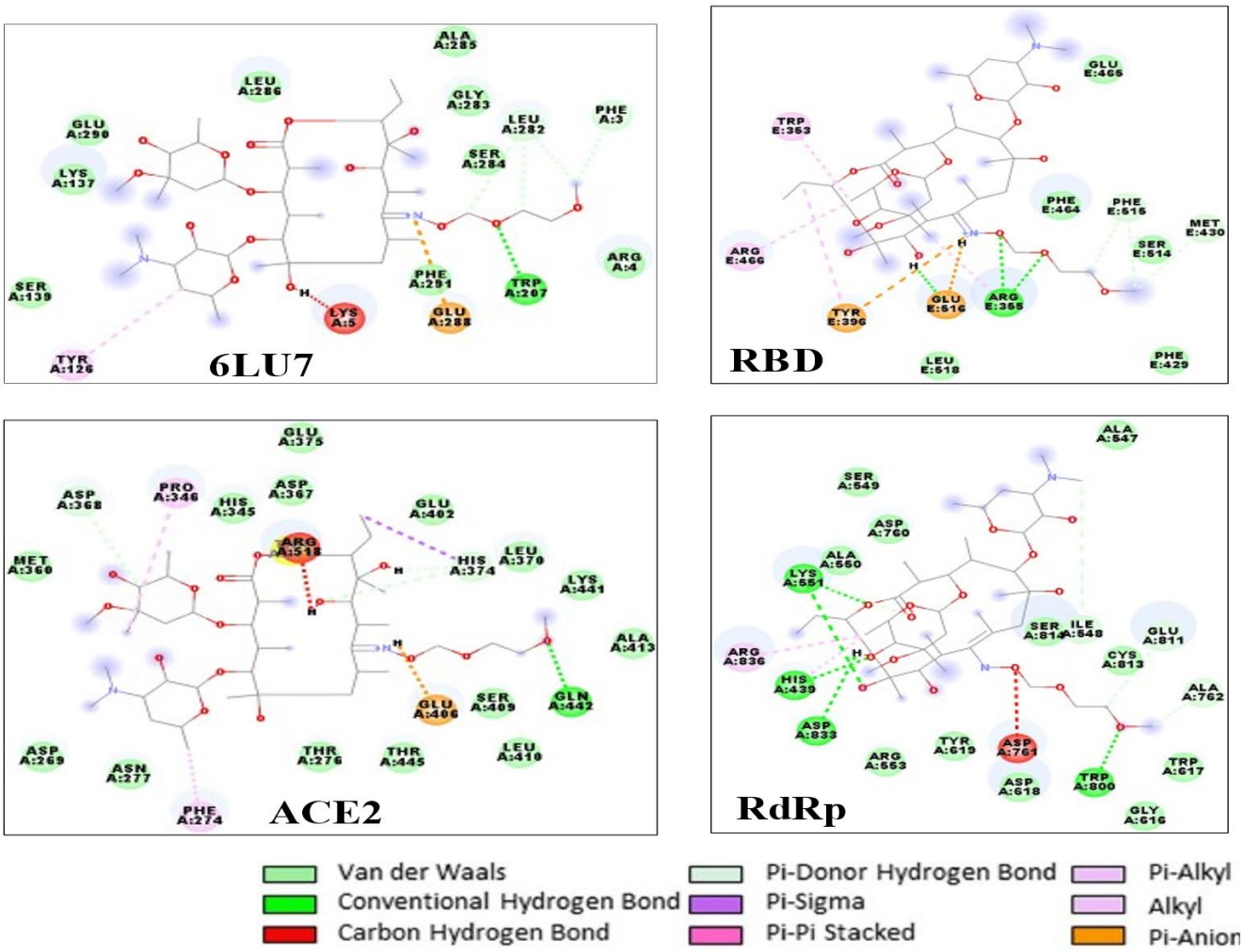

Figure 4.5. Interactions between Roxithromycin and angiotensin-converting enzyme 2 (ACE2), RNA-dependent RNA polymerase (RdRp), viral main protease (Mpro) and the receptor-binding domain (RBD). The dark green dashed lines represent the H-bonds, whilst other types of intermolecular forces are represented by the other dashed lines.

(e) Ivermectin (control)

In this study, Ivermectin showed H-bonds in all couplings, with RdRp having the most number of interactions (four): (ASN691, THR687, ARG56 and THR565). ACE2 (GLU406), M^{pro} (LYS137) and RBD(SER349) all had one interaction (Figure 4.6). The greater the number of hydrogen bonds, the greater the binding potential. Due to this, there is a presence of van der waals interactions, indicating a good binding potential with each of its receptors. In addition, these fittings showed other bonds including: alkyl and carbon hydrogen bonding. The presence of non-covalent interaction between aromatic rings is vital for its role in biological recognition as well as the organization of biomolecular structures(Li et al., 2013) . We used the results

obtained from Ivermectin (control) to compare with our macrolide antibiotics under investigation and this revealed promising results.

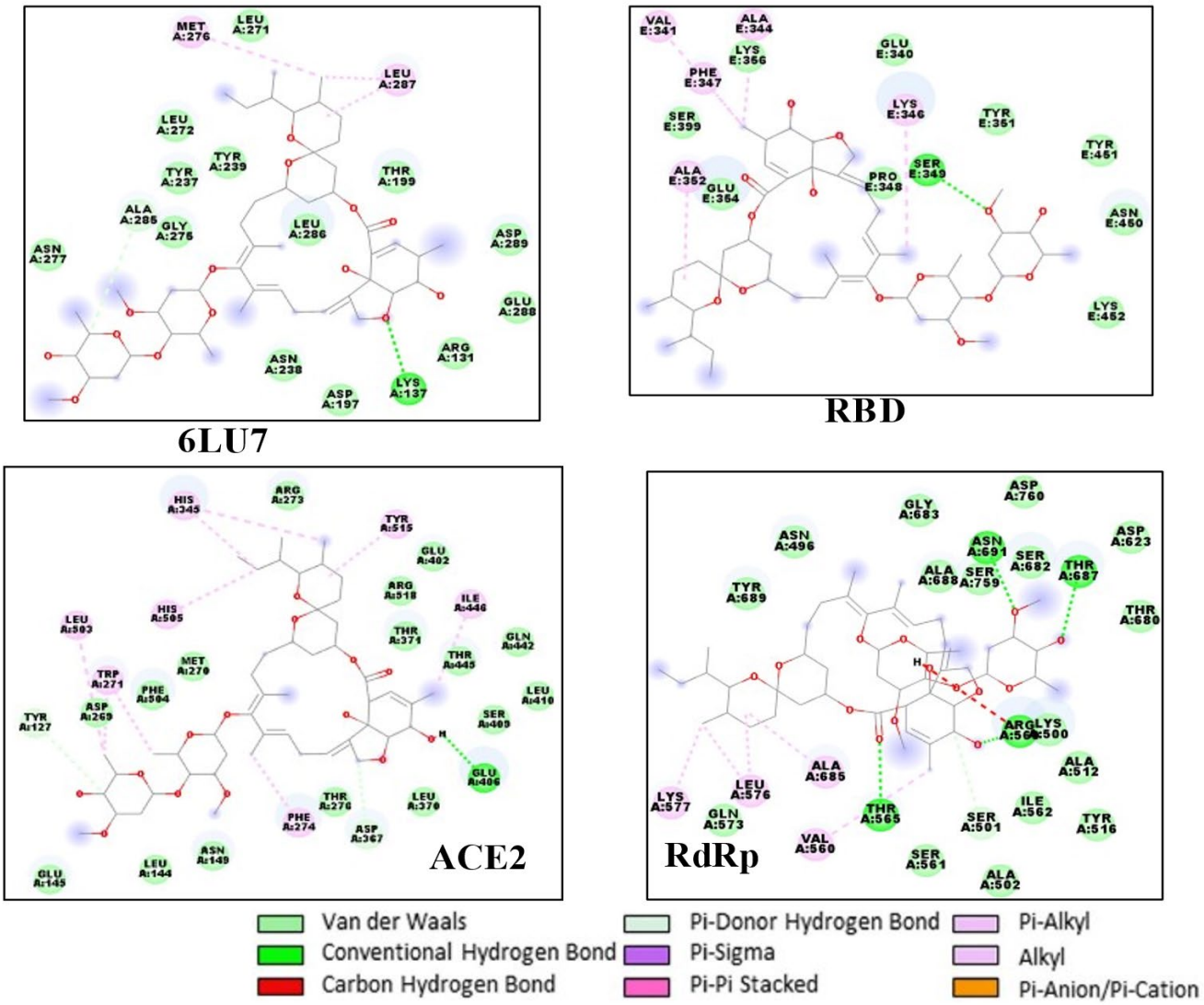

Figure 4.6. Interactions between Ivermectin and angiotensin-converting enzyme 2 (ACE2), RNA-dependent RNA polymerase (RdRp), viral main protease (Mpro) and the receptor-binding domain (RBD). The dark green dashed lines represent the H-bonds, whilst other types of intermolecular forces are represented by the other dashed lines.

(f) Remdesivir (control)

In this study, Remdesivir showed H-bonds in all couplings (Figure 4.7), with ACE2 and RdRp having the greatest number of interactions: five interactions in ACE2 (ARF514, ASP350,

HIS401, ASN394 and ASP382), and five interactions in RdRp (ASP218, ASN209, THR206, ASP36 and TYR38) RBD has four interactions (ARG355, GLU516, PHE515 and MET430). M^{pro} had 3 interactions (GLY143, CYS145 and LEU141). The greater the number of Hydrogen bonds, the greater the binding potential. Due to this, there is a presence of van der waals interactions, indicating a good binding potential with each of its receptors. In addition, these fittings showed other bonds including: alkyl and carbon hydrogen bonding. The presence of non-covalent interaction between aromatic rings is vital for its role in biological recognition as well as the organization of biomolecular structures (Li et al., 2013). We used the results obtained from Remdesivir (control) to compare with our macrolide antibiotics under investigation and this revealed promising results.

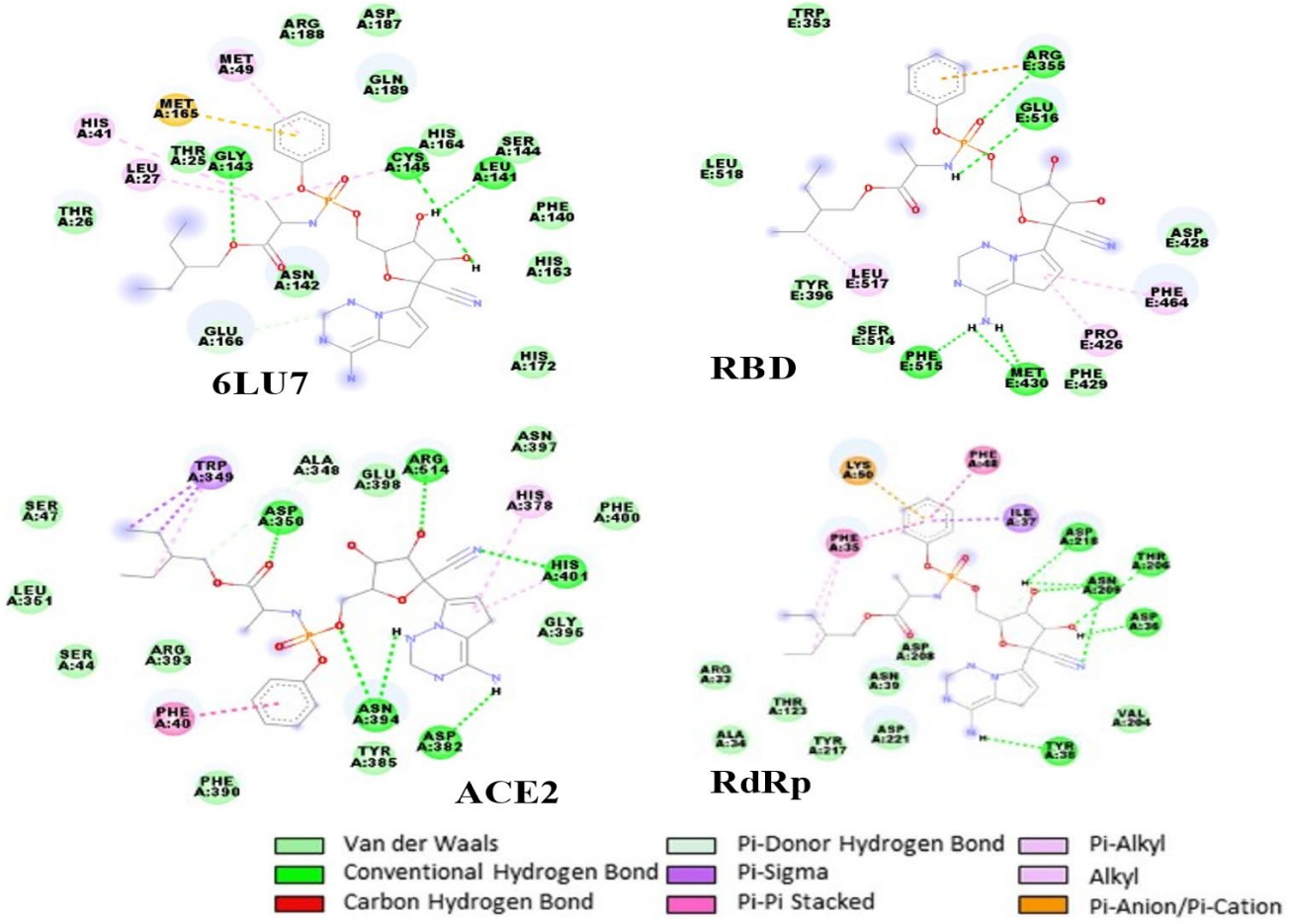

Figure 4.7. Interactions between Remdesivir and angiotensin-converting enzyme 2 (ACE2), RNA-dependent RNA polymerase (RdRp), viral main protease (Mpro) and the receptor-binding domain (RBD). The dark green dashed lines represent the H-bonds, whilst other types of intermolecular forces are represented by the other dashed lines.

(g) Best docked macrolide (AZM) comparison with controls

Figure 4.8 shows binding energies are all above -6.0 kcal/mol, making them best potential drug candidates for the treatment of SARS-CoV-2. The binding potential of all the macrolide antibiotics with each of their receptors, were very similar. However, AZM showed the greatest binding energies with each of the four receptors. AZM showed the greatest binding score from all four macrolides with M^{pro}, RBD and RdRp. However, it showed the second highest binding score for ACE-2.

AZM was then compared to both Ivermectin and Remdesivir (controls). It was shown that AZM shared similar binding affinity scoring with both Ivermectin and Remdesivir. There is a great number of Hydrogen bonds present in AZM, Ivermectin and Remdesivir, which made for stronger binding potential.

Best docked macrolide (AZM) comparison with controls

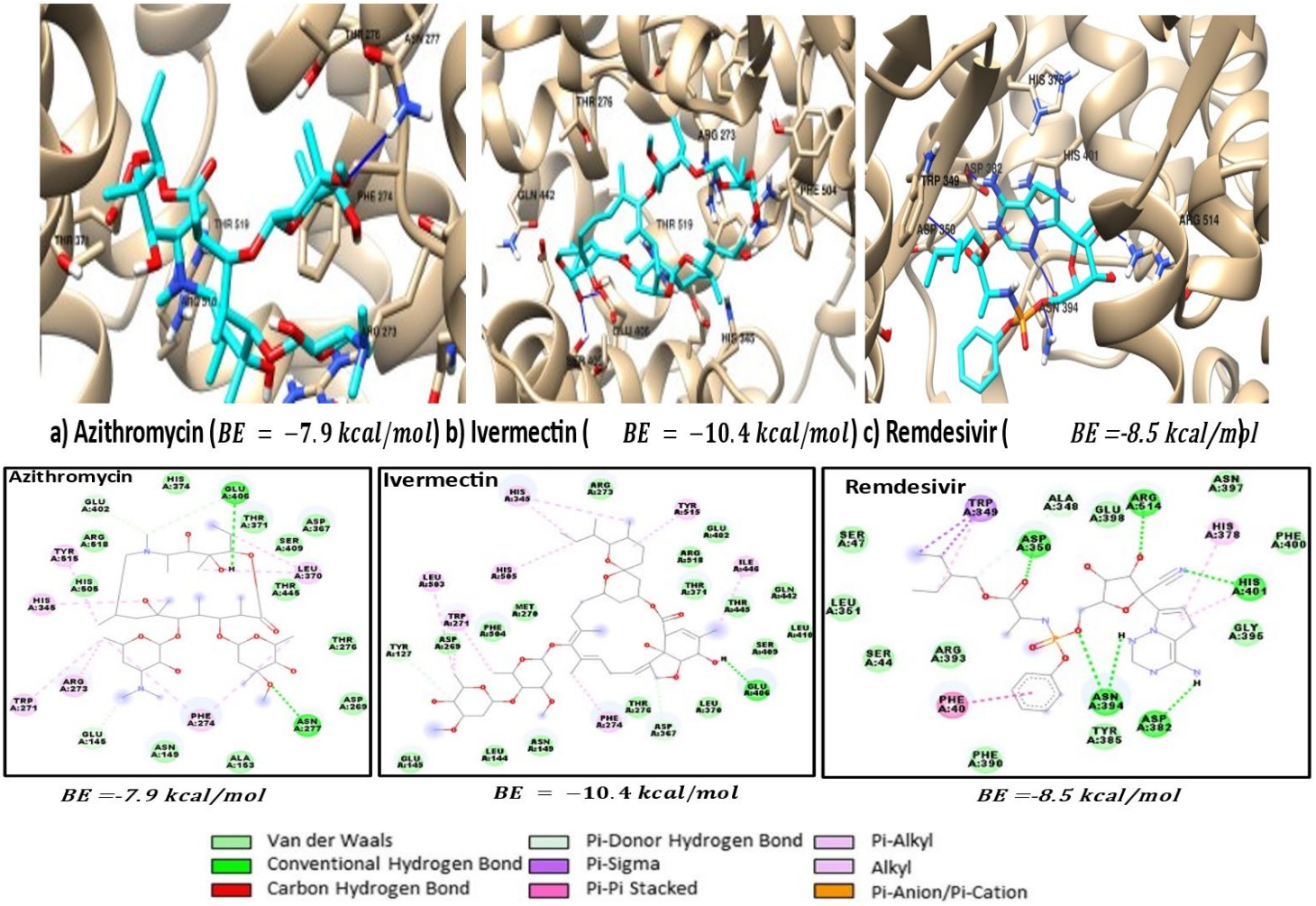

Figure 4.8. Best docked macrolide antibiotic (AZM) compared to controls (Ivermectin and Remdesivir)

4.3.2 Molecular dynamics

It has been shown that in order to increase the reliability of the ligand-protein energies, dynamics must be applied. In this study, MD simulations were performed for ACE2-Azithromycin, ACE2-Remdesivir, ACE2-Clarithromycin and ACE2-Erythromycin complexes, over 100ns under physiological conditions to observe any changes in the conformation, stability, or interactions of the drugs with amino acid residues over time. Using the generated MD trajectories, RMSD (Root Mean Square Deviation), RMSF (Root Mean Square Fluctuation), distance of the ligand inside the pocket and hydrogen bond interactions were computed as a function of time.

4.3.2.1 RMSD analysis

The stability profile of the abovementioned ligands, together with the ACE receptor, was determined using GROMACS command line gmx_rmsd (Figure 4.9) , to estimate their respective RMSD values throughout the simulation runs. (Al-Karmalawy et al., 2021) The RMSD value deviated the most between 0 -12.5ns for ACE2-Remdesivir, 37.5- 62.5ns for ACE2-Remdesivir, and 50-62.5ns for ACE2-AZM (Figure 4.10). The RMSD value deviated the most between 12.5- 37.5ns for ACE2-Erythromycin, and 75-100ns for both ACE2-Clarothromycin and ACE2-Erythromycin. Overall, the results shows that the Azithromycin, Clarithromycin, Erythromycin and Remdesivir complex did not influence the structural stability of the ACE2 protein and retained its structural integrity. However, the Azithromycin-ACE2 complex showed the least deviation to the ACE2 protein, and is therefore the most similar. With only limited fluctuations, the AZM RMSD tones focus attention to its preferential accommodation of the ACE2 binding site as compared with the other ligands under investigation.

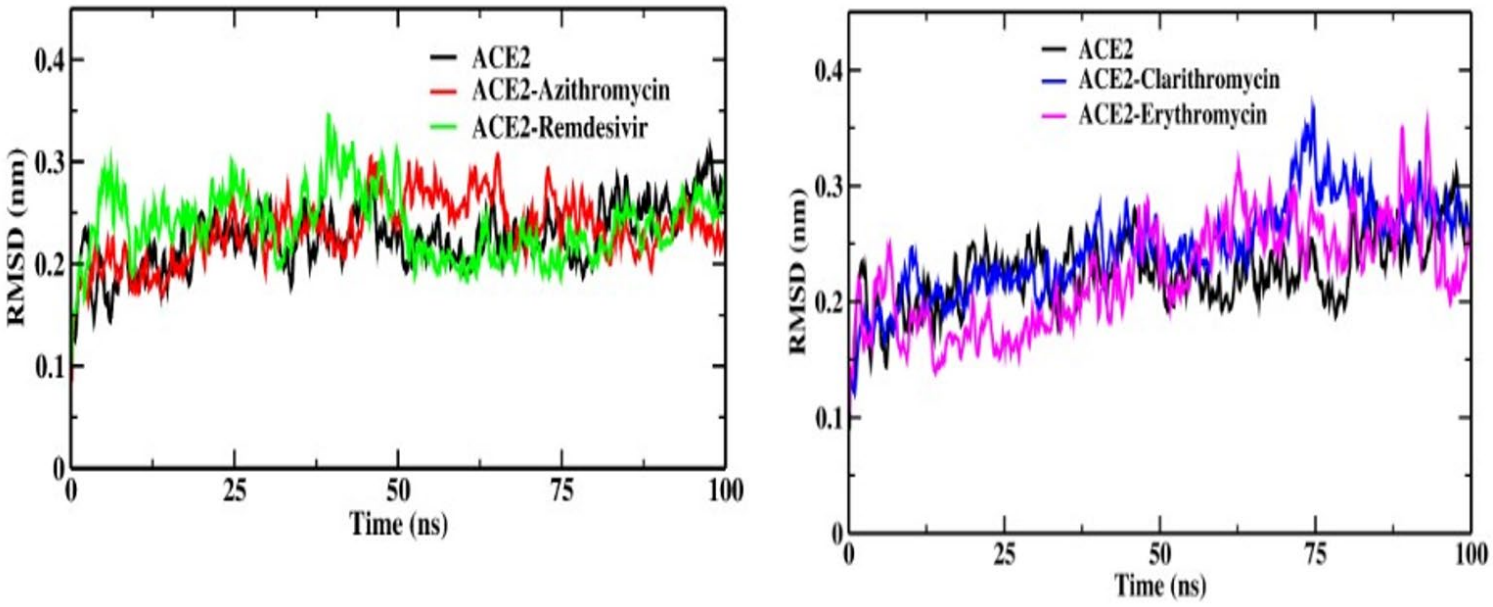

Figure 4.9. Plot of the RMSD value (nm) between ACE2 and Azithromycin, Remdesivir, Clarithromycin and Erythromycin over a period of 100ns

4.3.2.2 RMSF analysis

We gained more insight to the stability of the complex binding site, by estimating the RMSF profile for each ligand bound to the ACE2 protein (Figure 4.10). The individual backbone

RMSF of each protein was estimated using the GROMACS "gmx rmsf" command line. RMSF analysis is very similar to RMSD analysis, however, RMSF is a calculation of how much a particular residue moves/fluctuates during a simulation (Bahaman et al., 2021). The RMSF was analysed for the entire protein structure and ligands combined demonstrating each atom's mean displacement. The average RMSF indicates potential interactions of Azithromycin with the receptor protein (ACE2).

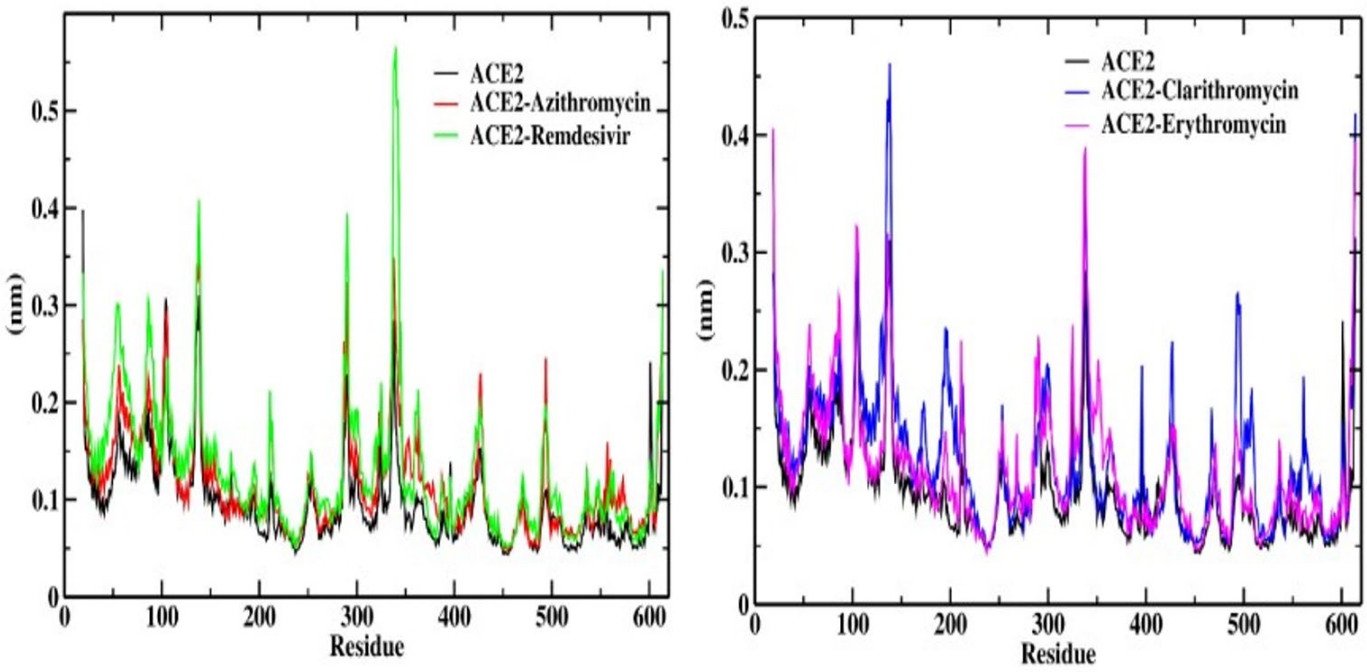

Figure 4.10. Molecular Dynamics of the RMSF value (nm) between ACE2 and Azithromycin, Remdesivir, Clarithromycin and Erythromycin as a function of simulation time.

4.3.2.3 Intermolecular Hydrogen Bonding

In order to understand the binding affinity of the ligand with the protein molecule, intermolecular H-bonding is one of the most crucial parameters to take into account (Pantsar and Poso, 2018). Formation or deformation of hydrogen bonds is a vital component during the MD simulation (Chen et al., 2016). A stronger binding affinity is signified by a larger number of H-bonds between the protein and the ligand (Chen et al., 2016). These results confirm that the macrolide antibiotics (Azithromycin, Clarithromycin and Erythromycin) have a significant number of H-bonds (Figure 4.11).

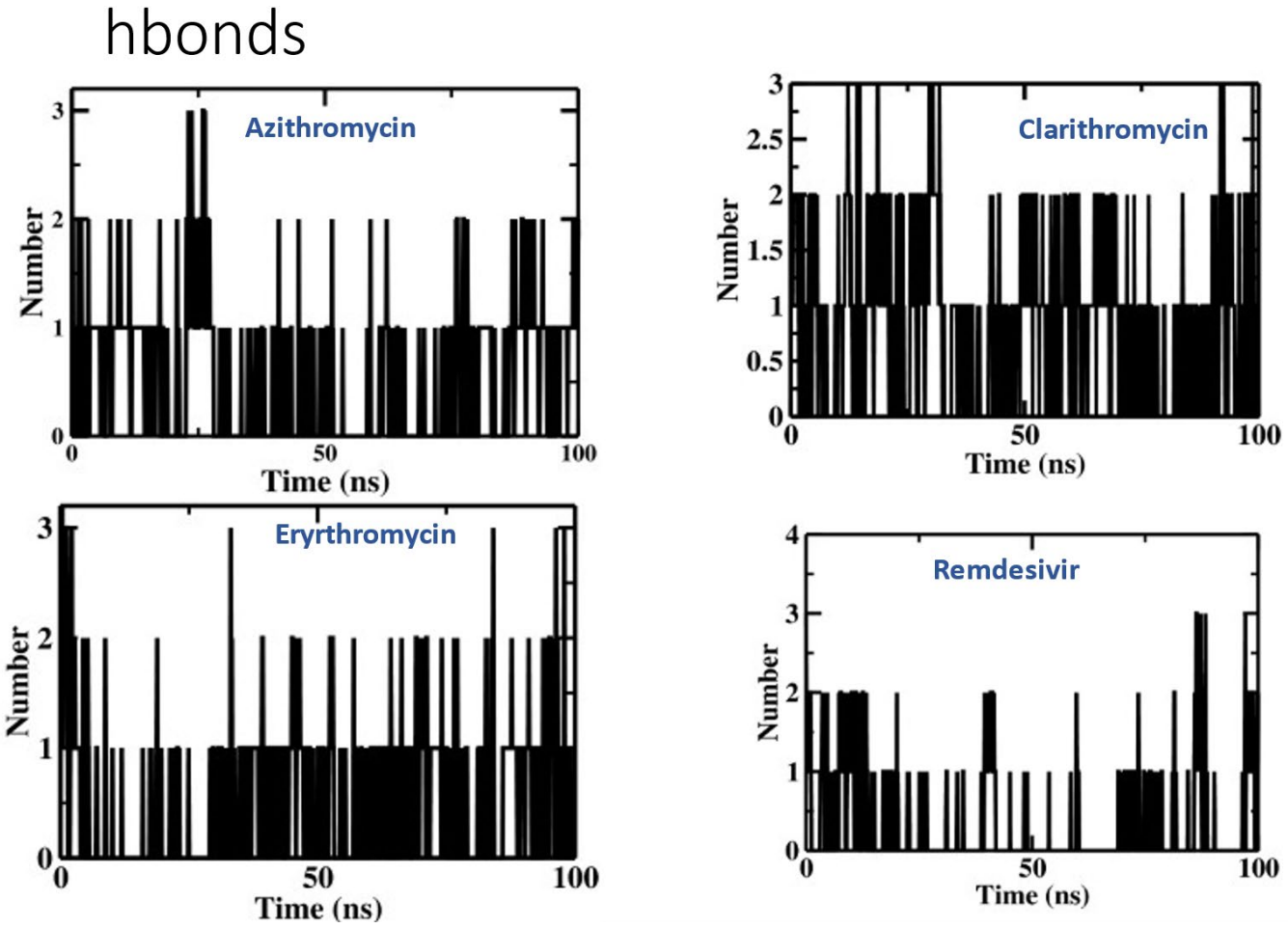

Figure 4.11. Molecular Dynamics of the number of hydrogen bonds present between ACE2 and Azithromycin, Clarithromycin, Erythromycin and Remdesivir over a specified time (100ns).

4.3.2.4 Distance of ligand while inside the pocket

Figure 4.12 shows that AZM started at 1.2nm at 0ns, and came closest at 75ns (0.9nm) to the protein inside the binding pocket, which indicates that AZM retained its binding pose but was out of the pocket before 75ns. Clarithromycin started at 0.8nm at 0ns, and remained <1nm in the first 12.5ns. Erythromycin started at 0.2nm at 0ns, and came closest at 25ns (<0.6nm). Remdesivir started at 0.125nm at 0ns, and came closest at 37.5ns (1nm). All three of which remained outside the pocket after 0ns.

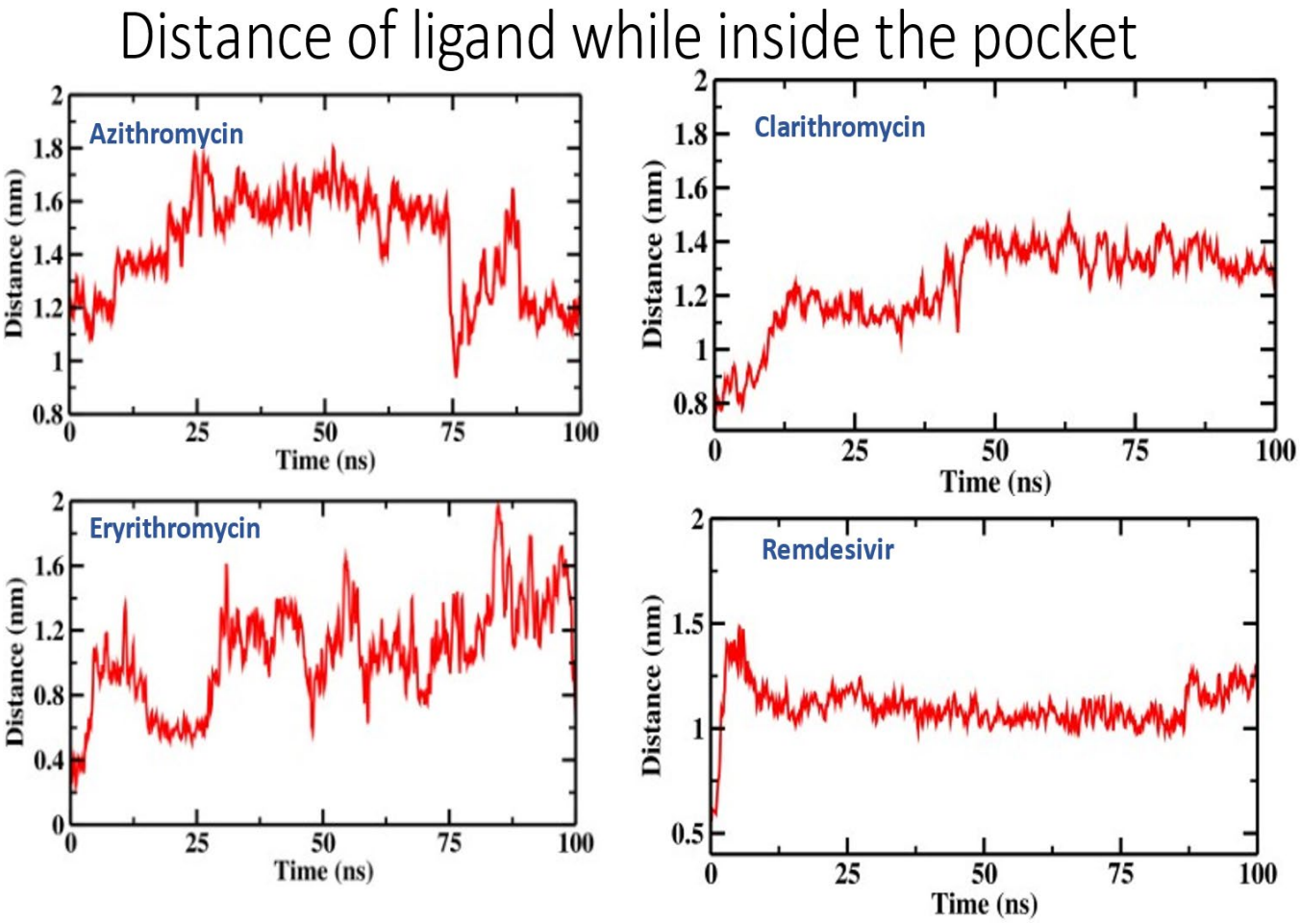

Figure 4.12. Molecular Dynamics of the distance of Azithromycin, Clarithromycin, Erythromycin and Remdesivir while inside the binding pocket, as a function of simulation time.

4.4 Results for MM-PBSA calculations

MMPBSA Calculations

	AZM	**ERY**	**CLA**	**REM**
van der Waal energy	-118.851 +/- 11.245 kJ/mol	-94.873 +/- 22.060 kJ/mol	-138.103 +/- 8.745 kJ/mol	-153.721 +/- 11.315 kJ/mol
Electrostattic energy	-9.889 +/- 11.683 kJ/mol	1.449 +/- 8.759 kJ/mol	-31.143 +/- 11.084 kJ/mol	-16.421 +/- 7.992 kJ/mol
Polar solvation energy	77.615 +/- 20.336 kJ/mol	65.053 +/- 20.515 kJ/mol	121.595 +/- 14.468 kJ/mol	99.329 +/- 14.151 kJ/mol
SASA energy	-17.913 +/- 1.260 kJ/mol	-13.003 +/- 1.869 kJ/mol	-19.409 +/- 1.083 kJ/mol	-18.088 +/- 0.993 kJ/mol
SAV energy	0.000 +/- 0.000 kJ/mol	0.000 +/- 0.000 kJ/mol	0.000 +/- 0.000 kJ/mol	0.000 +/- 0.000 kJ/mol
WCA energy	0.000 +/- 0.000 kJ/mol	0.000 +/- 0.000 kJ/mol	0.000 +/- 0.000 kJ/mol	0.000 +/- 0.000 kJ/mol
Binding energy	-69.037 +/- 17.113 kJ/mol	-41.373 +/- 11.979 kJ/mol	-67.060 +/- 14.989 kJ/mol	-88.902 +/- 12.723 kJ/mol

Table 4.1. Depicting MM-PBSA results

These calculations can predict good or bad binders as well as screening of large chemical libraries. However, limitations do exist, such as their poor ability to estimate the binding affinity of a complex and inability to provide information on time-lapse stability. In order to overcome these limitations, MD simulation was carried out to determine the time-lapse stability of the complex as well as the binding affinity of the ligands (evaluated using the MM-PBSA method). In order to ascertain the observed stability, binding free energy based on MM-PBSA was calculated to evaluate the affinity of the drugs in their active site. The binding free energy was calculated using a single trajectory, where a total of 200 snapshots were evenly extracted at predetermined intervals, between the first and last 40ns of the trajectory. The solvent dielectric constant was 80, the solute dielectric constant was 2, γ was 0.0226778kj/Mol/ Å2 and b was 3.84928kj/Mol, Pbsolver was used to solve the PB equation. MM-PBSA analysis showed that there were two factors that greatly contributed to the protein and ligand interactions i.e van der Waals and electrostatic energy.

4.5 Conclusion

To date, there is no known cure for covid-19. Vaccines have been tried and tested, and have proven to minimize severity of disease and fatality, globally. Due to constant new emerging strains, and constant spikes in the covid-19 infection cases, there is still a need for therapeutic intervention. This computational work was carried out to rapidly identify FDA approved drugs for immediate use as therapeutic intervention of SARS-CoV-2. A combination of molecular docking, molecular dynamic simulation and MM-PBSA free energy calculations were applied to improve computational accuracy. Our study shows that all drugs under investigation had more negative (better) binding energies with the ACE2 receptor. Furthermore, AZM, Remdesivir and Ivermectin have shown the greatest binding potential to the ACE2 receptor. Therefore, these drugs are good inhibitors of viral replication processes. However, further studies are required to test in vivo and in vitro response of these drugs to SARS-CoV-2. One of the fears that remain, and that may lead to another public health crisis, is the emergence of antimicrobial resistance due to the incorrect or overuse of antimicrobials in the treatment of Covid-19 or secondary infections. It is imperative that all healthcare professionals as well as patients, avoid dangerous misuse of antibiotics, and to educate and promote the rational use of antimicrobials.

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CHAPTER 5: Conclusion and Future Recommendations

CHAPTER 5

5.1 Conclusion and future recommendations

Outlined in this chapter is the general conclusion of the entire thesis based on our findings and recommendations for future studies.

5.2 General conclusion

This thesis focuses on the repurposed use of macrolide antibiotics for the treatment of SARS-CoV-2 virus. Viruses are structurally different to bacteria (Simmonds and Aiewsakun, 2018). They also replicate within a host cell and cannot survive outside this environment (Gillen and Nita-Lazar, 2019). Viruses reproduce by inserting their genetic material into the DNA of a human cell (Gillen and Nita-Lazar, 2019). Antibiotics cannot be used to kill viruses due to their different mechanisms and machinery used by them to survive and replicate (Uddin et al., 2021). Anti-viral medication and vaccines are used specifically for viruses (Seyfoori et al., 2021). The best way to help prevent Covid-19 is with a vaccine (Bhatti et al., 2020).

However, when covid-19 emerged and rapidly mutated to various strains, it brought about an urgent need for drug repurposing. This study dealt with the use of macrolide antibiotics in particular. Even though antibiotics aren't a treatment for viruses, due to the presence of their anti-viral activity, the macrolides under investigation showed promising results.

In this study. We used computational approaches to determine the mechanism in which macrolide antibiotics bind to and inhibit the SARS-CoV-2 protein. Our four macrolide antibiotics included in this study: Azithromycin, Erythromycin, Clarithromycin and Roxithromycin were compared to our two controls (Remdesivir and Ivermectin) during the molecular docking and molecular dynamic simulation. The drugs were assessed on how they bind to each of the four receptors: ACE-2, Mpro, RdRp and RBD.

The macrolide antibiotics under investigation showed very similar results to that of our two controls (Ivermectin and Remdesivir), of which Azithromycin showed the best results. Azithromycin showed the best binding potential and very minimal deviations were noted for Azithromycin-ACE2 complex

5.3 Recommendations and future studies

Multiple authors have carried out drug repurposing studies since the emergence of covid-19. This study showed promising results and the need for further evaluation research such as in vivo and in vitro. To date, there is no known cure for covid-19. Vaccines have been tried and tested, and have proven to minimize severity of disease and fatality, globally. Our study shows that all drugs under investigation had more negative (better) binding energies with the ACE2 receptor. Furthermore, AZM, Remdesivir and Ivermectin have shown the greatest binding potential to the ACE2 receptor. Therefore, these drugs are good inhibitors of the processes that contribute to viral replication.

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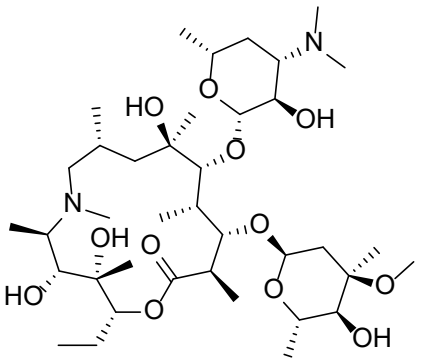
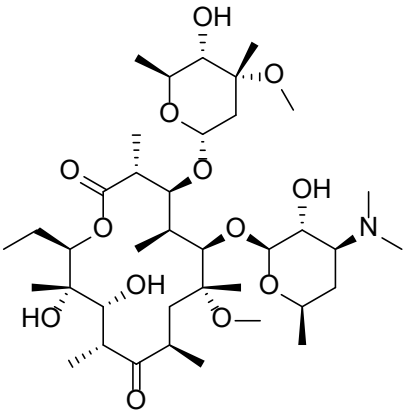
APPENDICES

Appendix 1. Input files of macrolide antibiotics



Azithromycin



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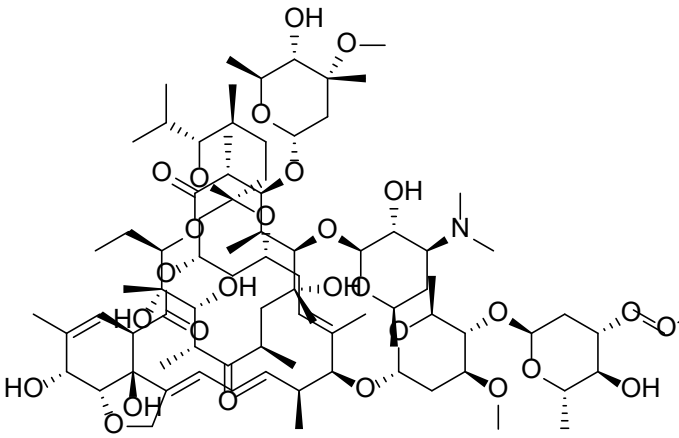
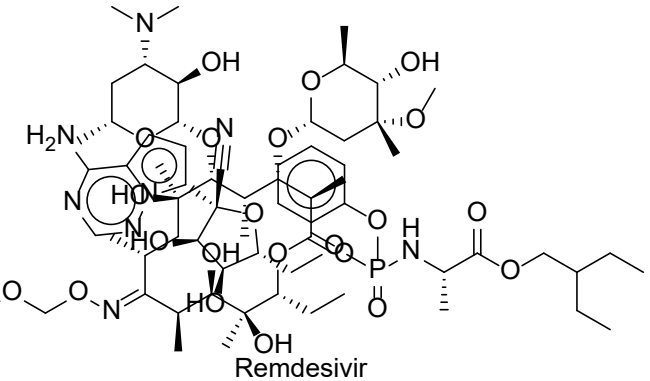


Erythromycin

Ivermectin

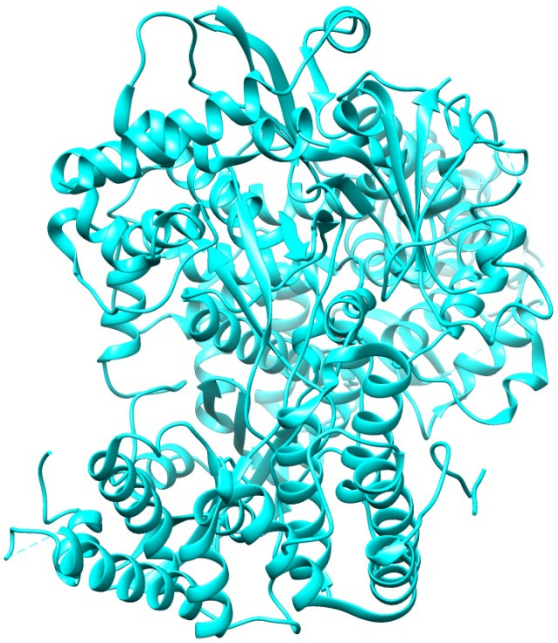


Roxithromycin

Remdesivir

Appendix 2. Input files of PDB structures

6ZLG



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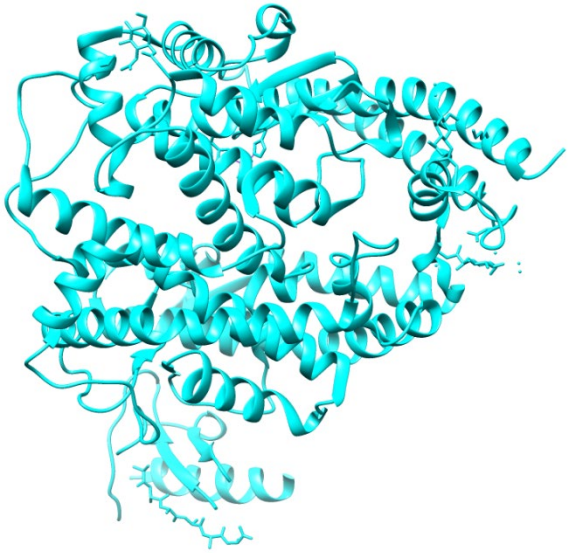
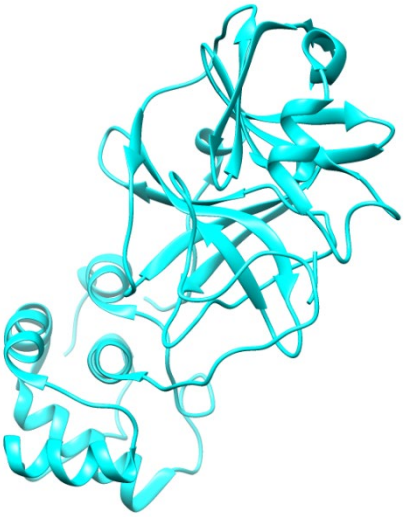


1R42



6LU7